

ULRR

The impact of carbon on microbial community dynamics in Irish grasslands

Item Type	Thesis
Authors	Fox, Aaron
Download date	2026-03-14 22:50:43
Item License	https://creativecommons.org/licenses/by-nc-sa/4.0/
Link to Item	https://doi.org/10.34961/researchrepository-ul.23954766



UNIVERSITY *of* LIMERICK

OLLSCOIL LUIMNIGH

Department of Biological Sciences, School of Natural Sciences

The Impact of Carbon on Microbial Community Dynamics in Irish Grasslands

A thesis submitted for the award of Doctor of Philosophy

Aaron Fox (BA TCD, MSc.)

Supervisors:

Dr. Achim Schmalenberger

Dr. Gary J. Lanigan

Prof. Rachel E. Creamer

Submitted to the University of Limerick, January 2017

Abstract:

Grasslands are recognised as a major European sink for the long term sequestration of carbon (C), helping to offset greenhouse gas emissions. Such a scenario is particularly attractive in an Irish context as grasslands comprise 90 % of the land devoted to agricultural production. Thus a greater understanding is required of how C influences both the composition and functionality of soil microbial community, as this represents the most important biological component of these systems. The results of this thesis demonstrated that both the physical protection of carbon (soil aggregates) as well as carbon addition through agricultural management (i.e, slurry addition) impacts on the soil microbiome in Irish agricultural grasslands. Specifically it was demonstrated that (i) aggregate size was a significant determinant of both bacterial and fungal community composition down the soil profile of Irish grasslands. (ii) The functionality of the soil microbiome responds in a staged manner to slurry derived carbon at the field scale while the composition of the microbiome is more influenced by seasonal succession. (iii) Over a 1 year experimental period, both aggregate size and slurry applied C significantly influenced potential extracellular enzymatic rates with this activity being temporally dynamic. A thorough comprehension of the spatial and temporal influence of both soil aggregates and agricultural fertilisation regimes on the constitution and activity of the soil microbiome is critical if we are to elucidate the microbially mediated mechanisms of terrestrial carbon cycling in Irish grasslands.

Declaration:

I, the undersigned, declare that the work in this project is entirely my own and to the best of my knowledge contains no material previously written, published or submitted for merit or award by this university or any other academic establishment, except where acknowledgment has been made in the text.

Signed: Aaron Fox

Aaron fox

Date: 9th January 2017

Dedication:

To my Parents. Whom I owe everything.

Acknowledgments:

Firstly, I must thank my supervisors Dr. Achim Schmalenberger, Dr. Gary Lanigan and Prof. Rachel Creamer for their invaluable guidance and endless encouragement.

I will always be grateful to Dr. Steve Wakelin in AgResearch and for making my all too short stay in New Zealand unforgettable. Not only did he impart an endless amount of knowledge to me but he was kind and generous with his time. I would also like to thank Dr. Glyn Francis, Dr. Maureen O'Callaghan, Sandra Jones and Dr. Bryony Dignam for doing so much to facilitate my visit.

I must also thank the researchers and technicians in Johnstown Castle who helped me so much in getting my work completed. To Pat Sills for helping me with the particle size analysis and teaching me all about the local history of Wexford, to Carmel for running endless samples for me on the LECO and Dr. Brian Reidy who helped me in the collection of soil samples. Being with Brian in the field was like getting a one to one master class in soil science and I was so fortunate to have had the opportunity to have worked with him. If it wasn't for the help, advice and support of Dr. Dote Stone I probably would not have made it through the first stage of my PhD. I will always be grateful to her. My thanks also must go to Miriam, who was never long about bringing me back down to Earth.

Thanks to Joanne, Olivia and Catriona who made my time in Johnstown so enjoyable and whom I have some great memories with. To Gemma for teaching me the ins and outs of soil fractionation and to Jessica for all the Thursday night pints that were often badly needed! Finally thanks to all the students and postdocs in Johnstown for the help and craic; Ian F, Steve, Phillip, Jose, Dara, Fran, Eamon, Lillian, Elisa and Guilia. Ye made the last few years infinitely better.

Table of Contents

Abstract.....	I
Declaration.....	II
Dedication.....	III
Acknowledgments.....	IV
Table of contents.....	V
Abbreviations.....	IX
List of Tables.....	X
List of Figures.....	XIII
Chapter I: Literature Review	
1.1.: Carbon in soils:.....	1
1.2: Soil aggregate formation:.....	5
1.3: The role of soil biology in soil aggregate formation.....	7
1.4: The role of the soil micro-biota in terrestrial C cycling.....	10
1.5: Microbial contributions to SOC.....	13
1.6: Response of the soil microbiota to carbon addition.....	21
1.7: Physical C protection mechanisms in soil.....	25
1.8: Spatial constraints on microbe-SOC interactions.....	30
1.9: Impact of agriculture on SOC.....	32
1.9.1: Livestock:.....	34
1.9.2: Tillage:.....	35
1.9.3: Slurry application:.....	38
1.10: Methods for studying soil microbial community structure:.....	41
1.11: Methods in enzymatic analysis:.....	44
1.12: Aggregate isolation through wet-sieving:.....	45
1.13: Motivation:.....	45
1.14: Hypothesis:.....	45
1.15: Objectives:.....	45
Chapter II: The community dynamics of bacteria and fungi in aggregate size fractions in grassland soils.....	46
2.1: Abstract.....	47
2.1.1: Highlights:.....	47
2.1.2: Keywords:.....	47
2.2: Introduction:.....	48
2.3: Materials and Methods:.....	50
2.3.1: Soil collection:.....	50
2.3.2: Soil aggregate isolation:.....	52
2.3.3: Aggregate coarse /fine sand determination:.....	53
2.3.4: DNA extraction:.....	53
2.3.5: PCR amplification of soil bacterial 16S and fungal ITS community:.....	54
2.3.6: Terminal Restriction Fragment Length Polymorphism (T-RFLP):.....	55
2.3.7: Edaphic and environmental properties:.....	55
2.3.8: Statistics:.....	56
2.3.8.1: Microbial community composition and richness:.....	56
2.3.8.2: Environmental variables:.....	56
2.3.8.3: Correlation of the microbial community with environmental variables:.....	57
3. Results:.....	57
3.1 Biological, chemical and physical characteristics of the BS with depth:.....	57
3.2: Bacterial Community structure within aggregate fractions:.....	60

3.3: Fungal community structure within aggregate fractions:.....	63
3.4: Effect of SOC on aggregate-size bacterial and fungal community composition.....	66
4. Discussion.....	66
5. Conclusion.....	71
Chapter III: The bacterial colonization patterns across aggregate-size fractions as determined by next generation sequencing.....	72
3.1: Abstract:.....	73
3.1.1: Keywords:.....	73
3.1.2: Highlights:.....	74
3.2: Introduction.....	74
3.3: Materials and Methods.....	76
3.3.1: Soil collection and soil aggregate isolation:.....	76
3.3.2: DNA extraction:.....	76
3.3.3: PCR amplification of soil bacterial 16S community:.....	76
3.3.4: Statistics:.....	77
3.4: Results:.....	78
3.4.1: Effect of horizon in the BS:.....	78
3.4.2: Effect of aggregate-size fraction:.....	81
3.4.3: The allocation of bacterial phyla and families between and within the various aggregate-sizes:	85
3.4.4: Interaction between horizon and aggregate-size fraction:.....	88
3.4.5: Effect of occluded SOC:.....	90
3.5: Discussion:.....	90
3.6: Conclusion:.....	95
Chapter IV: In-field slurry applications resulted in soil microbiota short term response to added carbon while microbial community structures were only sensitive to seasonal successions.....	96
4.1: Abstract:.....	97
4.1.2: Keywords:.....	97
4.1.3: Highlights:.....	97
4.2: Introduction.....	98
4.3: Materials and Methods.....	100
4.3.1: Field site set-up:.....	100
4.3.2: Sampling strategy:.....	102
4.3.3: Edaphic and environmental properties:.....	102
4.3.4: Enzymatic assays of C-cycling enzymes:.....	102
4.3.5: Determination of microbial biomass C and N and basal respiration:.....	103
4.3.6: Soil DNA extraction, amplification and analysis:.....	104
4.3.7: Quantitative PCR:.....	105
4.3.8: Statistics:.....	105
4.4: Results.....	106
4.4.1: Edaphic and environmental factors:.....	106
4.4.2: Enzymatic assays:.....	108
4.4.3: Microbial biomass-C, -N and Basal Respiration:.....	110
4.4.4: Bacterial and fungal community composition:.....	111
4.5: Discussion:.....	114
4.6: Conclusions.....	117
Chapter V: Response of various C-cycling enzymes in aggregate sized fractions to varying degrees of slurry application in two contrasting soil types.....	118
5.1: Abstract.....	119

5.1.1: Highlights.....	119
5.1.2: Keywords.....	119
5.2: Introduction.....	120
5.3: Materials and Methods.....	122
5.3.1: Soil and Slurry collection and preparation.....	122
5.3.2: Establishment of pot experiments.....	122
5.3.3: Soil Sampling.....	123
5.3.4: Enzymatic assays of C-cycling enzymes.....	124
5.3.5: Statistics.....	125
5.4: Results.....	126
5.4.1: Biological parameters of the bulk soil.....	126
5.4.2: The % proportion of aggregate-size to the bulk soil.....	130
5.4.3: Enzymatic activity within aggregates.....	134
5.4.4: Enzymatic activities at individual sampling points in the loam soil.....	137
5.4.5: Enzymatic activities at individual sampling points in the sand soil.....	140
5.5: Discussion.....	145
5.6: Conclusion.....	149
Chapter VI: General Discussion.....	150
6.1: Effect of aggregate size on microbial community composition.....	152
6.2: Impact of aggregate size and slurry application on the functionality of the soil microbiome.....	156
6.3: Conclusions and future work.....	161
6.3.1: Grassland disturbance:.....	162
6.3.2: Impact of soil type.....	163
Chapter VII: Bibliography.....	165
Chapter VIII: Appendix.....	193
Appendix A: Chapter II.....	194
A8 1: Bacterial 16S and fungal ITS t-RF richness.....	194
A8 2: Bacterial 16S t-RF richness.....	195
A8 3: Fungal ITS richness.....	196
A84: Irish SIS characteristics.....	197
A8 5: Bacterial 16S rRNA based community composition from aggregate size fractions from horizon 1, 2 and 3.....	199
A8 6: Fungal internal transcribed spacer region based community composition from the aggregates size fractions in horizon 1,2 and 3.....	200
Appendix B: Chapter III.....	202
B8 1: Table of phyla sequence abundance.....	202
B8 2: Graph of phyla sequence abundance.....	203
B8 3: Graph of family sequence abundance in Horizon 1.....	204
B8 4: Graph of family sequence abundance in Horizon 2.....	205
B8 5: Venn diagrams of shared/unique phyla.....	206
B8 6: Family sequence abundance and correlations to environmental variables.....	207
B8 7: Table of Margalef's richness index (d), Pielou's measure of species richness (J') and Shannon-weiner diversity index ($H' \log^e$).....	209
Appendix C: Chapter IV.....	210
C8 1: Field conditions prior to the establishment of field experiment.....	210
C8 2: Quantitative PCR for bacterial 16S rRNA gene and fungal ITS region.....	211
C8 3: Enzymatic activities throughout field experiment.....	212
C8 4: Microbial biomass C and N and basal respiration values throughout field experiment.....	213

C8 5: Bacterial community composition on day 5 of field experiment.....	214
C8 6: Rainfall amounts (mm) throughout field experiment.....	215
C8 7: Max air temperature (°C) throughout field experiment.....	216
Appendix D: Chapter V.....	217
D8 1: Biological parameters of the bulk soil.....	217
D8 2: pH from bulk soil.....	218
D8 3: % aggregate proportions.....	219
D8 4: Enzymatic activity in aggregate size fractions in loam soil at day 30.....	220
D8 5: Enzymatic activity in aggregate size fractions in loam soil at day 137.....	221
D8 6: Enzymatic activity in aggregate size fractions in loam soil at day 361.....	222
D8 7: Enzymatic activity in aggregate size fractions in sand soil at day 30.....	223
D8 8: Enzymatic activity in aggregate size fractions in sand soil at day 137.....	224
D8 9: Enzymatic activity in aggregate size fractions in sand soil at day 361.....	225
D8 10: Enzymatic activity in aggregate sized fractions in the loam and sand soils as a function of time (days).....	226
D8 11: % C from the bulk soil.....	227
D8 12: % C within aggregates sized fractions.....	228

List of abbreviations:

μ : Micron (10^{-6})

Bp: base pairs

BS: bulk soil

C: Carbon

Ca: Calcium

CEC: cation exchange capacity

dH₂O: distilled and deionized water

DNA: deoxyribose nucleic acid

dNTP: deoxyribose nucleotide triphosphate

e.g.: for example

EDTA: ethylene diamine tetra acetic acid

g: gram

hr(s): hour(s)

i.e.: that is

ITS: internal transcribed spacer region

K: potassium

Kb: kilobase

l/L: litre

LM: large macroaggregate

MAC: macroaggregate

Mg: magnesium

mg/ml: milligram/millilitre

mM: milli (10^{-3}) molar

NGS: next generation sequencing

μM : micro molar (10^{-6} molar)

μm : micro meter (10^{-6} meter)

MIC: microaggregate

Min(s): minute(s)

N: nitrogen

NGS: next generation sequencing

nm: nanometer

P: phosphorus

PCR: polymerase chain reaction

rRNA ribosomal deoxynucleic acid

SC: silt and clay

SOC: Soil organic carbon

SOM: Soil organic matter

TAE: tris-acetic acid-EDTA

TEMED: tetramethylethylenediamine

T-RFLP: terminal restriction fragment length polymorphism

V: volts

List of Tables:

Chapter II:

Table 1: Irish SIS sites descriptions.....51

Table 2: Measured environmental variables from the nine grassland sites.....58

Table 3: Summary statistics table of the effect of aggregate size and horizon on bacterial and fungal community composition.....64

Table 4: Summary statistics table of the effect of aggregate size within individual horizons.....65

Chapter III:

Table 5: Abundance of phyla sequences from bulk soil in horizon 1 and 2.....80

Table 6: Abundance of family sequences from aggregate sizes in horizon 1.....83

Table 7: Abundance of family sequences from aggregate sizes in horizon 2.....84

Table 8: Summary table of pairwise statistics between aggregate sizes from horizon 1 and 2.....89

Chapter IV:

Table 9: Table of environmental values from slurry and control treatment.....107

Table 10: Abundance of bacterial 16S rRNA gene and fungal internal transcribed spacer region from control and slurry treatment.....112

Table 11: Statistical correlations (DistLM) between environmental and biological parameters.....113

Chapter V:

Table 12: Enzymatic activities ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from the bulk soil in the loam...128

Table 13: Enzymatic activities ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from the bulk soil in the sandy soil.....129

Table 14: Summary table of pairwise statistical tests of potential enzymatic activity between aggregate sizes in the loam soil.....143

Table 15: Summary table of pairwise statistical tests of potential enzymatic activity between aggregate sizes in the sandy soil.....144

Chapter VIII:

Table 16: Summary statistics table of Bacterial 16S and fungal ITS t-RFs within aggregate size and horizon.....194

Table 17: Summary statistics table of bacterial 16S t-RFs within horizon 1, 2 and 3...195

Table 18: Summary statistics table of fungal ITS t-RFs within horizon 1, 2 and 3.....196

Table 19: Table of Irish SIS sites including reference number, soil type, drainage, horizon, horizon depth (cm), textural class, % sand, % silt and % clay.....	197
Table 20: Irish SIS site physiochemical characteristics including % C, % N, P, Al, Fe, Ph, Na, K, Mg, Ca and cation exchange capacity (CEC).....	198
Table 21: Abundance [%] of sequences allocated to major bacterial Phyla from aggregate sizes from horizon 1 and 2.....	202
Table 22: the abundance of sequences allocated to major families from the bulk soil from Horizon 1 and 2.....	207
Table 23: Margelef's richness index (d), Pielou's measure of species richness (J') and Shannon-weiner diversity index ($H' \log^e$) of both phyla and family from the four aggregate sizes from H1 and H2.....	209
Table 24: Field conditions prior to experimental establishment.....	210
Table 25: Enzymatic activities in control and slurry treatments.....	212
Table 26: Microbial biomass C and N and basal respiration values from control and slurry treatments.....	213
Table 27: Microbial biomass-C, -N and basal respiration from the bulk soil of both the loam and sandy soils from the control and three slurry treatments on day 30, 137 and 361.....	217
Table 28: pH of the bulk soil of each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.....	218
Table 29: % aggregate proportions to the bulk soil of each of the four aggregate size fractions; large macroaggregate on day 0, 30, 137 and 361 in the two soil types (loam and sandy).....	219
Table 30: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the loam soil 30 days after slurry application.....	220
Table 31: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the loam soil 137 days after slurry application.....	221
Table 32: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the loam soil 361 days after slurry application.....	222
Table 33: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the sandy soil 30 days after slurry application.....	223

Table 34: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the sandy soil 137 days after slurry application.....	224
Table 35: Activities of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction from the control and each of the slurry amendments; in the sandy soil 361 days after slurry application.....	225
Table 36: % C of the bulk soil of each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.....	227
Table 37: Averages (Avg.) and standard deviations (\pm) of the % C within aggregate sized fractions from each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.....	228

List of Figures:

Chapter I:

Figure 1: Model structure of humic acid.....	4
Figure 2: Aggregate-size hierarchy in soil.....	8
Figure 3: ‘Active’ and ‘stabilized’ pool concept.....	21
Figure 4: The physical nature of the soil microbial habitat.....	31
Figure 5: Overview of how soil organic carbon affects biological, chemical and physical parameters of agricultural soils.....	34
Figure 6: (a) Splash-plate slurry spreading (b) injection slurry application.....	40
Figure 7: Overview of steps required for T-RFLP assay.....	43

Chapter II:

Figure 8: Example of horizon profile from an Irish SIS site.....	51
Figure 9: Fractionation procedure as developed by Six et al. 1998.....	52
Figure 10: Proportion [%] each aggregate-size makes of the BS in Horizon1, 2 and 3....	59
Figure 11: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size and horizon on bacterial 16S rRNA gene based community composition.....	61
Figure 12: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size and horizon on fungal internal transcribed spacer region (ITS) community composition.....	62

Chapter III:

Figure 13: Abundance [%] of sequences allocated to major bacterial from the four aggregate sizes in Horizon 1 and 2.....	82
--	----

Figure 14: Venn diagrams indicating the families which are shared and unique among the aggregate-size fractions; from Horizon 1 and 2.....	87
--	----

Chapter IV:

Figure. 15: A: Experimental field layout. B: Simulated splash plate slurry spreading technique.....	101
---	-----

Figure 16: Enzymatic assays from the control and slurry treatment on day 5, 30 and 65 of the experiment.....	109
--	-----

Figure 17: Microbial biomass-N from the control and slurry amended treatments (dotted on day 5, 30 and 65.....	110
--	-----

Figure 18: Bootstrapped non-metric multidimensional scaling plots of 16S rRNA based bacterial (top) and ITS region based fungal (bottom) community composition on day 5, 30 and 65.....	112
---	-----

Chapter V:

Figure 19: Diagrammatic representation of the treatments used in pot experiment.....124

Figure 20: The % proportion that each aggregate size constitutes to the bulk soil at each sampling point in the loam soil.....132

Figure 21: The % proportion that each aggregate size constitutes to the bulk soil at each sampling point in the sand soil.....133

Figure 22: Bootstrapped non-metric multi-dimensional scaling (MDS) plots of the potential enzymatic activities of aggregate size fractions from all parameters during the course of the experiment from both the loam (top) and sand (bottom) soil.....135

Figure 23: Bootstrapped non-multidimensional scaling plots of the potential enzymatic activities from all parameters of aggregate size fractions associated with different experimental treatments in the loam (top panel) and the sandy (bottom panel).....136

Figure 24: MDS plots from day 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the loam soil.....138

Figure 25: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the loam soil from the three slurry amendments and the control.....139

Figure 26: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the sandy soil.....141

Figure 27: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the sandy soil from the three slurry amendments and the control.....142

Chapter VIII: Appendix

Figure 28: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on bacterial 16S rRNA gene based community composition from horizon 1.....199

Figure 29: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on bacterial 16S rRNA gene based community composition from horizon 2.....199

Figure 30: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on bacterial 16S rRNA gene based community composition from horizon 3.....200

Figure 31: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on fungal internal transcribed spacer region based community composition from horizon 1.....200

Figure 32: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on fungal internal transcribed spacer region based community composition from horizon 2.....	201
Figure 33: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate size on fungal internal transcribed spacer region based community composition from horizon 3.....	201
Figure 34: Bar-chart of averages of Abundance [%] of sequences allocated to major bacterial Phyla (cut-off 0.01 %) from the four aggregate sizes from horizon 1 and 2.....	203
Figure 35: Stacked bar-chart of averages of abundance [%] of sequences allocated to the major bacterial families (cut-off 0.001 %) from the four aggregate-size fractions in horizon 1.....	204
Figure 36: Stacked bar-chart of averages of abundance [%] of sequences allocated to the major bacterial families (cut-off 0.001 %) from the four aggregate-size fractions in horizon 2.....	205
Figure 37: Venn diagrams indicating the phyla which are shared and unique to the specific aggregate-size fractions from horizon 1 (top) and horizon 2 (bottom).....	206
Figure 38: Non-metric multidimensional scaling plots of 16S rRNA gene based bacterial community composition of the control (blue triangle) and slurry amended plot (red triangle) on day 5.....	214
Figure 39: Total daily rainfall (mm) recorded during field experiment.....	215
Figure 40: Maximum (Max.) daily air temperature (°C) recorded during field experiment.....	216
Figure 41: Bootstrapped multi-dimensional scaling plots displaying enzymatic activities in the loam and sand soils 30, 137 and 361 days after initial slurry application.....	226

Chapter I: Literature Review

1.1.: Carbon in soils:

The carbon cycle is the biogeochemical exchange of carbon between its five different reservoir pools through photosynthesis, respiration, decomposition and deposition. The largest of these pools is the Oceanic pool, containing 38,000 Pg of carbon, representing all of the carbon contained in the world's oceans. The carbon which is contained in rock (e.g, limestone) as well as coal, oil and gas is collectively referred to as the geologic pool. This represents the second largest reservoir pool of carbon (5000 Pg). Soil represents the third largest reserve of carbon (2500 Pg). It is this pedologic pool which shall be the subject of this review. It contains more than the total amount of carbon in the remaining pools, the atmospheric (760 Pg) and the biotic pool (560 Pg) (Eswaren 2000; Schlesinger 1995). All of the carbon pools are interconnected, with C capable of being transferred from one pool to the other.

The pedosphere is the thin covering of soil and sediment which lies on top of most of the habitable terrestrial land-masses (Voroney, 2007). It represents the biggest active terrestrial component in the global C cycle. Estimates as to the amount of organic C in the top 1 metre globally range from 1,220 Pg to approximately 1,550 Pg (Janzen 2005; Jobbágy and Jackson 2000). The pedosphere is integrally involved in the cycling of atmospheric gases (CO₂, CH₄ and NO₂) and nutrients essential to agro-ecosystem functioning, N, P, S and C. Approximately 3-5% of the global soil C cycle is designated as 'highly dynamic' with the flux of C into and out of soils calculated at around 75 Pg (Rumpel et al. 2012). Carbon (C) may be organically or inorganically bound in soil. Carbonates (such as calcium carbonate) are the predominant form of soil inorganic carbon (SIC). The amount of carbonates present in a soil is strongly dependant on soil type (Batjes 1996). High carbonate levels are often found in

soils with a calcareous parent material, while acidic soils tend to dissolve carbonates (Batjes 1996).

Generally, the amount of organic C in soil will be determined by C inputs in the form of plant litter and detritus (ie, roots and shoots) and C output via decomposition (Trumbore 1997). In Ireland, the total SOC was 2,021Mt in 2000 (47% in mineral soils and 53% in peat soils) a reduction from 2,048 Mt in 1990. This reduction is mainly as a consequence of industrial peat extraction (Tomlinson 2006). Soil organic carbon (SOC) is a general term which encompasses humic substances, labile carbon sources as well as macro-organisms and micro-organisms. The SOC portion of soil is essential to ecosystem functioning, being inherently involved in the cycling, retention and supply of bio-available nutrients. SOC is also understood to aid with soil structural stability, increasing resilience to soil compaction as well as enhancing soil biodiversity (Spink et al. 2010).

Humic substances are a heterogeneous mixture of high-molecular weight organic substances (Figure 1). It is the most abundant organic carbon substance in the terrestrial environment (Hayes. 1999; Lovley et al. 1996). It is essential to soil structure as well as the retention of water and nutrients due to its negative charge (Hayes. 1999). Schulten (1994) demonstrated that SOC was principally composed of polar and high molecular weight compounds, with aliphatic lipids (62%) phenols and benzyls predominating. There are three dominant fractions of humic substances, humic acid (HA), fulvic acid (FA) and humin (Hayes. 1999; Schulten 1994). These are operational definitions and do not represent pure compounds. The HA fraction is usually defined as the fraction which is usually precipitated at pH 1 from aqueous alkaline extracts of soil. While the FA fraction is defined as the component of alkaline soil extracts which remain in the soil after acidification. The humin fraction is not soluble in water at any pH. It represents more than 50% of the organic carbon in soil (Clapp et al.

2005.). The physiochemical formation of HS is the subject of on-going research. Many theories have been put forward such as the lingo-protein theory (Waksman 1932), the sugar-amine condensation theory and the polyphenol theory.

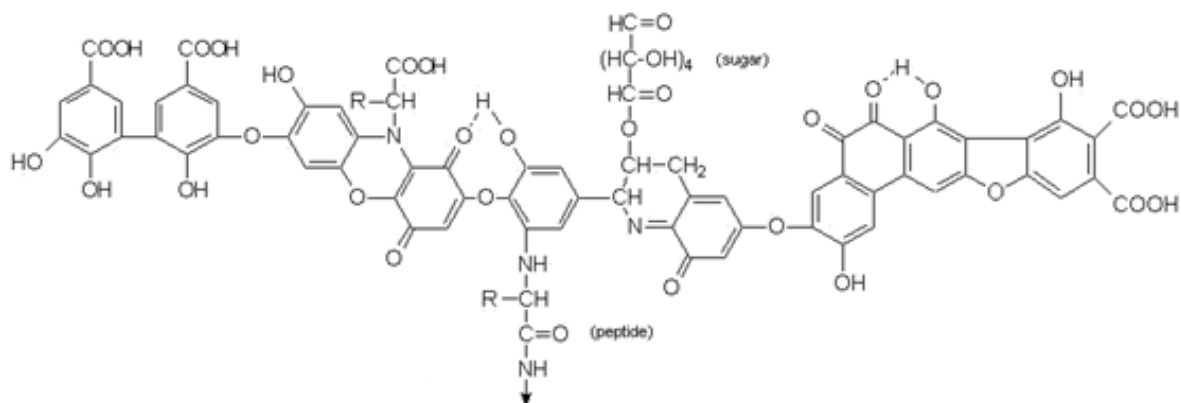


Figure 1: Model structure of humic acid (image taken from Stevenson, 1982).

The ligno-protein theory dominated thinking on this subject for decades. The theory proposes that a lignin unit reacting with amino acids which form the core humic molecule. The sugar-amine condensation theory involves the Maillard reaction where amino acids react with reducing sugars to form melanoidin (Maillard 1912). The reaction proceeds slowly but environmental processes such as freezing, thawing, wetting and drying may assist with condensation (Stevenson 1994). Studies of the last decade or so have highlighted difficulties with the theory. The Maillard reaction results in the formation of heterocyclic N, whereas amide N is the dominant N compound base in soil (Knicker 2004; Lovley et al. 1996). In the polyphenol theory lignin still plays an important role, but with a different mechanism. In this pathway phenolic aldehydes and acids which are released following microbiological attack are enzymatically converted to quinones which polymerise in the presence of amino compounds forming humic-like compounds (Stevenson 1994).

The organic C content of the sub-soil horizons is an important though often over-looked component of the terrestrial C cycle with estimates that the lower 70 cm may contain up to 45-65 % of the total C in the top 1 metre (Rumpel and Kögel-Knabner 2011). Subsoil C may also have a longer residence time (up to millennia) than the C in the upper portion of the profile indicated by its high radiocarbon age. Typically, the carbon to nitrogen ratio (C:N) decreased down the soil profile indicating that the C in the lower profiles are highly microbially processed. Indeed SOC in the sub-soil horizons are typically rich in C of microbial-origin and relatively depleted in plant-derived C, which is energy-rich (Boström et al. 2007; Rumpel and Kögel-Knabner 2011). The main recognised sources of C input into the sub-soil horizons are the root exudates and plant roots, dissolved carbon and translocation of particulate organic matter through bioturbation. In soils undergoing clay illuviation such as luvisols, clay-bound organic matter may also be transported down the soil profile (Rumpel and Kögel-Knabner 2011). As a consequence, there is a large spatial heterogeneity in the abundance of C with ‘hot-spots’ (containing a greater portion of ‘new’, more energy-rich C) being located around preferential flow pathways (vertical ‘tongues’ extending from higher up in the soil profile into the sub-soil). These hot-spots will also have a greater abundance of microbes and enzyme activities (Chabbi et al. 2009).

1.2: Soil aggregate formation:

Aggregates are secondary structures formed through the interactions of mineral particles as well as organic and inorganic substances (Bronick and Lal 2005; Tisdall and Oades 1982). They are grouped according to size, with large macroaggregates being considered >2 mm macro-aggregates generally being considered over 250 µm in size while microaggregates are less than 250 µm in size (Bronick and Lal 2005). Different binding mechanisms are acting at each stage of aggregate formation (Figure 2). The hierarchical theory of soil aggregates

developed by Tisdall and Oades (1982) put forward the idea that different binding agents are acting at each aggregate level. They proposed that silt sized ($>20\ \mu\text{m}$) aggregates are bonded together by humified organic matter, clay, and polyvalent cations to form microaggregates. Stable microaggregates can then join to form macroaggregates, which are typically bound together via plant roots and fungal hyphae and also (on $<50\ \mu\text{m}$ scale) by polysaccharides of both plant and microbial origin. These binding mechanisms are transient and are not as strong as the binding mechanisms acting on the microaggregates (Six et al. 2004). It is postulated that the roots and hyphae which bind macroaggregates together degrade into fragments they are covered in mucilage as a result of microbial decomposition. These fragments are then coated with clays and act as nucleation sites for microaggregate formation within the macroaggregate (Six et al. 2004). Microaggregate formation within macroaggregates is thought to result from the anaerobic (and therefore reducing) conditions which are found within the macroaggregate (Lavelle et al. 2004). Soil porosity is also related to aggregate formation with varying macro- and microporosity seen between aggregate sizes (Lavelle et al. 2004). The aggregate hierarchy theory would only hold in soils where organic matter is the main binding agent (Oades and Waters 1991). In oxide-rich soils, oxides rather than organic matter are the principle binding agents thus inhibiting the formation of an aggregate hierarchy, such soils however are not in Ireland and principally found in the tropics (Eswaren and Padmanabhan 2006).

The rate of aggregation is often controlled by soil type and conditions, with it generally being the case that the greater the amount of organic C and surface clay area the higher the aggregation rate (Bronick and Lal 2005). The cation exchange capacity (CEC) will also influence this rate as cations such as Al^{3+} , Fe^{3+} , Si^{4+} and Ca^{2+} act as bridges between primary organic particles and clay particles. These cations can also influence the precipitation of

compounds which act like bonding agents in aggregate structures (Bronick and Lal 2005). Increased soil pH is associated with large aggregates due to the high carbonate concentration (Boix-Fayos et al. 2001). A consequence of lime addition to agricultural soils (added to increase soil pH) has the additional effect of increasing plant growth (and thus root exudate release) as well as microbial activity. This results in greater SOC and thus aggregation (Bronick and Lal 2005).

1.3: The role of soil biology in soil aggregate formation

There are also a number of biological factors at play in aggregate formation. Fungal hyphae act as a 'sticky string bag' releasing organic compounds and enmeshing organic materials, clays and micro-aggregates together which may eliminate any spatial constraints on macro-aggregate formation (Bronick and Lal 2005; Rillig and Mummey 2006; Six et al. 2004). It has been demonstrated that inhibiting fungal growth and activity through the application of captan (a fungicide) resulted in reduced formation of macro-aggregates (Bossuyt et al. 2001). The application of a bacteriocide had no such effect on the formation of macro-aggregates, as polysaccharides from bacteria only act as binding agents for microaggregates (Lueders and Friedrich 2003). Fungi stabilize macroaggregates, decreasing their turnover rate. This will have the effect of aiding with microaggregate formation, though whether a correlation exists between fungal biomass and microaggregate formation in this way has yet to be investigated (Six et al. 2004). Arbuscular mycorrhizal fungi (AMF, fungi which form an intimate symbiosis with plant roots) are now known to produce the glycoprotein glomalin, which acting in combination with proteins of non-AMF origin (collectively referred to as glomalin related soil protein or GRSP), is hypothesised to act as a glue assisting in the formation and stabilization of macroaggregate structures (Rillig 2004). The GRSP protein complex is persistent in soil and may account for as much as 5% of soil C (Rillig et al. 2007; Rillig et al.

2001). Glomalin does not appear to be an active fungal secretion into the soil matrix. In a cultivation experiment, glomalin did not appear to be an active fungal secretion of *Glomus intraradices*, with 80 % of measured glomalin being in the fungal biomass rather than in the growth culture (Driver et al. 2005). Thus its secretion appears to be a secondary aspect, perhaps as a consequence of its long residence time in soil (Rillig and Mummey 2006).

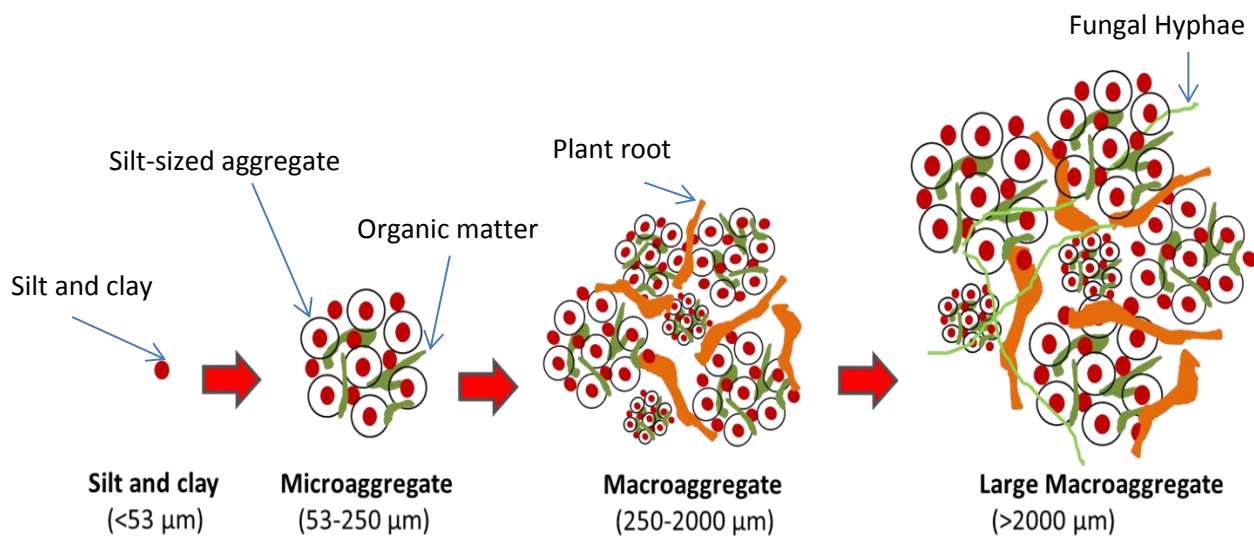


Figure 2: Aggregate-size hierarchy in soil. Image adapted from Torres-Sallan, 2016.

Earthworms play a key role in terrestrial C cycling through the transport of organic materials and plant litter from the soil surface and incorporation into the soil matrix (Martin 1991). They are also involved in aggregate formation through both burrowing and cast formation (Six et al. 2004). When an earthworm burrows through the soil it deposits mucus. This mucus will complex itself with clays along the burrow wall to form stable macroaggregate structures (Jeanson 1964). As well as promoting macroaggregate formation, studies suggest earthworm promote microaggregate formation. When they ingest organic matter it is mixed with inorganic materials in the gut and is excreted as a cast. Casts will augment microaggregate formation once they have aged and dried (Barois et al. 1993; Bossuyt et al. 2005; Jongmans et al. 2001; Marinissen and Dexter 1990; Shipitalo and Protz 1988) and are a major influence

on soil structure in the top 0-20 cm of soil where earthworms predominate (Parham et al. 2003). In temperate grasslands, 40-50 t ha⁻¹ of earthworm casts can be excreted in a year (Lee 1985), though this value is dependent on earthworm feeding activity and the quality of the organic matter ingested (Shipitalo and Protz 1988).

A higher degree of stability was also reported within the earthworm casts compared with the surrounding soil (Marinissen 1994; McKenzie and Dexter 1987; Shipitalo and Protz 1988). This may be due to the binding of the organic and inorganic material by polysaccharides which was exuded by the microbiota within the cast itself (Six et al. 2004). Stability may also occur as a consequence of entanglement of the casts by plant roots and fungal hyphae. A reduction in microbial activity has been reported within days of the cast being initially expelled (Litchman et al. 2015). This is associated with the decline of labile substrate and bio-available N, leaving more recalcitrant substrates to form organo-mineral complexes integrating into microaggregate structures (Shipitalo and Protz 1988; Six et al. 2004).

Soil aggregation and microbially-mediated C cycling can be affected by climatic, physiochemical and agronomic factors. The variation in temperature and moisture levels, resulting in freeze-thaw and dry-wet cycles can influence aggregate stability (Bronick and Lal 2005). Soil moisture is also an important consideration as it influences gas (such as O₂) diffusivity, which will affect microbial activity, with an optimal value for most soils being 55-60% water-filled space (Jones et al. 2006). Freeze-thaw cycles can reduce the aggregate stability as a function of soil moisture content and the number of freezing and thawing cycles it is subjected to (Wang et al. 2012). Macro-aggregates possess intra-aggregate pores which water can infiltrate and it is these aggregates which are most affected by freeze-thaw cycles (Benoit 1973; Wang et al. 2012). Wetting and drying cycles can have a varying impact on soil stability depending on clay type. Non-swelling clay tends to disperse when wet and form bridges and coating with other soil particles when drying. This is conducive to aggregate

formation as it leads to an enhanced contact between soil particles and increased soil bridging. While a swelling clay (such as smectite clay minerals) will tend to separate from other particles leading to reduced aggregate stability (Bronick and Lal 2005).

1.4: The role of the soil micro-biota in terrestrial C cycling

A classical tenet of environmental microbiology assumes that the soil microbiota is collectively capable of breaking down an unlimited range of organic molecules (Dungait et al. 2012). Heterotrophs, for example, utilise the nutrients present in SOC (i.e. carbohydrates, fats and proteins), simple carbohydrates found in exuded rhizodeposits, as well as decomposing plant tissue (Badri and Vivanco 2009) as a C substrate for aerobic respiration. Heterotrophic aerobic respiration in soil produces CO₂ through the tricarboxylic acid cycle (the Krebs cycle), which together with glycolysis and the mitochondrial electron transport process represent the three main pathways of microbial aerobic respiration (Ferne et al. 2004). While various biotic and abiotic factors affect the rate of heterotrophic respiration including the carbon: nitrogen (C:N) ratio of the substrate and soil physio-chemical properties such as texture, moisture, and pH, these are 'broad' processes which are carried out by a large range of micro-organisms (Cheng et al. 2013; Schimel and Schaeffer 2012).

Although microbial community composition is increasingly seen as the driving factor behind functional capabilities in soils (Allison and Martiny 2008), it has been widely believed in soil microbial ecology that the processes involved in C decomposition and sequestration are too universal to be affected by changes in microbial community diversity. It is assumed that in functionally very diverse communities any decline in function resulting from the loss of a microbial group will be taken up by another microbial group (Nannipieri et al. 2003). In ecosystem models such as CENTURY and TEM (terrestrial ecosystem model) microbial

community composition, diversity and abundance are entirely unimportant and have the working assumption that changes in the composition of microbial groups will have only minor effects on ecosystem processes. Such an assumption would only hold if the microbial community under study is resilient, resistant and/or functionally redundant (Condrón et al. 2010). In a review of the literature Allison and Martiny (2008) concluded that microbial communities are often sensitive to perturbations, such as agricultural management and organic matter amendment. They also suggest that disturbance induced changes in microbial community diversity can persist into the medium term (i.e, a few years after initial disturbance) and that these induced changes in diversity can influence ecosystem processes.

It has been proposed that the microbiota is functionally distinguishable at the family level up, with the genera and species level being relatively functionally redundant (Schimel and Schaeffer 2012). Functional diversity in relation to microbial C-turnover may be related to evolutionary life strategies. This is particularly seen when examining the roles of fungi and bacteria. In contrast with the aquatic environment, where bacteria are the dominant biological mediators of organic matter cycling, fungi have a number of distinct advantages over bacteria in the terrestrial environment (Boer et al. 2005). This is of a consequence of their different growth forms, fungi exhibit hyphal growth (long threads) while bacteria are individual cells. Hyphal threads are advantageous to the fungus as it allows the translocation of nutrients from a microsite where the nutrient is abundant to a site where the nutrient is limiting along the hyphal network. This network is often referred to as the hyphal bridge (Hendrix et al. 1986; Smith and Read 2008). As bacteria are unicellular they are unable to bridge the gap between air-filled pores in the soil matrix, limiting their motility (Boer et al. 2005). Bacteria and fungi also differ in their metabolic capabilities of decomposing organic substrates of differing

lability, with the competition and co-operation between the two for substrate access and breakdown being fundamental to terrestrial C cycling (Boer et al. 2005).

Plant above- and below ground biomass (litter) is the main source of C for the terrestrial microbiota. It contains a spectrum of C compounds which differ in their size, structure, aromaticity and nature of their chemical bonds, from low molecular weight ‘simple’ compounds (sugars, amino acids) to insoluble lipids ranging from the simple (cellulose) to the complex (e.g lignin) (Cotrufo et al. 2013). The rhizosphere, the soil in the immediate vicinity of the plant roots, has long been recognized as a ‘hot-spot’ of microbial abundance largely due to the sloughing of labile C compounds from the root surface (Curl and Truelove 1986). Bacteria have traditionally been seen as the major inhabitant of the rhizosphere as well as the main degraders of plant-root exudates (Boer et al. 2005). This is thought to be a consequence of their rapid growth rates, which is particularly the case for gram-negative bacteria (Butler et al. 2003). Saprotrophic fungi which utilize labile C compounds coupled with rapid growth rates and high sporulation capabilities have, however, been isolated from the rhizosphere (Bjørnlund et al. 2000). While the relative contribution of bacterial and fungal-mediated decomposition of root exudates is difficult to determine, the presence of these fungi in the rhizosphere must place a resource pressure on bacteria (Boer et al. 2005). Rhizobacteria are known to release anti-fungal inhibitory chemicals as well nutrient sequestering compounds (such as metal chelators) as a means of counteracting fungal activity (Whipps 2001). Fungi also have an array of anti-bacterial mechanisms, such as the manipulation of bacterial gene expression, suggesting a continually evolving ‘arms race’ for utilization of rhizospheric exudate C (Boer et al. 2005; Duffy et al. 2003). Studies have identified co-operation between bacteria and fungi in the breakdown of organically bound sulphur sources in soil (Gahan and Schmalenberger 2015; Martens et al. 1992), thus the implications of such interactions in SOC turnover needs further study.

The bacterial and fungal cellulolytic mechanisms are largely functionally analogous. Saying this, it is believed that fungi decompose the vast majority of cellulose entering soil (Boer et al. 2005; Lynd et al. 2002). This may be explainable by the fact that cellulose is usually entangled in a matrix of hemi-cellulose and lignin (a lingo-cellulose complex), as well as other structural polymers, and is rarely accessible on its own (Robyt 1998). In addition, cellulose has a fibrillar structure which can make access by hydrolytic enzymes difficult. Fungal hyphae can gain access to cellulose fibres by entering pores in the litter cell wall (Boer et al. 2005; Lynd et al. 2002). Fungi of the genus *Basidiomycota* are also capable of degrading the lignin-component of the lingo-cellulose complex and thus gaining access to the cellulose fibres (D'Souza et al. 1999; Lundell et al. 2010). Indeed the decomposition of lignin in the terrestrial environment is almost exclusively undertaken by the *Basidiomycota*, through laccases and peroxidases while bacterial lignin degradation is negligible (Boer et al. 2005; Kirk and Farrell 1987). Bacteria can act synergistically or antagonistically to the fungus during the breakdown of lignin. Bacteria can enhance the fungal accessibility of a substrate through the production of decomposer enzymes (i.e, pectinase). Bacteria can also actively compete with the fungus for the products (sugars and phenolic compounds) of lignin breakdown (Boer et al. 2005).

1.5: Microbial contributions to SOC

The C contained in the soil microbial biomass has been estimated to be equivalent to that contained in above-ground plant biomass with it containing as much as 10 times more N (Whitman et al. 1998). The microbial biomass is a major contributor to SOC, with this contribution increasing with soil depth and age (Rumpel and Kögel-Knabner 2011). While plant residues and exudates represent the primary source of soil C, the microbial transformation of it for incorporation into their biomass results in the NMR spectral

'fingerprint' of SOC having broad similarities with that of microbial biomass (Simpson et al. 2007). The C sequestration potential is thought to be correlated with the bacterial to fungal ratio of the soil, with a greater potential being attributed to fungal dominated communities than bacterially dominated ones. Two prevailing assumptions with regard to fungi is that they have a higher microbial growth efficiency (MGE) than bacteria and also that fungal necromass has a longer residence time in soils compared to bacterial necromass (Six et al. 2004; Six et al. 2006; Strickland and Rousk 2010).

The MGE for a microbe refers to the amount of substrate C that the microbe incorporates into its biomass against the amount of C that it respire as CO₂ (Six et al. 2006). The greater the substrate utilization the slower the turnover of C. Studies have reported lower qCO₂ (metabolic quotient) rates with higher amounts of fungal biomass, inferring a higher fungal MGE (Blagodatskaya and Anderson 1998; Miller and Dick 1995). A review by Six and colleagues (2006), however, questioned the validity of this conclusion based on the nature of the experimental measurement. He argued that fungi may appear to utilize less substrate per unit of measured biomass as many hyphal threads may be inactive at the time of measurement. He further argued that without assessing the amount of substrate metabolically utilised over the time- course of the experiment or measuring the amount of CO₂ respired as a function of new biomass growth you cannot accurately infer MGE. The review concluded that little to no evidence is currently in the literature to support the fact that fungi have a higher MGE than bacteria. A study subsequently reported that there was no difference in MGE for fungal and bacterially-dominated communities using ¹³C-labelled glucose (Thiet et al. 2006). Fungal biomass does, however, have a C: N ratio of about 10 while bacteria have a ratio of about 4. This indicates a greater proportion of C is retained per unit of fungal biomass

compared to that of bacteria (Six et al. 2006). Thus in a fungal-dominated community a greater proportion of C could be sequestered into the microbial biomass.

Fungal cell walls contain a number of complex polymers such as which are not found in bacterial cell walls, such as chitin. The amount and composition of these two components in the cell wall will vary with species and life strategy, i.e, ectomycorrhizal vs. saprotrophic (Fernandez and Koide, 2012). Upon fungal death, chitin and melanin are thought to confer a degree of recalcitrance to the necromass (Fernandez and Koide 2014; Hurst and Wagner 1969; Malik and Haider 1982; Treseder and Allen 2000). It has, however, been reported that chitin is not notably more recalcitrant than other components of the fungal cell wall, with increasing levels of chitin in the fungal necromass being associated with higher rates of decomposition (Fernandez and Koide 2011). This agrees with earlier assumptions that chitin is comparatively labile (Okafor 1966; Trofymow et al. 1983). Melanins contain phenolic and indolic monomers of which fungi biosynthesize four main types; 3,4-dihydroxyphenylalanine, γ -glutaminyl-4-hydroxybenzene, 1,8-dihydroxynaphthalene and catechol melanin. The former two compounds are synthesized in *Basidiomycetes* while the latter two are found in the *Ascomycetes* (Butler and Day 1998). This structural complexity does appear to impart on melanin a resistance to decomposition, requiring oxidative enzymes for their breakdown (Hurst and Wagner 1969; Malik and Haider 1982) with Fernandez and Koide (2014) concluding that fungal species with a high proportion of melanin in their cell walls can make a significant contribution to SOC. This is in comparison with the thin peptidoglycan (a polymer of amino acids and sugars) wall of gram-negative bacteria, though studies have reported that the thicker peptidoglycan wall of gram-positive bacteria may decompose at a rate similar to that of some fungi (Strickland and Rousk 2010).

Bacterial biomolecules, however, must make a significant long term contribution to SOC as NMR spectra for SOC implies this (Kelleher and Simpson 2006; Simpson et al. 2007). Furthermore, the C: N ratio of SOC in the fine particle fraction of soil has been reported in the region of 5-7 which is closer to the ratio found in bacterial biomass (Madigan MT 2006). Structural polymers present in the cell wall appear to be more structurally and chemically suited to protection and stabilisation than cytoplasmic constituents (Sollins et al. 1996). With this observation the 'patchy fragment formation cycle' was developed as a means of explaining the stabilisation of microbial (both bacterial and fungal) cell wall polymers (Miltner et al. 2012). This advanced the idea that when plant-derived substrate becomes limiting, starvation is induced in the microbiota. Individual cells die and fragment with the cytosol spewing into the soil matrix uniformly coating any surface in the immediate vicinity, becoming a food resource. Cell wall fragments, on the other hand, remain either isolated or attached to mineral surfaces becoming a long-term component of SOC. Completing the cycle, this SOC can be used as substrate for the soil microbiota. This hypothesis would explain the fact that only a small proportion (15%) of total fatty acids extracted from soil can be assigned to phospholipid fatty acids (PLFA) which are found only in extant microbial cells (Drenovsky et al. 2004). In addition, it provides a mechanism for relatively labile molecules to be stabilized in SOC. Indeed this has been reported for bacterial cell membrane lipids and amino sugars (Guggenberger et al. 1999; Lichtfouse et al. 1995) as well as microbial peptides (Fan et al. 2004) and polysaccharides (Kögel-Knabner 2002).

The exclusion hypothesis may also provide an explanation for bacterial persistence and thus contribution to SOC. The theory stipulates that microbial predators, such as protozoa and nematodes (diameter in the 5 μm to 15 μm range) would be unable to enter through small neck pores which would be accessible to bacteria (0.2-1.2 μm diameter) (Heijnen and

van Veen 1991). Microbivorous predators contribute to C mineralization as they affect both the structure of the microbial community but also microbial growth rates (Bengtsson et al. 1993; Griffiths, 1994 and Görres et al. 1999). This exclusion leads to reduced microbial growth rates as a greater proportion of the energy derived from substrate can be used for physiological growth and maintenance rather than predator evasion (Lützow et al. 2006). The porosity of an aggregate would represent ‘walls’ and ‘compartments’ which would restrict movement for mesofaunal predators. Additionally, a continuous water-film is needed to connect different aggregates to further aid their movement (Heijnen and van Veen 1991). This hypothesis may not always be suitable in explaining the grazing dynamics in soil as predators may become entrapped alongside microbes in soil pores where the neck diameter is less than that of the predator, the so called ‘enclosure hypothesis’(Bach and Hofmockel 2016). The different sizes of bacteria and fungi would logically imply that bacteria are afforded a greater degree of protection within the pore matrix. Any such difference, however, has not been experimentally determined (Six et al. 2004).

If all SOC compounds in soil are capable of being degraded by the soil microbiota it begs the question why is there any SOC in soil at all, not least SOC which has been radiocarbon dated as being thousands of years old (Allison 2006a)? Why has it not all simply been used up? There are a number of mechanisms at play which limit the soil microbiotas SOC utilization capacity, principally competition, nutrient availability, obstruction of extracellular enzyme stoichiometry via mineral sorption and humic acid hindrance (Allison 2006a).

SOC is a collection of polymeric, complex and randomly arranged molecules which microbes can’t assimilate into their biomass directly (Kögel-Knabner 2002). To overcome this, they release extracellular enzymes (EE) which break up SOC into simpler molecules suitable for microbial incorporation and metabolism (Allison and Jastrow 2006). As the SOC released

from the larger SOC molecule must make its way back to the microbe which initially produced the EE, the opportunity for other microbes to act as ‘cheaters’ arises. As EE are rich in C and N (as a consequence of being composed of amino acids) these opportunistic microbes may utilize them as an energy source. This may lead to localized areas of reduced SOC degradative capability (Allison 2005). The EE foraging strategy is probably linked to the spatial structure of the soil environment. The soil matrix contains a large degree of spatial heterogeneity, with microbes, minerals and organic molecules forming a multi-dimensional matrix of soil aggregates and pores of varying sizes. These spatial constraints will be a major influence on enzyme-substrate stoichiometry and could reduce the opportunistic threat from cheater microbes (Allison 2005).

In addition to cheating microbes, a number of other biological factors may be at play. Soils are abundant in proteases which may reduce EE concentrations and thus SOC utilization capabilities (Renella et al. 2002). Predation on decomposers may also act as a ‘top-down’ control on soil C preservation. Many detritivores are prey for both micro- and mesofauna (i.e, nematodes and collembola) with these predators consuming up to 60% of microbial populations in some soils. Studies have demonstrated the link between predation and decreased C mineralization (Birkhofer et al. 2008; Bouwman et al. 1994), it is though however, that predation plays only a weak or circumstantial role in C conservation with many studies identifying an increase in C cycling in soils (Allison and Jastrow 2006; Mikola and Setälä 1998).

Plant litter which becomes incorporated into soil is rich in plant structural compounds and represents C of a much lower quality than plant rhizodeposits, Furthermore, If litter is shed through senescence then the plant has re-absorbed much of its nutrients (Kögel-Knabner

2002). Plant material which is incorporated into the soil matrix post herbivore faecal shedding may also contain high concentrations of compounds which may resist decay (Liu et al. 2014). Thus nutrient availability is a major determinant in microbial C usage, particularly in relation to N. Low N availability can limit the ability of microbes to degrade SOC when the macromolecules in question are rich in C but poor in N (Allison 2006a). Two hypothesis have been proposed in relation to nutrient acquisition and SOC. The 'stoichiometric' hypothesis, which relates to the faster decomposition of organics with low C: to nutrient (typically N and P) ratios and the 'microbial mining' hypothesis which deals with the reduced depolymerisation of recalcitrant C forms for N and P under optimal nutrient conditions (Milcu et al. 2011).

Typically, the oldest and most abundant constituents of SOC are humic compounds. Humic materials such as humins and humic and fulvic acids are randomly organized into polymeric molecules which are rich in aromatic and aliphatic structures (Schulten and Schnitzer). The subunits of humic materials (lignins and tannins) are energy rich, releasing a similar amount of energy to glucose upon oxidation (Allison 2006a). However, the random chemical structure of many humic substances makes degradation by hydrolytic enzymes (i.e, those using a lock and key mechanism) difficult, as production of specific hydrolytic enzymes for each chemical configuration encountered would be extremely energetically costly. To overcome this, non-specific oxidative enzymes are favoured which depolymerize via a free radical mechanism. They do, however, tend to have low catalytic efficiencies (ten Have and Teunissen 2001). A number of other complicating factors add to the difficulty of SOC depolymerisation. Humic substances can hinder enzymatic access to a molecule through complexation, covalent bonding and steric hindrance (Allison 2006b). As oxidative enzymes are the main enzymatic strategy for SOC breakdown, O₂ limitations will be a major constraint by limiting aerobic respiration and growth (Allsion 2006b). Anaerobic conditions may prevail

in localized microsites in soils, particularly among the smaller aggregate-sized fractions (Wagner et al. 1996).

Soil minerals (e.g. allophane, ferrihydrite) have been shown to adsorb EE to their surface stabilizing them, with the reported effect of both increasing and decreasing enzymatic activity (Allison 2006; Naidja et al. 2000). It is hypothesized, however, that stabilized enzymes, while making up a large fraction of EE present in the soil are unable to interact efficiently with their substrate and possess low catalytic efficiencies (Allison 2006). The combined influences of humic inhibition and mineral stabilization led (Allison 2006b) to propose a multi-pool model in which enzymatic turnover rates in soils vary. Figure 3 (adapted from (Allison 2006)) is a figurative representation of the hypothesized activity dynamic of enzymes in the soil to added C substrate. This sees the EE as inhabiting two pools termed 'active' and 'stabilized'. Enzymes in the active pool contribute the most to the degradation of inputted SOC. Enzymes in the active pool may eventually become stabilized through mineral interaction or inhibited by humic acids and enter the stabilized pool, greatly reducing its catalytic potential. The interplay between the two pools is likely to vary among the different aggregate sized fractions and localized soil microsites. The stability of the enzyme will depend on the proximity of mineral surfaces to their sites of production and as to whether the EE are stabilized in newly forming humic macromolecules or are inactivated through interactions with already existing humic polymers.

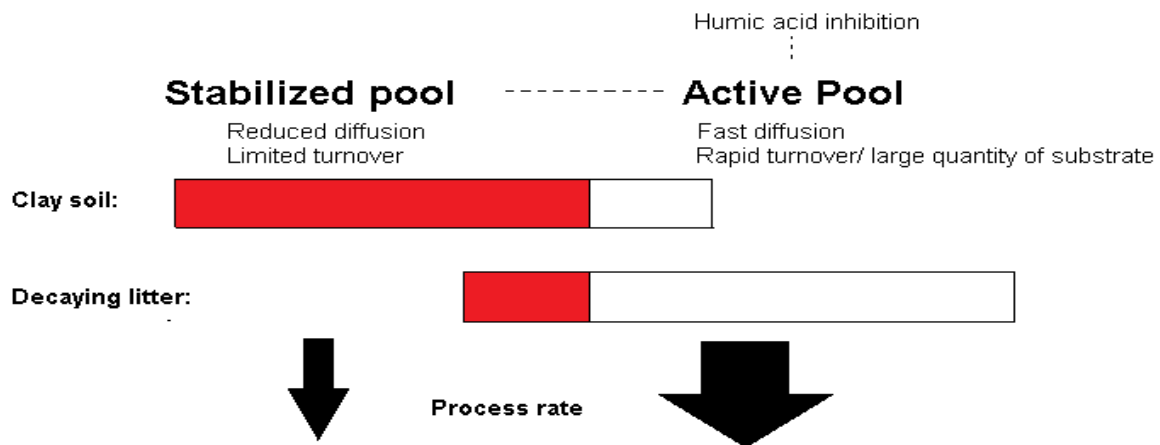


Figure 3: ‘Active’ and ‘stabilized’ pool concept developed by Allison 2006b.

1.6: Response of the soil microbiota to carbon addition

The carbon ‘priming effect’ (PE) is defined as the extra-decomposition of SOC following the addition of easily mineralizable C and N to soil (Bingeman et al. 1953; Blagodatskaya and Kuzyakov 2008; Dalenberg and Jager 1989). The PE may be ‘positive’ where the mineralization of SOC is accelerated upon substrate addition or ‘negative’ where SOC mineralization is hindered upon substrate addition (Kuzyakov et al. 2000). Positive PE have been reported following the addition of numerous C sources such as simple sugars (Kuzyakov and Bol 2006) and amino acids (Hamer and Marschner 2005). A further distinction is made between an ‘apparent’ PE and a ‘real’ PE. An apparent PE is the elevated CO₂ emitted in response to elevated microbial metabolism and biomass turnover. It is not related to SOC decomposition. A real PE on the other hand is where the emitted CO₂ is the result of SOC breakdown by the increased activity (i.e, enzyme production) of the soil microbiota (Blagodatskaya and Kuzyakov 2008).

A greater understanding of the mechanisms, persistence and inducibility of soil PE is crucial to further our understanding of the terrestrial carbon cycle. The functionality and structure of

microbial communities involved in PE has been widely discussed (Chen et al. 2014). It was suggested by De Nobili et al. (2001) that some microorganisms remain in a state of 'metabolic alertness' with them being more capable of reacting to new C additions than cells which have remained in dormancy. Contrastingly, Fontaine et al. (2003) hypothesised that the addition of readily degradable C substrate would select for the rapid growth of microorganisms capable of utilising the added substrate (autochthonous r-strategists, supposedly bacterial species), but were themselves not capable of utilizing the more complex C source which characterise SOC. The zymogenous K-strategists (supposedly slow growing fungal species), on the other hand, will utilize the more recalcitrant organic C. Thus, long-term PE may be explained by a successional increase in the bacterial/fungal ratio (Lundquist et al. 1999). Few studies have examined the role of fungi in PE, however, a reported inducement of PE after the incorporation into the soil of ^{14}C -labelled wheat straw was found to be predominantly fungal- rather than bacterially-mediated (Bell et al. 2003). The addition of the fresh substrate may activate previously dormant fungal spores, with the subsequent growth of fungal-hyphae being capable of reaching soil micro-sites which vegetative cells were incapable of reaching. Available SOC in these micro-sites would quickly be consumed by the fungi inducing a PE (Blagodatskaya and Kuzyakov 2008).

The response of the microbial community, however, is dependent on the type and amount of added C substrate. The addition of trace amounts of glucose may only stimulate the r-strategists, inducing an apparent PE and no change in diversity (De Nobili et al. 2001; Falchini et al. 2003). It is assumed this reflected the versatility of glucose as an organic substrate (it being a monomer of most plant-originating organic polymers) and thus a wide variety of microbial species would have evolved to metabolize it (Blagodatskaya and Kuzyakov 2008). The addition of substrates which have low availability (such as oxalic acid) may select for more specialised microbial species. These species may have remained dormant

in the soil but may adapt and come to dominate the microbial community and co-metabolize SOC inducing real PE (Blagodatskaya and Kuzyakov 2008). Fontaine and colleagues (2007) reported a significant increase in CO₂ production in deep soil layers upon the addition of fresh C substrate. This suggests the development of an oligotrophic microbial community, with high metabolic plasticity under conditions of sustained C limitation. With the addition of fresh C, this stimulates the production of a broad range of extra-cellular enzymes resulting in possible SOC decomposition (Blagodatskaya and Kuzyakov 2008).

The quality of the added carbon substrate will also differentially induce a PE. Kuzyakov and Bol (2006) reported that the priming effect occurred as a sequential mechanism, with the most preferential carbon source available being utilized first. This will activate the r-strategists. If the total amount of added substrate is sufficient, the total soil microbial community will increase (including the K-strategists). When the most easily utilizable substrate has been used up, the microbiota will then preferentially utilise the next most easily degradable substrate until there is no substrate available which is more easily utilized than that found in soil. At this point, the balance of when the differentially utilizable pools of SOC and the microorganisms which are responsible for their mineralization will return to the initial state which will depend on the amount of organic C in the soil and also its utilizability and can range from a few days to a few weeks (Kuzyakov and Bol 2006).

Any correlation between extracellular enzyme activity (EEA) and PE is unclear, yet any stimulation or repression of EEA may be essential to understand the relationship between it and PE intensity and direction (Blagodatskaya and Kuzyakov 2008). Any change in measured EEA has been proposed as either a consequence (where the addition of a substrate inducing PE stimulates EEA) or a cause (where the addition of a substrate acts as an energy source for EEA resulting in SOC decomposition) of PE. Fontaine and Barot (2005) hypothesized that

cellulose and lignin-degrading enzymes were responsible for SOC decomposition with higher levels of cellulolytic (i.e, cellulase) activity during the initial stage of PE with a shift to greater lignolytic activity during the later stage. This mechanism is an extension of r/K strategist theory (Fontaine et al. 2003). Cellulose-degrading enzymes are synthesized by a wide-range of bacterial species while lignin-degrading oxidoreductases are synthesized by fungal species (Carreiro et al. 2000; ten Have and Teunissen 2001). Measured increases in β -glucosidase activity have been linked to elevated rates of microbial respiration upon the addition of C substrate to soil (Bol et al. 2003a; Mondini et al. 2006). This could suggest enhanced microbially-mediated breakdown of an extra-cellular substrate (i.e, SOC) or metabolism of endocellular energy reserves. Thus, β -glucosidase may be a good indicator of PE though it does not allow the differentiation of the apparent and real responses of the effect (Blagodatskaya and Kuzyakov 2008; Mondini et al. 2006).

