Understanding the influence of infield biodiversity conservation on the environmental fate of crop protection products

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Abstract

Modern arable agriculture is in the majority dependent upon both tillage, and application of crop protection products. Increasingly the intensity of tillage is being reduced, for reasons including cost, carbon sequestration, and to improve the biodiversity of agricultural land. Reduced intensity tillage alters soil properties and microbial communities, and therefore may alter the environmental fate of crop protection products. Understanding influence of reduced tillage intensity is essential for ensuring the safe use of crop protection products.

The present study utilises high throughput amplicon sequencing of soil microbial communities, paired with ^{14}C radiolabelled compound degradation studies to assess the impact of varied tillage intensities at two field experiments.

Within the first field experiment, tillage intensity is found to significantly alter the community composition of soil eukaryotes. Additionally, soils underneath hedgerows are identified to host bacterial, eukaryotic and fungal communities that are distinct from infield communities. The rate of degradation of Mandipropamid and Simazine in intact soil cores collected from differently tilled field plots was similar in both soils, however the rate of degradation was more variable in soils under a lower intensity of tillage.

In a second, longer term field study, tillage intensity is identified to significantly differentiate soil bacterial, eukaryotic and fungal communities. The role of tillage in shaping the horizontal heterogeneity of the soil microbiome at different depths is explored.

In conclusion, tillage intensity is identified to alter both the composition of key soil microbial communities, and to affect the spatial variability of crop protection product degradation.

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Author's Declaration

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

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Chapter 1: General Introduction

1.1 Tillage

Tillage describes the range of actions that may be undertaken to prepare a seedbed for the sowing of crops. For much of the preceding 100 years, convention has been to till with a mouldboard plough, inverting the top soil (ca. 30 cm) (Lal et al., 2007). For the purposes of this thesis, such ploughing will be considered 'Inversion Tillage' (IT). IT is effective in the suppression of pest species and incorporating crop residues throughout the ploughed section of the soil profile. IT by ploughing may be followed by secondary treatments, such as harrowing (Morris et al., 2010).

Despite the efficacy of IT in the control of weed species, alternative methods of tillage, including both reduced intensity tillage (RT) and no tillage (NT) are increasingly being adopted in the UK and Europe (Holland, 2004, Morris et al., 2010). Development of less intensive, invasive and disruptive tillage methods was instigated by the Dustbowl phenomenon in the USA during the 1930s (Lal et al., 2007). RT and NT were first developed with a focus on reducing soil erosion. RT may utilise tillers or harrows, but does not uniformly invert the top soil, rather scraping at the soil surface. RT is frequently defined by the proportion of the soil surface that remains covered with crop residues following cultivation, where greater than 30 % coverage is required to be considered RT (Holland, 2004).

RT and NT methods spread widely throughout the Americas during the 20th century, however adoption within European farming has been substantially slower (Holland, 2004). Alskaf et al. (2019) estimate that in England, 47.6% and 7% of arable land is under RT and NT management respectively. Where RT has been adopted within the UK, the principal reason has been a reduction in cost relative to IT (Ingram, 2010, Morris et al., 2010) – both Townsend et al. (2016) and Alskaf et al. (2019) note that RT in England is more commonly practiced on larger farms, with the financial capital to transition to a RT system.

Significant co-benefits can be derived from RT, and these benefits are delivered across multiple scales. Lowering the intensity of tillage can increase soil porosity, reduce runoff from agricultural fields, reducing soil erosion, and contamination of watercourse from fertilisers and crop protection products (CPPs) (Holland, 2004). Lowering tillage intensity increases carbon storage within soils, with soil carbon becoming increasingly stratified (Haddaway et al., 2017). However this effect is small – Haddaway et al. (2017) found the average increase in soil organic carbon (SOC) to be 0.13 % ± 0.022 % (standard error) in RT relative to IT, considering only the upper 15 cm of soil.

Both increases in SOC, and improved soil structure may be attributed to an increased abundance of soil macroaggregates (Six et al., 2000). A reduction in tillage intensity is associated with a reduction in the turnover of soil macroaggregates, which reduces the turnover of soil microaggregates, and in turn reduces decomposition of particulate organic matter (POM), increasing SOC (Six et al., 2000, Six et al., 2004). Reduced turnover of soil macroaggregates under RT is attributable to reduced mechanical disturbance, increased presence/non-disruption of plant roots, and the increased abundance of hyphal and filamentous microorganisms (Six et al., 2000, Six et al., 2004, Blankinship et al., 2016, Hewins et al., 2017). Wilson et al. (2009) reported arbuscular mycorrhizal length to account for 68 % of variation in macroaggregate mass and 15 % of soil organic matter storage in grasslands (Figure 1.1).

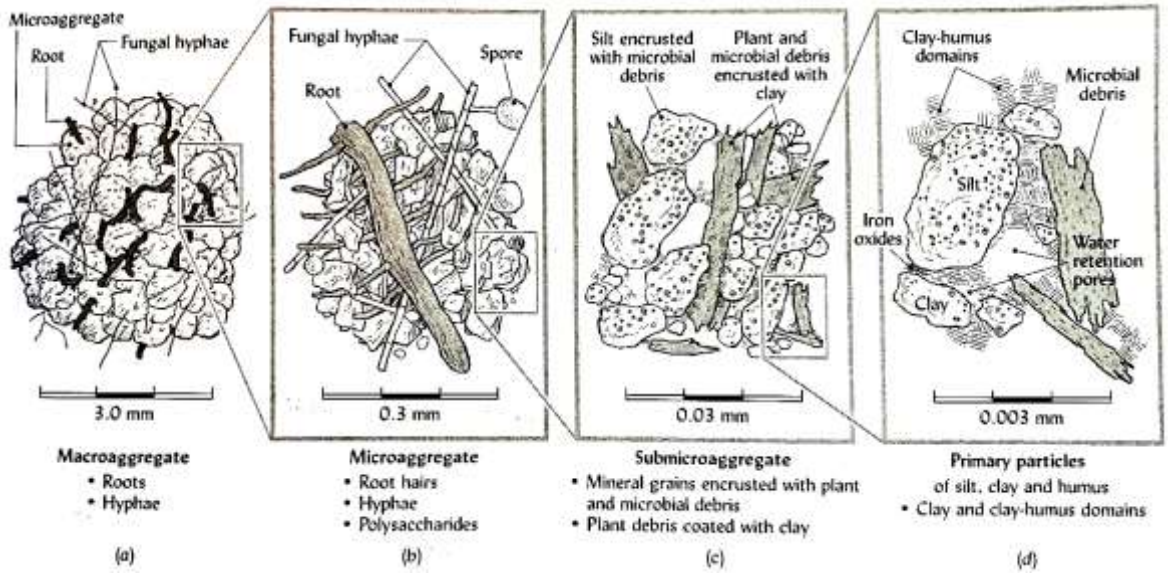


Figure 1.1 – Figure and figure caption adapted from Brady and Weil (2012). Soil macroaggregates are composed of microaggregates. This figure explores aggregate structure and formation in with four levels. The different factors important for aggregation at each level are noted. (a) Macroaggregates are formed from many microaggregates formed and held together by fine roots, and fungal hyphae (b) microaggregates of smaller clumps of mineral material and organic debris held together by fine roots, fungal hyphae, their exudates and microbial gum. (c) Submicroaggregates consist of fine mineral particles, coated with particulate organic matter, which in turn is coated with mineral material and Fe and/or Al oxides. (d) Clusters of clay platelets interacting with Fe and/or Al oxides and organic polymers at the smallest scale, these organoclay clusters bind to the surfaces of humus particles and the smallest of mineral grains.

Alteration of tillage intensity may have variable effect on crop yield, and is dependent on factors including field history, crop, and soil type (Ekebery and Riley, 1997, Holland, 2004, Morris et al., 2010, Armengot et al., 2014). Sans et al. (2011) reported both an increase in weed abundance, and a reduction in yields under RT relative to IT. Armengot et al. (2014) report increased weed abundance (230% higher than IT) in organic RT farming, but yields remained similar to organic IT

plots. Arvidsson (1998) reports deeper tillage to increase crop yields, and reduced tillage intensity to increase the occurrence of *Rhyncosporium secalis* (leaf scald).

In addition to the influence on crop and pest species, the intensity of agricultural management influences the infield soil biome. Cultivation is associated with a decline in the diversity of arbuscular mycorrhizal fungi, and increasing the relative abundance of bacteria to fungi, and lowering the abundance of earthworms (Helgason et al., 1998, Kuntz et al., 2013, Prendergast-Miller et al., 2021). In an meta-analysis of 62 studies, Zuber and Villamil (2016) report higher intensities of tillage to lower total microbial biomass, and enzyme activity. Kaurin et al. (2018) observed greater abundance of bacterial, crenarchaeal, and fungal communities at lower tillage intensities, the abundance of which was more sensitive to drought than in soils under IT, although this recovered following rewetting.

1.2 Crop Protection Products

The use of CPPs is widespread within modern agriculture. In Great Britain, the total weight of applied pesticides declined between 1996 (36 million kg) and 2016 (17 million kg) (FERA, 2021) (Figure 1.2). However the total treated area, defined as sum of areas applied to multiplied by number of applications, rose from 45 million ha in 1990 to in excess of 80 million ha in 2015 (FERA, 2021) (Figure 1.3). In 2016, fungicides accounted for 50 % of the total treated area, and 35 % of total weight of applied CPPs, herbicides 32 % and 46 % respectively, and insecticides 7.0 % and 1.9 % respectively; the remainder accounted for by products including molluscicides, rodenticides, and growth regulators (FERA, 2021). CPPs are expected to remain a significant factor in maintaining crop yields globally, to support the growing human population amidst the global environmental change forecast for the 21st century (Beddington, 2009, Popp et al., 2012, Fenner et al., 2013).

CPPs contain one or more active substances, one or more solvents, and adjuvants with a range of purposes, including surfactants, antifoaming agents and stabilisers (Krogh et al., 2003). An active ingredient is defined by EPA (2013) as:

"(1) in the case of a pesticide other than a plant regulator, defoliant, desiccant, or nitrogen stabilizer, an ingredient which will prevent, destroy, repel, or mitigate any pest;

(2) in the case of a plant regulator, an ingredient which, through physiological action, will accelerate or retard the rate of growth or rate of maturation or otherwise alter the behavior of ornamental or crop plants or the product thereof;

(3) in the case of a defoliant, an ingredient which will cause the leaves or foliage to drop from a plant;

(4) in the case of a desiccant, an ingredient which will artificially accelerate the drying of plant tissue; and

(5) in the case of a nitrogen stabilizer, an ingredient which will prevent or hinder the process of nitrification, denitrification, ammonia volatilization, or urease production through action affecting soil bacteria."

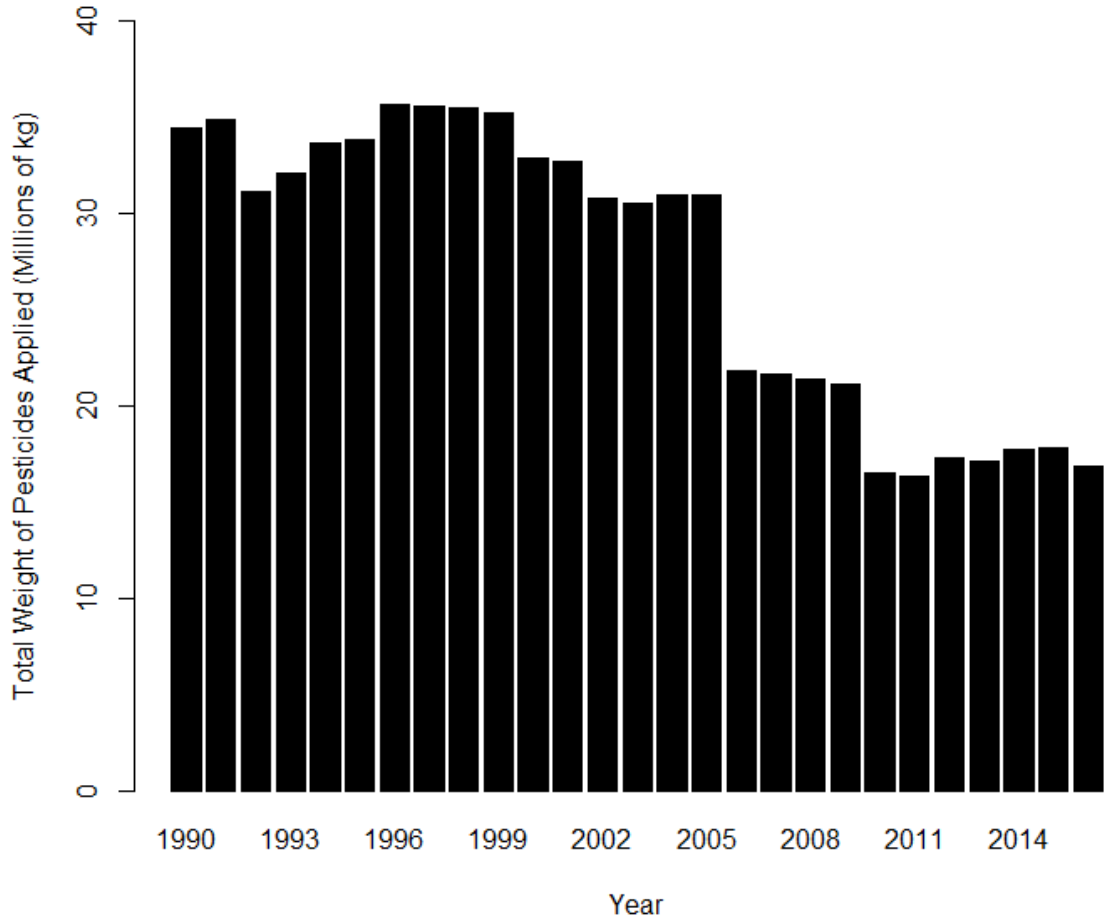


Figure 1.2 – Total weight (in millions of kg) of pesticides applied in Great Britain between 1990 and 2016. Data from FERA (2021).

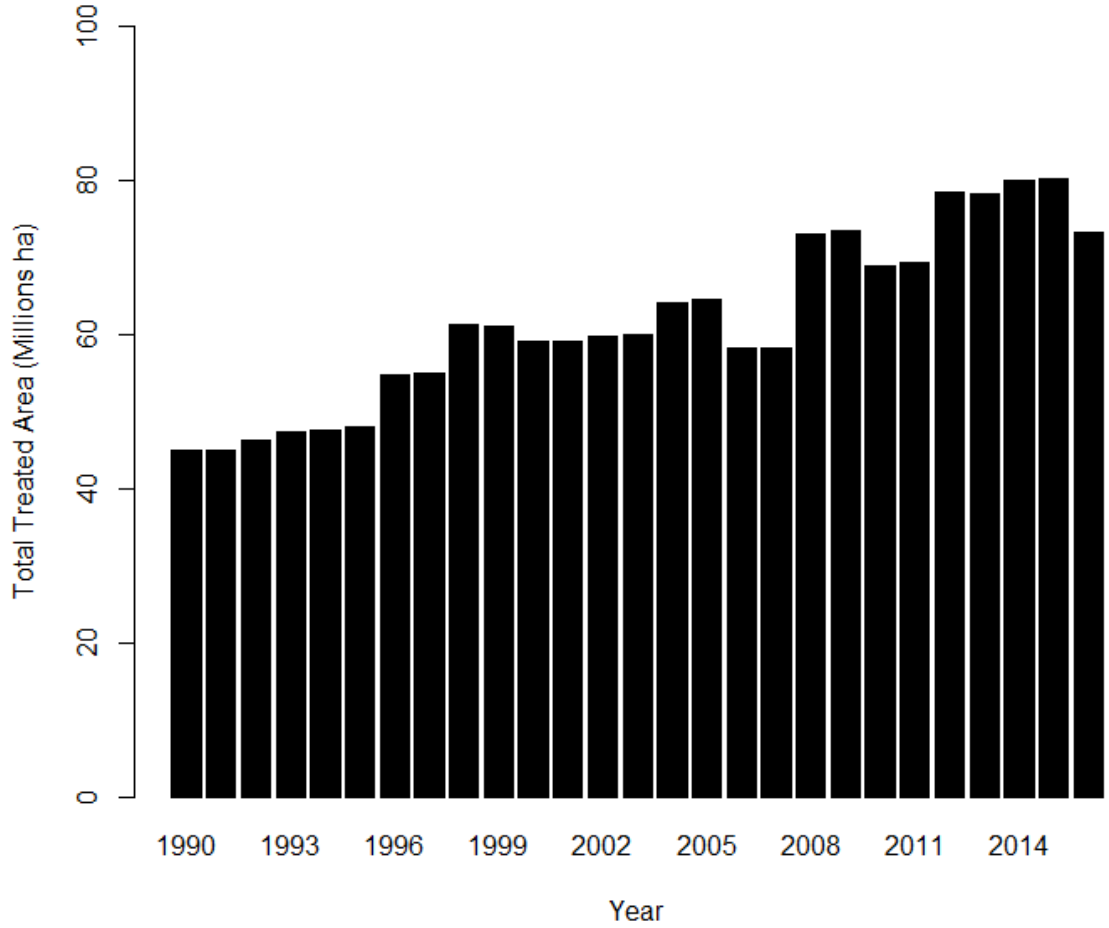


Figure 1.3 – Total area (in millions of ha) treated with pesticides in Great Britain between 1990 and 2016. Data from FERA (2021).

The environmental fate of a CPP is the sum of all processes that occur to a substance following its release into the environment, where the environment is all terrestrial, atmospheric and aquatic space, to the point at which the substance is mineralised, or irreversibly bound to another substance. Environmental fate within the present study is concerned with the fate of compounds applied outside, to cultivated-arable fields, and which are deposited or incorporated onto soils. Figure 1.4 details the major processes by which the environmental fate of CPP is determined within such a system.

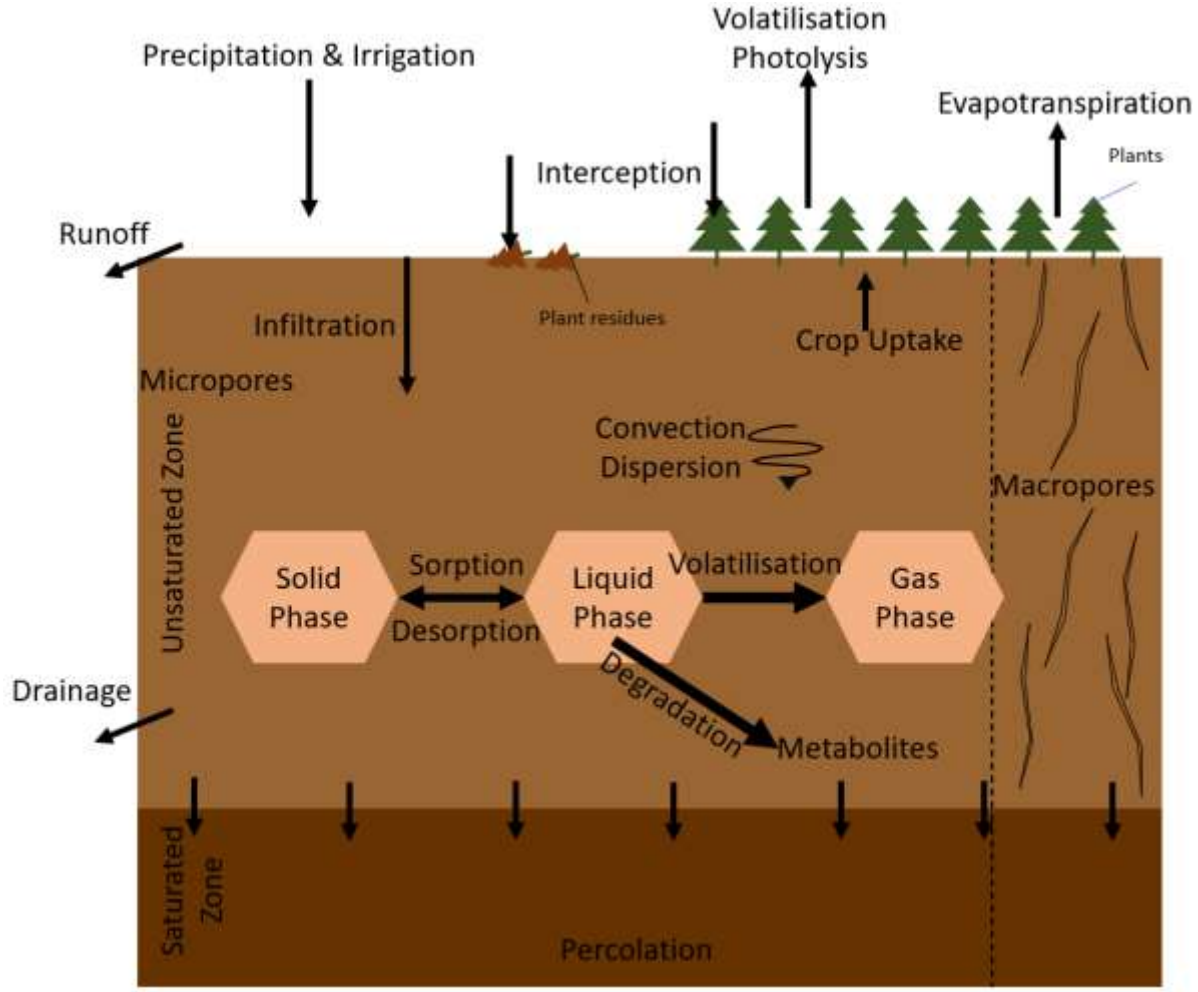


Figure 1.4 – Environmental fate and transport processes in soil, adapted from figure by Dubus (2012).

For the purposes of the present work, the environmental fate of CPPs is concerned principally with the fate of active substances. On differences of the environmental fate of active substances and formulated products, see for example Khan and Brown (2016), Gutowski et al. (2015a) and Gutowski et al. (2015b).

The environmental fate of a compound in soils is primarily predicted on the basis of its rate of degradation, and the sorption behaviour of the compound (Kah et al., 2007). The soil parameters that determine the rate of degradation of a compound vary dependent upon the properties of each compound, and therefore accurate universal predictions of degradation rates are not possible (Kah et al., 2007). However generally higher soil organic carbon, associated with higher metabolic activity, may be associated with higher rates of degradation (Kah et al., 2007). Routes of loss of compounds or metabolites, which tend to be more mobile than their parent compound (Boxall et al., 2004), may be via volatilisation, run-off, drainage, or the removal of residues in crops or plant products. Sorption

may temporarily remove compounds from availability for either metabolism or transport processes, however all sorption may be reversible (Suddaby et al., 2013, Suddaby et al., 2016).

Microbial metabolism (degradation) is a central process in the dissipation (to mineralisation or bound residues) of applied compounds, which may be highly stable abiotically – such as cleaving C-P bonds (Fenner et al., 2013). In soil, the microbial metabolism of compounds may be principally associated with bacteria (Fenner et al., 2013), however for some compounds, synergistic effects between soil bacteria and fungi have been observed (Levanon et al., 1994). Prokaryotes are suited to effectively degrade agrochemicals via their propensity to metabolise them for energy or nutrients, and not to detoxify them (Fenner et al., 2013). Further, the pace of evolution within bacterial populations, and horizontal transfer of genes within bacterial communities facilitate the (relatively) rapid development and dissipation of pathways to degrade xenobiotics within bacterial communities (de Souza et al., 1998b, Fenner et al., 2013). Phototrophic communities can have a significant impact upon the rate of CPP degradation (Thomas and Hand, 2012, Davies et al., 2013a), and light can structure the communities of phototrophs, bacteria and fungi on the surfaces of soils (Davies et al., 2013b). The relative role of crop residues in shaping microbial communities on soil surfaces is unclear.

Removal of residues via crop and plant products is dependent upon the uptake of such residues by plants. Briggs et al. (1982) found a relationship between the octanol:water partition coefficient (Kow) and plant uptake, however more recent hydroponics experiments have found there to be little to no relationship between Kow and plant uptake (EFSA, 2013). While plant uptake of CPPs may occur at a range of rates for different compounds, this may generally be considered a less determinant process than the most major processes such as microbial degradation. In EU and GB ground and surface water risk assessment of CPPs, the default position is that plant uptake is equivalent to 0.

1.3 Tillage Intensity and the Environmental Fate of Crop Protection Products

The crop residues that remain on the soil surface following RT are integral to the reduction in soil erosion achieved by RT farming, however these may intercept crop protection products (CPPs) applied within the field, altering the environmental fate of these compounds (Reddy et al., 1995, Locke and Bryson, 1997, Holland, 2004, Morris et al., 2010). Type and abundance of crop residue, and the time elapsed between tillage and CPP application alter the effect of crop residues on CPP fate (Alletto et al., 2010, Cassigneul et al., 2015). Cassigneul et al. (2015) report that increased crop residue degradation (to 56 days), increased sorption and retention of CPPs. Cassigneul et al. (2016) report the addition of supplementary crop residues to soil reduced the rate of glyphosate

mineralisation. Soluble glyphosate was found to be metabolised within the mulch fraction, but less efficiently than within the soil fraction. Glyphosate absorbed to soil remained available for microbial degradation, but that sorbed to crop resides did not (Cassigneul et al., 2016). The timing and mode of application of a CPP influences the relative impact of tillage intensity on CPP fate, for example, compound applied as seed treatments will not be subject to interception by crop residues, whilst preemergence broadcast applications may be most likely to be intercepted by crop residues (Alletto et al., 2010, Cassigneul et al., 2015).

The greater abundance of soil macroaggregates under RT and stratification of carbon within the soil profile (Six et al., 2000, Holland, 2004, Haddaway et al., 2017), may change the availability of CPPs and metabolites to degradation processes. Greater abundance of soil macroaggregates may increase the occurrence of non-equilibrium/aged sorption, which may occur by diffusion of compounds into soil aggregates (Beulke et al., 2015, Commission, 2021), and encapsulation via greater concentrations of soil carbon, which may occur either stratified to the upper soil layers, or throughout the soil profile (Beulke et al., 2015, Haddaway et al., 2017).

Altering tillage intensity may alter the rate and/or route of degradation of compounds applied to soils, influencing both abiotic and biotic degradation processes (Alletto et al., 2010). Abiotically, tillage intensity may alter standing water on field surfaces, and greater physical mixing of applied compounds by IT may reduce the occurrence of photolysis of compounds otherwise remaining on the soil surface. Biotically, crop residues on the soil surface may host distinct microbial communities that may have differing degradation potential than bulk soils, and the more heterogenous soil surface may influence the composition and function of the soil biological crust, which hosts important and distinct communities of compound degraders (Davies et al., 2013a, Davies et al., 2013b).

A reduction in tillage intensity has the potential to alter the transport of CPPs via processes including runoff, leaching and volatilisation (Alletto et al., 2010). Runoff of CPPs may be influenced both by a reduction in the volume of water flowing from a field, and by the reduction of suspended sediments within this flow from low intensity tillage plots (Alletto et al., 2010). Tebrugge and During (1999) report a reduction in runoff from plots under RT, however Logan et al. (1994) identified no difference in CPP runoff amongst four compounds. Similarly, the effect of tillage intensity on CPP leaching is variable, with reports of greater, no change, and lesser leaching with greater tillage intensity (Alletto et al., 2010, Okada et al., 2016). RT increasing leaching may be attributable to greater macroporosity due to the looser packing of aggregates, and lesser disturbance of earthworm and root channels by tillage (Alletto et al., 2010).

1.4 DNA Metabarcoding

The study of soil microbial communities has been transformed throughout the last decade by the development and application of omic technologies to study soil genomes, transcriptomes and metabolomes (Caporaso et al., 2011, Lindahl et al., 2013, Jansson and Baker, 2016). Application of high-throughput DNA sequencing platforms has facilitated the rise of metabarcoding studies of soil microbial communities (Caporaso et al., 2011, Hugerth and Andersson, 2017, Pollock et al., 2018). Metabarcoding studies amplify and sequence marker genes, or regions thereof, to provide qualitative, what sequences are present, and quantitative, relative abundance, data (Pollock et al., 2018).

Common target genes for metabarcoding studies of soil microbial communities include 16S rRNA, 18S rRNA and ITS genes (Lindahl et al., 2013, Pollock et al., 2018). These markers are frequently used to study prokaryotic communities including bacteria and archaea (via 16S), total eukaryotes (18S) and fungi (ITS) (White et al., 1990, Gardes and Burns, 1993, Stoeck et al., 2010, Ihrmark et al., 2012, Lindahl et al., 2013, Apprill et al., 2015, Parada et al., 2016, Pollock et al., 2018). The primers used to generate amplicons are subject to frequent revisions, as improvements are made to prevent omission or misrepresentation of members of microbial communities (Op De Beeck et al., 2014, Apprill et al., 2015).

Of equal if not greater importance to the generation of sequence data, the analysis of datasets from metabarcoding studies can be complex, with a range of methods applied to similar datasets asking similar questions, and an even greater range across different datasets and hypotheses (Callahan et al., 2017, Edgar, 2017a, Edgar, 2017b, Koo et al., 2017, Edgar, 2018, Knight et al., 2018, Pollock et al., 2018). Whilst historically, amplicon sequences have been clustered by sequence similarity, often at the level of 97 % sequence similarity (Hugerth and Andersson, 2017), this methodology is now being displaced. Use of an amplicon sequence variant (ASV) (also known as sequence variant (SV) or zero-radius operational taxonomic unit (ZOTU)) approach as opposed to operational taxonomic unit approach (OTU) methodology delivers benefits in computational cost, reduced batch effects, accuracy and reproducibility (Callahan et al., 2017, Edgar, 2017b). While ASV approaches to analysis of ITS sequence has been criticised for failure to recognise the occurrence of multiple distinct copies of the region within a genome (Lindner et al., 2013, Nilsson et al., 2018), such multiple copies are not guaranteed to cluster within a single OTU (Edgar, 2017b), and where the data is collapsed by taxon any inflation of seemingly unique features may be offset, provided a suitably accurate taxonomic assignment can be achieved.

Such taxonomic assignments are however dependent upon the quality of reference databases and the methods by which the classifications are assigned. Methods, such as BROCC (Dollive et al., 2012), VSEARCH (Rognes et al., 2016), and the RCP-Classifier (Wang et al., 2007), can assign nominal taxonomies to query sequences, working from reference databases that may be general, such as the NCBI nt database (NCBI, 1988), or smaller databases such as SILVA (Pruesse et al., 2007), Greengenes (DeSantis et al., 2006), and UNITE (Nilsson et al., 2015). The accuracy and precision of taxonomic assignments is variable dependent upon the method, reference database, and query sequences (Werner et al., 2012, Edgar, 2018), and may fail to accurately describe rare or novel organisms, which remain common (Elshahed et al., 2008).

1.5 Aims

Whilst the impact of tillage intensity has previously been studied upon constituents of the soil microbiome, and the environmental fate of crop protection products, such analyses have previously been independent from one another, may have relied on increasingly outdated approaches for assessing limited sections of soil microbial communities, and used methods that disrupt key differences between soils under different tillage intensities. The present work seeks to close key knowledge gaps, regarding both the impacts on soil microbial community composition, and its function in the degradation of CPPs.

Utilising two existing field experiments undertaken by the White Rose Sustainable Agriculture Consortium (WRSAC), this thesis attempts to address knowledge gaps: to understand at greater breadth (through studying soil archaea, bacteria, total eukaryotes, and soil fungi) and greater resolution (by application of high throughput amplicon sequencing (HTAS) the impact of tillage on soil microbial communities, both in relation to other tillage intensities, and also adjacent agricultural landscape components (hedgerows, field margins, and pastures). These analyses explore the relationship between tillage and the soil microbial community at different depths throughout the soil profile, and seek to understand how tillage may alter the distribution of functional genes associated with the degradation of CPPs. To directly assess the environmental fate of CPPs, non-conventional compound degradation studies are conducted, utilising an intact core design to preserve potential differences between soils under different tillage regimes, that may in previous studies been lost to common soil processing techniques.