The C:N ratio of the incorporated substrate is an important consideration in the priming of soil carbon. Microbial decomposers incorporate C and N into their biomass in a relatively stoichiometric manner. As a consequence these nutrients are typically mobilised together (Cleveland and Liptzin 2007). The C:N ratio, along with lignin content, has been traditionally used in the literature as a measure of residue ‘quality’, the ease at which the residue is decomposable (Fogel and Cromack Jr 1977; Taylor et al. 1989; Waksman 1924). Two theories exist in the literature to explain the role of C:N ratio in the priming effect, each assuming the opposite effect of N on SOC stability (Chen et al. 2014). The ‘stoichiometric decomposition theory’ states that microbial activity and decomposition rates will be highest when the C to N ratio matches the microbial demand (Chen et al. 2014; Janssen 1996; Knorr et al. 2005). If the assimilated residue has a high C:N ratio, the amount of N present may be insufficient for the micro-biotas metabolic requirements. In response, the ‘nitrogen-mining hypothesis’ the micro-biota will use the labile C as an energy source to decompose the

surrounding SOC to mineralizing organically bound nitrogen. This will increase the amount of organic N available, eventually meeting the micro-biotas requirement for N (Janssen 1996; Knorr et al. 2005). This N is not available for plant uptake until the microbial cells themselves are sloughed and decomposed.

While the two theories appear conflicting, they may co-exist in a system depending on the microbial availability of both C and N in different spatial and temporal scales (Chen et al. 2014). The intensity of PE is therefore determined by factors affecting the competitive ability of fast growing micro-organisms, namely the presence of C-rich substrate and N availability (Chen et al. 2014). The presence of available C and higher N availability will stimulate the growth of r-strategists, with the simultaneous availability of C and N promoting their mineralisation of organically-bound C (Blagodatskaya et al. 2007; Chen et al. 2014). On the other hand, added substrates with a low C:N ratio will favour the growth of the K-strategists, as they would be capable of mineralizing organic-N (Chen et al. 2014; Fontaine et al. 2011). The SOC pool in the past has been viewed as a homogenised C pool, whose C:N ratio regulates decomposition and soil N mineralisation and immobilisation (PiÑEiro et al. 2006). This is a simplified view-point, as SOC is composed of several C fractions of varying chemical complexity, composition and lability, with N availability differing among these fractions (Nguyen et al. 2004). An ‘active N pool’ has been proposed which is associated with the labile C fraction and a ‘slow N pool’ associated with the more recalcitrant C fraction (Wang et al. 2004).

1.7: Physical C protection mechanisms in soil

Plant-derived carbon compounds in soil are often characterised by their perceived ease of breakdown by the microbiota. Monomeric sugars (i.e, glucose) are termed ‘labile’ because they are easily degraded by microbes. On the other hand, lipids and complex polymers such

as lignin may take decades for microbes to breakdown and are thus termed 'recalcitrant' (Lützow et al. 2006; Sollins et al. 1996). This recalcitrance may be an intrinsic property of the molecule itself or may be acquired through polycondensation and complexation during decomposition (Gleixner et al. 2002; Six et al. 2002). Some of the noted biochemical features of molecules enhancing recalcitrance are molecular size, polarity, degree of aromaticity, its stereochemical complexity, the alkyl: O-alkyl ratio (aliphaticity) as well as amino functional groups (Bosatta and Ågren 1999; von Lützow et al. 2006). The concept of molecular recalcitrance, however, has been questioned and criticised as a vague concept (Kleber 2010). Kleber (2010) stated it more logical to consider an 'inherent recalcitrance' not on the basis of biochemical characteristics, but rather as a combined function of biological and soil physiochemical characteristics inherent to the soil. In a direct response to Kleber, (von Lützow and Kögel-Knabner 2010) challenged the rejection of recalcitrance as a biochemical property. They listed numerous studies where various molecular characteristics have been found to infer varying degrees of recalcitrance (Derenne and Largeau 2001; Kalbitz et al. 2003; Melillo et al. 1989).

The reported increase in the amount of aromatic and double-bonded hydrocarbons increase down the soil profile and the converse decrease in alkyl C hydrocarbons suggests that chemical recalcitrance plays some role in SOC preservation (Dungait et al. 2012). It appears to be the case, however, that if soil microorganisms have physical access to a substrate they can degrade it moderately quickly, with it is now recognised that physical protection mechanisms play a major role in protecting SOC against microbial attack regardless of its inherent chemical structure, with complex C components of litter not being preferentially stabilized in SOC (Dungait et al. 2012; Kleber 2010; Marschner et al. 2008; Spence et al. 2011). It has been postulated that microbial substrate use efficiency, defined as the proportion of substrate which is incorporated into the microbial biomass vs. the amount which is used

for respiration acts as a ‘filter’ controlling the integration of C and N into SOC (Cotrufo et al. 2013). In this reasoning, labile C compounds will be decomposed at a faster rate and will be more efficiently incorporated into the microbial biomass and products (e.g, proteins, extra-cellular polymers). Contrastingly lower quality C substrates will be degraded at a slower rate and more energy will be expended in breaking them down. The microbial demand for N will influence litter decomposition dynamics, as the amount of N in microbial biomass can be an order of magnitude higher than that present in the plant litter. This will particularly come into play when a high amount of N is present with C of a high quality, with the labile litter decomposing faster than low-N and low-C litter but due to microbial substrate use efficiency (MSUE) a higher proportion of the residue will incorporate into SOC over the long term.

Yet if all organic molecules can be used as substrate for microbial physiological purposes what are the mechanisms leading to SOC preservation in the soil? The identified mechanisms are principally (i) protection within aggregates and soil pores (ii) organo-mineral complex formation and (iii) Substrate-driven regulation of microbial activity (Dungait et al. 2012).

Occlusion within aggregates is widely seen as a mechanism of SOC protection. Freshly incorporated C has not yet associated with any polyvalent minerals and is seen as easily decomposable (Six et al. 2002). There is a large amount of evidence in the literature which has shown increased C mineralization when the aggregate structure is disrupted (Reicosky et al. 1997; Six et al. 2000; Six et al. 2006). Incorporation into an aggregate structure changes the composition of C, with alkyl C predominating over O-alkyl C. This suggests a selective preservation of the more easily degraded C (Six et al. 2002). The aryl-C/O-alkyl-C ratio is considered an indicator of the stage of organic matter decomposition in agricultural soils (Helfrich et al. 2006). The diffusivity of O₂ through the intra-aggregate matrix is limited thus

the potential for aerobic decomposition is reduced. The pore-size distribution (which characterises the soils 'pore space', the portion of the soil's volume which is not occupied or isolated by solid material) within the soil matrix as developed by Elliot and Coleman (1988) is also influential in occlusion of SOC (Lützow et al. 2006). Micro-aggregates are abundant in pores $<0.2 \mu\text{m}$ in diameter, which is considered the smallest habitable pore space for bacteria, thus SOC trapped within these pores are protected from microbial attack (Lützow et al. 2006). Estimates have shown that 15% of the total soil space is inaccessible to microbes in a sandy soil with this increasing to 52% for a clayey soil to 95% for a silt loam (Chenu and Stotzky 2002; Van Veen and Kuikman 1990). This does not exclude the possibility that exoenzymes can enter these pores or that SOC trapped within these pores do not diffuse out into more microbial-accessible parts of the soil matrix. It is not known, however, whether and at what rate these processes may occur (Lützow et al. 2006).

Protection within an organo-mineral complex is a more intricate mechanism than occlusion within the aggregate structure and is highly influenced by both the quantity and type of silt and clay present within the soil matrix. Numerous mechanisms may play a role in adsorption to mineral surfaces, ranging from weak interactions such as van der Waals forces and H-bonding to stronger interactions such as polyvalent cation bridges and ligand exchange (Lützow et al. 2006). In acidic soils, sorption of SOC onto phyllosilicates clays is aided by Al^+ , Fe^+ and Mn^+ oxides interacting through their large, charged surface. In more alkaline soils, polyvalent cations (e.g, Ca^{2+} in calcareous soils) form 'bridges' between phyllosilicates both of which are negatively charged (Cotrufo et al. 2013; Dungait et al. 2012). A conceptual model was developed in an attempt to explain organo-mineral interactions in soils by Kleber et al. (2007). They hypothesize that the amphiphilicity of many of these molecular fragments coupled to the polarity of the aqueous soil solution leads to SOC self-assembling into layered

structures when in contact with mineral surfaces. These self-assemblages are thought to contain three distinct ‘zones’, the contact zone, the hydrophobic zone and the kinetic zone (Kleber et al. 2007).

The contact zone is the point of connection between the SOC molecule and the mineral surface, the structural properties of which will depend on the pH of the soil solution, the type and density of the mineral as well as the chemical functionality of the presenting SOC molecule. The SOC molecular fragment which is directly attached to the mineral are protected from microbial attack, thus this portion of SOC may be protected for centuries or millennia. They also do not participate in any chemical interactions with the organic molecules in the soil solution. This is thought to be as a consequence of the ligand exchange bonding between amphiphilic molecules to the hydroxylated surface of the mineral it is attaching to. An amphiphilic molecule which is sorbed to a mineral surface has the propensity to shield the physiochemical properties of that surface, creating a ‘hydrophobic zone’ which is orientated outward. This is particularly the case when the surface of the mineral has a high density of reactive hydroxyls. This zone provides a suitable space for the sorption of hydrophobic moieties of amphiphilic molecules which will be present in the soil solution thus forming a ‘bi-layer’ on the surface of the mineral. This hydrophobicity would further reduce decomposition rates as the absence of water would impede microbial mobility and growth (Goebel et al. 2004; Jandl et al. 2004). The hydrophobic zone may not form in all cases (i.e, protein adsorption) thus is not an inevitable consequence of all SOC-mineral interactions. The outer most zone, the kinetic zone, is where further attachments of molecules from the soil solution may occur. The extent of this attachment is dependent on a number of external factors, such as pH and temperature and chemical factors such as stereochemical orientation and the degree of amphiphilicity. The organic matter in the kinetic zone is more portioned

than absorbed, thus is not as protected as the inner zones and will have a shorter residence time.

1.8: Spatial constraints on microbe-SOC interactions

The physical nature of soil can present physical constraints on soil microbial colonization and access to SOC for their metabolic requirements (Figure 4). For SOC decomposition to occur H₂O, O₂, C substrate and microbe must co-occur in relatively close proximity. Soil structure and aggregation will also regulate the accessibility of C to microbial decomposers. These spatial constraints will influence the diffusivity of the exo-enzymes and organisms in the soil matrix, thus protecting SOC in ‘biologically non-preferred soil spaces’ (Dungait et al. 2012; Ekschmitt et al. 2008). The ‘random-walk’ theory (Van Haastert and Bosgraaf 2009) states that under starvation conditions microbes/exo-enzymes will traverse the matrix randomly in search of SOC particles. The probability that a microbe/ exo-enzyme comes into contact with a SOC particle will be dependent on physio-chemical as well as biological variables. The length and tortuosity of the random route taken by the microbes will be dictated by the soil pore size as well as the energy and mobility capabilities of the organism. In the case of exo-enzymes SOC breakdown will depend on the activity of the enzyme, chemical steric hinderence as well as wheter there is a need for synergy with other enzymes (Dungait et al. 2012).

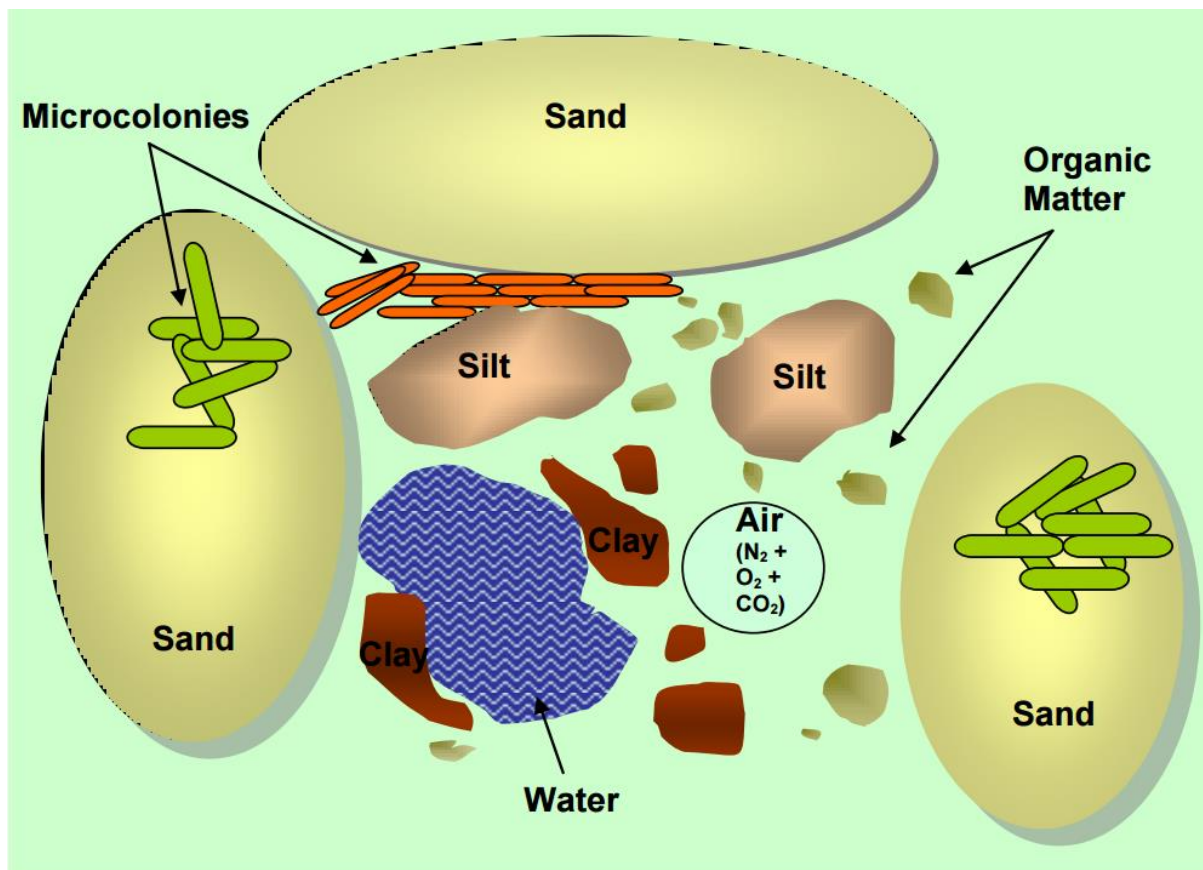


Figure 4: The physical nature of the soil microbial habitat. Physical constraints on colonization and access to SOC for metabolic requirements are challenges for the soil microbiome (Image taken from Madigan et al. (2009)).

The ‘microbial-efficiency matrix stabilization’ (MEMS) hypothesis was put forward by Cotrufo and colleagues (2013) as a mechanism of stable SOC formation. This postulation synergizes the ‘microbial filter’ mechanism previously mentioned with SOC physio-chemical stabilization. This MEMS framework depicts the decomposition, transformation and stabilization of SOC as a continuum. In it, labile C compounds are the major source of microbial products due to MSUE. These microbial products are the main originators of stable SOC via the promotion of soil aggregation and strong chemical bonding in the soil matrix.

The decomposition dynamic of SOC differ in the sub-soil horizons than the surface horizons (Sanauallah et al. 2011). In comparison to the surface horizons, sub-soils are typically colder,

anoxic environments which may be waterlogged for considerable periods of the year resulting in smaller, less active microbial communities (Ekelund et al. 2001; Ekschmitt et al. 2008; Fang and Moncrieff 2005). The abundance of soil fungi seems particularly affected by soil depth, with bacteria apparently dominating the microbial community below 50 cm depth (Ekschmitt et al. 2008; Taylor et al. 2002). With increasing depth, the substrate available for soil bacteria will consist of highly processed older SOC. This may be energetically expensive to metabolise (Kramer and Gleixner 2008). Indeed, it has even been suggested that microbial community activity is so low in sub-soils that even labile substrate may remain non-degraded for significant periods of time, as numerous studies have demonstrated that the sub-soil is rich in labile C compounds (Krull and Skjemstad 2003; Lal et al. 2011; Liang and Balsler 2008; Xiang et al. 2008). Studies have, however, shown that while the total microbial biomass declines with depth, sub-surface soils still possess a substantial microbial community which (when normalised to biomass size) is as metabolically active as the top-soil (Ajwa et al. 1998; Blume et al. 2002; Charnay et al. 2005; Taylor et al. 2002). While it represents a nutritionally impoverished environment, the sub-soil is not subjected to great variations in temperature and moisture and thus may also provide 'niche' habitats which the micro-biota can exploit (Sanaullah et al. 2011). The obstacles in the soil matrix which prevent the contact between substrate and the microbiota will be more pronounced in the sub-soil due to a greater bulk density (Dungait et al. 2012).

1.9: Impact of agriculture on SOC

Agricultural land management techniques can have a considerable influence on the SOC levels and CO₂ emissions in both the long and short term. The conditions of agricultural soil particularly the temperature, texture, moisture are of central importance to the cycling of SOC. Since 62% of Ireland's land surface is devoted to agricultural production, of which 3.4

million ha are in grassland and 0.4 million ha are in tillage, it is important to understand the effect that different land practices (such as ploughing) may have on SOC levels in Irish soils. In agro-ecosystems, reducing CO₂ emissions usually implies sequestering C into SOC. In Europe, grasslands are recognised as a CO₂ sink (Freibauer et al. 2004; Soussana and Lüscher 2007).

The difference between photosynthetically-fixed C entering the soil as exudate or residue form and C which is emitted from the soil as CO₂ following decomposition is small (Paustian et al. 2000). This variance, however, determines whether the soil in question will be a net CO₂ sink (sequestering C) or net source (emitting C). Europe's agricultural soils do not represent a continuous carbon sink, eventually agro-ecosystems will reach equilibrium and no further sequestration will occur (Smith 2004). The time-scale to reach this equilibrium after a change in land-use is highly variable, though it is estimated that it can take up to 100 years in temperate soils (Freibauer et al. 2004). Ireland (with the exception of New Zealand) is unique among developed countries in that the agricultural sector contributes very sizable contribution to total GHG emissions (Teagasc, 2011). This is largely due to the importance of the agricultural sector to the Irish economy as well as the fact that ruminant production (through both beef and dairy) is the main activity of the sector (O'Mara 2008). Agricultural practices contribute directly 29.1% to Ireland's total GHG emissions (Teagasc, 2011).

The benefits of carbon in agricultural soils are outlined in Figure 5. From an agronomic viewpoint, maintenance of an adequate level of SOC may also be required for consistent agricultural yields (Loveland and Webb 2003; Spink et al. 2010). A SOC level of 2% has been suggested as the base-line figure under which soil structural stability may be compromised (Kemper and Koch 1966). The notion of a relationship between SOC levels and

soil stability is, however, contentious and no consensus exists on the nature (if any) of the association (Spink et al. 2010). A comprehensive review of the literature by Spink et al. (2010) revealed that studies in the past have failed to observe a ‘critical limit’ between the two parameters, with other studies suggesting that there is no correlatory link. A review by Loveland and Webb (2003) did, however, cautiously conclude that if SOC decreases to ca.1% crop yield potential may be lowered.

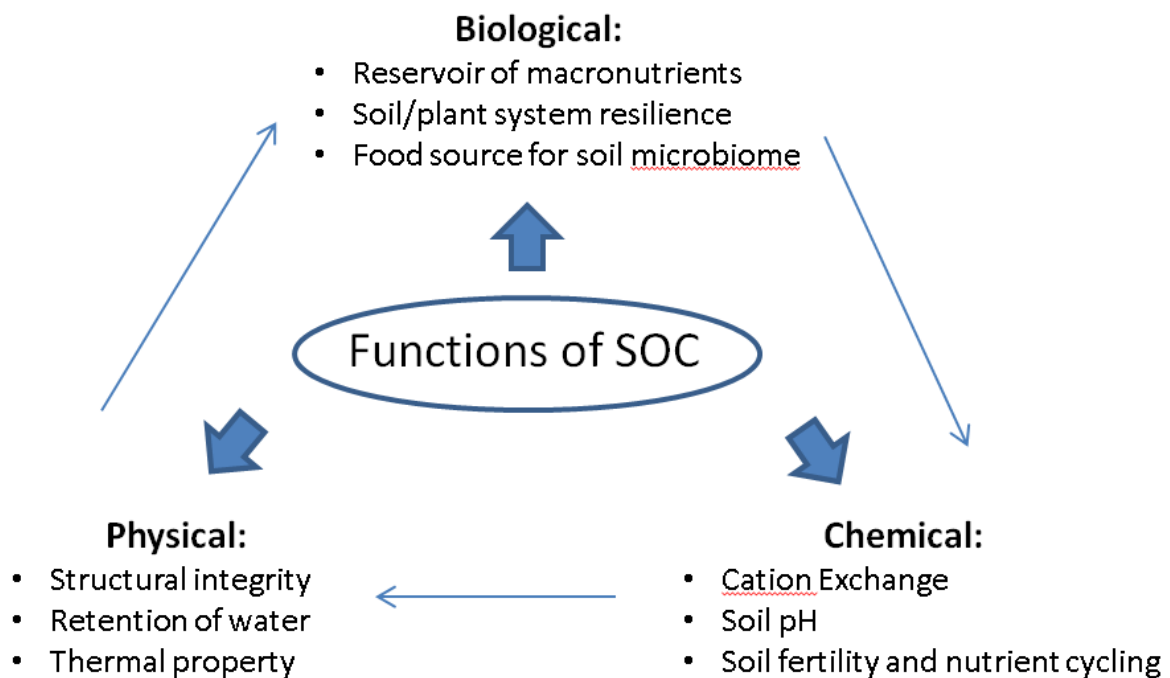


Figure 5: Overview of how soil organic carbon affects biological, chemical and physical parameters of agricultural soils.

1.9.1: Livestock:

Intensive grazing of livestock on grasslands can result in a large expulsion of urine. Up to 2 litres of urine can be excreted by a dairy cow in each urination event onto an area of 0.2 m².

This can occur up to 12 times a day, with a urine patch often being visible as darker green, vigorously growing pasture (Dennis et al. 2011). Urine depositions have been shown to alter the soil microbial community (Rooney et al. 2006; Williams et al. 2000) and increase microbial activity (Lovell and Jarvis 1996; Nunan et al. 2006; Rooney et al. 2006; Williams et al. 2000). Any increase in microbial activity could have important consequences for C concentrations in soil, with studies identifying declines in soil C under intensively grazed pastures (Schipper et al. 2007). A priming effect of soil C occurring upon urine deposition has been reported, leading to a rapid increase in soil CO₂ flux, indicating microbial mineralisation of urine C and possibly SOC (Kool et al. 2006; Lambie et al. 2013; Lovell and Jarvis 1996; Uchida et al. 2011). While these studies reported relatively modest losses of soil C, urine is deposited many times in an intensively grazed pasture and over time this can lead to a substantial decline of soil C (Moir et al. 2011).

1.9.2: Tillage:

The conversion of grassland soils to arable soils has a marked effect on soil SOC levels (Six et al. 2000). Tillage is the preparation of soil by mechanical agitation, i.e, ploughing. The depth, intensity and frequency of this agitation determines the effect of tillage on SOC (Paustian et al. 2000). The conventional method of tillage (such as the use of the mouldboard plough) involves upturning the soil to a depth of 20 cm or more, followed by a secondary agitation to breakup and homogenise the soil layer (Paustian et al. 2000; Spink et al. 2010). This method has a detrimental effect on soil aggregate structure causing a reduction in macroaggregates, which are C rich, and an increase in microaggregates which are comparatively C-depleted (Six et al. 2000). These aggregates contain large pores between the smaller, dense aggregates which encompass it. As a consequence, larger aggregates are

weaker and have a lower tensile strength. The pores act like planes of weakness when the aggregate is stressed (Elliott 1986). Oades and Waters (1991) explained the loss of SOC reported upon cultivation using the aggregate hierarchy theory. He explained that macroaggregates contain less processed and therefore more labile C than microaggregates. When the macroaggregates are disturbed, therefore, their constituent O-alkyl C is preferentially degraded by the soil microbiota.

Conservational tillage methods have been employed to help maintain soil structure and consequently SOC. Common methods include minimum tillage and no-till methods. Additionally, the straw from the previous harvest may be incorporated back into the soil (Spink et al. 2010). The benefits of conservation tillage have been most pronounced in arid and semi-arid regions such as the great plain of North America and Australia where soil moisture retention is an important consideration. Minimum-tillage is a non-inversion method of tillage with depths of 10-15 cm while no-till incurs drilling the seed directly into the stubble of the previous crop.

No-till enhances C sequestration in tillage areas through the promotion of macroaggregate formation (Six et al. 2000; Hutchinson et al. 2007). Six and colleagues (2000) developed a rationale to explain this, developing on earlier explanations (Oades 1984; Paustian et al. 1997). Incorporated C induces the formation of macroaggregates as described by Tisdall and Oades (1982). As the stability (and thus residence time) of macroaggregates increase, this coarse iPOM is fragmented and degraded into fine iPOM (Guggenberger et al. 1994). Fine iPOM act as nucleation sites for microaggregate formation within macroaggregates. This C is critical to C sequestration potential as microaggregates have a slower turnover time than macroaggregates (Jastrow 1996). With CT soils, the rate of macroaggregate turnover is increased, thus fewer microaggregates are formed compared with NT.

Studies suggest converting to zero-till and minimum tillage cultivation practices will result in a fungal-dominated microbial community (Frey et al. 1999; Sipilä et al. 2012). This is of a consequence of the different growth forms of bacteria and fungi. Fungi exhibit hyphal growth (long threads) while bacteria are individual cells. Hyphal threads are advantageous to the fungus as it allows the translocation of nutrients from a microsite where the nutrient is abundant to a site where the nutrient is limiting along the hyphal network (Hendrix et al. 1986; Smith and Read 2008). Conventional tillage would disturb these hyphal threads, even resulting in direct tissue damage while bacteria would be less disturbed (Strickland and Rousk 2010). As previously mentioned, conventional tillage homogenises the soil and its nutrients which negates the advantages associated with hyphal growth. Bacteria are also assumed to be more efficient utilisers of the carbon substrates made available post-till. The physiological differences between bacterial and fungal dominated communities have been previously mentioned in this review, though the most feasible mechanism of enhanced C sequestration as a consequence of fungal dominance would be in their role in macroaggregate stabilization (Six et al. 2006; Strickland 2009).

In a review of the literature, Baker et al. (2007) concluded that with the current evidence it cannot be concluded that adaption of conservation tillage practices will necessarily lead to increased SOC-C. They also pointed out that essentially all studies showing an increase in SOC with conservation tillage have only sampled to a depth of 30 cm (with many studies sampling considerably less). Contrastingly, studies which sampled below 30 cm reported no such increase in SOC. The review concluded that conservation tillage may differentially distribute SOC compared to conventional tillage as a consequence of alterations in thermal and physical conditions affecting the growth and distribution of plant roots (and thus plant root exudates). Furthermore, they suggested that factors other than tillage to be at work

resulting in the loss of SOC from the North American plains, principally the use of annual crops and land drainage (Baker et al. 2007).

The predominance of annual crops in modern agriculture is in contrast to the native perennial grasses prevalent in virgin grasslands. Between cropping periods, the land is left bare (fallow) with no input of plant root exudate. As previously stated, fresh SOC is critical in aggregate formation (Six et al. 2000). Leaving land fallow increases the rate of microbial SOC deposition with rates being 2 to 2.5 times faster than in a period of crop cultivation (Feng and Li 2001). Utilising a greater cropping intensity (i.e, reducing the frequency of bare fallow periods) will not also increase crop production but will increase the SOC input to soil (Peterson et al. 1998). Tillage with rotation of a cover crop such as perennial grasses or legumes can result in increased SOC fraction in cultivated soil due to the large increase in C from rhizodeposits and other plant residues. Rotation with a legume forage (such as clover) would also reduce the requirement of inorganic N fertilisers (Hutchinson et al. 2007). A 2% increase in SOC was reported over a 6 year period of over-winter cropping of *Lolium multiflorum* in the south-western part of Sweden (Blomblack et al. 2003). Plants which have been investigated in Ireland for their potential use as over-wintering cover crops include forage rape, rye oats, rye grass, mustard and a rye/pea mixture, though primarily to negate nitrate leaching rather than for SOC stability.

1.9.3: Slurry application:

Grass-fed livestock for beef and /or milk production is the main agricultural activity in Ireland (Harris et al. 2011; O'Mara, 2008). Animals are typically kept out on pasture from the months of March to October and are then over-wintered indoors. This is typically to protect the soil from trampling and compaction during the wetter winter months. This over-wintering produces a large quantity of manure (Harris et al. 2011). In 2003, the Irish national cattle herd

produced 37 million tonnes of manure, 29.3 million tonnes of which was liquid slurry, while the remainder was farm yard manure (FYM). Slurry is a heterogeneous mixture of animal feces, urine, small amounts of bedding and washings/rainwater and is typically stored in storage tanks on farms. The recycling of this slurry back onto the land is an imperative practice for the sustainability of the sector (Holden et al. 2004).

In Ireland, the ‘splash plate’ (Figure 6a) method of liquid slurry spreading is common practice where slurry is pressurized against a plate and spread uniformly on the land. This method however has been shown to lead to enhanced N₂O emissions, especially during the summer months (Dowling, Curran, Lanigan, 2009; Lalor and Schulte, 2008). The process appears to be as a consequence of a stimulation of microbial denitrification and nitrification processes shortly after the application of slurry due to the readily available labile C as well as high amounts of NH₄⁺-N (Morvan et al. 1996; Clemens and Huschka, 2001). A change in the application method to ‘injection’ spreading (Figure 6b) (i.e, trailing shoe, which applies slurry along 5cm bands on the ground and leads to a 79% reduction in the land covered by slurry compared to the splash-plate method) and optimising time of application (i.e, cooler spring days) are both recognised as effective methods of reducing N₂O and CO₂ emissions as lower temperatures would equate to lower metabolic activities of the soil micro-biota (Dowling, Curran, Lanigan, 2009; Lalor and Schulte, 2008).

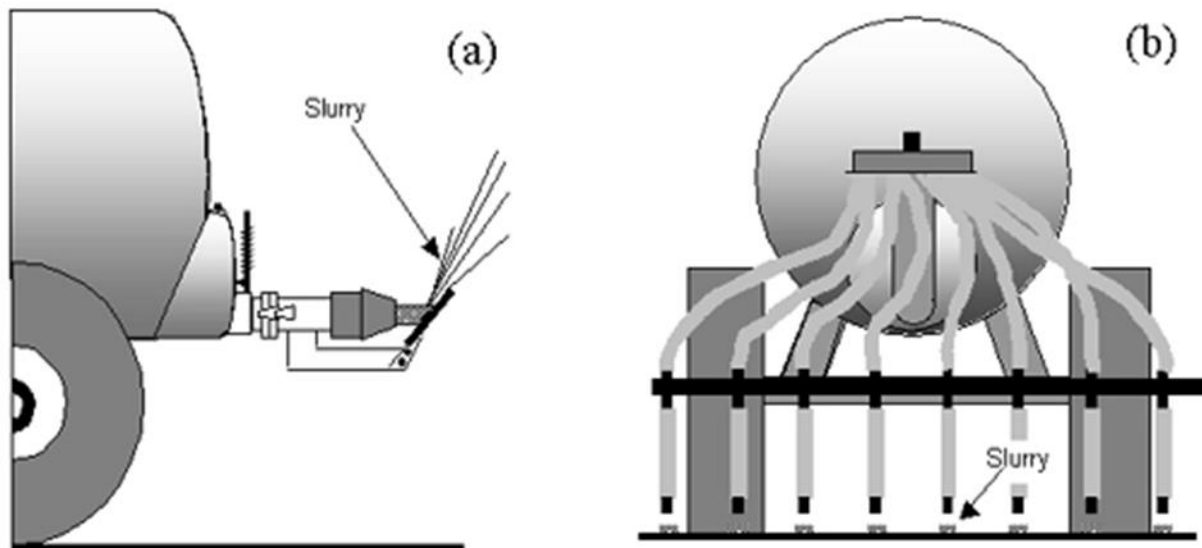


Figure 6: (a) Splash-plate slurry spreading (b) injection slurry application (adapted from Ryan, 2005)

Sequestration and loss of slurry derived C has been addressed in numerous studies in both laboratory (e.g., Saviozzi et al. 1997; Glaser et al. 2001; Bol et al. 2003a, 2003b, 2004; Kuzyakov and Bol, 2004, 2005, 2006; Fanguero et al. 2007) and field conditions (Rochette et al. 2000, 2004, 2006; Chantigny et al. 2001; Sauheitl et al. 2005; Jones et al. 2006). Slurry application has been shown to increase the soil microbial biomass through the supply of C-rich organic compounds as well as the addition of N, P, S and K (Saviozzi et al. 1997; Rochette et al. 2000; Bol et al. 2003a). Furthermore, this C addition may select for specific microbial taxa which utilise it as a nutritional substrate, altering the overall microbial community structure and functionality. The consequence of increased soil microbial biomass is an increase in respiration rate and soil CO₂ efflux (Flessa and Besse, 2000; Rochette et al. 2000, 2004, 2006; Chantigny et al. 2001; Bol et al. 2003b, Jones et al. 2005; 2006). The rate of CO₂ efflux peaks a few hours after application when slurry-derived C substrates, such as volatile fatty acids, are being metabolised by the soil microbiota (Chantigney et al. 2001; Kuzyakov and Bol, 2004). Further, Rochette et al. (2000) did show that the peak soil

microbial biomass C was correlated with the peak rate of respiration, which suggests that the micro-biota are a significant contributor to the CO₂ efflux. In addition, Sauheitl et al. (2005) reported significant microbial utilization of slurry-C within the first few days of application.

Bourdin (2012) suggested a ‘three-phase’ model of the decomposition of slurry-derived C, building on previous models (i.e. Rochette et al. 2000). The first phase occurs within the first few hours after slurry application, where dissolved CO₂ bicarbonates and carbonates are released to the atmosphere. The more liquid phase of the slurry percolates rapidly into the upper soil profile. This provides easily degradable molecules to the soil micro-biota, with supposed preferential utilisation by the r-strategists. These molecules are rapidly utilised and degraded. Over the next few days, simple C molecules, (i.e. sugars, amino acids and fatty acids) are released from the solid phase of slurry and gradually integrated into the soil. This second phase is characterised by the less mobile, particulate C fraction which is slowly integrated into the soil profile from the slurry solid phase. It has been suggested by some authors (e.g. Sauheitl et al. 2005) that this phase-dependant substrate utilization is due to the differing diffusional characteristics of the varying C fractions in slurry. Under this reasoning, smaller less complex particles will diffuse into the upper soil profile first, as the larger more complex particles will have to become smaller by decay at the soil surface before they will diffuse. After this slurry derived labile-C source has been fully exhausted, the soil micro-biota (presumably K-strategists) will shift from utilizing recalcitrant SOM to utilizing the more available organic compounds derived from the slurry.

1.10: Methods for studying soil microbial community structure:

Cultivation independent nucleic acid based methods for studying soil microbial community composition requires a robust and accurate (i.e, non-biased) DNA extraction technique.

Obtaining an adequate DNA yield is important as this will provide the most adequate description of the soil microbial community present (Whitman et al. 2016). Furthermore, the purity of the obtained DNA should be of a sufficient quality to facilitate its use in downstream molecular investigations (i.e, the removal of humic inhibitors; Peacock et al. 2011).

Community fingerprinting techniques can be used in the study of the diversity of the soil bacterial and fungal community. Such techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP) and Automated Intergenic Spacer analysis (ARISA). These methods involve the separation of targeted PCR-amplified DNA fragments based on a difference in length or base pair composition. In the fingerprinting method used in this thesis (T-RFLP; over-view provided in Figure 7) community marker genes (such as the bacterial 16S rRNA gene) are PCR-amplified with primers which have been fluorescently labelled. These PCR amplicons are then subjected to restriction digestion, where the enzymes employed will cleave the DNA strand at specific base pair sequences. Capillary electrophoresis on the restricted fragments can then assign the presence/ absence of a fragment size between samples. Rather than give specific identifications on microbial groups, fingerprinting methods provide an overview of soil microbial community composition. Such techniques are still invaluable in the study of microbial ecology as they allow for the rapid and inexpensive study of microbial community structure.

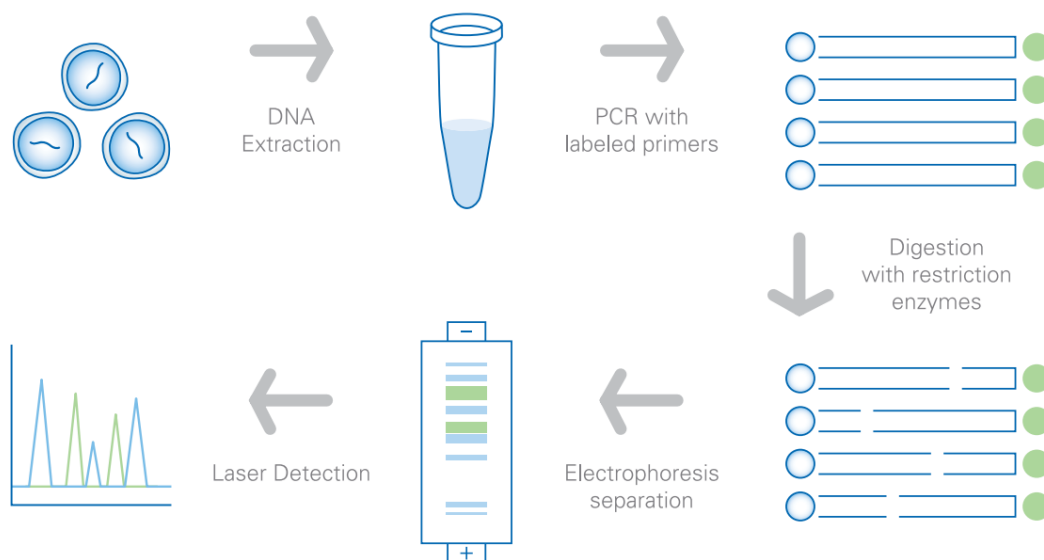


Figure 7: Overview of steps required for T-RFLP assay. Image courtesy of Applied Biosystems application note on T-RFLP 3130/ 3730

Next generation sequencing (NGS) is increasingly used in studies of microbial ecology. Illumina NGS, which was employed in this thesis, generates phylogenetic datasets of greater sequencing depth than older methods such as Sanger sequencing (Bol et al. 2003b). Amplified sequences obtained with this method are sufficiently long to provide robust phylogenetic information, specifically the presence/absence as well as the % relative abundance of microbial group. NGS will also provide details on lesser abundant groups thus giving a detailed picture of the composition and structure of the community under study than a fingerprinting technique (Fangueiro et al. 2007).

1.11: Methods in enzymatic analysis:

The two most commonly employed methods used in enzymatic assays are (i) the colorimetric method using p-nitrophenol-linked substrates or L-3,4-dihydroxyphenylalanine (L-DOPA) and (ii) the fluorometric method employing 4-methylumbelliferone (MUF) or 7-amino-4-

methylcoumarin (AMC) linked substrate (Burns et al. 2013). The adoption of microplates (which are usually 96 wells) over the last 15 years have meant that the fluorometric method has become more common as they can facilitate a more rapid measurement of a larger number of samples. It is also considered to be more sensitive than the colorimetric method (Burns et al. 2013).

1.12: Aggregate isolation through wet-sieving:

The concept of physical fractionation for aggregate isolation was developed from the conceptual model of aggregate hierarchy developed by Tisdall and Oades (1982). A wet sieving method developed by Six et al. (1998) (which itself was developed by Cambardella and Elliot, 1993) to evaluate the proportion of four aggregate sizes; the large macroaggregate (>2000 μm), macroaggregate (>250 μm), microaggregate (<250 μm) and silt and clay (53 μm). The procedure essentially involves the sequential slaking of soil through a series of sieves to obtain these four aggregate sizes. The re-wetting of the dried soil destabilizes the soil structure due to water rapidly entering the air-filled aggregate pore space.

Soil aggregate isolation by wet-sieving has been extensively employed to differentiate soil structure and consequent changes in carbon cycling potentials in soil. The method has also been widely used by soil microbial ecologists investigating differences in microbial community structure and functionality at the aggregate scale via extracellular enzymes (Allison 2006; Bach and Hofmockel 2014; Fansler et al. 2005) PCR fingerprinting (Mummey and Stahl 2004; Sessitsch et al. 2001), Phospholipid fatty acid analysis (Kong et al. 2011) and NGS (Davinic et al. 2012).

1.13: Motivation:

As grasslands comprise 4 million hectares of land area in Ireland (90% of the total area used for agricultural production), sequestration of SOC in grassland soils is seen as a promising strategy for offsetting the wider agricultural C footprint (O'Mara 2008). Understanding the influence of soil carbon on the most important biological parameter in these systems (the microbiome) is crucial if they are to be managed to optimise C sequestration.

1.14: Hypothesis:

Soil carbon, either through its physical protection (soil aggregates) or addition through agricultural management (slurry addition) will influence the diversity, relative abundance and activity of the soil microbiome.

1.15: Objectives:

- (i) To ascertain the influence of soil aggregates on the composition of bacterial and fungal communities through the varying horizons down the soil profile in grasslands (Chapter II).
- (ii) To gain further insight into how the relative abundance of specific bacterial taxonomic groups are affected by soil aggregation across horizons (Chapter III).
- (iii) To elucidate the short-term response of the composition and functionality of the soil microbiome to slurry derived carbon at the field scale (Chapter IV).
- (iv) To investigate the effect of soil aggregation, slurry application and time on the functionality of the soil microbiome in two contrasting soil types (Chapter V).

**Chapter II: The community dynamics of
bacteria and fungi in aggregate size fractions in
grassland soils**

2.1: Abstract

Determining soil microbial community composition at the soil aggregate scale may be a critical factor in understanding the terrestrial carbon cycle in grassland soils. This study aimed to determine the influence of horizon depth and aggregate-size on bacterial and fungal community composition in Irish agricultural grassland soils. Soil samples were taken from the top three horizons of the soil profile to approx. 75 cm depth, from nine grassland sites. Physical fractionation was used to obtain large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) fractions, respectively. After extraction of DNA and amplification of bacterial and fungal community marker genes, the structure of the microbial community was assessed via bacterial and fungal terminal restriction fragment length polymorphism. An effect of aggregate-size was detected on bacterial and fungal community structures, with the SC fraction being significantly ($P < 0.05$) distinct from the larger aggregates in each case. A significant effect of horizon depth was also observed on both bacterial and fungal community composition within aggregate-size fractions ($P < 0.05$). These results indicate that both horizon depth and aggregate-size fractions support distinct bacterial and fungal communities in grassland soils. Understanding these parameters is critical in our comprehension of terrestrial microbial ecology and its role in sustainability of our agroecosystems.

2.1.1: Highlights:

- Aggregate-size fraction has a significant effect on microbial community composition.
- Horizon depth significantly affected microbial community composition of aggregates.
- Mg had a strong significant correlation to bacterial community structure.
- Soil Organic Carbon was significantly correlated to microbial community composition of aggregates.

2.1.2: Keywords: Aggregate; T-RFLP; Bacteria; Fungi; Soil Organic Carbon

2.2: Introduction:

The microbial community residing in soil is rich in diversity and complexity, with an estimated 10^{10} prokaryotic cells per gram of soil (Torsvik et al. 1990). The heterogeneous nature of soil provides localized microenvironments of varying porosity, substrate and nutrient concentrations, pH, water, and O₂ permeability resulting in a multitude of habitats, that can affect microbial composition and supports the high richness of species (Negassa et al. 2015; Sessitsch et al. 2001). Despite this, the majority of soil microbial ecology studies are conducted on homogenized bulk soil samples. This approach obscures underlying heterogeneous patterns of microbial distribution occurring at small spatial scales (Vos et al. 2013).

Globally, soils contain 1550 Pg of organic C and 780 to 950 Pg of inorganic C (Batjes, 1996; Lal, 2008). As the pool of SOC is large and in flux with the soil biophysical environment, it is recognised as an important component of the global carbon (C) cycle (Janzen 2005). SOC is also important for sustainable soil function. The maintenance of adequate levels of SOC are required to support agricultural yields (Roper and Gupta 1995), and underpins soil structure and associated ecosystem services (Loveland and Webb 2003). Interactions of sand, silt, clay, and SOC result in the formation of aggregate-size fractions which are essential determinants of soil structure and act as a SOC storage mechanism. As grasslands comprise 4 million hectares of land area in Ireland (90% of the total area used for agricultural production), sequestration of SOC in grassland soils is seen as a promising strategy for offsetting the wider agricultural C footprint (O'Mara 2008).

Studies have elucidated patterns of (mainly bacterial) colonization across the aggregate-size spectrum (Davinic et al. 2012; Mummey et al. 2006; Mummey and Stahl 2004; Neumann et

al. 2013; Sessitsch et al. 2001). The bacterial to fungal proportions have also been shown to differ with decreasing aggregate size. In larger aggregate fractions (i.e. >250 μm) fungi make up the dominant proportion of microbial biomass with a rather simple bacterial community being present, while in smaller aggregate sizes (<250 μm) fractions, a complex and diverse bacterial community predominates (Gupta and Germida 1988; Kandeler et al. 2000; Poll et al. 2003; Schutter and Dick 2002). Thus, fungi and bacteria play differing roles in the formation and subsequent consolidation of differing aggregate sizes. Binding by fungal hyphae strands is a major binding mechanism in the formation of large macroaggregates (Six et al. 2004). In the smaller aggregate sizes, bacterial polysaccharides act to bind polyvalent metals, clay particles, and organo-metal complexes together (Rillig and Mummey 2006; Six et al. 2004).

Despite the pivotal role the soil microbiota play in both the formation and subsequent consolidation of soil aggregates, how the microbial community composition varies across the aggregate sizes and with horizon depth in grassland soils is not well characterized (Mummey et al. 2006). While studies exist which address bacterial colonization across the varying aggregate sizes, studies in relation to the other microbial components of the community, in particular fungi, are lacking (Gupta and Germida 2015). Understanding the impact of horizon depth and aggregate-size fraction on microbial diversity is critical in our comprehension of terrestrial microbial ecology and its role in biogeochemical cycling (particularly of C) in agroecosystems. Furthermore, a greater understanding of microbial diversity within aggregates will help with the integration of biotic data with the plethora of soil physiochemical information abounding in the literature. This has the potential to enhance our understanding of soil functioning (Mummey et al. 2006). This study aimed to determine the relationship between aggregate-size and horizon depth on microbial (bacterial and fungal) community composition in Irish agricultural grassland soils. Secondly, the study aimed to

understand the influence of environmental variables on microbial community structure of both the bulk soil and aggregate-size fractions.

2.3: Materials and Methods:

2.3.1: Soil collection:

Soil samples were collected from 9 sites in the North-West of the Republic of Ireland as part of the Irish Soil Information System (Irish SIS) soil survey (Creamer et al. 2014) (Table 1). At each sampling site a 1 m² by 1 m deep soil pit was dug. After the pit face was cleaned (i.e. loose soil removed), the soil profile was designated into distinct horizons following the FAO field handbook (WRB 2006). The top three horizons were sampled to a depth of approx. 70 cm, resulting in 26 samples in total (Figure 8; No sample could be gathered from the third horizon of RPM66BR01 due to the large amount of stony material present).

Approximately 300 g of soil was collected from each of the described horizons, across the nine soil profiles pits sampled. Sampling was conducted from the lowest horizon up to prevent soil from the upper horizons contaminating the lower horizons. Samples were collected under field-sterile conditions (i.e. use of 70% ethanol and sterile water to sanitise sampling equipment) between samplings. Soil samples were collected into sterile twist-seal bags and kept at 4°C in a cool box for transportation. Soils were not frozen at this stage to prevent damage to the constituent aggregate structures. Once back in the lab (within 48 hrs of collection), samples were homogenized and sieved (<8 mm) and a subsample immediately stored at -80°C. This would act as the bulk soil (BS) sample.

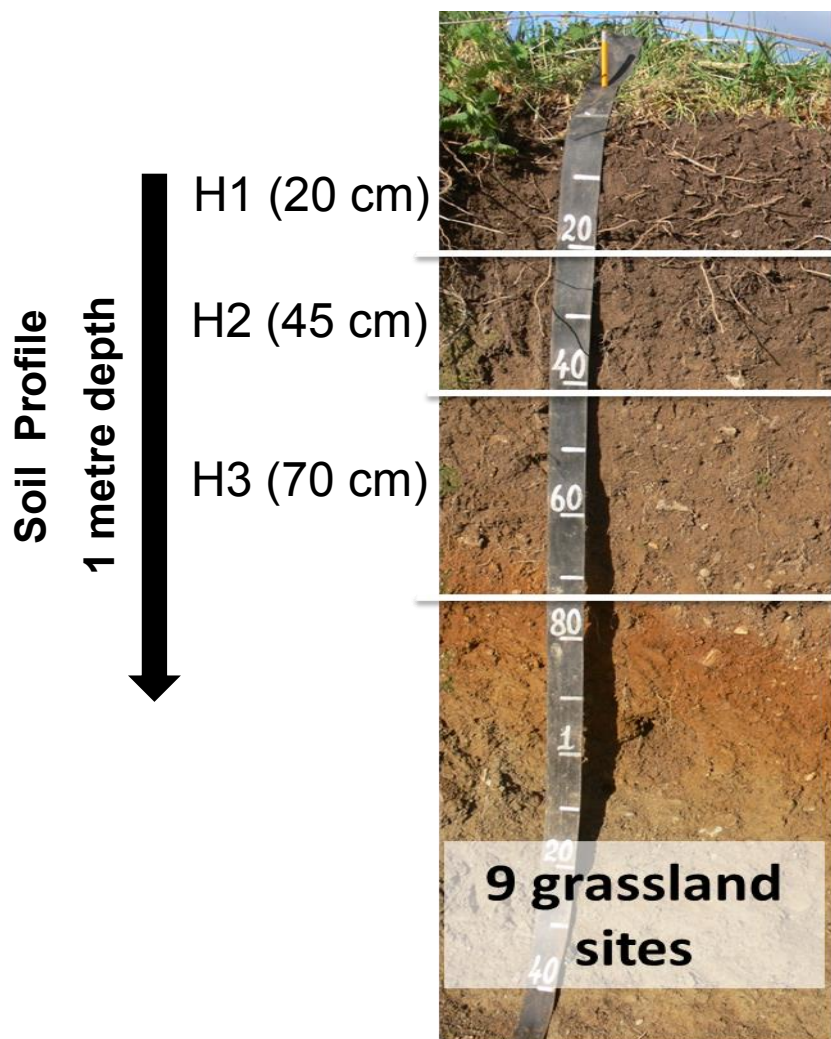


Figure 8: Example of horizon profile from an Irish SIS site. Average depth (cm) at each horizon is shown (rounded to the nearest whole number).

Table 1: Irish SIS reference code, soil sub group and wetness class of the 9 sites used in this study.

Irish SIS Reference Code	Soil Subgroup	Wetness Class
RPM45br01	Typical Luvisol	Moderately drained
RPR49br01	Typical Calcareous Brown Earth	Well Drained
RPG52BR01	Humo Ferric Podzol	Well Drained
RPG62br01	Typical Brown Earth	Well Drained
RPG63br01	Typical Brown Earth	Well Drained
RPM66br01	Stagnic Brown Podzolic	Moderately drained
RPM68br01	Typical Surface-water Gley	Poorly drained
RPM79br01	Humic Groundwater Gley	Poorly drained
RPM85br01	Typical Surface-water Gley	Poorly drained

2.3.2: Soil aggregate isolation:

The aggregate isolation procedure was based on that described by Six et al. (1998). Briefly, soil samples were dried at 40°C for 1 week. Wet-sieving, with the retention of the material on the sieve as an ‘operationally defined fraction’ and re-sieving of the soil which passed through the sieve was conducted. This process was sequential through 2 mm, 250 µm and 53 µm sieves which provided the large macroaggregate (LM), macroaggregate (MAC) and microaggregate (MIC) fractions. Material <53 µm was deemed the silt and clay fraction (SC). The LM, MAC and MIC fractions were collected into 100 ml containers. The SC fraction was collected along with the excess water from the procedure into two 500 ml containers (Figure 9). All material was dried at 50°C for 1 week, after which time the samples were weighed and the % proportion of each aggregate-size fraction was corrected relative to the BS (Six et al. 1998). Samples were homogenized via mixing and a subsample (approx. ½) was immediately stored at -80°C for later molecular work, while the remaining sample was used for the determination of SOC. All laboratory materials pertaining to the fractionation procedure were thoroughly washed in ethanol (70 % v/v) between each sample and autoclaved (121°C for 15 min) every 6th sample, to ensure truly sterile conditions.

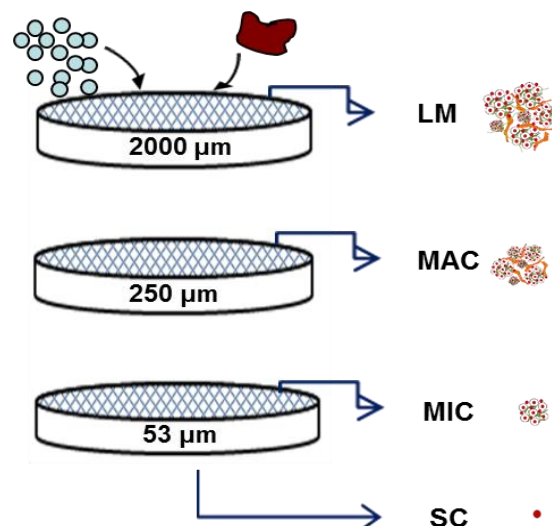


Figure 9: Sequential physical fractionation through 2000 µm, 250 µm, 53 µm sieves to obtain the large macroaggregate, macroaggregate, microaggregate and silt and clay. Image adapted from Torres-Sallan (2016).

2.3.3: Aggregate coarse /fine sand determination:

Stones which were >2 mm were removed from LM and associated mass subtracted. The amount of coarse and fine sand contained within the MAC and MIC fraction was also calculated, as it may distort the true proportion of these aggregates within the BS. Approximately 20 g of soil was placed into a 250 ml Erlenmeyer flask. To remove organic matter, 6 % (v/v) hydrogen peroxide (H₂O₂) was added covering the soil. The solution was boiled at 100°C with additional H₂O₂ being added to replace that which evaporated off. Organic matter was deemed to be completely removed when bubbles formed during the process turned clear. Any excess H₂O₂ was decomposed through the addition of 25 ml of 10 % ammonium hydroxide. Contents of the flasks were washed through a series of sieves (250 µm sieve on top and 53 µm sieve on the bottom) to collect the coarse (>250 µm) and fine (>53 µm) sands (Massey et al. 2014). Once dried, sands collected were weighted and the proportion of coarse sand was mathematically removed from the MAC fraction, and the proportion of fine sand was removed from the MIC fraction using the following equations:

$$\text{Corrected aggregate weight} = \frac{\text{Aggregate weight (MAC)}}{1 - (\text{coarse sand proportion})} \\ \frac{\text{Aggregate weight (MIC)}}{1 - (\text{fine sand proportion})}$$

2.3.4: DNA extraction:

DNA was extracted from the BS sample and each of the constituent aggregate-size fractions (0.25 g) using the Powerlyzer version of the Powersoil DNA isolation kit (MO BIO laboratories, Cupertino, CA). The protocol was undertaken as described in the manufacturer's instructions. The extraction step (bead beating) was undertaken on a Fast-PrepTM-24 instrument (MP Biomedicals, Santa Ana, CA) at a speed of 4 for 45 s. DNA extracts were quantified spectrophotometrically using a Nano-drop ND-1000 (Thermo Scientific, Waltham, MA). Extracts were then diluted with sterile dH₂O to a concentration of 10 ng/ µl.

2.3.5: PCR amplification of soil bacterial 16S and fungal ITS community:

The fluorescently labelled universal primer set 27F (5'-/5ATTO565N//i6-FAMK/AGA GTT TGA TCM TGG CTC AG'3) and 1492R (5'-/5ATTO565N//i6-FAMK/TAC GGY TAC CTT GTT ACG ACTT'3) (Lane 1991) were used to amplify the V3 region of the bacterial 16S rRNA gene. PCR was conducted in final volumes of 25 µl, containing 1x Dreamtaq buffer, 1 M Betaine, 0.2 mM dNTP-Mix, 0.4 pmol/µl of both the forward and reverse primer and 0.5 U Dreamtaq polymerase (Thermo Scientific, Waltham, MA). Each reaction received 1 µl of template eDNA at 10ng/ µl. PCR cycling conditions consisted of an initial denaturation step of 94°C for 4 min, followed by 35 cycles of '94°C denaturation for 45 s, 50°C annealing for 45 s, and 72°C extension for 2 min, followed by a final extension at 72°C for 5 min.

The internal transcribed spacer (ITS) region of the fungal rRNA gene region was used as a marker gene in the PCR amplification of the soil fungal community. Fungal PCR was undertaken using a nested approach. Non-fluorescently labelled fungal specific primers ITS-1F (5'CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns 1993) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990) were used in the initial amplification reaction. PCR reactions were conducted as described above applying the fungal primers instead of the 16S rRNA gene primers. Amplification conditions consisted of an initial denaturation step of 94°C 4 min, followed by 40 cycles of 'denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min', with a final extension step of 72°C for 10 min. A 1 µl aliquot of a 1:10 dilution of this first amplification was used as template DNA in the second PCR, using the same primers (labelled with the same fluorophores as the bacterial primers) same and amplification conditions as before.

2.3.6: Terminal Restriction Fragment Length Polymorphism (T-RFLP):

T-RFLP of bacterial 16S rRNA was conducted on 200 ng of purified PCR amplicons (GenElute PCR clean-up kit, Sigma Aldrich). These were subjected to a double restriction digestion using Alu I and Hha I at 37°C for 8 h (Penny et al. 2010). A sequential restriction digest was done on 200 ng of purified fungal ITS community PCR amplicon with an initial restriction reaction using Mae II at 65°C for 8 h (Alvarado and Manjón 2009). Reactions were allowed to cool at 4°C overnight, whereupon a restriction digest was performed with Hha I for a further 8 h at 37°C. All restriction digest reactions contained 5 U of enzyme per reaction in a 10X FastDigest buffer (all Thermo Scientific, Waltham, MA).

All restriction digests were 1:10 diluted and sent for fragment size and intensity analysis at MRC PPU DNA sequencing and Services (University of Dundee, Scotland, UK). Fragment sizes were determined on an applied Biosystems 3730 XL DNA analyser, using the LIZ500 size marker. Resultant electrophoretograms were imported into Genemapper (version 3.7, ABI, UK), and terminal restriction fragments (TRFs) binned with a 2 bp interval. T-RFs between 80 and 500 bp were included in the analysis and a presence/absence matrix was generated.

2.3.7: Edaphic and environmental properties

Edaphic and environmental parameters of the BS from each horizon soil sample were collected as part of the Irish SIS survey following the Irish SIS laboratory protocols (Massey et al. 2014). Parameters measured were: total carbon (C), total nitrogen (N), cation exchange capacity (CEC), pH, phosphorus (P), potassium (K), magnesium (Mg), and calcium (Ca).

Separately, soil organic matter (SOC) occluded within each aggregate-size fraction was measured on a LECO Truespec CN analyser. SOC samples were first fumigated with 10% hydrochloric acid (HCL) for 6 h under vacuum (Harris et al. 2001). The samples were then

neutralised to prevent corrosion to the instrument. SOC values for the MAC and MIC fractions were corrected for coarse and fine sand content, respectively.

2.3.8: Statistics:

2.3.8.1: Microbial community composition and richness:

The presence/absence matrix of the T-RFLP electrophoretograms from each aggregate-size fraction was imported into PRIMER-E (Version 7; Plymouth, UK) and Bray-Curtis resemblance matrix was constructed. A Bray-Curtis distance was chosen as it has broad suitability for representing distances (similarities) in biological data sets (Clarke and Warwick 2001). Permutational multivariate analysis of variance (PERMANOVA) (Monte Carlo, 9999 permutations) was used to test for differences in T-RFLP profiles between aggregate-size fractions, horizon samples, and sampling site. Similarities between samples were displayed using multi-dimensional scaling (mMDS), with bootstrapping to determine group variance and averages (300 per group, bootstrap region 95%). The Margelef's richness (d) for bacteria and fungi from each sample was calculated using the DIVERSE function in PRIMER to give an indication of T-RF richness (sensu OTUs).

2.3.8.2: Environmental variables:

Environmental variables data from the BS were imported into PRIMER-E, fourth-root transformed, normalized, and a resemblance matrix (Euclidian distance) made. The effect of horizon on each variable was determined via PERMONOVA analysis (Monte Carlo, 9999 permutations).

The effect of horizon and aggregate-size on SOC quantity was modelled using PROC MIXED in SAS (Version 9.3) to fit general linear models of main effects (aggregate-size and horizon) and interaction. Residual checks were made and SOC data was log transformed to ensure that the assumptions of the analysis were met.

The corrected % proportion of each aggregate-size fraction relative to the BS composition was square-root transformed in PRIMER-E and a resemblance matrix (Euclidian distance) was constructed. The effect of aggregate-size and horizon on the constituent makeup of the BS was evaluated via PERMANOVA analysis (Monte Carlo, 9999 permutations).

2.3.8.3: Correlation of the microbial community with environmental variables:

A distance based linear model (DistLM, 9999 permutations) test was run against the bacterial and fungal community composition presence/absence matrix (based on Bray-Curtis) and the resemblance matrix (based on Euclidian distance) of all the measured environmental variables (Clarke and Ainsworth 1993). Occluded SOC was also correlated against bacterial and fungal community composition (SOC first transformed to the fourth root).

Univariate and stepwise regressions were also run between the bacterial 16S and fungal ITS T-RF richness indices of the BS samples using Minitab v17 (Minitab Ltd). The variables SOC, N, P, K, Ca, and % clay were not normally distributed and were subjected to a Johnson transformation. Stepwise regression with all variables was also run against bacterial and fungal richness.

3. Results:

3.1 Biological, chemical and physical characteristics of the BS with depth:

There was a significant response of horizon on the bacterial community composition ($P=0.0007$) and richness in 16S T-RFs (*sensu* OTUs) ($P=0.0003$); in both cases, communities from H1 and H2 were significantly different from H3.

Fungal community composition did not vary significantly across horizons (P=0.18). There was, however, a significant effect of horizon on richness in fungal ITS T-RFs (P=0.03), with H1 and H2 significantly richer in T-RFs than H3 (P=0.02 and P=0.02, respectively).

There was a significant main effect of horizon on the concentrations of N (P=0.001), P (P=0.0003), and K (P=0.02) (Table 2). Other variables did, however, significantly differ between individual horizons. The concentrations of Mg was significantly reduced in H3 (0.37 mg/kg) compared with H1 (1.1 mg/kg; P=0.02). The pH value was also significantly higher (pH 6.99) in H3 than in H1 (pH 5.55; P=0.03). The % proportion of total C was significantly lower in H2 (2.54 %) than H1 (5.25 %; P=0.04), however, while there was a reduction in the quantity of total C between H1 and H3 (3.24 %) it was not significant.

Mg was also the sole variable significantly correlated to the variation (ecological) distances in the bacterial community composition (P<0.02, R²=0.08) in the BS. There were significant individual correlations (P<0.05) between the bacterial 16S T-RF richness and total C, N, P, pH, K, and CEC and a highly significant correlation to Mg (P<0.0001). Stepwise regression found variation in soil Mg content to be most strongly associated with 16S T-RF richness (P=0.001, R²=0.46). There were no significant individual correlations between fungal community composition or ITS T-RF richness and the measured environmental variables.

Table 2: Averages (Avg.) and standard deviations (\pm) of measured environmental parameters from Horizon 1 (H1), 2 (H2) and 3 (H3). Different symbols denote statistical significance (P<0.05)

	H1		H2		H3	
	Avg.	\pm	Avg.	\pm	Avg.	\pm
Mg (mg/kg)	1.11 ^a	0.73	0.56 ^{ab}	0.36	0.37 ^b	0.29
% C	5.25 ^a	2.28	2.54 ^b	1.91	3.24 ^{ab}	3.05
% N	0.71 ^a	0.70	0.17 ^b	0.16	0.07 ^b	0.06
% Clay	21.11 ^a	8.05	21.33 ^a	11	21.63 ^a	8.59
P (mg l⁻¹)	36.52 ^a	35.73	7.60 ^b	8.19	3.25 ^c	2.78
pH	5.55 ^a	0.80	5.99 ^b	1.03	6.99 ^{ab}	1.34
K (mg/kg)	0.39 ^a	0.22	0.23 ^{ab}	0.14	0.18 ^a	0.14
Ca (mg/kg)	7.27 ^a	4.49	6.68 ^a	7.75	5.63 ^a	3.41
CEC	10.29 ^a	6.33	6.36 ^a	3.68	6.59 ^a	2.33

A highly significant interaction was seen between the proportion each aggregate-size fraction contributed to the BS and horizon depth, with the % proportion LM, MIC, and SC fractions contributing to the composition of the BS being affected by horizon. The LM fraction significantly declined down the soil profile ($P < 0.0001$), while the MIC and SC fractions increased with depth ($P < 0.0001$ and $P = 0.0002$ respectively, Figure. 10).

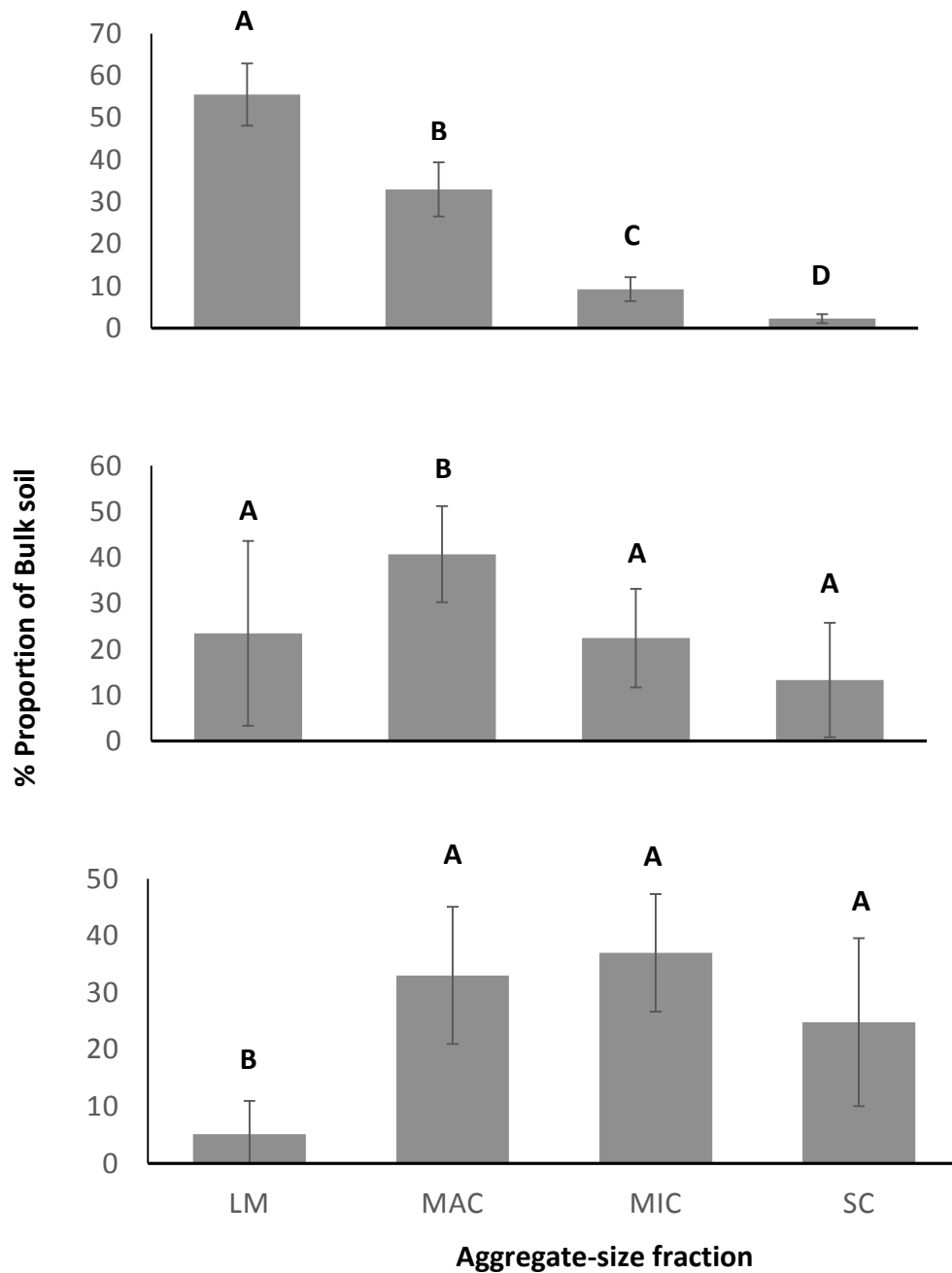


Figure 10: Proportion [%] each aggregate-size makes of the BS in Horizon1 (**Top panel**); 2 (**Middle panel**) and 3 (**Bottom panel**). Error bars are standard deviation. Different letters indicate statistical significance ($P < 0.05$).

3.2: *Bacterial Community structure within aggregate fractions:*

Bacterial community composition differed significantly among aggregate-size classes in the soil profile as a whole ($P < 0.0001$), with communities in the SC fraction being significantly different from LM, MAC and MIC (all $P < 0.0001$). Communities within LM, MAC, and MIC fractions were similar (Figure 11; top panel). A significant effect of aggregate-size was also observed on 16S T-RF richness ($P < 0.0001$), with the SC fraction having a significantly higher 16S T-RF richness than the LM ($P = 0.004$), MAC ($P < 0.0001$) and MIC ($P < 0.0001$) fractions (SM1).

The bacterial community composition of aggregates was also significantly influenced by horizon depth ($P < 0.0002$), with H1 being significantly different from both H2 ($P < 0.01$) and H3 ($P < 0.0001$), with H2 and H3 not differing significantly ($P < 0.17$) (Figure. 11; bottom panel). A significant interaction between horizon and aggregate-size fraction was also observed ($P = 0.007$), indicating that the bacterial community composition within aggregates were responding to the inherent changes in physiochemical parameters in each horizon. In addition to this, the bacterial community composition varied among sampling sites ($P < 0.0001$). Analysis of the variation apportioned to horizon, aggregate-size, and site did show that aggregate-size fraction explained the highest amount of variation in bacterial community composition (\sqrt{CV} : 19.09), followed by site (\sqrt{CV} : 14.47), and horizon (\sqrt{CV} : 11.7). 54.74% of the variation remained unexplained by the treatments tested (summarized in Table 3).

On the individual horizon level, there was a significant aggregate-size effect observed in both H1 and H2 on bacterial ($P < 0.0001$ and $P = 0.005$, respectively) community composition. In each case this was due to the SC fraction being significantly different from each of the larger aggregate sizes (summarized in Table 4). There was also a significant aggregate-size effect

on 16S T-RF richness for H1 ($P=0.0006$) and H2 ($P=0.0005$), in both instances SC being significantly richer in bacterial 16S T-RFs than the LM, MAC and MIC fraction sizes (SM2). There was no overall effect of aggregate-size on bacterial community structure in H3 (Table 4).

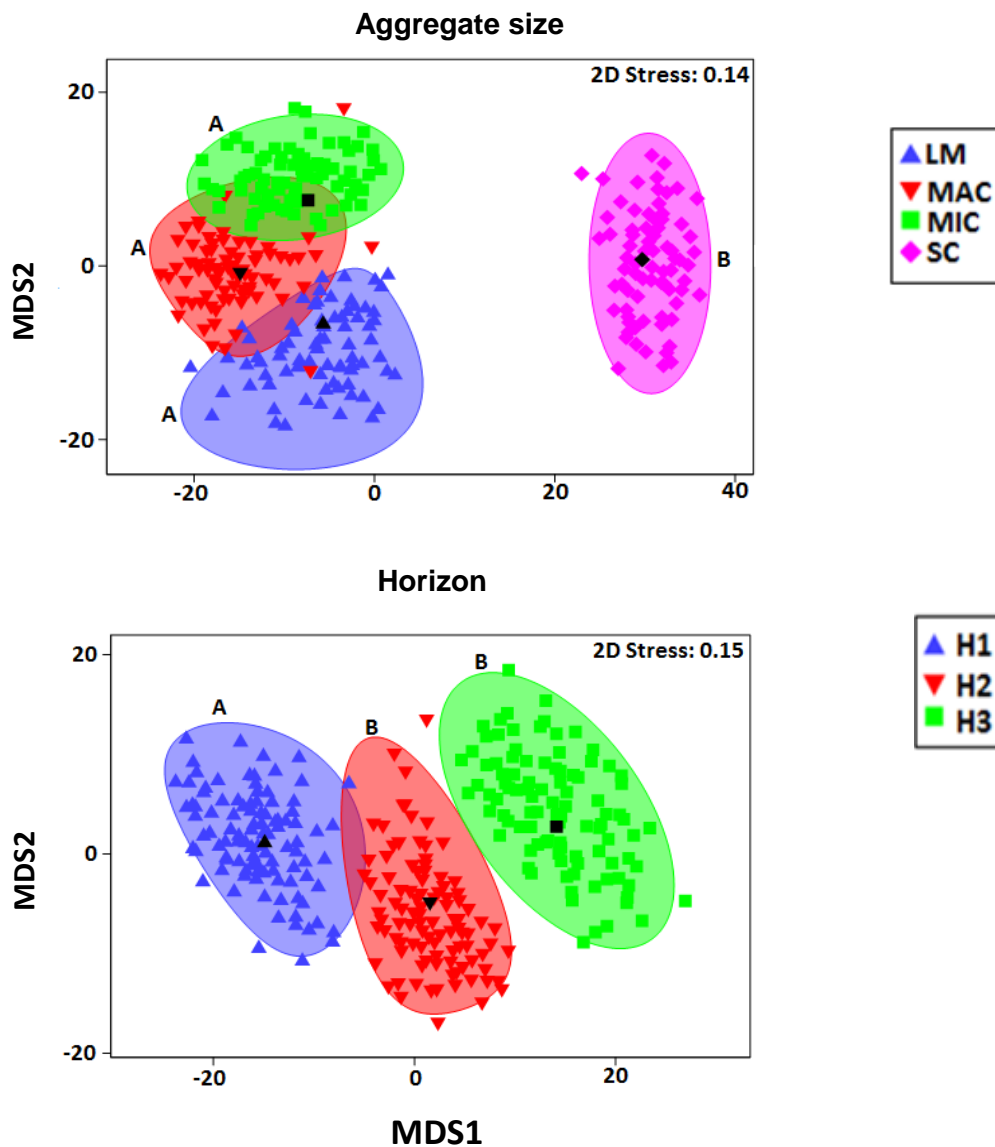


Figure 11: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size and horizon on bacterial 16S rRNA gene based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond). Horizons are as follows: Horizon 1(H1; blue triangle), Horizon 2 (H2; red triangle) and Horizon 3 (H3, green square). Different letters denote significant difference ($P < 0.05$). A 2D stress of < 0.2 on mMDS plots represents a good graphical representation of the data in two dimensions.

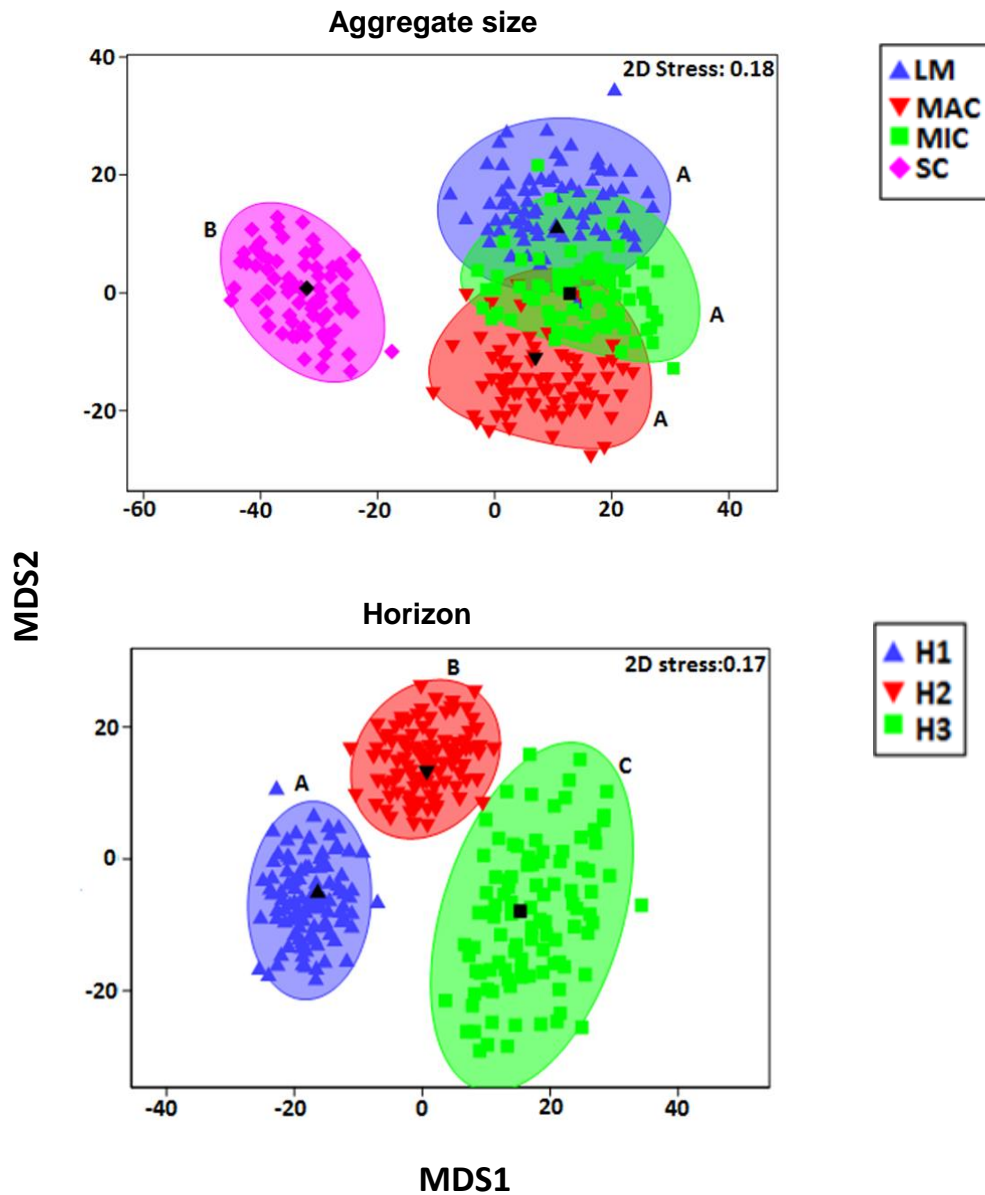


Figure 12: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size and horizon on fungal internal transcribed spacer region (ITS) community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond). Horizons are as follows: Horizon 1(H1; blue triangle), Horizon 2 (H2; red triangle) and Horizon 3 (H3, green square). Different letters denote significant difference ($P < 0.05$). A 2D stress of < 0.2 on mMDS plots represents a good graphical representation of the data in two dimensions.

3.3: *Fungal community structure within aggregate fractions:*

Aggregate-size also significantly influenced fungal community composition ($P < 0.0001$) in the soil profile as a whole, with again the SC fraction being significantly different from the larger aggregate-sizes (all $P < 0.0001$, Figure 12; top panel). The fungal community composition of aggregates was also affected by horizon ($P < 0.0001$) with H1 being significantly different from H2 ($P = 0.03$) and H3 ($P = 0.03$). Additionally, H2 and H3 were significantly distinct from each other ($P = 0.048$, Figure 12; bottom panel). A significant effect was also observed with site on fungal community composition ($P = 0.01$). Analysis of the variation apportioned to horizon, aggregate-size and site did indicate that aggregate-size did explain the greatest amount of the variation seen in the fungal community composition (\sqrt{CV} : 17.82) followed by horizon (\sqrt{CV} : 16.25) and site (\sqrt{CV} : 12.18). 53.75 % of the variation remained unexplained by the parameters tested (summarized in Table 3).

On the individual horizon level, there was a significant aggregate-size effect observed in both H1 and H2 on fungal ($P < 0.0001$ and $P = 0.03$, respectively) community composition, with the SC fraction being significantly different from each of the larger aggregate sizes in both cases (Table 4). Additionally, there was a significant aggregate effect on ITS T-RF richness ($P = 0.047$) in H1, with SC having a significantly lower level of ITS T-RF richness than LM ($P = 0.046$) and MIC ($P = 0.03$) (SM2). A significant overall fraction effect ($P = 0.04$) was also observed in the case of the fungal community composition in H3, with SC differing from MIC ($P = 0.03$) (Table 4).

Table 3: The effect of horizon, aggregate-size fraction and site on bacterial 16S and fungal ITS community composition in the soil profile as a whole. Statistical significance P<0.05

Community	Parameter			Pairwise	P-value
		P-value	\sqrt{CV}		
Bacterial 16S	Horizon	0.0002	11.7	H1 vs H2	0.01
				H1 vs H3	<0.0001
				H2 vs H3	0.17
	Aggregate-size fraction	<0.0001	19.09	LM vs MAC	0.36
				LM vs MIC	0.34
				LM vs SC	<0.0001
				MAC vs MIC	0.14
				MAC vs SC	<0.0001
	MIC vs SC	<0.0001			
Site	<0.0001	14.47			
Fungal ITS	Horizon	<0.0001	16.25	H1 vs H2	0.03
				H1 vs H3	0.03
				H2 vs H3	0.048
	Aggregate-size fraction	<0.0001	17.82	LM vs MAC	0.69
				LM vs MIC	0.98
				LM vs SC	<0.0001
				MAC vs MIC	0.42
				MAC vs SC	<0.0001
	MIC vs SC	<0.0001			
Site	0.01	12.18			

Table 4: The effect of aggregate size fraction on bacterial 16S rRNA and fungal internal transcribed spacer (ITS) region based community composition within the individual horizons. Statistical significance $P < 0.05$. * Large macroaggregate ITS PCR amplicons were not obtained in horizon 3 (H3).

Community	Horizon	Main effect P-value	Pairwise	P-value
Bacterial 16S	H1	<0.0001	LM vs MAC	0.89
			LM vs MIC	0.49
			LM vs SC	0.0001
			MAC vs MIC	0.15
			MAC vs SC	<0.0001
			MIC vs SC	<0.0001
	H2	0.005	LM vs MAC	0.37
			LM vs MIC	0.12
			LM vs SC	0.046
			MAC vs MIC	0.21
			MAC vs SC	0.01
			MIC vs SC	0.01
	H3	0.54	LM vs MAC	0.56
			LM vs MIC	0.37
			LM vs SC	0.04
MAC vs MIC			0.61	
MAC vs SC			0.04	
MIC vs SC			0.46	
Fungal ITS	H1	<0.0001	LM vs MAC	0.58
			LM vs MIC	0.996
			LM vs SC	<0.0001
			MAC vs MIC	0.7
			MAC vs SC	<0.0001
			MIC vs SC	<0.0001
	H2	0.03	LM vs MAC	0.67
			LM vs MIC	0.86
			LM vs SC	0.02
			MAC vs MIC	0.81
			MAC vs SC	0.01
			MIC vs SC	0.02
	H3*	0.04	MAC vs MIC	0.28
			MAC vs SC	0.07
			MIC vs SC	0.03

3.4: Effect of SOC on aggregate-size bacterial and fungal community composition

There was a highly significant effect of horizon ($P < 0.0001$) on the quantity of SOC associated with the four aggregate-sizes in the soil profile, with H1 being significantly higher from H2 ($P < 0.0001$) and H3 ($P < 0.0001$), with no difference between the two bottom horizons ($P = 0.83$). While an effect of aggregate-size was not seen on SOC quantity in the soil profile as a whole ($P = 0.57$) there was a significant interaction between horizon and aggregate-size ($P = 0.003$).

DistLM analysis showed a significant correlation between SOC within aggregate fractions and the bacterial community composition of those aggregates ($P = 0.04$, $R^2 = 0.03$). Teasing out the effect of SOC on the bacterial community composition of the aggregate-size fractions revealed that it was significantly correlated to the two largest aggregate sizes, LM and MAC ($P = 0.01$, $R^2 = 0.07$) but not the smaller aggregates, MIC and SC ($P = 0.6$). SOC was also significantly correlated to fungal community composition ($P = 0.03$, $R^2 = 0.03$) within aggregate-size fractions throughout the soil profile.

4. Discussion:

A highly significant effect of aggregate-size was observed on bacterial and fungal community composition. While an overall effect of aggregate-size was seen on community composition for both bacteria and fungi in the top two horizons, there was no such effect (as in the case for bacteria) or a greatly reduced effect (as with the fungi) in H3. This was a consequence of the reduction in aggregate stability with depth (particularly in relation to LM and MAC) and thus a reduction in the number of experimental replicates. In the soil profile as a whole, however, the SC fraction harboured a distinct bacterial and fungal community fingerprint when

compared to the LM, MAC and MIC fractions. This is indicative of the unique microhabitat this fraction constitutes in soil.

Heterogeneity found in the soil matrix results in gradients in the distribution of O₂, water, SOC and solutes which will differentially impact on certain microbial phyla (Davinic et al. 2012; Ruamps et al. 2013). The innermost part of the MIC fraction has been described as a difficult, oligotrophic environment for microbial cells (Mummey et al. 2006). The SC fraction will be dominated by microsites with anaerobic conditions and micro-porosity, resulting in higher moisture conditions coupled with limited O₂ availability. In addition, the presence of clay particles may locally influence soil physiochemical parameters; such as the exchangeability of cations, redox potentials and the availability of SOC (Husson 2013). Bacterial cells have also been shown to adhere to clay particles (Mueller 2015). Bacterial cells produce a polysaccharide secretion which penetrates the adjacent clay pores and forms a polymeric bridge between clay particles. This process has been suggested as both an important mechanism for aggregate formation as well as SOC storage (Alimova et al. 2009; Deneff and Six 2005; Mueller 2015). Lunsdorf et al. (2000) suggested that 'clay hutchies', which are described by the specific orientation of clay particles within a polysaccharide matrix, may provide a safe habitat for bacteria. This concept was supported by (Heijnen and van Veen 1991), who also proposed that that micro-porosity, such as that associated with SC, provides a safe environment for bacteria avoiding predation by their predators, such as bacterivorous nematodes. This often results in an increase in bacterial biomass in the SC fraction compared to larger fractions (Sessitsch et al. 2001). In this paper we report a significant increase in bacterial 16S T-RFs in the SC fraction, we also found that this micro porosity potentially restricts fungal hyphal penetration and colonization. This concurs with the findings of (Kandeler et al. 2000) who proposed that the microbial biomass of the larger

aggregates are largely fungal. Fungal hyphae are also known to harbour a diverse bacterial community (Gahan and Schmalenberger 2015).

The bacteria within the SC fraction will have a relatively simple metabolism and small size, contributing relatively little to overall measurable microbial activity, due to the restrictive micro-pore environment, limited gaseous exchange and SOC, which may be difficult for microbes to metabolize (Mummey and Stahl 2004), resulting in greater stability of SOC in this fraction.

In both the BS and the four aggregate fractions there was a significant effect of horizon on bacterial and fungal community composition. Soil depth has previously been shown to be a strong driver of microbial community structure in grassland soils (Will et al. 2010). In comparison to the surface horizons, sub-soils are typically colder and have lower rates of gas exchange as the abundance of air-filled pores declines with soil depth, resulting in an increase of anoxic microsites (Barber et al. 2004; Ekelund et al. 2001). Due to the high rainfall conditions prevalent in Ireland, these soils can be prone to waterlogging for considerable periods of the year resulting in smaller, less active microbial communities (Barber et al. 2004; Ekelund et al. 2001; Ekschmitt et al. 2008; Fang and Moncrieff 2005), and/or communities supported by anaerobic / microaerophilic biogeochemistry. In addition, with increasing depth, the substrate available for the soil microbiota decreases as a high proportion of SOC becomes stabilized by metal ions and bound to mineral surfaces (von Lutzow et al. 2006). Furthermore, the SOC available at depth will be more inherently recalcitrant (i.e, a lower C:N ratio) which may be more energetically expensive to metabolise (Kramer and Gleixner 2008). Indeed, it has even been suggested that microbial community activity is so low in sub-soils that even labile substrates may remain non-degraded for significant periods of time, as

numerous studies have demonstrated that the sub-soil is rich in otherwise potentially enzymatically labile C compounds (Krull and Skjemstad 2003; Lal et al. 2011; Liang and Balsler 2008; Xiang et al. 2008).

While significant, SOC was only weakly correlated to both bacterial and fungal community composition within aggregates in this study. SOC has previously been shown to influence the bacterial community found within aggregates (Davinic et al. 2012; Neumann et al. 2013). In this study, however, it is most likely the case that the same soil physiochemical parameters which are driving microbial community structure are also determining its activity in relation to SOC cycling. As SOC is a collection of polymeric, complex and randomly arranged molecules which microbes can't assimilate into their biomass directly (Kögel-Knabner 2002). To overcome this, they release extracellular enzymes (EE) which break up SOC into simpler molecules (Allison and Jastrow 2006). The spatial constraints inherent to aggregates will be a major influence on the enzyme-substrate stoichiometry (Allison 2005). In addition, soil minerals (e.g , allophane, ferrihydrite) have been shown to adsorb EE to their surface stabilizing them, with the reported effect of both increasing and decreasing enzymatic activity (Allison 2006; Naidja et al. 2000).

Previous studies have demonstrated that SOC concentration is a strong determinate of bacterial community composition with depth (Fierer et al. 2003). In the BS analysis of this study, however, it was Mg which was most strongly associated with bacterial community composition. Studies have shown a relationship between total microbial biomass and bacterial cell number in soil and concentrations of Mg (Allison et al. 2007; Wyszowska and Wyszowski 2002). The biological significance of the correlation in this study is unclear, as it is unlikely that Mg limitation is occurring in these sites (Brantley and Walter 2011). While

the association of Mg with bacterial community structure may have some mechanistic underpinning, it may be the result of a collinear relationship with some other environmental parameter not measured as part of this study or be representative of historical agricultural additions (i.e. lime or organic/inorganic fertilizers).

This study employed the ‘wet-sieving’ approach to fractionation, which is a commonly used method to assess variation in microbial community composition and functionality among aggregates (Bach and Hofmockel 2014; Davinic et al. 2012; Kong et al. 2011; Mummey and Stahl 2004). It offers advantages over other methods as it ensures a constant disruption energy allowing for more reproducibility among experimental replicates. While it can be assumed that the fractionation procedure induced changes in both the bacterial and fungal communities in the aggregates, we feel that the impact of any changes would be overcome by the methodology employed. All soil samples were collected in the same manner and all were subjected to the steps of the fractionation procedure (i.e. drying, re-wetting and slaking). As such, it can be reasonably assumed that the impact of the procedure on all aggregates was similar.

The strong effect of aggregate-size on microbial community structure highlights important avenues for further research investigating how these differences translate into microbial activities and functions. There are few studies available which have examined the differences in the abundance of functional marker genes of important biogeochemical cycles (i.e. *amoA* for nitrification, *nirK*, *nirS* and *nosZ* for denitrification, *phoD* for ester-bound phosphorus mobilisation, *asfA* for aromatic sulfonate desulfurization etc) across the aggregate sizes. Understanding the constraints on microbial colonization patterns and functionality is

important in relating ecological interactions within and between aggregate-size fractions and processes on the ecosystem scale (Schimel and Schaeffer 2012).

5. Conclusion

The strong effect of aggregate-size on microbial community structure makes it clear that colonization patterns within the soil matrix are not homogenous. Thus, this study highlights the need for microbial ecologists to focus efforts away from solely studying the bulk soil, as important configurations of microbial diversity are lost. In addition, studies in soil microbial ecology have largely focused on the top 10-20 cm of the soil profile, with the result that little is known about how soil depth influences the microbiota. This study highlights how differing horizons within the soil profile can harbour distinct microbial communities and the consequences this has for soil development and processes, such as structural aggregation and SOC cycling.

**Chapter III: The bacterial colonisation patterns
across aggregate size fractions as determined by
next-generation sequencing**

3.1: Abstract:

In chapter II, it was determined that aggregate-size and horizon depth had a significant influence on both bacterial and fungal community composition. The T-RFLP community fingerprinting technique does not provide additional information on how presence/abundance or the % relative abundance of specific bacterial groups change across the aggregate size spectrum. To achieve this, a next-generation sequencing technique was employed targeting the V4 region of the 16S rRNA gene of the bulk soil (BS) and each of its constituent aggregate-size fractions from the top two horizons of the nine grassland sites collected in Chapter II. Sequences were PCR amplified and the generated reads were run through the mothur command pipeline and sequence reads assigned to the phylum and family taxonomic levels. Bacterial community composition in the BS samples ($P < 0.05$) were significantly correlated to Mg ($P < 0.032$; $R^2 = 0.215$), C ($P < 0.037$; $R^2 = 0.0203$) and P ($P < 0.026$; $R^2 = 0.222$). Aggregate size had a significant effect on the bacterial community in both horizons at the family level and additionally on the phyla level in horizon 2 (H2). The MAC and MIC fraction significantly differed in their abundance of bacterial groups indicating differing predation pressures. An interaction between aggregate-size and horizon was seen ($P < 0.05$) with the community composition of the macroaggregate (MAC) fraction differing between horizon 1 (H1) and H2. There was no overall effect of occluded SOC on community composition, though lesser abundant bacterial groups were significantly correlated to this parameter. This study gained valuable additional information into the bacterial colonization patterns observed in chapter II.

3.1.1: Keywords: Bacteria, horizon, aggregate-size, next generation sequencing

3.1.2: Highlights:

- Significant correlation between Mg, C and P and bacterial community structure in BS samples
- Abundance of bacterial groups significantly different in MAC and MIC in H1
- Abundance of bacterial groups in MAC fraction significantly affected by horizon
- No overall main effect of occluded SOC on bacterial community within aggregates

3.2: Introduction

Soil is a tortuous physical network which determines the flow of substrates and solutes. This therefore provides varied physiochemical niches for microorganisms characterized by variations in nutrient, O₂, water as well as pore size distribution for habitation (Mummey and Stahl 2004). Soil can be differentiated into distinct aggregate-size fractions, which vary in their physical, chemical and structural characteristics (Ranjard et al. 2000). The stability, distribution and microarchitecture within and between soil aggregates will affect the composition and functionality of the soil microbiota (Mikha and Rice 2004).

These aggregate-size fractions represent distinct microhabitats for microbial colonization and substrate utilization. The large macroaggregate and macroaggregate fractions are enriched with labile C and N, predominantly of plant origin, and typically have higher fungal biomass than the smaller two fractions. Microaggregates are characterized by having lower concentrations of labile C and increased amounts of biochemically recalcitrant (i.e, higher C:N ratio) and physically protected C (Elliott 1986). In addition, the interior of microaggregates have been described as inherently oligotrophic, with low nutrient and O₂ availability resulting in reduced microbial activity (Mummey and Stahl 2004). The silt and clay fraction (SC) have relatively stable C and N and high levels of microbial biomass have

been reported within them (Elliott 1986; Sessitsch et al. 2001; vanGestel et al. 1996). Microbial acquisition of substrate is compounded by the sorption of extracellular to clay particles in the SC, with this process likely being responsible for the distinct bacterial and fungal community reported (Allison and Jastrow 2006; Mueller 2015).

This aggregate stratification on microbial community composition and functionality has important implications for soil organic carbon (SOC) cycling and retention in agroecosystems. Agricultural yields, soil structure and other associated ecosystem services are underpinned by SOC levels (Loveland and Webb 2003; Roper and Gupta 1995), with the interactions of soil particles, microbial community dynamics and SOC within aggregates acting as an important terrestrial C sequestration mechanism. Therefore, understanding the interplay between aggregate structure and microbial community dynamics and soil depth is vital to our comprehension of the terrestrial C cycle. It also has important implications for the preservation of soil biodiversity and the management of microbial communities for bio-control and plant disease suppression (Grundmann 2004).

Previous work on these grassland sites, employing the T-RFLP fingerprinting technique, did show significant effects of both horizon and aggregate-size on both bacterial and fungal community composition (Chapter II). The objective of this study was to gain further insights into the effects of these parameters on microbial community structure using next generation sequencing (Illumina Miseq). While our previous study examined both the bacterial and fungal community composition from the top three horizons in the soil profile, this study concentrated on the bacterial community from the first two horizons.

3.3: Materials and Methods

3.3.1: Soil collection and soil aggregate isolation:

Soils were collected from the top three horizons from 9 Irish grassland sites and soil aggregates were isolated as described in chapter II (section 2.3.1 and 2.3.2 respectively). Data pertaining to the environmental variables in this study were also reported in this chapter (Table 2).

3.3.2: DNA extraction:

DNA was extracted from the BS sample and each of the constituent aggregate-size fractions (0.25 g) using the Powerlyzer version of the Powersoil DNA isolation kit (MO BIO laboratories, Cupertino, CA). The protocol was undertaken as described in the manufacturer's instructions as described in Chapter II (section 2.3.4). DNA extracts were quantified spectrophotometrically using a Nano-drop ND-1000 (Thermo Scientific, Waltham, MA). Extracts were then diluted with sterile dH₂O to a concentration of 5 ng/ µl.

3.3.3: PCR amplification of soil bacterial 16S community:

The bacterial 16S rRNA gene region was amplified from each of the aggregate and horizon DNA samples using the universal primer pair 515F and 806rBC; these amplify the V4 region yielding an amplicon of approximately 300-350 bp. PCR reactions were 25 µl with 0.4 µM of each primer, 0.2 µM dNTPs, 1 × PCR buffer with 1.5 mM MgCl₂ and 0.5 U of TaKaRa Ex Taq polymerase. The PCR amplification conditions were conducted with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C (45 s), 50°C annealing (60 s) and 72°C extension (90 s) with a final extension step of 72°C for 10 min. The PCRs were multiplexed, such that each DNA sample was amplified with a unique 12-mer barcoded modified 806rBC primer (GoLay barcodes; Apprill et al., 2015 and references therein).

Individual PCR products for all samples were quantified using the high sensitivity Quant-iT PicoGreen spectroscopy method (Invitrogen), then equimolar amounts of each PCR were pooled into a single sample. The resultant mixed PCR product was purified using the GeneElute PCR purification kit (Sigma-Aldrich, St. Louis, MO) and quantified again as above. The amplicon mix was sequenced using 2 x 250 PE sequencing on an Illumina Miseq NGS platform (Illumina, San Diego, CA) at the University of Auckland.

The generated reads were processed, joined and analysed using the mothur (v.1.36.0) program following the commands described in the Miseq SOP (detailed: https://www.mothur.org/wiki/MiSeq_SOP) Sequences were matched and aligned with chimeras and mismatched sequences removed from the data-set. Rarefaction curves were also undertaken to deduce the sequencing depth achieved. Mothur was used to assign taxonomy to the sequences using the SILVA (release 102) database. Taxonomic information at the phylum and family taxonomic levels were consolidated from the Mothur output files.

3.3.4: Statistics:

The abundance of OTUs obtained from each sample were imported into PRIMER-E (Version 7, Plymouth, UK), standardized to the total number of sequences, log transformed and a Bray-Curtis resemblance matrix constructed. Statistical differences with individual phyla/families was done by first transforming to the fourth root. The effect of horizon, aggregate-size fraction (and the horizon x aggregate-size fraction interaction) and site was determined via permutational multivariate analysis of variance (PERMANOVA, Monte Carlo 9999 permutations). A number of diversity measurements were also calculated, namely Margelef's richness index (d), Shannon-weiner diversity index ($H \log^e$) and Pielou's measure of species richness (J') using the DIVERSE function in PRIMER-E (Clarke and Warwick 2001). A distance based linear model (DistLM, marginal tests, R² selection, 9999

permutations) was run against a resemblance matrix of the BS samples and a resemblance matrix (Euclidian distance, variables first fourth root transformed and normalized) of each of the environmental variables measured from these sites (% C, % N, P, K, Mg, Ca, pH, % clay and CEC). A distLM analysis was also ran against the community resemblance matrix of the aggregate-size fractions and a resemblance matrix of the SOC occluded within aggregates (data treated as above). Venn diagrams were constructed using the R package VennDiagram (Chen and Boutros 2011) to visualize the shared bacteria (taxonomy assigned to phylum and family levels) between the aggregate fractions.

3.4: Results:

3.4.1: Effect of horizon in the BS:

There was a significant effect of horizon on bacterial abundance in the BS samples on both the phyla and family levels ($P < 0.001$; respectively). A significant reduction ($P < 0.05$) in OTU abundance was seen for all bacterial phyla between H1 and H2 with the exception of the Firmicutes and the Gemmatimonadetes. The Firmicutes were the sole phyla whose abundance increased with soil depth, being 24.39 in H1 and 71.28% in H2 (Table 5). Of the 43 families with an abundance greater than 0.001% in both horizons, two classified families in the phylum Firmicutes (Paenibacillaceae, Incertae_Sedis_XVIII) increased in % abundance between H1 and H2. The abundance of six additional families in the phylum Firmicutes (Clostridiaceae, Peptococcaceae, Peptostreptococcaceae, Ruminococcaceae, Thermoactinomycetaceae and Veillonellaceae) did not change between horizons along with the Cystobacterineae family in the phylum Proteobacteria. The remaining 33 families all declined in abundance with horizon depth.

Marginal tests of the DistLM analysis revealed the Mg ($P=0.031$; $R^2=0.223$), C ($P=0.035$; $R^2=0.212$) and P ($P=0.032$; $R^2=0.216$) were significantly correlated to bacterial % relative abundance (Table 5). Two phyla which did show a sharp reduction in abundance with depth, Acidobacteria and Verrucomicrobia were significantly correlated to all three of these variables as were the phyla Chlamydiae and Planctomycetes. The most dominant phyla, the Firmicutes, was significantly correlated to both % C ($P=0.03$; $R^2=0.256$) and P ($P=0.05$; $R^2=0.223$) down the soil profile (Table 5). The next most abundant group, the Proteobacteria, was not significantly correlated to any environmental variable tested. Many of the families within these two phyla were significantly correlated, however, to at least one of these variables. Within the Firmicutes, Paenibacillaceae and Incertae_Sedis_XVIII which both increased with depth, were significantly correlated to Mg., % C and P. The Planococcaceae, which declined with depth, was significantly correlated to both % C ($P=0.043$; $R^2=0.237$) and P ($P=0.018$; $R^2=0.299$) while Erysipelotrichaceae was correlated to P ($P=0.007$; $R^2=0.408$). While in the Proteobacteria, Bradyrhizobiaceae, Hyphomicrobiaceae and an unclassified family were significantly correlated to the three variables (summary table provided in the supplementary materials).

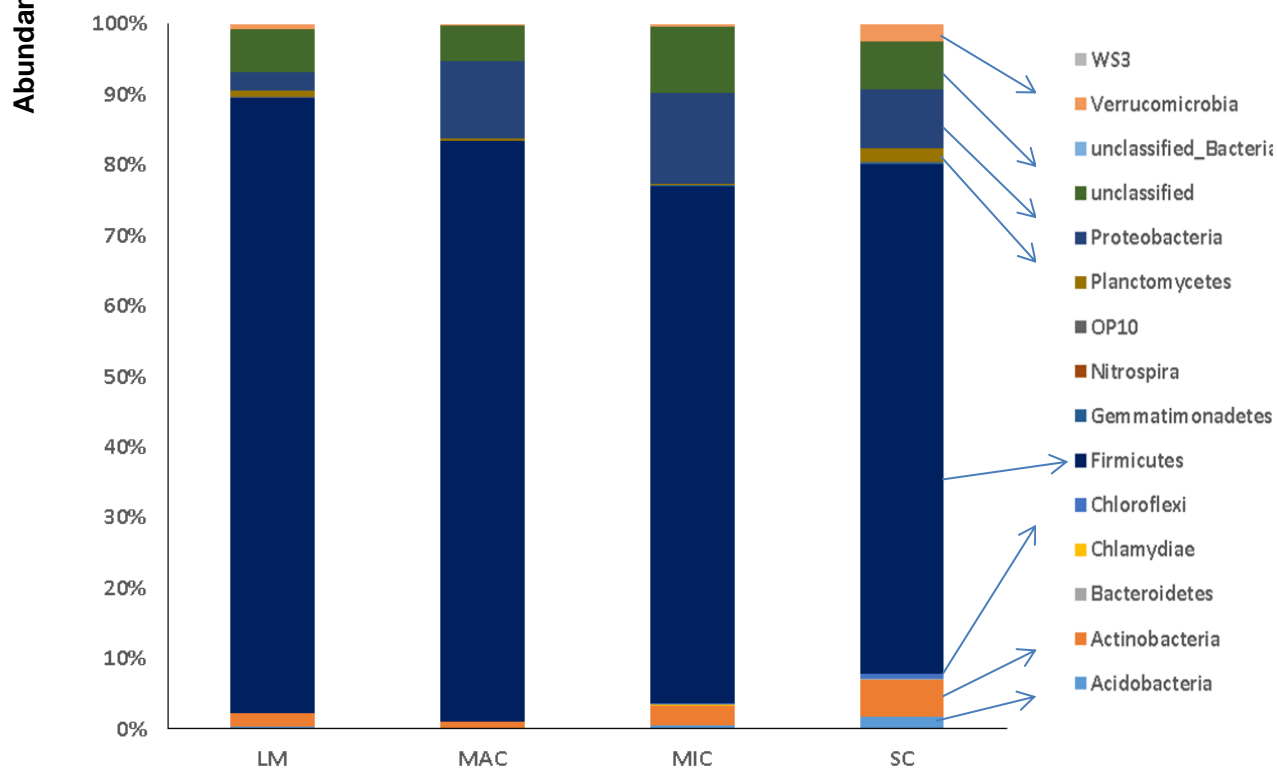
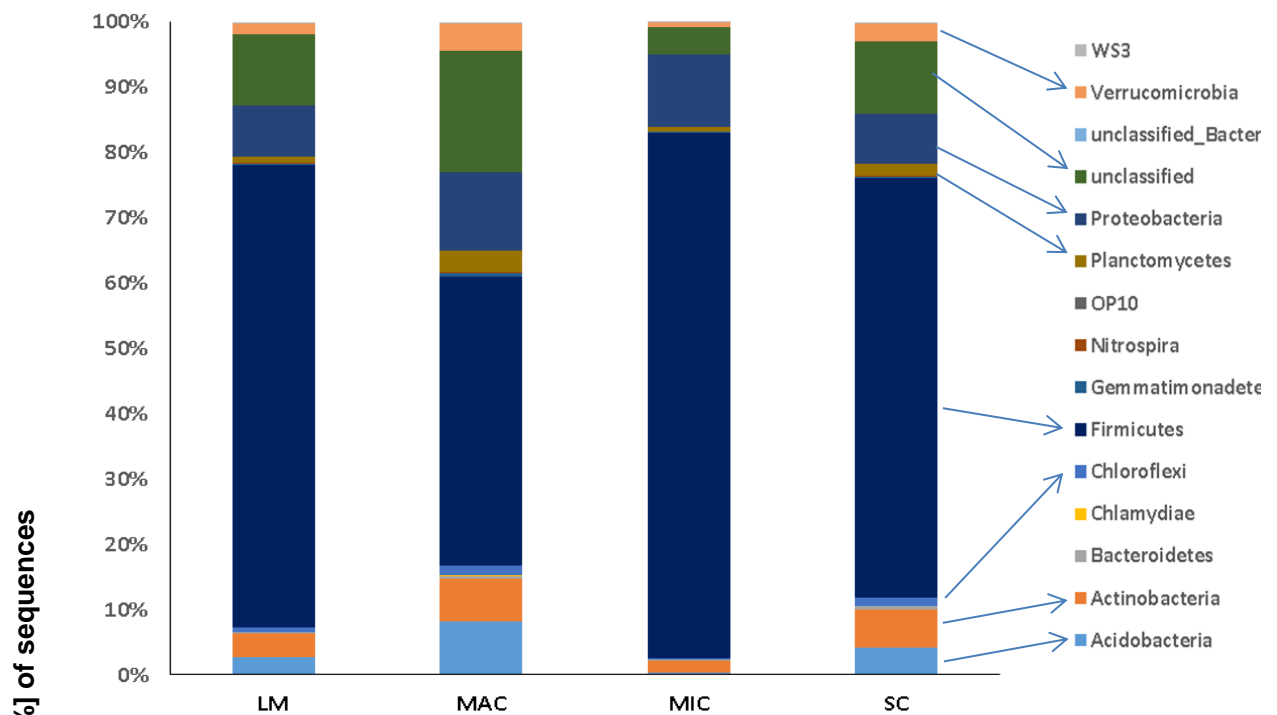
Table 5: Averages (Avg.) and standard deviations (\pm) of the abundance of sequences allocated to all the phyla from the bulk soil (BS) from Horizon 1 (H1) and Horizon 2 (H2). Different letters indicate statistical difference at $P < 0.05$. Statistical P-values and proportions (R^2) from distance based linear model (DistLM) tests between phyla and the environmental variables carbon, phosphorous and magnesium. Statistically significant correlations ($P < 0.05$) are highlighted in black.

	[%]		[%]		DistLM test		DistLM test		DistLM test	
	Abundance H1		Abundance H2		% C		Phosphorus		Magnesium	
	Avg.	\pm	Avg.	\pm	P-Value	R^2	P-Value	R^2	P-Value	R^2
Acidobacteria	11.419 ^a	4.989	0.351 ^b	0.390	0.006	0.406	0.029	0.263	0.0217	0.293
Actinobacteria	7.754 ^a	3.721	1.611 ^b	1.616	0.028	0.265	0.051	0.213	0.027	0.277
Bacteroidetes	2.109 ^a	1.317	0.550 ^b	1.576	0.136	0.139	0.266	0.077	0.068	0.196
BRC1	0.007 ^a	0.009	0.001 ^b	0.001	0.038	0.239	0.126	0.135	0.191	0.112
Caldiserica	0.00004	0.00015	n.d	n.d	0.22	0.155	0.664	0.015	0.28	0.092
Chlamydiae	0.381 ^a	0.204	0.041 ^b	0.048	0.014	0.322	0.041	0.248	0.025	0.274
Chlorobi	0.000137	0.00028	n.d	n.d	0.078	0.175	0.754	0.005	0.457	0.036
Chloroflexi	1.174 ^a	0.931	0.155 ^b	0.181	0.069	0.193	0.034	0.251	0.017	0.311
Cyanobacteria	0.072 ^a	0.126	0.002 ^b	0.003	0.03	0.229	0.227	0.08	0.089	0.197
Deferribacteres	0.001	0.002	n.d	n.d	0.394	0.046	0.62	0.015	0.01	0.325
Firmicutes	24.392 ^a	7.608	71.281 ^b	18.187	0.03	0.256	0.05	0.223	0.073	0.187
Gemmatimonadetes	0.272 ^a	0.157	0.089 ^a	0.091	0.060	0.199	0.217	0.096	0.028	0.267
Lentisphaerae	0.001	0.001	n.d	n.d	0.365	0.054	0.002	0.509	0.153	0.126
Nitrospira	0.295 ^a	0.300	0.011 ^b	0.014	0.247	0.081	0.009	0.359	0.007	0.379
OD1	0.001	0.002	n.d	n.d	0.128	0.141	0.857	0.002	0.044	0.229
OP10	0.027 ^a	0.014	0.004 ^b	0.007	0.064	0.2	0.048	0.224	0.317	0.062
OP11	0.005	0.007	0.000	0.000	0.048	0.224	0.187	0.1	0.123	0.151
Planctomycetes	2.551 ^a	1.268	0.178 ^b	0.196	0.014	0.327	0.016	0.315	0.016	0.314
Proteobacteria	26.521 ^a	13.895	19.909 ^b	16.594	0.62	0.016	0.344	0.057	0.871	0.002
Spirochaetes	0.004	0.004	n.d	n.d	0.146	0.127	0.021	0.292	0.153	0.12
SR1	0.00024	0.00049	n.d	n.d	0.09	0.174	0.628	0.015	0.28	0.073
TM7	0.003 ^a	0.003	0.00013 ^b	0.00026	0.024	0.271	0.786	0.005	0.266	0.077
Unclassified	12.830 ^a	4.624	5.602 ^b	3.152	0.373	0.05	0.29	0.071	0.136	0.135
unclassified_Bacteria	0.053 ^a	0.043	0.003 ^b	0.004	0.032	0.253	0.31	0.065	0.263	0.079
Verrucomicrobia	9.989 ^a	4.400	0.204 ^b	0.215	0.004	0.424	0.011	0.336	0.007	0.367
WS3	0.139 ^a	0.147	0.008 ^b	0.012	0.266	0.079	0.05	0.215	0.0114	0.356

3.4.2: *Effect of aggregate-size fraction:*

In the soil profile as a whole, no main effect of fraction was seen ($P=0.283$) on bacterial OTU abundance at the phylum level. Within individual horizons, there was a significant effect of fraction on bacterial OTU abundance ($P=0.039$ and $P=0.0174$ in H1 and H2 respectively). The MAC and MIC aggregates were significantly different in this regard in H1 ($P<0.001$, Figure 13; top). The MAC and MIC fractions in H1 also differed significantly in terms of H' (\log^e) ($P=0.003$) and J' ($P=0.005$). In contrast to H1, a significant main effect of aggregate-size was seen on the abundance of bacterial phyla between the MAC and SC fractions differing significantly ($P=0.036$, Figure 13; bottom). The bacterial communities within these two aggregate-sizes were also different in two of the diversity measures J' ($P<0.001$) and H' (\log^e) ($P<0.001$).

On the family level a significant main effect of aggregate-size was seen on bacterial abundance in the soil profile as a whole ($P=0.035$), as well as within each individual horizon (H1 $P=0.006$; H2 $P=0.002$). Within H1 the MIC fraction was significantly distinct from MAC ($P=0.002$), as was seen at the phylum level. In addition, MAC and MIC were distinct in two diversity measures J' and H' (\log^e) ($P=0.002$ and $P=0.001$). Furthermore, though not differing in abundance, LM and MAC within H1 were significantly distinct in both J' and H' loge diversity measurements. In H2, both LM and SC ($P=0.024$ and $P=0.002$ respectively) were significantly different from MAC, with these three also differing in terms of J' diversity measurement and the latter two also differing in terms of H' (\log^e) ($P=0.005$). In contrast to H1, no significant difference was seen between the MAC and MIC in abundance or on any diversity measure for either taxonomic level, for H2. There was also no significant effect of site on abundance or on any measurement of diversity.



Aggregate sized fraction

Figure 13: Bar-chart of averages of Abundance [%] of sequences allocated to major bacterial Phyla (cut-off 0.01 %) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in H1 (top) and H2 (bottom). Different letters indicate statistical significance (P<0.05). Arrows included to aid visualization.

Table 6: Abundance [%] of sequences allocated to major bacterial Families (cut-off 0.001%) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in Horizon 1. Different letters indicate statistical levels of significance between aggregate-size fractions (P<0.05).

Phylum	Family	LM		MAC		MIC		SC		
		% Avg.	±	% Avg.	±	% Avg.	±	% Avg.	±	
Acidobacteria	Holophagaceae	0.001 ^{ab}	0.001	0.016 ^a	0.023	0.001 ^b	0.001	0.032 ^{ab}	0.063	
	unclassified	2.714 ^a	5.921	8.238 ^b	7.068	0.372 ^a	0.274	4.216 ^{ab}	5.736	
Actinobacteria	Acidimicrobiales	0.190 ^{ab}	0.307	0.516 ^a	0.467	0.083 ^b	0.074	0.431 ^{ab}	0.547	
	Actinomycetales	1.378 ^{ab}	1.359	2.794 ^a	1.468	0.730 ^b	0.357	2.445 ^{ab}	2.563	
	Solirubrobacterales	1.691 ^a	2.424	2.434 ^a	2.376	0.936 ^a	0.955	2.395 ^a	2.690	
	unclassified	0.369 ^a	0.480	0.679 ^a	0.959	0.141 ^a	0.142	0.545 ^a	0.676	
Bacteroidetes	Chitinophagaceae	0.129 ^{ab}	0.216	0.252 ^a	0.254	0.015 ^b	0.012	0.136 ^{ab}	0.215	
Chloroflexi	unclassified	0.596 ^a	1.247	1.265 ^a	1.973	0.173 ^a	0.195	1.028 ^a	1.331	
Firmicutes	Alicyclobacillaceae	7.342 ^a	16.124	5.168 ^a	7.734	2.923 ^a	4.858	3.807 ^a	10.848	
	Bacillaceae	8.836 ^a	10.978	10.033 ^{ab}	8.641	19.683 ^{ab}	12.518	17.922 ^b	12.227	
	Clostridiaceae	2.006 ^a	2.793	1.176 ^a	1.422	4.013 ^a	2.775	2.632 ^a	1.814	
	Incertae_Sedis_XVIII	7.944 ^b	9.841	0.917 ^a	1.522	8.309 ^b	8.101	6.968 ^{ab}	11.918	
	Paenibacillaceae	22.603 ^{ab}	21.100	6.984 ^a	9.478	17.720 ^b	12.510	11.281 ^{ab}	12.573	
	Peptococcaceae	1.349 ^a	1.705	0.629 ^a	1.252	0.359 ^a	0.237	0.403 ^a	0.541	
	Peptostreptococcaceae	0.179 ^a	0.202	0.370 ^a	0.286	0.494 ^a	0.402	0.318 ^a	0.225	
	Planococcaceae	0.634 ^a	0.565	1.283 ^b	0.701	2.206 ^b	1.370	1.989 ^b	1.107	
	Ruminococcaceae	0.413 ^a	0.405	0.495 ^a	0.745	0.879 ^a	0.694	0.445 ^a	0.294	
	unclassified	19.170 ^a	11.652	16.609 ^a	14.653	23.279 ^a	8.986	17.842 ^a	13.320	
	Veillonellaceae	0.285 ^a	0.489	0.237 ^a	0.507	0.206 ^a	0.227	0.155 ^a	0.105	
	Gemmatimonadetes	Gemmatimonadaceae	0.185 ^a	0.211	0.500 ^a	0.412	0.236 ^a	0.328	0.265 ^a	0.244
	Planctomycetes	Planctomycetaceae	1.041 ^a	1.909	3.212 ^b	2.814	0.717 ^a	0.444	1.884 ^{ab}	1.967
	Proteobacteria	Burkholderiaceae	0.958 ^a	1.806	0.068 ^a	0.113	0.011 ^b	0.026	0.007 ^b	0.013
Cystobacterineae		0.198 ^a	0.233	0.429 ^a	0.620	0.194 ^a	0.151	0.337 ^a	0.327	
Hyphomicrobiaceae		0.726 ^a	0.867	1.806 ^a	1.486	0.651 ^a	0.426	1.194 ^a	1.082	
Nannocystineae		0.154 ^a	0.162	0.350 ^a	0.259	0.098 ^a	0.071	0.220 ^a	0.252	
Sinobacteraceae		0.128 ^a	0.321	0.554 ^b	0.820	0.017 ^a	0.019	0.076 ^{ab}	0.121	
Sorangiiineae		0.289 ^{ab}	0.228	0.450 ^a	0.292	0.208 ^b	0.116	0.334 ^{ab}	0.240	
unclassified		2.041 ^{ab}	3.744	6.171 ^a	5.340	0.768 ^b	0.277	3.497 ^{ab}	4.531	
Xanthomonadaceae		2.748 ^a	5.596	0.081 ^a	0.076	5.436 ^a	10.386	0.428 ^a	0.864	
unclassified		unclassified	11.019 ^{ab}	10.166	18.456 ^a	10.687	4.215 ^b	4.417	11.117 ^{ab}	10.000
Verrucomicrobia	unclassified	1.575 ^a	3.275	4.186 ^b	3.821	0.797 ^a	0.660	2.739 ^{ab}	3.382	
WS3	unclassified	0.176 ^a	0.435	0.188 ^a	0.286	0.002 ^b	0.002	0.197 ^a	0.370	

Table 7: Abundance [%] of sequences allocated to major bacterial Families (cut-off 0.001%) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in Horizon 2. Different letters indicate statistical significance in abundance between aggregate-size fractions (P<0.05).

	Family	LM		MAC		MIC		SC	
		% Avg.	±	% Avg.	±	% Avg.	±	% Avg.	±
Acidobacteria	Holophagaceae	0.001 ^a	0.001	0.001 ^a	0.001	0.006 ^a	0.018	0.002 ^a	0.003
	unclassified	0.364 ^{ab}	0.142	0.143 ^a	0.245	0.552 ^{ab}	0.482	1.702 ^b	2.683
Actinobacteria	Actinomycetales	0.626 ^{ab}	0.112	0.340 ^b	0.457	1.342 ^{ac}	1.025	2.474 ^c	2.224
	Solirubrobacterales	1.023 ^{ab}	0.967	0.391 ^a	0.619	1.109 ^{ab}	0.936	2.104 ^b	2.036
Chloroflexi	unclassified	0.125 ^a	0.107	0.083 ^a	0.172	0.151 ^a	0.133	0.616 ^a	0.950
Firmicutes	Alicyclobacillaceae	2.475 ^{ab}	4.272	16.008 ^a	20.001	5.691 ^{ab}	10.179	1.467 ^b	4.206
	Bacillaceae	31.640 ^a	11.355	4.975 ^b	6.717	8.754 ^b	8.604	22.898 ^a	10.494
	Clostridiaceae	5.086 ^a	1.332	1.620 ^b	3.380	3.002 ^{ab}	2.920	3.547 ^a	2.861
	Incertae_Sedis_XI	0.138 ^a	0.084	0.058 ^{ab}	0.128	0.016 ^b	0.018	0.058 ^a	0.043
	Incertae_Sedis_XVIII	0.367 ^a	0.496	15.649 ^b	13.045	5.463 ^{ab}	6.152	1.619 ^a	3.879
	Lachnospiraceae	0.311 ^a	0.017	0.092 ^b	0.192	0.113 ^{abc}	0.116	0.165 ^c	0.112
	Paenibacillaceae	9.936 ^a	7.584	13.905 ^a	7.930	19.977 ^a	13.921	15.029 ^a	13.084
	Peptococcaceae	0.511 ^b	0.075	0.156 ^a	0.309	0.972 ^{ab}	1.132	0.494 ^b	0.361
	Peptostreptococcaceae	0.700 ^b	0.151	0.139 ^a	0.212	0.253 ^{ab}	0.253	0.463 ^b	0.365
	Planococcaceae	5.712 ^a	6.502	0.703 ^b	0.867	1.127 ^b	0.628	2.430 ^a	1.151
	Ruminococcaceae	0.812 ^{ab}	0.106	0.408 ^a	0.839	0.499 ^{ab}	0.501	0.633 ^b	0.303
	unclassified	29.014 ^a	7.731	28.431 ^a	8.243	26.923 ^a	15.313	22.914 ^a	11.734
	Veillonellaceae	0.162 ^{ab}	0.047	0.054 ^a	0.097	0.338 ^{ab}	0.362	0.410 ^b	0.550
	Gemmatimonadetes	Gemmatimonadaceae	0.118 ^{ab}	0.098	0.016 ^a	0.012	0.165 ^{ab}	0.134	0.200 ^b
Planctomycetes	Planctomycetaceae	0.882 ^{ab}	0.239	0.318 ^b	0.491	0.279 ^b	0.147	2.039 ^a	2.815
Proteobacteria	Burkholderiaceae	0.877 ^a	1.752	1.434 ^a	2.468	0.079 ^a	0.226	0.734 ^a	2.195
	Cystobacterineae	0.145 ^b	0.021	0.044 ^a	0.075	0.120 ^{ab}	0.112	0.196 ^b	0.145
	Hyphomicrobiaceae	0.594 ^{ab}	0.272	0.317 ^a	0.328	0.657 ^{ab}	0.398	1.041 ^b	0.835
	Sorangineae	0.185 ^a	0.065	0.041 ^c	0.052	0.183 ^{ab}	0.113	0.338 ^b	0.140
	unclassified	0.662 ^a	0.289	0.666 ^a	0.683	0.996 ^a	0.985	1.712 ^a	2.279
unclassified	unclassified	6.046 ^a	4.669	4.954 ^a	4.739	9.416 ^a	8.754	6.884 ^a	4.608
Verrucomicrobia	unclassified	0.686 ^{ab}	0.345	0.259 ^a	0.384	0.425 ^{ab}	0.308	2.430 ^b	3.981

3.4.3: The allocation of bacterial phyla and families between and within the various aggregate-sizes:

Of the 21 phyla reported in H1, 16 were shared among all the aggregate sizes. Only the SC fraction harboured phyla which were not found in other aggregates (Deferribacteres and Spirochaetes), while the phyla BRC1 and OP11 were found within SC, LM and MAC with OD1 shared between SC and the MAC. Of the 123 families reported in H1, 83 were shared among all aggregates. Of the 40 families which are not shared among all fractions only seven (Desulfuromonadaceae, Enterobacteriaceae, Granulosicoccaceae, Incertae_Sedis_XIV, Rickettsiaceae and unclassified group in the family Chromatiales found in MAC and Alcaligenaceae found in MIC) were not found solely in the SC fraction or are shared with the SC fraction and one of the other aggregates. The SC fraction harboured eight unique families (Campylobacteraceae, Cryomorphaceae, Deferribacterales_incertae_sedis, Hydrogenophilaceae, Leptospiraceae, Methylophilaceae, Rikenellaceae and Spirochaetaceae) while sharing eleven with LM and MAC and eight solely with MAC. While only the Sporolactobacillaceae is shared with LM, MIC and SC five families (Caryophanaceae, Eubacteriaceae, GpI, Puniceicoccaceae Syntrophomonadaceae) were shared among SC, MIC and MAC (Figure 14; top panel).

No phyla were unique to any one of the aggregate-sizes in H2, with 14 of the 17 phyla detected shared among all of them. Nitrospira and BRC1 were shared among SC and the MIC and LM fractions respectively, with WS3 being shared among all three. A total of 106 families were detected in H2, 64 of them were shared among all the aggregate-sizes. Only eight of the remaining 42 families were not found in either solely in the SC or shared among the SC and the other aggregate-sizes, five of these were found exclusively in MIC; Campylobacteraceae, Methylophilaceae, Moraxellaceae, Rhodobacteraceae and an

unclassified group in the family Rhodospirillales. A total of seven (Alcaligenaceae, Cytophagaceae, Nitrosomonadaceae, Pseudomonadaceae, Saprospiraceae, Verrucomicrobiaceae and an unclassified group in the Flavobacteriales family) families were found exclusively in the SC. An additional 14 were shared between SC and MIC with Desulfobulbaceae shared between it and LM. A further four families (Caryophanaceae, Rhodocyclaceae, and unclassified groups in the Cyanobacteria and WS3 families) shared between these three aggregates (Figure. 14; bottom panel). All of the phyla and families which were not shared among all of the aggregate-sizes in both horizons had an average relative abundance of below 0.1%.