By application of HTAS metabarcoding, and ^{14}C compound degradation studies, to soils from two established field experiments, both containing IT and RT plots, the present thesis aims to:

1) Identify changes in the composition of soil microbial communities under different tillage intensities (Chapters 2 and 5)
2) Contextualise the composition of soil microbial communities in arable soils relative to other soils in an agricultural landscape (from pasture, field margins and hedgerows) (Chapter 2)
3) Identify differences in the rate of degradation of CPP in soils at different tillage intensities (Chapter 3)
4) Identify if tillage intensity alters the distribution of genes associated with CPP degradation within the soils (Chapter 4)
5) Identify the effects of the incubation of soil for degradation studies on soil microbial communities (Chapter 4)
6) Identify how tillage intensity alters the spatial variability of soil microbial communities (Chapter 5)

In Chapter 6, results from the chapters detailed above are discussed in context of the scientific literature, and recommendations for further work are made.

Chapter 2: The soil microbiome of agricultural landscapes

2.1 Introduction

2.1.1 Tillage in context of other environmental compartments

Modern agricultural landscapes are a complex patchwork of pasture and arable fields, divided by natural (such as watercourses) and man-made barriers (such as fences and roads), and bordered by non-agricultural spaces either urban, managed or natural. Within fields themselves, marginal areas may be distinct from the actively farmed space, with bare or vegetative borders between crops and field boundaries (Van Vooren et al., 2017, Haddaway et al., 2018). These field bordering features provide a wide range of ecosystem services, including reducing flow of overland and subterranean flow, harbouring for some taxonomic groupings more diverse and/or more abundant communities, and may improve the interconnectedness of (semi)natural spaces within the agricultural landscape (Davies and Pullin, 2007, Van Vooren et al., 2017, Haddaway et al., 2018, Holden et al., 2019, Prendergast-Miller et al., 2021).

Holden et al. (2019), Berdeni et al. (2021) and Prendergast-Miller et al. (2021) detail results from the SoilBioHedge experiment, which investigated a wide range of biological and physicochemical properties of an agricultural landscape in Britain, including research on the use of grass clover-leys to improve soil properties, and the role of hedgerows to provide biodiversity reservoirs of ecosystem engineering organisms (WRSAC, 2014). The experiment included three pasture fields that had arable crop strips cultivated within them, at two tillage intensities (IT and RT). Winter wheat was grown within the strips for two consecutive years (2015-17) (WRSAC, 2017).

2.1.2 Studying tillage methods

Assessment of tillage intensity on soil properties and microbial communities may best be achieved by adoption of a systems approach, to accurately capture the sum of effects that may be encountered by farmers changing practices (Derpsch et al., 2014). Such studies also benefit from long durations, in a recent metanalysis, Haddaway et al. (2017) included only results from experiments that had been established for ten or more years.

It is valuable to understand the impacts of varying tillage intensity on soil microbial communities, not only in context of other forms of tillage but also relative to adjacent environmental compartments, and land management techniques. Cultivation is known to lower the diversity of arbuscular mycorrhizal fungi relative to nearby woodlands (Helgason et al., 1998). Other

environmental compartments may host genetic reservoirs that may disperse into cultivated areas, either between instances of tillage, or following a reduction in tillage intensity. Prendergast-Miller et al. (2021) however report earthworm recruitment in arable to grass-ley conversion to be due to recruitment from infield communities, and not field boundaries.

2.1.3 Aims and Objectives

The present study aims to identify changes in the composition of the soil microbiome – considering archaea, bacteria, total eukaryotes and fungi under different intensities of tillage (IT and RT), and contextualise these relative to other agricultural land management areas – hedgerows, field margins, and pasture. It was hypothesised that increasing the intensity of tillage would alter the soil microbiome, with IT possibly being more homogenous than other infield settings which are not subject to mixing. It was also expected that the studied hedgerows would host a distinct soil microbiome, Holden et al. (2019) having reported a distinct fungal community within the roots of hedgerow plants – with the present study expanding the research to include archaea, bacteria, total eukaryotes and fungi from bulk soils.

2.2 Methodology

2.2.1 Field Site and Sampling

The field experiments of the SoilBioHedge project were conducted at the University of Leeds farm, England (53.874, -1.323). Holden et al. (2019) detail mean annual precipitation to be 674 mm, mean annual temperature of 9.2 °C, and the soils to be loamy calcareous brown earth (0.5-0.9 m in depth) above limestone. The present study draws soils from the three arable fields of the SoilBioHedge project; 'Paddock', 'Valley' and 'Warren' – Figure 2.1 farm layout, Figure 2.2 field layout.

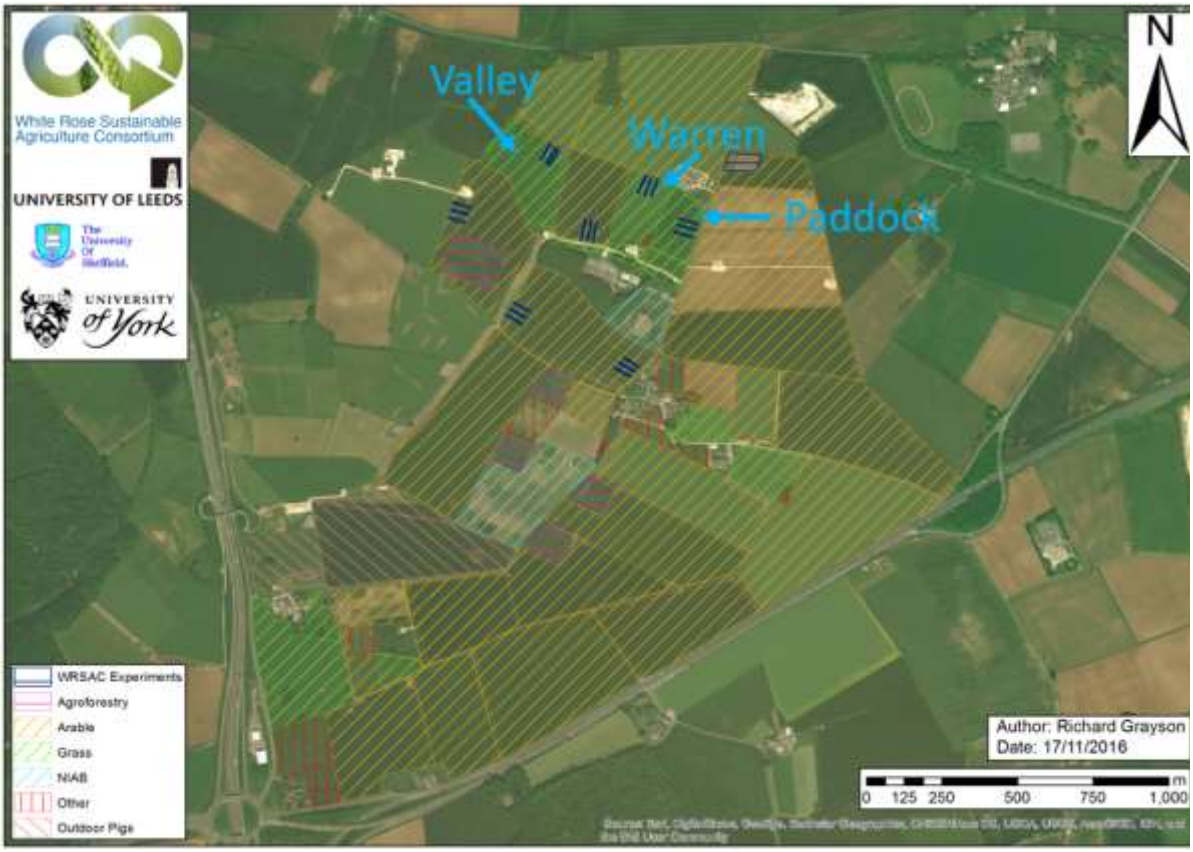


Figure 2.1 – University of Leeds farm layout. Adapted from (Grayson, 2016). From West to East the experimental strips are; in Valley RT, pasture, IT; in Warren IT, pasture, RT; in Paddock, from North to South, IT, pasture, RT.

Paddock and Valley fields have been pasture continuously from (at least) 2003. Warren field has been pasture since 2012, having previously had winter wheat (2003, 2004, 2006, 2007, 2009, 2010), potatoes (2005) and oil seed rape (2008) grown within the field. See Appendix 1 for details of previous CPP applications to all fields – Warren has a substantially larger range of compounds previously applied. See Appendix 2 for soil property data summarized from Grayson (2020).

Details of field experiments from Lappage (2015), Lappage (2016), Lappage (2017), Lappage (2018) and Holden et al. (2019).

Sampling was conducted on 5 occasions – April and August 2016, and February, April and July 2017. At each sampling event, bulk soil samples were collected as follows; pits of approximately 0.18 x 0.18 x 0.15 m in depth were dug, with a thin slice of this soil profile collected for analysis. Samples were frozen (-20 °C) within 24 hours of collection. Samples were collected in strips across each field representing the IT, RT and pasture treatments. These were collected at 2, 8, 16, 32 and 64 m into each field, with hedge and field margin samples collected in line with these samplings (see Figure 2.2). For the purposes of the present study, Holden et al. (2019)'s definition of a hedgerow as being a closely spaced area of trees and shrubs to form field boundaries will be adopted, with field margins being considered principally grassy features.

Note on sample naming – all samples in this Chapter are named following this scheme: four digits for month and year of sampling (for example 0416 for April 2016), two letter field code (PA for Paddock, VA for valley, and WA for Warren), two letter treatment code (CN for pasture control, IT for ploughed soils, and RT for lower intensity tillage), and one or two characters for strip location (H for hedge, M for margin, and digits for meters into the field 32 being for 32 meters into the field). Therefore 0416PACNH is a sample from April 2016 collected from the hedgerow in line with the pasture control strip, and 0717WAIT64 from July 2017 collected from Warren field 64 meters along the ploughed soil strip. ARC may be used as an abbreviation for archaea, BAC for bacteria, EUK for eukaryotes and FUN for fungi.

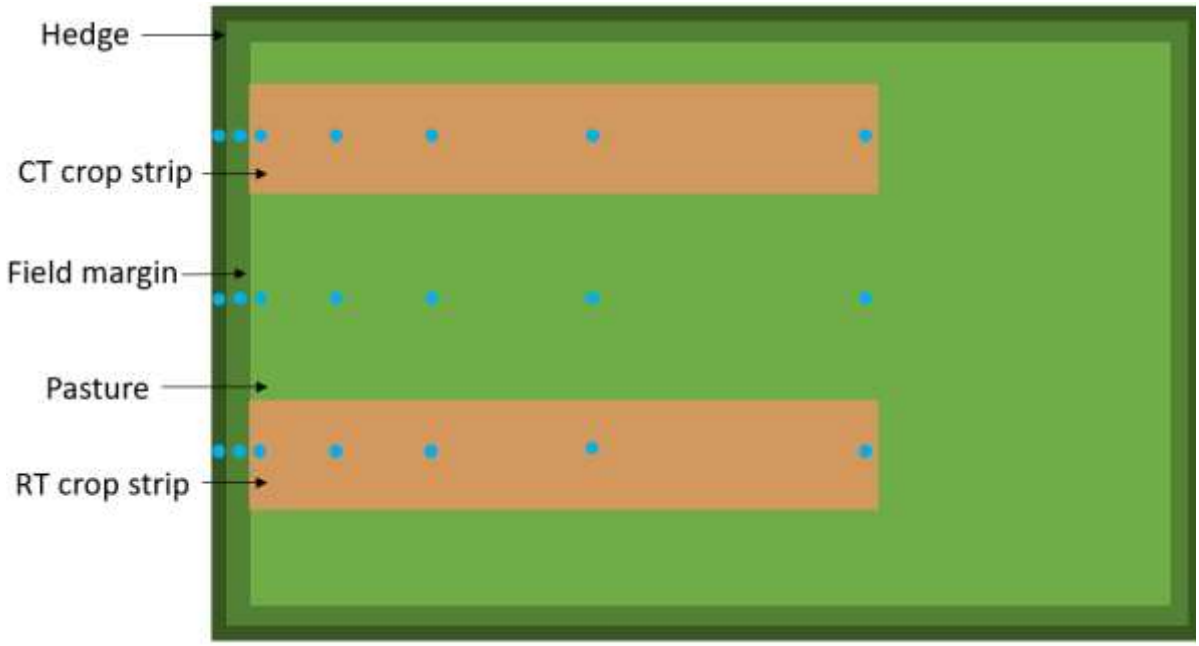


Figure 2.2 – Field layout for SoilBioHedge pasture fields with arable strips. Figures not to scale, strips established at approximately 64 m infield length, with an approximate width of 3m. Sampling locations marked in blue, being within the hedgerow, field-margin, 2 m, 8 m, 16 m, 32 m, and 64 m from field edge.

2.2.2 Molecular Methods

Samples were stored at -20 °C prior to freeze drying. Batches of samples were freeze dried for approximately 48 hours, until they were visibly dry, crumbled easily, and felt cool – not cold. Once dried, samples were homogenised by mixing within sample containers. 0.25 g aliquots were milled to fine powder. DNA was extracted using MoBio/Qiagen PowerSoil DNA Isolation Kits, per the manufacturer's instructions. DNA concentration was assessed using a Thermo Scientific Nanodrop 1000.

DNA amplicons were generated using the following primers; for archaea Arch349F and Arch806R (Takai and Horikoshi, 2000), for bacteria 515FY and 806rmod (Apprill et al., 2015, Parada et al., 2016), for eukaryotes TAReuk454FWD1 and TAReukREV3 (Stoeck et al., 2010) and for fungi gITS7 and ITS4 preceded by a 1TS1f and ITS4 preselect (White et al., 1990, Gardes and Burns, 1993, Ihrmark et al., 2012) (see Table 2.1 for primer sequences). Primers – excluding those for the fungal preselect – included adapters at the 5' end for Illumina library preparation. Bovine serum albumin (BSA) was included in all PCR reactions to reduce PCR inhibition – see Kreader (1996), and carried out in a total reaction volume of 25 µl. PCR reaction conditions: 200 µM dNTPs (Promega Corporation, WI, USA), 200 µM forward and reverse primers (515FY, 806rmod, gITS7, ITS1f, ITS4, TAReuk454FWD1 and TAReukREV3, IDT, IA, USA; ITS4-ill Sigma, MO, USA), 2 mM $MgCl_2$ (Promega

Corporation, WI, USA), 250 ng μl^{-1} BSA (New England Biolabs, MA, USA), 1x Colorless GoTaq Flexi Buffer (Promega Corporation, WI, USA), 0.025 U μl^{-1} GoTaq G2 Flexi DNA Polymerase (Promega Corpotation, WI, USA), made to volume with molecular biology grade water (Sigma, MO, USA). PCR template was 1 µl sample DNA, excluding the nested gITS7/ITS4 reaction, conducted with 1 µl of 1:100 dilution of the preceding PCR product. PCR conditions detailed Table 2.2.

PCR products were visually assessed on agarose gels for confirmation of product and to assess contamination. PCR products were purified using Agencout AMPure XP beads (Beckman Coulter, High Wycombe, UK); PCR product mixed in a 5:4 ratio with beads, washed 3 times with 80:20 ethanol: H_2O, and amplicons resuspended in H_2O. Purified PCR products were quantified using Quant-it dsDNA Assay Kit (Thermo Fisher, MA, USA), and either a BMG Labtech POLARstar OPTIMA or BMG Labtech Clariostar. PCR products were adjusted to 10 ng μl^{-1}. PCR products for each target pooled per sample in a ratio of 3:1:2:1 archaea:bacteria:eukaryotes:fungi. Library preparation and sequencing by Illumina MiSeq conducted by the University of York Technology Facility. 300 bp paired end reads were generated on an Illumina MiSeq platform.

Table 2.1 – PCR primer details. (Degenerate nucleotide codes: R = A,G ; Y = C,T; M = A,C ; K = G,T; S =C,G; W = A,T; V = A,C,G ; N = A,C,G,T)

Target	Region	Name	Sequence	Fragment Size	Reference
Archaea	16S	Arch349F	GYGCASCAGKCGMGAAW	457	Takai and Horikoshi (2000)
		Arch806R	GGACTACVSGGGTATCTAAT		
Bacteria	16S	515FY	GTGYCAGCMGCCGCGGTAA	291	Apprill et al. (2015), Parada et al. (2016)
		806rmod	GGACTACNVGGGTWTCTAAT		
Eukaryote	18S	TAReuk454FWD1	CCAGCASCYGCGGTAATTCC	ca. 460	Stoeck et al. (2010)
		TAReukREV3	ACTTTCGTTCTTGATYRA		
Fungi	ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	ca. 515-910	(White et al., 1990), Gardes and Burns (1993), Ihrmark et al. (2012)
		ITS4	TCCTCCGCTTATTGATATGC		
	ITS	gITS7	GTGARTCATCGARTCTTTG	ca. 370	
		ITS4	TCCTCCGCTTATTGATATGC		

Table 2.2 – PCR regimes

Primer Set	Initial denaturation	No. Cycles	Cycle details	Final extension
515FY / 806rmod	94 °C / 5 minutes	40	94 °C / 30 seconds 53 °C / 45 seconds 72 °C / 90 seconds	72 °C / 5 minutes
gITS7 / ITS4	95 °C / 5 minutes	25	94 °C / 30 seconds 55 °C / 45 seconds 72 °C / 90 seconds	72 °C / 10 minutes
ITS1F / ITS4	95 °C / 5 minutes	30	94 °C / 30 seconds 55 °C / 45 seconds 72 °C / 90 seconds	72 °C / 10 minutes
TAReuk454FWD1 / TAReukREV3	95 °C / 5 minutes	40	94 °C / 30 seconds 50 °C / 45 seconds 72 °C / 90 seconds	72 °C / 10 minutes

2.2.3 Data Analysis - Bioinformatics

Sequence data was received demultiplexed by sample as FASTQ files. Atropos (Didion et al., 2017), was first used to trim 13 bases from the forward reads containing indexing information. Atropos was run again on each of the files four times, trimming the paired end adapters from the relevant reads – those containing a specific primer pair – discarding untrimmed – non-target reads – to form files for each sample, containing reads associated with the desired primer pair. A minimum read length of 1 was specified.

Data was imported into Qiime 2 (version 2019.10) (Bolyen et al., 2018, Bolyen et al., 2019) .qza file format for analysis on a per target amplicon per run basis. Summaries were generated per target amplicon per run, and denoising parameters determined. DADA2 (Callahan et al., 2016) was used to denoise the data per target amplicon per run – note every instrument run should be denoised individually due to unique error profiles – varied DADA2 parameters detailed in Table 2.3, others default – maximum expected errors 2, chimera method consensus, minimum fold-parent-over-abundance 1. Parameters chosen to ensure sufficient overlap for paired end reads to be successfully merged, whilst discarding lower quality base calls at end of reads for archaea, bacteria and eukaryotes. A single end analysis for archaea was also carried out. For fungal ITS reads that are variable in length, read quality was used as the sole truncation criteria.

Table 2.3 – DADA2 parameters

Target Amplicon	Forward or Paired End	Forward read truncation length	Forward read truncation quality	Reverse read truncation length	Reverse read truncation quality
Archaea	Forward	250	2	NA	NA
	Paired End	265	2	215	2
Bacteria	Paired End	220	2	150	2
Eukaryotes	Paired End	220	2	200	2
Fungi	Paired End	NA	10	NA	10

Post denoising, data was pooled per target amplicon. Amplicon sequence variants (ASVs) were removed from frequency tables where total abundance of the ASV was <100 and/or where the ASV occurred in <3 samples. This was to remove spurious reads, and probable sequencing errors.

To assign a nominal taxonomic identity to the ASVs, classifiers were prepared based on reference databases – for archaea and bacteria, 16S data was imported into .qza format from the SILVA database release 132 (Pruesse et al., 2007, Bolyen et al., 2018, Bolyen et al., 2019), for eukaryotes SILVA release 132 18S reference data, and for fungi reference reads and taxonomy from UNITE 8.0 release (Nilsson et al., 2015). For each reference dataset, reads and reference taxonomic data were imported individually, for the most accurate possible assignment the complete dataset was used for fungi, whilst reads were extracted by primer sequence for archaea, bacteria and total eukaryotes, minimum and maximum lengths respectively constrained as 420/500, 250/330, 330/430. A naïve-Bayes classifier was trained on each reference dataset using the Qiime 2 operating on the default parameters (Pedregosa et al., 2011, Bolyen et al., 2018, Bolyen et al., 2019). The Qiime 2 (version 2019.10) hybrid taxonomic classification pipeline was used to assign the taxonomy – following a further filtering stage, VSEARCH was used to assign firstly exact match taxonomic classification, followed by a least common ancestor consensus assignment, sequence without an exact match were then assigned a taxonomy by the pretrained naïve-Bayes classifier. Classifier non default parameters – random seed set to 1 for reproducibility, confidence set to 0.95, prefilter identity 0.5 from a sample of 10000 reference reads. Reads not identified by the classification process to be within the target taxonomic domain – ie bacterial reads within archaeal dataset, or unclassifiable – to any taxonomic level – were discarded.

Note that following this process, only 13 ASVs of low abundance were identified within the archaeal dataset, with the majority being non-target bacterial data. This, and other issues identified

with the archaeal primers – amplification of nontarget 18 base pair long reads (Figure 2.3) – led to the discarding of the archaea dataset.

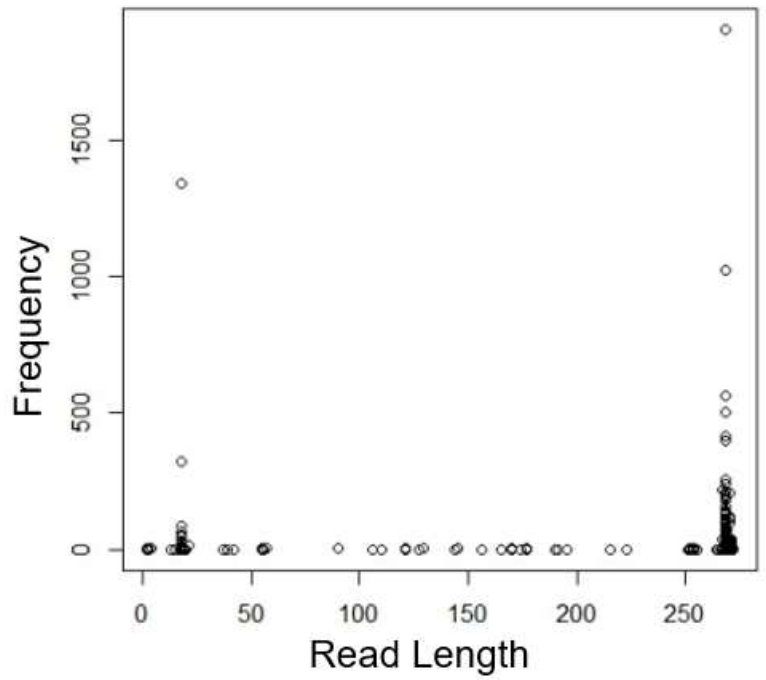


Figure 2.3 – Frequency of unique sequences relative to read length for archaea data

Table 2.4 details the normalization rates of the data, and the samples retained for each dataset. ASV – sample frequency tables were exported from Qiime 2 for analysis with R. Frequency tables with the data aggregated by assigned order, to compare the relative abundance of larger aggregations of data (conducted with R).

Table 2.4 – Data on normalization and retention of samples

	Normalization			Total Samples	Samples retained		
Study	Bacteria	Eukaryotes	Fungi		Bacteria	Eukaryotes	Fungi
SBH	15095	9572	9881	314	314	294	310

2.2.4 Data Analysis – Statistics

All statistical analysis were conducted using R (R_Core_Team, 2013). Analysis was conducted on both only IT and RT samples, and the complete dataset.

Detrending correspondence analysis (DCA) (Hill and Gauch, 1980) as implemented within the R package Vegan (Oksanen et al., 2018) – command decorana – was used to generate ordinations of Wisconsin double standardized ASVs abundance. All DCAs generated with 4 axes. Plots of ordinations, movement of a sample location by time within the ordination, pirate-plots (Phillips, 2018) of distance from a median centroid of data points calculated by field, treatment, sampling, and location type were generated, and heatmaps of the abundance of most determinant ASVs plotted. Pirate-plots show mean, highest density interval (95%), data distribution, and individual data points (Phillips, 2018).

Prior to statistically testing by PERMANOVA (permutational multivariate analysis of variance), the R function betadisper (implemented with the R package vegan), analogous to Levene's Test of equality of variance (Oksanen, 2015), was used to assess the homogeneity of dispersion of the sample-taxon table, by Field, Treatment, Location and Sampling. For all target communities, significant ($P<0.05$) heterogeneity of dispersion was observed, for both the complete and RT/IT datasets only. Anderson and Walsh (2013) suggest that where experimental design is balanced, PERMANOVA remains a reliable test where the data are heterogeneous in dispersion. Therefore, PERMANOVA is an acceptable method for the analysis of the RT/IT dataset. For the complete dataset, PERMANOVA is less sensitive to the over dispersion of the data than either ANOSIM or the Mantel test (Anderson and Walsh, 2013). Therefore, the results of PERMANOVA testing are presented for all communities for both the complete and IT/RT dataset, but results close to the limit of statistical significance should be interpreted with care. PERMANOVA was conducted using the adonis function in the R package vegan (Oksanen et al., 2018), using the Bray-Curtis method (Bray and Curtis, 1957) to generate the dissimilarity indices. All PERMANOVA analysis conducted with 10000 permutations.

ASV richness and Simpson's Index of diversity (Simpson, 1949) (as implemented in vegan command 'diversity', 'index=simpson') are calculated. Relative abundance of aggregated assigned taxa processed in Qiime 2 (Bolyen et al., 2018, Bolyen et al., 2019) were aggregated (mean taken) by metadata categories, with the ten most abundant taxonomic groups retained alongside 'Other' – the sum of all of taxonomic groupings. Relative abundance as a percentage was calculated, and plotted.

2.3 Results

2.3.1 Analysis of cultivated soils

Considering only cultivated soils – from the IT and RT – strips of the SoilBioHedge experiment:

For bacteria, PERMANOVA revealed significant effects of field, sampling and location (DF=2 F=45 P<0.001, DF=4 F=4.9 P<0.001, DF=8 F=3.7 P<0.001). Tillage method did not have a significant effect on bacterial community composition (DF=1 F=1.5 P>0.1). Field is the most determinant variable of bacterial community composition accounting for 34 % variance (as percentage of total sum of squares), with treatment, sampling and location accounting for 0.5 %, 7.4 % and 5.6 % respectively (residuals 52 %, all to 2 significant figures). Figures 2.4A and 2.4B show the DCA ordinations of the bacterial community composition grouped by field and treatment respectively – the dominant effect of field, and the minimal effect of treatment can be noted. Median distance from the centroid of the group for treatment and location within a field reveal bacterial communities within RT soils to be marginally more variable in composition than under IT (Figure 2.5), and for variability within community composition to decrease with distance from the field boundary (Figure 2.6). Figures 2.7 and 2.8 show relative abundance of taxonomic groups of bacteria grouped by field and treatment respectively – in aggregate, treatment can be observed to have a minimal effect upon the relative abundance of the ten most commonly observed taxonomic groupings (Figure 2.8), whilst Paddock can be observed to host relatively more *Chthoniobacteraceae* and fewer *Chloroflexi* than Valey and Warren (Figure 2.7).

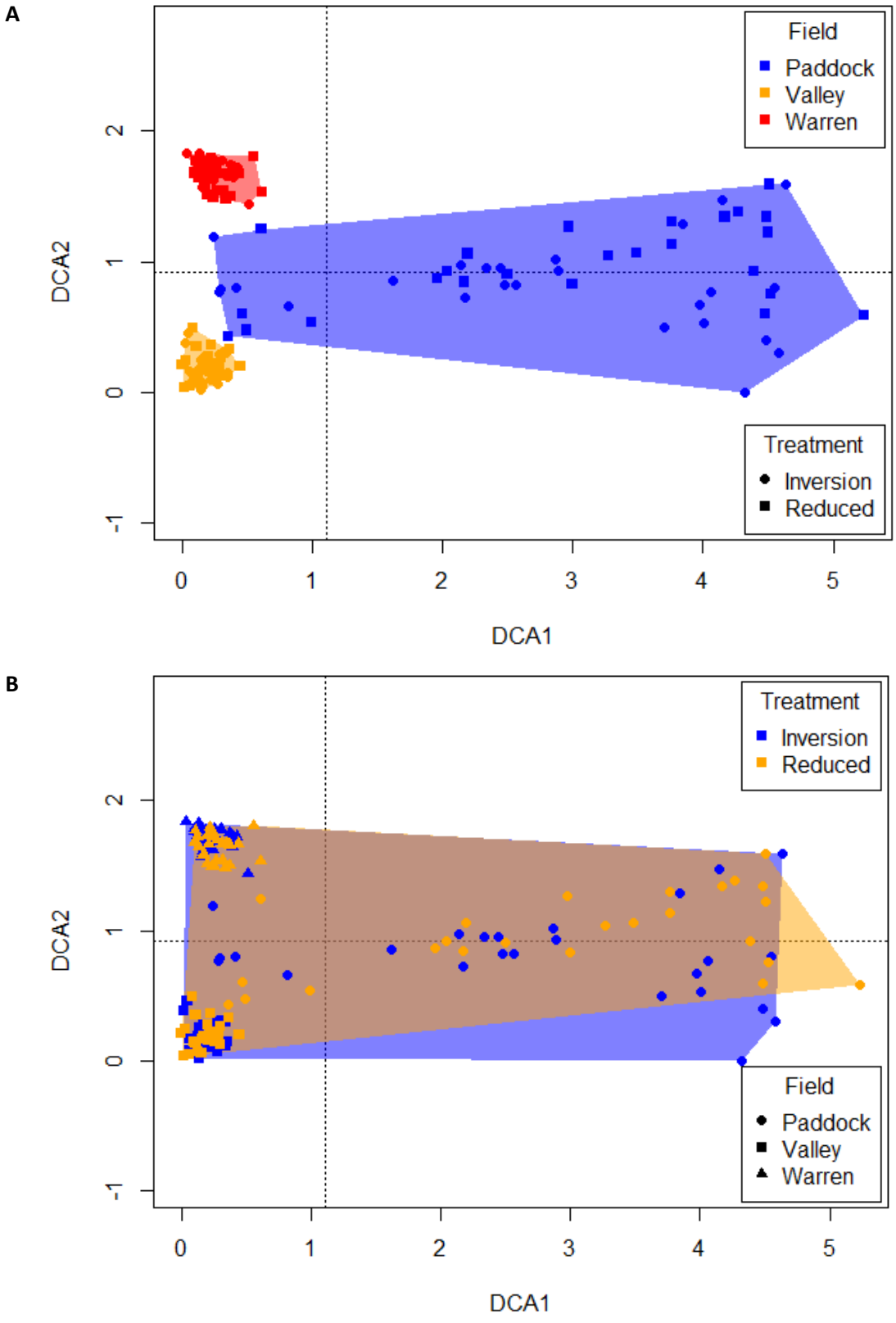


Figure 2.4 – DCA sample scores (axis 1 and 2) for bacterial community from IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from IT (blue), and RT (yellow).