3.4.4: Interaction between horizon and aggregate-size fraction:

Horizon depth had a significant effect on bacterial OTU abundance of the aggregate-size fractions at both taxonomic levels (phylum $P=0.037$; family $P=0.004$). Analysis of the variation in abundance at the family level apportioned to horizon and aggregate-size did indicate that horizon did explain a greater amount of the variation (\sqrt{CV} : 10.35) than did fraction (\sqrt{CV} : 9.99). There was a significant interaction between aggregate-size and horizon in terms of abundance on both the phylum ($P=0.004$) and family levels ($P=0.001$).

The MAC fraction was most influenced by horizon, with there being a significant change in the abundance of bacterial OTUs in this fraction between horizons for both taxonomic levels, with a significant change in all phyla (over 0.01% abundance) with the exception of OP10 and Proteobacteria. There was also a significant difference in most diversity measures in MAC between horizons at both taxonomic levels, with the exception being Margelef's richness at the phylum level. Indeed, the MAC fraction in H2 did differ in bacterial abundance in both of the taxonomic levels from SC in both horizons. Furthermore, the MAC fraction from H1 differed from the MIC fraction in H2 in terms of abundance of OTUs (phyla: $P=0.01$; family: $P=0.009$). A summary of the pairwise tests in the abundance of bacterial OTUs between different aggregate-sizes within horizons is provided in Table 8, with an additional table summarizing the pairwise tests of the diversity measurements provided in the supplementary materials.

Table 8: Summary table showing the level of statistical difference between the aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in horizon 1 (H1) and horizon 2 (H2). Statistically significant ($P < 0.05$) pairwise tests are highlighted in bold.

Pairwise	Taxonomic level	
	Phylum P-value	Family P-value
LM H1 vs. MAC H1	0.0839	0.082
LM H1 vs. MIC H1	0.437	0.372
LM H1 vs. SC H1	0.7001	0.364
LM H1 vs. LM H2	0.5351	0.128
LM H1 vs. MAC H2	0.0902	0.213
LM H1 vs. MIC H2	0.9251	0.964
LM H1 vs. SC H2	0.7823	0.226
MAC H1 vs. MIC H1	0.0004	0.002
MAC H1 vs. SC H1	0.197	0.216
MAC H1 vs. LM H2	0.0285	0.014
MAC H1 vs. MAC H2	0.0011	0.002
MAC H1 vs. MIC H2	0.0067	0.010
MAC H1 vs. SC H2	0.067	0.052
MIC H1 vs. SC H1	0.1592	0.132
MIC H1 vs. LM H2	0.6336	0.158
MIC H1 vs. MAC H2	0.1671	0.056
MIC H1 vs. MIC H2	0.3872	0.616
MIC H1 vs. SC H2	0.1063	0.159
SC H1 vs. LM H2	0.3277	0.334
SC H1 vs. MAC H2	0.0362	0.006
SC H1 vs. MIC H2	0.2433	0.181
SC H1 vs. SC H2	0.709	0.900
LM H2 vs. MAC H2	0.105	0.024
LM H2 vs. MIC H2	0.2521	0.111
LM H2 vs. SC H2	0.2389	0.331
MAC H2 vs. MIC H2	0.0777	0.139
MAC H2 vs. SC H2	0.0048	0.002
MIC H2 vs. SC H2	0.1447	0.084

3.4.5: *Effect of occluded SOC:*

There was no significant main effect of the quantity of occluded SOC within aggregates on bacterial community structure in either H1 or H2. Lesser abundant phyla, however, were significantly correlated namely the Chlorobi ($P=0.037$; $R^2=0.236$) in H1. On the family level in H1, the Chlorobiaceae ($P=0.034$; $R^2=0.236$) and Methylobacteriaceae ($P=0.049$; $R^2=0.115$) were significantly correlated to SOC. No individual phyla were correlated to occluded SOC in H2, though on the family level the Burkholderiaceae ($P=0.049$; $R^2=0.147$), Halomonadaceae ($P=0.036$; $R^2=0.21$), Puniceicoccaceae ($P=0.036$; $R^2=0.16$), Acidomicrobia_incertae_sedis ($P=0.033$; $R^2=0.166$), Bacteriovoracaceae ($P=0.0464$; $R^2=0.139$), Commonadaceae ($P=0.0499$; $R^2=0.131$) and Natranaerobiaceae ($P=0.036$; $R^2=0.21$) were.

3.5: Discussion:

A significant effect of horizon was observed in the BS samples of this study, as has been previously reported in bacterial community structures in grassland soils using a next-generation sequencing technique (Will et al. 2010). The relative abundance of most phyla declined between H1 and H2 with only the Firmicutes increasing in abundance with depth. Subsoils are typically colder with lower rates of gaseous exchange (Barber et al. 2004) compared to the topsoil. As a result bacterial numbers decline and preferentially colonize at or around air-pores (Nunan et al. 2003). The significant correlation to C was unsurprising as SOC becomes increasingly metabolically expensive to breakdown due to the increase in inherent recalcitrance (Kramer and Gleixner 2008) with total C previously being reported to significantly decline with depth in these sites (Chapter II).

Additionally, the bacterial community was correlated to soil P and Mg levels. Soil bacteria are known to be important in P transformations in grasslands (Fox et al. 2014), with P fertilizer additions being shown to alter bacterial community composition (Leff et al. 2015). Thus, the reduction in soil P with depth will impact on the abundance of certain bacterial groups as they may no longer be able acquire sufficient P to meet their physiological requirements (Čapek et al. 2016; Chapter II). As seen in chapter II with the T-RFLP data a significant correlation to Mg and bacterial community structure was seen, with this most likely the result of legacy effects of previous agricultural management and nutrient additions or a collinear relationship of some other environmental variable. The most prevalent phyla, the Firmicutes was significantly correlated to both % C and P indicating that dominant bacterial groups are sensitive to prevailing soil physiochemical conditions to the same extent as lesser bacterial groups, which would contrast with the suggestion put forward by (Davinic et al. 2012).

In the BS and each of the constituent aggregate size fractions in both horizons, the phylum Firmicutes is highly prevalent in terms of its abundance. While the extent of the abundance of the Firmicutes was a surprise, it is not without precedent in grassland soils. In a study of a peaty, acid grassland in the Netherlands, 65% of all bacterial ribosomes from a clone library study originated from this phylum, with the *Bacillus* being seen as the most active component of the bacterial community (as determined by bacterial 16S rRNA)(Felske et al. 1998). A later study at the same Dutch site also confirmed the high prevalence of Firmicutes (Felske et al. 2000). The Firmicutes were also the most abundant bacterial phylum in two Dutch chalk grasslands at approx. 20-25 % (as measured by 16S rRNA-gene based microarrays)(Kuramae et al. 2010), similar to its abundance in the BS of H1 in this study. The sites used in this study were predominantly used for extensive grazing of livestock. Thus the long term addition of livestock excreta may also have played a role in the abundance of Firmicutes in these sites, as

they make up a large component of the bacterial community in ruminant waste (Durso et al. 2010; Rice et al. 2012).

Aggregate-size fraction did significantly impact the bacterial community composition, as was reported in our last study using a PCR fingerprinting technique. In H1, the relative abundance of the Firmicutes declines in the MAC compared to the MIC fraction. This is likely due to increased predation within MAC. Despite being gram-positive (which are believed to be less preferred by protozoa due to their protective cell wall) the abundance of Firmicutes has been shown to decrease upon the addition of an amoeba predator (Rosenberg et al. 2009). While nematodes have been previously reported not to predate the most abundant bacteria present in soil (Ladygina et al. 2009), the tortuosity of the inner MAC fraction may allow the lesser abundant bacteria to escape predation by occupying niches inaccessible to the bacterivorous predators (Heijnen et al. 1993). The dominance of the Firmicutes would imply that they occupy all niches, thus being easily predated. The MIC fraction, in contrast, is characterized by a low predation pressure (Ranjard and Richaume 2001). Furthermore, the Acidobacteria were found to be enriched in the MAC fraction compared to the MIC, which concurs with the study from Mummey et al. (2004). Bacterivorous predation is known to be a major driver in the community structure of the Acidobacteria (Naether et al. 2012) and it may be that they are benefitting from the reduced abundance of the Firmicutes. Furthermore, studies have demonstrated that the MIC represents an operationally defined microenvironment for the soil microbiota (Kong et al. 2011; Poly et al. 2001). While the inner MIC microhabitat has been described as an oligotrophic environment due to limited O₂ permeability (Mummey et al. 2006), it does have a relatively stable water potential, and has restricted access for external toxic elements (Poly et al. 2001).

The majority of bacterial OTUs from both taxonomic levels were shared among all aggregate-sizes, with all the phyla and families not shared among the four aggregates having a relative abundance of less than 0.1%. It has been previously suggested that the lesser abundant bacterial groups are more important at differentiating the bacterial community at the aggregate scale (Davinic et al. 2012), and that is indeed the case here. The majority of the 'unique' bacterial groups were found either exclusively within the SC or shared with the SC and one or more of the larger aggregates-sizes, likely derived from the silt and clay proportion within these aggregates. Previous studies have reported a distinct bacterial (and fungal) community within the SC (Sessitsch et al. 2001; Chapter II), with the distinct microhabitat the SC provides in soil influencing microbial community structure.

The Deferribacteres, exclusive to the SC fraction in H1, are characterised by their preference for anaerobic respiration (Alauzet and Jumas-Bilak 2014). Some bacterial groups within the Spirochaetes (also found in exclusively in SC) are also reportedly anaerobic and have been implicated in the breakdown of complex organic polymers (Droge et al. 2006). Of the eight families unique to this fraction, only the Leptospiraceae and Methylophilaceae are not known to be anaerobic (or contain anaerobic genera) (Doronina et al. 2014; Picardeau 2014). This suggests that many of the bacterial groups unique to SC may be physiologically adapted to deal with the specificities of the SC microenvironment, characterized as it is by highly protected SOC and limited O₂ availability (Sexstone et al. 1985). In H2, more families were shared between MIC and SC than any of the other aggregate-sizes indicating that with increasing depth these two aggregates may become increasingly physio-chemically analogous. With the majority of bacterial groups being shared across all aggregate-sizes, these results indicate that the aggregate spectrum has a greater influence on bacterial abundance than on harbouring distinct bacterial groups.

The significant interaction between aggregate-size and horizon indicates that horizon is a strong determinant of bacterial community structure in aggregates as it is in the BS. This corroborates with our previous study which reported the same effect. The influence of horizon was solely felt in the MAC fraction. The increase in the abundance of the Firmicutes in the MAC fraction in H2 is possibly as a consequence of the decline in bacterivorous predators reducing predation pressure. Protozoan abundance has been shown to decline rapidly with depth, more so than bacteria (Ekelund et al. 2001). The significant reduction in most of the other phyla in MAC with depth indicates that they are being out-competed by the Firmicutes for resource acquisition and habitable niche space.

Soil C cycling rates are influenced by aggregate disruption (e.g. grassland conversion to tillage, aggregate slaking due to frost), temporal fluctuations in temperature and soil moisture content as well as the structure soil food-web network and how this interacts with soil structure (Bronick and Lal 2005; Zahn et al. 2016). The higher rates of predation within the MAC fraction would lead to greater C turnover within this fraction compared to MIC, in line with the ‘microbial-loop’ concept in relation to nutrient mineralization (Adl and Gupta 2006). The oligotrophic conditions within both MIC and SC would lead to reduced rates of microbial activity, favouring the presence of slower degradative enzymatic pathways and thus slower SOC decomposition (Davidson and Janssens 2006). This, coupled with increased steric hindrance (and in the case of SC clay sorption) leading to lower enzyme efficiency, leaves the C within these fractions less prone to microbial attack. There was no significant main correlation of occluded SOC on the bacterial community composition within aggregates. It has previously been reported that SOC composition rather than quantity is a greater determinant of bacterial community structure within aggregates (Davinic et al. 2012). Some lesser abundant bacterial phyla and families (<0.1%) were significantly correlated to

SOC quantity, however, indicating C availability within aggregates is more of a constraint on these groups than on more abundant bacterial groups (Davinic et al. 2012).

3.6: Conclusion:

This study was a follow on from the earlier work done in Chapter II. The findings reported here largely corroborate this earlier study, with a significant effect of aggregate size and horizon being seen as well as significant interaction between the two. The study highlights, however, the importance of utilizing next generation sequencing techniques over the more traditional fingerprinting methods to garner in-depth information on bacterial communities in the soil environment. Studies in the future need to take an interdisciplinary approach to elucidating mechanisms of terrestrial C sequestration; incorporating both biological and physiochemical parameters. Such studies should include the functionality and composition (bacteria, fungi and archaea) of the soil microbiome, food web interactions between the different trophic levels, soil chemistry (in particular C, N and P), structure (aggregation and depth) and processes (e.g, clay illuviation).

**Chapter IV: In-field slurry applications
resulted in soil microbiota short term response
to added carbon while microbial community
structures were only sensitive to seasonal
successions.**

4.1: Abstract:

This study investigated the incorporation of slurry derived carbon (C) into the soil matrix and its effects on the composition and activity of the soil bacteria and fungi (microbiota). A 180 m² area field site was divided into 36 random treatment plots (2 m x 1.5 m). Slurry (8% dry matter) was splash-plate applied at a rate of 30 t ha⁻¹. Sampling was conducted 5, 30 and 65 days after application. The activity of the soil microbes was examined using eight C-cycling enzymes (α -mannosidase, β -xylosidase, cellobiosidase, α -arabinosidase, β -galactosidase, β -glucosidase, α -glucosidase and β -N-acetyl-glucosaminidase). Microbial diversity was analyzed via bacterial 16S rRNA gene and fungal internal transcribed spacer region (ITS) based PCR and subsequent terminal restriction fragment length polymorphism (T-RFLP). A significant increase in enzymatic activity with slurry treatment was reported on days 5 and 65, indicating a staged response of the microbiota to slurry-derived C with the utilization of labile C on day 5 and the more stable C on day 65. In contrast, T-RFLP data revealed that only bacterial community structures were significantly affected by slurry application on day 5, while bacterial and fungal communities significantly shifted their structure through the progressing season (day 5, 30 and 65). These findings suggest that soil microbial communities are responding to carbon supply from slurry applications via enhanced microbial activity but remain largely stable in their structure whereas other environmental factors have a greater impact.

4.1.2: Highlights:

- Staged response in enzymatic activity to slurry addition
- Microbial biomass-N (but not -C) significantly affected by slurry addition
- Microbial community composition largely unresponsive to slurry application.

4.1.3: Keywords: Bacteria, Fungi, T-RFLP, soil enzymatic activity, carbon

4.2: Introduction

Pasture-based livestock production is the most practiced agricultural activity in Ireland. Animals are typically kept out on pasture between March and November and are then over-wintered indoors. This is typically to protect the soil from trampling and compaction during the wetter winter months. This over-wintering produces a large quantity of slurry. Slurry is a heterogeneous mixture of animal faeces, urine, small amounts of bedding and washings/rainwater. The application of this slurry back onto the land is an imperative practice for the recycling of nutrients in Irish agricultural systems (Holden et al. 2004).

Slurry derived C is typically classified into labile and recalcitrant fractions. These fractions are differentially incorporated into the soil matrix (Rochette et al. 2000). The labile C fraction (e.g. simple carbohydrates, amino and organic acids) is incorporated within a few days after slurry application while the more recalcitrant C fraction (cellulose, lignin) is incorporated over a period of weeks (Dungait et al. 2009; Rochette et al. 2000). This dynamic will correspondingly affect the soil microbial community. Initially it is hypothesized that there will be a large increase in microbial (particularly bacterial) biomass as the autochthonous r-strategists utilise the labile C fraction as an easily available energy source (Fontaine et al. 2003). As this energy source becomes exhausted, the microbial community will shift towards the zymogenous K-strategists who will utilise the recalcitrant C source. It is these K-strategists which may be responsible for the observed 'priming effect' after labile C additions, in which soil organic carbon (SOC) is utilized in addition to the added C (Chen et al. 2014). Thus, the fate of slurry derived C and the dynamic of the SOC pool is heavily mediated by the soil microbiota.

The soil-slurry-micro-biota interface is largely unknown. Harris and colleagues (2011) synergised the varying interconnected aspects of this interface and the consequences for biogeochemical cycling. A major hypothesis is that the site input history (i.e. N, P and K fertilizer regime, previous slurry application) will influence the composition of the soil microbial community as well as its functional capacity. Long-term inputs into a site will alter the r/K strategist abundance ratio. This resulting microbial community configuration will be a determining factor in the immediate microbial response to slurry input, with differing community configurations responding differently to the same or differing slurry types. Further, the C substrate quality, the C:N ratio and the constituent microbial community of the applied slurry will be determining factors in slurry induced priming (Morvan et al. 1996). Soil physiochemical parameters such as soil moisture, pH, temperature and soil organic C content are also integral factors to be considered.

The effect of slurry application to the bacterial and fungal community structure is poorly reported in the literature and this is especially the case for field applications. The effect of cow slurry on bacterial community structures in soils under laboratory conditions has been recently reported, where changes in the communities were seen. However, these effects were either not reported with adequate replication (Peacock et al. 2001) or at application rates (66 g kg^{-1}) which exceeded agricultural practices (Yousefi et al. 2008). Consequently, the effect of slurry applications to the field under agricultural practice conditions on the soil bacteria and fungi is still largely elusive.

This experiment aimed to determine the temporal response of both the composition and functionality of the soil microbiome to the application of slurry derived carbon on the field scale. As slurry will only become incorporated in the top 2-5 cm of the soil profile (Bourdin,

2014) coupled with the fact that the bulk of microbial activity will be seen in the first 10 cm (Richter, 2016), this experiment (and subsequently Chapter V) did not sample the soil profile in the manner described in Chapters II and III.

4.3: Materials and Methods:

4.3.1: Field site set-up:

The experimental field site was located at Teagasc Johnstown castle, Wexford, Ireland (52°18'N; 6°30'W). The site is a well-drained coarse loam over fine loam, classified as Haplic Cambisol (WRB 2006) (Typical Brown Earth; Simo et al. 2014), with an established (>10 yr) and uniform ryegrass (*Lolium perenne*) sward. The average rainfall and temperature at this site (averaged over 25 yr 1978-2003) are 1044 mm and 10°C (Bourdin et al. 2014). In 2009, site was used in experimental field trials regarding slurry application (Bourdin et al. 2014). From 2010-2013, site was used for the light grazing of livestock. In 2010, there were two applications of N (CAN) at 27 kg h⁻¹ (May) and 20 kg h⁻¹ (July). In September 2012, the plot received 14.18 kg h⁻¹ of N (CAN). In April 2013, 30.3 kg h⁻¹ of urea was applied to the plot, while the following September it received a further 27.63 kg h⁻¹ of N (CAN). Prior to the start of the experiment (April 2014), soil samples (10 cm depth) were taken from around the perimeter of the designated experimental area to determine particle size distribution, pH, C and N content as well as levels of phosphate (PO₄³⁻) and sulphate (SO₄²⁻).

In May 2014, grass was cut to 5 cm height on a 180 m² area which was divided into 40 plots (2 m x 1.5 m with a 0.5 m gap between each; Bourdin et al., 2014; Figure 15 A). The site was left for 1 week to allow time for the soil microbial community to stabilize after grass cutting. Plots were set out in a randomized block design. On the 9th of May 2014, slurry (8.18 % dry matter) was spread simulating the splash-plate method (Bourdin et al. 2014; Figure 15 B) at a

rate of 30 t ha⁻¹ on 18 of these plots. Slurry (41.80 % C; 2.50 % N; 49.5 ppm PO₄³⁻ and 11.1 ppm SO₄²⁻) applied was collected from beef cattle fed a ryegrass silage which had a C/N ratio of 16.72 and pH of 7.61. The remaining plots received no slurry and acted as controls. The variable in this experiment was the time after slurry addition that the plots were sampled. There were three sampling times: 5 days, 30 days and 65 days after slurry application. At each sampling event, six of the slurry-amended plots were sampled in conjunction with six control plots.

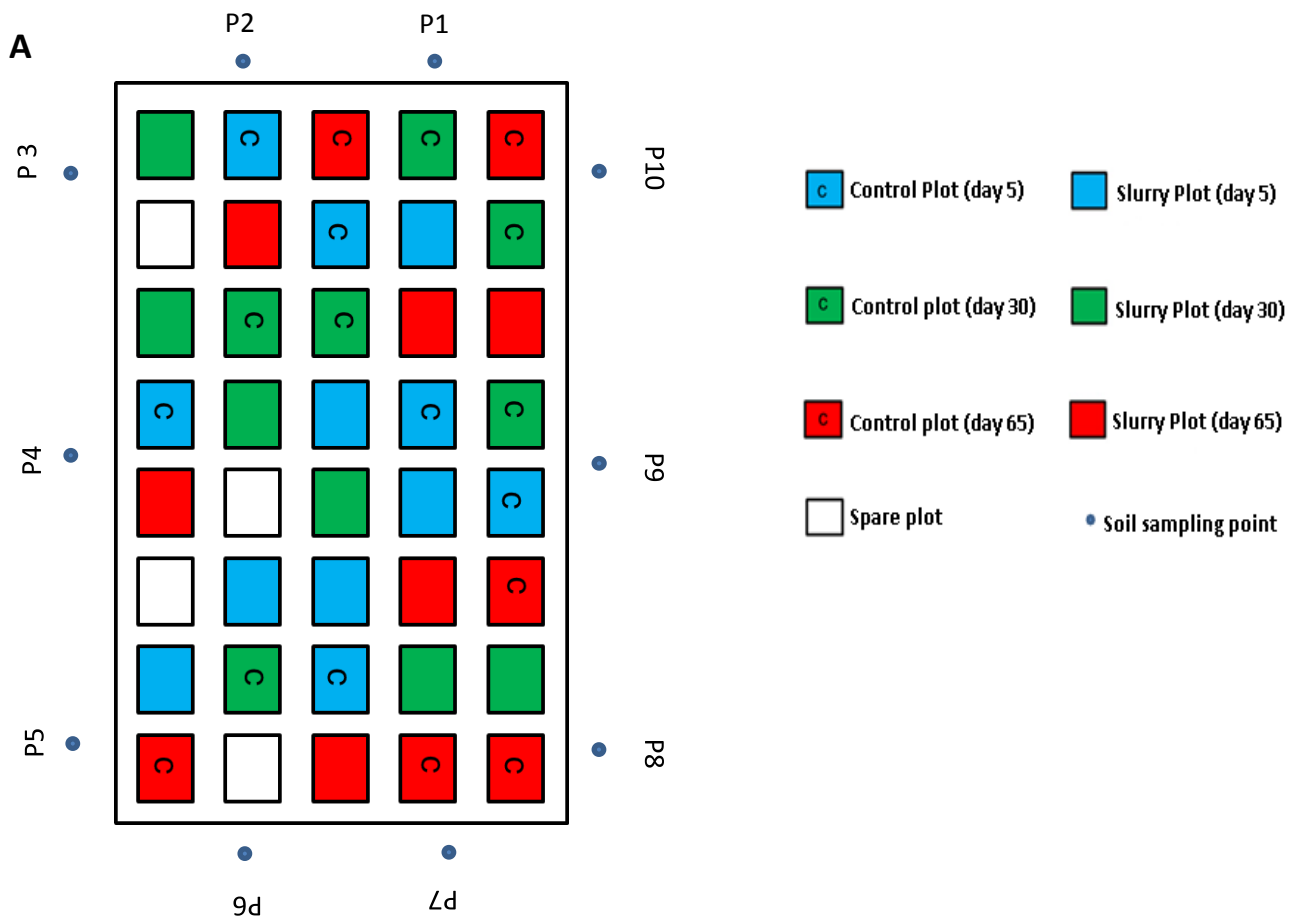


Figure. 15: **A:** Experimental field layout. Points P1-P10 were sampled before experiment to determine field characteristics. **B:** Simulated splash plate slurry spreading technique (image courtesy of Nynke Hoekstra).

4.3.2: *Sampling strategy:*

After above ground vegetation was removed, each plot was sampled in five separate points in a 'W' pattern using a 7 cm Dutch auger to a depth of 10 cm. Approximately 600 g of soil was collected from each plot and placed in sterile bags. The auger was disinfected after being washed thoroughly with 70 % (v/v) ethanol and sterile water between samplings. These were stored on ice until transported back to the lab (max. 2 h) where samples were stored at 4°C. In the lab, samples were mixed and sieved (2 mm) and a sub-sample was frozen at -80°C for subsequent molecular work.

4.3.3: *Edaphic and environmental properties:*

Measurements of total carbon (% C), total nitrogen (% N), pH and gravimetric soil water content (θ_d) were conducted as per the protocols developed by the Irish SIS laboratory protocols (Massey et al. 2014). The quantity of SOC was also measured on a LECO Truespec CN analyser. SOC samples were first fumigated with 10% hydrochloric acid for 6 h under vacuum (Harris et al. 2001). Water extractable phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) was determined via ion chromatography using a Dionex ICS1100 with an AS23 column and a carbonate mobile phase as recommended by the manufacturer (Sunnyvale, CA; Fox et al. 2016).

4.3.4: *Enzymatic assays of C-cycling enzymes:*

Extra-cellular enzymatic activity was determined on 8 different fluorogenic model substrates; 4-Methylumbelliferyl (4-MUF)- α -D-mannopyranoside, 4-MUF- α -L-arabinopyranoside, 4-MUF- β -D-Cellobioside, 4-MUF- β -D-xylopyranoside, 4-MUF-N-acetyl- β -D-glucosaminide, 4-MUF- β -D-galactopyranoside, 4-MUF- β -D-glucopyranoside and 4-MUF- α -D-glucopyranoside (all Sigma Aldrich, St. Louis, MO). 5 g of fresh soil was placed into 50

ml of sterile dH₂O and enzymes were extracted by shaking on a Gyrotory shaker (New Brunswick Scientific) at 150 rpm for 10 min, followed by centrifugation at 750 rpm for 10 min at 4°C. 200 µl of this supernatant was pipetted into individual wells of black microtiter plates (VWR, Radnor, PA). The plates had initially received 10 µl of MOPS (3-(N-morpholino) propanesulfonic acid sodium salt) buffer (pH 7.4) as well as 40 µl of the analogous MUF-substrate (at a final concentration of 50 µM). For standard curve calculations, MUF-salt (4-methylumbelliferone) in four concentrations was added (Hendriksen et al. 2016; Johansen et al. 2005). Plates were incubated at 25°C and allowed to settle for 30 min before the fluorescence derived from the liberated substrates was measured (wavelength at 360 nm excitation and 460 nm emission) on a modulus microplate reader (Promega, Madison WI) at 15 min intervals for a further 60 min. Extracellular enzymatic activity (µmol h⁻¹ g⁻¹ dry soil) was calculated as described in Hendriksen et al. (2016).

4.3.5: Determination of microbial biomass C and N and basal respiration:

A 10 g of subsample of fresh soil was subjected to chloroform fumigation for 24 h, with a duplicate sample not being subject to fumigation (Vance et al. 1987). Microbial biomass- C (MBC) and -N (MBN) were extracted from both samples using 4:1 (vol/wt) of 0.5 M K₂SO₄. Total organic carbon (TOC) and total N of the extracts was measured on a Shimadzu TOC-V CPH Organic Carbon and Nitrogen Analyser (Shimadzu Corporation, Japan). The MBC and MBN values were then calculated by the subtraction of the extracted C and N from the fumigated and un-fumigated samples. This value was then divided by a predefined conversion factor (K_{ec}) with a value of 0.45 and 0.54 being used for MBC and MBN respectively (Brookes et al. 1985; Wu et al. 1990).

Samples were pre-incubated at 25°C for 1 week prior to basal respiration (BR) analysis (Creamer et al. 2014). After this time had elapsed, 10 g of soil was added to a 100 ml serum bottle and capped with a rubber septum and the system was pressurized using 20 cm³ of laboratory air. Bottles were incubated at 25°C for 6 h with a 7 cm³ sample being taken from the head-space after 2 and 6 h and inserted into pre-evacuated vials. The gas vials were analysed using a Varion CP-3800 Gas Chromatograph (Varion Creek, Walnut Creek, CA). The CO₂ concentration of each sample was calculated using Star Chromatography Workstation (2004), with the rate of CO₂ formation being determined using the calculations set out in Creamer et al. (2014).

4.3.6: Soil DNA extraction, amplification and analysis:

DNA was extracted using the Powerlyzer version of the Powersoil DNA isolation kit (MO BIO laboratories, Cupertino, CA). The bacterial 16S rRNA gene and fungal internal transcribed spacer region (ITS) were amplified for terminal restriction fragment length polymorphism (T-RFLP) using the primer pair 27F and 1492R (Lane 1991) and ITS-1F and ITS-4 (Gardes and Bruns 1993; White et al. 1990), respectively using PCR and restriction digest conditions described in chapter II (sections 2.3.4; 2.3.5 and 2.3.6).

Fragment sizes were determined on an applied Biosystems 3730 XL DNA analyser, using the LIZ500 size marker. Resultant electrophoretograms were imported into Genemapper (version 3.7, ABI, UK), and terminal restriction fragments (TRFs) binned with a 2 bp interval. TRFs between 80 and 500 bp were included in the analysis and a presence/absence matrix was generated.

4.3.7: *Quantitative PCR:*

Quantitative PCR (qPCR) was conducted to quantify the number of 16S rRNA gene and ITS spacer region copies per gram of soil. Quantification of 16S rRNA genes was performed using the eubacterial primers 341F (5'CCTACGGGAGGCAGCAG'3) and 518R (5'-ATTACCGCGGCTGCTGG-3'; Muyzer et al. 1993) while quantification of the fungal ITS region was achieved using the primer pair ITS-1 and ITS-4. PCR reactions were carried out with a Maxima SYBR green master mix (Fermentas, Germany) in a qPCR microtitre plate (Sarstedt, Nuembrecht, Germany) using a Lightcycler[®] 480 II (Roche Diagnostics Ltd. Burgess Hill, UK) under conditions described in the supplementary materials. Standards (10^2 - 10^8 molecules per reaction) were prepared as described previously (Schmalenberger et al. 2013). Results were expressed as gene copy number per gram of soil, normalized to soil DNA extraction yield.

4.3.8: *Statistics:*

All statistics were performed in PRIMER-E (Version 7, Plymouth, UK). Individual environmental variables were Log (X+1) transformed and a resemblance matrix based on Euclidian distance was constructed. The effect of treatment and time on these variables was determined via permutational multivariate analysis of variance (PERMANOVA) (Monte Carlo 9999 permutations). The data obtained from the 8 enzymatic assays were imported together and transformed (fourth root) and normalized, while the data from MBC, MBN and BR were imported separately and transformed (Log X+1). A resemblance matrix was made of each (Euclidian distance), and the PERMANOVA analysis of the tested parameters was conducted. Separate Bray-Curtis resemblance matrices were made of the bacterial and fungal T-RFLP (non-transformed) and qPCR (Log X+1 transformed) data and they were also subjected to the PERMANOVA analysis (Clarke and Warwock 2001). A distance based linear

model (DistLM, marginal tests, 9999 permutations) was run against a resemblance matrix (Euclidian distance) of the environmental variables (all variables imported together, 4th root transformed and normalized) against each of the biological variables.

4.4: Results

4.4.1: Edaphic and environmental factors:

There was a main effect of slurry application on pH ($P < 0.001$) throughout the course of the experiment, with the control and slurry treatment differing on day 5 (4.98 and 5.29 respectively; $P = 0.0001$), day 30 (4.96 and 5.12 respectively; $P < 0.001$) and day 65 (4.94 and 5.12 respectively; $P = 0.003$). There was also a significant interaction between treatment and time on soil pH ($P = 0.012$), while the pH of the control treatment was not significantly altered over the course of the experiment, the pH of the slurry amended treatment did significantly shift between day 5 and day 30 ($P < 0.001$) and day 5 and 65 ($P = 0.004$). There was also a significant interaction between time and treatment for soil θ_d ($P = 0.001$). On day 65, the control treatment had a significantly lower θ_d than it did on both day 5 and day 30 (both $P = 0.001$), while the slurry treatment had a significantly altered θ_d at each sampling time (all $P = 0.001$). While there was no main effect of treatment for this variable, the θ_d value was significantly influenced by treatment on day 5 ($P = 0.024$).

There was a significant main effect of slurry application on soil phosphate (PO_4^{3-} ; $P = 0.017$), with a significant increase reported in the slurry amended soil (11.44 mg/g) over the control (6.61 mg/g) on day 5 ($P = 0.002$). No significant difference was reported between the plots which received slurry and the control on days 30 or 65. The levels of sulphate (SO_4^{2-}) in soil did not significantly differ between the slurry and control plots on any of the sampling days. There was, however, a highly significant temporal effect on this variable ($P < 0.001$), with the

levels from both the slurry treated and the control plots on day 65 (47.99 and 50.22 mg/g, respectively) and day 30 (42.7 and 46.24 mg/g, respectively) significantly differing from day 5 (17.28 and 21.54 mg/g, respectively). Additionally, while there was no main effect of treatment or time on these variables throughout the course of the experiment, there was a significant effect of slurry application on day 5 for % C (P=0.045), SOC (P=0.036) and % N (P=0.036). The C/N ratio was also not significantly affected by treatment or time in this experiment (Table 9).

Table 9: Averages (Avg.) and Standard deviations (\pm) of environmental variables: gravimetric water content (θ_d), pH, total Carbon (% C), total Nitrogen (% N), soil organic carbon (SOC), carbon to nitrogen ratio (C/N), phosphate (ppm PO_4^{3-}) and sulfate (ppm SO_4^{2-}) from replicate field plots from both the control and slurry treatment on day 5, 30 and 65. Different letters indicate statistical difference (P<0.05) between slurry treated and control plots at one sampling period.

	Day 5				Day 30				Day 65			
	Control		Slurry		Control		Slurry		Control		Slurry	
	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
θ_d	0.36 ^a	0.02	0.4 ^b	0.03	0.35 ^a	0.04	0.29 ^a	0.06	0.2 ^a	0.02	0.19 ^a	0.03
pH	4.98 ^a	0.05	5.29 ^b	0.05	4.96 ^a	0.05	5.12 ^b	0.05	4.94 ^a	0.06	5.12 ^b	0.07
% C	3.8 ^a	0.35	4.3 ^b	0.43	4.09 ^a	0.31	4.06 ^a	0.66	3.83 ^a	0.14	3.97 ^a	0.5
% N	0.34 ^a	0.02	0.39 ^b	0.04	0.37 ^a	0.03	0.37 ^a	0.05	0.35 ^a	0.02	0.35 ^a	0.04
% SOC	3.63 ^a	0.35	4.06 ^b	0.33	3.97 ^a	0.39	3.72 ^a	0.32	3.65 ^a	0.12	3.82 ^a	0.53
C/N	10.56 ^a	0.75	10.53 ^a	0.41	10.77 ^a	0.67	10.12 ^a	1.61	10.51 ^a	0.53	11.04 ^a	1.04
ppm PO_4^{3-}	6.61 ^a	1.89	11.44 ^b	2.37	9.22 ^a	1.87	10.04 ^a	1.67	8.69 ^a	0.85	8.89 ^a	2.51
ppm SO_4^{2-}	21.54 ^a	4.68	17.28 ^a	3.08	46.24 ^a	5.99	42.7 ^a	4.54	50.22 ^a	5.22	47.99 ^a	5.5

4.4.2: Enzymatic assays:

There was a significant main effect of slurry application on total enzymatic activity (TEA) throughout the course of this experiment ($P=0.0009$). A significant increase in TEA upon slurry application was reported on both day 5 of the experiment and again after 65 days (both $P=0.0001$). No significant increase, however, was seen in TEA in the slurry amended treatment above the control on day 30 ($P=0.155$).

When looking at enzymes individually, β -glucosidase ($P=0.0218$), cellobiosidase ($P=0.0478$) and α -mannosidase ($P=0.0001$) showed a significant enzymatic activity increase in the slurry amended treatment compared to the control on day 5 (Figure. 16; top panel). No individual enzyme showed a significant increase in activity between the two treatments on day 30 of the experiment ($P>0.05$, Figure. 16; middle panel). While on day 65, β -glucosidase ($P=0.02$), β -N-acetyl-glucosaminidase ($P=0.002$), cellobiosidase ($P=0.03$), α -mannosidase ($P<0.001$) and β -galactosidase ($P=0.03$) displayed a significant increase in activity in the slurry amended treatment above the control (Figure. 16; bottom panel).

A highly significant effect of time ($P=0.0002$) was also reported on TEA, with the activity rate on day 5 being significantly different from that seen on day 30 ($P=0.0104$) and day 65 ($P=0.0035$). Additionally, there was also a significant difference seen in the enzymatic activity rate between day 30 and 65 ($P=0.0001$). Time explained a greater amount of variation seen in TEA (\sqrt{CV} : 1.95) than did treatment (\sqrt{CV} : 1.66) with the TEA being significantly correlated to θ_d , pH and PO_4^{3-} (summarized in Table 11).

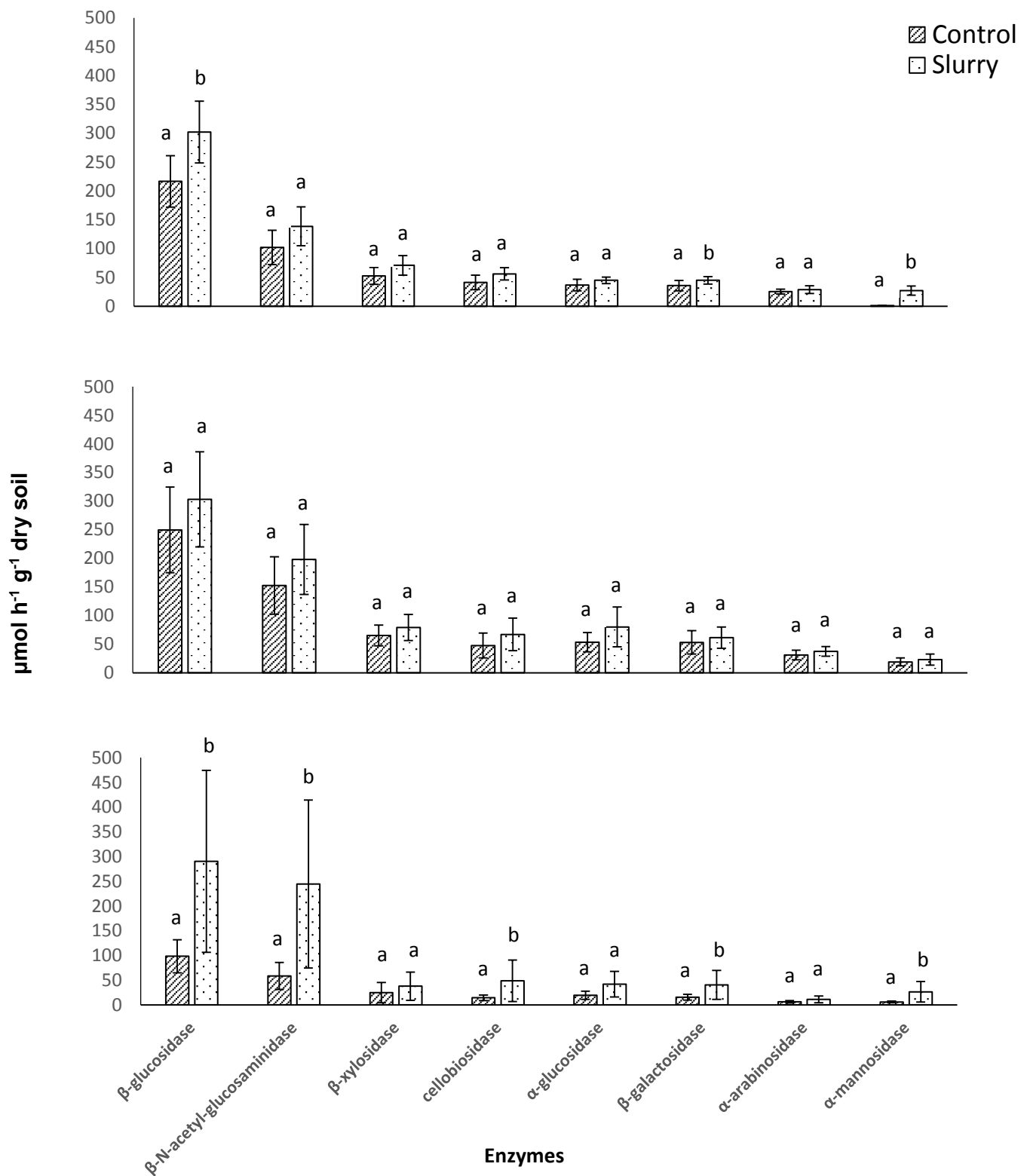


Figure. 16: Enzymatic assays of β -glucosidase, β -N-acetyl-glucosaminidase, β -xylosidase, cellobiosidase, α -glucosidase, β -galactosidase, α -arabinosidase, and α -mannosidase, from the control (grey bars) and slurry (striped bars) amended from day 5 (top panel), 30 (middle panel) and 65 (bottom panel). Error bars are standard deviation. Different letters indicate statistical difference between slurry treated and control plots for each enzyme

4.4.3: Microbial biomass-C, -N and Basal Respiration:

An overall main effect of slurry application was not observed on MBN ($P=0.43$, Figure 17), MBC ($P=0.4258$) or BR rate ($P=0.2699$). A significant increase in MBN however, was observed in the slurry amended treatment over the control on day 5 ($P=0.0311$), while it was significantly lower than the control on day 65 ($P=0.046$). Both MBC and BR were significantly influenced by time ($P=0.001$ and $P=0.0162$, respectively), while MBN on the other hand was not ($P=0.256$). Neither MBC nor the BR measurement differed significantly between day 5 and day 30 ($P=0.2219$ and $P=0.6708$, respectively), both did however differ between days 5 and 65 ($P=0.0002$ and $P=0.0048$, respectively) and days 30 and 65 ($P=0.0001$ and $P=0.0093$, respectively). All three variables were significantly correlated to θ_d , with MBC and MBN additionally correlated to SO_4^{2-} . There was also a significant correlation between MBN and pH (summarized in Table 11).

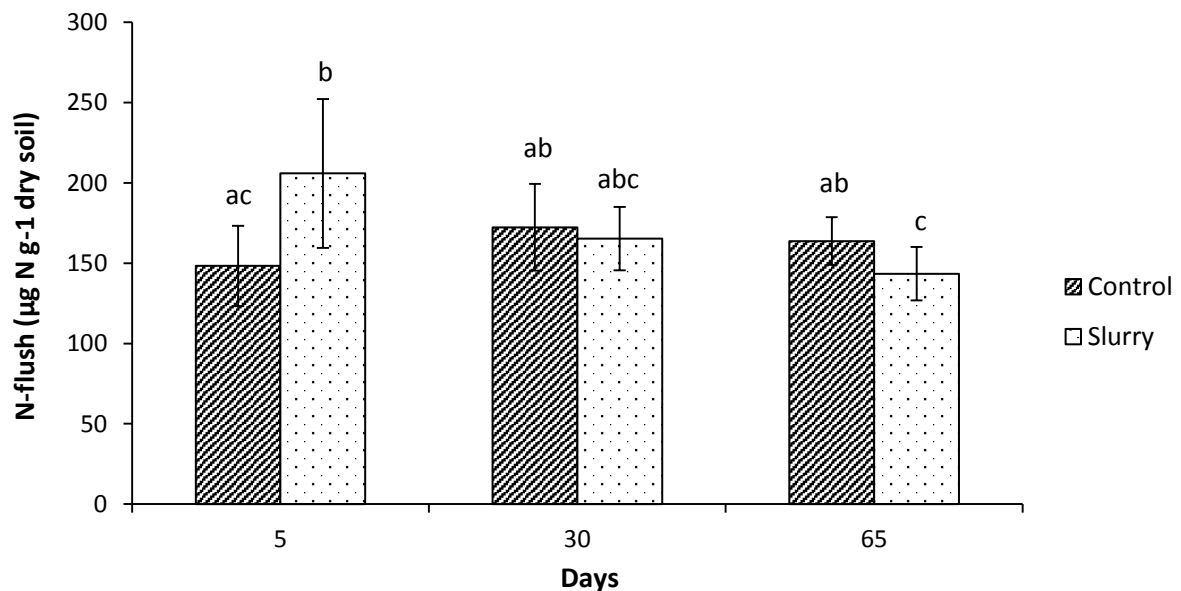


Figure. 17: Microbial biomass-N from the control (stripped bars) and slurry amended treatments (dotted bars) on day 5, day 30 and day 65. Error bars are standard deviation. Different letters indicate statistical difference ($P<0.05$) between slurry treated and control plots and different sampling times.

4.4.4: Bacterial and fungal community composition:

There was no significant main effect of slurry amendment on either bacterial or fungal community composition. Nevertheless, a significant shift in bacterial community composition between the slurry amended and control treatments on day 5 ($P=0.042$) was observed, though no significant change was reported between the two on day 30 ($P=0.268$) or day 65 ($P=0.308$) (Figure. 18; top panel). Fungal community composition was not significantly affected by slurry treatment at any time point (Figure. 18; bottom panel). Microbial community structure, however, was affected by time as both bacterial and fungal communities were significantly different on day 5 compared to day 65 ($P=0.001$ and $P=0.024$, respectively).

There was a significant increase in bacterial 16S gene copy number (GCN) in the slurry amended plots ($1.16 \times 10^{10} \text{ g}^{-1}$) over the control ($9.37 \times 10^9 \text{ g}^{-1}$) seen on day 5 ($P=0.049$). No such increase was seen between the two on day 65 ($P=0.2646$). Fungal ITS GCN was also not significantly affected by slurry application on day 5 ($P=0.9212$) or 65 ($P=0.054$) (Table 3). There was a significant effect of seasonal progression both bacterial 16S and fungal ITS GCN (both $P<0.001$), though both responded to this differently. There was a significant increase in the 16S GCN in both the control and slurry amended plots between day 5 and 65 (both $P<0.001$). Contrastingly, while there was no significant change in ITS GCN in the control plots between days 5 and 65. There was, however, a highly significant reduction seen in the slurry amended plots between day 5 ($3.90 \times 10^7 \text{ g}^{-1}$) and 65 ($1.23 \times 10^7 \text{ g}^{-1}$; $P<0.001$). Both bacterial and fungal community composition and GCN were significantly correlated to both θ_d and SO_4^{2-} (results summarized in Table 11).

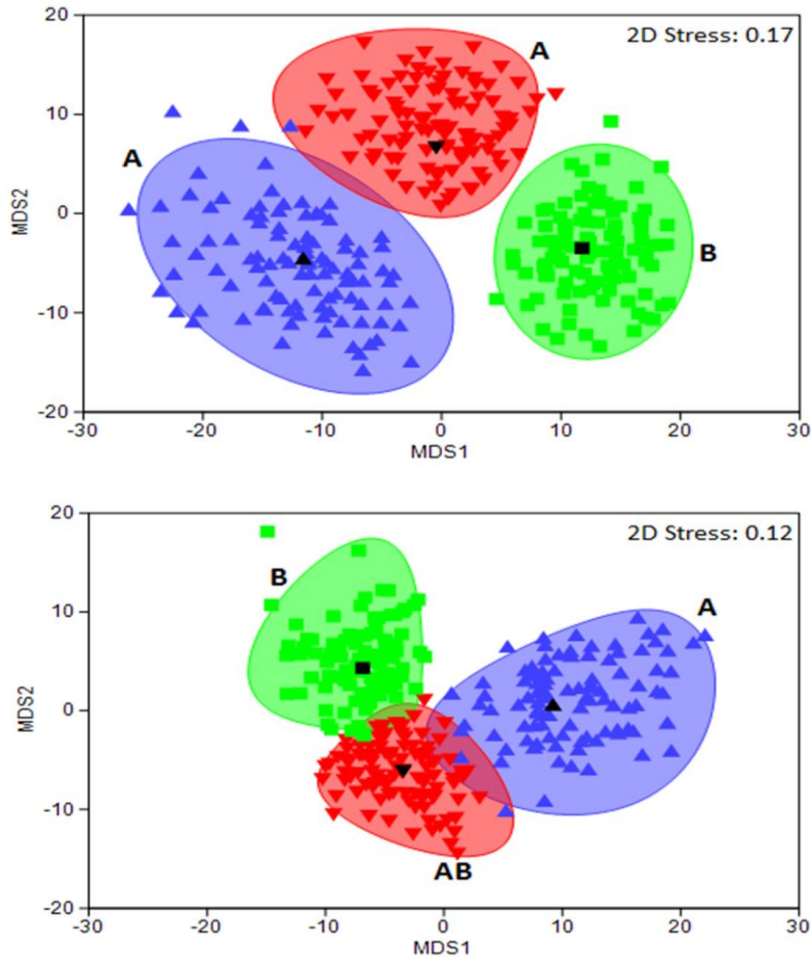


Figure 18: Bootstrapped non-metric multidimensional scaling plots of 16S rRNA based bacterial (top) and ITS region based fungal (bottom) community composition on day 5 (blue triangle), 30 (red triangle) and 65 (green squares). Different letters indicate statistical differences ($P < 0.05$). Black dots represent averages. A 2D stress of < 0.2 represents good graphical representation in two dimensions.

Table 10: Averages (Avg.) and standard deviations (\pm) of bacterial 16S rRNA and fungal internal transcribed spacer region gene copy number from both the slurry amended and control plots on day 5 and 65. Different letters indicate statistical difference between slurry treated and control plots at one sampling period.

	Day 5				Day 65			
	Control		Slurry		Control		Slurry	
	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
16S	9.37E+09 ^a	2.17E+09	1.16E+10 ^b	1.02E+09	1.87E+10 ^a	1.55E+09	1.99E+10 ^a	1.66E+09
ITS	4.23E+07 ^a	1.61E+07	3.90E+07 ^a	1.07E+07	2.66E+07 ^a	1.77E+07	1.23E+07 ^a	7.87E+06

Table 11: A distance based linear model (DistLM) correlations of the biological variables: microbial biomass C (MBC), microbial biomass N (MBN), basal respiration (BR), total enzymatic activity (TEA), bacterial 16S rRNA gene based community composition (BCC), fungal ITS region copy number (ITS GCN) (ITS) region based community composition (FCC), bacterial 16S rRNA gene copy number (16S GCN) and fungal ITS region copy number (ITS GCN) against the environmental variables measured in this study: gravimetric water content (θ_d), pH, total soil Carbon (% C), total soil Nitrogen (% N), soil organic Carbon (SOC), Carbon to Nitrogen ratio (C/N), water extractable phosphate (PO_4^{3-}) and water extractable sulfate (SO_4^{2-}). Significant correlations ($P < 0.05$) and the corresponding R^2 values are highlighted in bold.

	θ_d	pH	% C	% N	SOC	C/N	PO_4^{3-}	SO_4^{2-}								
	P-value	R^2	P-value	R^2	P-value	R^2	P-value	R^2								
MBC	0.0001	0.538	0.362	0.024	0.533	0.011	0.996	>0.001	0.605	0.008	0.566	0.01	0.34	0.028	0.0005	0.31
MBN	0.017	0.154	0.04	0.119	0.222	0.044	0.125	0.069	0.188	0.05	0.753	0.003	0.179	0.05	0.046	0.112
BR	0.0004	0.327	0.784	0.002	0.297	0.032	0.46	0.016	0.532	0.011	0.883	<0.0001	0.578	0.009	0.057	0.102
TEA	0.013	0.133	0.003	0.183	0.098	0.066	0.1094	0.062	0.271	0.035	0.812	0.008	0.007	0.153	0.114	0.006
BCC	0.03	0.058	0.372	0.03	0.204	0.038	0.245	0.036	0.581	0.024	0.764	0.019	0.814	0.017	0.006	0.075
FCC	0.047	0.079	0.592	0.028	0.755	0.021	0.437	0.036	0.863	0.016	0.591	0.027	0.166	0.058	0.003	0.125
16S GCN	0.0001	0.697	0.5584	0.016	0.4313	0.029	0.799	0.003	0.4491	0.027	0.4293	0.028	0.104	0.115	0.0001	0.747
ITS GCN	0.0015	0.371	0.7265	0.006	0.1	0.118	0.0573	0.151	0.2793	0.052	0.2972	0.048	0.9054	0.001	0.0031	0.340

4.5: Discussion:

The functionality of the microbiota responded in a staged manner to slurry derived C. The increase in TEA represents the microbial community utilizing the labile easily degradable slurry derived C on day 5 (Bol et al. 2003a; Sauheidl et al. 2005), with this C source being exhausted by day 30, with no significant difference being seen in SOC levels between the control and slurry amended plots. A further increase in enzymatic activity was seen on day 65. Previous studies have reported significant priming effects upon slurry addition (Bol et al. 2003b; Kuzyakov and Bol 2006). Indeed, a previous study on this site reported a significant native SOC loss upon slurry application (Bourdin et al. 2010). This suggests that by day 65, the soil microbiota has likely shifted to utilizing less labile, more complex slurry C or SOC (Rochette et al. 2000), and the increase in cellobiosidase suggests this. The microbiota N requirement may be a key driver in this response. The significant increase in MBN seen in the slurry amended plots on day 5 suggest that the microbiota are utilising the labile slurry-derived organic matter as a source of both C and N. Likewise the reduction in MBN in the slurry amended plots seen on day 65, suggests increased competition for N between the soil microbiota and the overlying ryegrass (Ghorbani et al. 2008), which may be driving the microbiota to mine the more complex slurry C and the SOC.

Such a scenario is in line with the the ‘nitrogen-mining hypothesis’ in which the microbiota will use the labile C as an energy source to decompose the surrounding SOC to mineralize organically bound nitrogen. This will increase the amount of organic N available, eventually meeting the microbial requirement for N (Chen et al. 2014; Janssen 1996; Knorr et al. 2005). A shift towards the mineralization of labile organic N after slurry application has been reported previously (Muller et al. 2011). This N acquisition is likely driven by the bacterial

component of the microbiota in this study as there was a significant correlation between bacterial 16S GCN and total soil N.

The MBC in both the control and slurry amended plots was significantly increased on day 65 compared with the two previous sampling points. The grass sward on the plots had re-established itself after the initial cutting in the latter month of the experiment, and this would stimulate an increased flux of rhizobial exudates which the soil microbiome can utilize as a labile C source (Meharg and Killham 1995). The increase in MBC on day 65 indicates that the microbiota may preferentially utilize this C source for incorporation into their biomass and ‘mine’ other C sources (such as slurry derived and native soil C) for other macronutrients such as N. Indeed, it has been previously reported that rhizodeposits act as the preferential substrate for microbes involved in SOM turnover and N cycling (Phillips et al. 2011).

Significant changes in bacterial community composition, bacterial 16S GCN and associated pH changes were observed between the control and slurry amended treatment on day 5. This may have affected bacterial abundance, as an increase in soil pH has been reported to be associated with increased microbial biomass (Aciego Pietri and Brookes 2008). While slurry derived bacteria may also be responsible for this change in community structure, it has been shown previously that populations of faecal bacteria have declined rapidly after manure application (Unc and Goss 2004). This trend is variable, however, with significant populations of faecal bacteria being shown to survive in soils for periods longer than the duration of this experiment (70 days; Unc and Goss, 2004). The short-term nature of this response though (coupled with the increase in MBN and TEA) suggests the change is most likely due to the proliferation of native soil microbes utilizing the labile C source, as a source of N (i.e, r-strategist shift; Fontaine et al. (2003)). In addition, the lack of a significant

increase in BR upon slurry addition at this time-point would indicate that the native soil bacteria are responding to the addition of slurry C through the proliferation of numbers rather than through the excessive utilization of substrate. This suggests a larger microbial community may be more metabolically efficient at decomposing C sources (Hopkins et al. 2014).

Previous studies reported an increase in soil microbial biomass C through the supply of C-rich organic compounds though this effect is not always significant, as was seen in this study. (Bol et al. 2003a; Rochette et al. 2000; Rochette et al. 2004a; Rochette et al. 2004b; Saviozzi et al. 1997). The high existing level of MBC in these soils may have accounted for this. There is evidence, however, that the cumulative effect of organic fertilizer on MBC can persist for several years after the last addition (Ginting et al. 2003). Furthermore, while a staged response to slurry application was seen in the enzymatic activity, there was no significant difference in the microbial community structure between the slurry treatment and control on day 30 or 65 (ie. No K-strategist shift). It therefore appears that due to the previous management history of this site (with slurry and inorganic N fertilizer additions in the years preceding the experiment), the soil microbial community may be already compositionally adapted to exploit external nutrient sources. Such a scenario has been suggested by Harris et al. (2011).

A main effect of time was seen on all biological parameters in this study (with the exception of MBN) and all were significantly correlated to θ_d , indicating that seasonal progression was a stronger driver of biological parameters than slurry application. The reduction in soil water content reported in this study will restrict the diffusion of nutrients within the soil pore space (Robertson et al. 1997). This will also limit the physiological capabilities of soil microbes,

thus driving microbial community structure (Ma et al. 2015). Soil water content fluctuation associated with season may thus be a strong driver of microbial community composition and functionality with respect to terrestrial biogeochemical cycling in grassland agroecosystems. Biological parameters (with the exception of TEA and BR) were also correlated to SO_4^{2-} . The increase in this variable seen on day 30 and 65 is thought to be due to the release of excess SO_4^{2-} following the breakdown organo-S sources (Gahan and Schmalenberger 2014). Thus the correlation seen here may be circumstantial.

4.6: Conclusions

A number of factors influence the functionality and composition of the soil microbiota to slurry applied C. Chief among these is the microbiotas own nutrient demand, but also it seems the previous management history and season (which determines soil moisture levels). Soil biology has been largely overlooked in the considerations of slurry application. These results indicate, however, that the soil microbiota play a key role in the utilization and incorporation of slurry derived nutrients into the soil matrix. Thus, further understanding and consideration of this role has the potential to significantly improve slurry management in pasture based systems.

**Chapter V: Response of various C-cycling
enzymes in aggregate sized fractions to varying
degrees of slurry application in two contrasting
soil types**

5.1: Abstract:

Pot experiments of ryegrass (*Lolium perenne*) were established on contrasting soil types (loam and sandy). Pots were treated with either a high (HS; 9%), medium (MS; 5%) or low (LS; 3.25%) dry matter slurry. A slurry free treatment acted as a control. Pots were sampled prior to the slurry being applied (day 0) and subsequently 30, 137 and 361 days after application. Soils were physically fractionated in order to obtain the large macroaggregate (2000 μm), macroaggregate (>250 μm), microaggregate, (<250 μm) and silt and clay (>53 μm) fractions respectively. Enzyme assays (β -xylosidase, cellobiosidase, α -arabinosidase, β -galactosidase, β -glucosidase, α -glucosidase and β -N-acetyl-glucosaminidase) were conducted on each isolated aggregate from each treatment. These results indicate a distinct level of potential extracellular enzymatic (EE) activity inherent to each aggregate-sized fraction, with the highest levels of potential EE activity being seen in the larger aggregates (i.e. >250 μm). This potential EE activity was also influenced by slurry application, with the corresponding increase in activity reflecting the incorporation of the added C into soil aggregates. Aggregate potential EE activity was also significantly influenced by experimental sampling point. These results highlight the need to integrate soil structural, management and temporal parameters when considering the microbially mediated cycling of carbon in agroecosystems.

5.1.1: Highlights:

- Elevated extracellular enzymatic activity was seen upon slurry amendment in both soil types in the bulk soil.
- Aggregate size had a significant impact on potential extracellular enzymatic activity.
- Slurry application also influenced the potential extracellular enzymatic activity within aggregates, with the activity reflecting the incorporation dynamic of the added carbon.
- Potential enzymatic activity was significantly influenced by sampling time.

5.1.2: Keywords: soil enzymatic activity, aggregate size fractions, carbon, slurry

5.2: Introduction

Chapters 2 and 3 of this thesis demonstrated that differing aggregate sizes do represent distinct microsites of microbial colonization. The spatial and temporal conditions driving the change in community composition will also be major determinants of microbial activity and by extension the turnover of terrestrial carbon (C). The localization of microbial extracellular enzymatic (EE) activity has also attracted much attention, with many studies in agreement that it is not uniform throughout the soil matrix (Allison and Jastrow, 2006; Bach and Hofmockel, 2014; 2016; Fansler et al. 2005; Marx et al. 2005). The intra-aggregate pore space environment represents the physio-chemical parameters which govern microbial activity (and thus biogeochemical cycling) primarily through substrate availability and O₂ and water permeability (Bach and Hofmockel 2014). This varied distribution of SOC and microbes caused by soil aggregation is a key determinant in the regulation of organic matter mineralization. While it has been well known for some time that it is difficult to interpret soil EE activities with respect to activities occurring in soil (Dick 1992), microbial EE activity are potentially sensitive indicators of changes to the biogeochemical makeup of SOC as well as the total C content of the soil (Fansler et al. 2005). Microbial EE activities can be sensitive indicators of the relationship between changes in soil physiochemistry and microbial metabolism, and thus C turnover (Bach and Hofmockel 2016).

Which aggregate-size fraction the C is stored in determines its long-term sequestration in grasslands (Fansler et al. 2005). According to the theory of aggregate formation, the most labile C pools will be associated with the larger aggregate sizes, whilst micro-aggregate and silt/clay associated C is more recalcitrant due to increased anaerobiosis and chemical bonding associated with these aggregate sizes (Oades et al, 1984). Large macroaggregates are rich in particulate residues derived from plants and fungi and may be highly susceptible to decomposition. The smaller aggregate fractions (the microaggregates and the silt and clay),

on the other hand, are characterised by increased humification and C:N ratios and thus will persist in soil for longer (Marx et al. 2005). The degree of C decomposition differs in the aggregate hierarchy, with C turnover times of about 15-50 years in larger (>250 µm fractions) and 100-300 years in microaggregates (von Lützow et al. 2007). This hierarchy would also induce a hierarchy of microbial activity at the spatial scale of the soil aggregate and it has been recognised that understanding biological processes at this is fundamental to optimise ecosystem function (Lavelle et al. 2004). It has been proposed that physiochemical factors are at play limiting the enzymatic potential in smaller aggregates (>250 µm, Allison and Jastrow, 2006). Accessibility to C substrate by either microbe or enzyme is an imperative requirement for decomposition (von Lützow et al. 2007). The physical structure of the aggregates themselves (i.e, the porosity) may result in the separation of EE from their substrates. Additionally, sorption of EE onto mineral surfaces may reduce actual enzymatic activity in soil in comparison to the enzyme assay measurements measured in the laboratory (Allison and Jastrow 2006).

Elucidating the mechanisms of soil C sequestration is essential if we are to optimise the management of our agroecosystems in terms of C sink capacity. The structural homogenization (i.e, sieving) of bulk soil samples employed in most studies in soil microbial ecology, however, obfuscates the influence the soil aggregates on microbial activity (Young and Crawford, 2004). Only a very small number of studies have examined aggregate enzymatic in managed agroecosystems (Bach and Hofmockel 2016; Lagomarsino et al. 2012; Schutter and Dick 2002). This experiment aimed to establish the relationship between aggregate size and the activity rates of 7 C-cycling enzymes in two contrasting soil types, a loam and sandy soil. A further aim was to clarify the influence that the addition of carbon (C)

through agricultural management (i.e, slurry addition) and temporal variation would have on the enzymatic activity rates within aggregates.