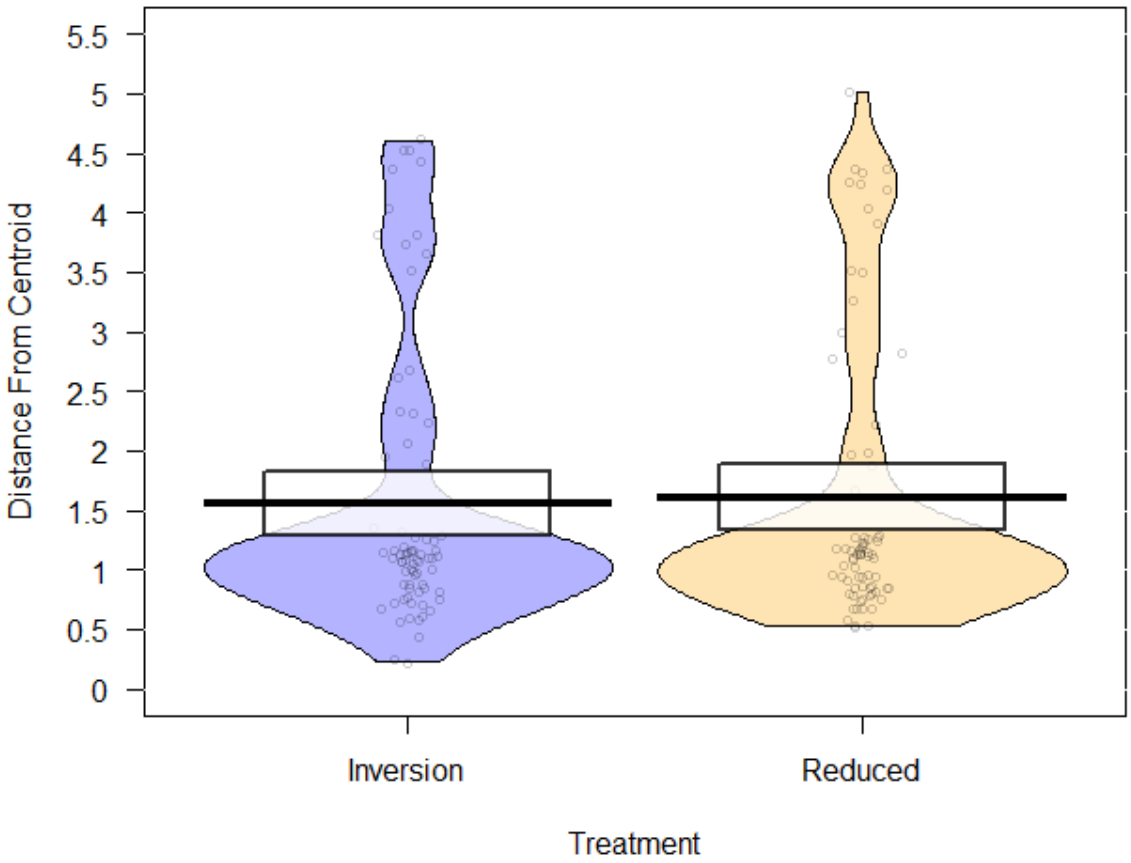


Figure 2.5 – Distance from median sample score (across all 4 DCA axis) for bacterial composition in soils under IT (blue) and RT (yellow).

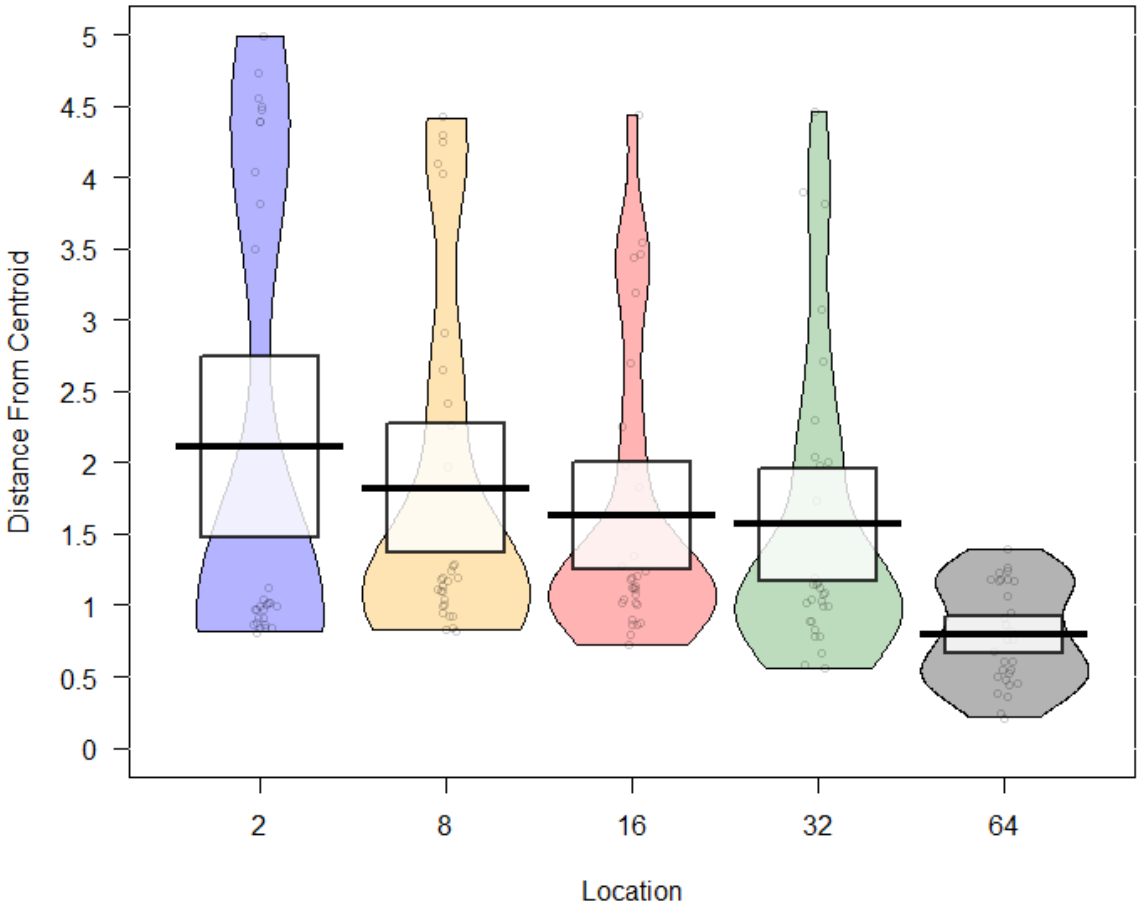


Figure 2.6 - Distance from median sample score (across all 4 DCA axis) for bacterial composition in soils at distances (in meters) from field boundary.

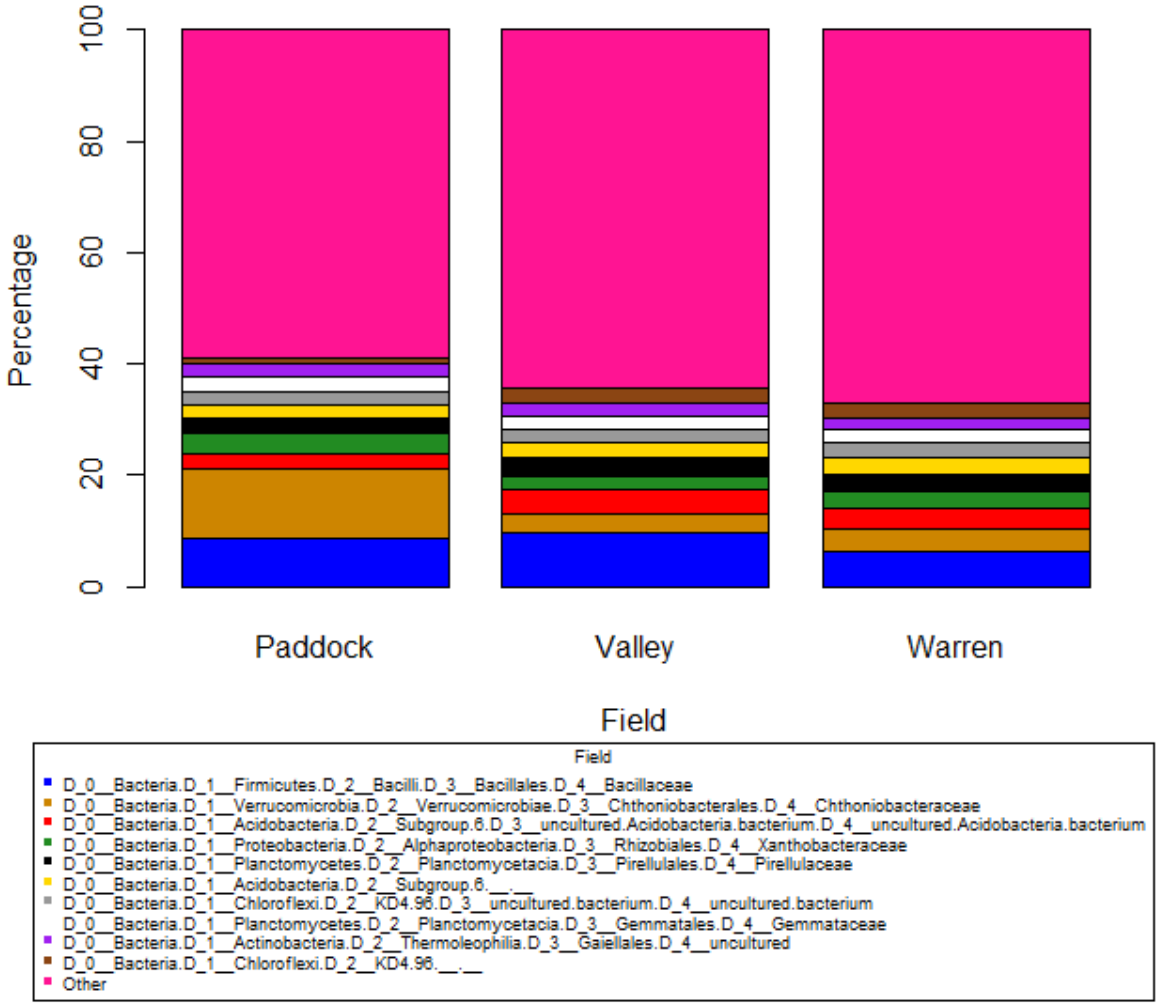


Figure 2.7 – Bacterial community composition in soils under IT and RT. Grouped by field.

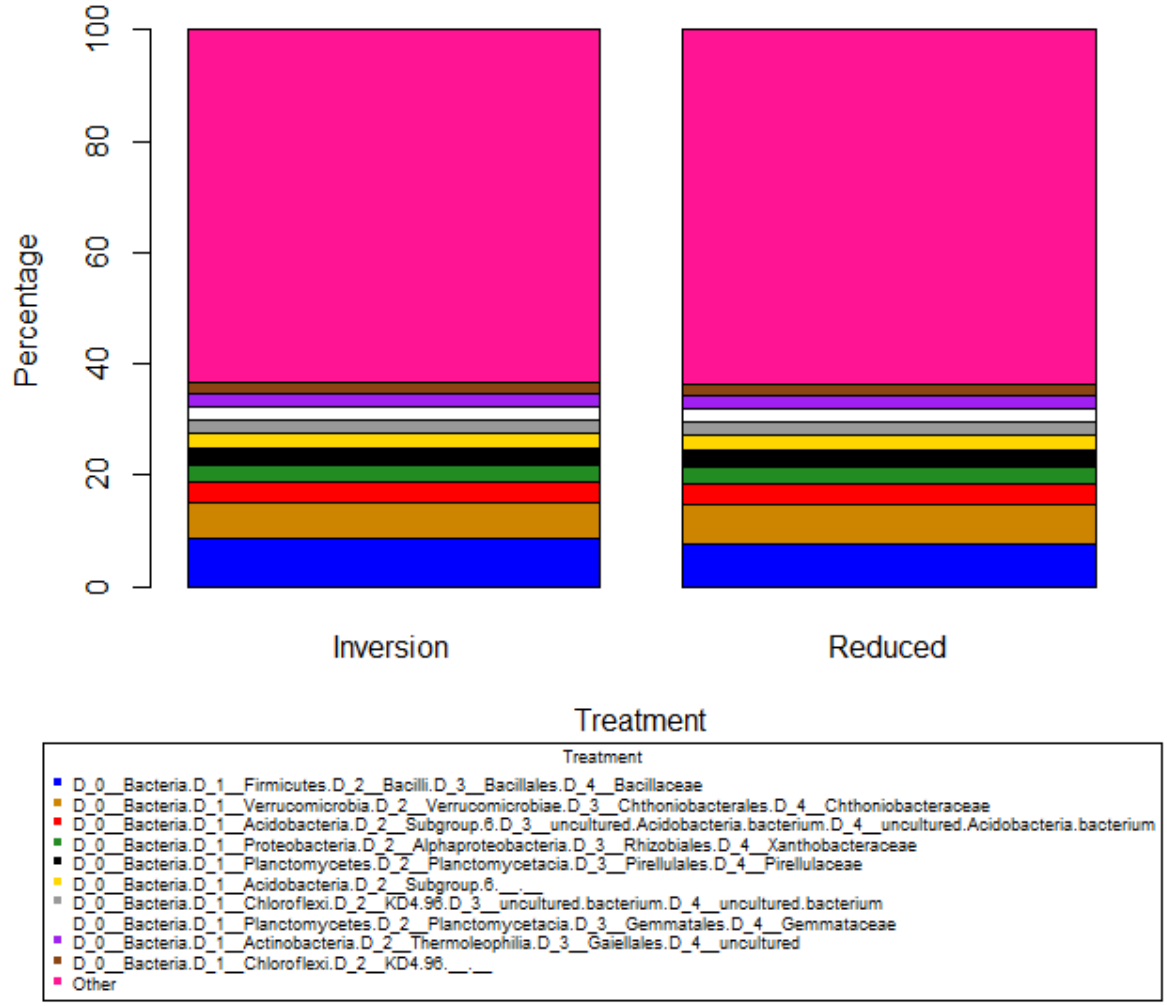


Figure 2.8 – Bacterial community composition in soils under IT and RT. Grouped by treatment.

For total eukaryotes, PERMANOVA revealed significant effects of field, treatment, sampling and location (DF=2 F=15 P<0.001, DF1= F=1.9 P<0.01, DF=4 F=4.8 P<0.001, DF=8 F=1.9 P<0.001 respectively). Field is the most determinant variable of eukaryote community composition accounting for 17 % variance (as percentage of total sum of squares), with treatment, sampling and location accounting for 1.0 %, 1.0 % and 4.1 % respectively (residuals 58 %, all to 2 significant figures). Figures 2.9A and 2.9B show the DCA ordinations of the eukaryotic community composition grouped by field and treatment respectively – the dominant effect of field, and the minimal effect of treatment can be noted. Median distance from the centroid of the group for treatment reveal eukaryotic communities furthest from field boundaries (64 m) are less variable than those closer to the field boundary (≤32 m) (Figure 10), and within RT soils to be marginally more variable in composition than under IT (Figure 2.11). Figures 2.12 and 2.13 show relative abundance of taxonomic groups of eukaryotes grouped by field and treatment respectively, with the most ten

most common taxonomic groups substantially more represented within Valley than Warren, where *Charophyta poales* has a greater relative abundance than within Paddock and Valley (Figure 2.12). Similarly, *Charophyta poales* have a greater relative abundance within RT plots than IT.

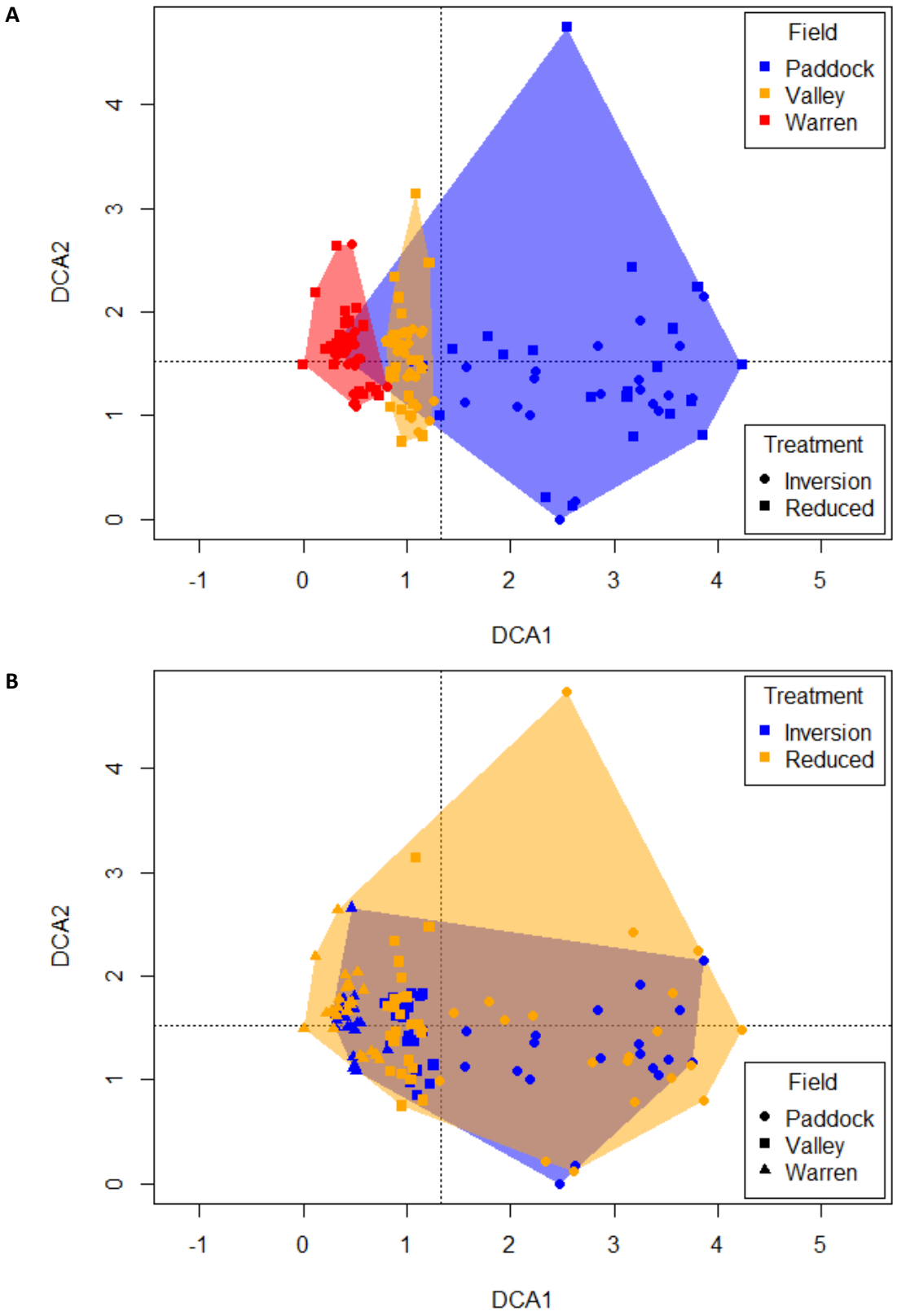


Figure 2.9 – DCA sample scores (axis 1 and 2) for eukaryotic community from IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from IT (blue), and RT (yellow).

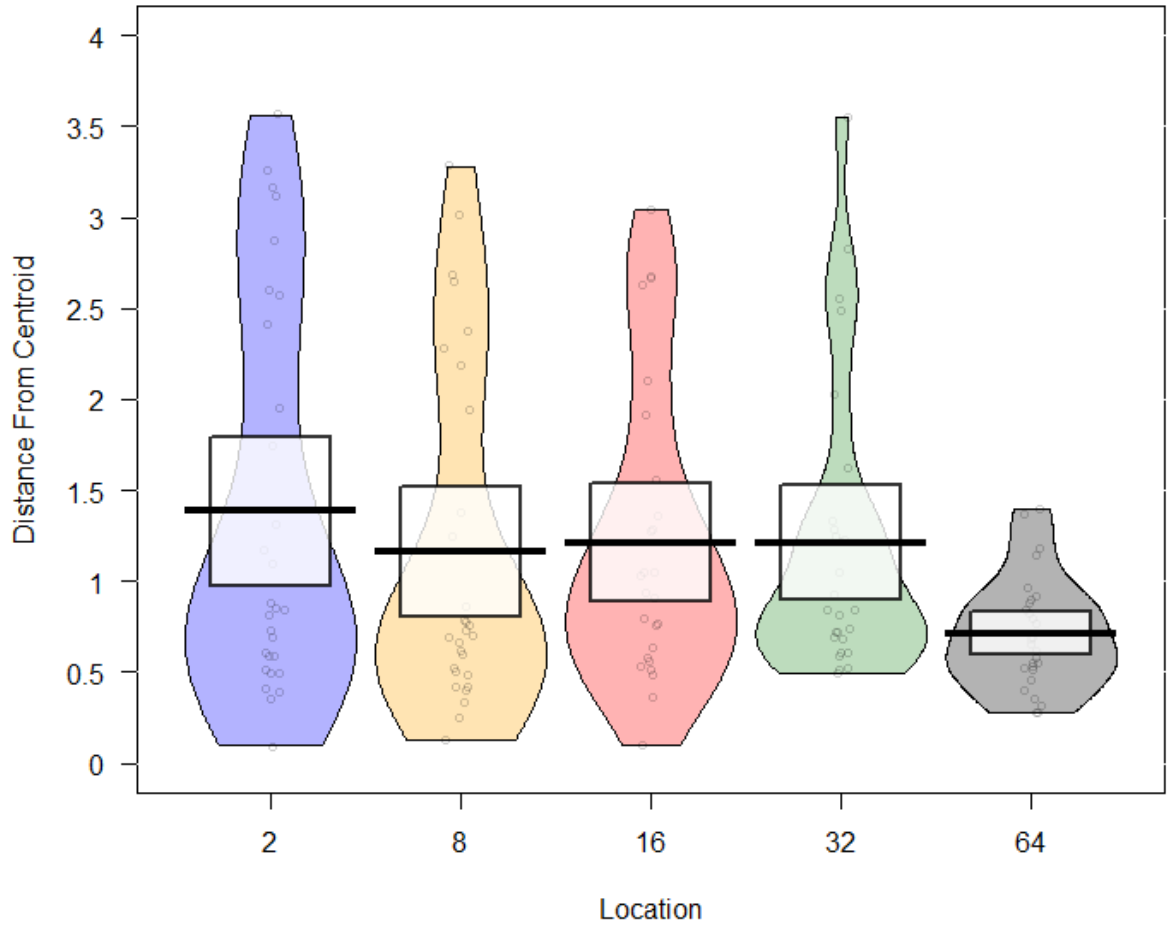


Figure 2.10 - Distance from median sample score (across all 4 DCA axis) for eukaryotic composition in soils at distances (in meters) from field boundary.

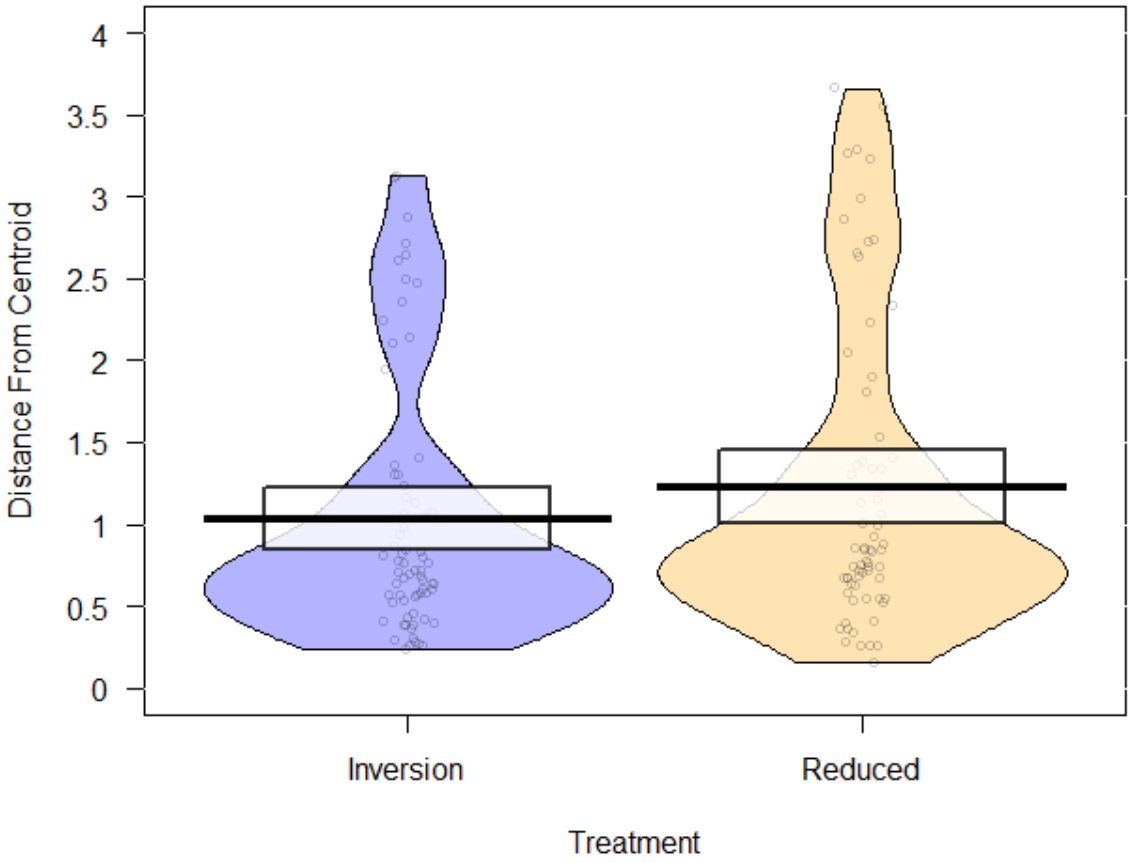


Figure 2.11 – Distance from median sample score (across all 4 DCA axis) for eukaryotic composition in soils under IT (blue) and RT (yellow).

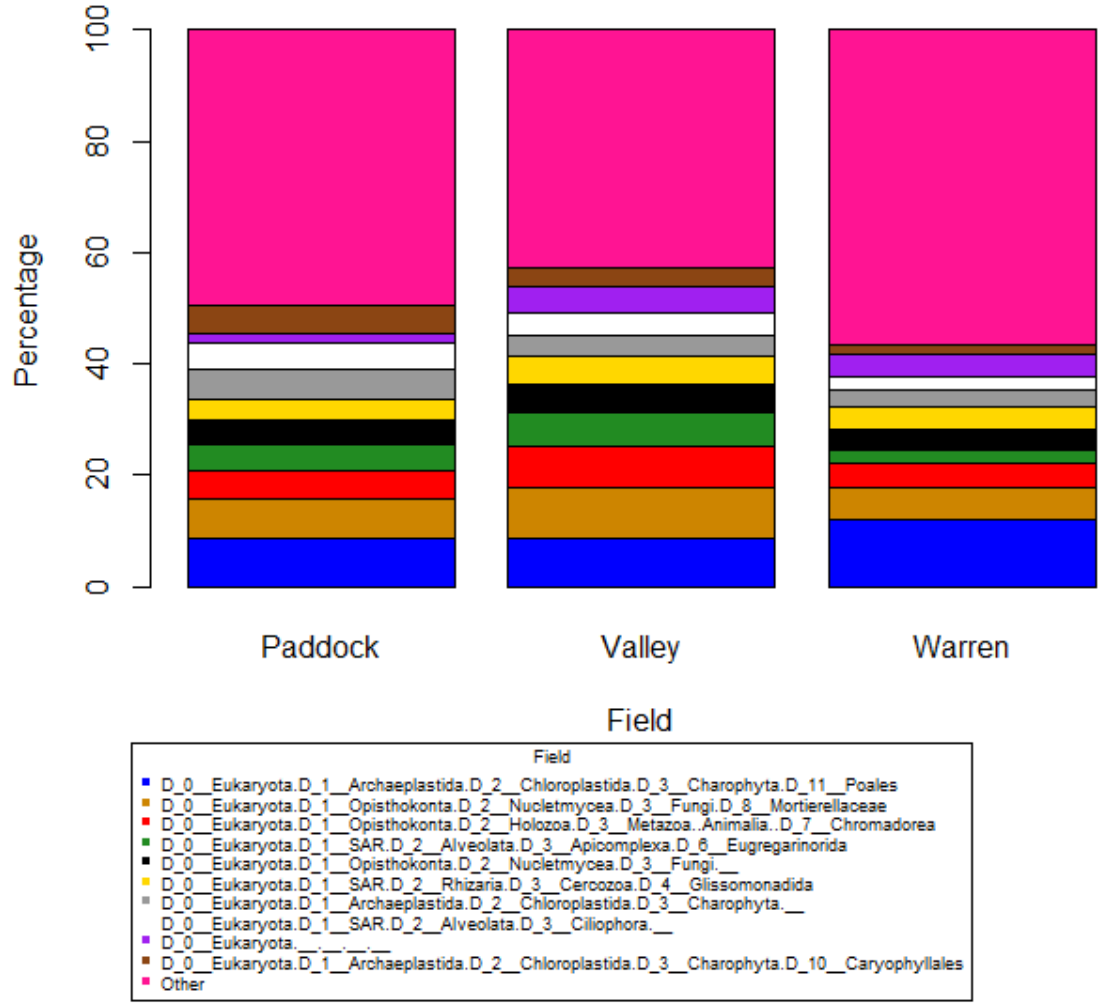


Figure 2.12 – Eukaryotic community composition in soils under IT and RT. Grouped by field.

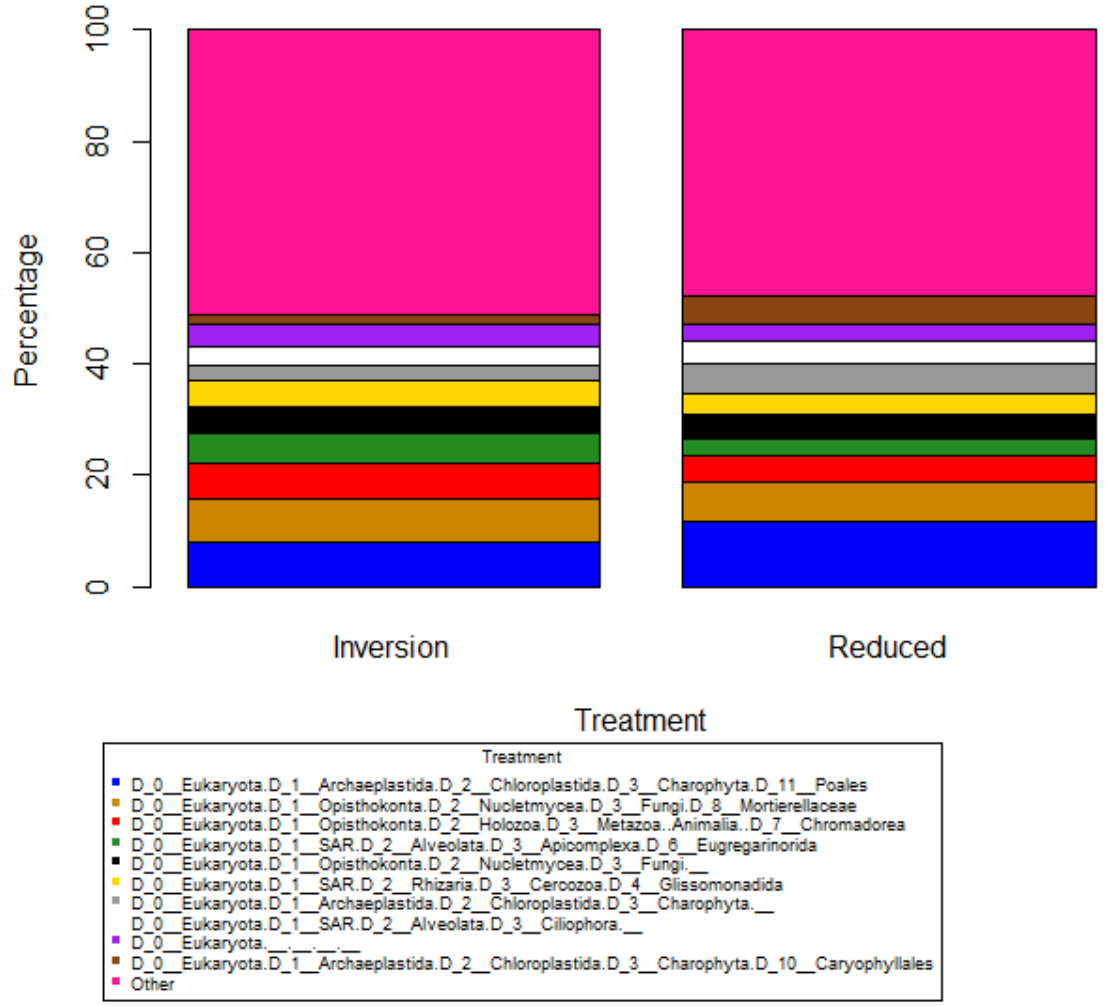


Figure 2.13 – Eukaryotic community composition in soils under IT and RT. Grouped by treatment.

For fungi, PERMANOVA revealed significant effects of field, sampling and location (DF=2 F=24 P<0.001, DF=4 F=4.5 P<0.001, DF=8 F=2.7 P<0.001). Tillage method did not have a significant effect on fungal community composition (DF=1 F=1.4 P>0.1). Field is the most determinant variable of fungal community composition accounting for 22 % variance (as percentage of total sum of squares), with treatment, sampling and location accounting for 0.6 %, 8.4 % and 5.0 % respectively (residuals 64 %, all to 2 significant figures). Figures 2.14A and 2.14B show the DCA ordinations of the fungal community composition grouped by field and treatment respectively – the dominant effect of field, and the minimal effect of treatment can be noted. Median distance from the centroid of the group, for location within a field reveal fungal community composition further from a field margin to be less variable than those close to field boundaries (Figure 2.15), and for treatment show composition under RT to be more variable than under IT (Figure 2.16). Figures 2.17 and 2.18 show relative abundance of taxonomic groups of fungi grouped by field and treatment respectively. When

aggregated by treatment, few difference can be observed between the differently tilled areas (Figure 2.18), suggesting that at environmentally relevant scales (ie. Landscape or catchment scale) difference between smaller plots (fields or sections thereof) may be negated. Where individual fields are considered (Figure 2.17), *Ascomycota* are generally dominant within fungal community, being 9 of the 10 most common taxonomic groupings. Within which *Plectosphaerellaceae, Nectriaceae, Chaetomiaceae* and *Microasceae* are have higher relative abundance in Warren field, relative to Paddock and Valley, where *Herpotrichiellaceae* and *Sporomiaceae* are relatively more abundant.

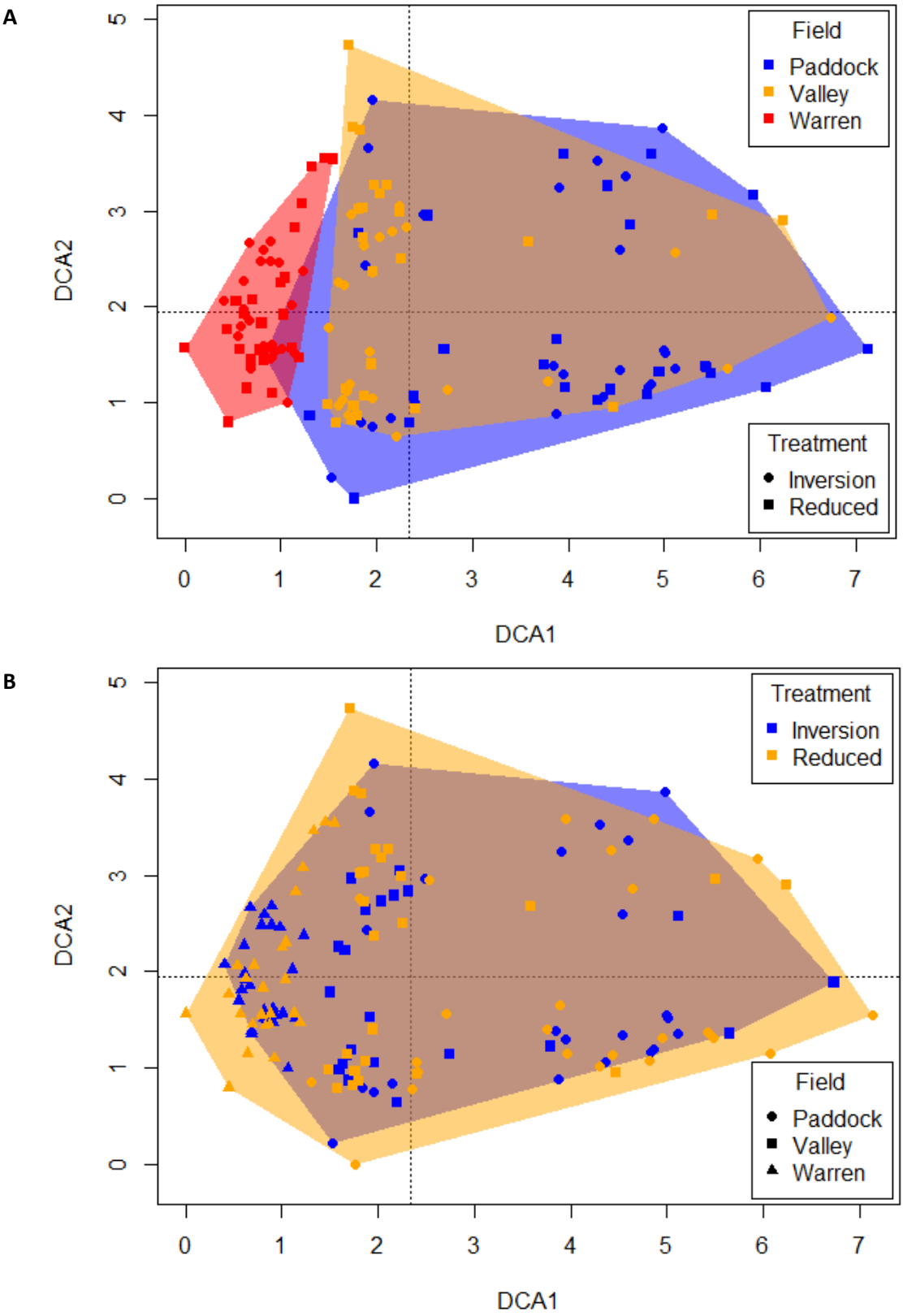


Figure 2.14 – DCA sample scores (axis 1 and 2) for fungal community from IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from IT (blue), and RT (yellow).

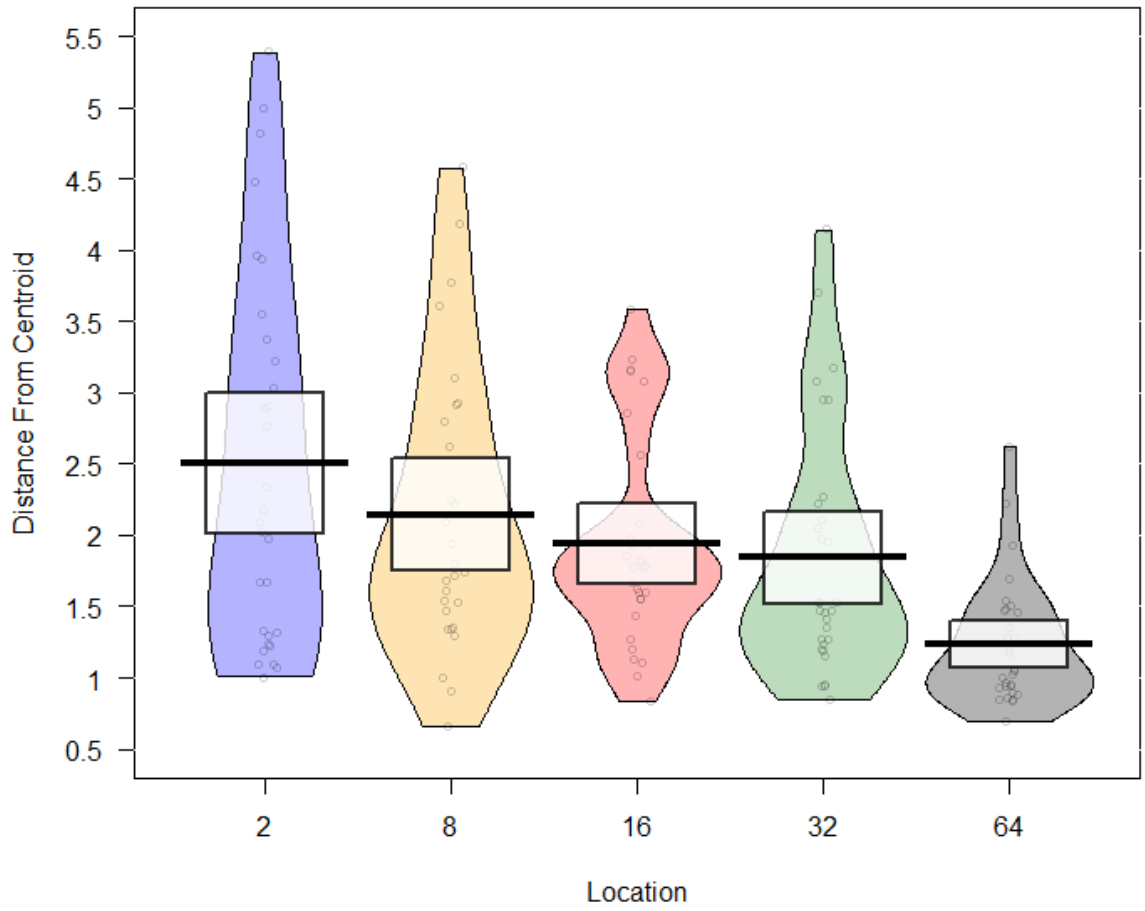


Figure 2.15 - Distance from median sample score (across all 4 DCA axis) for fungal composition in soils at distances (in meters) from field boundary.

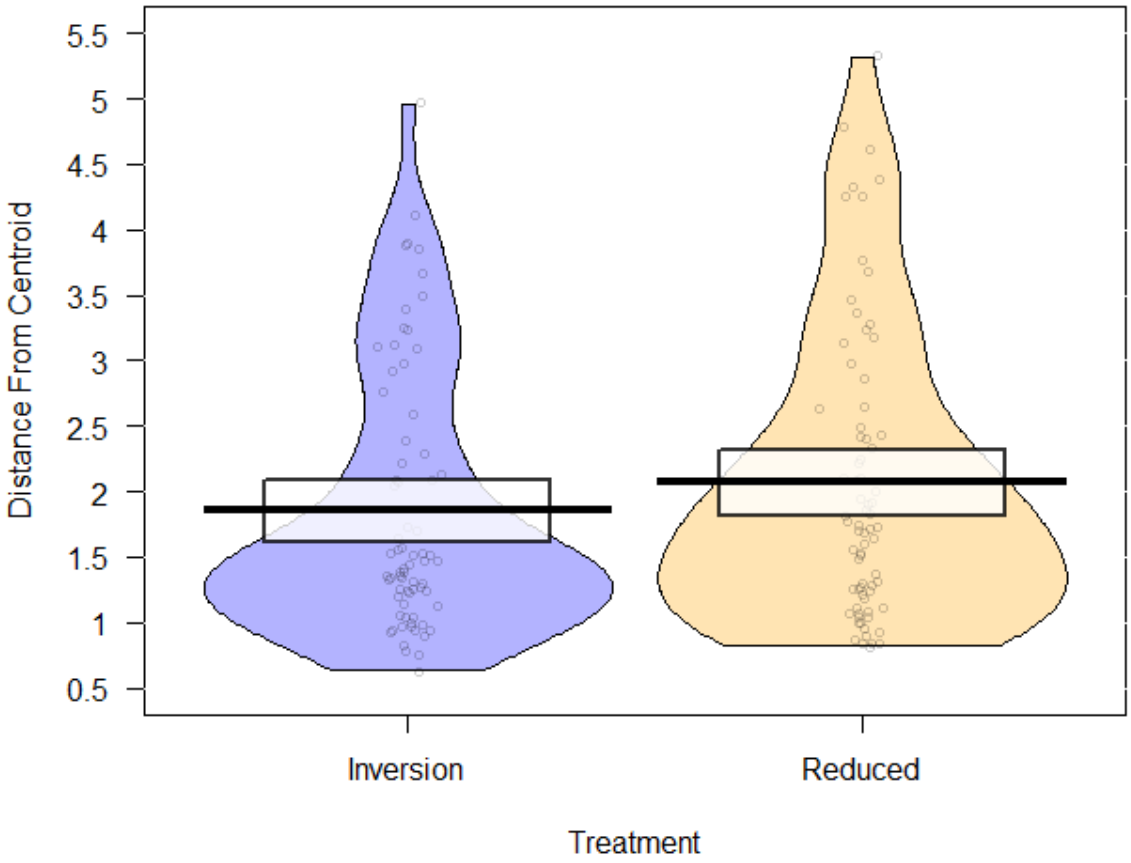


Figure 2.16 – Distance from median sample score (across all 4 DCA axis) for fungal composition in soils under IT (blue) and RT (yellow).

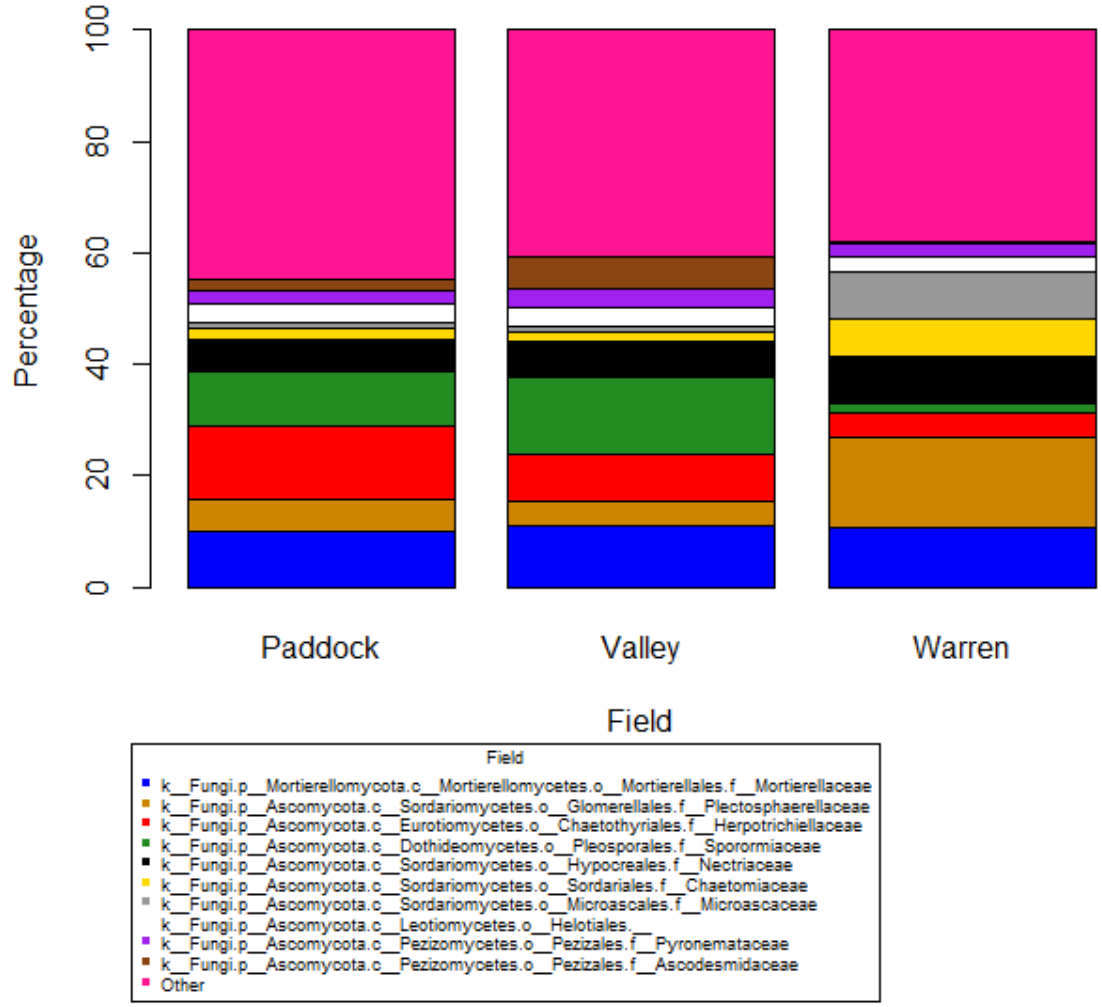


Figure 2.17 – Fungal community composition in soils under IT and RT. Grouped by field.

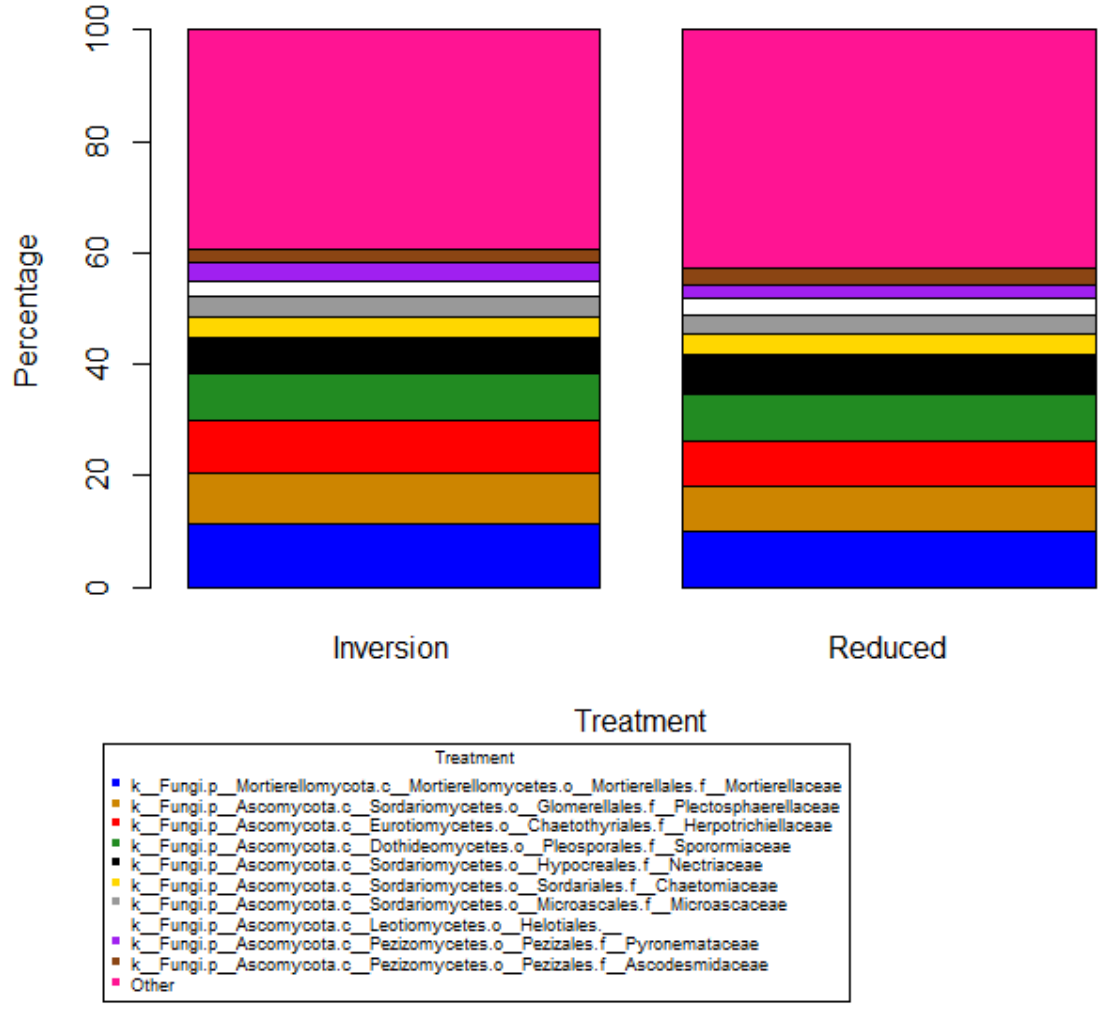


Figure 2.18 – Fungal community composition in soils under IT and RT. Grouped by treatment.

2.3.2 Analysis of all soil types

Considering soils from all areas – hedge, margin, pasture, IT and RT – of Paddock, Valley and Warren fields:

For bacterial community composition, PERMANOVA revealed significant effects of field, treatment, sampling and location (DF=2 F=69 P<0.001, DF=4 F=11 P<0.001, DF=4 F=9.2 P<0.001, DF=12 F=2.2 P<0.001 respectively). Field is the most determinant variable of bacterial community composition accounting for 26 % variance (as percentage of total sum of squares), with treatment, sampling and location accounting for 8.1 %, 6.9 % and 5.0 % respectively (residuals 54 %, all to 2 significant figures). Figures 2.19A and 2.19B show the DCA ordinations of the bacterial community composition grouped by field and treatment respectively – the dominant effect of field, and lower (on average) variability amongst samples from hedgerows than other treatment types (see also Figure 2.20). Figure 2.20 also show that variability within bacterial communities decreases with

distance into the field, with communities at 64m from hedgerows being substantially more homogenous than those ≤32 m of the field boundary. Figures 2.21 and 2.22 show relative abundance of taxonomic groups of bacteria grouped by field and treatment respectively, hedgerows host fewer of the ten most common taxonomic groups, and in particular few *Bacillaceae*, aggregated, the short-term RT and IT tillage plots can be observed to host similar bacterial communities to the untilled pastures (Figure 2.22). Between fields, Paddock contains a greater relative abundance of *Chthoniobacteraceae* than Valley and Warren (Figure 2.21). Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 2.23, hedges having lower ASV richness than infield communities, but greater diversity, due to greater evenness of ASVs.

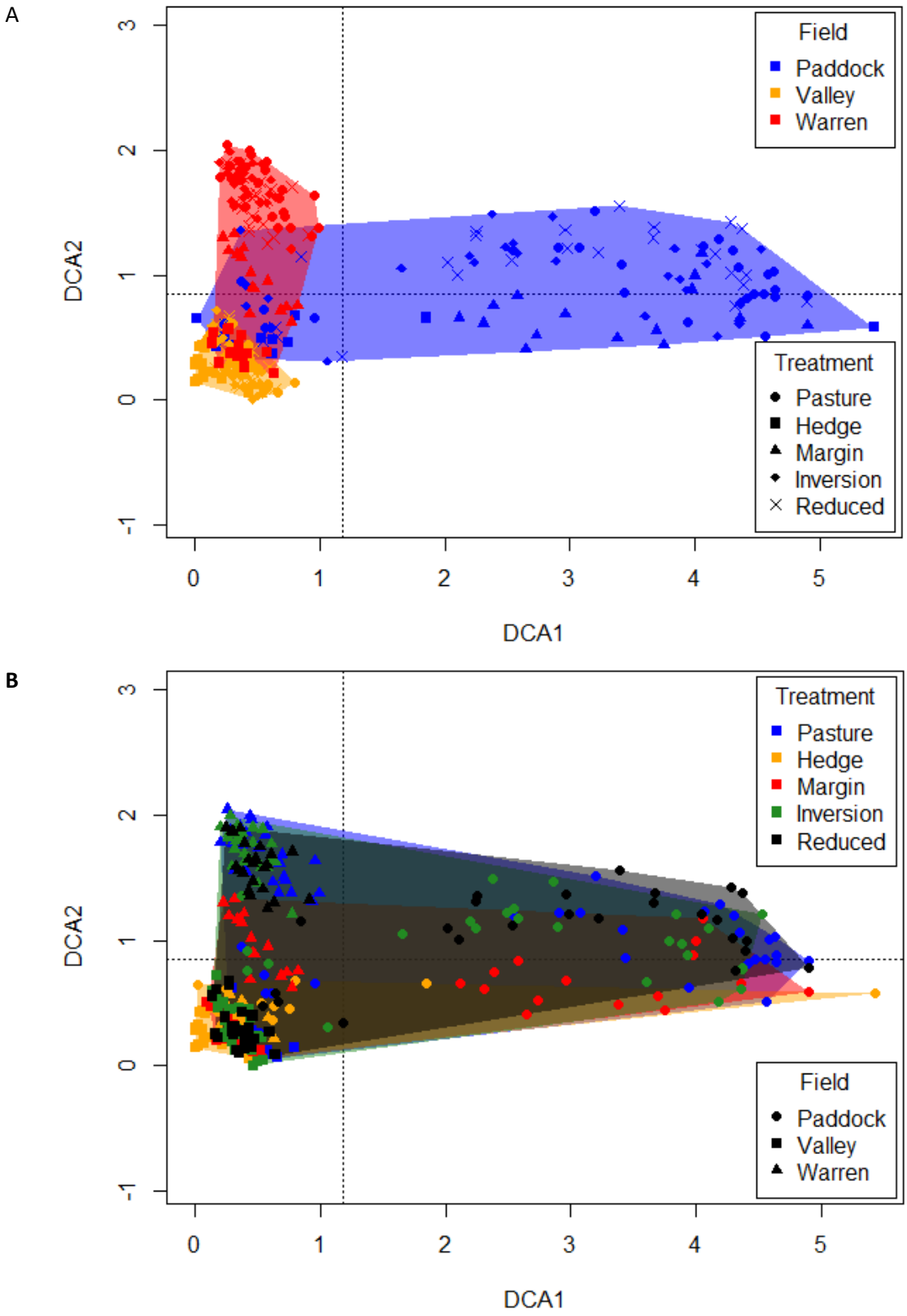


Figure 2.19 – DCA sample scores (axis 1 and 2) for bacterial community from hedge, margin, pasture, and IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from pasture (blue), margin (red), IT (green), RT (black) and hedge (yellow).

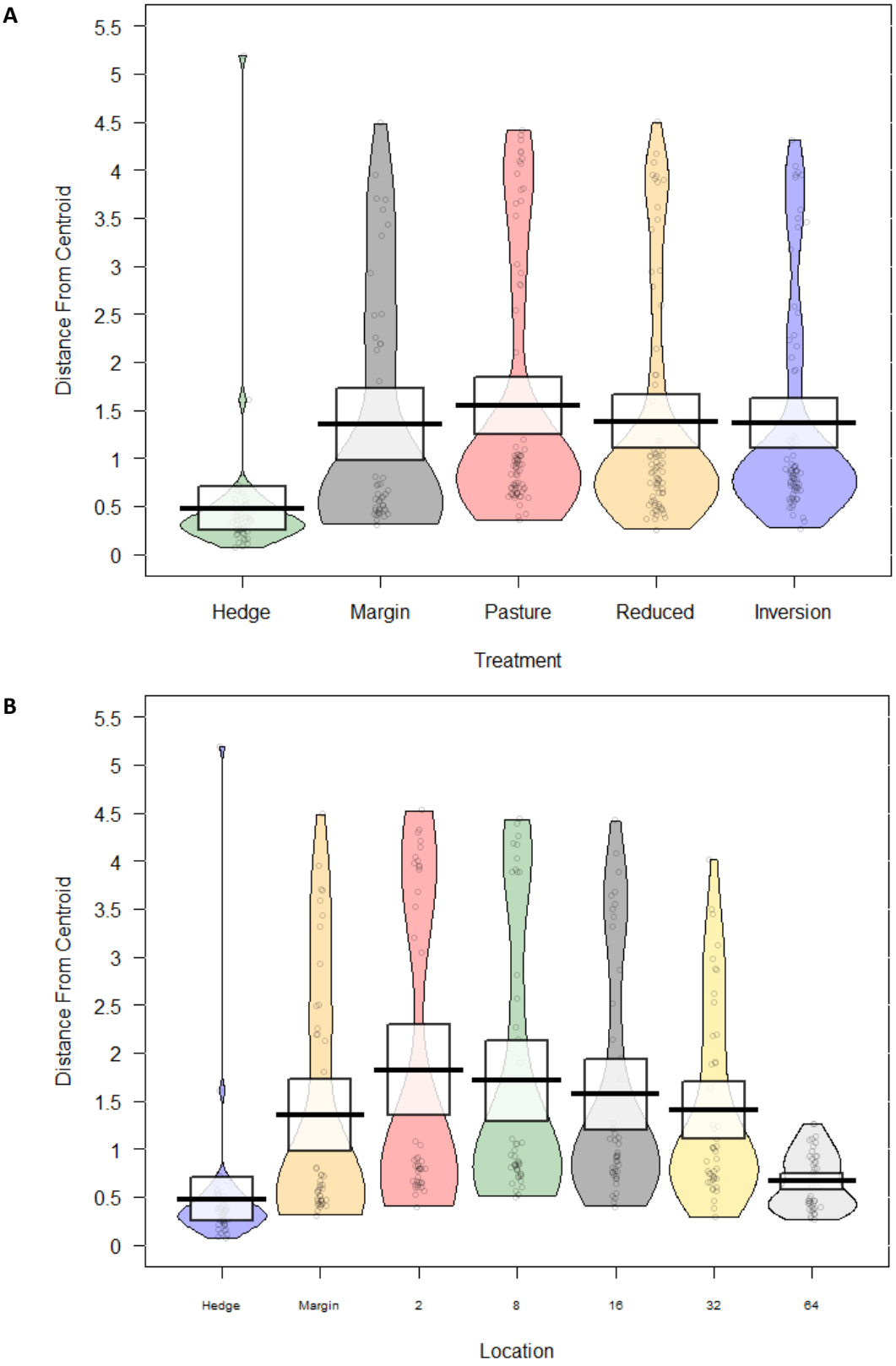


Figure 2.20 – Distance from median sample score (across all 4 DCA axis) for bacterial composition in soils from hedgerows, margin, pasture, RT, and IT. (A) Samples grouped by treatment (hedgerows (green), margin (black), pasture (red), RT (yellow) and IT (blue)), and (B) samples grouped by location in field (hedge (blue), margin (orange), 2 m (red), 8 m (green), 16 m (black), 32 m (yellow) and 64 m (grey).

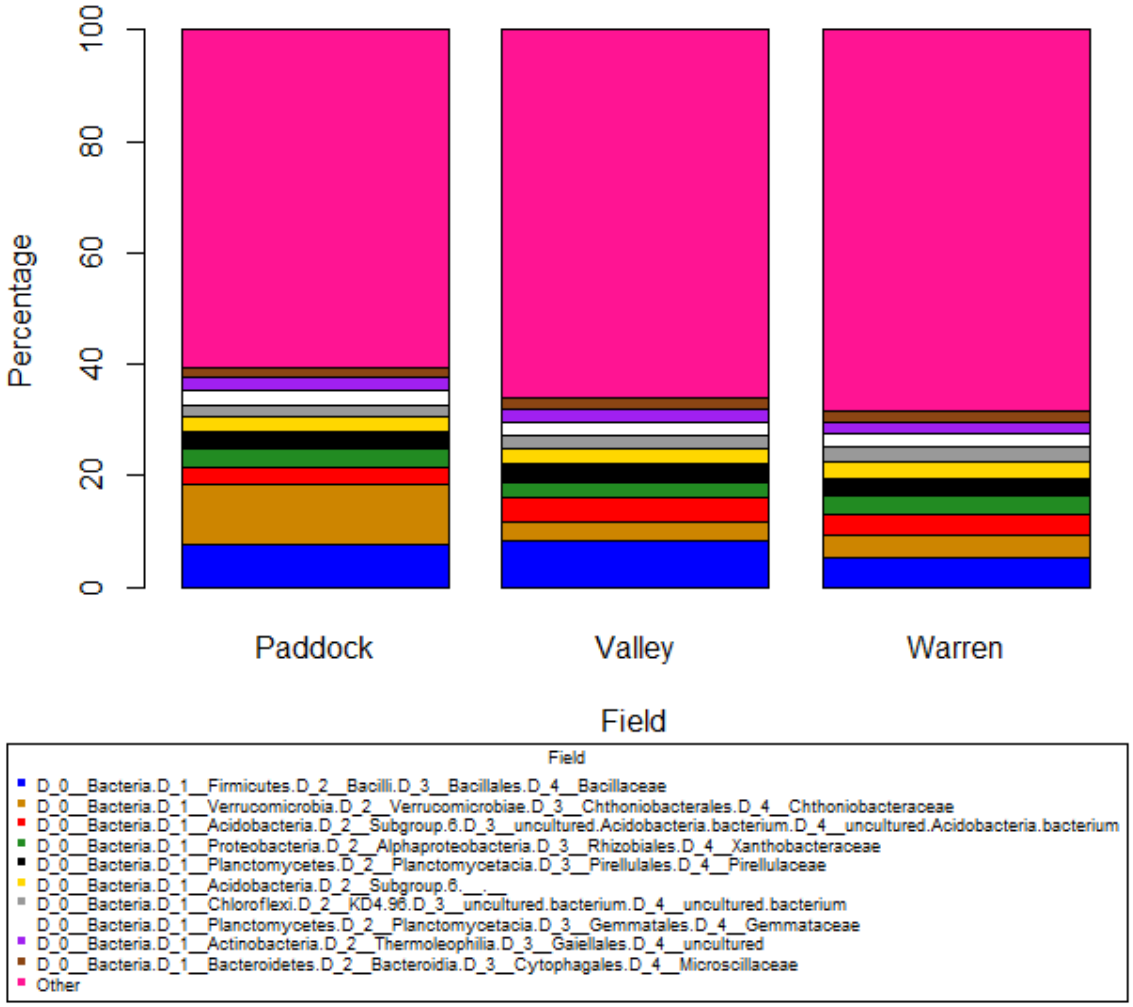


Figure 2.21 – Soil bacteria community composition, grouped by field.