5.3: Materials and Methods

5.3.1: Soil and Slurry collection and preparation

Two contrasting soil types were collected from the research farm at Johnstown Castle, Co. Wexford, Ireland (52° 16'N, 06° 30'W) in May 2015. The sandy soil consisted of 75% coarse (2-0.2 mm) sand, 14 % fine (0.2-0.05 mm) sand 4% silt (0.05-0.002 mm) and 7% clay (>0.002 mm). In contrast, the loam soil consisted of 27% coarse sand, 22% fine sand, 32 % silt and 19% clay. Upon soil collection, soil clods were broken up and plant root material was removed by hand and sieved (8 mm). Slurry (3.5 L) was collected from beef cattle fed a diet of ryegrass silage in May 2015. Slurry DM and total C and N content were calculated as described previously. The initial slurry (9.13 % DM) was designated the 'high slurry'. This slurry was thoroughly homogenized and a 1 L subsample was diluted in 1 litre dH₂O (1:1 vol/vol). This resultant slurry was termed the 'medium slurry' (5.53 % DM). This slurry was in turn vigorously homogenized and a 1 L subsample was again diluted in 1 L dH₂O (1:1 vol/vol). The resultant slurry from this dilution was designated the 'low slurry' (3.25% DM). Slurries were stored at 4°C for two weeks prior to application.

5.3.2: Establishment of pot experiments

Pot experiments were established on the two contrasting soil types, with 900 g of soil being added per pot. Each pot was sown with 0.8 g of *Lolium perenne* seed. Pots were given 45 days in which the *Lolium* sward was allowed to become established. Once established, the

sward was cut back to a height of 5 cm, pots were left for an additional 5 days before the experiment commenced. Pots were either kept slurry-free as a control or treated with 60 g of the high (HS), medium (MS) or low (LS) DM slurry in replicates of four. Pots were kept in a glasshouse from May 2015-June 2016 and were subjected to ambient Irish temperatures (Figure 19). Pots were watered (100 cm³ per pot) at a rate of once every three days from May 2015-October 2015, once every seven days from November 2015-February 2016 and again once every three days from March-June 2016. Each treatment from each sampling point had replicate pots.

5.3.3: Soil Sampling

Pots were sampled prior to the slurry being applied (day 0; 3rd July 2015) and subsequently 31 (4th August 2015), 137 (17th November 2015) and 361 (June 28th 2016) days after this application. At each sampling point, the above-ground plant material was removed. The soil was sieved (8 mm) and large root materials were also removed. Soil was further homogenized (cone and quartering technique) and 150 g was subsampled for biological and chemical analysis of the bulk soil (BS). The remaining soil was dried at 40°C for 1 week. Measurement of total carbon (% C), total nitrogen (% N), pH and gravimetric soil water content (θ_d) in the BS were measured as per the protocols developed by the Irish SIS laboratory protocols (Massey et al. 2014). The aggregate isolation procedure was based on the 'wet-sieving' technique developed by Six et al. (1998) and described in greater detail in chapter II. Samples were fractionated twice, once to ascertain the % proportion each aggregate size constituted to the BS and secondly to isolate aggregates for enzymatic assays. Soil aggregates isolated for enzymatic assays were not subjected to the final drying step. Instead isolated aggregates were placed into sterilized centrifuge containers and centrifuged at 750 rpm for 10 min at 4°C, after which the supernatant was discarded.

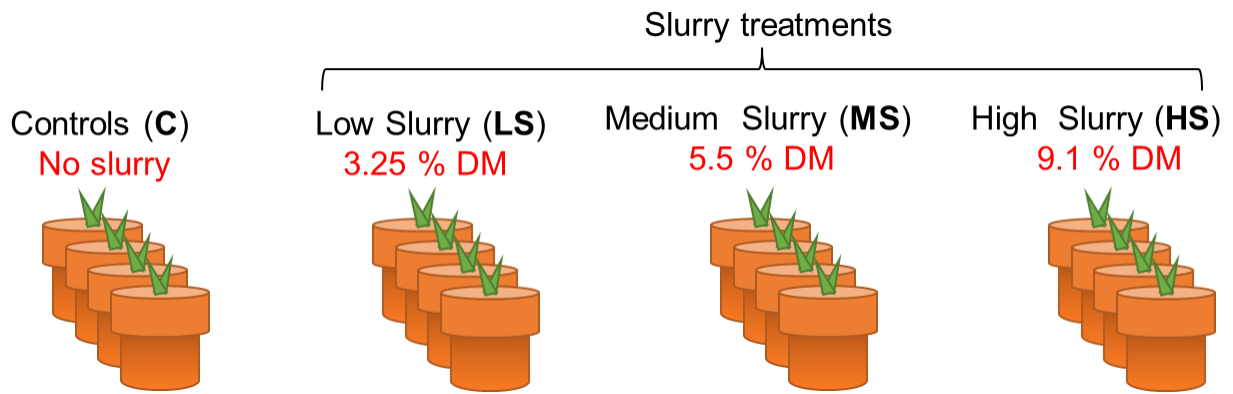


Figure 19: Diagrammatic representation of the treatments used in this experiment. Insert is a picture of the pot experiment on day 280.

5.3.4: Enzymatic assays of C-cycling enzymes

Extra-cellular enzymatic activity (EEA) was determined on 7 different fluorogenic model substrates; 4-MUF- α -L-arabinopyranoside, 4-MUF- β -D-Cellobioside, 4-MUF- β -D-xylopyranoside, 4-MUF-N-acetyl- β -D-glucopyranoside, 4-MUF- β -D-galactopyranoside, 4-MUF- β -D-glucopyranoside and 4-MUF- α -D-glucopyranoside (all Sigma Aldrich, St. Louis, MO). Assays were done on the BS and each of its constituent aggregate size fractions from each treatment. Enzymatic assays were conducted as described in chapter IV (section 4.3.4),

with the exception that 3 g of fresh soil was placed in 30 ml of sterile dH₂O. The EE activity from

both the BS and each aggregate size was expressed as $\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil as per the calculations outlined in Hendriksen et al. (2016). The potential aggregate EE activity was also expressed as a proportion of the activity of the whole soil activity using the following equation:

$$\text{EEA}_{(\mu\text{mol h}^{-1} \text{g}^{-1} \text{ dry soil})} * P_{(\text{aggregate fraction})}$$

Where EEA is the activity per gram of dry aggregate soil and $P_{(\text{aggregate fraction})}$ is the proportion of the whole soil mass comprised within the aggregate of interest (Fansler et 2005).

The levels of microbial biomass-C (MBC), -N (MBN) were conducted on BS samples using the chloroform fumigation extraction methodology described in chapter IV (section 4.3.5). Furthermore, basal respiration (BR) analysis was done on BS samples as per the protocol described in Creamer et al. (2014), further details of which have been previously provided.

5.3.5: Statistics

All statistics were performed in PRIMER-E (Version 7, Plymouth, UK). The MBC, MBN and BR variables from the BS were $\text{Log}(X+1)$ transformed. The % proportion each aggregate size made to the BS at each sampling point was also imported. A resemblance matrix based on Euclidian distance was constructed and the effect of treatment and sampling point on these variables was determined via permutational multivariate analysis of variance (PERMANOVA; Monte Carlo 9999 permutations). The data obtained from the 7 enzymatic assays from each were imported together and transformed (square root) and normalized, a resemblance matrix was made (Euclidian distance), and a PERMANOVA analysis of the

tested parameters (aggregate size, treatment and sampling point) was conducted (Clarke and Warwock 2001).

5.4: Results

5.4.1: Biological parameters of the bulk soil

In the loam soil, there was a significant effect of slurry treatment on enzymatic activity in the bulk soil on days, 30, 137 and 361 ($P < 0.05$). The three slurry amended treatments had significantly higher rates of enzymatic activity compared to the control treatment at each of the three time points, with the exception that there was no significant difference in activity between the LS and control treatment on day 361. Furthermore, there was a significantly increased level of enzymatic activity in the HS treatment over MS on day 30, and over the LS treatment on day 137. On day 361, each of the three slurry treatments had significantly different levels of enzymatic activity in the BS, with the highest activity seen in the HS (Table 12).

There was no difference in MBC between the control treatment and the three slurry amended treatments at on day 30. There was a significant increase, however, in MBN in the HS treatment compared with the control. On day 137, there was an increase in MBC in both the MS and HS treatment compared with the control, though no increase in MBN was seen between any of the slurry treatments and the control. At the final sampling point, no significant difference was seen in the MBC between any of the treatments, an increase in MBN was seen in MS and HS over LS (both $P = 0.001$), though not over the control. There was no difference in the rate of BR between the control and the three slurry amendments at any time-point, with the exception of an increase in MS over the control on day 361 ($P = 0.001$).

There was a significant increase in enzymatic activity in the bulk soil between the control and each of the three slurry amendments on days 30 and 137 (all $P=0.001$) for the sandy soil. On day 361, there was a higher level of enzymatic activity only in the HS compared with the control ($P=0.001$). The three slurry treatments did not differ in enzymatic activities on day 30, though a higher level of activity was reported in HS over LS on day 137 ($P=0.031$). At the final sampling point, both MS and HS had significantly higher activity levels when compared to LS ($P=0.001$ and $P=0.03$; Table 13). There was no difference in MBC or MBN in the bulk soil between any of the treatments on day 30. On day 137, there was a significant increase in both LS and MS compared to the control ($P=0.033$ and $P=0.001$ respectively) though there was no difference in MBN between all treatments at this time point. There was an increase in MBC in the MS and HS over the control on day 361 (T3, both $P=0.001$). There was also a significant increase in MBN in each of the slurry treatments over the control. There was no difference in BR between the control and the slurry amendments throughout the course of the experiment with the exception of a significant increase in LS over the control on day 30 ($P=0.001$).

Table 12: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from the bulk soil of the loam soil. Enzymatic assays were conducted on samples from the control treatment (C) and three slurry applications, low slurry (LS), medium slurry (MS) and high slurry (HS) at three sampling points 30, 137 and 361 days after slurry application. Different letters indicate significant difference at $P < 0.05$.

Time (days)	Treatment Enzyme	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
30	β -N-acetyl-glucosaminidase	8.688 ^a	2.963	66.322 ^b	35.953	71.131 ^b	36.082	71.097 ^b	22.158
	α -glucosidase	1.688 ^a	0.733	33.641 ^b	23.182	31.247 ^b	32.941	73.478 ^b	39.083
	α -arabinosidase	1.549 ^a	0.897	16.917 ^b	7.465	9.959 ^b	3.435	97.243 ^c	37.866
	cellobiosidase	0.804 ^a	0.381	14.631 ^b	5.431	11.083 ^b	6.041	2.546 ^c	0.423
	β -xylosidase	1.135 ^a	0.559	8.960 ^b	2.055	5.263 ^c	1.823	8.737 ^{bc}	2.844
	β -glucosidase	9.580 ^a	3.570	127.542 ^b	56.546	114.132 ^b	55.695	112.063 ^b	42.471
	β -galactosidase	2.162 ^a	0.551	27.072 ^b	11.900	19.300 ^b	7.258	25.394 ^b	10.650
137	β -N-acetyl-glucosaminidase	80.715 ^a	24.917	72.716 ^a	10.673	80.687 ^a	25.850	135.334 ^a	63.815
	α -glucosidase	33.343 ^{ab}	39.624	22.281 ^a	9.121	57.915 ^{ab}	42.528	66.707 ^b	37.179
	α -arabinosidase	8.192 ^a	2.738	8.930 ^a	5.053	6.673 ^a	3.350	7.058 ^a	5.155
	cellobiosidase	11.390 ^a	10.834	13.231 ^a	1.589	23.302 ^{ab}	14.201	45.142 ^b	25.034
	β -xylosidase	6.307 ^a	1.889	18.819 ^b	7.824	21.228 ^b	6.294	26.445 ^b	11.366
	β -glucosidase	19.499 ^a	5.060	42.526 ^b	10.687	83.741 ^{ab}	42.333	119.510 ^b	45.526
	β -galactosidase	9.470 ^a	2.245	26.396 ^{ab}	23.290	19.450 ^b	10.456	21.229 ^{ab}	10.481
361	β -N-acetyl-glucosaminidase	204.656 ^a	30.613	305.712 ^{ab}	101.283	322.076 ^b	30.643	847.700 ^b	718.892
	α -glucosidase	41.882 ^a	28.447	65.134 ^{ab}	41.398	111.083 ^{bc}	45.516	175.866 ^c	60.448
	α -arabinosidase	31.961 ^a	1.919	25.210 ^b	3.884	27.712 ^b	4.102	43.019 ^b	22.221
	cellobiosidase	30.520 ^a	21.427	32.539 ^a	16.010	54.663 ^a	15.672	189.324 ^b	62.189
	β -xylosidase	64.471 ^a	18.782	56.656 ^a	14.920	88.489 ^b	9.788	135.085 ^b	36.133
	β -glucosidase	164.474 ^a	63.265	281.273 ^{ab}	54.877	195.347 ^a	32.830	477.924 ^b	227.094
	β -galactosidase	35.140 ^a	6.677	43.344 ^{ab}	12.966	57.690 ^b	7.675	109.430 ^b	82.347

Table 13: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from the bulk soil of the sand soil. Enzymatic assays were conducted on samples from the control treatment (C) and three slurry applications, low slurry (LS), medium slurry (MS) and high slurry (HS) at three sampling points 30, 137 and 361 days after slurry application. Different letters indicate significant difference at $P < 0.05$.

Time(days)	Treatment Enzyme	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
30	β -N-acetyl-glucosaminidase	19.089 ^a	4.707	55.340 ^b	12.181	46.129 ^b	5.526	48.449 ^b	6.111
	α -glucosidase	9.796 ^a	4.368	31.128 ^b	11.473	38.591 ^b	18.276	71.402 ^b	28.505
	α -arabinosidase	8.420 ^a	4.776	11.085 ^a	2.168	11.086 ^a	3.216	9.094 ^a	2.242
	cellobiosidase	3.255 ^a	1.826	10.014 ^{ab}	4.894	9.817 ^{ab}	5.038	13.432 ^b	7.593
	β -xylosidase	1.296 ^a	0.879	6.627 ^b	2.104	7.056 ^b	1.115	3.471 ^c	1.252
	β -glucosidase	26.262 ^{ab}	30.987	104.169 ^a	33.013	66.797 ^b	10.717	87.336 ^{ab}	45.832
	β -galactosidase	24.219 ^a	5.468	9.431 ^b	5.411	19.705 ^{ab}	10.998	15.075 ^{ab}	6.126
137	β -N-acetyl-glucosaminidase	23.189 ^a	20.128	108.805 ^{ab}	73.973	179.642 ^b	49.258	118.968 ^b	30.860
	α -glucosidase	22.878 ^a	27.246	45.640 ^b	4.805	61.803 ^{bc}	17.480	91.901 ^c	19.900
	α -arabinosidase	2.437 ^a	1.532	8.567 ^{bc}	1.952	17.837 ^b	9.483	5.560 ^{ac}	2.519
	cellobiosidase	10.460 ^a	8.045	27.692 ^b	7.566	42.884 ^b	18.452	41.967 ^b	17.744
	β -xylosidase	19.935 ^a	23.167	43.410 ^a	20.413	33.336 ^a	12.519	72.200 ^a	33.533
	β -glucosidase	47.782 ^a	45.875	104.981 ^a	35.798	340.017 ^b	61.969	196.623 ^b	26.382
	β -galactosidase	5.635 ^a	4.400	32.527 ^b	10.822	33.340 ^b	12.984	28.520 ^b	9.569
361	β -N-acetyl-glucosaminidase	163.046 ^a	48.721	217.815 ^a	22.831	304.665 ^a	92.638	677.234 ^b	229.070
	α -glucosidase	78.600 ^a	26.971	85.399 ^a	19.727	93.125 ^a	34.682	434.760 ^b	315.245
	α -arabinosidase	18.311 ^a	8.116	17.833 ^a	2.117	29.213 ^{ab}	16.431	58.056 ^b	28.792
	cellobiosidase	35.572 ^a	6.963	43.542 ^a	16.723	70.024 ^a	30.109	204.443 ^b	40.102
	β -xylosidase	58.709 ^a	18.263	62.080 ^a	20.338	103.371 ^a	49.323	186.377 ^b	44.636
	β -glucosidase	279.359 ^{ab}	104.763	193.525 ^a	35.531	353.754 ^{ab}	118.691	735.748 ^b	374.654
	β -galactosidase	50.218 ^a	14.314	51.015 ^a	17.527	70.306 ^{ab}	36.238	238.639 ^b	194.815

5.4.2: The % proportion of aggregate-size to the bulk soil

When the % proportion each aggregate size made to that of the bulk soil from all samples in the experiment were analysed together, there was a significant interaction between slurry application and aggregate size ($P=0.046$) in the loam soil. The LM fraction contributed a greater % proportion of the bulk soil in the HS slurry treatment (avg. 31.7 %) than in the control (avg. 21.74 %; $P=0.027$). Consequently, the MAC fraction contributes significantly less in the makeup of the BS in the HS slurry treatment (avg. 31.3 %) than it does in the control (avg. 35.52 %; $P=0.044$). There was also a highly significant interaction between % proportion of each aggregate size and sampling point ($P<0.001$, Figure 20), with significant aggregate turnover being seen between sampling times. The LM fraction had an average % proportion of 23.27 % at day 0, increasing to 32.58 % at day 30 ($P<0.001$). It decreased again on day 137 to 14.65 % and increased again at the final sampling point to 26.36 % ($P<0.001$). The high turnover rate of the LM fractions governs the % proportion of all the other aggregate sizes, especially MIC and SC, with the % proportion of these aggregates increasing when the LM decreases. The average % proportion of both MIC and SC decreases from day 0 (27.91 % and 16.38 % respectively) to day 30 (21.52 % and 8.59 % respectively; both $P<0.001$). Both MIC and SC increase again at day 137 (33.12 % and 24.12 % respectively) before declining again at day 361 (24.05 % and 10.97 %; both $P<0.001$).

There was no interaction between % proportion of aggregates and slurry application in the sandy soil ($P=0.615$). As was observed in the loam, there was an interaction between the % proportion of aggregates and sampling point ($P=0.001$; Figure 21), with a significant change in the dynamic of aggregate proportions to the BS being seen throughout the course of the experiment. A large amount of turnover was seen in the LM fraction throughout the 1 year

experimental period. The % proportion of LM was low at both day 0 and 30 (9.96 % and 7.14 % respectively) before rising to 37.86 % at day 137 and 40.05 % at day 361 (all $P < 0.001$). The most pronounced effect of this LM turnover is on the MAC and MIC fractions. The % proportion of MAC was 41.88 % at day 0 increasing to 49.88 % at day 30 ($P < 0.001$) and declined to 26.9 % and 29.65 % at days 137 and 361 respectively (both $P < 0.001$). The average % proportion of MIC was 44.67 % at day 0 and declined at each subsequent sampling time until it represented 26.54 % of the bulk soil at day 361 (all $P < 0.001$).

% proportion of aggregate sizes to the BS in the Loam soil

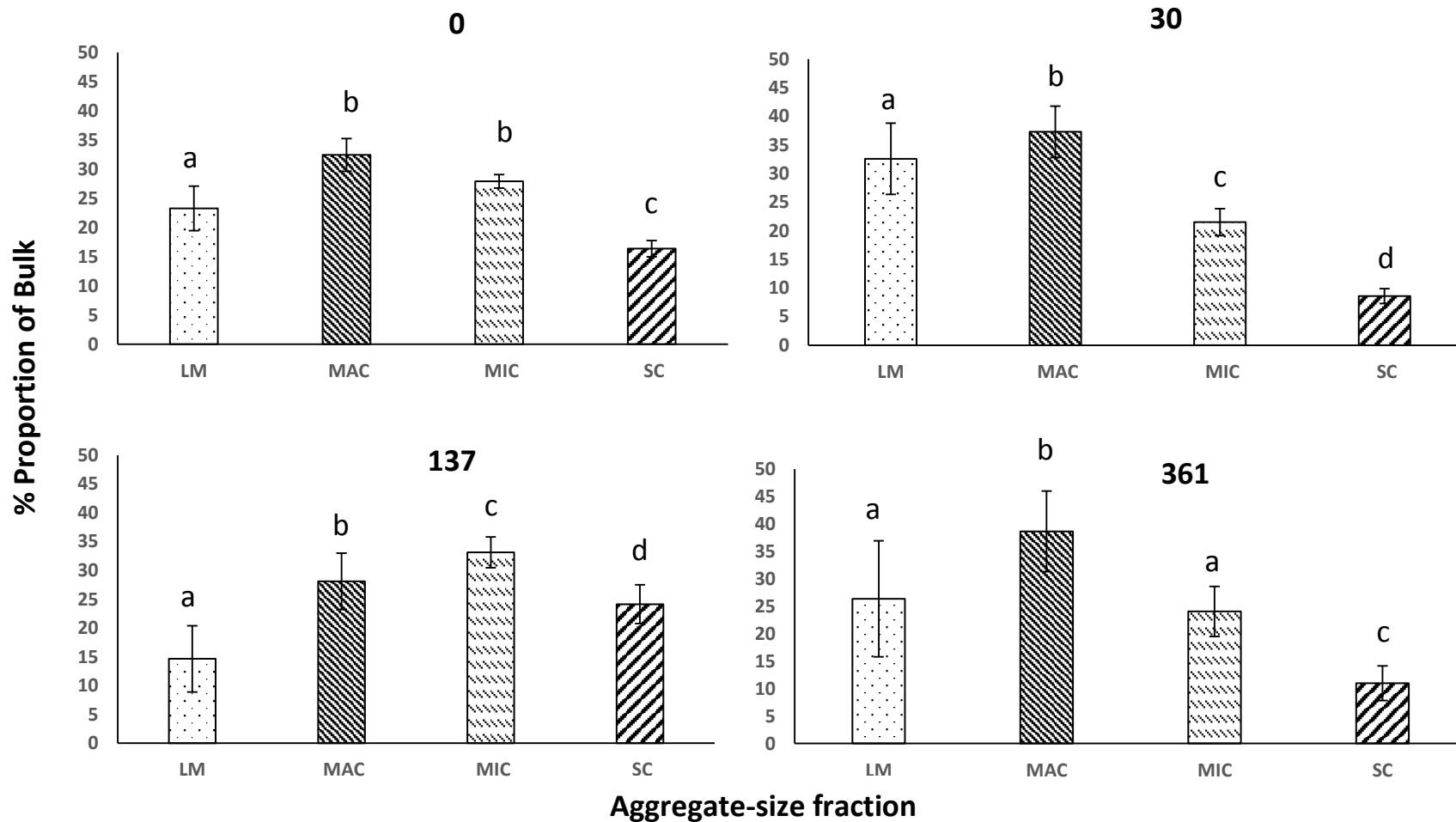


Figure 20: The % proportion that the large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay fraction constitutes to the bulk soil at each sampling points in the loam soil. Sampling points were prior to the commencement of the experiment (T0) and subsequently 30, 137 and 361 days after the application of slurry treatments. Figures from T1, T2 and T3 represent the % proportion of aggregates from the four treatments combined. Different letters indicate statistical differences ($P < 0.05$) in proportions within each time point.

% proportion of aggregate sizes to the Bulk soil in the Sand

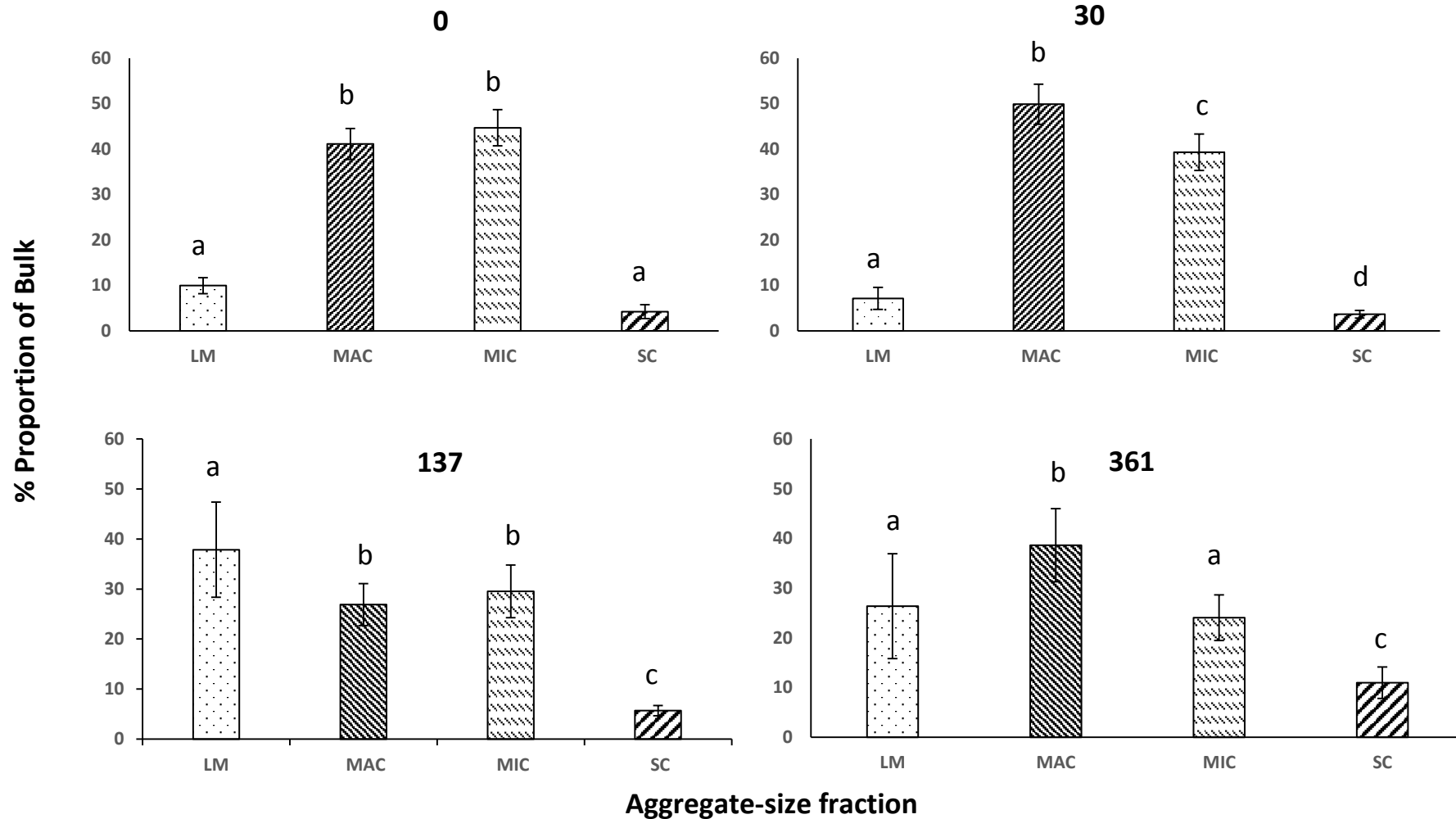


Figure 21: The % proportion that the large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay fraction constitutes to the bulk soil at each sampling points in the sand soil. Sampling points were prior to the commencement of the experiment (day 0) and subsequently 30, 137 and 361 days after the application of slurry treatments. Figures from days 30, 137 and 361 represent the % proportion of aggregates from the four treatments combined. Different letters indicate statistical differences ($P < 0.05$) in proportions within each time point.

5.4.3: Enzymatic activity within aggregates

When all treatments and sampling points are analysed together, a highly significant effect of aggregate size was seen on potential EE activity ($P=0.001$), with each of the aggregate size having a distinct level of activity in the loam soil (Figure 22; top panel). The LM fraction had the highest level of activity with the lowest activity observed in the SC fraction. There was also an effect of slurry application ($P=0.003$), with the HS treatment having significantly higher levels of activity when compared with the control ($P=0.003$), LS ($P=0.007$) and MS ($P=0.035$) treatments (Figure 23: top panel). Sampling point also had a highly significant impact on potential EE activity within aggregates ($P<0.001$), with the level of observed activity differing at each sampling point and a further significant interaction seen between aggregate size and sampling point ($P<0.001$) as well as treatment and sampling point ($P<0.001$). Analysis of variation apportioned to aggregate-size, treatment and sampling point did indicate that aggregate size explained the greatest amount of variation seen in enzymatic activity (\sqrt{CV} : 1.362) followed by time (\sqrt{CV} :1.271) and treatment (\sqrt{CV} :0.676).

When all parameters were analysed together in the sandy soil, significant effects of aggregate-size ($P<0.001$), treatment ($P=0.003$) and time ($P<0.001$) were observed on potential EE activity within aggregates. Only the LM and MAC fractions did not differ in their enzymatic activities ($P=0.081$), with both fractions having significantly higher levels of activity when compared with MIC and SC (Figure 22; bottom panel). There were significantly higher levels of potential EE activity in soil aggregates in the LS ($P=0.008$), MS ($P=0.006$) and HS ($P<0.001$) treatments compared to the control (Figure 23: Bottom panel). As with the loam soil, each of the sampling points differed from each other in terms of their EE activities, with significant interactions between aggregate size and time ($P<0.001$) but no significant interaction between treatment and time ($P=0.474$). When analysis of variation was

apportioned to aggregate-size, treatment and time did indicate it was seen that aggregate size explained the greatest amount of variation seen in enzymatic activity (\sqrt{CV} : 1.643) followed by time (\sqrt{CV} :1.058) and treatment (\sqrt{CV} :0.432).

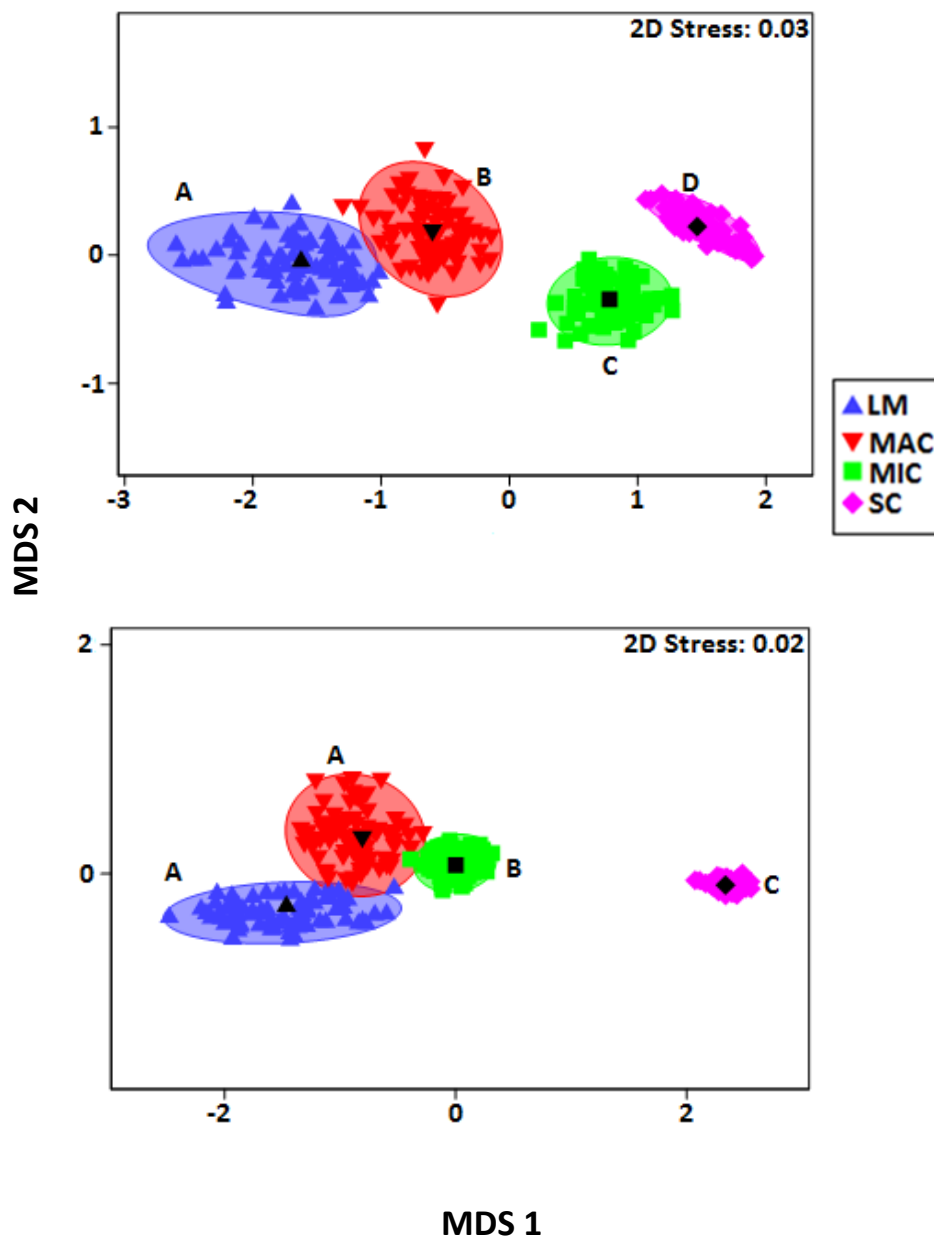


Figure 22: Bootstrapped non-metric multi-dimensional scaling (MDS) plots of the potential enzymatic activities of aggregate size fractions from all parameters throughout the course of the experiment from both the loam (top) and sand (bottom) soil. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond). Different letters indicate statistical difference at $P < 0.05$.

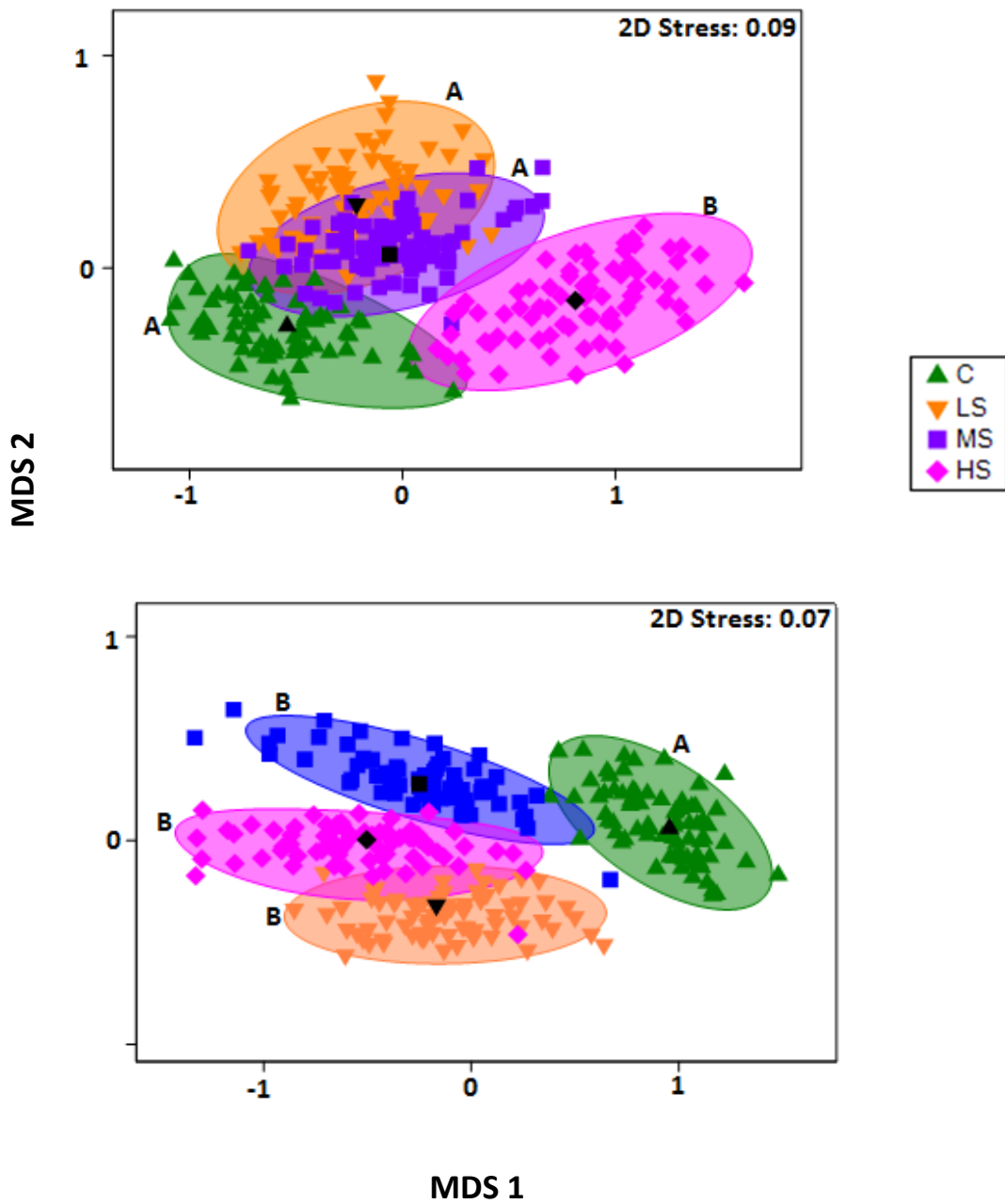


Figure 23: Bootstrapped non-multidimensional scaling plots of the potential enzymatic activities from all parameters of aggregate size fractions associated with different experimental treatments in the loam (top panel) and the sandy (bottom panel). Treatments were as follows: Control (green triangle), Low slurry treatment (orange triangle), medium slurry (blue squares) and the high slurry treatment (purple triangles). Different letters indicate statistical significance at $P < 0.05$.

5.4.4: Enzymatic activities at individual sampling points in the loam soil

At each of the four sampling times there was a highly significant effect of aggregate size on potential EE activity (all $P=0.001$) in the loam soil. There was also an effect of slurry application on days 30 and 137 (both $P=0.001$ respectively), however, no such effect was seen at T3 ($P=0.132$).

When analysing the effect of aggregate size on potential EE activity from the all treatments, it was seen that the dynamic of the activity between aggregates did differ across time. At all four time points, the LM fraction had significantly higher levels of EE activity when compared to the MIC and SC fractions. Furthermore, while LM had significantly higher rates of activity when compared with MAC on days 0 and 30 ($P=0.001$ and $P=0.023$, respectively), this was not the case at both day 137 ($P=0.312$) and day 361 ($P=0.225$). The comparable enzymatic activity between MAC and the two smallest aggregate-sizes sequentially changes from no difference in activity rates to significantly higher levels of activity between the four sampling points, with no difference seen between MAC and MIC and MAC and SC at either day 0 ($P=0.597$ and $P=0.393$ respectively) or day 137 ($P=0.098$ and $P=0.078$ respectively). A significantly higher rate of potential EE activity, however, was observed between the MAC and MIC and the MAC and SC on days 30 (both $P=0.001$) and 361 (both $P=0.001$). Additionally, while no difference in the potential activity of C-cycling enzymes was seen between MIC and SC on days 30 ($P=0.086$) or 137 ($P=0.077$), significantly higher levels of activity were observed in MIC when compared to SC at days 0 and 361 (both $P=0.001$; Figure 24; summarised in Table 14).

There was no difference in EE activity within aggregates between the controls and the three slurry amendments at day 30, though significantly higher levels of activity were observed in HS when compared to MS ($P=0.024$). On day 137, significantly higher potential EE activity

rates were seen in each of the slurry treatments when compared to the control. Moreover, each of the slurry treatments differed in their aggregate EE activity rates, with the highest activity seen in the HS treatment. At the final sampling point in the experiment, no difference is seen in EE activity within aggregates between the four treatments (Figure 25; Table 14).

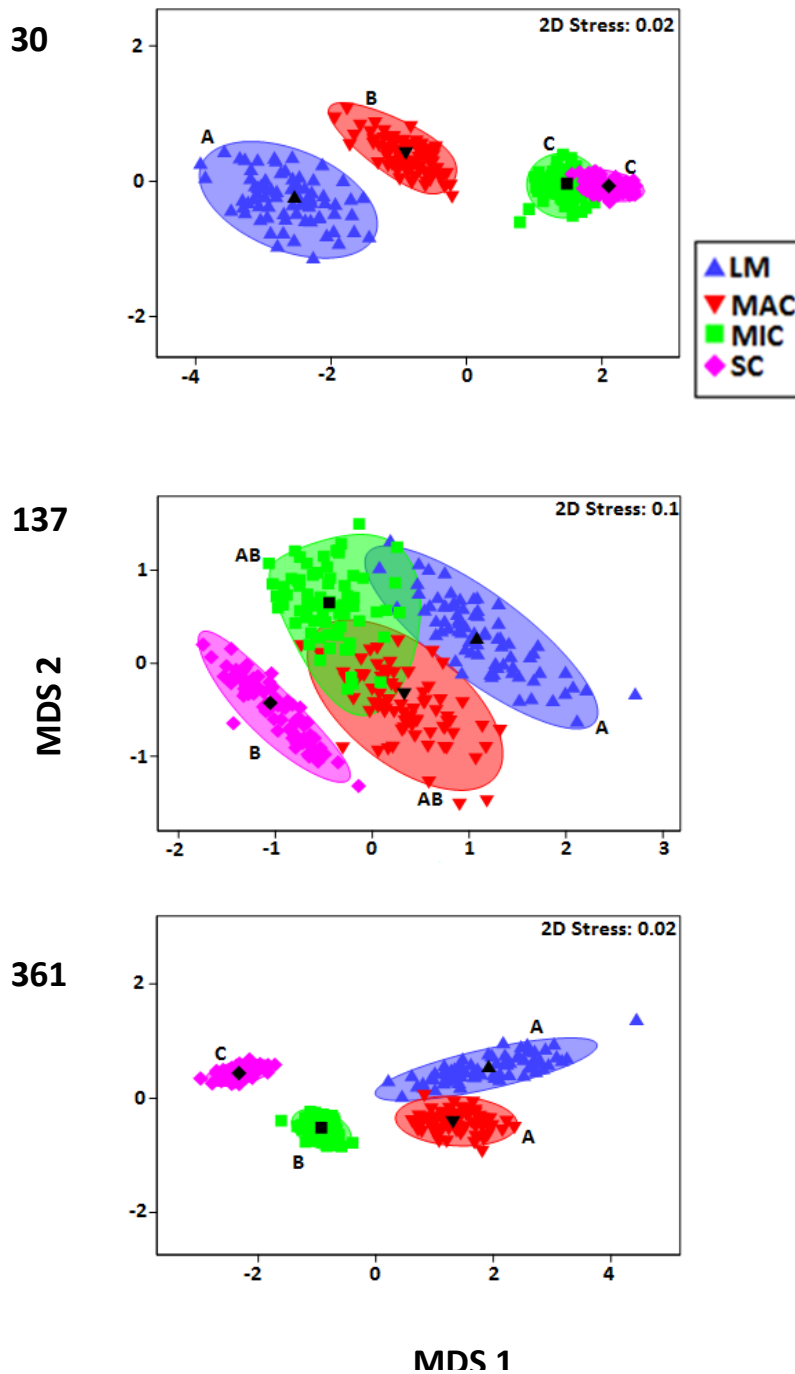


Figure 24: MDS plots from day 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the loam soil. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond). Different letters indicate statistical difference at $P < 0.05$.

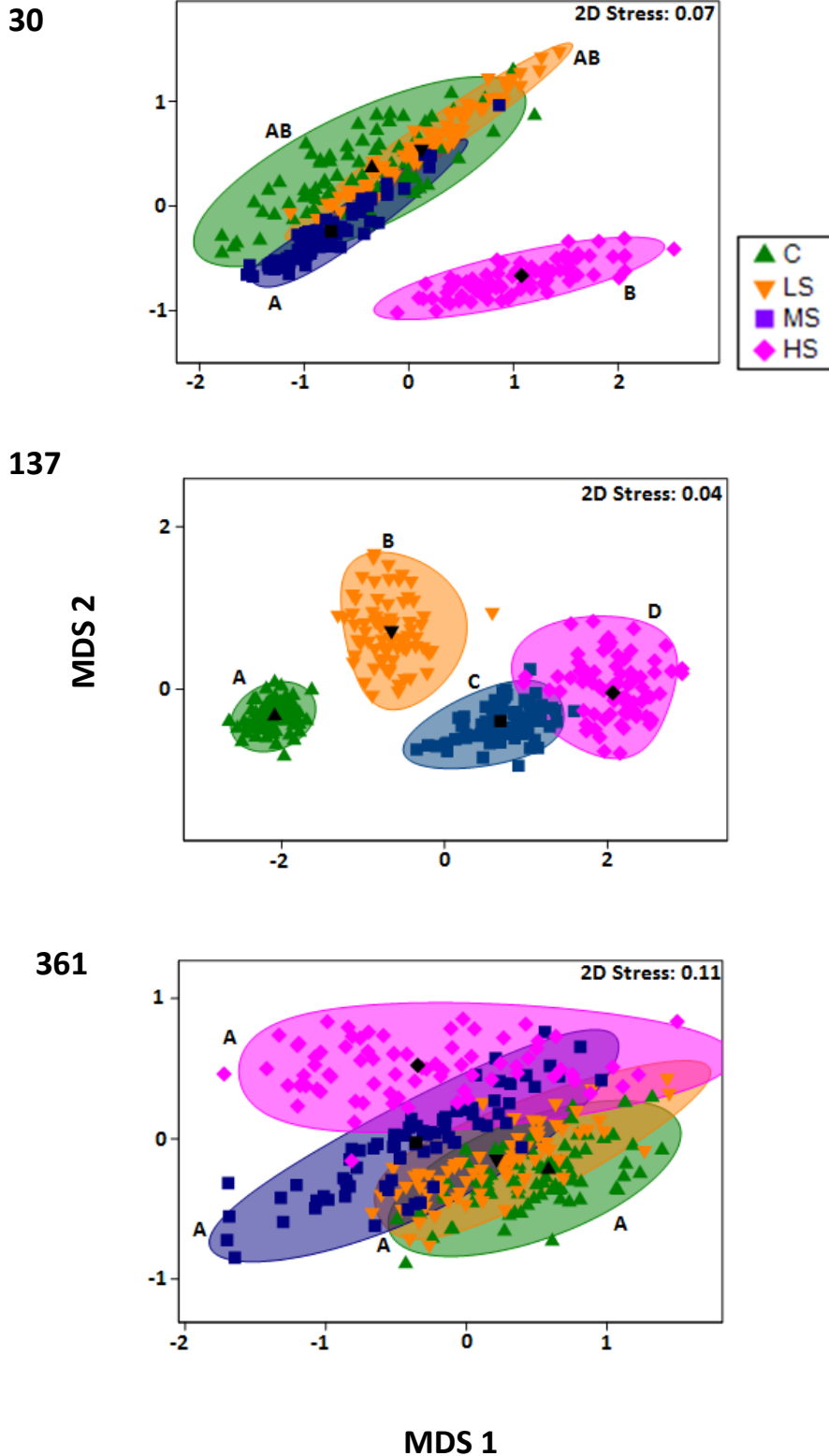


Figure 25: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the loam soil from the three slurry amendments and the control. Treatments are as follows: Control (green triangle), Low slurry treatment (orange triangle), medium slurry (blue squares) and the high slurry treatment (purple triangles). Different letters indicate statistical significance at $P < 0.05$.

5.4.5: Enzymatic activities at individual sampling points in the sandy soil

In the sandy soil, a significant effect of aggregate-size was seen at days 30, 137 and 361 ($P=0.001$) though not at day 0 ($P=0.1028$). There was also an effect of slurry application on the enzymatic activity rates within aggregates on days 30 and 137 (both $P=0.001$) but not at day 361 ($P=0.097$).

The dynamics of EE activity between aggregates differed between sampling points when all treatments were analysed together, as was seen with the loam. No significant difference in activity was seen between LM and the other three aggregate-sizes at day 0. Contrastingly at day 30, both the MAC and MIC have significantly higher levels of activity compared with LM, though SC has lower levels of activity compared to LM. At day 137, LM has the highest level of potential EE activity of all the aggregates and at day 361 has a higher activity rate compared with MIC and SC (both $P=0.001$) though not MAC ($P=0.066$). At each of the four sampling points, MAC had a significantly higher level of activity compared to SC (all $P=0.001$) though did not differ from MIC at either day 0 ($P=0.801$), 30 ($P=0.133$) or 137 ($P=0.266$) but at day 361 had a higher EE activity rate ($P=0.001$; Figure 26; summarised in table 15). The two smallest aggregates, MIC and SC differed significantly from each other on days 30, 137 and 361 (all $P=0.001$), with MIC having a higher EE activity rate at each occasion. They did not, however, differ on day 0.

Looking at the effect of slurry application on the enzymatic activity rates within aggregates, it was observed that the control treatment had a significantly lower level of activity when compared with MS ($P=0.027$) though not with LS ($P=0.067$) or HS ($P=0.087$) on day 30. Contrastingly at day 137, the control has significantly lower levels of activity when compared to LS ($P=0.04$) and HS ($P=0.012$), though not MS ($P=0.099$). There was no significant difference in enzymatic activity within aggregates between the treatments at day 361 (Figure 27; Table 15).

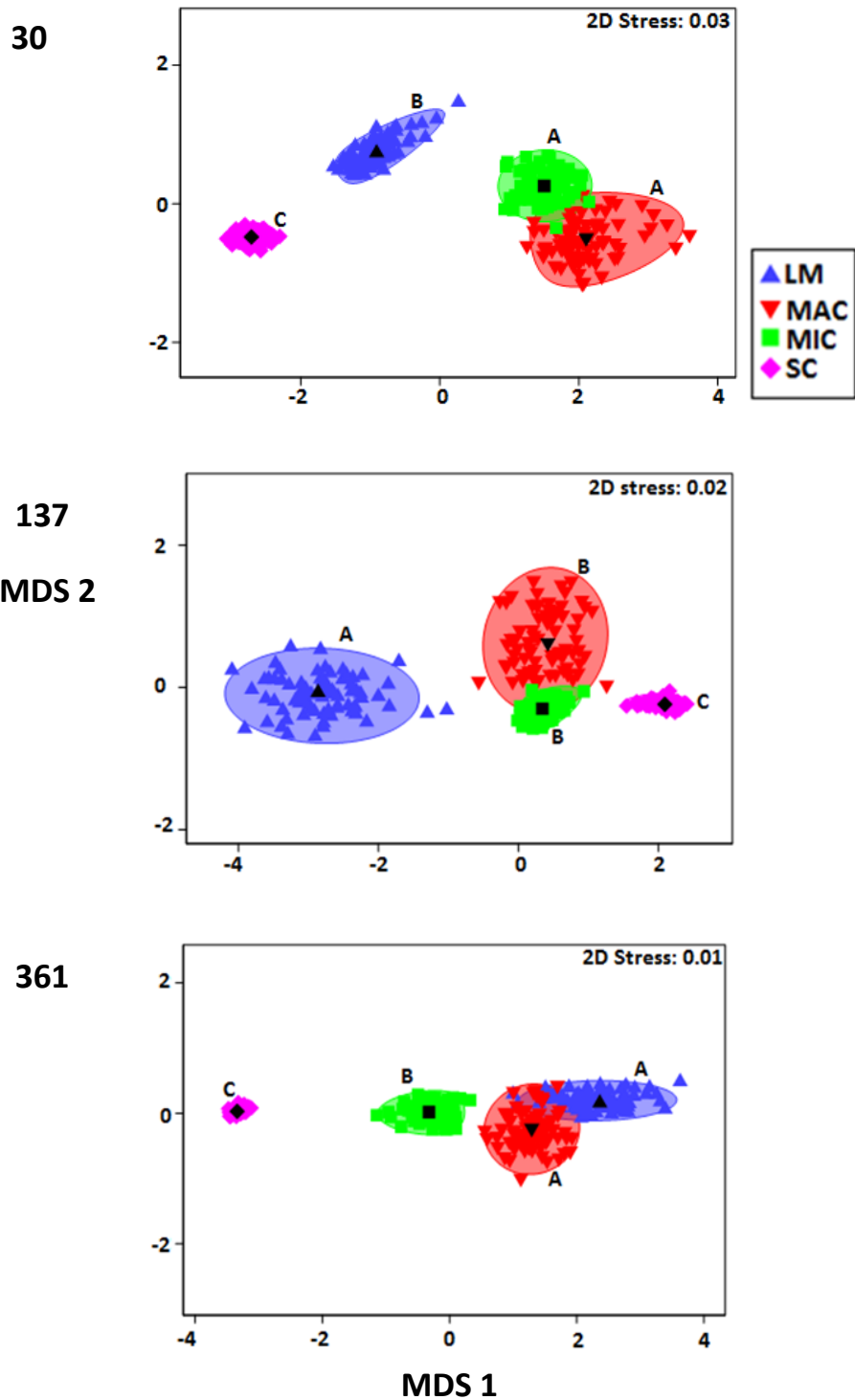


Figure 26: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the sandy soil. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond). Different letters indicate statistical difference at $P < 0.05$.

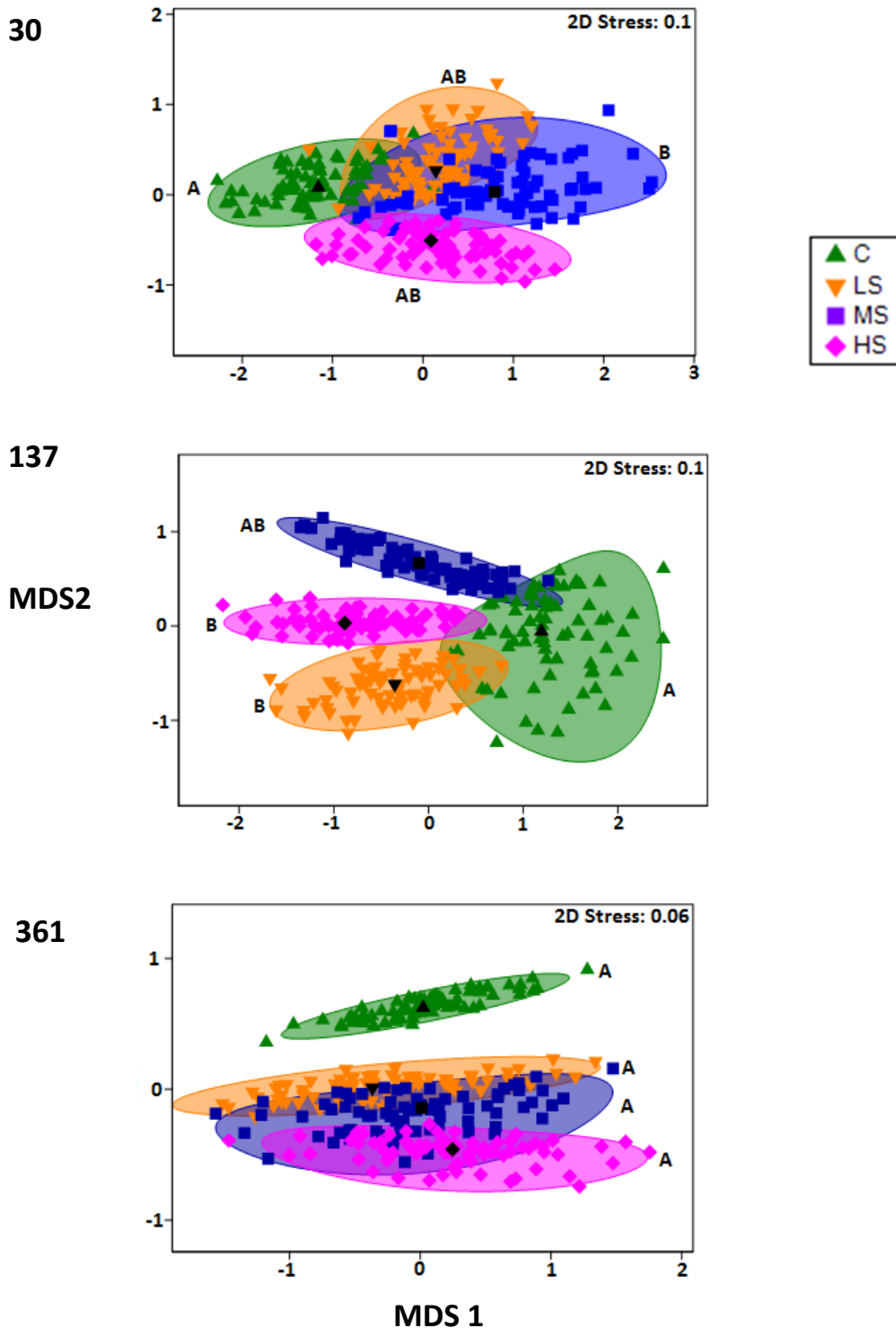


Figure 27: Bootstrapped multidimensional scaling (MDS) plots from days 30 (top panel), 137 (middle panel) and 361 (bottom panel) sampling points of the potential aggregate enzymatic activity from 7 C-cycling enzymes in the sandy soil from the three slurry amendments and the control. Treatments are as follows: Control (green triangle), Low slurry treatment (orange triangle), medium slurry (blue squares) and the high slurry treatment (purple triangles). Different letters indicate statistical significance at $P < 0.05$.

Table 14: Summary table of pairwise statistical tests of differences in potential enzymatic activity in both aggregate size (LM= large macroaggregate; MAC= macroaggregate, MIC= microagggregate and SC= silt and clay) and treatment (C= control, LS= low slurry, MS= medium slurry and HS= high slurry) from each sampling day in the loam soil. Pairwise tests detailing statistical difference between sampling points are also shown. Statistical difference $P < 0.05$.

Sampling day	Parameter	Pair-wise	P-value
0	Aggregate-size	LM vs MAC	>0.001
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	0.597
		MAC vs SC	0.393
		MIC vs SC	>0.001
30	Aggregate-size	LM vs MAC	0.023
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	>0.001
		MAC vs SC	>0.001
		MIC vs SC	0.086
	Treatment	C vs LS	0.557
		C vs MS	0.38
		C vs HS	0.072
		LS vs MS	0.217
137	Aggregate-size	LM vs MAC	0.312
		LM vs MIC	0.04
		LM vs SC	0.019
		MAC vs MIC	0.098
		MAC vs SC	0.078
		MIC vs SC	0.077
	Treatment	C vs LS	>0.001
		C vs MS	>0.001
		C vs HS	>0.001
		LS vs MS	0.0013
361	Aggregate-size	LM vs MAC	0.225
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	>0.001
		MAC vs SC	>0.001
		MIC vs SC	>0.001
	Treatment	C vs LS	0.4
		C vs MS	0.302
		C vs HS	0.295
		LS vs MS	0.302
Sampling point	LS vs HS	0.385	
	MS vs HS	0.502	
	T0 vs T1	0.003	
	T0 vs T2	0.004	
	T0 vs T3	0.018	
	T1 vs T2	>0.001	
	T1 vs T3	>0.001	
T2 vs T3	0.0011		

Table 15: Summary table of pairwise statistical tests of differences in potential enzymatic activity in both aggregate size (LM= large macroaggregate; MAC= macroaggregate, MIC= microagggregate and SC= silt and clay) and treatment (C= control, LS= low slurry, MS= medium slurry and HS= high slurry) from each sampling day in the sand soil. Pairwise tests detailing statistical difference between sampling points are also shown. Statistical difference $P < 0.05$.

Sampling day	Parameter	Pair-wise	P-value
0	Aggregate-size	LM vs MAC	0.105
		LM vs MIC	0.495
		LM vs SC	0.103
		MAC vs MIC	0.801
		MAC vs SC	<0.001
		MIC vs SC	0.303
30	Aggregate-size	LM vs MAC	>0.001
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	0.133
		MAC vs SC	>0.001
		MIC vs SC	>0.001
	Treatment	C vs LS	0.067
		C vs MS	0.027
		C vs HS	0.087
		LS vs MS	0.553
		LS vs HS	0.317
		MS vs HS	0.335
137	Aggregate-size	LM vs MAC	>0.001
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	0.266
		MAC vs SC	>0.001
		MIC vs SC	>0.001
	Treatment	C vs LS	0.04
		C vs MS	0.099
		C vs HS	0.012
		LS vs MS	0.209
		LS vs HS	0.342
		MS vs HS	0.249
361	Aggregate-size	LM vs MAC	>0.066
		LM vs MIC	>0.001
		LM vs SC	>0.001
		MAC vs MIC	>0.001
		MAC vs SC	>0.001
		MIC vs SC	>0.001
	Treatment	C vs LS	0.354
		C vs MS	0.426
		C vs HS	0.263
		LS vs MS	0.65
		LS vs HS	0.442
	Sampling point	MS vs HS	0.708
		T0 vs T1	>0.001
		T0 vs T2	>0.001
		T0 vs T3	>0.001
		T1 vs T2	>0.001
		T1 vs T3	>0.001
		T2 vs T3	0.0208

5.5: Discussion

The results of this study clearly indicate that (i) enzymatic activity within the bulk soil remains elevated for a significant period of time after the application of slurry (ii) soil aggregates (i.e, soil structure) influences microbial enzymatic activity in relation to C-cycling in grasslands. (iii) the addition of C through agricultural management influences this activity and (iv) enzymatic activity within aggregates fluctuates over time.

The higher rates of enzymatic activity seen in the BS of one or more of the slurry amendments compared with the control in both soil types throughout the course of this experiment agrees with the findings presented in chapter 4, where an elevated enzymatic activity was still seen in slurry amended field plots 2 months after the initial application. Additionally, other studies have reported a priming effect upon the application of slurry to a grassland soil, particularly in the top 5 cm (Bol et al. 2003; Kuzyakov and Bol 2006) and the elevated microbial EE activity within the bulk soil is an important underlying mechanism for this change in terrestrial SOC dynamic.

It has previously observed been demonstrated in chapters II and III of this thesis that soil aggregates are an important determinant of microbial community composition. The physical fractionation of soil aggregates allows the study of localized enzymatic activity rates and this study has shown that soil aggregates are key determinants in potential EE activity. Independent of treatment and time both LM and MAC had significantly higher levels of potential EE activity when compared to the two smaller aggregates. Such changes in activity are indicative of the differing microenvironments each aggregate size represents. The larger microbial investment in enzyme production in larger aggregates (250 μm) is reflective of the ease of accessibility to substrate (due to the macro porosity)

of the greater requirement for decomposition due to the larger amount of organic matter (Fansler et al. 2005). The tortuosity of the micro pore in MIC will be a significant hindrance on the enzyme-substrate stoichiometry with physical protection being a dominant process influencing soil functions (Allison 2005; Lagomarsino et al. 2012). Furthermore, the oligotrophic conditions within both MIC would lead to reduced microbial metabolism and activity (Mummey and Stahl 2004).

Slurry application significantly impacted the potential EE activity within aggregates in both soil types. The temporal dynamic of this treatment associated increase in potential aggregate associated EE activity is reflective of the rate of incorporation of slurry associated-C into soil aggregates. It was observed in the loam soil that 30 days after the initial application of slurry there was no increase in aggregate EE activity in the three slurry amended treatments when compared to the control. This would indicate insufficient time for the slurry derived-C to decompose and become incorporated into aggregates which the associated microbes could subsequently utilize. A similar situation occurred in the sandy soil at this time point, though the significant increase in potential aggregate EE activity in the MS over the control suggests a degree of variation could be expected in the incorporation of external C sources into soil aggregates dependant on soil type and the amount of C applied. Slurry C did infuse into the soil matrix by this time point (as indicated by the increase in EE activity in the BS for both soil types), thus the soil microbiota in the bulk soil are playing a crucial role in the decomposition of external C sources and in their subsequent incorporation into aggregates (Six et al. 2004). When microbes decompose C sources they themselves produce polysaccharides which act as binding agents between the C source, soil minerals and existing soil aggregates leading to enhanced soil aggregation (Yousefi et al. 2008).