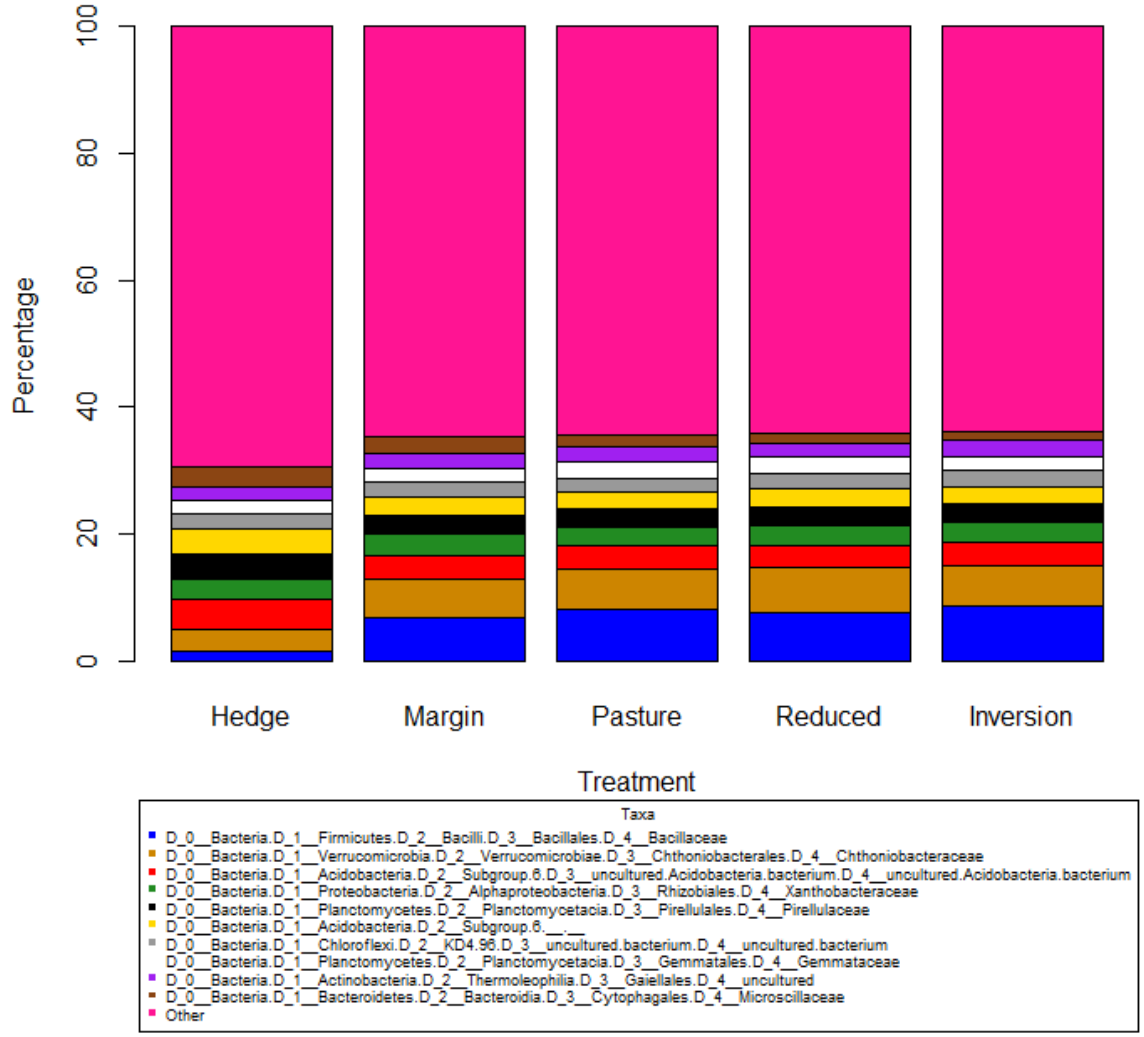


Figure 2.22 – Soil bacteria community composition, grouped by treatment.

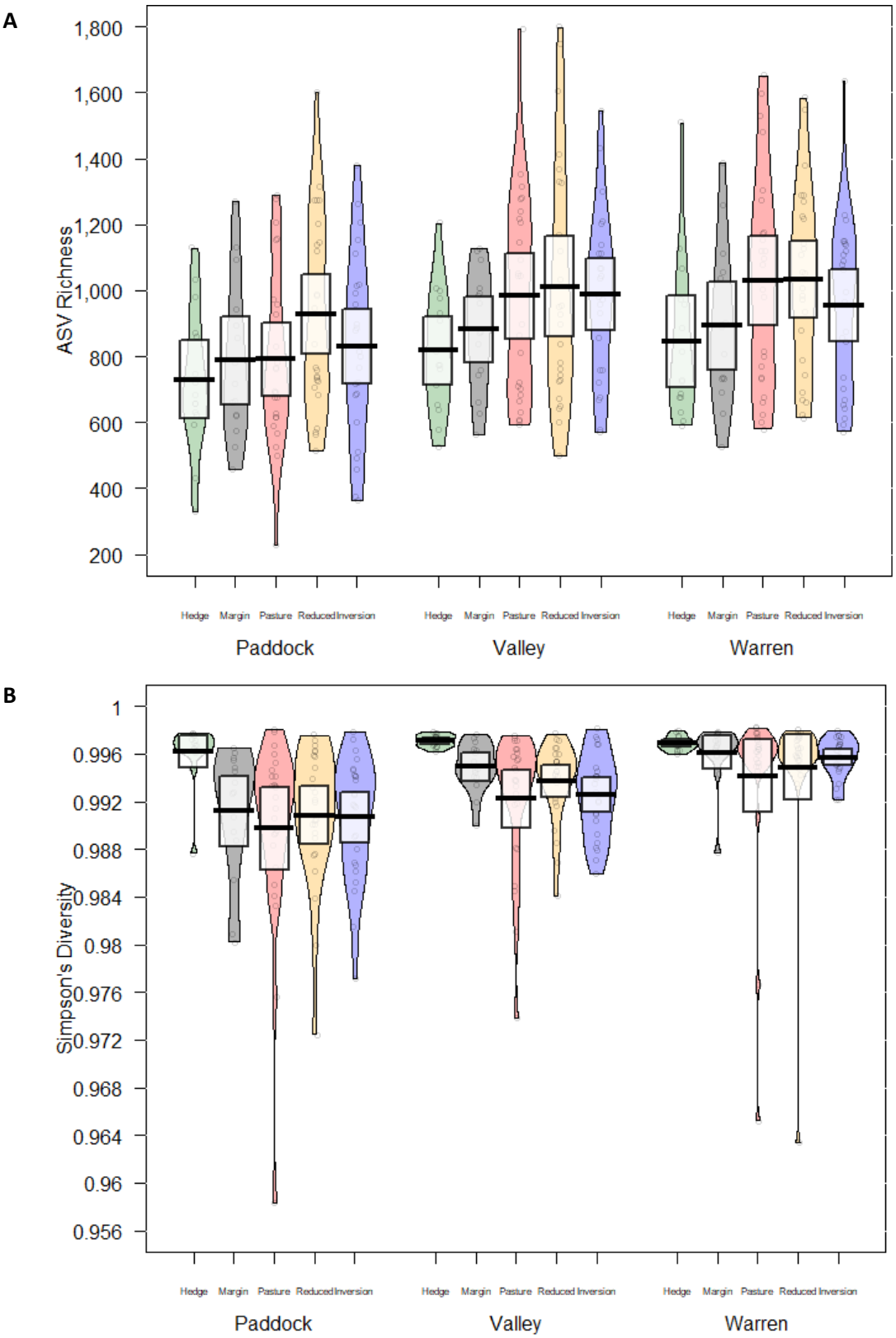


Figure 2.23 – (A) Bacterial ASV richness per sample. (B) Bacterial diversity per sample. Hedge (green), margin (black), pasture (red), RT (orange) and IT (blue).

For eukaryote community composition, PERMANOVA revealed significant effects of field, treatment, sampling and location (DF=2 F=21 P<0.001, DF=4 F=16 P<0.001, DF=4 F=5.9 P<0.001, DF=12 F=1.3 P<0.001 respectively). Treatment is the most determinant variable of eukaryote community composition accounting for 15 % variance (as percentage of total sum of squares), with field, sampling and location accounting for 10 %, 5.7 % and 3.7 % respectively (residuals 65 %, all to 2 significant figures). Figures 2.24A and 2.24B show the DCA ordinations of the eukaryotic community composition grouped by field and treatment respectively – the dominant effect of field, and the distinctness of soils from hedgerows is apparent (see also Figure 2.25). Figures 2.26 and 2.27 show relative abundance of taxonomic groups of eukaryotes grouped by field and treatment respectively, aggregated by field, the results are broadly similar (Figure 2.26), however a significant treatment effects separating tilled and untilled soils is apparent in Figure 2.27). All infield (pasture, RT and IT plots) are distinct from hedgerows and field margins by greater relative abundance of *Poales,* and tilled plots are then further distinguished from the pasture by substantially lower relative abundances of *Charophyta*. This difference in relative abundance is accounted for by growth of the 'other' (non-top-ten most common) taxa. Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 2.28, with both richness and diversity broadly similar, despite the differences in relative abundance described in Figures 2.26 and 2.27.

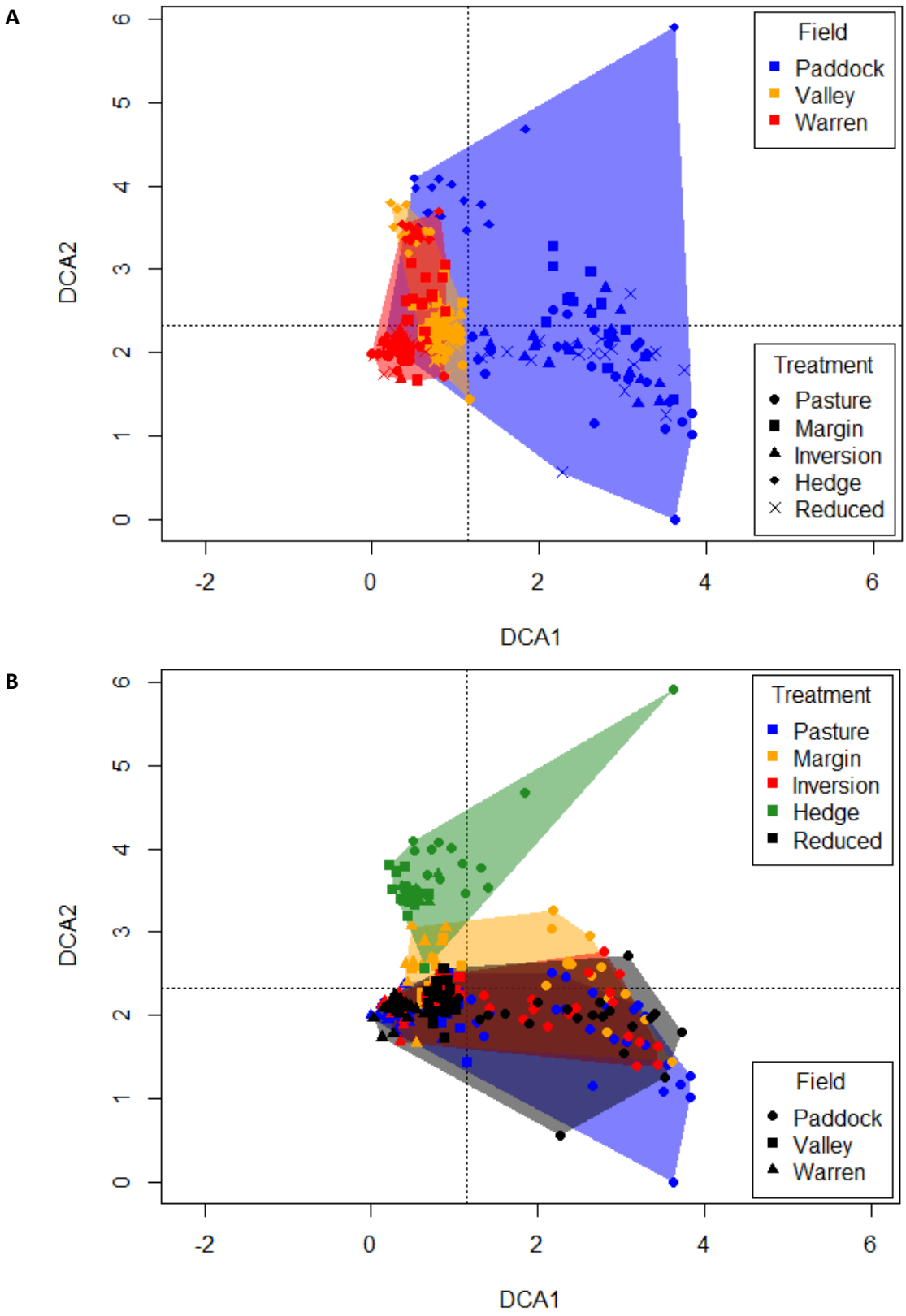


Figure 2.24 – DCA sample scores (axis 1 and 2) for eukaryote community from hedge, margin, pasture, and IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from pasture (blue), margin (red), IT (green), RT (black) and hedge (yellow).

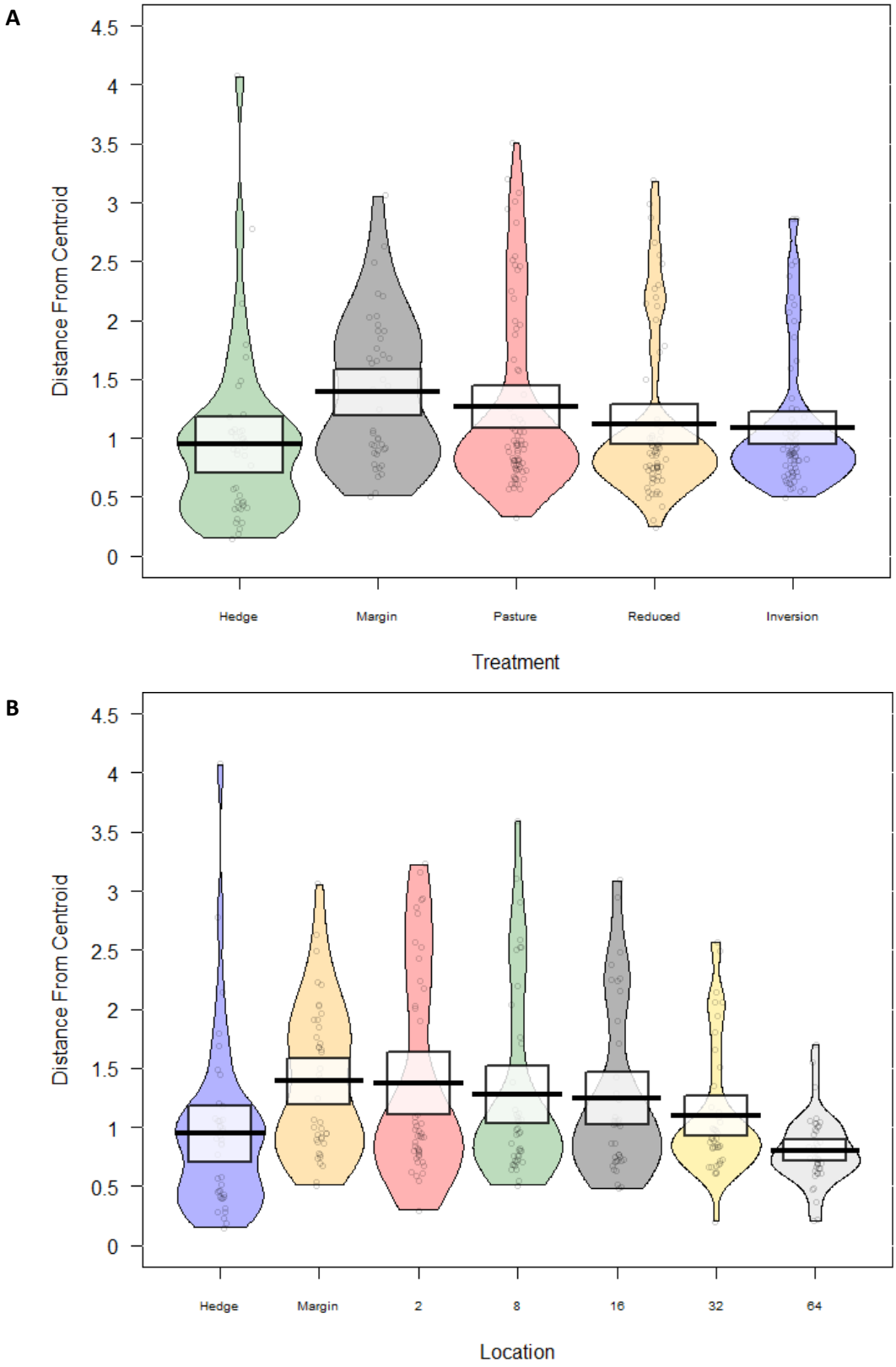


Figure 2.25 – Distance from median sample score (across all 4 DCA axis) for eukaryote composition in soils from hedgerows, margin, pasture, RT, and IT. (A) Samples grouped by treatment (hedgerows (green), margin (black), pasture (red), RT (yellow) and IT (blue)), and (B) samples grouped by location in field (hedge (blue), margin (orange), 2 m (red), 8 m (green), 16 m (black), 32 m (yellow) and 64 m (grey).

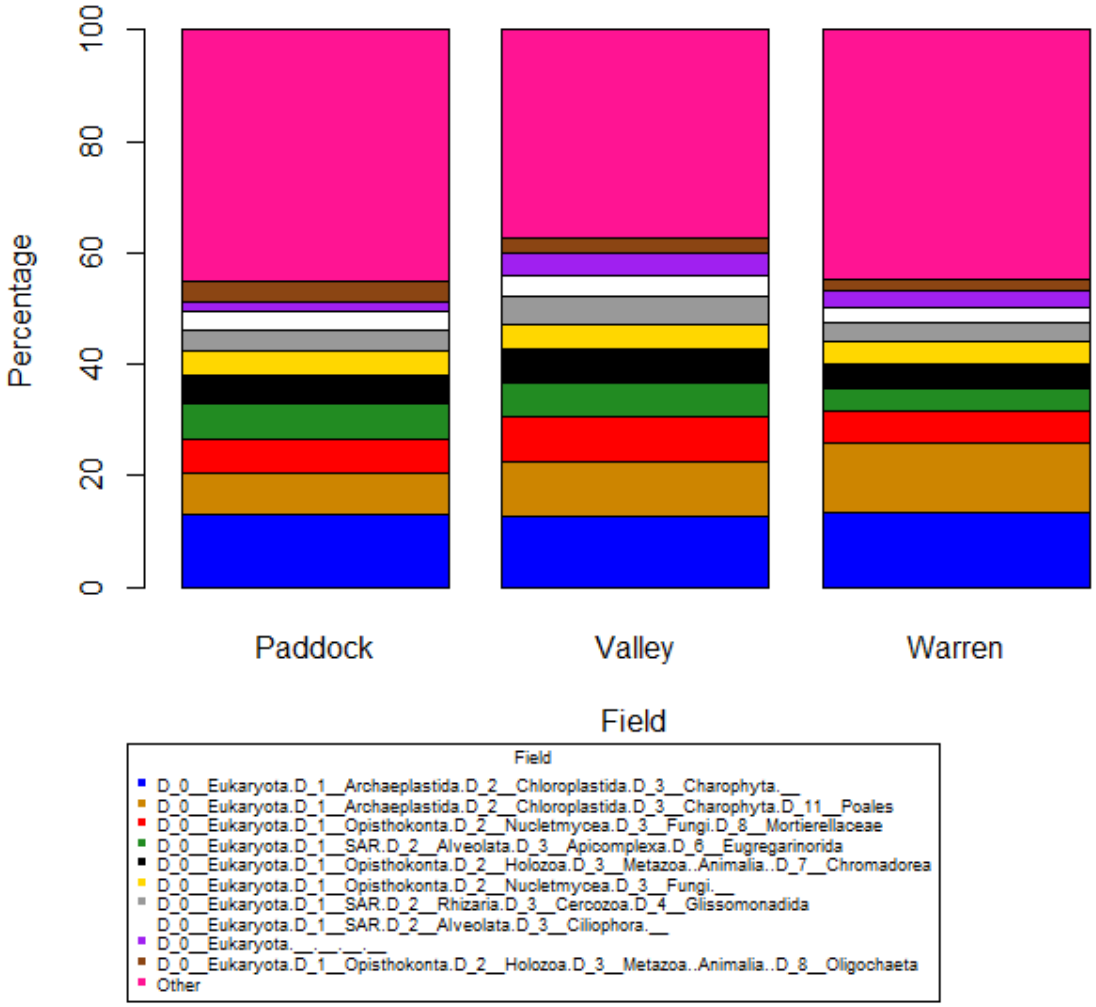


Figure 2.26 – Soil eukaryote community composition, grouped by field.

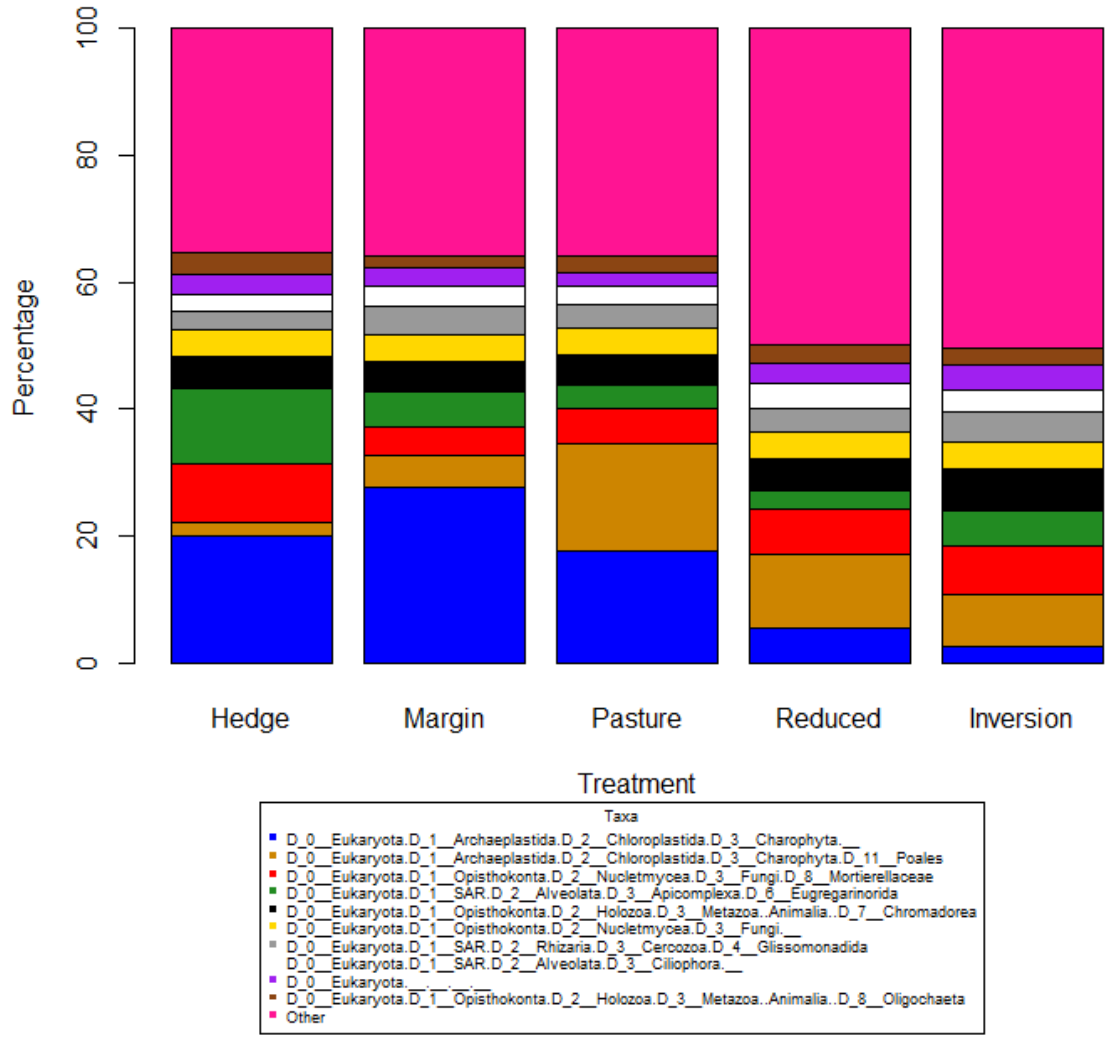


Figure 2.27 – Soil eukaryote community composition, grouped by treatment.

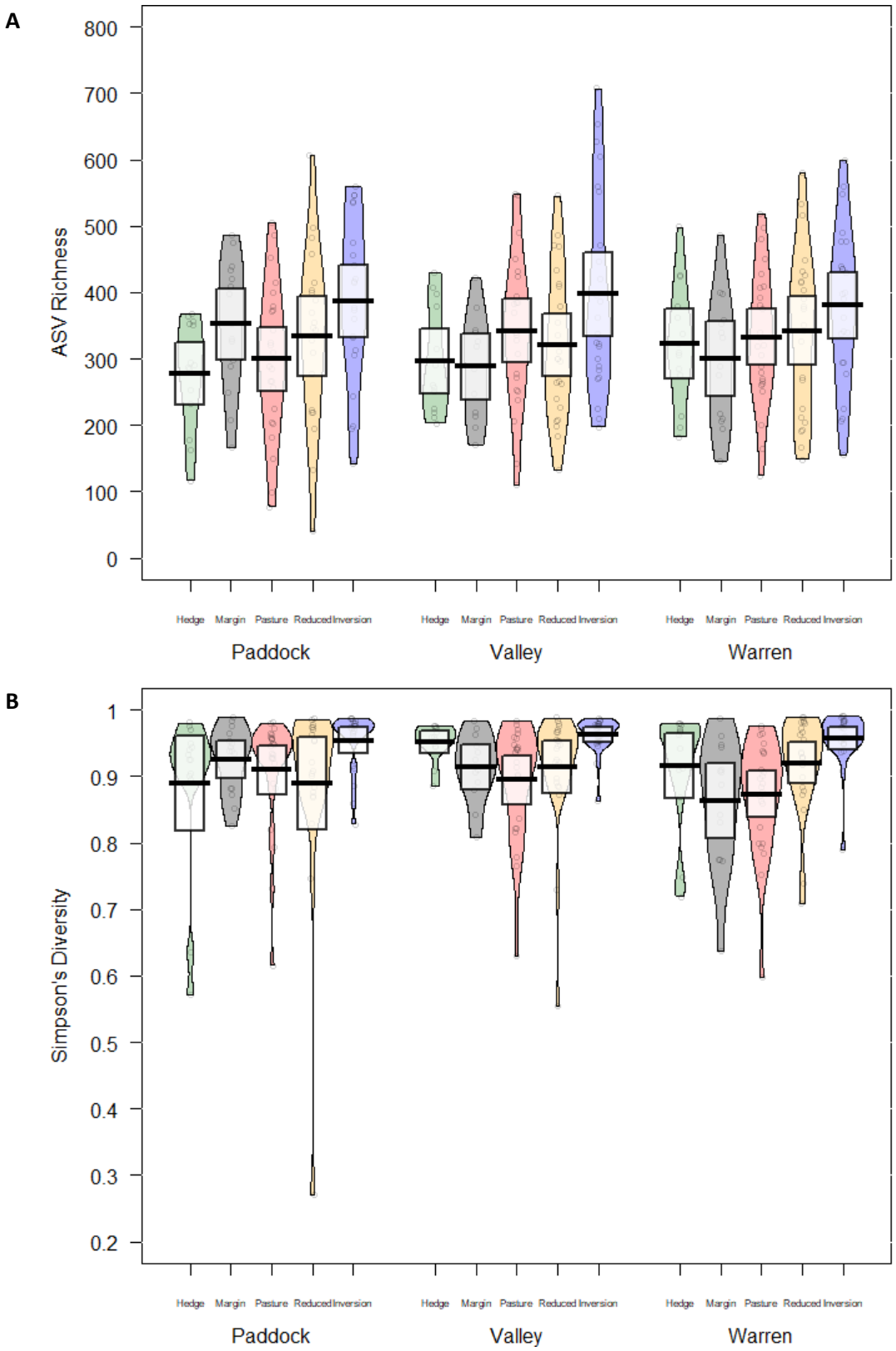


Figure 2.28 – (A) Eukaryote ASV richness per sample. (B) Eukaryote diversity per sample. Hedge (green), margin (black), pasture (red), RT (orange) and IT (blue).

For fungal community composition, PERMANOVA revealed significant effects of field, treatment, sampling and location (DF=2 F=36 P<0.001, DF=4 F=9.6 P<0.001, DF=4 F=5.5 P<0.001, DF=12 F=1.7 P<0.001 respectively). Field is the most determinant variable of fungal community composition accounting for 16 % variance (as percentage of total sum of squares), with treatment, sampling and location accounting for 8.7 %, 5.0 % and 4.6 % respectively (residuals 65 %, all to 2 significant figures). Figures 2.29A and 2.29B show the DCA ordinations of the fungal community composition grouped by field and treatment respectively – the dominant effect of field, and the distinctness of soils from hedgerows is apparent (see also Figure 2.30). In Figure 2.30, it can be noted that communities at the greatest distance (64 m) are less variable than those in closer to field boundaries. Figures 2.31 and 2.32 show relative abundance of taxonomic groups of fungal communities grouped by field and treatment respectively. At a field level, Warren is distinct from Paddock and Vallety by means of greater relative abundances of *Plectosphaerellaceae* and *Microascacese*, whilst relatively fewer *Sporomiaceae* and *Herpotrichiellaceae* compared to Paddock and Valley (Figure 2.31). Warren's higher relative abundance of *Plectosphaerellaceae* suggests it is in general closer in composition to hedgerows than the other fields (Figure 2.32), and this is supported by the near absence of *Helotiales* in hedgerows, which are of lower relative abundance in Warren than in other fields (Figure 2.31). Infield communities (pasture, RT and IT) are generally consistent relative to field boundary (Hedge and Margin) fungal communities. Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 2.33, on average Valley contains a lower richness of fungal ASVs, but Simpson's diversity values are similar relative to Paddock and Warren.

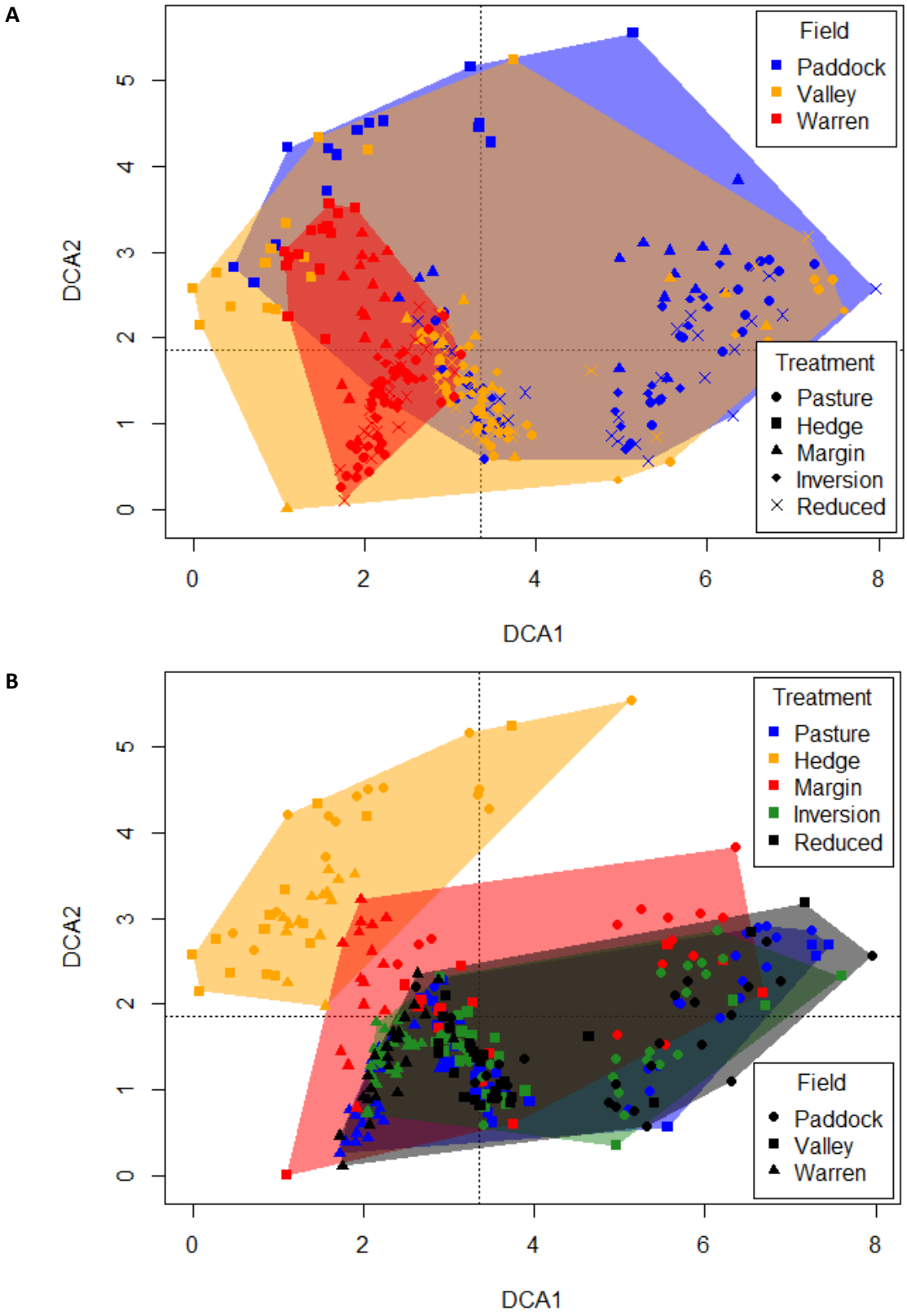


Figure 2.29 – DCA sample scores (axis 1 and 2) for fungal community from hedge, margin, pasture, and IT and RT soils. (A) Coloured hulls show the range of all samples from Paddock (blue), Valley (yellow) and Warren (red). (B) Coloured hulls show the range of all samples from pasture (blue), margin (red), IT (green), RT (black) and hedge (yellow).

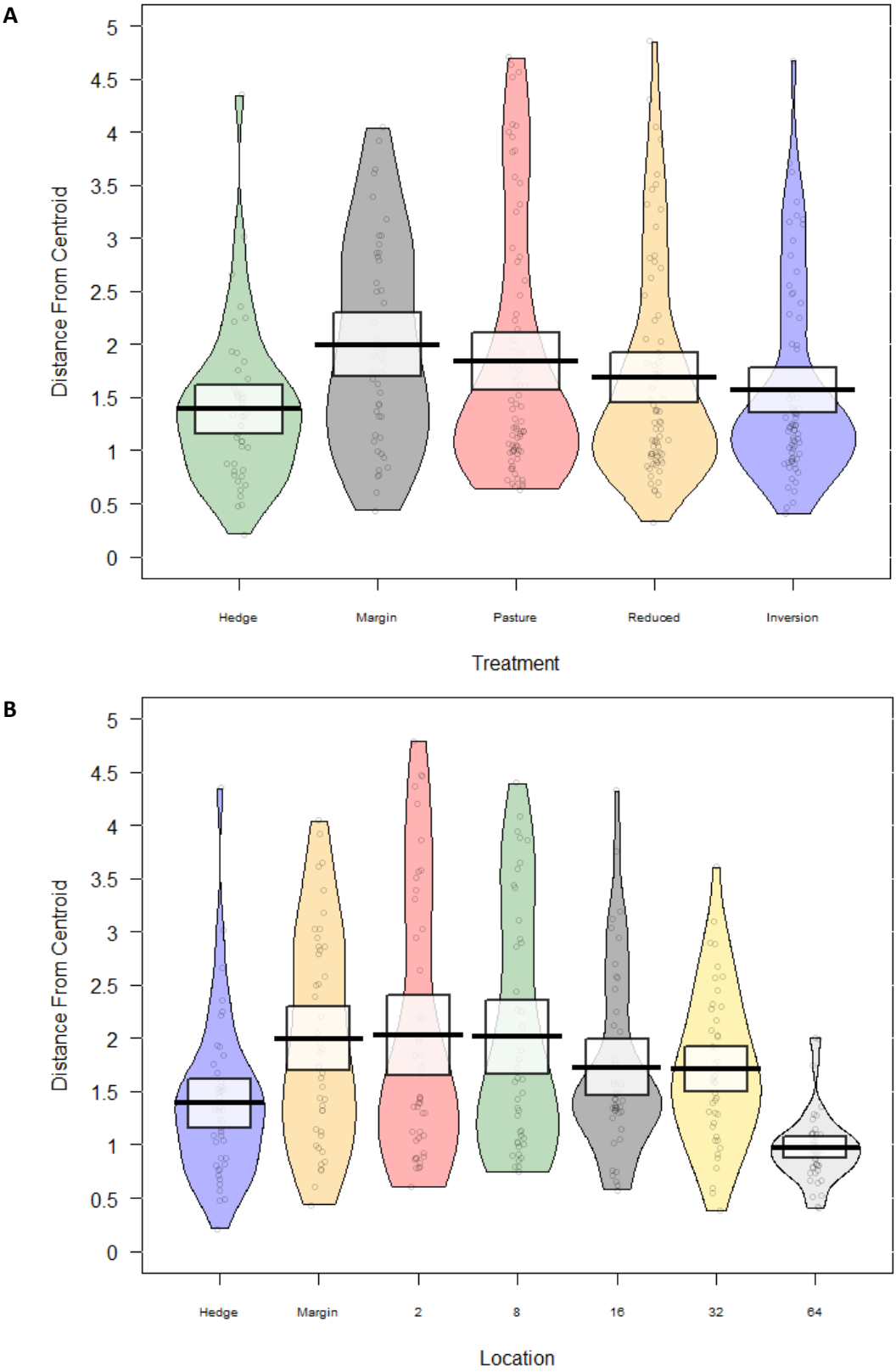


Figure 2.30 – Distance from median sample score (across all 4 DCA axis) for fungal composition in soils from hedgerows, margin, pasture, RT, and IT. (A) Samples grouped by treatment (hedgerows (green), margin (black), pasture (red), RT (yellow) and IT (blue)), and (B) samples grouped by location in field (hedge (blue), margin (orange), 2 m (red), 8 m (green), 16 m (black), 32 m (yellow) and 64 m (grey).

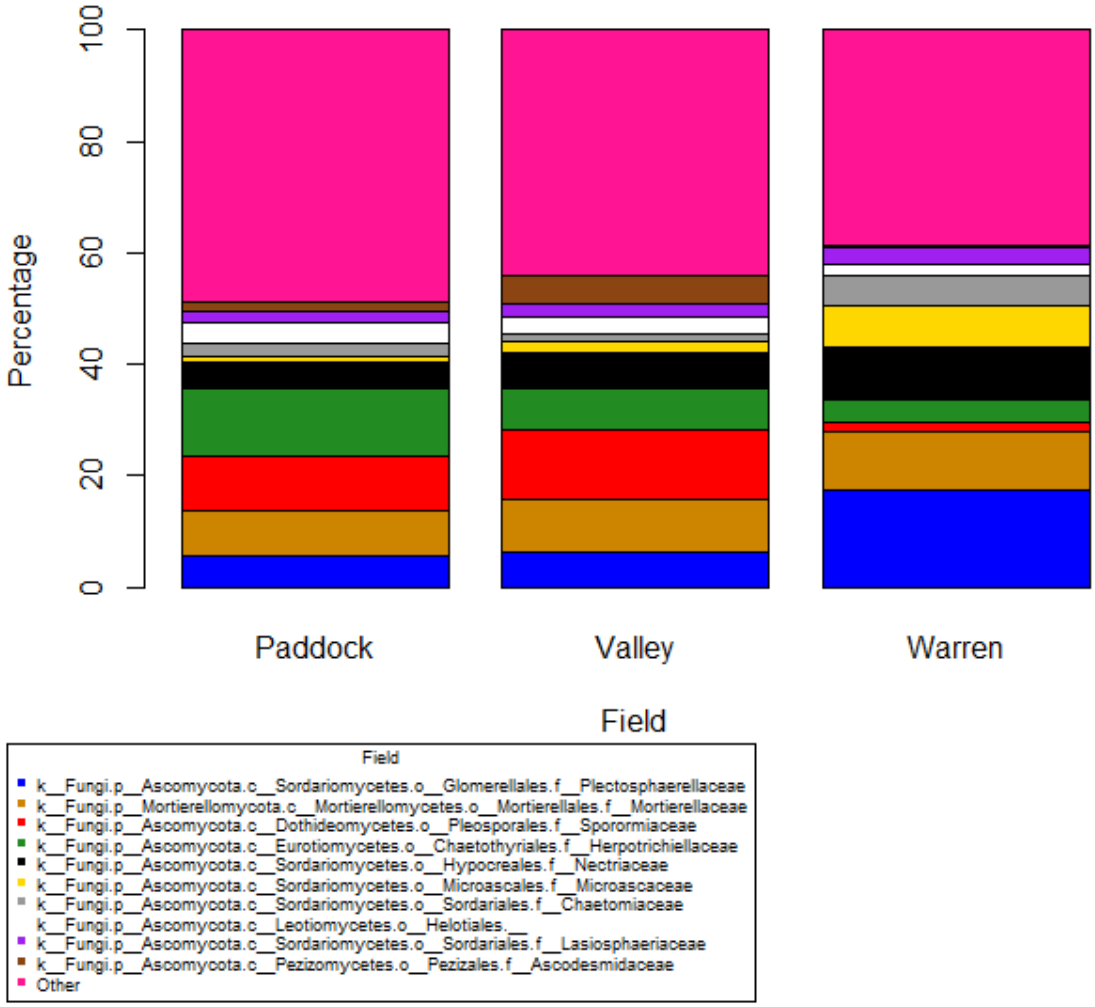


Figure 2.31 – Soil fungal community composition, grouped by field.

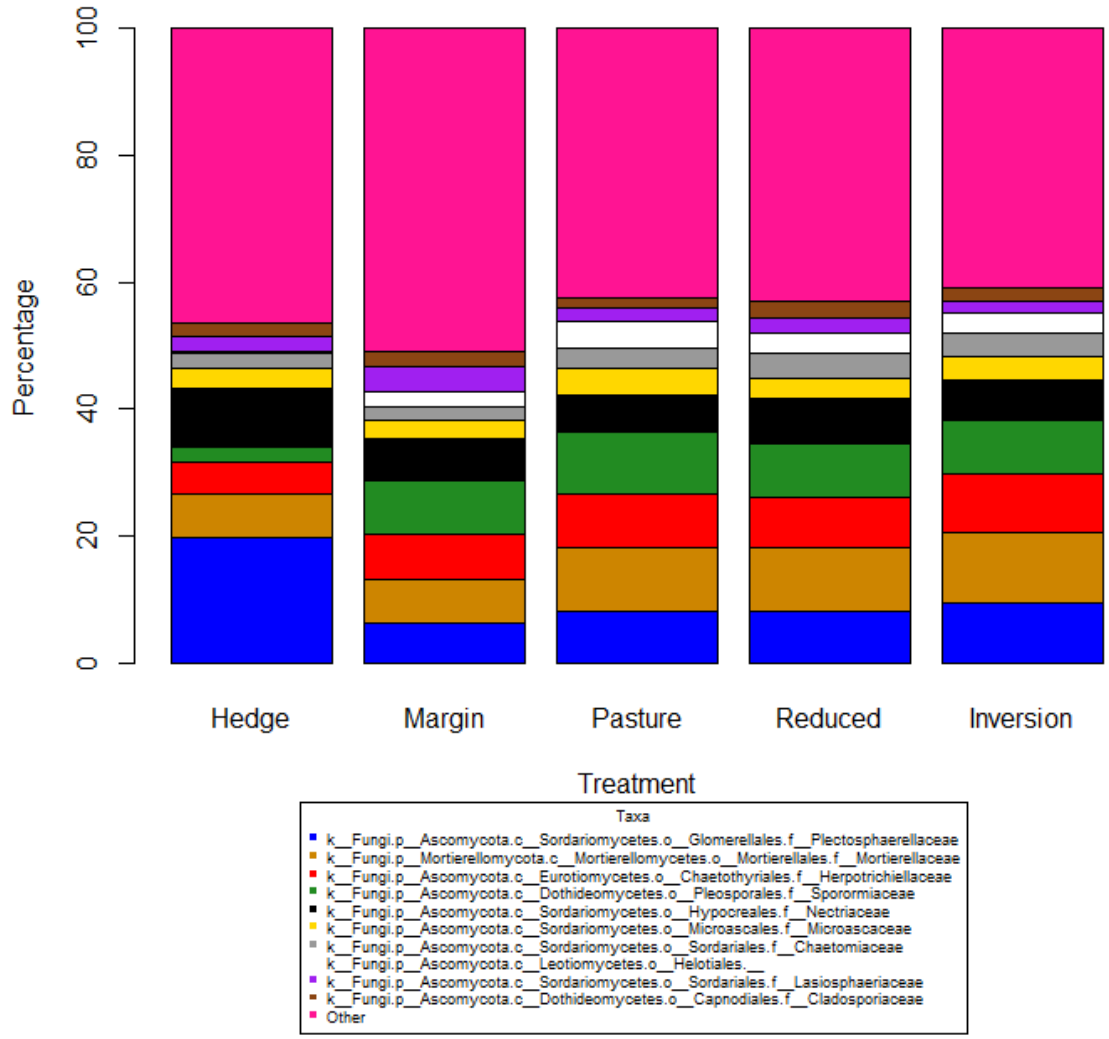


Figure 2.32 – Soil fungal community composition, grouped by treatment.

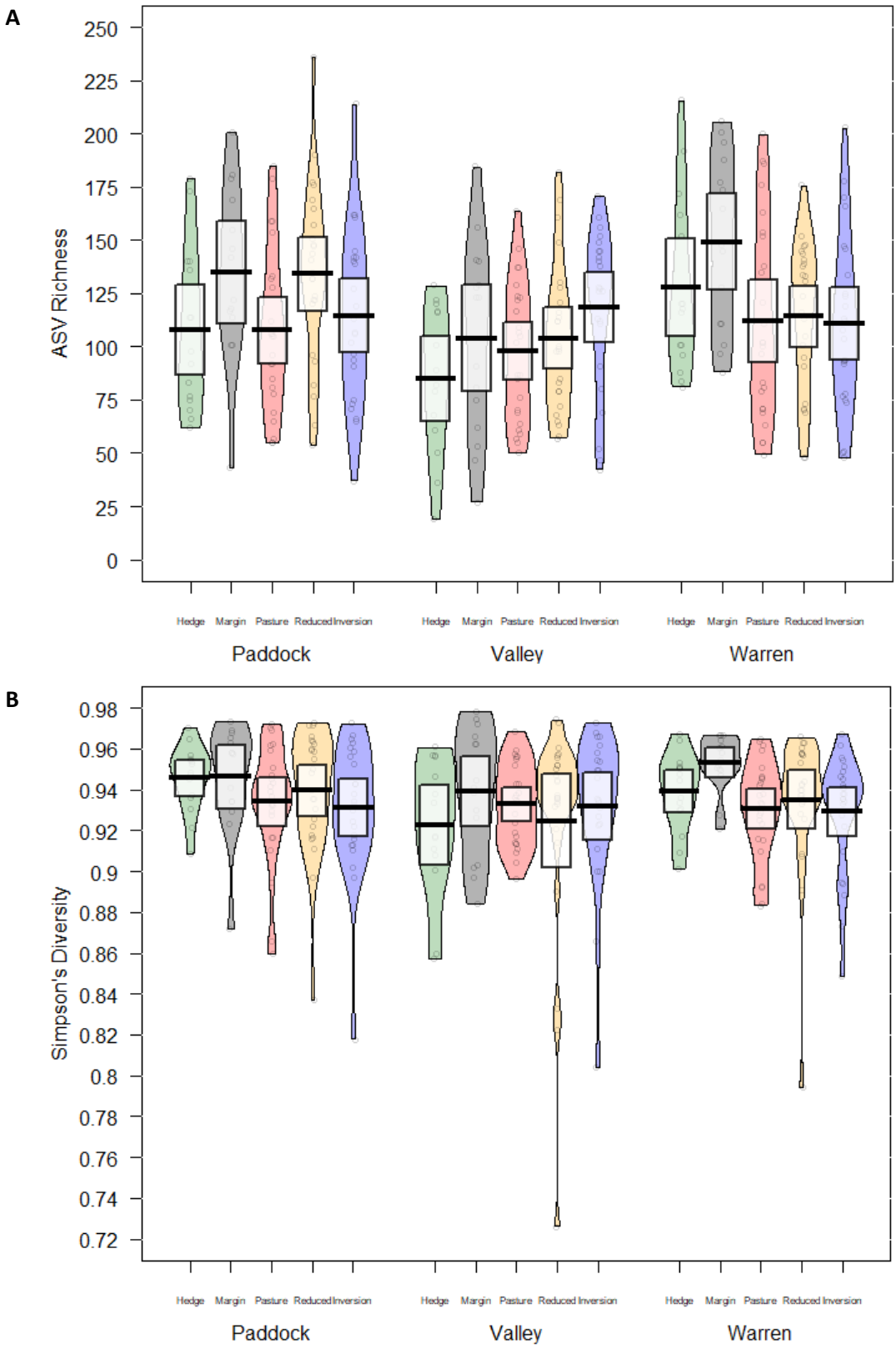


Figure 2.33 – (A) Fungal ASV richness per sample. (B) Fungal diversity per sample. Hedge (green), margin (black), pasture (red), RT (orange) and IT (blue).

2.4 Discussion

2.4.1 Field as the greatest determinant of infield soil microbiomes

For both only cultivated soils (IT and RT), and the complete dataset (including hedge, margin, and pasture), field is the most significant determinant – of field, treatment, sampling, location within a field – of all studied components of the microbial community composition (PERMANOVA results, Figures 2.4, 2.9, 2.14, 2.19, 2.24, and 2.29). The heterogeneity among samples from a given field varies substantially – Figures 2.4A and 2.19A, 2.9A and 2.24A show Paddock to have much greater variability in bacterial and eukaryote (respectively) composition relative to Valley and Warren, whilst Figures 2.14A and 2.29A show variability in fungi to be greater in both Paddock and Valley than Warren.

It is therefore important to consider what *field* as a variable means – most simply each field is a spatially distinct unit from neighbouring fields, however each field has unique natural influences – including topography, climate, hydrology, pedosphere and underlying lithosphere. Further, each field is and has been subject to human interventions – including defining field boundaries, cropping cycles, stocking with livestock and chemical, both CPP and fertiliser, inputs. Known differences in the intrinsic and human influences on the studied fields are detailed in Holden et al. (2019), and Appendices 1 and 2.

For bacteria and eukaryotes, Paddock field has a greater degree of variability in community composition than either Valley or Warren (Figures 2.4A, 2.9A, 2.19A, 2.24A). The total bacterial community composition of Paddock contains a much higher relative abundance of *Verrucomicrobia*, and considering only tilled areas Paddock also a higher relative abundance of *Firmicutes* (Figures 2.22 and 2.7). None of the ten most abundant taxonomic groupings of eukaryotes obviously explain the difference in variation between Paddock, and Valley and Warren – these differences may be driven by the ~40 % of ASVs grouped as 'other', or by ASV variation within the most abundant taxa. In a study of soils in prairie-wetlands, Griffin et al. (2020) found the period a sample spends saturated to be the most strongly correlated variable (of saturation, PH or depth) with variability in bacterial community composition and diversity. Paddock field may be characterised as generally more acidic than Valley and Warren (Appendix 2), with untilled areas also being more variable in pH than in other fields. Whilst this variability may be less impactful than saturation (Griffin et al., 2020), it may explain partially the greater variance observed within this field for bacterial and eukaryotic communities (Figures 2.4A, 2.9A, 2.19A, 2.24A). That this pattern does not follow for fungal communities (Figures 2.14A and 2.29A) may suggest these to be less sensitive to pH than bacteria or the total eukaryotic community.

For fungi, Paddock and Valley are more greatly variable in community composition than Warren (Figures 2.14A and 2.29A). Warren with less variability has previously had fungicides applied (Appendix 1), however with all sampled hedgerow soils being less dissimilar to Warren field than the composition of fungi from Paddock and Valley, it is considered unlikely the reduced variability is solely due to fungicide applications. Additionally, it can be noted that the vast majority of fungicide applied to Warren field would be expected to have degraded or dissipated prior to the present study, with half-lives of less than a year (Lewis et al., 2016), and none within four years of sampling (Appendix 1). All fungal data is dominated by *Ascomycota* (Figures 2.17 and 2.31), with Warren having a higher relative abundance of *Plectosphaerllacese* and *Sordariales,* and Paddock and Valley having higher relative abundances of *Sporomiaceae* and *Herpotrichiellaceae* (Figure 2.31).

There remain high levels (in all cases >52%) of residual variance as percentage of sum of squares that is not attributable in the current analysis to Field, Treatment, Sampling or Location. Some variance may be attributed to small scale experimental spatial variation in sampling – no identical soil volume can be extracted from twice. The scales at which both the composition and functional variance of the soil microbiome is greatest have been considered to be both at sampling distance within millimetres and at landscape scales (Bending et al., 2006, Dechesne et al., 2014, Martiny et al., 2006, Martiny et al., 2011, O'Brien et al., 2016). Sample variance associated with Location may also be related to heterogeneity of vegetation within experimental plots.

2.4.2 Hedgerows are special

For bacteria, eukaryotes, and fungi (Figures 2.19B, 2.20, 2.24B, 2.25, 2.29B and 2.30) microbial communities growing under hedgerows are less dissimilar from each other than from the infield microbial communities. The studied hedgerows are distinct (not shared by any studied field) and not continuous – being separated by gateways and access points (Warren and Valley), and a farmyard and buildings (Paddock to Warren and Valley) (Figure 1). Hedge samples contain on average a lower richness of ASVs (Figures 2.23A, 2.28A and 2.33A), contain a similar diversity to infield eukaryote and fungal communities (Figures 2.28B and 2.33B) and have a higher and less variable level of diversity within bacterial communities (Figure 2.23B). That these samples cluster strongly despite sharing many variables from within their assigned study fields implies certain shared traits are more significant in microbial community composition than those that vary by space – at this scale. This may be due to the greater vegetative cover – implying greater root abundance – leading to an increase in soil carbon, stabilized in a greater abundance of soil macroaggregates, and/or lower moisture contents occurring under hedgerows due to interception and transpiration (Prendergast-Miller et al., 2021). Hedgerows were observed to have greater soil conductivity than in

field areas (Appendix 2), again reflecting the lower moisture content, and greater salinity of hedgerow soils Brady and Weil (2012). The greater relative sensitivity of soil bacteria to salts relative to fungi (Smith and Doran, 1997, Brady and Weil, 2012), may contribute to the greater dissimilarity of fungal communities within hedgerow soils, as opposed to bacterial communities, that within hedgerow soils are more homogenous (Figures 2.20 and 2.30). Holden et al. (2019) report soils under hedgerows to be less compacted, and therefore less dense than infield soils. Hedges have lower abundances of *Bacillaceae* and *Chthoniobacteracea* and a greater abundance of *Microscillaceae* than infield bacterial communities, and lower abundance of *Helotiales* and greater abundance of *Plectosphaerellaceae* and *Nectriaceae* than infield fungal communities. The present study extends upon Holden et al. (2019)'s finding of soils under hedgerows hosting a distinct soil community to include soil bacteria and eukaryotes, and expands the evidence base for distinct fungal communities.

2.4.3 The soil microbiome within IT and RT crop strips

Previous studies on the effect of tillage on soil microbial communities have often focussed upon enzyme activity, biomass and other abundance metrics – see Zuber and Villamil (2016) and Kaurin et al. (2018) – with community composition and diversity studied often for a single taxonomic grouping – such as Yin et al. (2010) for bacteria, and Helgason et al. (1998) for arbuscular mycorrhizal fungal – or not by amplicon marker-gene or metagenomics approaches – see Wang et al. (2017) for phospholipid fatty acids analysis. However, Somenahally et al. (2018) include community composition analysis for both bacterial and fungal communities under different tillage techniques, although this was a comparison of RT and NT techniques.

Intensity of tillage did not have a significant effect on the community composition of bacteria and fungi (PERMANOVA (DF=1 F=1.5 P>0.1, DF=1 F=1.4 P>0.1)), accounting for 0.5 % and 0.6 % of variance respectively (as percentage of total sum of squares) (Figures 2.4B and 2.13B). These results are consistent with the findings of Somenahally et al. (2018), which does not include analysis of total soil eukaryotes. In the present study, tillage was identified to have a significant effect (PERMANOVA (DF1= F=1.9 P<0.01)) on total eukaryotes community composition, although this is very slight, accounting for 1.0 % variance – this difference is hard to observe within the DCA ordination (axis 1 and 2, Figure 2.9B). The most notable difference between IT and RT composition is a greater relative abundance of *Chloroplastida*, green-algal soil protists (Figure 2.13) within RT soils – however both IT and RT communities have a lower relative abundance of *Chloroplastida* than hedge, margin, and pasture samples (Figure 2.27). For all studied groups, community composition under IT is less variable than under RT.

Within the studied crop strips, it can be observed that variance within the composition of bacterial, eukaryotic, and fungal communities declines with distance from the field boundary (Figures 2.6, 2.10 and 2.15). Why this occurs is unclear, but may be due to the more marginal locations receiving microorganisms dispersing into each field from hedgerows or other surrounding areas – it may also imply more even or intense farming management within the central zone of a field, whereas the outer regions of a field may be less tightly managed, due to a perceived lower value (Ingram, 2010, Morris et al., 2010). Where the 'movement' of a given sample location is plotted through time, it can be observed that the motion is cyclical through each year, and that there is no clear or singular direction as a location spends a longer time period under tillage (data not shown).

2.4.4 Shortcomings

Two of the most significant shortcomings within the present study relate to time – firstly the duration of the field experiment (see below) and secondly the failure to account for relic-DNA, and any possible role it has in screening both seasonal effects, and divergence between treatments. Relic-DNA, as described by Carini et al. (2016) is DNA that is extracellular at the time of sampling. Such extracellular DNA may have persisted in the sampled soils for (up to) years, and yet may be included in present studies and analyses the same as that from organisms intact at the time of sampling. Within the present study, relic-DNA may in particular account for the low levels of dissimilarity observed between IT, RT, and pasture treatments, due to the IT and RT plots being in a created from (and therefore containing relic-DNA) of pasture treatments. Particularly, whilst fungal communities were not observed to be significantly different between IT and RT plots (PERMANOVA (DF=1 F=1.4 P>0.1), Figure 2.14B), potentially, this may reflect relic-DNA from hyphal networks which may have been destroyed or disrupted at a greater rate under IT treatments, but from which relic-DNA may obscure effects that may be predicted from the literature (Helgason et al., 1998). Whilst Carini et al. (2016) describe how relic-DNA may be removed from samples using propidium monoazide, the applicability of these techniques to the scale of sampling undertaken by large field experiments, such as the present study are questionable. This is because in freezing (and particularly in freeze drying, as in the present study) necessary due to the volume of samples to be processed, damage to live at the time of sampling organism may lead to non-relic-DNA being removed as such.

The short – relative to continuous cropping of much arable land – duration of the experimental tillage plots (less than 24 months from first of two incidences of cultivation (of pasture) to final sampling) confound the applicability of the results to areas under sustained cultivation. That the treatment effect of different tillage regimes is apparent within this timeframe may be due to

strong difference from the different tillage techniques, or reflect different stages of a transition from pasture to arable conditions. Changes in soil organic carbon (SOC) content following changes in tillage technique may be expected to only become evident following 10 years of divergent treatment (Söderström et al., 2014, Haddaway et al., 2015). As changes in SOC are commonly strongly linked to changes in the soil microbiome (Six et al., 2000, Brady and Weil, 2012), this may be indicative to the duration of tillage experiments necessary to observe substantial changes in the soil microbial community

Additionally, the present study assesses the composition of the community, but does not address the size of the communities in question. This is a deficiency both within a single studied community, preventing exploration of further research questions, such as is there a greater abundance of bacteria under hedgerows relative to cultivated soils (as there is for earthworms (Prendergast-Miller et al., 2021)). And in comparison between taxonomic groupings, such as investigating the often reported shift in bacterial:fungal biomass with increasing tillage intensity (Zuber and Villamil, 2016, Kaurin et al., 2018). General criticisms of metabarcoding studies, sequencing by synthesis and reference taxonomic databases can be found in the general discussion/introduction.

Chapter 3: Degradation of Mandipropamid and Simazine in soils under inversion and reduced tillage

3.1 Introduction

3.1.1 Risk assessment of CPPs

The majority of regulatory assessment of the environmental fate and behaviour of chemicals is based upon the OECD Guidelines for Testing Chemicals Section 3 (OECD, 2020). OECD 307 (OECD, 2002) details the experimental design for evaluating the degradation of compounds in soils. In brief, compounds are applied to a sieved and homogenised soil substrate, and incubated in the dark, at a constant temperature and soil moisture. Volatile products are collected for analysis by absorption. At intervals, samples undergo extraction, and analysis for the parent compound and any degradants. Use of ^{14}C radiolabelled compound allows for the calculation of a mass balance, the identification of non-extractable residues, and determination of the mineralisation rate of the study compound. From OECD 307 style studies, degradants of concern (>5% of total compound applied) may be identified, and the half-life of compound (DT50) established. Regulators of different regions require different ranges of soils to be used as substrate in studies, which are included in the application dossiers for compound approval (OECD, 2002). See regulations EC 283/2013 and 284/2013 for data requirements for active substance and formulated CPP product applications within the EU (Commission, 2013a, Commission, 2013b).

Regulators may also require field testing of compounds prior to approval for use, guidance for which is also published by the OECD (2016). Compounds are applied to study plots, at rates representative of intended use. Whereas there are multiple tests detailed in Section 3 of the OECD Guidelines for the Testing of Chemicals, the field study guidelines contain multiple modules that may be used to determine degradation, runoff, volatilisation, leaching and plant uptake. To establish the rate of degradation, at intervals throughout the experiments, soil cores are removed from the study plot and analysed for parent compound and transformation products. It is of note that the guidelines outline that for compounds to be applied to soils under RT, bare ground plots are necessary (OECD, 2016).

With the substantial difference between soils under IT and RT occurring in the physical structure and profile of the soil – carbon stratification, aggregate and pore size – meaningful study of compound degradation in soils under different tillage regimes should seek to preserve the structure of soils. Regulatory laboratory study of the degradation of compounds, such as OECD 307 based studies utilise sieved soils (OECD, 2002), and are deficient for identifying the effect of such differences in compound degradation. Dougan et al. (2013) demonstrate how studies conducted with intact soil cores may identify differences in compound degradation in the same soils, subject to different incubation conditions – static and variable moisture, and dark/light cycling.

3.1.2 Study Compounds

Lamberth et al. (2008) report the discovery of Mandipropamid, a carboxylic acid amide, with fungicidal properties. In particular, Mandipropamid is highly effective in the control of oomycetes including *Phytophthora infestans* (potato and tomato late blight) and *Plasmopara viticola* (grape downy mildew), both significant pests of commercially important crops. Mandipropamid inhibits cell wall biosynthesis, inhibiting the cellulose synthase-like PiCesA3 (Blum et al., 2010, Blum et al., 2012).

Simazine is an s-triazine herbicide, and has been used globally since 1955 (Kodama et al., 2001), although its agricultural application has been prohibited within European Union countries since 2004 (Scherr et al., 2017). S-triazine herbicides inhibit many weed species at concentrations that do not significantly inhibit crop species (Wackett, 2002). S-triazines inhibit photosynthetic electron transport, by binding to the quinone-binding protein in photosystem-II (Wackett, 2002). Simazine may be persistent in some soils, and can, with its degradation products, still be detected in European soils (Scherr et al., 2017, Hvezdova et al., 2018). This may be a consequence of persistence, illegal application, or Simazine as an impurity of Terbuthylazine (Hvezdova et al., 2018).

3.1.3 Aims and objectives

The present study aims to assess if Mandipropamid and Simazine degrade at different rates in soils under IT and RT. Given the probability of disrupting key differences – microbial community, macro-aggregate abundance and turnover, carbon distribution within the soil profile – between soils from IT and RT systems under OECD 307 guidelines (see Miranda (2019) on effects of OECD 307 on microbial communities), an intact soil core approach was chosen to attempt to preserve any differences between studied soils. It is possible that predicted increases in soil carbon and soil macroaggregates in RT soils may reduce compound availability for degradation (by sorption and aged sorption), and therefore the rate of degradation. Spatial heterogeneity (of microbiota, and soil

minerals and organic matter) in soils under RT may be greater than those under IT, due to reduced mixing – this may increase variability in degradation rates.