The slurry derived C had become incorporated into the aggregate structures at the next sampling point (137 days after slurry application) with treatment associated increases in potential aggregate EE activity being seen in both soil types. The exception being the MS treatment in the sandy soil, suggesting the labile C incorporated by day 30 had become fully exhausted. This increase in potential aggregate activity could represent a combination of both the utilization of slurry derived C and a priming effect on native aggregate associated C. This incorporated C had become fully utilized by the end of the experiment (day 361). Interestingly, elevated treatment associated EE activity was still observed in the BS at this time point. The microbiome in the BS may be benefiting from increased rhizodeposits from more vigorously growing ryegrass which got added macro-nutrients from the slurry. It may also suggest that the priming potential of aggregate associated C is comparatively low in comparison to the BS.

A significant degree of aggregate turnover was observed in both soil types over the 1 year time course of this experiment, with this almost solely due to the breakdown and re-formation of the LM fraction. The temporal turnover of soil aggregates has previously been documented, with estimates of a 27 day turnover time for MAC (Plante and McGill 2002), while MIC have been found to be stable on the decades to centuries scale (Oades 1984). Seasonal soil aggregate turnover has also been reported in grasslands managed for bioenergy production (Bach and Hofmockel 2016). In natural soils LM typically breakdown as a result of precipitation and freeze-thaw cycles and will re-build over time as a result of root and fungal enmeshment as well as through the activities of the mega-, meso- and micro- fauna (Oades 1993). The variations in soil structure seen in this experiment will have resulted from a multitude of factors (i.e, interactions with plant roots and arbuscular mycorrhizal fungi, fluctuations in soil temperature and soil moisture

etc) and the two soil types may have responded in a different manner to these (Six et al. 2004). The significant increase in the % proportion of the LM fraction in the HS slurry treatment over the control in the loam soil suggests that C inputs through agricultural management will also influence soil structure. This may be as a consequence of greater root and fungal hyphal growth leading to a greater consolidation of soil particles into LM structures.

The significant interaction between the potential EE activities within aggregates and sampling point in this experiment will be reflective of this aggregate turnover. Large macroaggregate breakdown results in the release of formerly physically protected C which can then be accessed and broken down by the soil microbiota through the exudation of EEs (Sollins et al. 1996). Bach and Hofmockel (2016) have reported that large macroaggregate breakdown corresponds to increases in extracellular enzymatic activity. They also observed that potential soil aggregate EE activity exhibited seasonal peaks which corresponded to aggregate disruption. Variations in soil temperature, moisture content and root litter inputs will also result in fluxuations in microbial EE activity over time (Bach and Hofmockel 2016; Poll et al. 2006). The significant interaction between the potential EE activity within aggregates from the various treatments and sampling point would also indicate the fluxuations in aggregate turnover and soil phyiochemical conditions is an important determinant in the decomposition of C into a grassland system.

A comprehensive understanding of the interactions between soil structural, temporal and agricultural management on the microbial EE activity is key if we are to elucidate the mechanisms underpinning C sequestration in agroecosystems as well as utilizing SOC as a balanced nutrient mineralization in agricultural grassland soils. Foodwise 2025 lays out

ambitious targets for increasing Ireland's agricultural output on one hand but also to achieve this intensification of production in a sustainable manner. The methods involved in increased agricultural output will correspondingly result in an greater levels of soil management and manipulation. Thus a greater understanding of the impacts of fertilisation regimes on aggregate structure and the microbial activities within these aggregates is vital in the maintenance of soil health and fertility as well as in aiding the soil in the deliverables of essential ecosystem services.

5.6: Conclusion

Both aggregate size and the application of slurry derived C were significant determinants of potential EE activity in both the loam and sandy soils. Understanding this interplay between soil structure, agricultural fertilisation and temporal variations in the structural and chemical parameters within the soil matrix are vital towards the comprehension of the C cycle in agricultural grasslands.

Chapter VI: General discussion

The study of the structure, diversity and dynamics of soil microbial communities and their role in biogeochemical cycles is a fast moving and exciting area of research (Litchman et al. 2015). Recent work has established the influence of soil aggregates on carbon storage in Irish grasslands (Torres-Sallan 2016). This study focused on the biological aspects of C cycling in these systems. Specifically, it investigated how the physical protection of C (soil aggregates) and C addition (slurry application) influenced i) the composition and diversity ii) the relative % abundance and iii) the activity (as measured by extracellular enzymes) of the soil microbial community.

Main findings:

Composition : Aggregate size significantly influenced the composition of both bacterial and fungal communities as determined by terminal restriction fragment length polymorphism (T-RFLP). Horizon depth also significantly influenced the microbial communities associated with these aggregates.

Relative % abundance: The relative % abundance of both bacterial phyla and families were both influenced by aggregate size as determined by next generation sequencing (Illumina Miseq). The relative % abundance of both the phyla and the families within these aggregates was also significantly affected by horizon depth, with a significant interaction being seen between these two parameters.

Activity: Soil extracellular enzymatic activity responded in a step-wise manner to the application of in field slurry derived C, though only the bacterial community structure responded to its immediate addition. Seasonal succession had a more pronounced impact on most biological parameters measured than did slurry application.

Aggregate size significantly impacted potential extracellular enzymatic activity, as did the addition of slurry derived C in two contrasting soil types (loam and sandy). Time of sampling was also a highly significant determinant of observed activity.

6.1: Effect of aggregate size on microbial community composition

Soil microbial diversity is positively correlated with both soil health and fertility (Fox et al. 2014; Ghorbani et al. 2008). The heterogeneous nature of soil structure resulting from the amalgamation of organic matter and soil minerals into varying sized soil aggregates results in localized microenvironments which has been shown to influence microbial community composition (Negassa et al. 2015; Sessitsch et al. 2001). Despite it being established that the soil microbiota plays a crucial role in both the formation and stabilisation of soil aggregates, no studies to date have investigated how the two main components of the soil microbiome (bacteria and fungi) are influenced by aggregate size down the soil profile of grassland soils. This thesis looked at the differences in both bacterial and fungal diversity in varying sized aggregates from the top three horizons from nine Irish grassland sites using both the T-RFLP fingerprinting technique and Illumina Miseq NGS technique.

Using the T-RFLP fingerprinting technique, the SC fraction was found to harbour a significantly distinct bacterial and fungal community. Bacterial biomass is often higher in the SC fraction compared to larger aggregates where it can reach levels of 30-80 % higher than that found in the macroaggregate sizes (>250 μm) which concurs with the observation in this study which revealed a significant increase in the number of unique bacterial t-RFs in the SC fraction. By contrast fungal biomass is mainly found in the macroaggregate sizes, where the microporosity in the SC will limit fungal growth, as suggested by the significant reduction in fungal ITS t-RFs seen in H1 (Orgiazzi et al. 2016). The high proportion of clay in the SC fractions will also have been a key influencing factor on the compositional dynamic of the bacteria community observed in

this study (Mueller, 2015). The significant effect of horizon depth on both bacterial and fungal communities in both the BS and within aggregates is reflective of the changing soil physiochemical conditions characterized by an increase in anaerobic conditions, increased C substrate recalcitrance, decreases in soil temperature and in certain cases waterlogging (Barber et al. 2004; von Lutzow et al. 2006).

A significant effect of both aggregate size and horizon depth was seen on the % relative abundance of both bacterial phyla and families, with a significant interaction seen between the two parameters, using Illumina Miseq. This study highlighted the significant change in the % relative abundance of both phyla and family bacterial taxonomic levels between the MAC and MIC aggregates in H1. This change in bacterial community structure is reflective of the differing microenvironments both aggregates represent. The MIC fraction has been defined in the literature as an operationally stable microenvironment which is inherently oligotrophic with a low predation pressure (Kong et al. 2011; Poly et al. 2001; Mummey et al. 2006). This is in contrast with the MAC fraction whose macro-porosity will allow bacterivorous predators (i.e, nematodes) to gain access to and predate on bacteria (Jiang and Sun, 2016). Additionally, the significant change in the % relative abundance of both bacterial taxonomic levels in the MAC fraction between H1 and H2 is reflective of the influence of soil depth on this predator-prey dynamic in the soil matrix, with the decline in bacterivorous predators being more pronounced than bacteria with depth (Ekelund et al. 2001). The influence of horizon on MAC may also be indicative of the effect soil depth will have on the physiochemistry of this aggregate, influencing as it will the permeability of both water and O₂.

While both the T-RFLP and Illumina Miseq both detected the significant influence of both tested parameters on the bacterial community in this study, differences in the specifics of these effects were seen. Such disparities are reflective of the analytical methodologies inherent to both techniques. The T-RFLP technique provides a profile of community structure and is considered to have higher resolution and greater reproducibility compared to other fingerprinting methods, such as denaturing gradient gel electrophoresis (Lueders and Friedrich 2003; Okubo and Sugiyama 2009; Thies 2008). T-RFLP analyses polymorphic lengths of restriction fragments which have been generated by the enzymatic digestion of PCR amplicons (Okubo and Sugiyama 2009). Thus the technique provides information on the top 40 or so phylotypes with the result that most of the diversity present in a microbial community is not taken into account.

A major aim in soil microbial ecology is to identify specific microbial groups and their relative abundances. This can't be adequately achieved with a fingerprinting technique and instead a next generation sequencing (NGS) technique must be employed. Despite the inherent limitations, T-RFLP still has a number of advantages over many NGS techniques. It can provide a robust analysis of microbial community structure from a large amount of samples, as the technique allows comparability between sample runs (Nocker et al. 2007), which is not possible with an NGS technique such as Illumina Miseq. Thus going forward T-RFLP will be best used in analysing patterns from a large number of environmental samples and this information can then be used to inform further NGS analysis.

The Illumina platform is a favoured NGS platform due to its affordability and comparability of results when using shorter reads (Bartram et al. 2011; Caporaso et al. 2011). The continuing reductions in the running times and costs of next generation sequencing analysis now makes it an essential tool in the study of microbial ecology (Gloor et al. 2010). Massive parallel sequencing/ next generation sequencing has generated an enormous amount of knowledge on bacterial diversity but only reveals indirect information on bacterial functions (Schmalenberger and Fox 2016). The continued development of sequencing platforms, throughput and capabilities in bioinformatics will make it more affordable to concentrate instead on functional genes (metagenomics) and gene expression (metatranscriptomics) (Teeling and Glöckner 2012). Therefore future studies on the quantification of gene expression and abundance across the aggregate sizes will give important indications as to the microbial functional stratification which may be inherent in the aggregate hierarchy. A re-emphasis on bacterial isolation and cultivation in order to gain a greater in-depth understanding of the metabolic capabilities of microbial groups is needed in soil microbial studies (Schmalenberger and Fox 2016) and such analysis should be conducted on aggregate associated bacteria.

The studies into microbial community composition in this thesis represent an important step towards understanding the impact the two most important structural parameters in the soil profile (aggregation and horizon) have on the two dominant components of the soil microbial community, the bacteria and fungi. Microorganisms exist throughout the soil profile, microbial ecologists however have largely focused their efforts on the composition and functional capacity of the microbiome occupying the uppermost portion of the profile. While most microbial biomass, diversity and activity may be found in the

top 10-30 cm it is imperative to focus on the collective soil profile (and its inherent structural complexity) as a microbial habitat as soils are not static systems (Bundt et al. 2001; Eilers et al. 2012; Fierer et al. 2003; Murphy et al. 1998). Soil processes such as clay illuviation, the vertical movement of carbon, stagic properties and nutrient leaching will all impact on microbial community composition and activity. Additionally, agricultural management practices such as ploughing, grass re-seeding, drainage and their consequences (i.e, soil compaction, aggregate breakdown etc) will all impact on these soil processes and thus the dynamism of the microbial community structure and activity (Drenovsky et al. 2010; Fontaine et al. 2007; Qiu et al. 2013; Unger et al. 2009). Soil structure is also a key factor controlling the predator-prey dynamic between the micro-, meso- and mega-fauna and this has important implications on the nutrient cycling capabilities as well as on soil health and fertility (Young and Crawford 2004; Young and Ritz 2000). It is the soil profile as a collective which delivers the ecosystem service of carbon sequestration and thus future studies into soil microbial ecology reflect this reality (Dilling and Kaiser 2002; Fierer et al. 2005; Rumpel and Kögel-Knabner 2011; Trumbore 2000).

6.2: Impact of aggregate size and slurry application on the functionality of the soil microbiome

As well as influencing microbial community structure (as was seen in chapters II and III) soil aggregates will also be important determinants of microbial EE activity (Allison and Jastrow 2006; Bach and Hofmockel 2014; Bach and Hofmockel 2016; Fansler et al. 2005; Marx et al. 2005). Few studies have examined the potential aggregate associated EE activity in soil managed for agricultural purposes (Lagomarsino et al. 2012; Schutter and Dick 2002) or over time (Bach and Hofmockel 2016). Additionally, while studies have

examined the response of microbial respiration rates, extracellular enzymatic activity, microbial biomass carbon and microbial community structure to the application of slurry derived C in the bulk soil (Bol et al. 2003; Chantigny et al. 2001; Moynihan et al. 2015; Plaza et al. 2004; Rochette et al. 2000; Saviozzi et al. 1997), studies which have examined its effects at the aggregate scale are lacking. There is also an inadequate understanding of the comparable effect of slurry derived C on the functionality of the soil microbiome against the short- to medium term influences of seasonally induced changes to the prevailing soil physiochemical parameters at both the bulk soil and aggregate scales.

Both chapters IV and V demonstrated the effect of slurry application on the EE activity of the soil microbiome. The step-wise response in the functionality of the soil microbiome seen in chapter IV reflects the sequential incorporation of the slurry derived C into the bulk soil. The increase in β -glucosidase activity 5 days after slurry application suggests the rapid utilization of labile C (Bol et al. 2003; Sauheitl et al. 2005). This observation is in agreement with the increase in the activity of this enzyme seen in the 2 week period after the application of cattle slurry seen by Bol et al. (2003); though this study also reported increases in β -cellobiohydrolase and β -N-acetyl-glucosaminidase. The significant increase in EE activity 65 days after C addition is indicative of the soil microbiome shifting to utilizing more recalcitrant slurry derived C or SOC. Such sustained increases in activity after slurry amendment have been reported in an incubation experiment which lasted for 31 weeks (Martens et al. 1992), and in the bulk soil of both a loam and sandy soil up to 1 year after slurry application seen in chapter V of this thesis. This sequential degradation of slurry derived C and a resultant priming effect of SOC in

the bulk soil has been previously suggested (Bourdin 2012; Fangueiro et al. 2007; Kuzyakov and Bol 2006).

A temporally specific activity pattern in response to slurry derived C was also observed at the aggregate scale in both the loam and sandy soil types in chapter V. In the loam soil, no increase in potential aggregate EE activity in any of the three slurry treatments over the control was observed 30 days after slurry application. At 137 days after slurry application, a significant increase in potential EE was seen in each of the three slurry amendments over the control. This indicates that the slurry derived C had become incorporated into the soil aggregates and that the aggregate associated microbiome was now utilizing this as a C source or is reflective of an inner aggregate priming effect on native SOC. Any slurry associated increase in potential EE activity was no longer observed 1 year after the initial application, indicating the slurry derived C source had become exhausted. A similar activity pattern over the 1 year experimental period was also observed in the sandy soil. While studies have examined the sequential incorporation of slurry derived C into the bulk soil (e.g. Dungait et al. 2009; Bourdin 2012), this study highlights the need to further these initial investigations to examine the sequential incorporation of C into soil aggregates.

The strong temporal influence on all biological parameters seen in Chapter IV (with the exception of MBN) is reflective of the deterministic underpinning of the prevailing soil physio-chemistry on the biological parameter of soil. This study indicates that soil moisture content (which declined with progressing season) is a more important driver of soil microbial community structure and function than C amendment through slurry. Several studies exist which have highlighted soil moisture as a key factor in influencing soil microbial community structure and enzymatic activities (Brockett et al. 2012;

Gömöryová et al. 2006; Ma et al. 2015; Zhao et al. 2016). Seasonal associated large macroaggregate turnover was also an important determinant of potential aggregate EE activity in both soil types in chapter IV as has been reported previously at the field scale (Bach and Hofmockel, 2016). Thus the spatial and temporal fluctuations in soil structural and physiochemical conditions and its effects on microbial activity is an important consideration in relation to C turnover in Irish grassland soils.

Understanding the relationship between microbial community structure and function is a major research objective in microbial ecology. Shifts in microbial community structure have been reported following the addition of livestock wastes to field trials (Parham et al. 2003; Peacock et al. 2001) and the staged response seen in enzymatic activity seen in chapter IV would suggest a consequential r/K strategist dynamic (i.e, a rapid increase in bacteria followed slow growing fungal species) to be seen in microbial community structure (Fontaine et al. 2003; Lundquist et al. 1999). The significant change in bacterial community composition and abundance as well as the increase in MBN indicate a rapid bacterial response to the added substrate as a source of N. The secondary increase in EE activity did not correspond with a change in either bacterial or fungal community composition or abundance. The rather muted response of microbial community structures to slurry application seen in this study however is perhaps a consequence of the microbiome being compositionally adapted to utilize external resources which may due to the previous management history of the site (Xue et al. 2013).

Enzyme assays have traditionally been conducted on large size samples (i.e, the bulk soil) while microbial community interactions may be more pertinent at the aggregate scale (Bailey et al. 2013). Thus linking the function of the microbial community with its

compositional structure at the aggregate scale is the logical research progression to the work undertaken in Chapter V. It is known that significant functional redundancy in relation to C turnover exists in soils (Schimel and Schaeffer 2012) though the scale at which this redundancy operates is unclear. Early studies into this topic do suggest, however, that β -glucosidase activity within macroaggregates was expressed by a specific taxonomic group (Bailey et al. 2013).

Metatranscriptomics gives in situ activities of the microbial community in soil through analysing mRNA (messenger ribonucleic acid) expression. Such molecular methods for studying aggregate activity will be compounded by the difficulties in obtaining high quality mRNA for analysis due to the abundance of nucleases which abound in soil (Cai et al. 2006) but also due to the effects resulting from the aggregate isolation procedure. The most practical experimental approach to linking community structure in relation to C turnover is through stable isotope tracing (SIP) (Griffiths et al. 2004).

In such an experimental setup a ^{13}C labelled carbon source would become incorporated into soil aggregates and become utilized by the active soil microbiome and incorporated into the cellular components (i.e, DNA, phospholipids). These can then be extracted from soil and analysed. Labelled phospholipids can be extracted by the Bligh and Dyer (1959) method and identified via gas chromatography mass spectroscopy-isotope ratio mass spectroscopy (GCMS-IRMS)(Denef et al. 2009). An alternative analysis would be DNA-SIP in which nucleic acids which have incorporated the ^{13}C label can be separated from non-labelled nucleic acids using a CsCl/EtBr density gradient ultracentrifugation and phylogenetic analysis can then be done on the separated labelled DNA (Neufeld et al. 2007). Such an experiment would be logistically difficult to achieve with a livestock

slurry (the animal would have to be fed a pure ^{13}C source) and would be expensive to achieve the required ^{13}C enrichment in the resultant slurry. An easier experimental alternative would be to trace ^{13}C labelled root exudates (from a plant which has photosynthetically incorporated ^{13}C labelled CO_2) into soil aggregates and subsequent incorporation into aggregate associated soil microbes.

As slurry application is an important practice in the recycling of nutrients back into agricultural grasslands (Holden et al. 2004), the understanding of its effect EE activity in both the bulk soil and the aggregate scale is an important consideration in the management of soil C stocks. The need for a greater consideration of the soil microbiota in slurry management has been highlighted in the literature (Harris et al. 2011), and this study highlighted the temporal response of the microbiome to applied cattle slurry. If we are to manipulate the soil microbiome to optimise slurry management in agricultural grasslands then a greater synergy of research strands is required. Chief among these considerations is an evaluation of how the previous management of systems affects the functionality of the microbiome and its subsequent response to nutrient addition. The timing of application (i.e, spring vs autumn) and the disparity of the nutrient content between that of the slurry and soil (C:N:P ratio) will also be important determinants of this response.

6.5: Conclusions and future work:

Soil is a non-renewable resource whose degradation and nutrient exhaustion is a major global challenge. With an ever increasing world population, we are expecting our soils to both increase food and fibre production but also to maximally deliver a range of

ecosystem services such as C sequestration. This thesis has demonstrated that soil structure (i.e, soil aggregates, the physical protection of C), physio-chemistry (i.e, % C, soil moisture), agricultural management (C addition through slurry) and time are important determinants of the diversity, % relative abundance and activity of the soil microbiome, with important implications for the C cycling dynamic in grassland systems. Research into microbially mediated mechanisms of such sequestration is very timely, with the recent adoption of the 4 per mill initiative at COP21, in Paris (Nov 2015). The EU member states commitment in COP21 to reduce CO2 emissions also highlights the need for the Irish agricultural sector to bring forward workable solutions in achieving this aim (European Commission, 2016). Increasing C stocks in grasslands represents a rare ‘win-win’ strategy, coupling as it does the removal of unwanted C from the atmosphere and storing it in soils where it increases their fertility and health status.

6.5.1: Grassland disturbance:

There is currently a large emphasis placed on soils to sequester C and reduce greenhouse gas emissions. How this can be achieved in practice is an on-going debate between agricultural management practices and land-use change scenarios. The Foodwise 2025 report has set out ambitious targets for both increasing agricultural output while doing so in a sustainable manner. These objectives coupled with the recent abolition of the milk quota system (a tax established by the European Commission in 1984 as a mechanism to cap milk production levels in response to over –production) in April 2015 will have a significant impact on how grassland utilized for dairy production will be managed. This intensification in agricultural production will only be achieved through drainage of grassland soils, increased nutrient inputs, pasture re-seeding and increases in the animal

stocking rates (Teagasc 2015). Understanding how these changes will impact the microbially mediated turnover of terrestrial C and how these changes can be managed to optimise soil fertility and functionality is now an imperative area of study.

The research questions which will need to be addressed include:

- How will grassland disturbance affect aggregate structure as well as the composition and activity within soil aggregates?
- What will be the consequence of altered land management activities on this sequestered C in grasslands?
- How will grassland disturbance influence soil processes (i.e, clay illuviation, the vertical movement of C etc) and what affect will this have soil aggregation rates and their constituent microbiomes?

6.5.2: Impact of soil type:

Little attention has been paid to the fact that some soil types in Ireland have the potential to sequester C within their subsoils to a considerable degree. Recently, a study on Irish grasslands has shown that soils that undergo clay illuviation (the translocation of clay particles from surface horizons to subsoil horizons) in deep horizons have an increased proportion of SOC in the smaller aggregate sizes (microaggregate and silt /clay) (Torres-Sallan, 2016). Taking into account that incorporation of SOC to small aggregate sizes is considered C sequestration (Denef et al. 2004), it seems that these soil types have a greater proportion of sequestered C when compared to those soils without clay illuviation (Typical Brown Earths (Cambisols)).

Studies have looked at the effect of soil type on bacterial community composition and functionality across Irish soil types (Richter 2016). More in-depth studies, however, focusing specifically on the aggregate associated microbial community in the major soil types in Ireland (i.e, Brown Earth, Ground/ Surface water gley, podzol, luvisol etc) and their associated processes (i.e, clay illuvation, vertical movement of C and stagnic properties) is required. This would allow us to identify which soils matter in terms of long term storage (sequestration) of C and such information will inform management decisions to help mitigate climate change. This would be just one of many measures needed in Ireland to ensure a future of climate smart agriculture, but one of the easiest to facilitate.

The research questions to be addressed in such a study include:

- What affect does soil type have on these aggregate microbial communities?
- Do certain soil types and management practices support higher C sequestration capacity?
- Is this due to their constraints on microbial colonisation and activity? What are the mechanistic processes of this sequestration?
- Does the microbial community composition found in these soils reflect the physico-chemical recalcitrance of carbon?

Chapter VII: Bibliography

- Aciego Pietri JC, Brookes PC (2008) Relationships between soil pH and microbial properties in a UK arable soil *Soil Biol Biochem* 40:1856-1861
- Adl MS, Gupta VS (2006) Protists in soil ecology and forest nutrient cycling *Can J For Res* 36:1805-1817
- Ajwa HA, Rice CW, Sotomayor D (1998) Carbon and Nitrogen Mineralization in Tallgrass Prairie and Agricultural Soil Profiles *Soil Sci Soc Am J* 62:942-951
- Alauzet C, Jumas-Bilak E (2014) The Phylum Deferritales and the Genus *Caldithrix*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 595-611.
- Alimova A, Katz A, Steiner N, Rudolph E, Wei H, Steiner JC, Gottlieb P (2009) Bacteria-clay interaction: structural changes in smectite induced during biofilm formation *Clays Clay Miner* 57:205-212
- Allison SD (2005) Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments *Ecol Lett* 8:626-635
- Allison SD (2006) Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes *Biogeochemistry* 81:361-373
- Allison SD, Jastrow JD (2006) Activities of extracellular enzymes in physically isolated fractions of restored grassland soils *Soil Biol Biochem* 38:3245-3256
- Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities *Proc Natl Acad Sci U S A* 105:11512-11519
- Allison VJ, Yermakov Z, Miller RM, Jastrow JD, Matamala R (2007) Using landscape and depth gradients to decouple the impact of correlated environmental variables on soil microbial community composition *Soil Biol Biochem* 39:505-516
- Alvarado P, Manjón JL (2009) Selection of enzymes for terminal restriction fragment length polymorphism analysis of fungal internally transcribed spacer sequences *Appl Environ Microbiol* 75:4747-4752
- Apprill A, McNally S, Parsons R, Weber L. 2015. Minor revision to V4 region of SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*. 75:129-137
- Bach EM, Hofmockel KS (2014) Soil aggregate isolation method affects measures of intra-aggregate extracellular enzyme activity *Soil Biol Biochem* 69:54-62
- Bach EM, Hofmockel KS (2016) A time for every season: soil aggregate turnover stimulates decomposition and reduces carbon loss in grasslands managed for bioenergy *Glob Change Biol Bioen* 8: 588-599

- Badri DV, Vivanco JM (2009) Regulation and function of root exudates *Plant Cell Environ* 32:666-681
- Bailey VL, Fansler SJ, Stegen JC, McCue LA (2013) Linking microbial community structure to β -glucosidic function in soil aggregates *ISME J* 7:2044-2053
- Baker JM, Ochsner TE, Venterea RT, Griffis TJ (2007) Tillage and soil carbon sequestration—What do we really know? *Agric, Ecosyst Environ* 118:1-5
- Barber KR, Leeds-Harrison PB, Lawson CS, Gowing DJG (2004) Soil aeration status in a lowland wet grassland *Hydrol Process* 18:329-341
- Barois I, Villemin G, Lavelle P, Toutain F (1993) Transformation of the soil structure through *Pontoscolex corethrurus* (Oligochaeta) intestinal tract *Geoderma* 56:57-66
- Bartram AK, Lynch MD, Stearns JC, Moreno-Hagelsieb G, Neufeld JD (2011) Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end illumina reads *Appl Environ Microbiol* 77:3846-3852
- Batjes NH (1996) Total carbon and nitrogen in the soils of the world *Eur J Soil Sci* 47:151-163
- Bell JM, Smith JL, Bailey VL, Bolton H, Jr. (2003) Priming effect and C storage in semi-arid no-till spring crop rotations *Biol Fertility Soils* 37:237-244
- Bengtsson G, Hedlund K, Rundgren S (1993) Patchiness and compensatory growth in a fungus-Collembola system *Oecol* 93:296-302
- Benoit GR (1973) Effect of freeze-thaw cycles on aggregate stability and hydraulic conductivity of three soil aggregate sizes *Soil Sci Soc Am J* 37:3-5
- Bingeman CW, Varner JE, Martin WP (1953) The Effect of the Addition of Organic Materials on the Decomposition of an Organic Soil *Soil Sci Soc Am J* 17:34-38
- Birkhofer K et al. (2008) Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity *Soil Biol Biochem* 40:2297-2308
- Bjørnlund L, Ekelund F, Christensen S, Jacobsen CS, Krogh PH, Johnsen K (2000) Interactions between saprotrophic fungi, bacteria and protozoa on decomposing wheat roots in soil influenced by the fungicide fenpropimorph (Corbel®): a field study *Soil Biol Biochem* 32:967-975
- Blagodatskaya EV, Anderson T-H (1998) Interactive effects of pH and substrate quality on the fungal-to-bacterial ratio and qCO_2 of microbial communities in forest soils *Soil Biol Biochem* 30:1269-1274
- Blagodatskaya EV, Blagodatsky SA, Anderson TH, Kuzyakov Y (2007) Priming effects in Chernozem induced by glucose and N in relation to microbial growth strategies

- Blagodatskaya E, Kuzyakov Y (2008) Mechanisms of real and apparent priming effects and their dependence on soil microbial biomass and community structure: critical review *Biol Fertility Soils* 45:115-131
- Blomblack K, Eckersten H, Lewan E, Aronsson H (2003) Simulations of soil carbon and nitrogen dynamics during seven years in a catch drop experiment *Agric Syst* 76:95-114
- Blume E, Bischoff M, Reichert JM, Moorman T, Konopka A, Turco RF (2002) Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season *Appl Soil Ecol* 20:171-181
- Boer W, Folman LB, Summerbell RC, Boddy L (2005) Living in a fungal world: impact of fungi on soil bacterial niche development *FEMS Microbiol Rev* 29:795-811
- Boix-Fayos C, Calvo-Cases A, Imeson AC, Soriano-Soto MD (2001) Influence of soil properties on the aggregation of some Mediterranean soils and the use of aggregate size and stability as land degradation indicators *CATENA* 44:47-67
- Bol, R., Amelung, W. and Friedrich, C. (2004) Role of aggregate surface and core fraction in the sequestration of carbon from dung in a temperate grassland soil *Eur J Soil Sci*, 55: 71-77
- Bol R, Kandeler E, Amelung W, Glaser B, Marx MC, Preedy N, Lorenz K (2003a) Short-term effects of dairy slurry amendment on carbon sequestration and enzyme activities in a temperate grassland *Soil Biol Biochem* 35:1411-1421
- Bol R, Moering J, Kuzyakov Y, Amelung W (2003b) Quantification of priming and CO₂ respiration sources following slurry-C incorporation into two grassland soils with different C content *Rapid Commun Mass Spectrom* 17:2585-2590
- Bosatta E, Ågren GI (1999) Soil organic matter quality interpreted thermodynamically *Soil Biol Biochem* 31:1889-1891
- Bossuyt H, Denef K, Six J, Frey SD, Merckx R, Paustian K (2001) Influence of microbial populations and residue quality on aggregate stability *Appl Soil Ecol* 16:195-208
- Bossuyt H, Six J, Hendrix PF (2005) Protection of soil carbon by microaggregates within earthworm casts *Soil Biol Biochem* 37:251-258
- Boström B, Comstedt D, Ekblad A (2007) Isotope fractionation and ¹³C enrichment in soil profiles during the decomposition of soil organic matter *Oecol* 153:89-98
- Bourdin F (2012) Assessing the balance between greenhouse gases and ammonia emissions from Irish pastures amended with cattle slurry. Cranfield University
- Bourdin F, Lanigan GJ, Sakrabani R, Kibblewhite M (2010) Field assessment of the balance between greenhouse gases and ammonia emissions from grassland under

various N-management regimes. Paper presented at the Advances in Animal Bioscience (Proceedings of the British Society of Animal Science and the Agricultural Research Forum), Belfast, UK

- Bourdin F, Sakrabani R, Kibblewhite MG, Lanigan GJ (2014) Effect of slurry dry matter content, application technique and timing on emissions of ammonia and greenhouse gas from cattle slurry applied to grassland soils in Ireland *Agric, Ecosyst Environ* 188:122-133
- Bouwman LA, Bloem J, Boogert PHJF, Bremer F, Hoenderboom GHJ, Ruiten PC (1994) Short-term and long-term effects of bacterivorous nematodes and nematophagous fungi on carbon and nitrogen mineralization in microcosms *Biol Fert Soils* 17:249-256
- Brantley SLM, J. P. Scatena, F. N. Balogh-Brunstad, Z. Barnes, R. T. Bruns, M. A. Van Cappellen, P. Dontsova, K. Hartnett, H. E. Hartshorn, A. S. Heimsath, A. Herndon, E. Jin, L. Keller, C. K. Leake, J. R. McDowell, W. H. Meinzer, F. C. Mozdzer, T. J. Petsch, S. Pett-Ridge, J. Pregitzer, K. S. Raymond, P. A. Riebe, C. S. Shumaker, K. Sutton-Grier, A., Walter RY, K. (2011) Twelve testable hypotheses on the geobiology of weathering *Geobiology* 9:140-165
- Brockett BFT, Prescott CE, Grayston SJ (2012) Soil moisture is the major factor influencing microbial community structure and enzyme activities across seven biogeoclimatic zones in western Canada *Soil Biol Biochem* 44:9-20
- Bronick CJ, Lal R (2005) Soil structure and management: a review *Geoderma* 124:3-22
- Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil-nitrogen-A rapid direct extraction method to measure microbial biomass nitrogen in soil *Soil Biol Biochem* 17:837-842
- Bundt M, Widmer F, Pesaro M, Zeyer J, Blaser P (2001) Preferential flow paths: biological 'hot spots' in soils *Soil Biol Biochem* 33:729-738
- Burns RG et al. (2013) Soil enzymes in a changing environment: Current knowledge and future directions *Soil Biol Biochem* 58:216-234
- Butler JL, Williams MA, Bottomley PJ, Myrold DD (2003) Microbial Community Dynamics Associated with Rhizosphere Carbon Flow *Appl Environ Microbiol* 69:6793-6800
- Butler MJ, Day AW (1998) Fungal melanins: a review *Can J Microbiol* 44:1115-1136
- Cai P, Huang Q-Y, Zhang X-W (2006) Interactions of DNA with Clay Minerals and Soil Colloidal Particles and Protection against Degradation by DNase *Environ Sci Technol* 40:2971-2976
- Čapek P, Kotas P, Manzoni S, Šantrůčková H (2016) Drivers of phosphorus limitation across soil microbial communities *Funct Ecol* 30:1705-1713

- Caporaso JG et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample Proc Natl Acad Sci USA 108 Suppl 1:4516-4522
- Carreiro MM, Sinsabaugh RL, Rebert DA, Parkhurst DF (2000) Microbial enzyme shifts explain litter decay responses to stimulated nitrogen deposition Ecology 81:2359-2365
- Chabbi A, Kögel-Knabner I, Rumpel C (2009) Stabilised carbon in subsoil horizons is located in spatially distinct parts of the soil profile Soil Biol Biochem 41:256-261
- Chantigny MH, Rochette P, Angers DA (2001) Short-term C and N dynamics in a soil amended with pig slurry and barley straw: a field experiment Can J Soil Sci 81:131-137
- Charnay M-P, Tuis S, Coquet Y, Barriuso E (2005) Spatial variability in ¹⁴C-herbicide degradation in surface and subsurface soils Pest Manage Sci 61:845-855
- Chen H, Boutros P (2011) VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R BMC Bioinformatics 12
- Chen R et al. (2014) Soil C and N availability determine the priming effect: microbial N mining and stoichiometric decomposition theories Glob Change Biol 20:2356-2367
- Cheng F et al. (2013) Soil microbial biomass, basal respiration and enzyme activity of main forest types in the Qinling Mountains PLoS One 8:e67353
- Chenu C, Stotzky G (2002) Interactions between microorganisms and soil particles. An overview. Interactions Between Soil Particles and Microorganisms Wiley-VCH-Verlag, Weinheim.
- Clapp CE, M. H. B. Hayes, A. J. Simpson, Kingery WL (2005.) Chemistry of soil organic matter. Chemical processes in soils. Soil Science Society of America, Madison, Wisc
- Clarke KR, Ainsworth M (1993) A method of linking multivariate community structure to environmental variables Mar Ecol Prog Ser 92:205-219
- Clarke KR, Warwock RM (2001) Changes in marine communities: an approach to statistical analysis and interpretation. 2nd edn. PRIMER-E, Plymouth
- Clemens, J. and Huschka, A. (2001) ‘The effect of biological oxygen demand of cattle slurry and soil moisture on nitrous oxide emissions’, *Nutr Cycl Agroecosyt*, 59, 193-198
- Cleveland C, Liptzin D (2007) C:N:P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass? Biogeochemistry 85:235-252
- Clough TJ, Sherlock RR, Cameron KC, Ledgard SF (1996) Fate of urine nitrogen on mineral and peat soils in New Zealand Plant Soil 178:141-152

- Condon L, Stark C, O'Callaghsn M, Clinton P, Huang Z (2010) The role of microbial communities in the formation and decomposition of soil organic matter. *Soil Microbiology and Sustainable Crop Production*. Springer, London, UK
- Cotrufo MF, Wallenstein MD, Boot CM, Deneff K, Paul E (2013) The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Glob Change Biol* 19:988-995
- Creamer R et al. (2014) Irish Soil Information System (2007 S CD 1 S1) EPA STRIVE Programme 2007 2013, Synthesis Report
- Creamer RE et al. (2014) Measuring basal soil respiration across Europe: Do incubation temperature and incubation period matter? *Ecol Indicators* 36:409-418
- Curl EA, Truelove B (1986) *The rhizosphere*. Springer, Berlin
- D'Souza TM, Merritt CS, Reddy CA (1999) Lignin-modifying enzymes of the white rot basidiomycete *Ganoderma lucidum* *Appl Environ Microbiol* 65:5307-5313
- Dalenberg JW, Jager G (1989) Priming effect of some organic additions to ¹⁴C-labelled soil *Soil Biol Biochem* 21:443-448
- Davidson EA, Janssens IA (2006) Temperature sensitivity of soil carbon decomposition and feedbacks to climate change *Nature* 440:165-173
- Davinic M et al. (2012) Pyrosequencing and mid-infrared spectroscopy reveal distinct aggregate stratification of soil bacterial communities and organic matter composition *Soil Biol Biochem* 46:63-72
- Deneff K, Roobroeck D, Manimel Wadu MCW, Lootens P, Boeckx P (2009) Microbial community composition and rhizodeposit-carbon assimilation in differently managed temperate grassland soils *Soil Biol Biochem* 41:144-153
- Deneff K, Six J (2005) Clay mineralogy determines the importance of biological versus abiotic processes for macroaggregate formation and stabilization *Eur J Soil Sci* 56:469-479
- Deneff K, Six J, Merckx R, Paustian K (2004) Carbon Sequestration in Microaggregates of No-Tillage Soils with Different Clay Mineralogy *Soil Sci Soc Am J* 68:1935-1944
- De Nobili M, Contin M, Mondini C, Brookes PC (2001) Soil microbial biomass is triggered into activity by trace amounts of substrate *Soil Biol Biochem* 33:1163-1170
- Dennis SJ, Moir JL, Cameron KC, Di HJ, Hennessy D, K R (2011) Urine patch distribution under dairy grazing at three stocking rates in Ireland. *Irish J Agr Food*

- Derenne S, Largeau C (2001) A review of some important families of refractory macromolecules. Composition, origin and fate in soils and sediments. *Soil Sci* 166:833-847
- Dick RP (1992) Biotic Diversity in Agroecosystems A review: long-term effects of agricultural systems on soil biochemical and microbial parameters *Agric, Ecosyst Environ* 40:25-36
- Dilling J, Kaiser K (2002) Estimation of the hydrophobic fraction of dissolved organic matter in water samples using UV photometry *Water Res* 36:5037-5044
- Doronina N, Kaparullina E, Trotsenko Y (2014) The Family Methylophilaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) *The Prokaryotes – Alphaproteobacteria and Betaproteobacteria*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 869-880.
- Dowling, C., Curran, T. and Lanigan.G.J. (2008) The effect of application technique and climate conditions on ammonia emissions from cattle slurry *In: 13th Int. RAMIRAN proceedings, Albena, Bulgaria*
- Drenovsky RE, Elliott GN, Graham KJ, Scow KM (2004) Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities *Soil Biol Biochem* 36:1793-1800
- Drenovsky RE, Steenwerth KL, Jackson LE, Scow KM (2010) Land use and climatic factors structure regional patterns in soil microbial communities *Glob Ecol Biogeogr* 19:27-39
- Driver JD, Holben WE, Rillig MC (2005) Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi *Soil Biol Biochem* 37:101-106
- Droge S, Frohlich J, Radek R, Konig H (2006) *Spirochaeta coccoides* sp nov., a novel coccoid spirochete from the hindgut of the termite *Neotermes castaneus* *Appl Environ Microbiol* 72:392-397
- Duffy B, Schouten A, Raaijmakers JM (2003) Pathogen self-defense: Mechanisms to Counteract Microbial Antagonism *Annu Rev Phytopathol* 41:501-538
- Dungait JAJ, Bol R, Bull ID, Evershed RP (2009) Tracking the fate of dung-derived carbohydrates in a temperate grassland soil using compound-specific stable isotope analysis *Org Geochem* 40:1210-1218
- Dungait JAJ, Hopkins DW, Gregory AS, Whitmore AP (2012) Soil organic matter turnover is governed by accessibility not recalcitrance *Global Change Biol* 18:1781-1796
- Durso LM et al. (2010) Animal-to-Animal Variation in Fecal Microbial Diversity among

- Eilers KG, Debenport S, Anderson S, Fierer N (2012) Digging deeper to find unique microbial communities: The strong effect of depth on the structure of bacterial and archaeal communities in soil *Soil Biol Biochem* 50:58-65
- Ekelund F, Rønn R, Christensen S (2001) Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites *Soil Biol Biochem* 33:475-481
- Ekelund F, Rønn R, Christensen S (2001) Distribution with depth of protozoa, bacteria and fungi in soil profiles from three Danish forest sites *Soil Biol Biochem* 33:475-481
- Ekschmitt K et al. (2008) Soil-carbon preservation through habitat constraints and biological limitations on decomposer activity *J Plant Nutr Soil Sci* 171:27-35 doi:10.1002/jpln.200700051
- Elliott ET (1986) Aggregate Structure and Carbon, Nitrogen, and Phosphorus in Native and Cultivated Soils *Soil Sci Soc Am J* 50:627-633
- Eswaren H, Padmanabhan E (2006) *Soils of the Tropics and Subtropics vol 2nd. Encyclopedia of Soil Science, 2nd edn. Taylor & Francis Group, 270 Madison Avenue, NY*
- Eswaren H, Reich, P., Kimble, J.M., Beinroth, F.H., Padamnabhan, E and Moncharoen, P. (2000) Global Carbon stocks. *Global Climate Change and Pedogenic Carbonates. CRC/Lewis, Boca Raton, FL*
- Falchini L, Naumova N, Kuikman PJ, Bloem J, Nannipieri P (2003) CO₂ evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation *Soil Biol Biochem* 35:775-782
- Fan TWM, Lane AN, Chekmenev E, Wittebort RJ, Higashi RM (2004) Synthesis and physico-chemical properties of peptides in soil humic substances *J pept Sci* 63:253-264
- Fang C, Moncrieff JB (2005) The variation of soil microbial respiration with depth in relation to soil carbon composition *Plant Soil* 268:243-253
- Fangueiro D, Chadwick D, Dixon L, Bol R (2007) Quantification of priming and CO₂ emission sources following the application of different slurry particle size fractions to a grassland soil *Soil Biol Biochem* 39:2608-2620
- Fansler SJ, Smith JL, Bolton H, Bailey VL (2005) Distribution of two C cycle enzymes in soil aggregates of a prairie chronosequence *Biol Fertility Soils* 42:17-23
- Felske A, Wolterink A, Van Lis R, Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands)

Appl Environ Microbiol 64:871-879

Felske A, Wolterink A, Van Lis R, De Vos WM, Akkermans ADL (2000) Response of a soil bacterial community to grassland succession as monitored by 16S rRNA levels of the predominant ribotypes Appl Environ Microbiol 66:3998-4003

Feng Y, Li X (2001) An Analytical Model of Soil Organic Carbon Dynamics Based on A Simple "Hockey Stick" Function Soil Sci 166:431-440

Fernandez CW, Koide RT (2011) The role of chitin in the decomposition of ectomycorrhizal fungal litter Ecology 93:24-28

Fernandez CW, Koide RT (2014) Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter Soil Biol Biochem 77:150-157

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport Curr Opin Plant Biol 7:254-261

Fierer N, Chadwick OA, Trumbore SE (2005) Production of CO₂ in Soil Profiles of a California Annual Grassland Ecosystems 8:412-429

Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles Soil Biol Biochem 35:167-176

Flessa, H. and Besse, F. (2000) Laboratory estimates of trace gas emissions following surface application and injection of cattle slurry *J Environ Qual*, 29:262-268

Fogel R, Cromack Jr K (1977) Effect of habitat and substrate quality on Douglas fir litter decomposition in western Oregon Can J Bot 55:1632-1640

Fontaine S, Barot S, Barre P, Bdioui N, Mary B, Rumpel C (2007) Stability of organic carbon in deep soil layers controlled by fresh carbon supply Nature 450:277-280

Fontaine S et al. (2011) Fungi mediate long term sequestration of carbon and nitrogen in soil through their priming effect Soil Biol Biochem 43:86-96

Fontaine S, Mariotti A, Abbadie L (2003) The priming effect of organic matter: a question of microbial competition? Soil Biol Biochem 35:837-843

Fox A, Kwapinski W, Griffiths BS, Schmalenberger A (2014) The role of sulfur- and phosphorus-mobilizing bacteria in biochar-induced growth promotion of *Lolium perenne* FEMS Microbiol Ecol 90:78-91

Fox A, Gahan J, Ikoyi I, Kwapinski W, O'Sullivan O, Cotter PD, Schmalenberger A (2016) *Miscanthus* biochar promotes growth of spring barley and shifts bacterial community structures including phosphorus and sulfur mobilizing bacteria. *Pedobiologia* 59:195-202

- Freibauer A, Rounsevell MDA, Smith P, Verhagen J (2004) Carbon sequestration in the agricultural soils of Europe *Geoderma* 122:1-23
- Frey SD, Elliott ET, Paustian K (1999) Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients *Soil Biol Biochem* 31:573-585
- Gahan J, Schmalenberger A (2015) Arbuscular mycorrhizal hyphae in grassland select for a diverse and abundant hyphospheric bacterial community involved in sulfonate desulfurization *Appl Soil Ecol* 89:113-121
- Gahan J, Schmalenberger A (2014) The role of bacteria and mycorrhiza in plant sulfur supply *Front Plant Sci* 5: 723
- Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts *Mol Ecol* 2:113-118
- Ghorbani R, Wilcockson S, Koocheki A, Leifert C (2008) Soil management for sustainable crop disease control: a review *Environ Chem Lett* 6:149-162
- Ginting D, Kessavalou A, Eghball B, Doran JW (2003) Greenhouse gas emissions and soil indicators four years after manure and compost applications *J Environ Qual* 32:23-32
- Glaser, B., Bol, R., Preedy, N., McTiernan, K., Clark, M. and Amelung, W. (2001) Tracing slurry-derived carbon and nitrogen in a temperate grassland using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ natural abundance *J Plant Nutr Soil Sc* 164: 467-474
- Gleixner G, Poirier N, Bol R, Balesdent J (2002) Molecular dynamics of organic matter in a cultivated soil *Org Geochem* 33:357-366
- Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, MacPhee R, Reid G (2010) Microbiome Profiling by Illumina Sequencing of Combinatorial Sequence-Tagged PCR Products *PLoS One* 5:e15406.
- Goebel M-O, Bachmann J, Woche SK, Fischer WR, Horton R (2004) Water Potential and Aggregate Size Effects on Contact Angle and Surface Energy *Soil Sci Soc Am J* 68:383-393
- Gömöryová E, Gregor J, Pichler V, Gömöry D (2006) Spatial patterns of soil microbial characteristics and soil moisture in a natural beech forest *Biologia* 61:S329-S333
- Görres J, Savin M, Neher D, Weicht T, Amador J (1999) Grazing in a porous environment: 1. The effect of soil pore structure on C and N mineralization *Plant Soil* 212:75-83
- Griffiths BS (1994) Soil nutrient flow. Soil Protozoa. CAB International Wallingford, Oxon, UK

- Griffiths RI, Manefield M, Ostle N, McNamara N, O'Donnell AG, Bailey MJ, Whiteley AS (2004) $^{13}\text{CO}_2$ pulse labelling of plants in tandem with stable isotope probing: methodological considerations for examining microbial function in the rhizosphere *J Microbiol Methods* 58:119-129
- Grundmann GL (2004) Spatial scales of soil bacterial diversity - the size of a clone *FEMS Microbiol Ecol* 48:119-127
- Guggenberger G, Christensen BT, Zech W (1994) Land-use effects on the composition of organic matter in particle-size separates of soil: I. Lignin and carbohydrate signature *Eur J Soil Sci* 45:449-458
- Guggenberger G, Frey SD, Six J, Paustian K, Elliott ET (1999) Bacterial and Fungal Cell-Wall Residues in Conventional and No-Tillage Agroecosystems *Soil Sci Soc Am J* 63:1188-1198
- Gupta V, Germida JJ (1988) Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation *Soil Biol Biochem* 20:777-786
- Gupta V, Germida JJ (2015) Soil aggregation: Influence on microbial biomass and implications for biological processes *Soil Biol Biochem* 80:A3-A9
- Hamer U, Marschner B (2005) Priming effects in soils after combined and repeated substrate additions *Geoderma* 128:38-51
- Harris D, Horwath WR, van Kessel C (2001) Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis *Soil Sci Soc Am J* 65:1853-1856
- Harris JA et al. (2011) Does soil biology hold the key to optimized slurry management? A manifesto for research *Soil Use Manage* 27:464-469
- Clapp CE and Hayes MHB (1999) Characterization of humic substances isolated from clay- and silt-sized fractions of a corn residue-amended agricultural soil *Soil Sci* 164:899-913
- Heijnen CE, Chenu C, Robert M (1993) Micro-morphological studies on clay-amended and unamended loamy sand, relating survival of introduced bacteria and soil structure *Geoderma* 56:195-207
- Heijnen CE, van Veen JA (1991) A determination of protective microhabitats for bacteria introduced into soil *FEMS Microbiol Lett* 85:73-80
- Helfrich M, Ludwig B, Buurman P, Flessa H (2006) Effect of land use on the composition of soil organic matter in density and aggregate fractions as revealed by solid-state ^{13}C NMR spectroscopy *Geoderma* 136:331-341
- Hendriksen NB, Creamer RE, Stone D, Winding A (2016) Soil exo-enzyme activities across Europe—The influence of climate, land-use and soil properties *Applied*

- Hendrix PF, Parmelee RW, Crossley DA, Coleman DC, Odum EP, Groffman PM (1986) Detritus Food Webs in Conventional and No-tillage Agroecosystems *Bioscience* 36:374-380
- Holden NM, Fitzgerald D, Ryan D, Tierney H, Murphy F (2004) Rainfall climate limitation to slurry spreading in Ireland *Agricultural and Forest Meteorology* 122:207-214
- Hopkins FM, Filley TR, Gleixner G, Lange M, Top SM, Trumbore SE (2014) Increased belowground carbon inputs and warming promote loss of soil organic carbon through complementary microbial responses *Soil Biol Biochem* 76:57-69
- Hurst HM, Wagner GH (1969) Decomposition of ¹⁴C-Labeled Cell Wall and Cytoplasmic Fractions from Hyaline and Melanic Fungi *Soil Sci Soc Am J* 33:707-711
- Husson O (2013) Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: a transdisciplinary overview pointing to integrative opportunities for agronomy *Plant Soil* 362:389-417
- Hutchinson JJ, Campbell CA, Desjardins RL (2007) Some perspectives on carbon sequestration in agriculture *Agr Forest Meteorol* 142:288-302
- Jandl G, Leinweber P, Schulten HR, Eusterhues K (2004) The concentrations of fatty acids in organo-mineral particle-size fractions of a Chernozem *Eur J Soil Sci* 55:459-470
- Janssen BH (1996) Nitrogen mineralization in relation to C:N ratio and decomposability of organic materials *Plant Soil* 181:39-45
- Janzen HH (2005) Soil carbon: A measure of ecosystem response in a changing world? *Can J Soil Sci* 85:467-480
- Jastrow JD (1996) Soil aggregate formation and the accrual of particulate and mineral-associated organic matter *Soil Biol Biochem* 28:665-676
- Jeanson C (1964) Micromorphology and experimental soil zoology: contribution to the study, by means of giant-sized thin section, of earthworm-produced artificial soil structure. *Soil Micromorphology Proceedings of the Second International Work Mett on Soil Micromorphology Arnhem, the Netherlands*. Elsevier Publishing Company, Amsterdam
- Jiang Y and Sun, B (2016) Nematode grazing promotes bacterial community dynamics within soil aggregates 16th International Symposium on Microbial Ecology, ISME 16, Montreal, Canada.
- Jobbágy EG, Jackson RB (2000) The vertical distribution of soil organic carbon and its relation to climate and vegetation *Ecol Appl* 10:423-436

- Johansen A et al. (2005) Non-target effects of the microbial control agents *Pseudomonas fluorescens* DR54 and *Clonostachys rosea* IK726 in soils cropped with barley followed by sugar beet: a greenhouse assessment *Soil Biol Biochem* 37:2225-2239
- Jones CA et al. (2006) Conservation agriculture in Europe: an approach to sustainable crop production by protecting soil and water? SOWAP. . Jealott's Hill International Research Center, Bracknell, Berkshire.
- Jongmans AG, Pulleman MM, Marinissen JCY (2001) Soil structure and earthworm activity in a marine silt loam under pasture versus arable land *Biol Fertility Soils* 33:279-285
- Kandeler E, Tscherko D, Bruce KD, Stemmer M, Hobbs PJ, Bardgett RD, Amelung W (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil *Biol Fertil Soils* 32:390-400
- Kalbitz K, Schmerwitz J, Schwesig D, Matzner E (2003) Biodegradation of soil-derived dissolved organic matter as related to its properties *Geoderma* 113:273-291
- Kelleher BP, Simpson AJ (2006) Humic Substances in Soils: Are They Really Chemically Distinct? *Environ Sci Technol* 40:4605-4611
- Kemper WD, Koch EJ (1966) Aggregate stability of soils from the western portions of the United states and Canada. 1355, United States Government printing office, Washington, D.C
- Kirk TK, Farrell RL (1987) Enzymatic "Combustion": The Microbial Degradation of Lignin *Annu Rev Microbiol* 41:465-501
- Kleber M (2010) What is recalcitrant soil organic matter? *Envir Chem* 7:320-332
- Kleber M, Sollins P, Sutton R (2007) A conceptual model of organo-mineral interactions in soils: self-assembly of organic molecular fragments into zonal structures on mineral surfaces *Biogeochemistry* 85:9-24
- Knicker H (2004) Stabilization of N-compounds in soil and organic-matter-rich sediments—what is the difference? *Mar Chem* 92:167-195
- Knorr M, Frey SD, Curtis PS (2005) Nitrogen additions and litter decomposition: A meta-analysis *Ecology* 86:3252-3257 doi:10.1890/05-0150
- Kögel-Knabner I (2002) The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter *Soil Biol Biochem* 34:139-162
- Kong AY, Scow KM, Cordova-Kreylos AL, Holmes WE, Six J (2011) Microbial community composition and carbon cycling within soil microenvironments of conventional, low-input, and organic cropping systems *Soil Biol Biochem* 43:20-

- Kool DM, Hoffland E, Abrahamse S, van Groenigen JW (2006) What artificial urine composition is adequate for simulating soil N₂O fluxes and mineral N dynamics? *Soil Biol Biochem* 38:1757-1763
- Kramer C, Gleixner G (2008) Soil organic matter in soil depth profiles: Distinct carbon preferences of microbial groups during carbon transformation *Soil Biol Biochem* 40:425-433
- Krull ES, Skjemstad JO (2003) $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ profiles in ¹⁴C-dated Oxisol and Vertisols as a function of soil chemistry and mineralogy. *Geoderma* 112: 1–29
- Kuramae EE et al. (2010) Microbial secondary succession in a chronosequence of chalk grasslands *ISME J* 4:711-715
- Kuzyakov, Y. and Bol, R. (2004) Using natural ¹³C abundance to differentiate between three CO₂ sources during incubation of a grassland soil amended with slurry and sugar *J Plant Nutr Soil Sci* 167: 669-677
- Kuzyakov, Y. and Bol, R. (2005) 'Three sources of CO₂ efflux from soil partitioned by ¹³C natural abundance in an incubation study', *Rapid Commun Mass Spec*, 19, 1417-1423
- Kuzyakov Y, Bol R (2006) Sources and mechanisms of priming effect induced in two grassland soils amended with slurry and sugar *Soil Biol Biochem* 38:747-758
- Kuzyakov Y, Friedel JK, Stahr K (2000) Review of mechanisms and quantification of priming effects *Soil Biol Biochem* 32:1485-1498
- Ladygina N, Johansson T, Canback B, Tunlid A, Hedlund K (2009) Diversity of bacteria associated with grassland soil nematodes of different feeding groups *FEMS Microbiol Ecol* 69:53-61
- Lagomarsino A, Grego S, Kandeler E (2012) Soil organic carbon distribution drives microbial activity and functional diversity in particle and aggregate-size fractions *Pedobiologia* 55:101-110
- Lal R, Delgado JA, Groffman PM, Millar N, Dell C, Rotz A (2011) Management to mitigate and adapt to climate change *J Soil Water Conserv* 66:276-285
- Lal, R., 2008. Sequestration of atmospheric CO₂ in global carbon pools. *Energy Environ. Sci.* 1, 86.
- Lalor, S.T.J. and Schulte, R.P.O. (2008) Low-ammonia-emission application methods can increase the opportunity for application of cattle slurry to grassland in spring in Ireland *Grass Forage Sci*, 63(4): 531-544
- Lambie SM, Schipper LA, Balks MR, Baisden WT (2012) Carbon leaching from undisturbed soil cores treated with dairy cow urine *Soil Research* 50:320-327

- Lambie SM, Schipper LA, Balks MR, Baisden WT (2013) Priming of soil decomposition leads to losses of carbon in soil treated with cow urine *Soil Research* 51:513-520
- Lane DJ (1991) *Nucleic acid techniques in Bacterial systematics*. John Wiley&Sons,
- Lavelle P, Bignell D, Austen MC, Brown VK, Behan-Pelletier V, Garey JR (2004) Vulnerability of ecosystem services at different scales: role of biodiversity and implications for management *Sustaining Biodiversity and Functioning in soils and sediments*. Island Press, New York
- Lee KE (1985) *Earthworms. Their Ecology and Relationships with land Use*. Academic Press, N.Y
- Leff JW et al. (2015) Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe *PNAS* 112:10967-10972
- Liang C, Balser TC (2008) Preferential sequestration of microbial carbon in subsoils of a glacial-landscape toposequence, Dane County, WI, USA *Geoderma* 148:113-119
- Lichtfouse É, Berthier G, Houot S, Barriuso E, Bergheaud V, Vallaeyts T (1995) Stable carbon isotope evidence for the microbial origin of C¹⁴–C¹⁸ n-alkanoic acids in soils *Org Geochem* 23:849-852
- Litchman E, Edwards KF, Klausmeier CA (2015) Microbial resource utilization traits and trade-offs: implications for community structure, functioning, and biogeochemical impacts at present and in the future *Front Microb* 6
- Liu YP, Tan HM, Deng QL, Cao LX (2014) Characterization of fibrolytic and lipid accumulating fungi isolated from fresh cattle feces *Environ Sci Pollut Res* 21:9228-9233
- Loveland P, Webb J (2003) Is there a critical level of organic matter in the agricultural soils of temperate regions: a review *Soil and Tillage Research* 70:1-18
- Lovell RD, Jarvis SC (1996) Effects of urine on soil microbial biomass, methanogenesis, nitrification and denitrification in grassland soils *Plant Soil* 186:265-273
- Lovley DR, Coates JD, Blunt-Harris EL, Phillips EJP, Woodward JC (1996) Humic substances as electron acceptors for microbial respiration *Nature* 382:445-448
- Lueders T, Friedrich MW (2003) Evaluation of PCR Amplification Bias by Terminal Restriction Fragment Length Polymorphism Analysis of Small-Subunit rRNA and mcrA Genes by Using Defined Template Mixtures of Methanogenic Pure Cultures and Soil DNA Extracts *Appl Environ Microbiol* 69:320-326
- Lundell TK, Makela MR, Hilden K (2010) Lignin-modifying enzymes in filamentous basidiomycetes--ecological, functional and phylogenetic review *J Basic Microbiol* 50:5-20

- Lundquist EJ, Jackson LE, Scow KM, Hsu C (1999) Changes in microbial biomass and community composition, and soil carbon and nitrogen pools after incorporation of rye into three California agricultural soils *Soil Biol Biochem* 31:221-236
- Lützw Mv, Kögel-Knabner I, Ekschmitt K, Matzner E, Guggenberger G, Marschner B, Flessa H (2006) Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions – a review *Eur J Soil Sci* 57:426-445
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology *Microbiol Mol Biol Rev* 66:506-577
- Ma L, Guo C, Lu X, Yuan S, Wang R (2015) Soil moisture and land use are major determinants of soil microbial community composition and biomass at a regional scale in northeastern China *Biogeosciences* 12:2585-2596
- Madigan MT MJ (2009) Brock biology of microorganisms. 11th edn. Pearson Education Inc., Prentice Hall, New Jersey
- Maillard LC (1912) Action des acides amines sur les sucres: formation des melanodines par voie methodique *CRHebdSeancesAcadSci* 154:66-68
- Malik KA, Haider K (1982) Decomposition of ¹⁴C-labelled melanoid fungal residues in a marginally sodic soil *Soil Biol Biochem* 14:457-460
- Marinissen JCY (1994) Earthworms and stability of soil structure: a study in a silt loam soil in a young Dutch polder *Agric, Ecosyst Environ* 51:75-87
- Marinissen JCY, Dexter AR (1990) Mechanisms of stabilization of earthworm casts and artificial casts *Biol Fertility Soils* 9:163-167
- Marschner B et al. (2008) How relevant is recalcitrance for the stabilization of organic matter in soils? *J Plant Nutr Soil Sci* 171:91-110
- Martens DA, Johanson JB, Frankenberger WT (1992) Production and persistence of soil enzyme with repeated addition of organic residues. *Soil Sci* 153:53-61
- Martin A (1991) Short- and long-term effects of the endogeic earthworm *Millsonia anomala* (Omodeo) (Megascolecidae, Oligochaeta) of tropical savannas, on soil organic matter *Biol Fertil Soils* 11:234-238
- Marx MC, Kandeler E, Wood M, Wermbter N, Jarvis SC (2005) Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions *Soil Biol Biochem* 37:35-48
- Massey P et al. (2014) Irish SIS Final Technical Report 7: Irish Soil Information System: Laboratory Standard Operating Procedures. Environmental Protection Agency Ireland
- McKenzie BM, Dexter AR (1987) Physical properties of casts of the earthworm