3.2 Methodology

3.2.1 Field Site

Samples were collected from Warren field of the SoilBioHedge experiment, as detailed in Chapter 2, and by Holden et al. (2019).

Under OECD (2002) guideline OECD 307, laboratory assessments of the transformation of chemicals in soil, soils used for such studies should not have been treated with the test substance or its structural analogues within the previous four years. Previous CPP applications to Warren field are detailed in Appendix 1. Neither Mandipropamid, Simazine, or any structural analogues of these compounds were applied to Warren field in the four years previous to sampling. Therefore, the sampled soils may be regarded as appropriate under OECD 307 for use in the present study.

3.2.2 Sampling

Sampling was conducted on the 4th of September 2017. Sampling was conducted at approximately 48 m from the field margin. Immediately prior to sampling, crop residues from the recent harvest and approximately 2 cm of the uppermost soil was removed. Sampling included the collection (per tillage type) of 90 soil cores for incubation (dimensions 73 mm internal diameter, 75 mm in height), 2 cores for determination of water holding capacity, and approximately four kg of bulk soil, from between the cores. Soil cores were collected from a region of the crop strip uncompacted by the passage of vehicles.

Soils were stored in the dark at ambient temperatures, separated by treatment type, prior to transport to Jealott's Hill International Research Centre on the 6th of September 2017. Thereafter soils were stored in the dark, at 20 ° C. Cores for determination of soil water holding capacity, and bulk soil for characterisation, were shipped to Agvise Laboratories (North Dakota, USA) (results Table 3.1).

Table 3.1 – Bulk soil characterisation of IT and RT crop strips of Warren field (Agvise, USA)

Parameter			Reduced tillage		Inversion tillage	
% Sand			51		49	
% Silt			28		30	
% Clay			21		21	
Texture class			Loam		Loam	
Disturbed density (g/cm^3)			1.14		1.14	
% Organic matter			4.7		4.7	
% Organic carbon			2.7		2.8	
CEC (meq/100g)			16.6		17.1	
pH (1:1 in water)			7.2		7.1	
pH (1:2 $CaCl_2$)			7.1		6.9	
Base saturation data						
Calcium	%	ppm	60.5	2010	60.7	2070
Magnesium	%	ppm	23.0	459	23.2	474
Sodium	%	ppm	1.4	52	0.4	15
Potassium	%	ppm	1.2	76	1.2	80
Hydrogen	%	ppm	14	23	14.5	25
Disturbed soil moisture						
% Moisture at 1/10 bar			30.0		29.8	
% Moisture at 1/3 bar			20.6		22.5	
% Moisture at 15 bar			12.7		12.9	
Undisturbed soil moisture						
Mean % moisture at pF2			33.5		30.7	
Mean % moisture at 1/10 bar			33.5		30.7	

3.2.3 Experimental set up and moisture control

72 soil cores per tillage treatment were selected to undergo incubation, avoiding cores with visible stones, voids or substantial cracks. Cores selected for incubation were placed on moistened autoclaved sand (prepared as 80 g sand, 20 g water) (Figure 3.1). Triplicate cores per tillage treatment were selected for moisture control, each probed with a WaterScout SM 100 Soil Moisture Sensor (Spectrum Technologies Inc.), data logged by WatchDog 1000 series Micro Stations (Spectrum Technologies Inc.). Watering was conducted to maintain a mean moisture content of 25 %

volumetric water content (VWC). Figure 3.2 shows the 24 hour mean %VWC per tillage treatment throughout the sample incubation.

To account for the loss of moisture from the sand layer, three dummy cores containing a sealed mass of dry sand were incubated, and the average loss of weight from these used to estimate the moisture required to remoisten the sand layer.

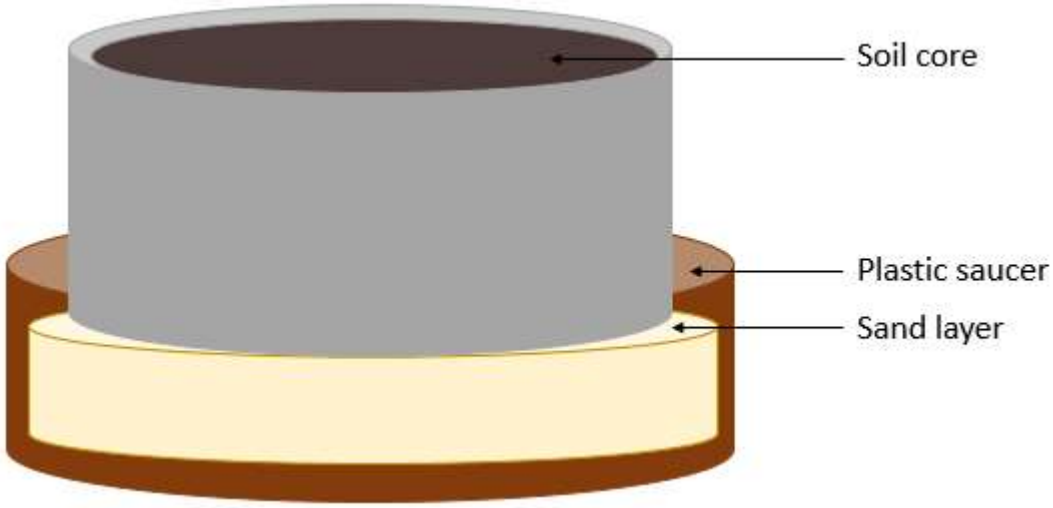


Figure 3.1 – Soil core incubation design

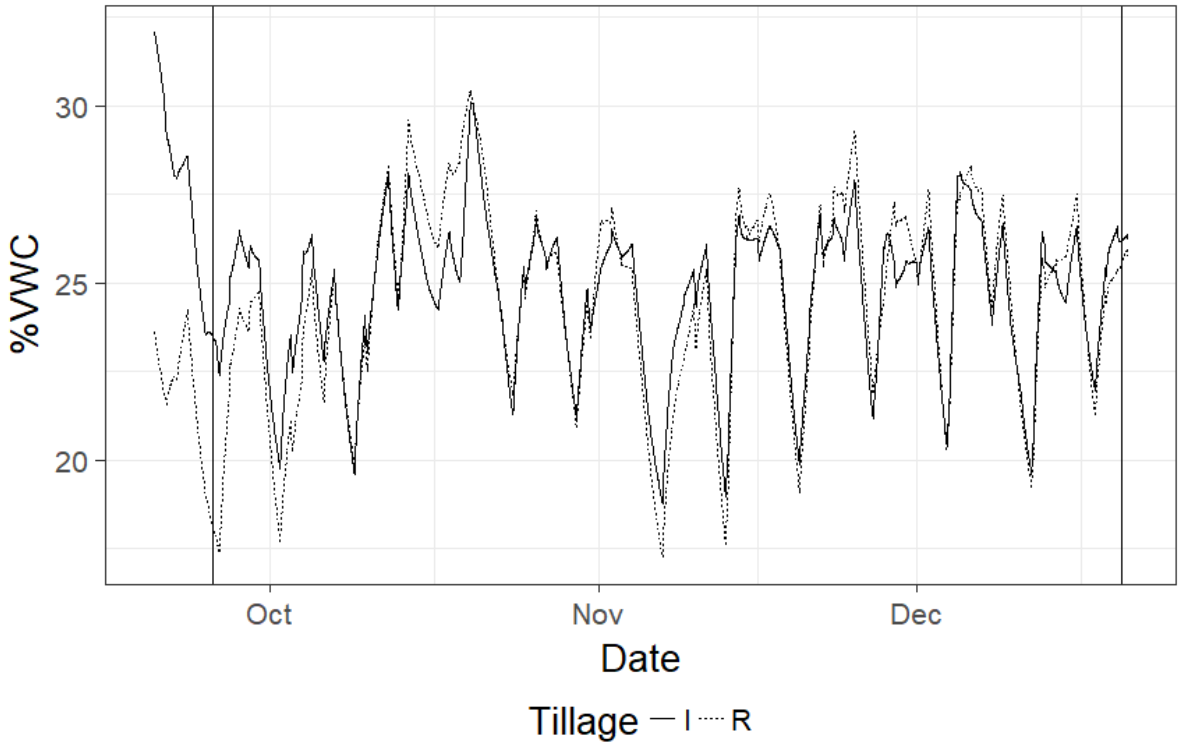


Figure 3.2 – 24 hour mean moisture content of control cores. Date of treatment and final sampling marked.

Following the incubation, each of the six moisture control soil cores were homogenised, and three sub-samples taken. The moisture content of each sub-sample was then determined using either a Mettler LJ16 Moisture Analyser, Mettler PM480 Delta Range Balance with Mettler LP16 Heater-Unit, or Sartorius MA45. Figure 3.3 shows the mean percentage %VWC measured by weight, relative to the final probe measurements of water content. The mean difference between %VWC measured by probe and by mass was 3.6%, with the probes generally reporting the soil to have a higher water content than determined by mass.

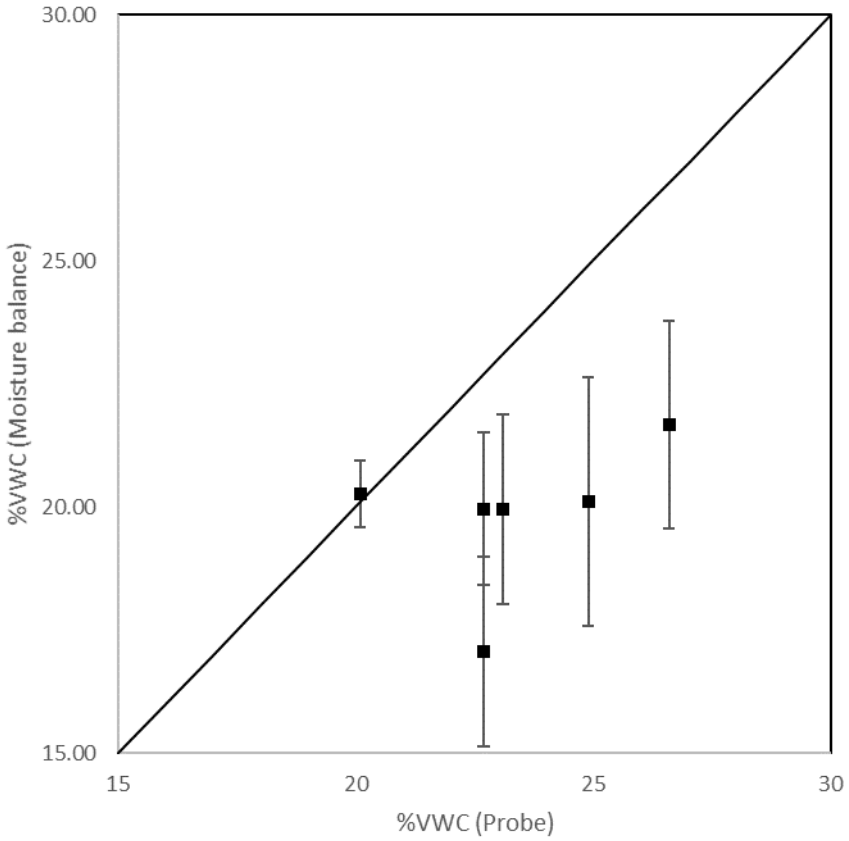


Figure 3.3 – VWC of moisture control cores, moisture probe readings relative to moisture balance readings ± SD.

3.2.4 Preparation of treatment solutions

All stock solutions were stored at -20 °C.

A batch of NOA446510 (Mandipropamid) (chlorophenyl-U-^{14}C) (structure Figure 3.4, properties Table 2) in acetonitrile (MeCN), was received with a specific activity of 1.96 MBq mg^{-1}. The received material was quantitatively transferred to a 15 ml volumetric flask and made to volume

with MeCN. For radiochemical stock check analysis by liquid scintillation counting (LSC), in duplicate 25 µl was transferred to 10 ml volumetric flasks. The volumetric flasks were made to volume with MeCN. Duplicate 250 µl aliquots of each solution were transferred to LSC vials, 5 ml of scintillation cocktail (ProSafe+, Meridian Biotechnologies Ltd) was added, and radioactivity quantified on a Packard Tri Carb 3100TR Liquid Scintillation Analyser (using Quanta Smart software). The total received radiochemical stock had an activity of 25.009 MBq.

Mandipropamid consists of two enantiomers, and is synthesised and applied with an isomer ratio of 1:1 (EFSA, 2012). Parent compound enantiomer-selective degradation has been demonstrated in soils (DT_{50} of the S enantiomer being 20-70 % greater than the R enantiomer) (European Food Safety Authority, 2012), however all methods in the present study consider only the total behaviour of both enantiomers.

The desired rate of application for Mandipropamid was 400 g ai ha^{-1}. Each soil core had a surface area of 4.19 X 10^{-3} m^2, requiring an application of 167.4 µg ai per core, equivalent to 0.328 MBq per core. The intended application volume was 833 µl for which 60 volumes were prepared.

Following preparation, the concentration of the Mandipropamid solution was checked by LSC, in duplicate, 50 µl was transferred to 10 ml volumetric flasks. The volumetric flasks were made to volume with MeCN. Duplicate 250 µl aliquots of each solution were transferred to LSC vials, 5 ml of scintillation cocktail (ProSafe+, Meridian Biotechnologies Ltd) was added, and radioactivity quantified on a Packard Tri Carb 3100TR Liquid Scintillation Analyser (using Quanta Smart software). The Mandipropamid treatment solution had a total activity of 19.46 MBq, 98.85 % of the desired activity. The application volume was revised to 843 µl.

The radiochemical purity of the Mandipropamid treatment solution was assessed by HPLC with radiodetection. The HPLC method used for Mandipropamid had previously been developed (Table 3.4). A 25 µl aliquot from a treatment solution quantification solution was used to assess stock purity. HPLC system details: Agilent 1100 Series HPLC G1311A Quaternary Pump, Agilent 1100 Series HPLC G1313A Autosampler, Agilent 1100 Series HPLC G1316A Column Compartment, Agilent 1100 Series G1315B Diode Array Detector, and β-Ram Model 5C Radiodetector – software LAURA 4.1.14.96. The radiochemical purity of the Mandipropamid treatment solution was 100 %.

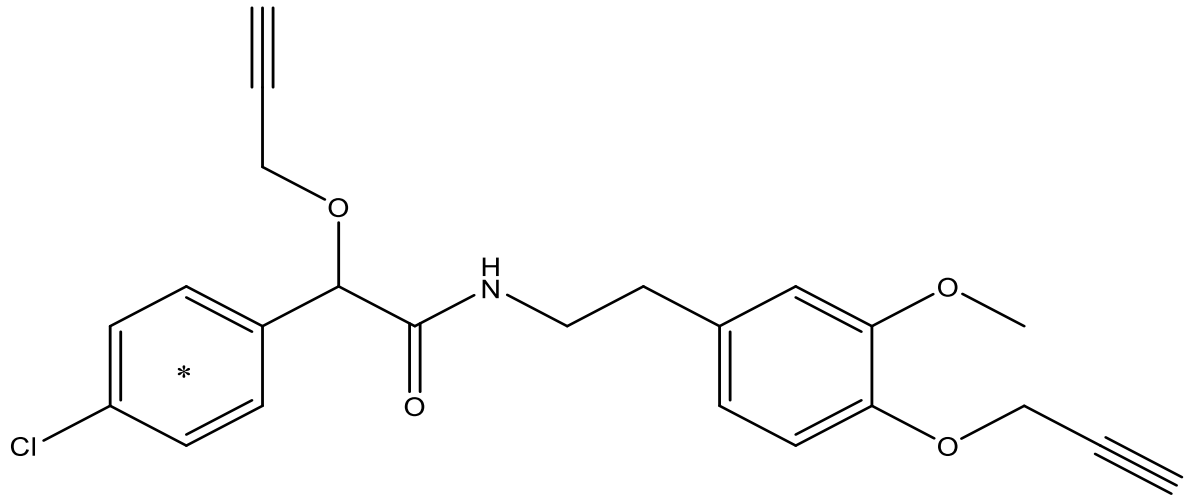


Figure 3.4 – Structure of Mandipropamid, location of ^{14}C labelled ring indicated (*).

Table 3.2 – Properties of Mandipropamid (Lewis et al., 2016)

IUPAC Name	(*RS*)-2-(4-chlorophenyl)-*N*-[3-methoxy-4-(prop-2-ynyloxy)phenethyl]-2-(prop-2-ynyloxy)acetamide
CAS Number	374727-62-2
Molecular Formula	$C_{23}H_{22}ClNO_4$
Molecular mass (g mol^{-1})	411.9
Solubility in water at 20 ° C (mg l^{-1})	4.2
Vapour pressure at 25 ° C (mPa)	9.4 x 10^{-4}
Henry's law constant at 25 ° C (Pa m^3 mol^{-1})	9.2 x 10^{-5}
Log P	3.2

A batch of G27692 (Simazine)(triazinyl-U-^{14}C) (structure Figure 3.5, properties Table 3.3) in 11 ml of tetrahydrofuran (THF), was received with a specific activity of 1.63 MBq mg^{-1}. The received material was quantitatively transferred to a 25 ml volumetric flask and made to volume with THF. For radiochemical stock check analysis, in duplicate 25 µl was transferred to 10 ml volumetric flasks. The volumetric flasks were made to volume with THF. Duplicate 250 µl aliquots of each solution were transferred to LSC vials, 5 ml of scintillation cocktail (ProSafe+, Meridian Biotechnologies Ltd) was added, and radioactivity quantified on a Packard Tri Carb 3100TR Liquid Scintillation Analyser

(using Quanta Smart software). The total received radiochemical stock had an activity of 21.310 MBq.

A stock solution of non-radiolabelled Simazine was prepared, 30 mg of technical standard were transferred to a 10 ml volumetric flask and made to volume with THF.

To minimise the use of radiochemicals, the Simazine treatment solution was prepared with 45.8 % radiolabelled Simazine, and 54.2 % unlabelled Simazine. Following preparation, the concentration of the Simazine solution was checked by LSC, in duplicate, 25 µl was transferred to 10 ml volumetric flasks. The volumetric flasks were made to volume with THF. Duplicate 250 µl aliquots of each solution were transferred to LSC vials, 5 ml of scintillation cocktail (ProSafe+, Meridian Biotechnologies Ltd) was added, and radioactivity quantified on a Packard Tri Carb 3100TR Liquid Scintillation Analyser (using Quanta Smart software). The Simazine treatment solution had a total activity of 17.92 MBq, 95.54 % of the desired activity. The application volume was revised to 872 µl.

The desired rate of application for Simazine was 1000 g ai ha^{-1} (where ai is active ingredient). Each soil core had a surface area of 4.19 X 10^{-3} m^2, requiring an application of 436.2 µg ai per core, equivalent to 0.341 MBq per core (54.2% of applied Simazine being cold material). The intended application volume was 833 µl for which 60 volumes were prepared.

The radiochemical purity of the Simazine treatment solution was assessed by HPLC with radiodetection. The HPLC method used for Simazine had previously been developed (Table 3.5). A 25 µl aliquot from a treatment solution quantification solution was used to assess stock purity. HPLC system details: Agilent 1200 Series HPLC G1311A Quaternary Pump, Agilent 1200 Series HPLC G1329A Autosampler, Agilent 1200 Series HPLC G1316A Column Compartment, Agilent 1200 Series HPLC G1315D Diode Array Detector, β-Ram Model 4C Radiodetector and SoFie Stop-Flow System – software LAURA 4.1.14.96. The radiochemical purity of the Simazine treatment solution was 93.02 %. The purity of the cold Simazine stock solution was assessed by HPLC with UV detection at 254 nm, using the same HPLC system and method. The cold Simazine stock solution was 100 % pure.

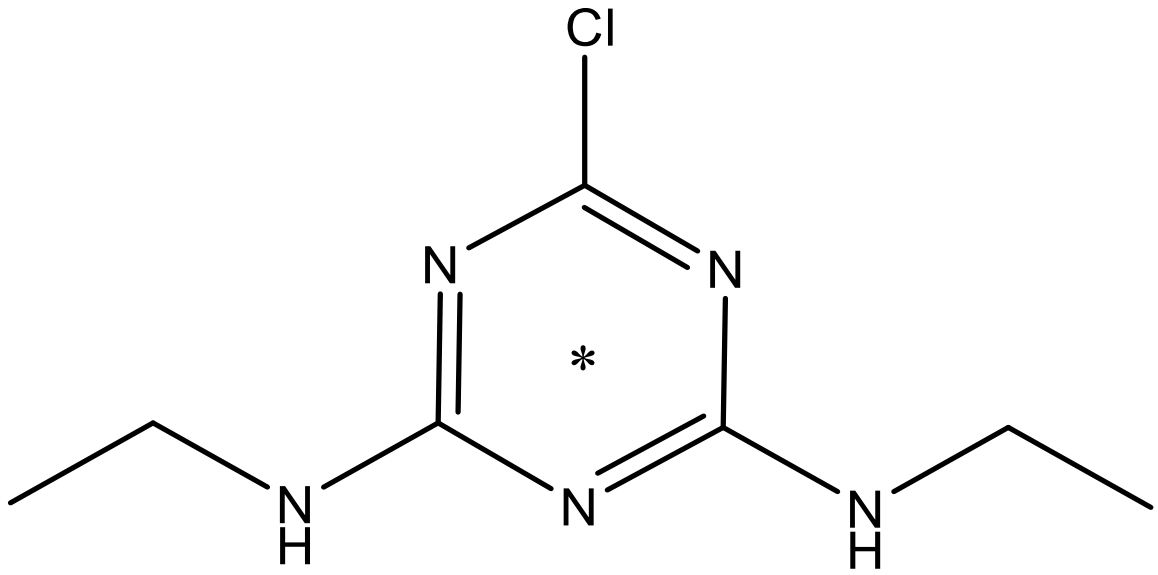


Figure 3.5 – Structure of Simazine, location of ^{14}C labelled ring indicated (*).

Table 3.3 – Properties of Simazine (Lewis et al., 2016)

IUPAC Name	6-chloro-*N2*,*N4*-diethyl-1,3,5-triazine-2,4-diamine
CAS Number	122-34-9
Molecular Formula	$C_7H_{12}ClN_5$
Molecular mass (g mol^{-1})	201.66
Solubility in water at 20 ° C (mg l^{-1})	5
Vapour pressure at 25 ° C (mPa)	8.1 x 10^{-4}
Henry's law constant at 25 ° C (Pa m^3 mol^{-1})	5.6 x 10^{-5}
Log P	2.3

3.2.5 Treatment

Application was conducted on the 25th of September 2017. Treatment solutions were applied dropwise to the surface of the soil cores at the specified rate – Mandipropamid 843 µl per core equivalent to 400 g ha^{-1} ai, Simazine 872 µl equivalent to 1000 g ha^{-1} ai. Pre and post application checks were conducted for both compounds – the application volume being pipetted into a 10 ml volumetric flask prior to the first application, application made to all treated cores without adjustment of the pipette, and a final volume pipetted into a second 10 ml volumetric flask. The pre

and post treatment flasks were made to volume with MeCN for Mandipropamid, and with THF for Simazine. Radioactivity within 250 µl aliquots of the pre and post application checks were quantified by LSC. The quantified average application rate for Mandipropamid was 0.343 MBq per core, equivalent to 419 g ha^{-1} ai. The average quantified application rate for Simazine was 0.345 MBq per core, equivalent to 1106 g ha^{-1} ai.

3.2.6 Extraction regime and quantification of extracts

For Mandipropamid, triplicate samples per tillage treatment were analysed at 0, 4, 14, 28, 63 and 85 days after treatment (DAT) and duplicate samples at 43 DAT. For Simazine, triplicate samples per tillage treatment were analysed at 0, 15, 29, 36, 50, 64 and 86 DAT. Each soil core was divided into approximate thirds by depth – the upper 25 mm becoming subsection A, the mid 25 third subsection B, and the lower third, including any sand bound to the samples when lifted from the plastic saucer subsection C. Where necessary, large stones were removed from the subsections during processing. Each subsection was placed into an extraction vessel, and 150 ml extraction solvent (80:20 MeCN:H_2O) applied. Samples were shaken at 270 rpm on an orbital shaker for 1 hour, then centrifuged for 10 minutes at 2500 rpm using either a Heraeus Sepatech Varifuge 3.0 or Heraeus Sepatech Varifuge 3.0RS. The supernatant from each sample was decanted to a Duran bottle. The extraction process was repeated twice (total 3 solvent applications), with the supernatant pooled per subsample.

Total extract weight was recorded. All extracts were thoroughly mixed prior to analysis. For each subsamples combined extracts, ^{14}C recovery was quantified by LSC of duplicate 1 ml aliquots (scintillation cocktail ProSafe+ (Meridian Biotechnologies Ltd), instrument – Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 2920 TR or Packard Tri Carb 3100TR Liquid Scintillation Analyzer (using Quanta Smart software)).

For subsamples containing ≥ 5 % of applied radioactivity aliquots were concentrated to quantify the parent fraction of the recovered radioactivity. Aliquots were blown to dryness under a stream of nitrogen, and then suspended in 50:50 H_2O:MeCN. Recovery through the concentration process was quantified by LSC with duplicate 25 µl aliquots of the concentrated solution (scintillation cocktail ProSafe+ (Meridian Biotechnologies Ltd), instrument – Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 2920 TR or Packard Tri Carb 3100TR Liquid Scintillation Analyzer (using Quanta Smart software)).

The purity of Mandipropamid, and parent fraction of sub-sample combined extracts at 0, 4, 14, and 28 DAT were assessed by HPLC. HPLC system details: Agilent 1100 Series HPLC G1311A

Quaternary Pump, Agilent 1100 Series HPLC G1313A Autosampler, Agilent 1100 Series HPLC G1316A Column Compartment, Agilent 1100 Series G1315B Diode Array Detector, and β-Ram Model 5C Radiodetector – software LAURA 4.1.14.96. HPLC method Table 3.4.

The parent fraction of Mandipropamid in sub-sample combined extracts at 43, 63 and 85 DAT were assessed by HPLC. HPLC system details: Agilent 1200 Series HPLC G1311A Quaternary Pump, Agilent 1200 Series HPLC G1329A Autosampler, Agilent 1200 Series HPLC G1316A Column Compartment, Agilent 1200 Series HPLC G1315D Diode Array Detector, β-Ram Model 4C Radiodetector and SoFie Stop-Flow System – software LAURA 4.1.14.96. HPLC method Table 3.4.

The purity of Simazine (unlabelled and radiolabelled stock) and parent fraction of sub-sample combined extracts at 0, 15, 29, 36, 50, 64, and 86 DAT were assessed by HPLC. HPLC system details: Agilent 1200 Series HPLC G1311A Quaternary Pump, Agilent 1200 Series HPLC G1329A Autosampler, Agilent 1200 Series HPLC G1316A Column Compartment, Agilent 1200 Series HPLC G1315D Diode Array Detector, β-Ram Model 4C Radiodetector and SoFie Stop-Flow System – software LAURA 4.1.14.96. HPLC method Table 3.5.

Table 3.4 – HPLC Chromatographic Conditions for Mandipropamid

Method	TK0294824-M
Gradient	TK0294824-M
Mobile phase A	Acetonitrile
Mobile phase B	Water + 0.1 % Trifluoroacetic acid
Scintillation cocktail	Meridian Proflow G+
Elute flow rate (ml minute^{-1})	1
Scintillation cocktail flow rate (ml minute^{-1})	1
Column Packing	Synergi 4u Hydro-RP 80A
Column Dimensions	250 x 4.6 mm, 4 µm
Column Temperature (°C)	25
Detection	Radiochemical

Gradient Timetable

Time (minutes)	% A	% B
0	5	95
4	5	95
24	60	40
31	95	5
34	95	5
36	5	95
40	5	95

Table 3.5 – HPLC Chromatographic Conditions for Simazine

Method	TK0294824-S
Gradient	TK0294824-S
Mobile phase A	Acetonitrile
Mobile phase B	Water + 0.1 % Formic acid
Scintillation cocktail	Meridian Proflow G+
Elute flow rate (ml minute^{-1})	1
Scintillation cocktail flow rate (ml minute^{-1})	1
Column Packing	Phenomenex Luna 3u C18(2) 100 A
Column Dimensions	150 x 4.6 mm, 3 µm
Column Temperature (°C)	25
Detection	Radiochemical and UV at 254 nm

Gradient Timetable

Time (minutes)	% A	% B
0	5	95
2	5	95
20	95	5
24	95	5
28	5	95
30	5	95

3.2.7 Post extraction solids

Post extraction solids (PES) for subsamples from which ≥ 5 % of applied radioactivity were recovered were analysed, post extraction solids for subsamples with < 5 % of applied radioactivity were discarded. Analysed PES samples were air dried, initially within extraction vessels, then removed to plastic trays, and extraction vessels rinsed with acetone, and further air dried. Dried samples were homogenised using a pestle and mortar, and PES subsample mass recorded. Per subsample, triplicate aliquots of known mass were weighed into paper cones (lined with an additional lid to aid combustion) and capped. Samples were combusted with a Perkin Elmer Model A307 Sample Oxidiser, with a combustion duration of 2-3 minutes. Prior to oxidisation, samples were moistened with water (approximately 2 drops) to slow combustion, and treated with (approximately 3 drops) of CombustAid (Perkin Elmer) to aid combustion. Carbon dioxide emitted from the oxidised samples was adsorbed to 5 ml of Carbo-sorb E (Perkin Elmer), with 15 ml of the scintillation cocktail Permaflour E+ (Perkin Elmer). Quantification of evolved ^{14}C by LSC on a Perkin Elmer Tri Carb 2910 TR.

3.2.8 Modelling and analysis

Single first order (SFO) kinetic models were calculated for the degradation of parent compound, % parent applied defined as % recovery x % ROI (including only subsamples with ≥ 5 % recovery by solvent extraction), using Computer Assisted Kinetic Evaluation (CAKE) version 3.3 software (Tessella Software). SFO kinetic models were also calculated for dissipation (defined as the decline in total extractability over time). SFO model equation:

$$C_t = C_0 e^{-kt}$$

Where C_t = concentration at time t, C_0 = concentration at time 0, e = base e, k = rate of constant decline 1/days, and t = time.

CAKE fit settings for all modelling – convergence tolerance 1 x 10^{-5}, maximum iterations 1 x 10^5, SANN max iterations 1 x 10^5, optimiser – iteratively reweighted least squares, maximum reweightings 1 x 10^2, error variance tolerance 1 x 10^{-5}.

3.3 Results

3.3.1 Mandipropamid

The SFO modelled time period required for 50 % degradation (DT_{50}) of Mandipropamid applied to IT soils was 143 days, and to RT soils was 125 days (chi-squared 1.53 and 2.00 respectively). SFO models and observations Figure 3.6, showing very similar rates of degradation for both soils. IT SFO parameters Parent_0 95.56 and k_Parent 0.004853. RT SFO parameters Parent_0 96.57, and k_Parent 0.005566. The difference in the rate of degradation of Mandipropamid in soils under RT and IT is not considered significant.

Figure 3.7 shows the modelled (SFO) and observed data for the dissipation of Mandipropamid; the time period required for 50 % dissipation ($DIST_{50}$) in IT soils is 170 days, and in RT soils 143 days (chi-squared 1.97 and 2.26 respectively). The rate of dissipation in both soils can be seen to be very similar (Figure 3.7), although RT observations are more variable. IT SFO parameters Parent_0 99.17 and K Parent 0.004834. RT SFO parameters Parent_0 101, and K_Parent 0.004068. The difference in the rate of dissipation of Mandipropamid in soils under IT and RT is not considered significant.