Aporrectodea rosea Biol Fertility Soils 5:152-157

- Meharg AA, Killham K (1995) Loss of exudates from the roots of perennial ryegrass inoculated with a range of microorganisms Plant Soil 170:345-349
- Melillo J, Aber J, Linkins A, Ricca A, Fry B, Nadelhoffer K (1989) Carbon and nitrogen dynamics along the decay continuum: Plant litter to soil organic matter Plant Soil 115:189-198
- Mikha MM, Rice CW (2004) Tillage and manure effects on soil and aggregate-associated carbon and nitrogen Soil Sci Soc Am J 68:809-816
- Mikola J, Setälä H (1998) No evidence of trophic cascades in an experimental microbial-based soil food web Ecology 79:153-164
- Milcu A, Heim A, Ellis RJ, Scheu S, Manning P (2011) Identification of General Patterns of Nutrient and Labile Carbon Control on Soil Carbon Dynamics Across a Successional Gradient Ecosystems 14:710-719
- Miller M, Dick RP (1995) Dynamics of soil C and microbial biomass in whole soil and aggregates in two cropping systems Applied Soil Ecology 2:253-261
- Miltner A, Bombach P, Schmidt-Brücken B, Kästner M (2012) SOM genesis: microbial biomass as a significant source Biogeochemistry 111:41-55
- Moir JL, Cameron KC, Di HJ, Fertsak U (2011) The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system The Journal of Agricultural Science 149:473-485
- Mondini C, Cayuela ML, Sanchez-Monedero MA, Roig A, Brookes PC (2006) Soil microbial biomass activation by trace amounts of readily available substrate Biol Fertility Soils 42:542-549
- Morvan T, Leterme P, Mary B (1996) Quantifying nitrogen flux after application of N-15-labelled pig slurry on triticale in the late autumn Agronomie 16:541-552
- Morvan, T., Nicolardot, B. and Penn, L. (2006) Biochemical composition and kinetics of C and N mineralisation of animal wastes: a topological approach *Biol Fert Soils*, 42: 513-522
- Moynihan EL, Richards KG, Brennan FP, Tyrrel SF, Ritz K (2015) Enteropathogen survival in soil from different land-uses is predominantly regulated by microbial community composition Applied Soil Ecology 89:76-84
- Mueller B (2015) Experimental Interactions Between Clay Minerals and Bacteria: A Review Pedosphere 25:799-810
- Muller C, Laughlin RJ, Christie P, Watson CJ (2011) Effects of repeated fertilizer and cattle slurry applications over 38 years on N dynamics in a temperate grassland

soil *Soil Biol Biochem* 43:1362-1371

- Mummey D, Holben W, Six J, Stahl P (2006) Spatial stratification of soil bacterial populations in aggregates of diverse soils *Microb Ecol* 51:404-411
- Mummey DL, Stahl PD (2004) Analysis of soil whole- and inner-microaggregate bacterial communities *Microb Ecol* 48:41-50
- Murphy DV, Sparling GP, Fillery IRP (1998) Stratification of microbial biomass C and N and gross N mineralisation with soil depth in two contrasting Western Australian agricultural soils *Soil Research* 36:45-56
- Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA *Appl Environ Microbiol* 59:695-700
- Naether A et al. (2012) Environmental Factors Affect Acidobacterial Communities below the Subgroup Level in Grassland and Forest Soils *Appl Environ Microbiol* 78:7398-7406
- Naidja A, Huang PM, Bollag J-M (2000) Enzyme-clay interactions and their impact on transformations of natural and anthropogenic organic compounds in soil *J Environ Qual* 29
- Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions *Eur J Soil Sci* 54:655-670
- Negassa WC, Guber AK, Kravchenko AN, Marsh TL, Hildebrandt B, Rivers ML (2015) Properties of soil pore space regulate pathways of plant residue decomposition and community structure of associated bacteria *PLoS One* 10:1-22
- Neufeld JD, Vohra J, Dumont MG, Lueders T, Manefield M, Friedrich MW, Murrell JC (2007) DNA stable-isotope probing *Nat Protocols* 2:860-866
- Neumann D, Heuer A, Hemkemeyer M, Martens R, Tebbe CC (2013) Response of microbial communities to long-term fertilization depends on their microhabitat *FEMS Microbiol Ecol* 86:71-84
- Nguyen BV, Olk DC, Cassman KG (2004) Characterisation of humic acid fractions improves estimates of nitrogen mineralization kinetics for lowland rice soils *Soil Sci Soc Am J* 68:1266-1277
- Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: a critical technical review *Microb Ecol* 54:276-289
- Nunan N et al. (2006) Sheep-urine-induced changes in soil microbial community structure *FEMS Microbiol Ecol* 56:310-320

- Nunan N, Wu KJ, Young IM, Crawford JW, Ritz K (2003) Spatial distribution of bacterial communities and their relationships with the micro-architecture of soil FEMS Microbiol Ecol 44:203-215
- O'Mara F (2008) Country pasture/forage resource profiles: Ireland. Food and agriculture organization of the United Nations (FAO), Rome, Italy
- Oades JM (1984) Soil organic matter and structural stability- mechanisms for implications and management Plant Soil 76: 319-337
- Oades JM (1993) The role of biology in the formation, stabilization and degradation of soil structure Geoderma 56:377-400
- Oades JM, Waters A (1991) Aggregate hierarchy in soils Soil Research 29:815-828
- Okafor N (1966) Estimation of the decomposition of chitin in soil by the method of carbon dioxide release Soil Sci 102:140-142
- Okubo A, Sugiyama S-i (2009) Comparison of molecular fingerprinting methods for analysis of soil microbial community structure Ecol Res 24:1399
- Orgiazzi A et al. (2016) Global soil biodiversity atlas. European Commission, Publications Office of the European Union, Luxembourg
- Parham JA, Deng SP, Da HN, Sun HY, Raun WR (2003) Long-term cattle manure application in soil. II. Effect on soil microbial populations and community structure Biol Fertility Soils 38:209-215
- Paustian K, Collins HP, Paul EA (1997) Management controls on soil carbon. . Soil Organic Matter in Temperate Agroecosystems: Long-term Experiments in North America. CRC Press, Boca Raton, FL
- Paustian K, Six J, Elliott ET, Hunt HW (2000) Management options for reducing CO₂ emissions from agricultural soils Biogeochemistry 48:147-163
- Peacock AD, Mullen MD, Ringelberg DB, Tyler DD, Hedrick DB, Gale PM, White DC (2001) Soil microbial community responses to dairy manure or ammonium nitrate applications Soil Biol Biochem 33:1011-1019
- Penny C, Nadalig T, Alioua M, Gruffaz C, Vuilleumier S, Bringel F (2010) Coupling of denaturing high-performance liquid chromatography and terminal restriction fragment length polymorphism with precise fragment sizing for microbial community profiling and characterization Appl Environ Microbiol 76:648-651
- Perez Mateos M, Gonzalez Carcedo S Effect of fractionation on location of enzyme activities in soil structural units Biol Fertility Soils 1:153-159

- Peterson GA, Halvorson AD, Havlin JL, Jones OR, Lyon DJ, Tanaka DL (1998) Reduced tillage and increasing cropping intensity in the Great Plains conserves soil C *Soil Till Res* 47:207-218
- Phillips RP, Finzi AC, Bernhardt ES (2011) Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO₂ fumigation *Ecol Lett* 14:187-194
- Picardeau M (2014) The Family Leptospiraceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 711-729.
- Plante AF, McGill WB (2002) Intra-seasonal Soil Macroaggregate Dynamics in Two Contrasting Field Soils Using Labeled Tracer Spheres The Research for this paper was performed at the Department of Renewable Resources, University of Alberta *Soil Sci Soc Am J* 66:1285-1295
- PiÑeiro G, Oesterheld M, Batista WB, Paruelo JM (2006) Opposite changes of whole-soil vs. pools C : N ratios: a case of Simpson's paradox with implications on nitrogen cycling *Glob Change Biol* 12:804-809
- Plaza C, Hernández D, García-Gil JC, Polo A (2004) Microbial activity in pig slurry-amended soils under semiarid conditions *Soil Biol Biochem* 36:1577-1585
- Poll C, Ingwersen J, Stemmer M, Gerzabek MH, Kandeler E (2006) Mechanisms of solute transport affect small-scale abundance and function of soil microorganisms in the detritusphere *Eur J Soil Sci* 57:583-595
- Poll C, Thiede A, Wermbter N, Sessitsch A, Kandeler E (2003) Micro-scale distribution of microorganisms and microbial enzyme activities in a soil with long-term organic amendment *Eur J Soil Sci* 54:715-724
- Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ (2001) Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties *Appl Environ Microbiol* 67:2255-2262
- Qiu S et al. (2013) Effects of open drainage ditch design on bacterial and fungal communities of cold waterlogged paddy soils *Braz J Microbiol* 44:983-991
- Ranjard L, Poly F, Combrisson J, Richaume A, Gourbiere F, Thioulouse J, Nazaret S (2000) Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA) *Microb Ecol* 39:263-272
- Ranjard L, Richaume A (2001) Quantitative and qualitative microscale distribution of bacteria in soil *Res Microbiol* 152:707-716
- Reicosky DC, Dugas WA, Torbert HA (1997) Tillage-induced soil carbon dioxide loss

from different cropping systems *Soil Till Res* 41:105-118

- Renella G, Landi L, Nannipieri P (2002) Hydrolase activities during and after the chloroform fumigation of soil as affected by protease activity *Soil Biol Biochem* 34:51-60
- Rice WC, Galyean ML, Cox SB, Dowd SE, Cole NA (2012) Influence of wet distillers grains diets on beef cattle fecal bacterial community structure *BMC Microbiol* 12:13
- Richter A (2016) Integrating soil characteristics, land management and soil microbial communities. University College Dublin
- Rillig M, Caldwell B, Wösten HB, Sollins P (2007) Role of proteins in soil carbon and nitrogen storage: controls on persistence *Biogeochemistry* 85:25-44
- Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure *New Phytol* 171:41-53
- Rillig M, Wright S, Nichols K, Schmidt W, Torn M (2001) Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils *Plant Soil* 233:167-177
- Rillig MC (2004) Arbuscular mycorrhizae, glomalin, and soil aggregation *Can J Soil Sci* 84: 355–363
- Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure *New Phytol* 171:41-53
- Robertson GP, Klingensmith KM, Klug MJ, Paul EA, Crum JR, Ellis BG (1997) Soil resources, microbial activity, and primary production across an agricultural ecosystem *Ecol Appl* 7:158-170
- Robyt JF (1998) *Essentials of Carbohydrate Chemistry*. Springer,, New York, USA.
- Rochette P, Angers DA, Côté D (2000) Soil Carbon and Nitrogen Dynamics Following Application of Pig Slurry for the 19th Consecutive Year I. Carbon Dioxide Fluxes and Microbial Biomass *Carbon Soil Sci Soc Am J* 64:1389-1395
- Rochette P, Angers DA, Chantigny MH, Bertrand N, Côté D (2004a) Carbon Dioxide and Nitrous Oxide Emissions following Fall and Spring Applications of Pig Slurry to an Agricultural Soil *Soil Sci Soc Am J* 68:1410-1420
- Rochette P, Angers DA, Chantigny MH, Bertrand N, Côté D (2004b) Carbon Dioxide and Nitrous Oxide Emissions following Fall and Spring Applications of Pig Slurry to an Agricultural Soil *Soil Sci Soc Am J* 68:1410-1420
- Rochette, P., Angers, D.A., Chantigny, M.H., Gagnon, B. and Bertrand, N. (2006) 'In situ mineralization of dairy cattle manures as determined using soil-surface carbon dioxide fluxes', *Soil Sci Soc Am J*, 70, 744-752

- Rooney D, Kennedy N, Deering L, Gleeson D, Clipson N (2006) Effect of sheep urine deposition on the bacterial community structure in an acidic upland grassland soil *Appl Environ Microbiol* 72:7231-7237
- Roper MM, Gupta V (1995) Management-practices and soil biota *Aust J Soil Res* 33:321-339
- Rosenberg K, Bertaux J, Krome K, Hartmann A, Scheu S, Bonkowski M (2009) Soil amoebae rapidly change bacterial community composition in the rhizosphere of *Arabidopsis thaliana* *ISME J* 3:675-684
- Ruamps LS, Nunan N, Pouteau V, Leloup J, Raynaud X, Roy V, Chenu C (2013) Regulation of soil organic C mineralisation at the pore scale *FEMS Microbiol Ecol* 86:26-35
- Rumpel C, Chabbi A, Marschner B (2012) Carbon Storage and Sequestration in Sub-soil Horizons: Knowledge, Gaps and Potentials. *Recarbonization of the Biosphere Ecosystems and Global Carbon Cycle*. Springer Academic Press,
- Rumpel C, Kögel-Knabner I (2011) Deep soil organic matter—a key but poorly understood component of terrestrial C cycle *Plant Soil* 338:143-158
- Sanaullah M, Chabbi A, Leifeld J, Bardoux G, Billou D, Rumpel C (2011) Decomposition and stabilization of root litter in top- and subsoil horizons: what is the difference? *Plant Soil* 338:127-141
- Sauheitl L, Glaser B, Bol R (2005) Short-term dynamics of slurry-derived plant and microbial sugars in a temperate grassland soil as assessed by compound-specific $\delta^{13}\text{C}$ analyses *Rapid Commun Mass Spectrom* 19:1437-1446
- Saviozzi A, Levi-Minzi R, Riffaldi R, Vanni G (1997) Role of chemical constituents of wheat straw and pig slurry on their decomposition in soil *Biol Fertility Soils* 25:401-406
- Schimel JP, Schaeffer SM (2012) Microbial control over carbon cycling in soil *Front Microbiol* 3:11
- Schipper LA, Baisden WT, Parfitt RL, Ross C, Claydon JJ, Arnold G (2007) Large losses of soil C and N from soil profiles under pasture in New Zealand during the past 20 years *Glob Change Biol* 13:1138-1144
- Schlesinger WH (1995) An over-view of the carbon cycle. *Soils and Global Change*. Lewis Publishers, Boca Raton
- Schmalenberger A, O'Sullivan O, Gahan J, Cotter PD, Courtney R (2013) Bacterial communities established in bauxite residues with different restoration histories *Environ Sci Technol* 47:7110-7119
- Schmalenberger A, Fox A (2016) Chapter Three - Bacterial Mobilization of Nutrients From Biochar-Amended Soils. In: Sima S, Geoffrey Michael G (eds) *Adv Appl Microbiol*, vol Volume 94. Academic Press, pp 109-159

- Schulten H-R, Schnitzer M Three-dimensional models for humic acids and soil organic matter *Naturwissenschaften* 82:487-498
- Schulten H (1994) A chemical structure for humic acid. Pyrolysis-gas chromatography and pyrolysis-soft ionization mass spectroscopy evidence. *Humic substances in the Global environment and implications for human health*. Elsevier, Amsterdam
- Schutter ME, Dick RP (2002) Microbial community profiles and activities among aggregates of winter fallow and cover-cropped soil *Soil Sci Soc Am J* 66:142-153
- Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H, Kandeler E (2001) Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment *Appl Environ Microbiol* 67:4215-4224
- Sexstone AJ, Revsbech NP, Parkin TB, Tiedje TM (1985) Direct measurement of oxygen profiles and denitrification rates in soil aggregates *Soil Sci Soc Am J* 49:645-651
- Shipitalo MJ, Protz R (1988) Factors Influencing the Dispersibility of Clay in Worm Casts *Soil Sci Soc Am J* 52:764-769
- Simpson AJ, Simpson MJ, Smith E, Kelleher BP (2007) Microbially Derived Inputs to Soil Organic Matter: Are Current Estimates Too Low? *Environ Sci Technol* 41:8070-8076
- Sipilä TP, Yrjälä K, Alakukku L, Palojärvi A (2012) Cross-Site Soil Microbial Communities under Tillage Regimes: Fungistasis and Microbial Biomarkers *Appl Environ Microbiol* 78:8191-8201
- Six J, Bossuyt H, Degryze S, Deneff K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics *Soil Till Res* 79:7-31
- Six J, Conant RT, Paul EA, Paustian K (2002) Stabilization mechanisms of soil organic matter: Implications for C-saturation of soils *Plant Soil* 241:155-176
- Six J, Elliott ET, Paustian K (1999) Aggregate and soil organic matter dynamics under conventional and no-tillage systems *Soil Sci Soc Am J* 63:1350-1358
- Six J, Elliott ET, Paustian K (2000) Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture *Soil Biol Biochem* 32:2099-2103
- Six J, Elliott ET, Paustian K, Doran JW (1998) Aggregation and soil organic matter accumulation in cultivated and native grassland soils *Soil Sci Soc Am J* 62:1367-1377

- Six J, Frey SD, Thiet RK, Batten KM (2006) Bacterial and Fungal Contributions to Carbon Sequestration in Agroecosystems *Soil Sci Soc Am J* 70:555-569
- Smith P (2004) Soils as carbon sinks: the global context *Soil Use Manage* 20:212-218
- Smith SE, Read D (2008) *Mycorrhizal symbiosis*. 3rd edn. Academic press, San Diego, CA, USA
- Sollins P, Homann P, Caldwell BA (1996) Stabilization and destabilization of soil organic matter: mechanisms and controls *Geoderma* 74:65-105
- Soussana JF, Lüscher A (2007) Temperate grasslands and global atmospheric change: a review *Grass Forage Sci* 62:127-134
- Spence A et al. (2011) The degradation characteristics of microbial biomass in soil *Geochim Cosmochim Acta* 75:2571-2581
- Spink J, Hackett R, Forristal D, Creamer R (2010) Soil Organic Carbon: A review of 'critical' levels and practices to increase levels in tillage land in Ireland Teagasc, Oak Park, Carlow
- Stevenson FJ (1982) *Humus chemistry: Genesis, Composition, Reactions*. 2nd Ed. edn. Wiley & Sons, New York,
- Strickland MS (2009) *Ecosystem carbon cycling: Relationships to soil microbial community structure*. University of Georgia
- Strickland MS, Rousk J (2010) Considering fungal:bacterial dominance in soils – Methods, controls, and ecosystem implications *Soil Biol Biochem* 42:1385-1395
- Taylor BR, Parkinson D, Parsons WFJ (1989) Nitrogen and Lignin Content as Predictors of Litter Decay Rates: A Microcosm Test *Ecology* 70:97-104
- Taylor JP, Wilson B, Mills MS, Burns RG (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques *Soil Biol Biochem* 34:387-401
- Teagasc (2011), In: *Irish agriculture, greenhouse gas emissions and climate change: opportunities, obstacles and proposed solutions*, (Eds.) Schulte, R.P.O and Lanigan, G. Carlow, Ireland
- Teagasc (2015) *Dairy Farming Systems Post Milk Quota Abolition*.2015 Series No 1. Dublin, Ireland.
- Teeling H, Glöckner FO (2012) Current opportunities and challenges in microbial metagenome analysis—a bioinformatic perspective *Briefings in Bioinformatics* 13:728-742
- ten Have R, Teunissen PJM (2001) *Oxidative Mechanisms Involved in Lignin*

- Thies JE (2008) Molecular Methods for Studying Microbial Ecology in the Soil and Rhizosphere. In: Nautiyal CS, Dion P (eds) Molecular Mechanisms of Plant and Microbe Coexistence. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 411-436.
- Thiet RK, Frey SD, Six J (2006) Do growth yield efficiencies differ between soil microbial communities differing in fungal:bacterial ratios? Reality check and methodological issues Soil Biol Biochem 38:837-844
- Torres-Sallan G (2016) Physical and Biochemical Characterisation of Soil Organic Carbon in the Topsoil and Subsoil of Irish Grasslands. University of Limerick
- Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soils J Soil Sci 33:141-163
- Tomlinson RW (2006) Climate change – Land Use, Land-Use Change and Carbon Stocks. Environmental Protection Agency, Johnstown Castle Wexford
- Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria Appl Environ Microbiol 56:782-787
- Treseder KK, Allen MF (2000) Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO₂ and nitrogen deposition New Phytol 147:189-200
- Trofymow JA, Morley CR, Coleman DC, Anderson RV (1983) Mineralization of cellulose in the presence of chitin and assemblages of microflora and fauna in soil Oecologia 60:103-110
- Trumbore S (1997) Potential responses of soil organic carbon to global environmental change Proc Natl Acad Sci U S A 94:8284-8291
- Trumbore S (2000) Age of soil organic matter and soil respiration: Radiocarbon constraints on belowground C dynamics Ecol Appl 10:399-411
- Unc A, Goss MJ (2004) Transport of bacteria from manure and protection of water resources Appl Soil Ecol 25:1-18
- Uchida Y, Clough T, Kelliher F, Hunt J, Sherlock R (2011) Effects of bovine urine, plants and temperature on N₂O and CO₂ emissions from a sub-tropical soil Plant Soil 345:171-186
- Unger IM, Kennedy AC, Muzika R-M (2009) Flooding effects on soil microbial communities Appl Soil Ecol 42:1-8
- Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C Soil Biol Biochem 19:703-707 d
- vanGestel M, Merckx R, Vlassak K (1996) Spatial distribution of microbial biomass in microaggregates of a silty-loam soil and the relation with the resistance of

microorganisms to soil drying *Soil Biol Biochem* 28:503-510

- Van Haastert PJM, Bosgraaf L (2009) The local cell curvature guides pseudopodia towards chemoattractants *HFSP Journal* 3:282-286 doi:10.2976/1.3185725
- Van Veen JA, Kuikman PJ (1990) Soil structural aspects of decomposition of organic matter by micro-organisms *Biogeochemistry* 11:213-233
- von Lützw M, Kögel-Knabner I (2010) Response to the concept paper: 'What is recalcitrant soil organic matter?' by Markus Kleber *Envir Chem* 7:333-335
- von Lützw M, Kögel-Knabner I, Ekschmitt K, Flessa H, Guggenberger G, Matzner E, Marschner B (2007) SOM fractionation methods: Relevance to functional pools and to stabilization mechanisms *Soil Biol Biochem* 39:2183-2207
- von Lützw M, Kögel-Knabner I, Ekschmitt K, Matzner E, Guggenberger G, Marschner B, Flessa H (2006) Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions - a review *Eur J Soil Sci* 57:426-445
- Voroney, R. (2007) *The Soil Habitat. Soil Microbiology, Ecology and Biochemistry*, Paul, A. (eds) 3rd edn. Academic Press, London, UK
- Vos M, Wolf AB, Jennings SJ, Kowalchuk GA (2013) Micro-scale determinants of bacterial diversity in soil *FEMS Microbiol Rev* 37:936-954
- Wagner C, Grieshammer A, Drake HL (1996) Acetogenic capacities and the anaerobic turnover of carbon in a Kansas prairie soil *Appl Environ Microbiol* 62:494-500
- Waksman SA (1924) Influence of microorganisms upon the carbon-nitrogen ratio in the soil *The Journal of Agricultural Science* 14:555-562
- Waksman SA (1932) Contribution to our knowledge of the chemical nature and origins of humus: I. On the synthesis of the humus nucleus *Soil Sci* 34: 43-70
- Wang E, Cruse RM, Chen X, Daigh A (2012) Effects of moisture condition and freeze/thaw cycles on surface soil aggregate size distribution and stability *Can J Soil Sci* 92:529-536
- Wang WJ, Smith CJ, Chen D (2004) Predicting Soil Nitrogen Mineralization Dynamics with a Modified Double Exponential Model *Soil Sci Soc Am J* 68:1256-1265
- Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere *J Exp Bot* 52:487-511
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: a guide to methods and applications*. Academic Press, New York, USA
- Whitman T, Pepe-Ranney C, Enders A, Koechli C, Campbell A, Buckley DH, Lehmann J

- (2016) Dynamics of microbial community composition and soil organic carbon mineralization in soil following addition of pyrogenic and fresh organic matter *ISME J* 10:2918-2930
- Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority *Proc Natl Acad Sci U S A* 95:6578-6583
- Will C et al. (2010) Horizon-specific bacterial community composition of german grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes *Appl Environ Microbiol* 76:6751-6759
- Williams B, Grayston S, Reid E (2000) Influence of synthetic sheep urine on the microbial biomass, activity and community structure in two pastures in the Scottish uplands *Plant Soil* 225:175-185
- WRB IWG (2006) World Reference Base for Soil Resources – a framework for international classification, correlation and communication. World Soil Resources Reports 103. ISSN 0532-0188, FAO Rome, 128pp.
- Wu J, Joergensen RG, Pommerening B, Chaussod R, Brookes PC (1990) Measurement of soil microbial biomass C by fumigation-extraction—an automated procedure *Soil Biol Biochem* 22:1167-1169
- Wyszkowska J, Wyszkowski M (2002) Effect of cadmium and magnesium on microbiological activity in soil *Pol J Environ Stud* 11:585-591
- Xiang S-R, Doyle A, Holden PA, Schimel JP (2008) Drying and rewetting effects on C and N mineralization and microbial activity in surface and subsurface California grassland soils *Soil Biol Biochem* 40:2281-2289
- Xue K et al. (2013) Functional Gene Differences in Soil Microbial Communities from Conventional, Low-Input, and Organic Farmlands *Appl Environ Microbiol* 79:1284-1292
- Young IM, Crawford JW (2004) Interactions and Self-Organization in the Soil-Microbe Complex *Science* 304:1634
- Young IM, Ritz K (2000) Tillage, habitat space and function of soil microbes *Soil and Tillage Research* 53:201-213
- Yousefi M, Hajabbasi M, Shariatmadari H (2008) Cropping system effects on carbohydrate content and water-stable aggregates in a calcareous soil of Central Iran *Soil Till Res* 101:57-61
- Zahn G, Wagai R, Yonemura S (2016) The effects of amoebal bacterivory on carbon and nitrogen dynamics depend on temperature and soil structure interactions *Soil Biol Biochem* 94:133-137
- Zhao C et al. (2016) Soil microbial community composition and respiration along an experimental respiration gradient in a semi arid steppe *Sci Rep* 6: 24317

Chapter VIII: Appendix

Appendix A: Chapter I

A8 1: Bacterial and Fungal t-RF richness:

Table 16: Averages (Avg.) and standard deviations (\pm) of bacterial 16S and fungal ITS t-RF richness within horizon, aggregate-size fraction and site. Statistical significance defined as $P < 0.05$.

Community	Parameter	P-value	\sqrt{CV}	Pairwise	t-RF Richness Avg.	\pm	Pairwise	P-value
Bacterial 16S	Horizon	0.09		H1	4.39	0.87	H1 vs H2	0.72
				H2	4.32	0.96	H1 vs H3	0.17
				H3	4.07	0.76	H2 vs H3	0.36
	Aggregate-size fraction	0.0001	6.84	LM	4.3	0.79	LM vs MAC	0.01
				MAC	3.65	0.64	LM vs MIC	0.2
				MIC	4.02	0.61	LM vs SC	0.004
				SC	5.04	0.8	MAC vs MIC	0.051
							MAC vs MIC	<0.0001
							MIC vs SC	<0.0001
	Site	0.9682						
Fungal ITS	Horizon	0.28		H1	5.23	1.93	H1 vs H2	0.19
				H2	5.92	2.01	H1 vs H3	0.9
				H3	5.31	2.32	H2 vs H3	0.4
	Aggregate-size fraction	0.22		LM	6.38	1.73	LM vs MAC	0.03
				MAC	4.84	1.72	LM vs MIC	0.48
				MIC	5.82	2.29	LM vs SC	0.15
				SC	5.33	2.11	MAC vs MIC	0.18
							MAC vs MIC	0.46
							MIC vs SC	0.48
	Site	0.26						

A8 2: Bacterial 16S t-RF richness:

Table 17: Averages (Avg.) and standard deviations (\pm) of bacterial 16S gene based t-RF richness of aggregate –size fractions within Horizon 1 (H1), 2 (H2) and 3 (H3). Statistical significance defined as $P < 0.05$.

Community	Horizon	Main effect P-value	Aggregate-size fraction	t-RF Richness	\pm	Pairwise	P-value
Bacterial 16S	H1	0.0006	LM	4.34	1.3	LM vs MAC	0.93
			MAC	4.17	0.88	LM vs MIC	0.64
			MIC	4.35	0.76	LM vs SC	0.04
			SC	6.01	0.48	MAC vs MIC	0.6
						MAC vs MIC	0.002
						MIC vs SC	0.001
	H2	0.0005	LM	4.23	0.67	LM vs MAC	0.085
			MAC	3.44	0.78	LM vs MIC	0.93
			MIC	4.06	0.6	LM vs SC	0.007
			SC	5.41	0.44	MAC vs MIC	0.19
						MAC vs MIC	0.0004
						MIC vs SC	0.005
	H3	0.27	LM	4.34	0.53	LM vs MAC	0.35
			MAC	3.99	0.55	LM vs MIC	0.045
			MIC	3.67	0.47	LM vs SC	0.76
SC			4.4	0.1	MAC vs MIC	0.62	
					MAC vs MIC	0.16	
					MIC vs SC	0.13	

A8 3: Fungal ITS richness:

Table 18: Averages (Avg.) and standard deviations (\pm) of fungal internal transcribed spacer (ITS) region based t-RF richness of aggregate –size fractions within Horizon 1 (H1), 2 (H2) and 3 (H3). Statistical significance defined as $P < 0.05$. *Large macroaggregate ITS PCR amplicons were not obtained in horizon 3 (H3).

Community	Horizon	Main effect P-value	Aggregate-size fraction	t-RF Richness	\pm	Pairwise	P-value
Fungal ITS	H1	0.047	LM	6.13	1.83	LM vs MAC	0.18
			MAC	5.05	2.26	LM vs MIC	0.83
			MIC	6.23	2.07	LM vs SC	0.046
			SC	3.84	0.6	MAC vs MIC	0.33
						MAC vs MIC	0.43
						MIC vs SC	0.03
	H2	0.4	LM	6.62	1.76	LM vs MAC	0.09
			MAC	4.95	1.58	LM vs MIC	0.83
			MIC	6.37	2.44	LM vs SC	0.6
			SC	6.06	2.27	MAC vs MIC	0.22
						MAC vs MIC	0.27
						MIC vs SC	0.81
	H3*	0.42	MAC	4.27	1.62	MAC vs MIC	0.92
			MIC	4.33	2.48	MAC vs MIC	0.18
			SC	6.47	2.27	MIC vs SC	0.2

A8 4: Irish SIS site characteristics

Table 19: Table of Irish SIS sites including reference number, soil type, drainage, horizon, horizon depth (cm), textural class, % sand, % silt and % clay

Profile codes	Soil Type	Drainage	Horizon	Depth (cm)	Textural class	Sand (200-005 mm % w/w)	Silt (0002-005 mm % w/w)	Clay <0002 mm % w/w
RPM79br01	Humic Ground Water Gley	Poor	1 (Apg)	0-15	Clay loam	20	41	39
			2 (AB)	15-34	Clay	17	36	47
			3 (Btg)	34-46	Loam	46	35	19
RPM68br01	Typical Surface-water Gley	Poor	1 (Apg)	0-31	Loam	43	39	38
			2 (Cg)	31-70	Clay loam	34	33	27
			3 (Ck)	70-99	Clay loam	23	28	35
RPM66br01	Stagnic Brown Podzolic	Moderate	1 (Apg)	0-28	Sandy loam	61	30	9
			2 (EB)	28-60	Sandy loam	58	27	15
RPR49br01	Typical calcareous brown earth	Well	1 (Ap)	0-14	Loam	51	30	19
			2 (Bw)	14-38	Sandy clay loam	56	23	21
			3 (BC)	38-72	Sandy loam	70	16	16
RPM45br01	Typical Luvisol	Moderate	1 (Ap)	0-20	loam	39	38	23
			2 (Bw)	20-53	loam	42	39	19
			3 (Ck)	53-99	Sandy clay loam	52	15	33
RPM85br01	Typical Surface-water Gley	Poor	1 (Ap)	0-27	Loam	51	34	15
			2 (Ea)	27-41	Sandy Loam	55	34	11
			3 (Bs)	41-59	Loam	52	28	20
RPG62BR02	Typical Brown Earth	Well	1 (Ah)	0-17	Sandy clay loam	62	18	20
			2 (Bw)	17-35	Sandy loam	68	21	11
			3 (Bs)	35-58	Sandy loam	73	18	9
RPG52BR01	Humo Ferric Podzol	Well	1 (Ap)	0-14	Loam	48	30	22
			2 (Bs)	14-52	Loam	51	28	21
			3 (Bc)	42-74	Loam	39	39	22
RPG63br01	Typical Brown Earth	Well	1 (Ap1)	0-12	Loam	50	30	20
			2 (Ap2)	12-30	Loam	52	29	19
			3 (Bs)	30-55	Loam	51	30	19

Table 20: Irish SIS site physiochemical characteristics including % C, % N, P, Al, Fe, Ph, Na, K, Mg, Ca and cation exchange capacity (CEC)

Profile codes	Horizon	% C	% N	Mehlich P (mg/L ⁻¹)	Mehlich Al (mg/L ⁻¹)	Fe (g Kg ⁻¹)	pH	Na	K	Mg	Ca	CEC (c mol Kg ⁻¹)
RPM79br01	1 (Apg)	9	0.908	23.58	631.843	354.33	5.28	0.163	0.496	1.632	15.31	20.946
	2 (AB)	5.37	0.557	9.246	623.077	259.825	5.75	0.109	0.133	1.19	26.046	12.564
	3 (Btg)	0.648	0.082	2.623	505	127.65	6.77	0.05	0.00711	0.228	7.416	1.835
RPM68br01	1 (Apg)	4.22	0.315	16.862	1074.66	312.75	5.5	0.085	0.13	0.487	3.464	9.188
	2 (Cg)	0.591	0.053	4.685	895.583	161.159	6.65	0.074	0.184	0.522	5.897	7.382
	3 (Ck)	3.58	0.022	1.353	22.337	90.897	8.29	0.086	0.182	0.376	10.029	6.553
RPM66br01	1 (Apg)	4.39	0.302	19.659	586.36	350.333	6.38	0.127	0.197	0.549	7.811	9.403
	2 (EB)	1.06	0.05	2.395	1058.25	479.887	5.77	0.090	0.113	0.3	2.265	2.912
RPR49br01	1 (Ap)	3.38	0.351	18.755	727.99	186.706	6.49	0.0471	0.262	2.513	11.636	17.153
	2 (Bw)	5.87	0.061	4.13	105.412	52.438	7.99	0.182	0.164	0.795	6.191	8.704
	3 (BC)	8.11	0.022	2.477	42.888	33.45	8.35	0.08	0.109	0.43	3.392	6.436
RPM45br01	1 (Ap)	4.76	0.431	15.731	805.005	284.195	6.14	0.168	0.423	0.692	10.204	14.615
	2 (Bw)	2.73	0.198	5.33	931.935	193.302	6.33	0.143	0.321	0.507	8.742	9.449
	3 (Ck)	7.17	0.011	1.548	10.857	17.204	8.33	0.211	0.132	0.011	6.25	7.028
RPM85br01	1 (Ap)	2.88	2.52	40.072	797.773	321.934	5.71	0.263	0.321	0.277	4.236	5.864
	2 (Ea)	0.85	0.063	8.175	782.871	276.746	5.84	0.101	0.251	0.096	1.724	5.922
	3 (Bs)	0.642	0.068	9.525	1032.87	174.122	6.05	0.178	0.18	0.12	2.742	9.221
RPG62BR02	1 (Ah)	9.21	0.574	5.096	508.148	287.439	3.82	0.273	0.331	0.975	1.7	9.454
	2 (Bw)	2.83	0.179	1.81	1122.07	354.403	4.1	0.293	0.154	0.128	0.357	4.996
	3 (Bs)	4.1	0.187	1.57	1650.73	230.94	4.85	0.332	0.108	0.312	1.02	5.207
RPG52BR01	1 (Ap)	4.61	0.484	73.104	953.75	362.888	5.22	0.205	0.876	1.102	3.501	5.498
	2 (Bs)	1.76	0.195	4.181	948.955	245.276	5.47	0.238	0.574	0.602	2.596	5.151
	3 (Bc)	0.86	0.092	1.939	895.459	245.589	5.79	0.093	0.486	0.968	3.986	7.482
RPG63br01	1 (Ap1)	4.81	0.486	115.821	856.078	345.648	5.38	0.227	0.426	1.727	7.534	0.486
	2 (Ap2)	1.76	0.163	28.473	765.313	256.354	6	0.11	0.21	0.927	6.34	0.163
	3 (Bs)	0.774	0.06	4.934	522.073	128.443	7.47	0.092	0.208	0.544	10.233	8.919

A8 5: Bacterial 16S rRNA based community composition from aggregate size fractions from horizon 1, 2 and 3

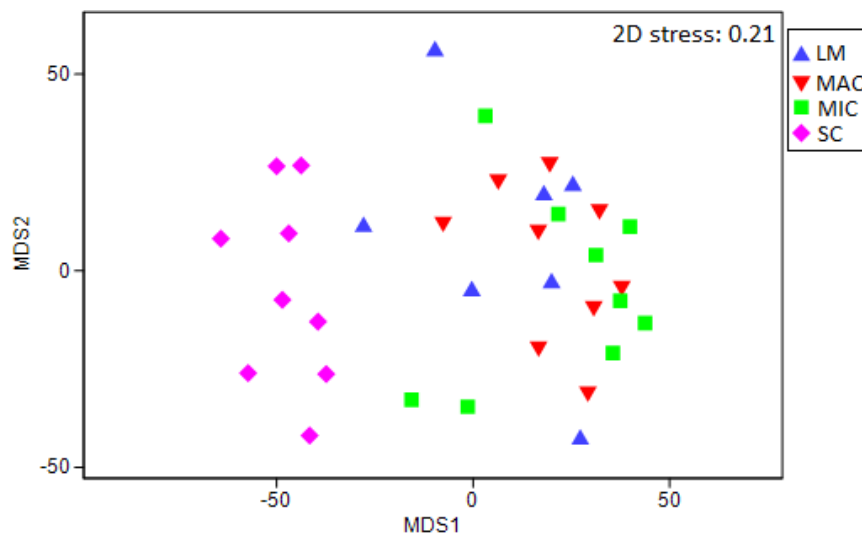


Figure 28: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on bacterial 16S rRNA gene based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond) from horizon 1.

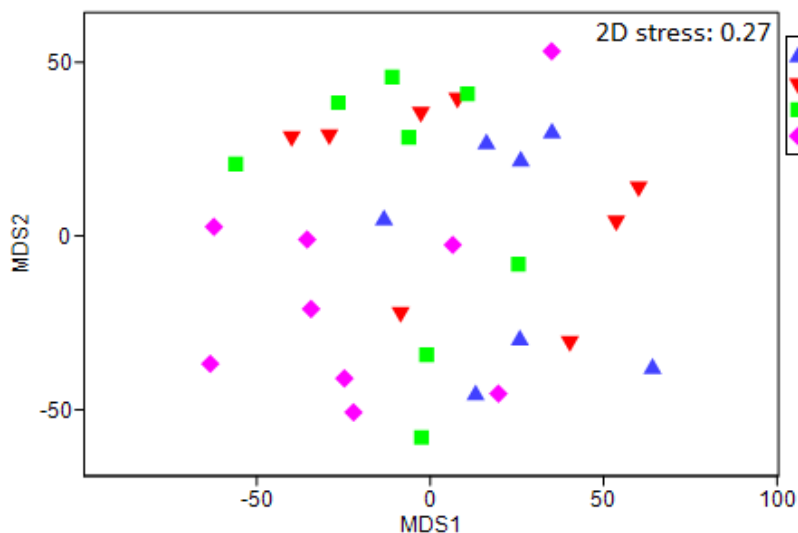


Figure 29: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on bacterial 16S rRNA gene based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond) from horizon 2.

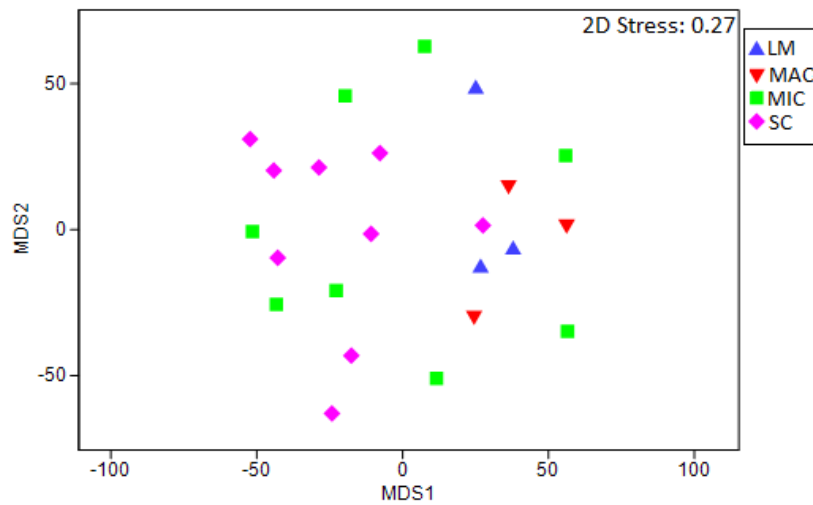


Figure 30: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on bacterial 16S rRNA gene based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond) from horizon 3.

A8 6: Fungal internal transcribed spacer region based community composition from the aggregates size fractions in horizon 1,2 and 3

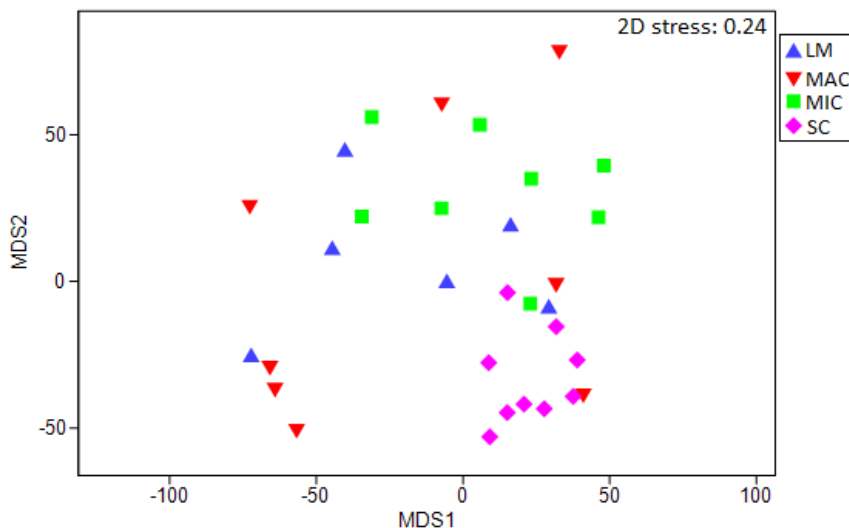


Figure 31: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on fungal internal transcribed spacer region based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond) from horizon 1.

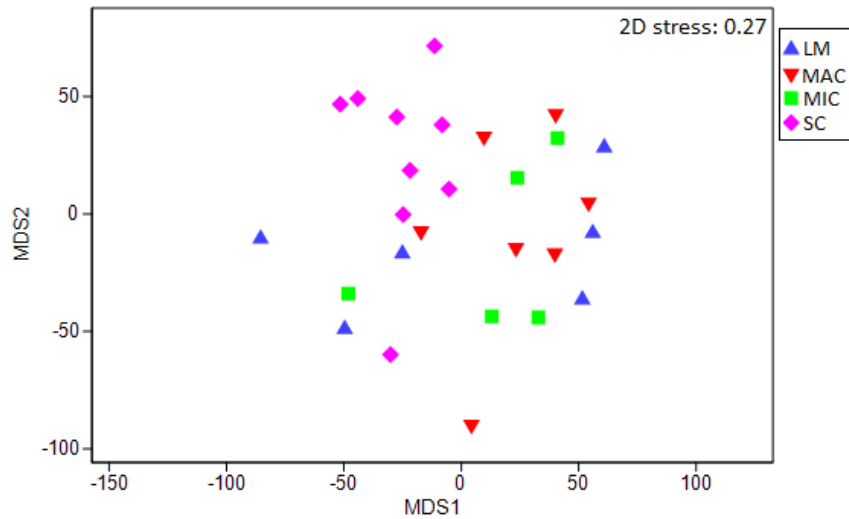


Figure 32: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on fungal internal transcribed spacer region based community composition. Aggregate sizes are as follows: large macroaggregate (LM; blue triangle), macroaggregate (MAC; red triangle), microaggregate (MIC; green square) and silt and clay (SC; purple diamond) from horizon 2.

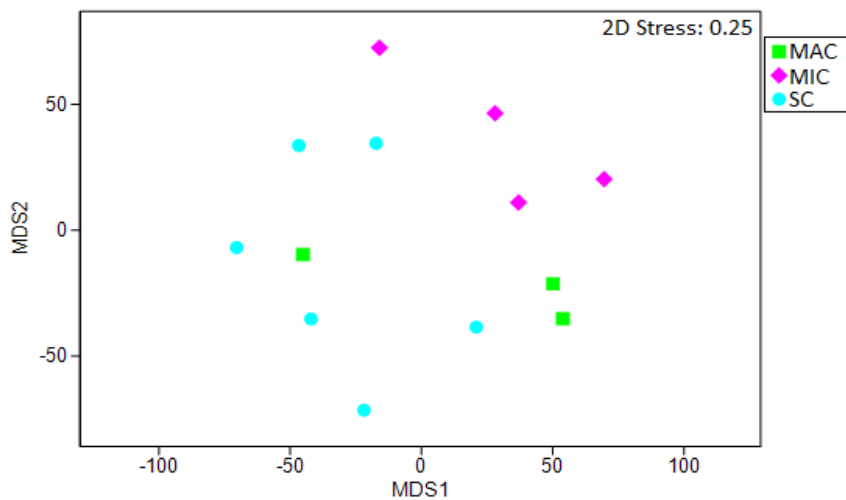


Figure 33: Metric Multi-dimensional scaling (MDS) plots of the effect of aggregate-size on fungal internal transcribed spacer region based community composition. Aggregate sizes are as follows: macroaggregate (MAC; green square), microaggregate (MIC; purple diamond) and silt and clay (SC; blue circle) from horizon 3.

Appendix B: Chapter III

B8 1: Table of phyla sequence abundance

Table 21: Abundance [%] of sequences allocated to major bacterial Phyla (cut-off 0.01%) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in Horizon 1 (Top Table) and Horizon 2 (Bottom table). Different letters indicate statistical difference between aggregate-size fractions (P<0.05).

Phylum	LM		MAC		MIC		SC	
	Avg.	±	Avg.	±	Avg.	±	Avg.	±
H1								
Acidobacteria	2.715 ^a	5.921	8.255 ^b	7.069	0.373 ^a	0.273	4.248 ^{ab}	5.786
Actinobacteria	3.635 ^{ab}	4.360	6.435 ^a	4.861	1.891 ^b	1.371	5.824 ^{ab}	6.090
Bacteroidetes	0.189 ^{ab}	0.291	0.416 ^b	0.367	0.039 ^a	0.028	0.432 ^{ab}	0.628
Chlamydiae	0.065 ^{ab}	0.063	0.199 ^b	0.162	0.020 ^a	0.013	0.090 ^a	0.132
Chloroflexi	0.657 ^a	1.366	1.473 ^a	2.283	0.192 ^a	0.214	1.239 ^a	1.673
Firmicutes	70.900 ^{ab}	30.282	44.266 ^b	36.628	80.537 ^a	16.014	64.199 ^{ab}	36.683
Gemmatimonadetes	0.185 ^a	0.211	0.500 ^a	0.412	0.236 ^a	0.328	0.265 ^a	0.244
Nitrospira	0.064 ^a	0.137	0.178 ^a	0.263	0.001 ^b	0.001	0.143 ^a	0.257
OP10	0.015 ^a	0.016	0.072 ^a	0.114	0.015 ^a	0.018	0.038 ^a	0.062
Planctomycetes	1.041 ^b	1.909	3.212 ^a	2.814	0.717 ^b	0.444	1.884 ^{ab}	1.967
Proteobacteria	7.736 ^a	7.091	12.072 ^a	9.095	10.947 ^a	18.598	7.518 ^a	7.816
unclassified	11.019 ^{ab}	10.166	18.456 ^b	10.687	4.215 ^a	4.417	11.117 ^b	10.000
Unclassified_bacteria	0.016 ^{ab}	0.014	0.035 ^a	0.030	0.005 ^b	0.006	0.022 ^{ab}	0.033
Verrucomicrobia	1.581 ^b	3.286	4.234 ^a	3.850	0.799 ^b	0.661	2.760 ^{ab}	3.410
WS3	0.176 ^b	0.435	0.188 ^b	0.286	0.002 ^a	0.002	0.197 ^{ab}	0.370
H2								
Acidobacteria	0.364 ^{ab}	0.142	0.144 ^a	0.245	0.559 ^{ab}	0.480	1.705 ^b	2.683
Actinobacteria	1.815 ^{ab}	1.043	0.834 ^a	1.222	2.787 ^b	1.416	5.273 ^b	4.317
Bacteroidetes	0.015 ^{ab}	0.009	0.006 ^a	0.005	0.039 ^{ab}	0.034	0.199 ^b	0.465
Chlamydiae	0.011 ^a	0.008	0.006 ^a	0.008	0.081 ^b	0.069	0.034 ^b	0.019
Chloroflexi	0.142 ^a	0.107	0.091 ^a	0.183	0.169 ^a	0.149	0.674 ^a	1.008
Firmicutes	87.104 ^a	4.052	82.288 ^a	18.808	73.277 ^a	17.319	72.240 ^a	21.104
Gemmatimonadetes	0.118 ^{ab}	0.098	0.016 ^a	0.012	0.165 ^{ab}	0.134	0.200 ^b	0.150
OP10	0.035 ^a	0.022	0.017 ^b	0.038	0.017 ^b	0.038	0.030 ^{ab}	0.038
Planctomycetes	0.882 ^{ac}	0.239	0.318 ^{ab}	0.491	0.279 ^b	0.147	2.039 ^c	2.815
Proteobacteria	2.758 ^a	1.990	11.047 ^a	17.407	12.753 ^a	13.158	8.208 ^a	7.878
unclassified	6.046 ^a	4.669	4.954 ^a	4.739	9.416 ^a	8.754	6.884 ^a	4.608
Verrucomicrobia	0.686 ^{ab}	0.345	0.259 ^a	0.384	0.427 ^{ab}	0.309	2.440 ^b	3.999

B8 2: Graph of phyla sequence abundance:

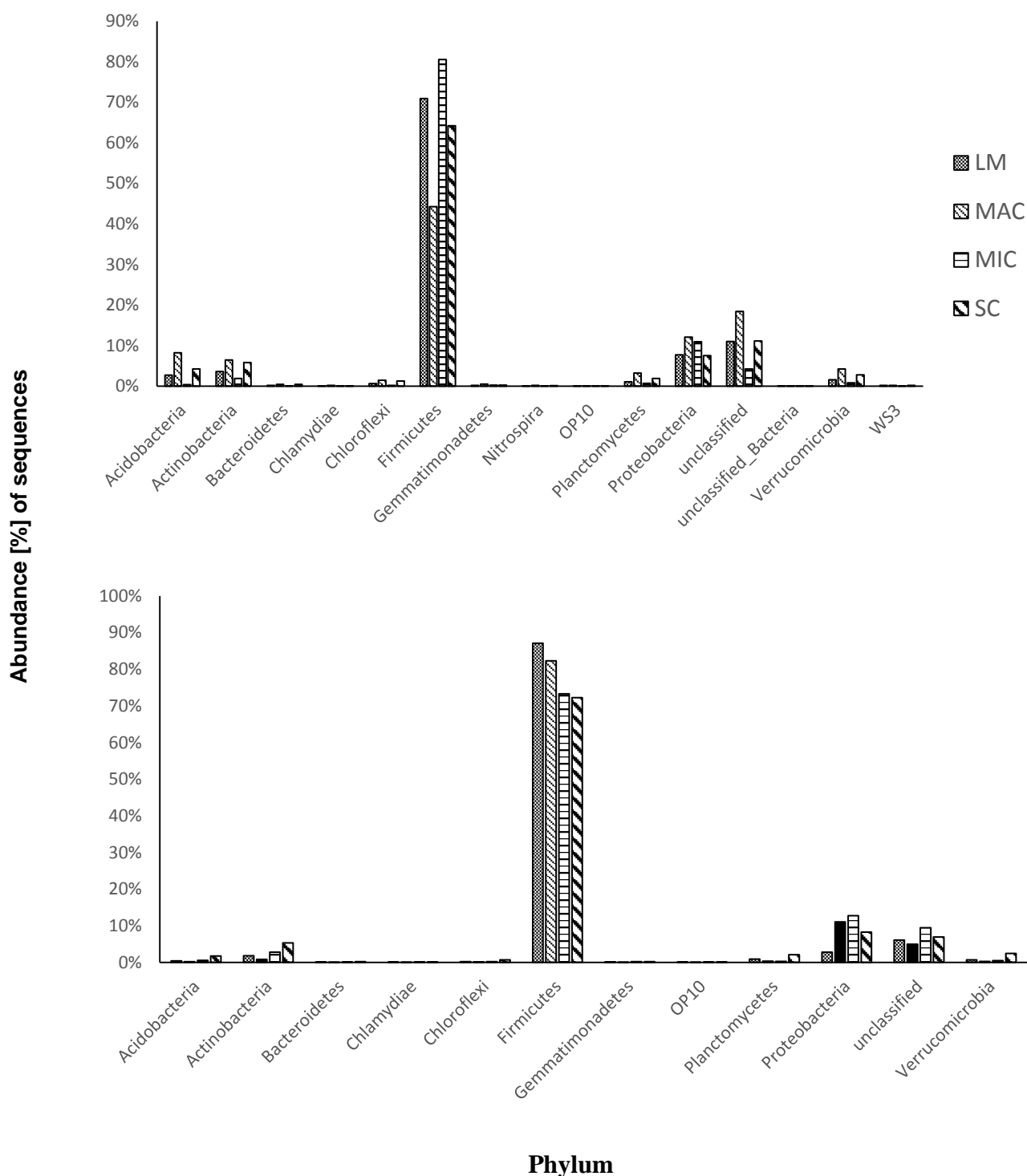


Figure 34: Bar-chart of averages of Abundance [%] of sequences allocated to major bacterial Phyla (cut-off 0.01 %) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in Horizon 1 (top) and Horizon 2 (H2)

B8 3: Graph of family sequence abundance in Horizon 1:

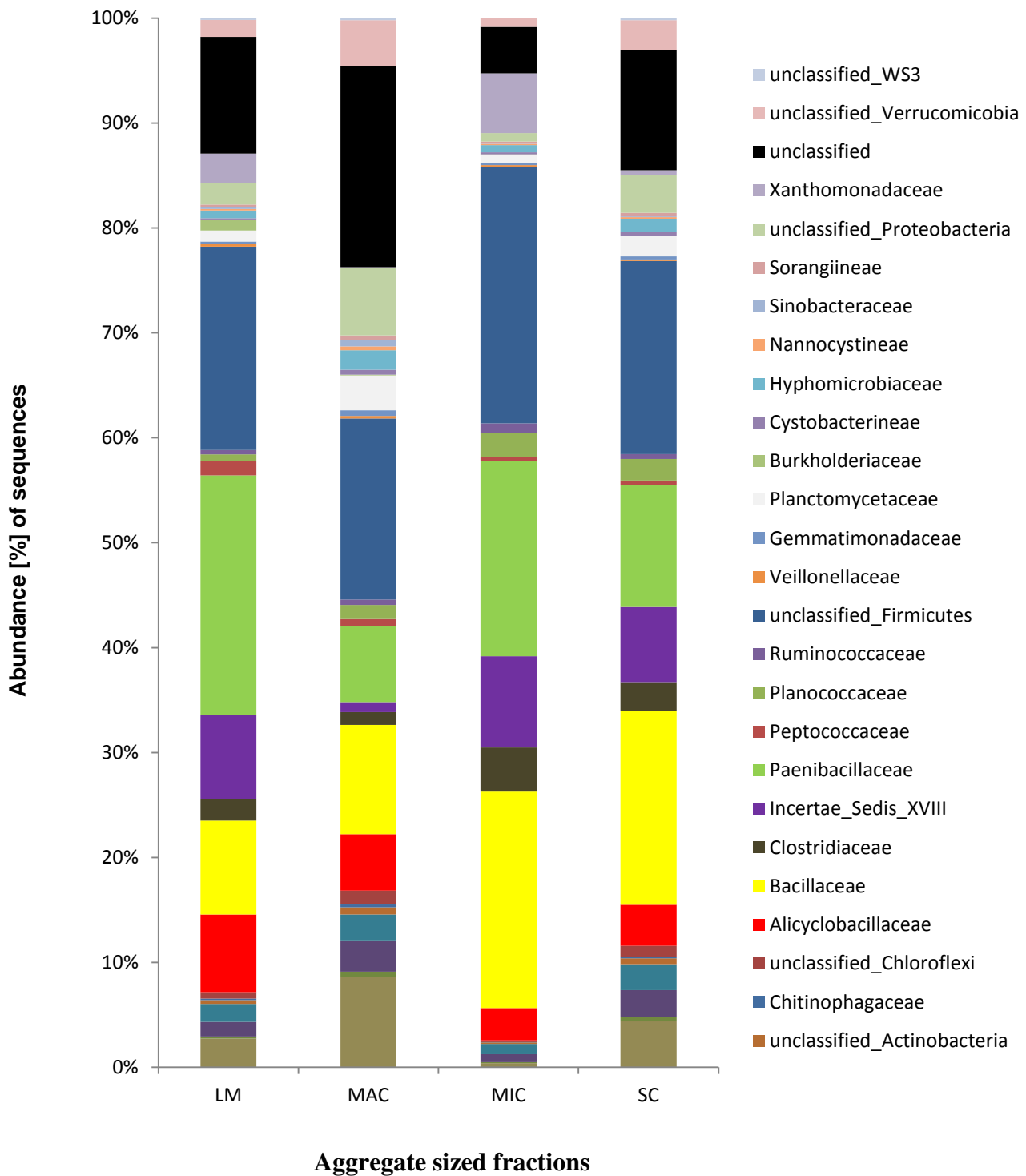


Figure 35: Stacked bar-chart of averages of abundance [%] of sequences allocated to the major bacterial families (cut-off 0.001 %) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in horizon 1.

B8 4: Graph of family sequence abundance in Horizon 2:

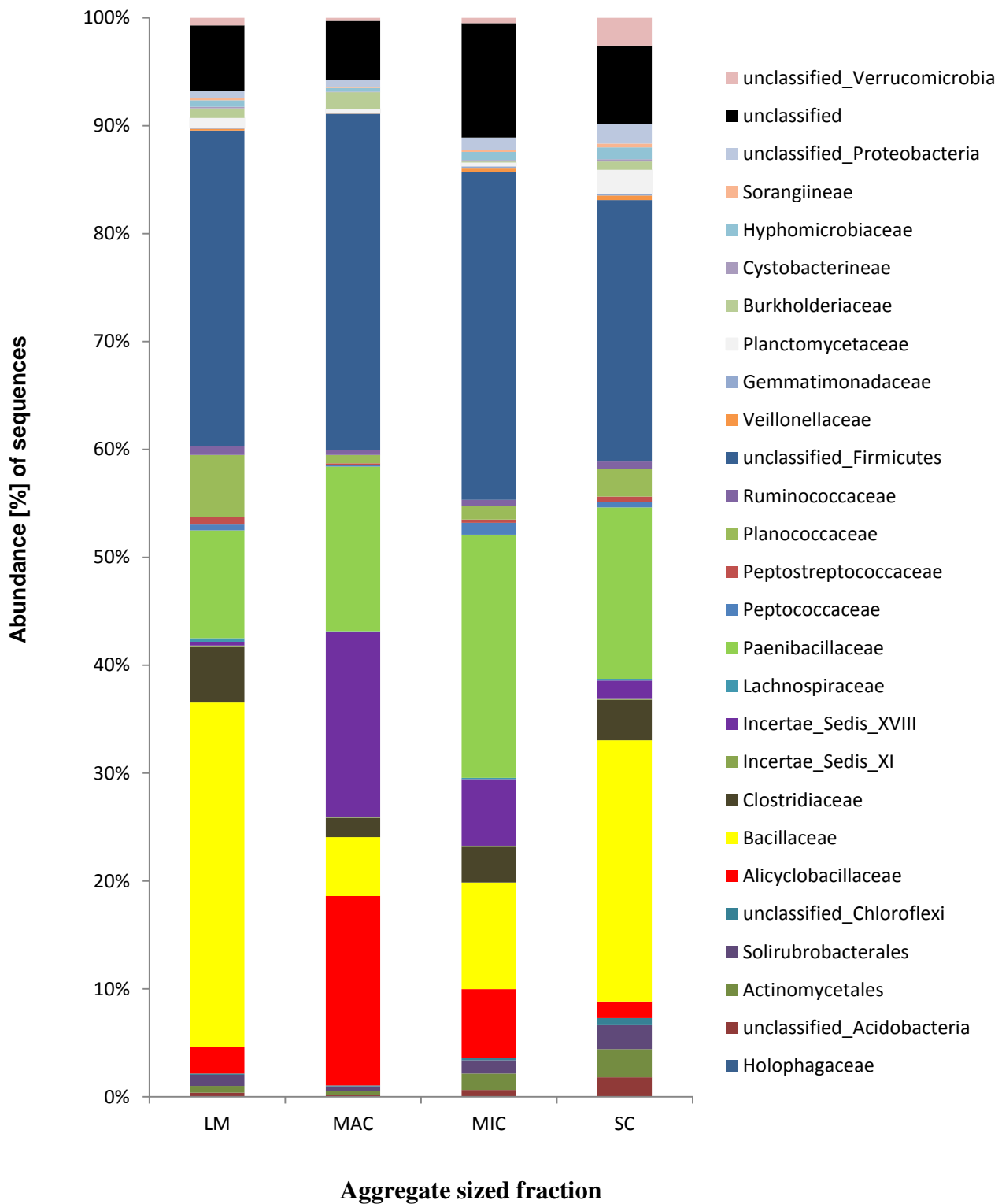


Figure 36: Stacked bar-chart of averages of abundance [%] of sequences allocated to the major bacterial families (cut-off 0.001 %) from the four aggregate-size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in horizon 2.

B8 5: Venn diagrams of shared/unique phyla:

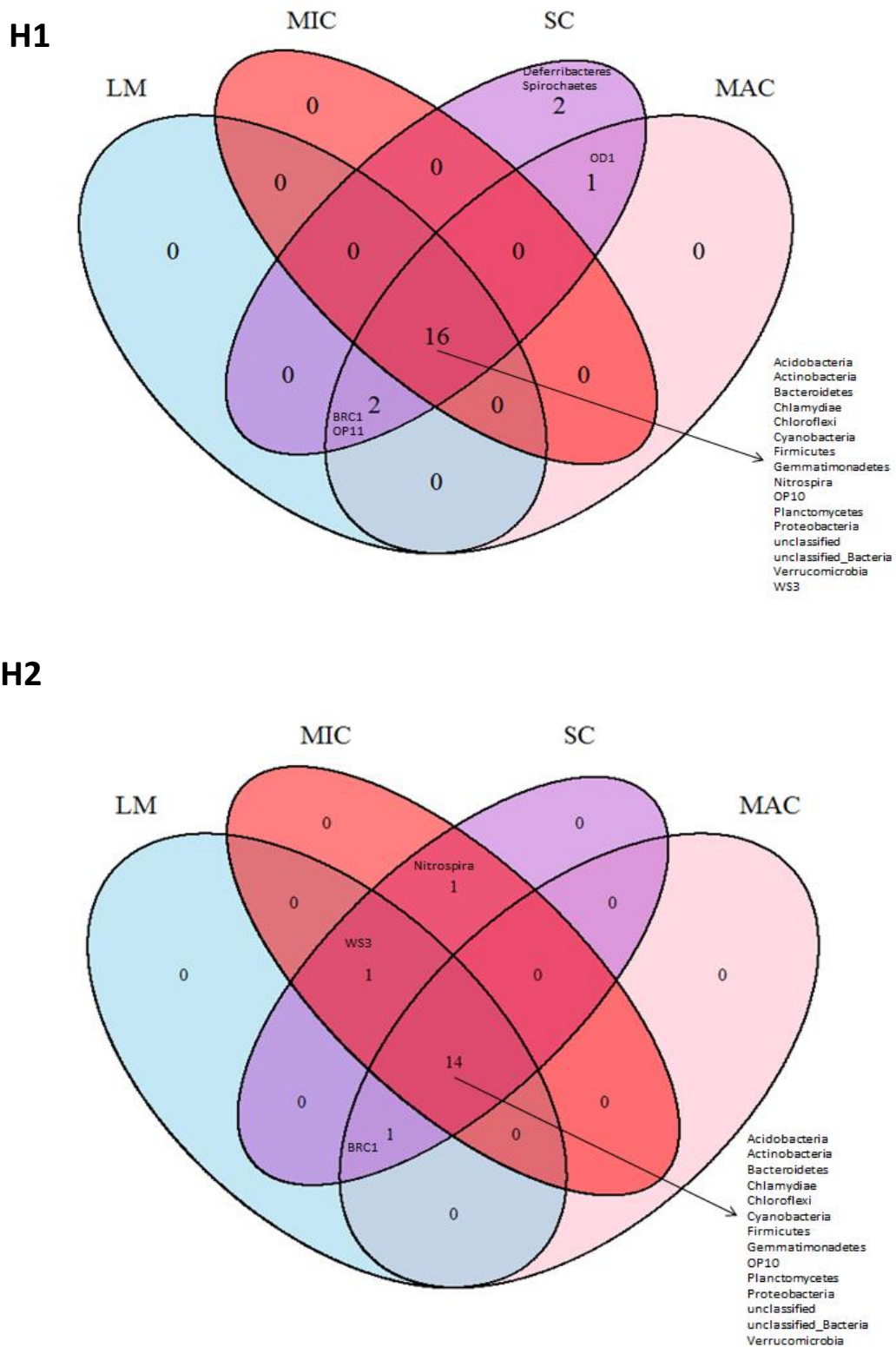


Figure 37: Venn diagrams indicating the phyla which are shared and unique to the specific aggregate-size fractions; the large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from horizon 1 (H1, top) and horizon 2 (H2, bottom).

B8 6: Family sequence abundance and correlations to environmental variables

Table 22: Averages (Avg.) and standard deviations (\pm) of the abundance of sequences allocated to families (cut-off 0.001%) from the bulk soil (BS) from Horizon 1 (H1) and Horizon 2 (H2). Statistical P-values and proportions (R^2) from distance based linear model (DistLM) marginal tests between phyla and the environmental variables carbon, phosphorous and magnesium. Statistically significant correlations ($P < 0.05$) are highlighted in black.

Phylum	Family	Horizon				DistLM test % C		DistLM test Phosphorus		DistLM test Magnesium	
		H1		H2		P-Value	R^2	P-Value	R^2	P-Value	R^2
		Avg.	\pm	Avg.	\pm						
Acidobacteria	unclassified	11.379 ^a	4.987	0.351 ^b	0.390	0.004	0.406	0.031	0.263	0.018	0.293
Actinobacteria	Acidimicrobiales	0.565 ^a	0.324	0.082 ^b	0.086	0.015	0.315	0.041	0.236	0.017	0.316
	Actinomycetales	3.411 ^a	1.628	0.461 ^b	0.495	0.004	0.400	0.047	0.228	0.031	0.267
	Solirubrobacterales	2.948 ^a	1.852	0.787 ^b	0.865	0.064	0.199	0.053	0.213	0.023	0.286
Bacteroidetes	unclassified	0.804 ^a	0.492	0.279 ^b	0.326	0.181	0.111	0.065	0.193	0.015	0.326
	Chitinophagaceae	1.156 ^a	0.814	0.536 ^b	1.580	0.377	0.050	0.545	0.024	0.194	0.100
	unclassified	0.659 ^a	0.342	0.010 ^b	0.014	0.007	0.380	0.008	0.338	0.002	0.440
Chlamydiae	Parachlamydiaceae	0.232 ^a	0.129	0.030 ^b	0.036	0.032	0.250	0.013	0.333	0.019	0.300
Chloroflexi	Anaerolineaceae	0.276 ^a	0.267	0.013 ^b	0.020	0.032	0.251	0.047	0.227	0.015	0.318
	unclassified	0.865 ^a	0.677	0.135 ^b	0.164	0.086	0.177	0.053	0.212	0.026	0.278
Firmicutes	Bacillaceae	10.448 ^a	5.061	4.178 ^b	5.838	0.133	0.137	0.216	0.093	0.493	0.030
	Clostridiaceae	1.001 ^a	0.748	1.614 ^a	2.692	0.715	0.008	0.851	0.002	0.801	0.004
	Erysipelotrichaceae	0.053 ^a	0.058	0.008 ^b	0.010	0.198	0.103	0.007	0.408	0.137	0.136
	Incertae_Sedis_XI	0.071 ^a	0.082	0.010 ^b	0.019	0.267	0.078	0.051	0.222	0.444	0.037
	Incertae_Sedis_XVIII	0.012 ^a	0.035	13.408 ^b	9.197	0.007	0.385	0.040	0.244	0.019	0.305
	Lachnospiraceae	0.102 ^a	0.078	0.038 ^b	0.053	0.184	0.105	0.150	0.124	0.599	0.018
	Paenibacillaceae	1.893 ^a	3.695	23.120 ^b	12.846	<0.001	0.546	0.034	0.244	0.017	0.313
	Peptococcaceae	0.112 ^a	0.095	0.497 ^a	0.996	0.555	0.022	0.815	0.003	0.861	0.002
	Peptostreptococcaceae	0.302 ^a	0.199	0.241 ^a	0.425	0.652	0.012	0.527	0.025	0.405	0.043
	Planococcaceae	2.719 ^a	1.670	0.468 ^b	0.618	0.043	0.237	0.018	0.299	0.160	0.121
	Ruminococcaceae	0.273 ^a	0.196	0.217 ^a	0.290	0.663	0.012	0.642	0.014	0.778	0.005
Thermoactinomycetaceae	0.043 ^a	0.069	0.025 ^a	0.043	0.786	0.005	0.960	0.000	0.950	0.000	
unclassified	6.052 ^a	1.952	22.400 ^b	13.477	0.270	0.077	0.022	0.293	0.197	0.101	

	Veillonellaceae	0.086 ^a	0.063	0.141 ^a	0.192	0.666	0.012	0.776	0.005	0.901	0.001
Planctomycetes	Planctomycetaceae	2.551 ^a	1.268	0.178 ^b	0.196	0.015	0.327	0.015	0.315	0.017	0.314
Proteobacteria	Acetobacteraceae	0.635 ^a	0.442	0.552 ^b	1.169	0.103	0.154	0.831	0.003	0.929	0.001
	Bradyrhizobiaceae	1.175 ^a	0.582	0.006 ^b	0.007	0.005	0.395	0.049	0.222	0.039	0.240
	Burkholderiales_incertae_sedis	0.128 ^a	0.094	0.003 ^b	0.003	0.142	0.127	0.020	0.292	0.015	0.320
	Caulobacteraceae	0.079 ^a	0.121	0.002 ^b	0.002	0.023	0.257	0.855	0.002	0.409	0.039
	Cystobacterineae	0.505 ^a	0.344	0.226 ^a	0.283	0.624	0.015	0.312	0.063	0.380	0.049
	Geobacteraceae	0.398 ^a	0.393	0.053 ^b	0.112	0.276	0.074	0.004	0.456	0.010	0.333
	Hyphomicrobiaceae	3.043 ^a	1.363	0.379 ^b	0.447	0.015	0.323	0.029	0.265	0.022	0.285
	Methylocystaceae	0.132 ^a	0.247	0.001 ^b	0.002	0.403	0.040	0.395	0.042	0.884	0.001
	Nannocystineae	0.415 ^a	0.342	0.061 ^b	0.063	0.185	0.108	0.002	0.489	0.003	0.420
	Sinobacteraceae	0.932 ^a	2.081	0.012 ^b	0.013	0.031	0.235	0.901	0.001	0.266	0.077
	Sorangiiineae	0.448 ^a	0.307	0.119 ^b	0.129	0.130	0.138	0.031	0.261	0.013	0.332
	Syntrophorhabdus	0.029 ^a	0.039	0.000 ^b	0.001	0.188	0.110	0.173	0.111	0.401	0.044
	unclassified	10.179 ^a	3.937	1.264 ^b	1.500	0.004	0.422	0.035	0.245	0.033	0.256
	unclassified_Rhizobiales	0.256 ^a	0.417	0.000 ^b	0.001	0.013	0.281	0.799	0.004	0.342	0.055
	Xanthobacteraceae	0.514 ^a	0.389	0.009 ^b	0.012	0.038	0.245	0.019	0.294	0.102	0.159
unclassified	unclassified	12.830 ^a	4.624	5.602 ^b	3.152	0.034	0.253	0.304	0.065	0.252	0.079
unclassified_Bacteria	unclassified	0.053 ^a	0.043	0.003 ^b	0.004	0.003	0.422	0.013	0.338	0.008	0.367
Verrucomicrobia	unclassified	9.873 ^a	4.365	0.202 ^b	0.214	0.260	0.079	0.050	0.215	0.009	0.356

B8 7: Table of Margelef's richness index (d), Pielou's measure of species richness (J') and Shannon-weiner diversity index ($H' \log^e$):

Table 23: Averages (Avg.) and standard deviations (\pm) Margelef's richness index (d), Pielou's measure of species richness (J') and Shannon-weiner diversity index ($H' \log^e$) of both phyla and family of the large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) in Horizon 1 (H1) and Horizon 2 (H2). Different letters indicate statistical difference ($P < 0.05$) between aggregate-size fractions within both horizons.

Phylum	LM				MAC				MIC				SC			
	H1 Avg.	Std.	H2 Avg.	Std.	H1 Avg.	Std.	H2 Avg.	Std.	H1 Avg.	Std.	H2 Avg.	Std.	H1 Avg.	Std.	H2 Avg.	Std.
d	6.178 ^a	0.872	6.441 ^a	0.525	6.046 ^a	0.463	6.070 ^a	1.224	6.098 ^a	0.703	5.856 ^a	0.783	6.596 ^a	0.662	5.995 ^a	0.493
J'	0.615 ^a	0.120	0.613 ^a	0.023	0.725 ^b	0.065	0.477 ^d	0.107	0.604 ^{ac}	0.089	0.605 ^{ab}	0.081	0.648 ^{abc}	0.107	0.657 ^{abc}	0.072
$H' \log^e$	1.703 ^a	0.377	1.698 ^{ac}	0.069	2.087 ^b	0.248	1.261 ^d	0.337	1.645 ^{ac}	0.280	1.637 ^{ac}	0.270	1.873 ^{abc}	0.416	1.834 ^{abc}	0.280
Family																
d	23.379 ^{ab}	3.374	23.283 ^{ab}	2.349	25.684 ^a	3.453	20.490 ^b	4.443	24.114 ^{ab}	3.267	23.632 ^{ab}	3.947	25.756 ^{ab}	5.041	24.959 ^{ab}	4.173
J'	0.634 ^a	0.079	0.662 ^{abcd}	0.019	0.706 ^c	0.038	0.574 ^d	0.090	0.647 ^{ab}	0.026	0.630 ^{abd}	0.070	0.671 ^{abc}	0.054	0.669 ^{ab}	0.034
$H' \log^e$	2.724 ^{abc}	0.424	2.841 ^b	0.061	3.148 ^a	0.257	2.366 ^c	0.494	2.807 ^b	0.204	2.712 ^{bc}	0.407	2.969 ^{ab}	0.408	2.942 ^{ab}	0.295

Appendix C: Chapter IV

C8 1: Field conditions prior to experimental establishment:

Table 24: Particle size distribution, (% of course and fine sand, silt and clay), pH, C and N content from the first 10 cm of experimental site (P1-P10). Data collected in April 2014 prior to the establishment of experiment

Variable	Avg.	Std.
% Course Sand (2-0.2 mm)	32.1	2.64
% Fine sand (0.2 mm-0.05 mm)	26.1	3.28
% Silt (0.05-0.002 mm)	27.2	2.66
% Clay <0.002 mm	14.6	1.84
pH	5.27	0.12
% C	3.46	0.38
% N	0.33	0.03
C/N	10.39	0.21
ppm PO ₄ ³⁻	8.69	4.07
ppm SO ₄ ²⁻	10.96	2.69

C8 2: Quantitative PCR for bacterial 16S rRNA gene and fungal ITS region:

Both bacterial 16S and fungal ITS qPCR reactions were carried out with a Maxima SYBR green master mix (Fermentas) in a qPCR microtitre plate (Sarstedt) using a lightcycler 480 (Roche). Reactions contained 0.3 pmol of each primer and 1 µl of DNA template (10 ng/ µl). Bacterial 16S amplification conditions were 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Fungal amplification conditions were 50 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 60 s.

C8 3: Enzymatic activities in control and slurry treatments:

Table 25: Averages (Avg.) and standard deviations (\pm) of enzymatic activities ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from replicate field plots from both the control and slurry treatments on Day 5, 30 and 65.

	Day 5				Day 30				Day 65			
	Control		Slurry		Control		Slurry		Control		Slurry	
	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
β-glucosidase	216.63 ^a	44.53	301.95 ^b	53.51	249.6 ^a	74.82	302.95 ^a	83.16	98.17 ^a	33.42	290.2 ^b	184.12
β-N-acetyl-glucosaminidase	102.18 ^a	29.71	138.52 ^b	33.65	152.54 ^a	50.23	198.15 ^a	61.14	58.44 ^a	27.39	244.27 ^b	169.92
β-xylosidase	52.75 ^a	14.56	70.97 ^a	16.89	65.17 ^a	18.27	79.01 ^a	22.87	24.73 ^a	20.51	37.81 ^a	28.59
cellobiosidase	41.33 ^a	12.42	56.12 ^b	10.7	47.61 ^a	21.59	67.05 ^a	28.51	14.27 ^a	5.59	48.48 ^b	42.03
α-glucosidase	36.64 ^a	9.98	45.08 ^a	5.64	53.42 ^a	16.75	80.05 ^a	34.79	19.22 ^a	8.4	41.85 ^a	25.98
β-galactosidase	35.79 ^a	9.13	44.99 ^a	6.53	53.15 ^a	20.32	61.42 ^a	18.47	15.52 ^a	5.73	40.46 ^b	29.31
α-arabinosidase	25.43 ^a	4.33	28.84 ^a	6.67	31.11 ^a	8.52	37.16 ^a	8.44	5.98 ^a	2.46	11.13 ^a	6.84
α-mannosidase	1.03 ^a	0.3	27.22 ^b	8.03	18.85 ^a	6.91	23.04 ^a	9.64	5.89 ^a	1.63	26.39 ^b	20.87

C8 4: Microbial biomass C and N and basal respiration values from control and slurry treatments:

Table 26: Averages (Avg.) and standard deviation (\pm) of microbial biomass-C, Microbial biomass-N and basal respiration from the control and slurry treatment from day 5, 30 and 65. Different letters indicate statistical difference ($P < 0.05$) between slurry treatment and control and between sampling days

	Day 5				Day 30				Day 65			
	Control		Slurry		Control		Slurry		Control		Slurry	
	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Microbial Biomass-C	1687.34 ^a	248.82	2059.57 ^{ab}	389.53	1964.38 ^b	175.63	1938.9 ^{ab}	246.62	3653.55 ^c	251.35	3424.25 ^c	224.43
C-flush ($\mu\text{g C g}^{-1}$ dry soil)												
Microbial Biomass-N	148.26 ^{ac}	24.94	205.84 ^b	46.32	172.27 ^{ab}	26.99	165.26 ^{abc}	19.67	163.71 ^{ab}	14.83	143.42 ^c	16.67
N-flush ($\mu\text{g N g}^{-1}$ dry soil)												
Basal Respiration	0.065 ^{ab}	0.015	0.061 ^a	0.017	0.058 ^a	0.0076	0.073 ^{ab}	0.017	0.077 ^b	0.009	0.079 ^b	0.0082
$R_{\text{CO}_2 \text{ T}_6\text{-T}_2}$ ($\text{mg CO}_2 \text{ g}^{-1} \text{ h}^{-1}$)												

C8 5: Bacterial community composition on day 5 of experiment:

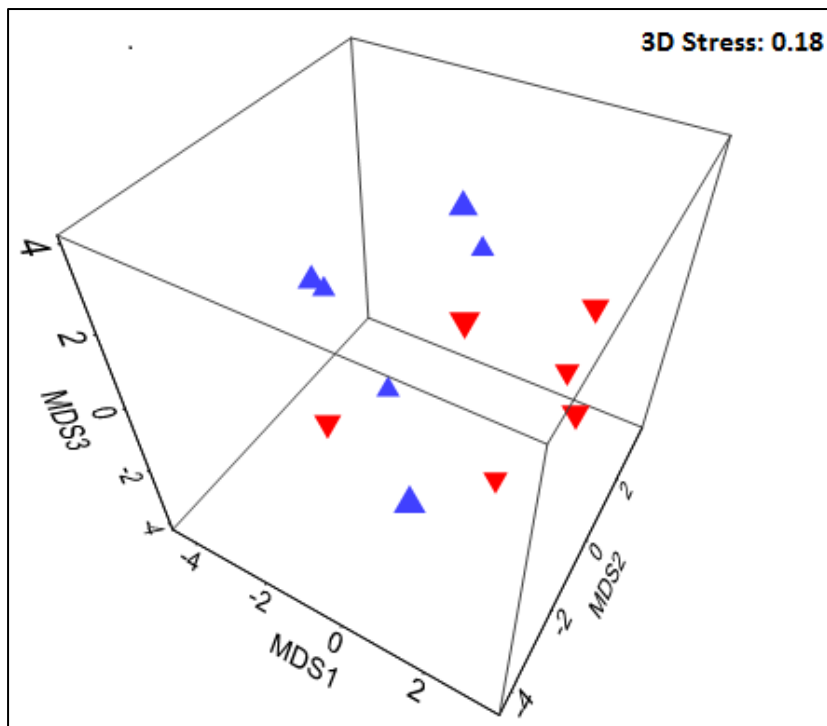


Figure 38: Non-metric multidimensional scaling plots of 16S rRNA gene based bacterial community composition of the control (blue triangle) and slurry amended plot (red triangle) on day 5 of the experiment.

C8 6: Rainfall amounts (mm) throughout experiment:

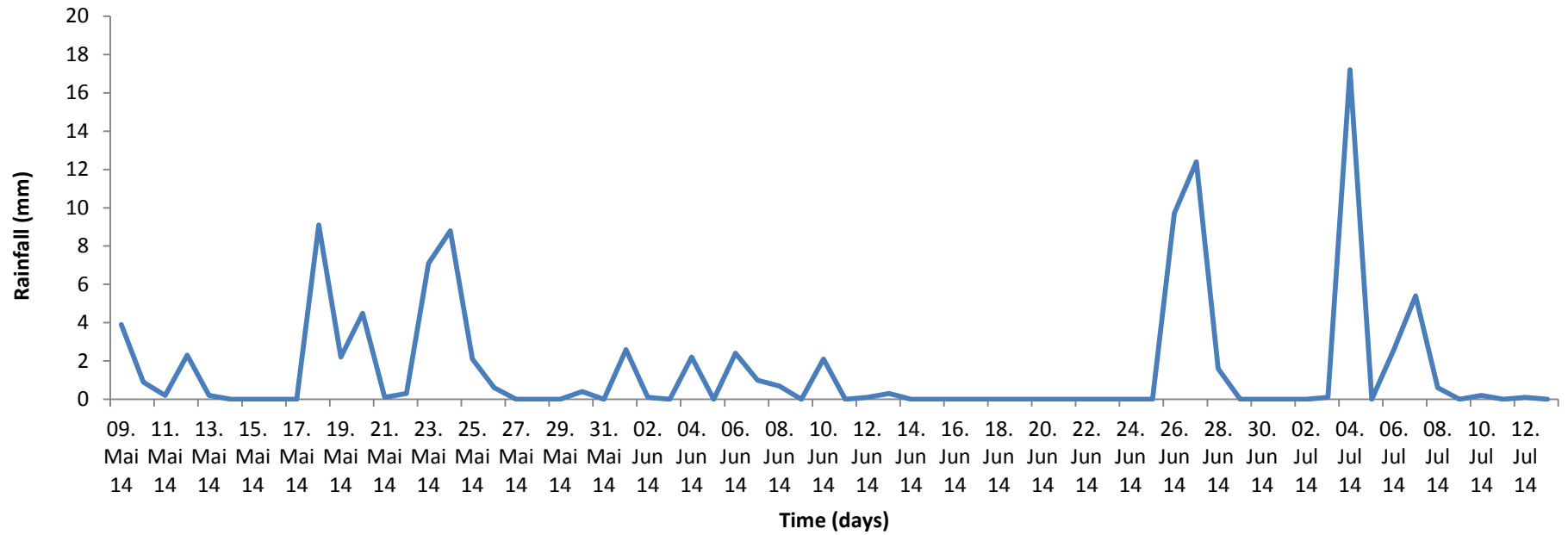


Figure 39: Total daily rainfall (mm) recorded at the Johnstown Castle automatic weather station for each day of the experiment (9th May 2014- 13th July 2014)

C8 7: Max air temperature (°C) throughout field experiment:

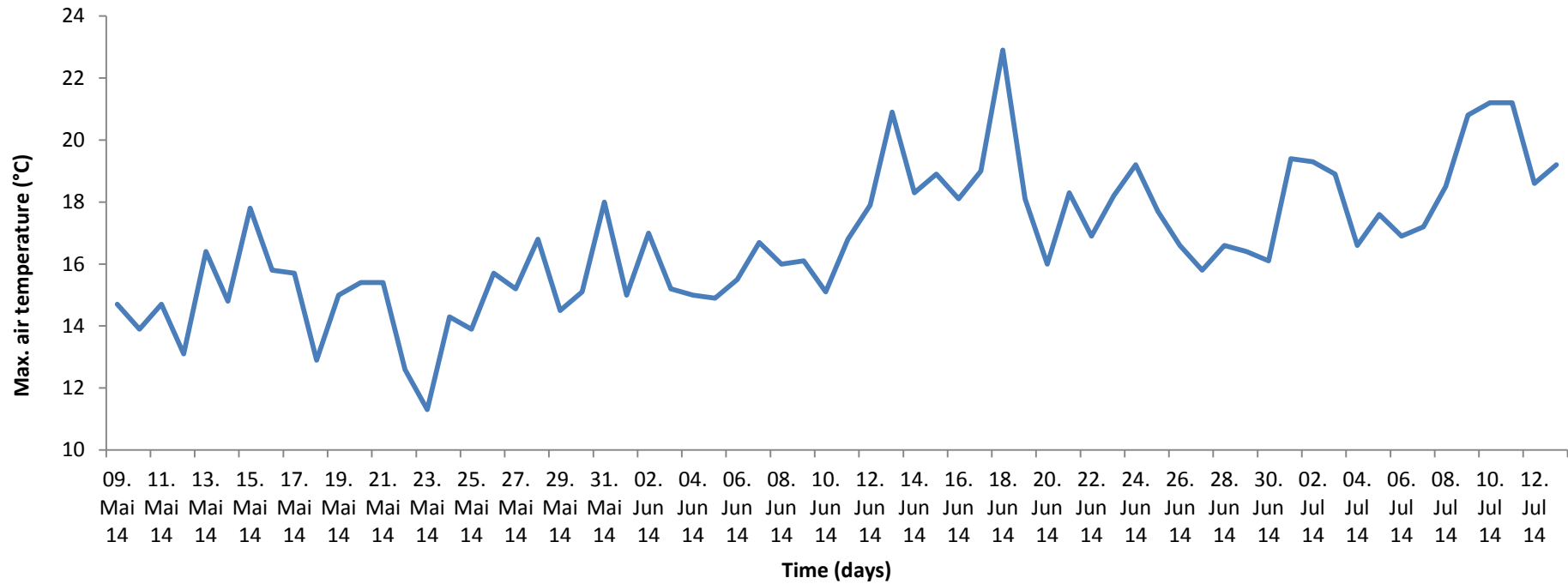


Figure 40: Maximum (Max.) daily air temperature (°C) recorded at the Johnstown Castle automatic weather station for each day of the experiment (9th May 2014-13th July 2014)

Appendix D: Chapter V

D8 1: Biological parameters of the bulk soil.

Table 27: Averages (Avg.) and standard deviations (\pm) of microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and basal respiration from the bulk soil of both the loam (top) and sandy (bottom) from the control (C) and three slurry treatments; low slurry (LS), medium slurry (MS) and high slurry (HS) 30, 137 and 361 days after slurry application. Different letters indicate statistical difference at $P < 0.05$.

Loam									
Time	Treatment Assay	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
30	MBC	1315.794 ^{ab}	101.521	1089.657 ^b	182.375	1115.306 ^b	110.114	1633.421 ^a	346.148
	MBN	93.071 ^a	10.719	91.893 ^{ab}	27.655	94.091 ^a	6.643	143.050 ^b	35.311
	Basal Respiration	0.073 ^{ab}	0.013	0.057 ^b	0.021	0.070 ^{ab}	0.030	0.128 ^b	0.039
137	MBC	784.070 ^a	44.532	920.671 ^{ab}	73.945	1041.768 ^b	125.785	996.354 ^b	189.977
	MBN	56.628 ^a	11.965	72.697 ^a	14.097	74.375 ^a	18.953	71.787 ^a	19.834
	Basal Respiration	0.127 ^a	0.010	0.145 ^a	0.039	0.152 ^a	0.024	0.225 ^a	0.097
361	MBC	982.422 ^a	244.303	948.565 ^a	339.350	1328.562 ^a	268.517	1545.111 ^a	964.569
	MBN	124.436 ^{ab}	33.442	103.441 ^a	11.068	178.810 ^b	28.854	186.774 ^b	86.025
	Basal Respiration	0.021 ^a	0.003	0.038 ^{ab}	0.014	0.046 ^b	0.018	0.024 ^{ab}	0.009

Sandy									
Time	Treatment Assay	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
30	MBC	298.280 ^a	213.457	349.332 ^a	129.263	391.849 ^a	121.317	291.749 ^a	50.403
	MBN	18.820 ^a	9.554	31.007 ^a	16.851	37.860 ^a	10.141	28.826 ^a	6.995
	BR	0.122 ^a	0.044	0.277 ^b	0.062	0.253 ^a	0.111	0.175 ^a	0.037
137	MBC	812.236 ^a	64.180	1012.828 ^b	98.002	1002.995 ^b	103.685	926.742 ^{ab}	155.995
	MBN	27.607 ^a	6.118	37.287 ^a	10.119	39.169 ^a	10.726	38.895 ^a	8.005
	BR	0.178 ^a	0.050	0.160 ^a	0.049	0.196 ^a	0.041	0.263 ^a	0.120
361	MBC	529.889 ^a	91.095	772.343 ^{ab}	198.895	1020.789 ^b	405.546	1960.422 ^b	1110.945
	MBN	68.518 ^a	7.599	119.046 ^b	25.645	166.930 ^{bc}	56.524	214.239 ^c	94.582
	BR	0.033 ^{ab}	0.011	0.022 ^a	0.004	0.043 ^b	0.019	0.025 ^{ab}	0.010

D8 2: pH from the bulk soil.

Table 28: Averages (Avg.) and standard deviations (\pm) of the pH of the bulk soil of each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.

Soil	Day	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Loam	0	5.244	0.048						
	30	5.375 ^a	0.031	5.680 ^b	0.029	5.878 ^c	0.057	6.39 ^d	0.188
	137	6.035 ^a	0.026	6.173 ^b	0.080	6.248 ^b	0.186	6.660 ^c	0.223
	361	6.748 ^a	0.096	6.998 ^b	0.101	7.198 ^b	0.096	7.428 ^c	0.083
Sand	0	5.515	0.064						
	30	5.743 ^a	0.039	6.215 ^b	0.077	6.430 ^c	0.088	6.318 ^{bc}	0.059
	137	6.313 ^a	0.117	6.413 ^a	0.150	6.708 ^b	0.102	6.655 ^b	0.124
	361	7.093 ^a	0.093	7.180 ^a	0.077	7.465 ^b	0.091	7.540 ^b	0.268

D8 3: % aggregate proportions.

Table 29: Averages (Avg.) and standard deviations (\pm) of the % aggregate proportions to the bulk soil of each of the four aggregate size fractions; large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) on day 0, 30, 137 and 361 days of the experiment in the two soil types (loam and sandy).

Loam Time	Aggregate size Treatment	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
0		23.272	3.830	32.440	2.807	27.912	1.165	16.376	1.404
30	Control	29.489	5.149	39.272	3.999	22.778	1.109	8.461	0.278
	LS	27.674	5.663	41.282	3.406	22.335	1.578	8.708	1.304
	MS	35.628	5.272	35.689	1.954	20.381	2.271	8.302	1.447
	HS	37.529	4.198	33.002	3.859	20.574	3.646	8.895	2.031
137	Control	10.675	2.166	31.391	2.450	34.399	2.152	23.535	0.874
	LS	15.557	6.429	26.630	3.693	33.101	2.770	24.712	2.361
	MS	13.648	1.962	26.122	4.685	34.449	1.556	25.781	4.873
	HS	18.714	8.333	28.262	7.475	30.568	2.810	22.456	4.292
361	Control	23.902	10.637	38.196	3.329	25.621	5.581	12.282	3.341
	LS	19.328	6.320	42.026	8.932	26.392	2.977	12.254	4.081
	MS	23.319	8.228	41.630	7.728	24.605	5.153	10.447	3.265
	HS	38.882	6.469	32.616	6.580	19.586	0.706	8.915	0.811
Sandy Time	Aggregate size Treatment	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
0		9.959	1.779	41.134	3.408	44.673	3.978	4.234	1.517
30	Control	5.182	1.235	52.189	7.297	38.933	5.926	3.697	0.667
	LS	7.815	1.574	47.129	3.674	40.966	3.126	4.090	1.462
	MS	8.481	2.143	50.784	2.007	37.383	0.707	3.351	0.561
	HS	7.079	3.568	49.424	2.860	39.939	5.029	3.558	0.270
137	Control	27.286	10.216	31.342	4.098	35.124	5.576	6.248	1.313
	LS	38.737	7.043	25.788	2.327	29.661	4.064	5.814	0.729
	MS	44.257	3.052	22.899	1.296	27.321	3.183	5.522	0.417
	HS	41.170	8.459	27.561	3.737	26.054	4.169	5.214	1.405
361	Control	31.333	9.611	31.726	5.660	32.034	4.213	4.906	0.347
	LS	50.047	8.913	25.455	6.550	21.428	2.480	3.070	0.559
	MS	40.038	24.726	32.400	16.753	24.115	7.767	3.446	0.702
	HS	38.798	5.168	29.007	6.140	28.571	6.260	3.624	0.288

D8 4: Enzymatic activity in aggregate size fractions in loam soil at day 30.

Table 30: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) in the loam soil 30 days after slurry application.

Treatment	Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	8.902 ^a	3.097	6.892 ^a	2.256	1.340 ^b	0.568	0.700 ^b	0.076
	α -glucosidase	6.134 ^a	3.471	6.404 ^a	1.949	1.213 ^b	0.161	0.713 ^b	0.282
	α -arabinosidase	5.684 ^a	3.587	5.926 ^a	5.550	0.691 ^b	0.493	0.427 ^b	0.077
	cellobiosidase	2.835 ^a	1.561	3.661 ^a	2.065	1.769 ^{ab}	1.865	0.248 ^b	0.180
	β -xylosidase	2.085 ^a	1.083	3.252 ^a	1.948	0.690 ^b	0.191	0.439 ^b	0.157
	β -glucosidase	29.837 ^a	19.876	13.013 ^{ab}	8.995	4.817 ^b	2.442	1.484 ^c	0.265
	β -galactosidase	2.979 ^a	1.520	3.927 ^a	1.911	0.800 ^b	0.069	0.658 ^b	0.124
LS	β -N-acetyl-glucosaminidase	6.546 ^a	4.039	5.735 ^a	1.987	1.438 ^b	0.160	1.884 ^b	0.741
	α -glucosidase	7.597 ^{bc}	6.566	6.098 ^b	1.969	1.788 ^a	0.619	1.639 ^{ac}	1.138
	α -arabinosidase	6.031 ^a	5.514	4.628 ^a	4.159	0.787 ^b	0.303	1.013 ^b	0.663
	cellobiosidase	4.596 ^a	4.627	3.033 ^{ab}	2.490	0.569 ^c	0.190	0.763 ^{bc}	0.623
	β -xylosidase	3.307 ^a	2.583	3.025 ^{ac}	2.441	0.780 ^b	0.405	0.902 ^{bc}	0.521
	β -glucosidase	34.755 ^a	10.930	17.523 ^a	8.908	3.533 ^b	1.418	3.503 ^b	1.453
	β -galactosidase	5.989 ^a	3.687	4.560 ^a	2.915	1.188 ^b	0.564	1.241 ^b	0.613
MS	β -N-acetyl-glucosaminidase	6.935 ^a	5.849	4.062 ^a	1.552	1.720 ^b	0.752	1.154 ^b	0.322
	α -glucosidase	4.380 ^{ab}	3.780	2.808 ^a	0.810	1.704 ^{ab}	0.868	1.026 ^b	0.445
	α -arabinosidase	3.532 ^a	3.749	1.649 ^a	0.853	0.822 ^{ab}	0.451	0.457 ^b	0.130
	cellobiosidase	2.955 ^a	3.651	0.702 ^{ab}	0.286	0.371 ^{bc}	0.261	0.292 ^c	0.122
	β -xylosidase	2.196 ^{ab}	2.415	1.682 ^a	0.731	1.227 ^a	0.305	0.475 ^b	0.131
	β -glucosidase	26.876 ^a	17.650	10.167 ^a	4.239	4.248 ^b	1.770	2.754 ^b	2.068
	β -galactosidase	4.164 ^a	2.867	2.541 ^{ab}	0.860	1.500 ^{bc}	0.646	0.891 ^c	0.320
HS	β -N-acetyl-glucosaminidase	16.493 ^a	5.637	4.309 ^b	1.516	2.360 ^{bc}	1.317	1.810 ^c	0.447
	α -glucosidase	10.141 ^a	4.679	4.650 ^{ab}	1.179	4.446 ^{ab}	2.319	2.949 ^b	1.280
	α -arabinosidase	19.949 ^a	2.986	7.443 ^b	2.774	6.184 ^{bc}	3.525	2.287 ^c	2.163
	cellobiosidase	1.153 ^a	0.650	1.052 ^a	0.466	0.533 ^a	0.091	0.509 ^a	0.175
	β -xylosidase	2.023 ^a	0.688	1.017 ^b	0.230	0.764 ^b	0.382	0.692 ^b	0.196
	β -glucosidase	45.925 ^a	13.073	10.992 ^b	3.870	4.830 ^{bc}	2.657	3.314 ^c	1.069
	β -galactosidase	8.363 ^a	1.971	2.464 ^b	0.495	1.713 ^b	1.031	1.514 ^b	0.657

D8 5: Enzymatic activity in aggregate size fractions in loam soil at day 137.

Table 31: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) 137 days after slurry application.

Treatment	Aggregate size Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	7.840 ^a	3.780	6.620 ^a	4.612	11.218 ^a	6.691	1.798 ^b	0.732
	α -glucosidase	2.969 ^a	1.799	4.716 ^a	1.264	3.642 ^a	1.697	3.766 ^a	2.928
	α -arabinosidase	0.425 ^a	0.293	1.175 ^{ab}	0.720	1.018 ^{ab}	0.834	0.889 ^b	0.165
	cellobiosidase	10.487 ^{ab}	4.624	9.795 ^a	4.890	8.211 ^b	0.629	4.251 ^{ab}	1.987
	β -xylosidase	0.990 ^{ab}	0.693	1.120 ^{ab}	0.468	0.505 ^a	0.244	0.694 ^b	0.563
	β -glucosidase	2.095 ^{ab}	0.586	3.494 ^{ab}	1.523	3.936 ^a	4.146	2.357 ^b	0.934
	β -galactosidase	0.738 ^a	0.292	1.078 ^a	0.574	0.915 ^a	0.250	0.476 ^a	0.239
LS	β -N-acetyl-glucosaminidase	11.969 ^a	6.886	11.456 ^a	1.864	14.601 ^a	10.398	3.590 ^b	1.309
	α -glucosidase	6.861 ^a	3.065	6.160 ^a	3.915	2.827 ^a	1.542	3.564 ^a	1.123
	α -arabinosidase	1.579 ^a	0.604	1.309 ^a	0.329	1.388 ^a	0.433	1.072 ^a	0.140
	cellobiosidase	23.776 ^a	16.819	12.946 ^{ab}	2.957	12.225 ^b	3.158	6.998 ^b	1.153
	β -xylosidase	1.383 ^a	0.372	1.631 ^a	1.308	0.570 ^{ab}	0.494	0.784 ^b	0.104
	β -glucosidase	11.153 ^a	10.247	32.684 ^a	38.461	7.186 ^a	9.038	13.994 ^a	22.461
	β -galactosidase	3.514 ^a	2.029	2.765 ^a	0.571	2.065 ^a	0.755	1.440 ^a	0.274
MS	β -N-acetyl-glucosaminidase	20.407 ^a	11.508	17.142 ^a	8.088	5.374 ^b	1.900	6.824 ^b	1.759
	α -glucosidase	10.088 ^a	5.174	9.742 ^a	7.349	7.601 ^a	7.498	15.348 ^a	6.139
	α -arabinosidase	1.234 ^a	0.437	1.618 ^a	0.404	2.032 ^a	1.044	2.910 ^a	1.405
	cellobiosidase	32.912 ^{ab}	17.000	28.193 ^a	8.050	12.849 ^b	14.774	17.422 ^{ab}	11.172
	β -xylosidase	6.897 ^a	8.887	4.050 ^a	2.047	1.234 ^a	1.716	2.683 ^a	0.900
	β -glucosidase	4.168 ^a	1.699	3.233 ^a	0.283	2.293 ^a	3.471	6.729 ^a	2.771
	β -galactosidase	3.121 ^{ab}	1.567	3.066 ^a	0.177	2.938 ^{ab}	1.300	3.781 ^b	1.255
HS	β -N-acetyl-glucosaminidase	24.201 ^a	11.308	18.350 ^{ab}	14.878	34.250 ^{ab}	25.782	7.882 ^b	3.506
	α -glucosidase	17.688 ^a	9.062	20.369 ^a	11.247	21.250 ^a	17.841	23.723 ^a	12.481
	α -arabinosidase	1.787 ^a	0.663	3.986 ^a	1.979	1.999 ^a	1.182	2.308 ^a	0.559
	cellobiosidase	44.706 ^a	20.137	28.299 ^{ab}	16.899	15.183 ^c	4.936	13.372 ^b	4.417
	β -xylosidase	11.725 ^a	5.714	8.694 ^{ab}	7.198	2.491 ^{ab}	1.150	4.812 ^b	1.617
	β -glucosidase	6.132 ^a	3.618	7.702 ^{ab}	5.543	8.792 ^b	8.114	8.046 ^b	3.433
	β -galactosidase	6.334 ^a	3.362	3.619 ^a	1.838	2.889 ^a	0.572	2.940 ^a	0.432

D8 6: Enzymatic activity in aggregate size fractions in loam soil at day 361.

Table 32: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) in the loam soil 361 days after slurry application.

Treatment	Aggregate size Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	30.273 ^a	17.004	24.997 ^a	1.337	28.367 ^a	18.906	4.085 ^b	1.970
	α -glucosidase	13.866 ^a	8.349	5.650 ^{ab}	2.378	3.262 ^b	0.978	3.907 ^b	1.264
	α -arabinosidase	3.936 ^{ab}	2.278	3.377 ^a	0.533	2.038 ^b	0.911	1.148 ^c	0.867
	cellobiosidase	2.168 ^a	1.719	0.850 ^{ab}	0.573	0.499 ^{ab}	0.218	0.389 ^b	0.138
	β -xylosidase	5.803 ^{ab}	3.904	3.892 ^a	1.172	2.961 ^a	1.141	1.342 ^b	0.489
	β -glucosidase	41.978 ^a	28.664	27.254 ^a	8.606	16.418 ^a	4.890	8.345 ^b	2.964
	β -galactosidase	9.873 ^a	7.468	6.137 ^a	2.792	3.921 ^a	1.259	3.116 ^a	1.256
LS	β -N-acetyl-glucosaminidase	38.287 ^a	30.718	35.935 ^a	7.548	25.609 ^a	8.436	6.774 ^b	3.138
	α -glucosidase	10.553 ^a	9.657	10.212 ^a	2.650	6.476 ^a	3.309	6.008 ^a	3.677
	α -arabinosidase	2.450 ^{ab}	1.818	3.587 ^a	0.807	1.815 ^b	0.714	1.535 ^b	0.894
	cellobiosidase	2.117 ^a	2.825	1.258 ^a	0.721	0.598 ^a	0.588	0.484 ^a	0.145
	β -xylosidase	4.541 ^{ab}	5.135	3.889 ^a	0.688	1.980 ^b	0.965	1.433 ^b	0.813
	β -glucosidase	61.543 ^{ab}	59.685	54.418 ^a	1.328	26.167 ^{bc}	8.390	12.294 ^c	6.745
	β -galactosidase	7.533 ^{ab}	6.999	7.808 ^a	2.752	3.846 ^b	1.318	3.358 ^{ab}	1.864
MS	β -N-acetyl-glucosaminidase	31.384 ^{ab}	17.576	53.925 ^a	8.513	24.236 ^b	16.017	5.114 ^c	3.863
	α -glucosidase	12.573 ^a	4.014	17.474 ^a	7.237	4.354 ^b	1.894	4.757 ^b	3.975
	α -arabinosidase	2.313 ^a	0.813	5.040 ^b	2.209	1.537 ^a	1.077	1.198 ^a	0.815
	cellobiosidase	2.037 ^a	1.060	1.368 ^{ab}	0.329	0.358 ^c	0.385	0.604 ^{bc}	0.426
	β -xylosidase	5.734 ^a	2.659	9.200 ^a	3.769	2.573 ^b	1.092	1.452 ^b	1.048
	β -glucosidase	38.045 ^a	17.328	54.770 ^a	17.045	14.923 ^b	7.013	6.346 ^c	4.518
	β -galactosidase	9.815 ^{ab}	5.343	16.868 ^a	5.983	5.364 ^b	2.198	4.140 ^b	2.724
HS	β -N-acetyl-glucosaminidase	54.053 ^b	19.167	24.246 ^a	12.898	17.524 ^a	5.312	4.144 ^c	0.470
	α -glucosidase	28.863 ^a	19.674	9.247 ^b	3.330	5.978 ^b	1.326	3.394 ^c	0.614
	α -arabinosidase	4.308 ^a	1.937	2.381 ^{ab}	0.815	1.559 ^b	0.180	0.800 ^c	0.128
	cellobiosidase	7.223 ^a	6.511	2.058 ^a	2.727	0.898 ^a	0.803	0.894 ^a	0.671
	β -xylosidase	10.369 ^a	7.013	4.326 ^{ab}	1.641	2.552 ^b	0.420	1.120 ^c	0.239
	β -glucosidase	85.528 ^a	47.891	30.775 ^b	8.968	16.076 ^c	2.136	6.635 ^d	0.427
	β -galactosidase	14.262 ^a	6.845	6.265 ^{ab}	1.972	4.642 ^b	1.409	2.434 ^c	0.923

D8 7: Enzymatic activity in aggregate size fractions in sandy soil at day 30.

Table 33: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) in the sandy soil 30 days after slurry application.

Treatment	Aggregate size Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	4.044 ^a	1.890	8.299 ^b	1.386	5.111 ^a	1.794	1.167 ^c	0.244
	α -glucosidase	1.620 ^a	0.788	8.130 ^b	2.062	7.486 ^b	1.352	1.460 ^a	0.403
	α -arabinosidase	1.540 ^b	0.651	5.088 ^a	1.469	3.805 ^a	0.818	0.702 ^c	0.123
	cellobiosidase	1.410 ^a	0.750	2.568 ^a	1.077	2.289 ^a	0.654	0.461 ^b	0.140
	β -glucosidase	15.152 ^{ab}	13.500	17.598 ^a	9.582	9.424 ^a	4.998	2.518 ^b	0.368
	β -galactosidase	2.453 ^{ab}	1.959	9.055 ^a	7.514	5.553 ^a	6.498	0.495 ^b	0.250
	β -xylosidase	0.208 ^a	0.071	1.701 ^b	0.908	0.881 ^b	0.147	0.173 ^c	0.075
LS	β -N-acetyl-glucosaminidase	3.920 ^a	2.012	9.438 ^b	2.651	9.867 ^b	2.293	1.978 ^a	1.074
	α -glucosidase	3.981 ^a	1.078	7.778 ^b	1.774	9.880 ^b	1.399	2.666 ^a	1.381
	α -arabinosidase	3.236 ^a	1.396	4.266 ^a	1.283	4.881 ^a	0.670	0.858 ^b	0.254
	cellobiosidase	3.498 ^a	2.311	2.435 ^a	1.156	3.041 ^a	0.657	0.703 ^b	0.311
	β -glucosidase	27.429 ^{ab}	25.417	43.061 ^a	41.469	23.628 ^a	8.261	3.806 ^b	1.542
	β -galactosidase	0.720 ^a	0.703	21.447 ^a	23.311	8.823 ^a	10.501	1.472 ^a	1.262
	β -xylosidase	1.378 ^b	0.468	2.599 ^a	0.707	3.165 ^a	0.791	0.556 ^c	0.159
MS	β -N-acetyl-glucosaminidase	4.193 ^a	1.570	24.253 ^b	13.781	12.820 ^b	1.019	2.820 ^a	1.800
	α -glucosidase	4.122 ^a	1.041	13.740 ^b	4.593	11.115 ^b	1.912	2.550 ^a	1.354
	α -arabinosidase	2.503 ^b	0.458	7.330 ^a	3.716	4.782 ^a	0.391	0.838 ^c	0.436
	cellobiosidase	2.860 ^a	1.573	4.406 ^a	2.654	2.437 ^a	0.771	0.600 ^b	0.375
	β -glucosidase	12.668 ^a	5.454	84.924 ^{ab}	92.293	22.147 ^b	4.226	3.086 ^c	1.336
	β -galactosidase	1.813 ^a	0.383	6.157 ^b	1.417	5.689 ^b	0.508	1.175 ^a	0.533
	β -xylosidase	1.225 ^a	0.141	5.326 ^a	1.788	3.522 ^a	0.391	0.553 ^a	0.237
HS	β -N-acetyl-glucosaminidase	5.518 ^{abc}	4.917	13.173 ^b	1.797	14.543 ^{ab}	3.832	2.445 ^c	1.140
	α -glucosidase	5.868 ^{ac}	6.002	14.780 ^{ab}	4.441	20.026 ^b	9.155	2.803 ^c	1.288
	α -arabinosidase	2.347 ^a	1.821	4.286 ^{ab}	0.892	5.239 ^b	1.683	0.647 ^c	0.130
	cellobiosidase	2.493 ^a	2.145	1.997 ^a	0.479	3.800 ^a	1.697	0.489 ^b	0.137
	β -glucosidase	17.510 ^a	17.940	23.343 ^a	2.939	23.546 ^a	9.729	3.245 ^b	1.052
	β -galactosidase	0.936 ^{ac}	0.615	12.407 ^b	15.372	5.309 ^{ab}	5.226	0.498 ^c	0.333
	β -xylosidase	0.618 ^a	0.480	2.299 ^b	0.381	2.205 ^b	0.748	0.261 ^a	0.054

D8 8: Enzymatic activity in aggregate size fractions in sandy soil at day 137.

Table 34: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) in the sandy soil 137 days after slurry application.

Treatment	Aggregate size Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	8.071 ^a	5.105	1.760 ^{bc}	1.602	3.832 ^{ab}	3.259	0.396 ^c	0.140
	α -glucosidase	4.874 ^a	2.703	1.250 ^b	1.080	3.928 ^a	2.050	0.569 ^c	0.528
	α -arabinosidase	2.361 ^a	1.619	48.380 ^{ab}	90.786	0.928 ^a	0.241	0.243 ^b	0.139
	cellobiosidase	20.557 ^a	13.737	3.045 ^{bc}	1.290	6.970 ^b	2.513	1.017 ^c	0.758
	β -glucosidase	6.694 ^a	4.505	1.052 ^{ab}	0.700	1.401 ^a	0.555	0.350 ^b	0.178
	β -galactosidase	23.535 ^a	11.600	2.296 ^{ab}	2.010	7.479 ^a	4.325	2.470 ^b	0.589
	β -xylosidase	42.767 ^a	31.006	8.328 ^a	4.846	21.584 ^a	15.033	2.649 ^b	1.638
LS	β -N-acetyl-glucosaminidase	15.070 ^a	8.580	7.546 ^a	4.781	7.014 ^{ab}	5.212	1.853 ^b	0.802
	α -glucosidase	13.694 ^a	7.071	5.221 ^{ab}	3.308	5.689 ^{ab}	1.112	1.428 ^b	0.679
	α -arabinosidase	3.450 ^a	2.133	4.372 ^a	3.854	1.921 ^a	0.465	0.665 ^b	0.381
	cellobiosidase	25.508 ^a	18.927	6.297 ^{ab}	2.437	6.846 ^b	3.415	4.230 ^c	1.986
	β -glucosidase	17.986 ^a	12.608	10.007 ^{ab}	6.443	4.932 ^b	2.029	1.219 ^c	0.693
	β -galactosidase	27.028 ^a	22.363	10.071 ^{abc}	6.948	7.913 ^b	8.596	2.383 ^c	1.038
	β -xylosidase	77.520 ^a	31.035	26.707 ^a	18.024	13.198 ^{ab}	7.066	4.069 ^b	0.963
MS	β -N-acetyl-glucosaminidase	10.955 ^a	6.807	1.592 ^a	0.307	2.494 ^{ab}	0.727	1.190 ^b	0.672
	α -glucosidase	9.573 ^a	5.704	1.832 ^a	0.411	2.678 ^a	1.051	1.935 ^b	1.473
	α -arabinosidase	3.239 ^a	1.382	0.794 ^b	0.161	2.107 ^{ab}	1.497	0.615 ^b	0.274
	cellobiosidase	30.187 ^a	17.130	5.675 ^a	1.694	7.614 ^a	2.716	6.288 ^b	5.173
	β -glucosidase	17.933 ^a	11.425	2.981 ^b	0.847	2.211 ^{bc}	1.132	1.517 ^c	1.573
	β -galactosidase	72.164 ^a	61.241	9.928 ^a	5.035	9.974 ^a	3.754	5.001 ^b	0.964
	β -xylosidase	117.381 ^a	40.131	23.016 ^b	5.496	20.869 ^b	11.931	9.619 ^b	6.033
HS	β -N-acetyl-glucosaminidase	23.510 ^a	18.777	6.131 ^{bc}	4.490	8.256 ^b	2.448	2.161 ^c	0.728
	α -glucosidase	11.421 ^a	2.632	6.338 ^{ab}	3.199	5.326 ^b	1.420	1.694 ^c	0.578
	α -arabinosidase	2.071 ^a	1.227	1.149 ^{ab}	0.987	0.868 ^{ab}	0.666	0.519 ^b	0.133
	cellobiosidase	34.561 ^a	18.899	12.688 ^{bc}	7.140	9.309 ^b	0.858	3.979 ^c	1.715
	β -glucosidase	10.011 ^a	4.503	7.617 ^{ab}	7.795	3.338 ^b	0.957	1.075 ^c	0.497
	β -galactosidase	33.388 ^a	1.013	20.497 ^{ab}	15.528	13.422 ^b	8.299	4.620 ^c	1.760
	β -xylosidase	69.698 ^a	14.990	42.725 ^{ab}	27.255	21.905 ^a	7.434	7.658 ^b	2.676

D8 9: Enzymatic activity in aggregate size fractions in sandy soil at day 361.

Table 35: Averages (Avg.) and standard deviations (\pm) of 7 C-cycling enzymes ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) from each aggregate-size fraction large macroaggregate (LM), macroaggregate (MAC), microaggregate (MIC) and silt and clay (SC) from the control and each of the slurry amendments; low slurry (LS), medium slurry (MS) and high slurry (HS) in the sandy soil 361 days after slurry application.

Treatment	Aggregate size Enzyme	LM		MAC		MIC		SC	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Control	β -N-acetyl-glucosaminidase	21.585 ^a	3.652	26.765 ^a	17.645	14.595 ^a	4.602	2.194 ^b	0.744
	α -glucosidase	21.890 ^a	10.406	18.942 ^a	9.356	16.682 ^a	4.090	1.720 ^b	0.702
	α -arabinosidase	4.654 ^a	0.496	4.670 ^{ab}	2.332	3.407 ^b	0.897	0.363 ^c	0.135
	cellobiosidase	3.306 ^a	0.807	3.459 ^{ab}	2.824	1.630 ^b	0.817	0.193 ^c	0.085
	β -glucosidase	99.819 ^a	15.991	124.087 ^a	56.393	65.734 ^b	13.544	6.116 ^c	2.037
	β -galactosidase	11.438 ^a	2.154	13.455 ^a	5.088	11.554 ^a	5.802	1.054 ^b	0.542
	β -xylosidase	8.434 ^a	2.281	7.541 ^a	6.411	4.485 ^a	1.927	0.472 ^b	0.201
LS	β -N-acetyl-glucosaminidase	44.705 ^a	18.068	18.755 ^b	6.188	7.767 ^c	2.395	1.207 ^d	0.496
	α -glucosidase	49.566 ^b	13.002	16.738 ^a	16.281	5.634 ^a	2.605	0.849 ^c	0.528
	α -arabinosidase	6.014 ^b	1.282	2.837 ^a	1.413	1.430 ^a	0.536	0.170 ^c	0.036
	cellobiosidase	5.724 ^a	2.090	3.080 ^{ab}	1.455	1.269 ^b	0.901	0.196 ^c	0.125
	β -glucosidase	100.855 ^a	31.793	33.083 ^b	9.242	17.806 ^c	5.322	1.538 ^d	0.763
	β -galactosidase	26.880 ^a	3.487	11.208 ^{ab}	8.510	5.181 ^b	2.509	0.560 ^c	0.239
	β -xylosidase	12.264 ^a	4.340	5.611 ^b	3.182	2.406 ^c	0.725	0.296 ^d	0.114
MS	β -N-acetyl-glucosaminidase	46.100 ^a	30.659	38.216 ^a	29.605	11.423 ^a	6.826	2.765 ^b	1.370
	α -glucosidase	40.645 ^a	42.091	17.101 ^a	7.472	9.684 ^a	4.022	1.210 ^b	0.371
	α -arabinosidase	5.373 ^a	5.053	4.779 ^a	1.958	2.119 ^a	1.101	0.215 ^b	0.080
	cellobiosidase	5.998 ^a	5.367	8.199 ^a	6.451	3.070 ^a	4.011	0.157 ^b	0.057
	β -glucosidase	99.690 ^{ab}	87.405	71.546 ^a	28.003	26.854 ^b	17.284	2.268 ^c	0.268
	β -galactosidase	28.335 ^a	26.851	13.551 ^a	6.082	7.552 ^a	3.677	0.744 ^b	0.249
	β -xylosidase	8.230 ^a	5.196	7.591 ^a	5.784	2.885 ^a	1.882	0.210 ^b	0.175
HS	β -N-acetyl-glucosaminidase	51.535 ^a	22.565	36.330 ^{ab}	11.237	19.580 ^b	9.735	0.818 ^c	0.216
	α -glucosidase	54.602 ^a	45.680	30.845 ^a	9.812	17.619 ^a	7.269	0.739 ^b	0.373
	α -arabinosidase	4.000 ^a	2.318	3.317 ^a	1.093	2.329 ^a	1.545	0.075 ^b	0.015
	cellobiosidase	7.480 ^a	4.981	9.036 ^a	7.123	3.703 ^a	1.539	0.154 ^b	0.120
	β -glucosidase	94.494 ^a	50.911	52.157 ^a	15.687	41.550 ^a	19.632	0.989 ^b	0.334
	β -galactosidase	20.917 ^a	15.674	20.313 ^a	5.208	9.716 ^a	5.755	0.300 ^b	0.191
	β -xylosidase	9.122 ^a	5.552	5.815 ^a	2.337	5.383 ^a	2.696	0.177 ^b	0.088

D8 10: Enzymatic activity in aggregate sized fractions in the loam and sand soils as a function of time (days)

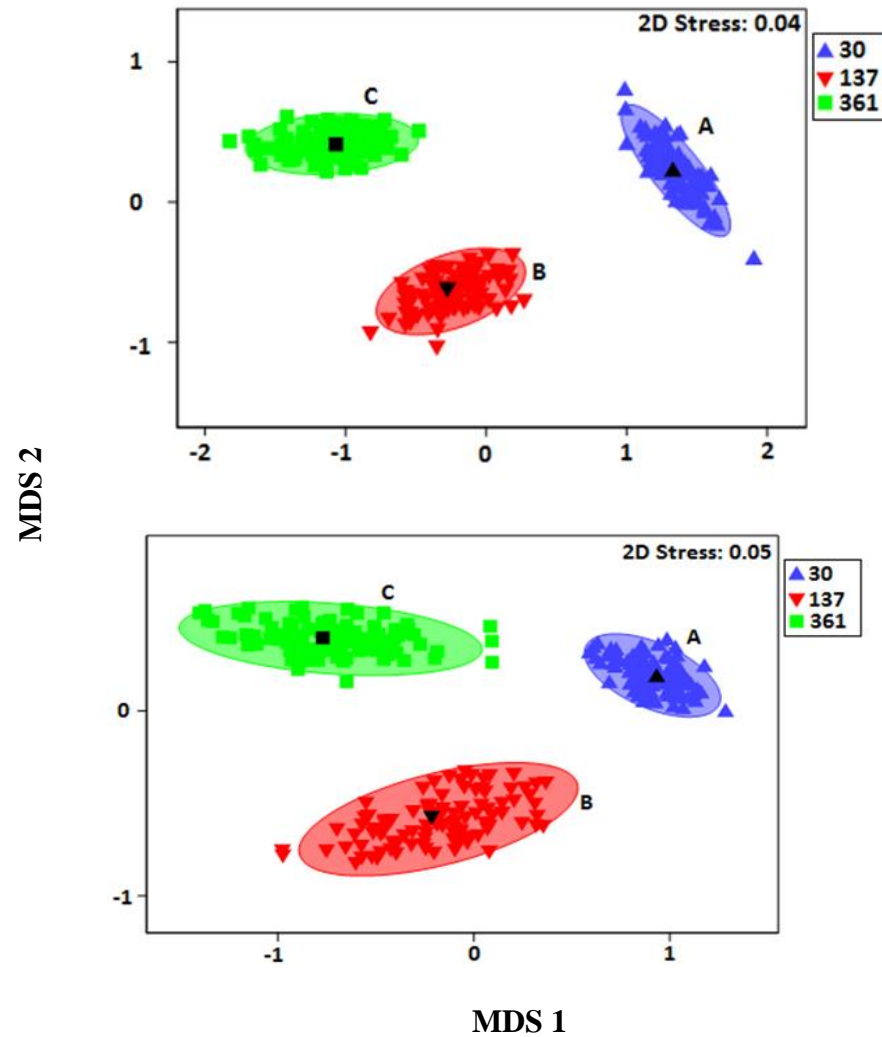


Figure 41: Bootstrapped MDS plots displaying enzymatic activities ($\mu\text{mol h}^{-1} \text{g}^{-1}$ dry soil) in the loam (top) and sand (bottom) soils 30 (blue triangle), 137 (red triangle) and 361 (green square) days after initial slurry application. Different letters indicate statistical difference at $P < 0.05$.

D8 11: % C from the bulk soil.

Table 36: Averages (Avg.) and standard deviations (\pm) of the % C of the bulk soil of each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.

Soil	Day	C		LS		MS		HS	
		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
Loam	0	2.378	0.022						
	30	2.328 ^a	0.03	2.288 ^a	0.03	2.578 ^b	0.033	2.48 ^c	0.039
	137	2.310 ^a	0.039	2.38 ^a	0.048	2.625 ^b	0.137	2.648 ^b	0.046
	361	2.268 ^a	0.066	2.355 ^a	0.062	2.355 ^b	0.062	2.495 ^c	0.033
Sand	0	1.433	0.043						
	30	1.393 ^a	0.013	1.323 ^a	0.044	1.608 ^b	0.017	1.505 ^b	0.062
	137	1.453 ^a	0.061	1.573 ^{ab}	0.412	1.683 ^b	0.036	1.755 ^b	0.052
	361	1.525 ^a	0.204	1.655 ^a	0.064	1.685 ^a	0.019	1.765 ^a	0.120

D8 12: % C within aggregate sized fractions.

Table 37: Averages (Avg.) and standard deviations (\pm) of the % C within aggregate sized fractions from each of the four sampling points (day 0, 30, 137 and 361 days) and four treatments (control, C, low slurry; LS, medium slurry; MS and high slurry; HS) in the experiment from both the loam and sand soil.

Loam	Aggregate size	LM		MAC		MIC		SC	
Time		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
0		3.014 ^a	0.065	3.307 ^b	0.035	2.680 ^c	0.067	2.244 ^d	0.061
30	Control	2.653 ^a	0.097	3.147 ^a	0.528	2.670 ^{ab}	0.346	2.230 ^b	0.360
	LS	2.895 ^a	0.217	3.542 ^b	0.175	2.599 ^{ac}	0.096	2.588 ^c	0.053
	MS	3.185 ^a	0.227	3.648 ^a	0.306	2.452 ^b	0.197	2.358 ^b	0.156
	HS	3.605 ^a	0.192	3.400 ^a	0.166	2.632 ^b	0.241	2.338 ^b	0.202
137	Control	3.140 ^a	0.222	2.992 ^{ab}	0.242	2.708 ^b	0.155	2.905 ^{ab}	0.149
	LS	3.293 ^a	0.669	3.924 ^a	0.952	2.996 ^a	0.216	3.145 ^a	0.121
	MS	3.348 ^a	0.511	3.331 ^a	0.152	2.970 ^a	0.241	3.080 ^a	0.248
	HS	4.988 ^a	1.384	3.484 ^b	0.201	3.168 ^{bc}	0.134	2.935 ^c	0.159
361	Control	2.570 ^a	0.049	3.113 ^b	0.101	2.481 ^a	0.231	2.321 ^{ab}	0.772
	LS	3.020 ^{ab}	0.321	3.454 ^a	0.196	2.995 ^{ab}	0.485	2.533 ^b	0.249
	MS	3.205 ^a	0.410	3.814 ^a	0.254	2.469 ^b	0.060	2.508 ^b	0.088
	HS	2.659 ^{bc}	0.147	3.439 ^a	0.115	2.599 ^b	0.025	2.816 ^c	0.175
Sand	Aggregate size	LM		MAC		MIC		SC	
Time		Avg.	\pm	Avg.	\pm	Avg.	\pm	Avg.	\pm
0		3.107 ^a	0.235	2.742 ^{ab}	0.269	2.433 ^b	0.073	5.206 ^c	0.068
30	Control	2.795 ^a	0.674	3.105 ^a	0.460	2.550 ^a	0.277	5.673 ^b	0.327
	LS	3.888 ^a	0.147	3.718 ^{ab}	1.140	2.731 ^b	0.387	5.713 ^c	0.354
	MS	4.280 ^a	0.313	3.012 ^b	0.078	2.530 ^c	0.307	6.035 ^d	0.576
	HS	4.625 ^a	0.926	2.877 ^b	0.510	2.591 ^b	0.562	5.735 ^a	0.602
137	Control	2.173 ^a	0.521	2.627 ^a	0.206	2.718 ^a	0.106	5.867 ^b	0.137
	LS	2.095 ^a	0.371	3.437 ^{ab}	2.241	2.845 ^b	0.365	6.235 ^c	0.473
	MS	2.103 ^a	0.269	2.455 ^{ab}	0.254	2.627 ^b	0.109	6.750 ^c	0.209
	HS	2.225 ^a	0.484	3.139 ^a	1.233	2.548 ^a	0.221	6.710 ^b	0.122
361	Control	1.853 ^a	0.209	2.659 ^b	0.285	2.474 ^b	0.153	6.463 ^c	0.703
	LS	1.965 ^a	0.279	4.976 ^{bc}	3.520	2.582 ^b	0.556	6.365 ^c	0.128
	MS	2.370 ^a	0.371	3.624 ^{ab}	1.036	2.529 ^a	0.512	5.093 ^b	2.018
	HS	3.350 ^a	1.765	3.087 ^a	0.919	2.903 ^a	0.377	5.910 ^b	0.394