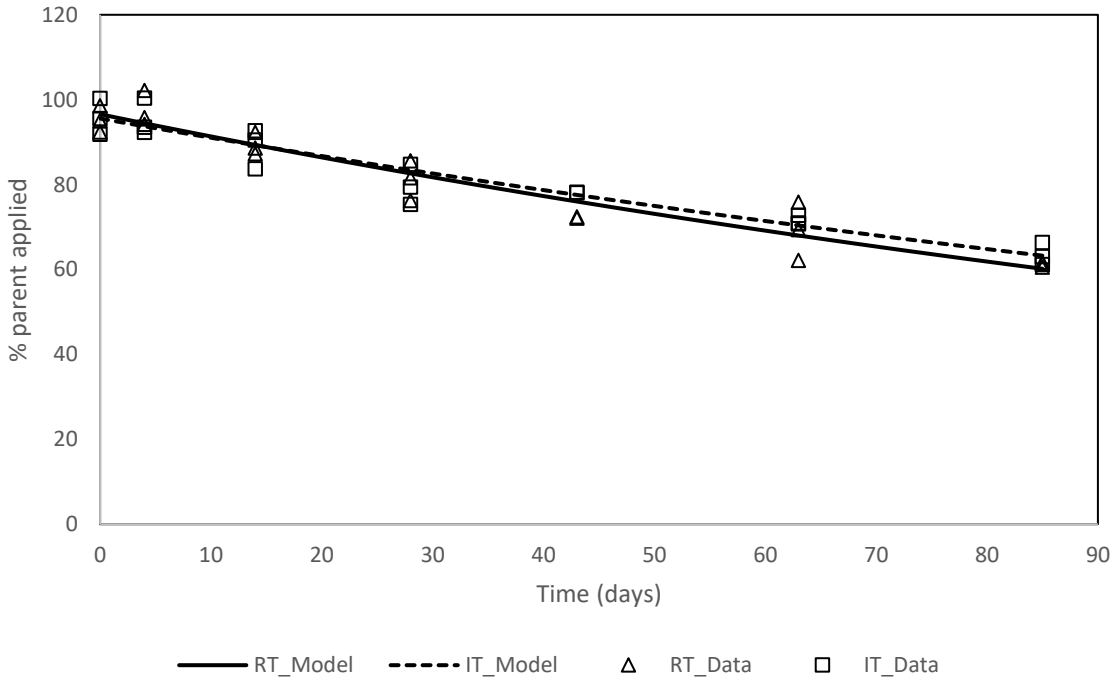


Figure 3.6 – Degradation of Mandipropamid

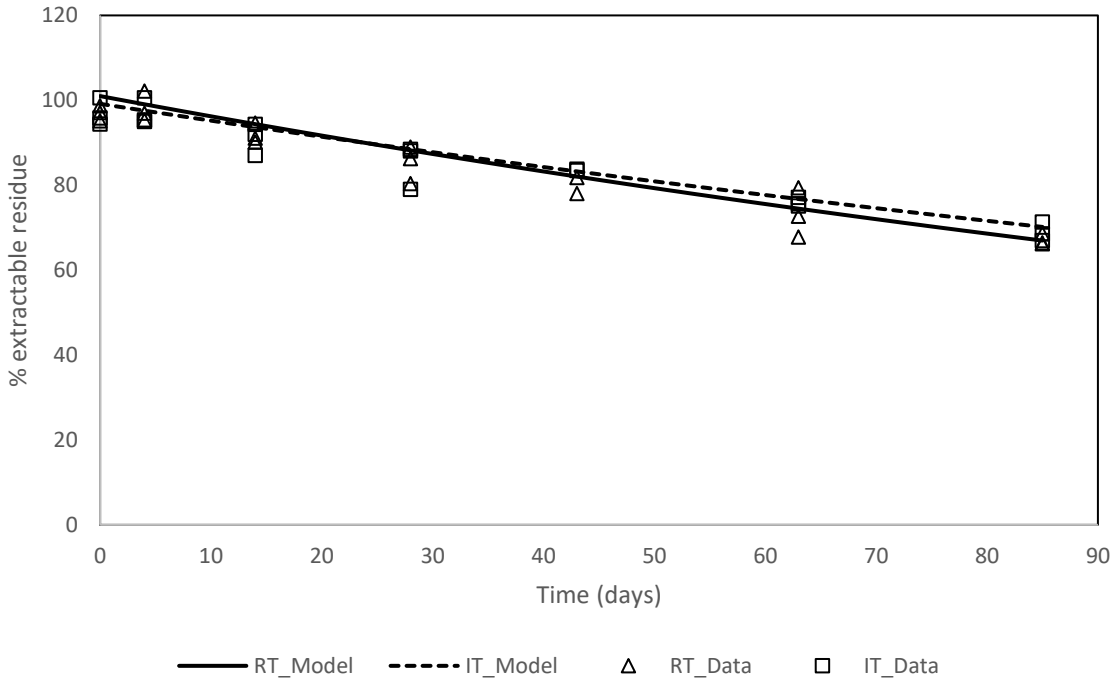


Figure 3.7 – Dissipation of Mandipropamid

Throughout the experiment, the majority of the extractable residue remained parent compound, with degradant(s) accounting for a maximum (in a single core) of 11.2 % extractable residues (Figure 3.8). As expected in a non-sealed system, the mass balance declined to 88.22 % in soils under IT, and 86.53 % for RT (Figure 3.9), due to an assumed loss of volatiles (including CO_2). A small fraction of material that may not have been accounted for is that lost as non extracted residues in the B and C subsections from which extracts contained <5 % of applied radioactivity. However, there is little evidence of parent compound, or degradants being mobile within the soil cores (Figure 3.11). This is consistent with the known low mobility of the parent compound (EFSA, 2012). There is an increase of residues remaining bound to the PES throughout the experiment to a mean of 19.29 and 19.44 % in soils under IT and RT respectively at 85 DAT, however there is a slight decrease in values at 43 DAT (Figures 3.9 and 3.10).

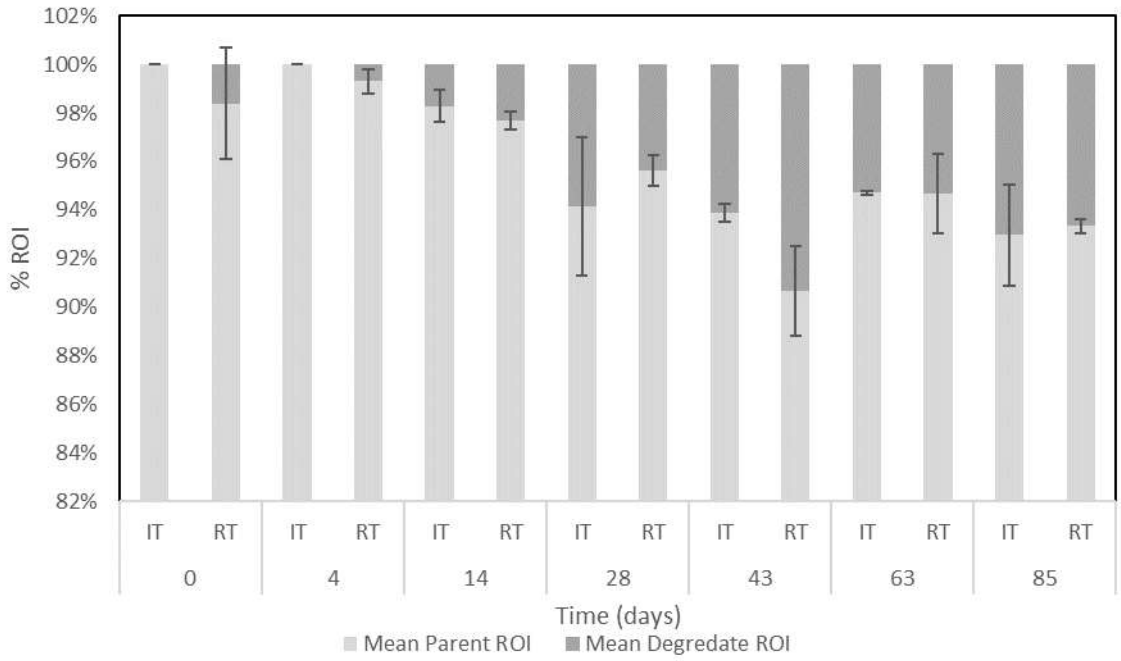
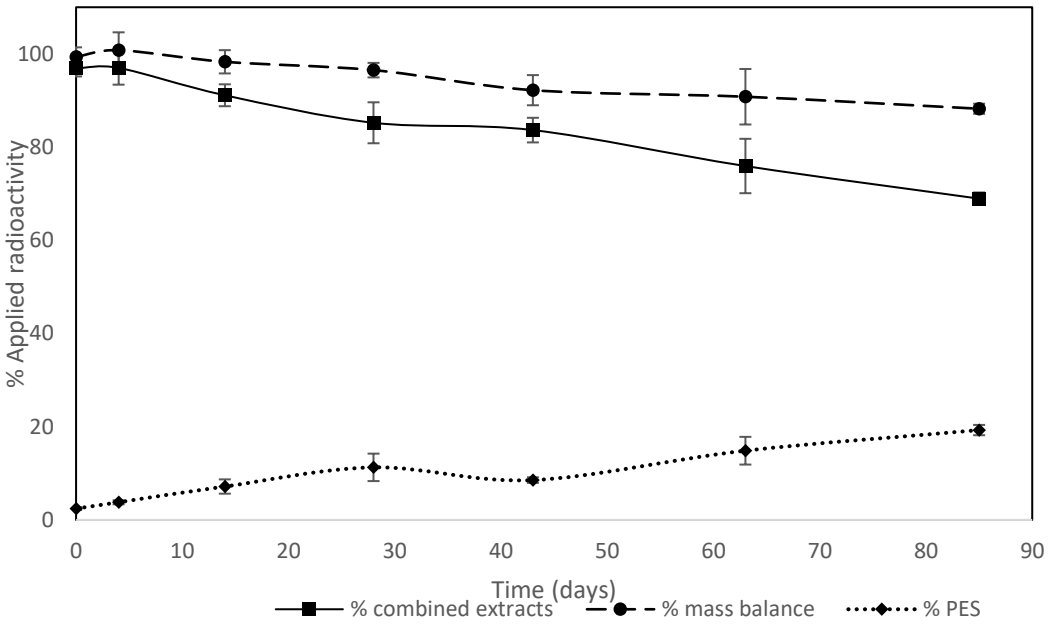


Figure 3.8 – Mean parent Mandipropamid fraction of extractable residues for soils under IT and RT. Error bars 1 standard deviation. % ROI is equivalent to % area of either parent or degradant peaks in HPLC chromatogram

A



B

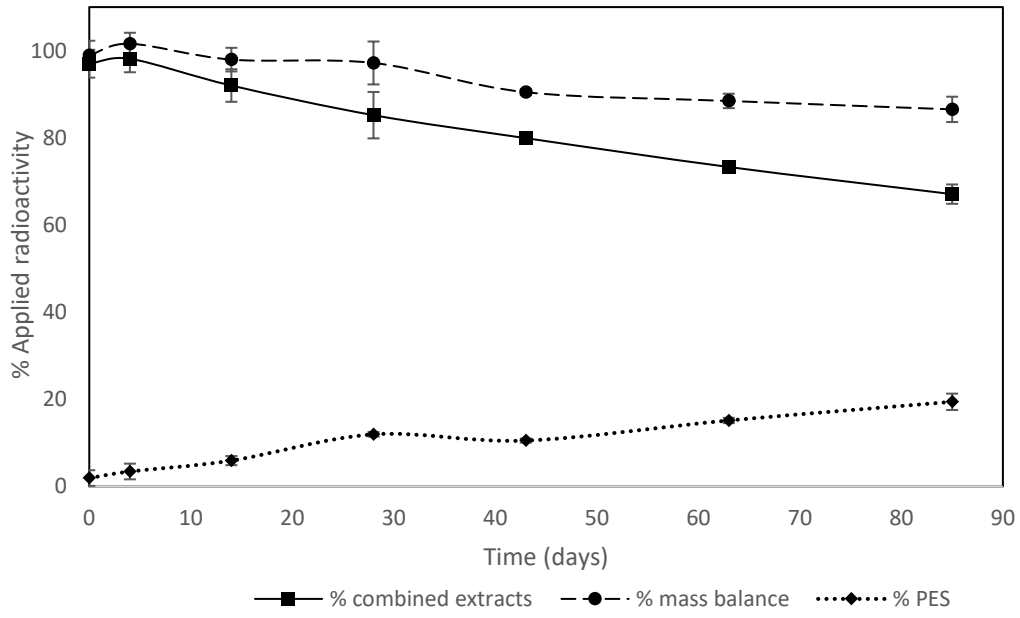


Figure 3.9 – Mean mass balance (shown ± S.D.) of Mandipropamid degradation experiment for soils under (A) IT and (B) RT

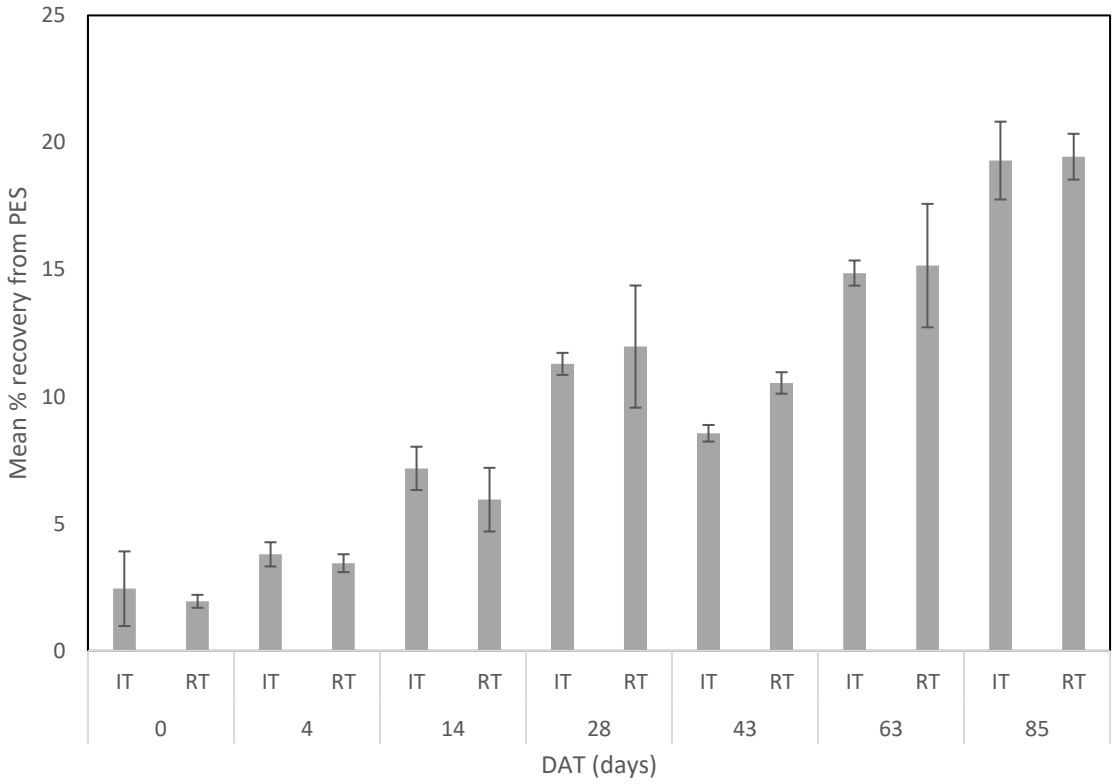


Figure 3.10 – Mean recovery (shown ± S.D.) of applied radioactivity recovered from PES for degradation of Mandipropamid in soils under IT and RT

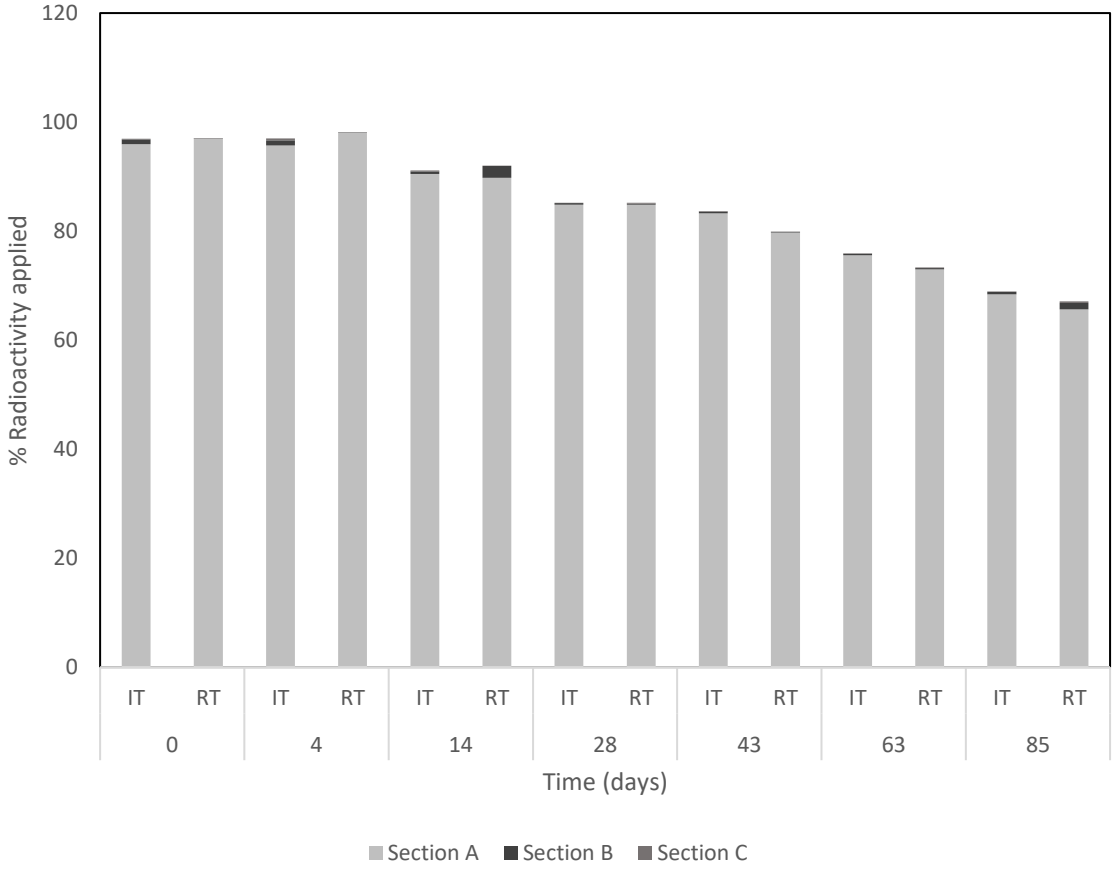


Figure 3.11 – Mean distribution of applied radioactivity per section combined extract for Mandipropamid. Where Section A is the upper section of each core, Section B the middle, and Section C the lowest.

3.3.2 Simazine

The SFO DT_{50} of Simazine applied to IT soils was 43.9 days, and to RT soils was 48.1 days (chi-squared 8.51 and 11.9 respectively). SFO models and observations Figure 3.12, the similar rates of degradation within the two soils is apparent, as is the higher variability of the RT observations. IT SFO parameters Parent_0 91.01 and k_Parent 0.01579. RT SFO parameters Parent_0 92.23, and k_Parent 0.01442. The difference in the rate of degradation of Simazine in soils under RT and IT is not considered significant.

For the dissipation of Simazine, the $DIST_{50}$ in IT soils is 80.5 days, and in RT soils 80.3 days (chi-squared 4.05 and 5.25 respectively). IT SFO parameters Parent_0 97.98 and K Parent 0.008614. RT SFO parameters Parent_0 93.3, and K_Parent 0.00863. SFO models and observations Figure 3.13. The near identical rate of dissipation of Simazine within the two soil types is apparent in Figure 3.13, despite the greater variability within the RT observations. The difference in the rate of dissipation of

Simazine in soils under IT and RT is not considered significant. The material that was recoverable for Simazine included a higher proportion of degradant(s), which in total was more variable than that for Mandipropamid (Figure 3.14).

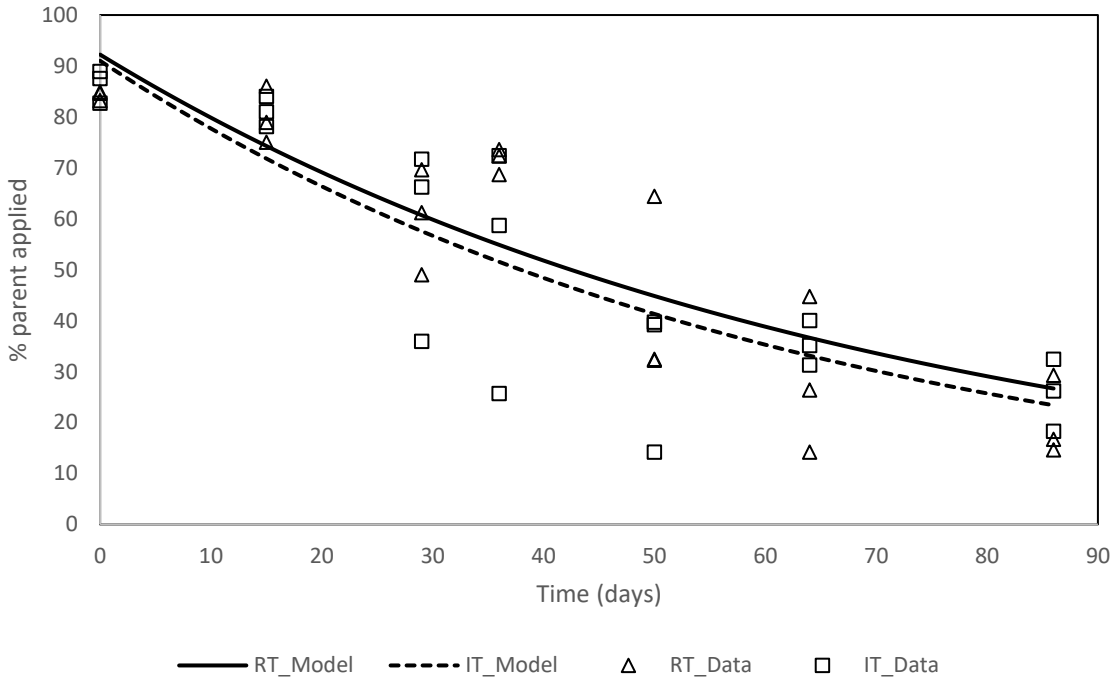


Figure 3.12 – Degradation of Simazine

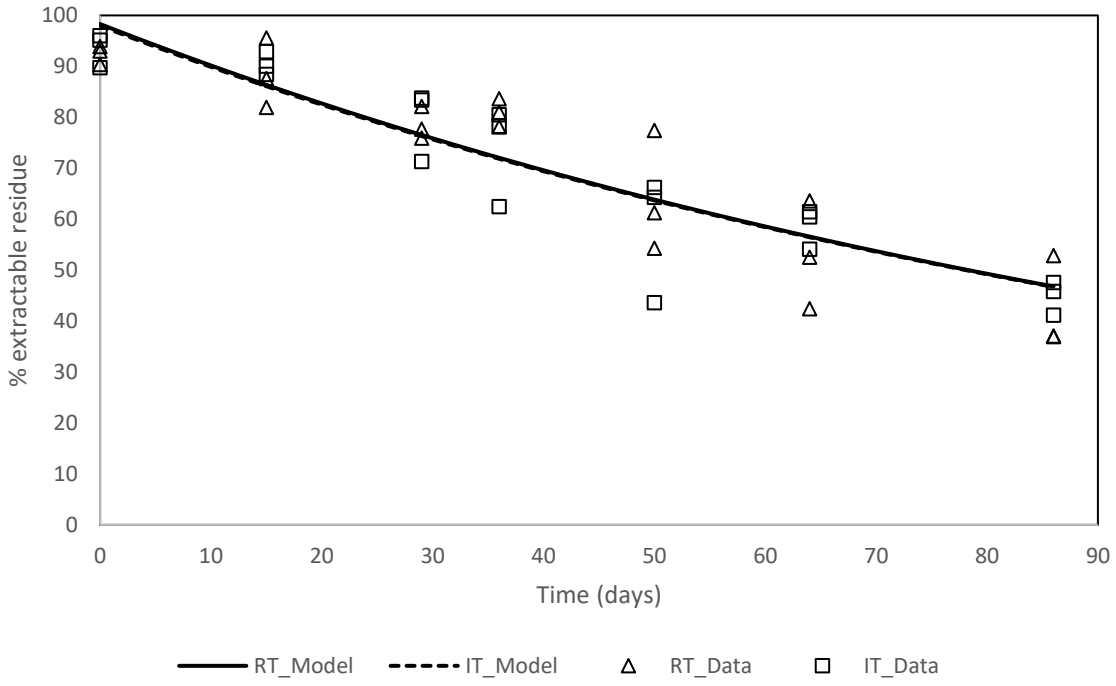


Figure 3.13 – Dissipation of Simazine

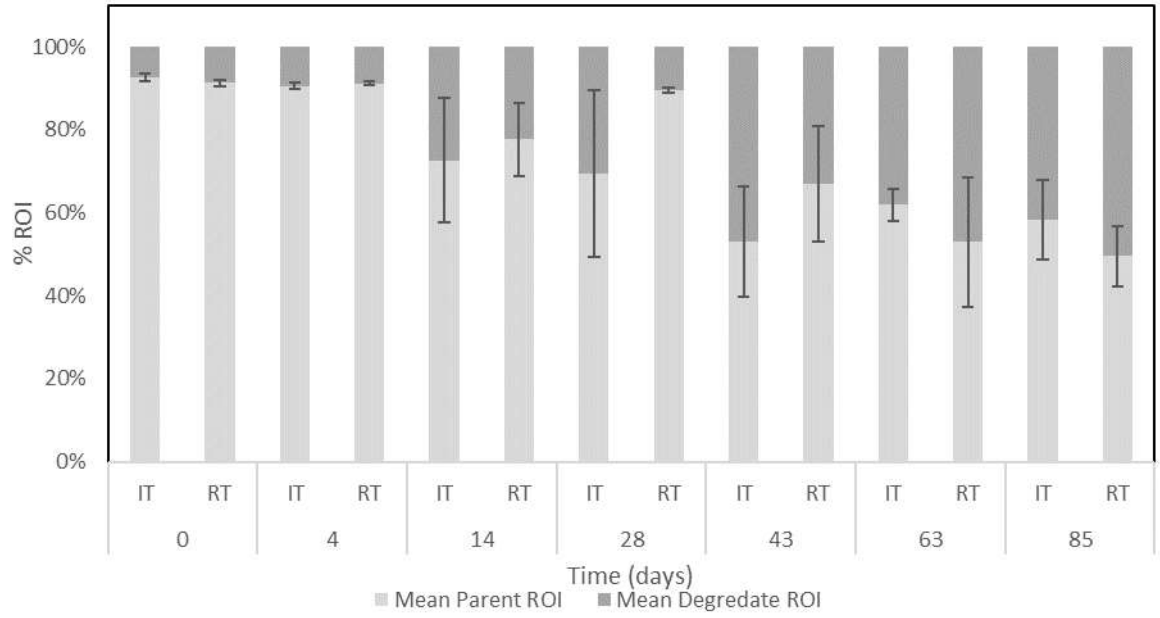
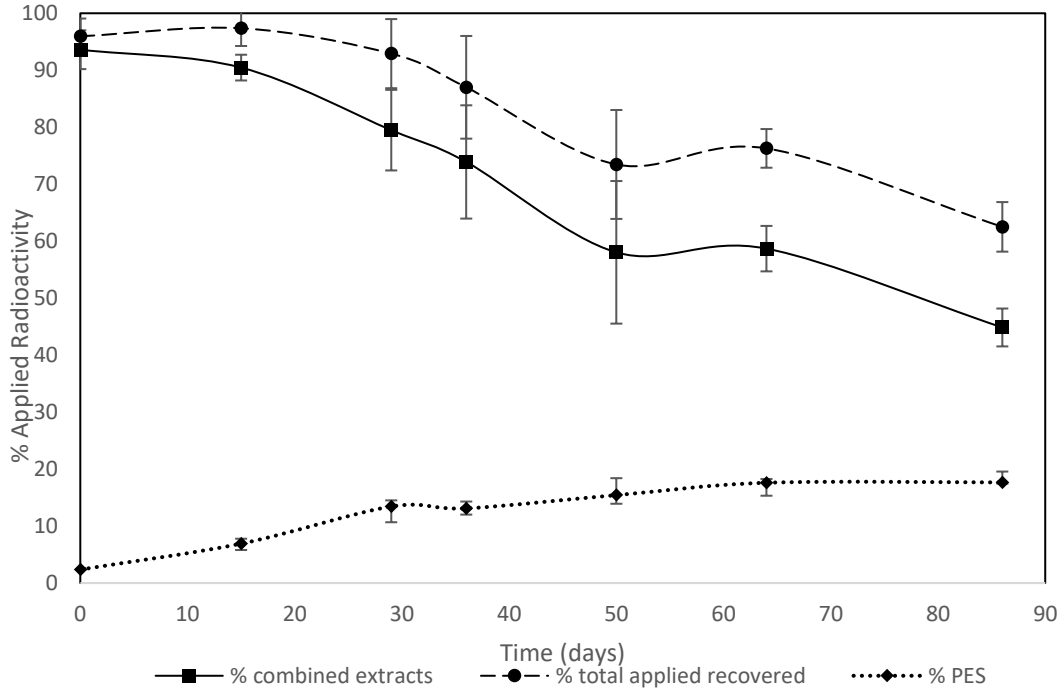


Figure 3.14 – Mean parent Simazine fraction of extractable residues for soils under IT and RT. Error bars one standard deviation. % ROI is equivalent to % area of either parent or degradant peaks in HPLC chromatogram.

Total recovery for Simazine declined to 62.51 and 59.48 % for IT and RT respectively at 86 DAT. As with Mandipropamid, very little material is associated with the lower core sections (Figure

3.17) and unaccounted for radioactivity was likely emitted as volatiles (including CO_2). Non extractable residue recovery from PES increase rapidly to 29 DAT (mean 13.43 and 14.29 % recovery from IT and RT respectively) but then accumulate at a lower rate, reaching a mean recovery of 17.66 and 17.19 % from IT and RT respectively at 86 DAT (Figures 3.15 and 3.16). This bimodal behaviour is not currently explained.

A



B

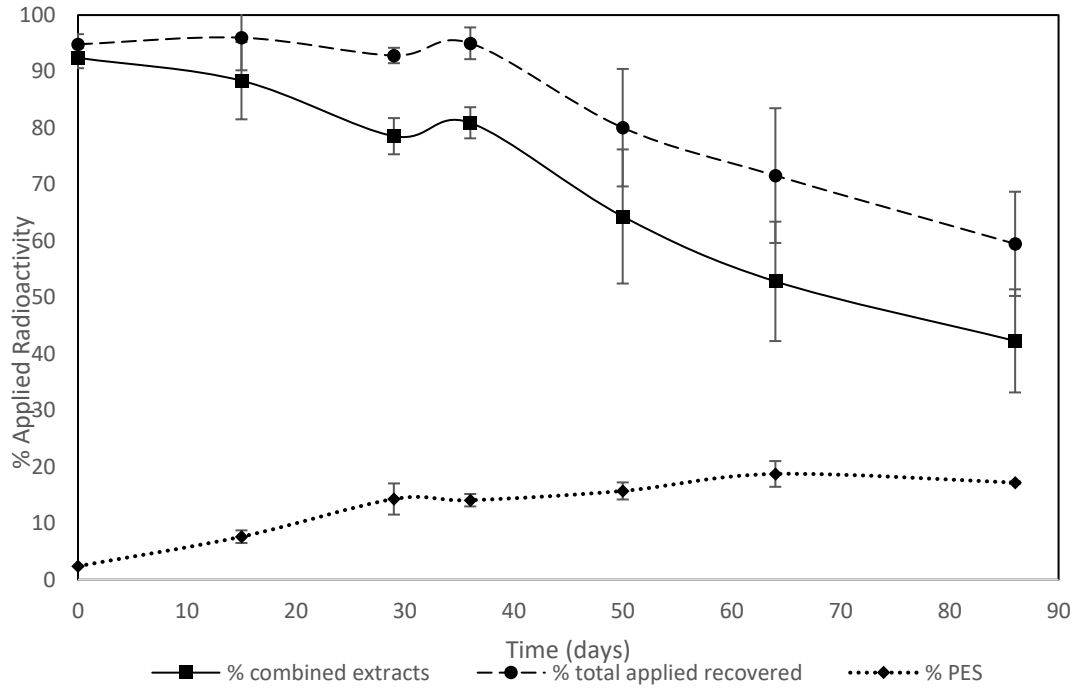


Figure 3.15 – Mean mass balance (shown ± S.D) of Simazine degradation experiment for soils under (A) IT and (B) RT

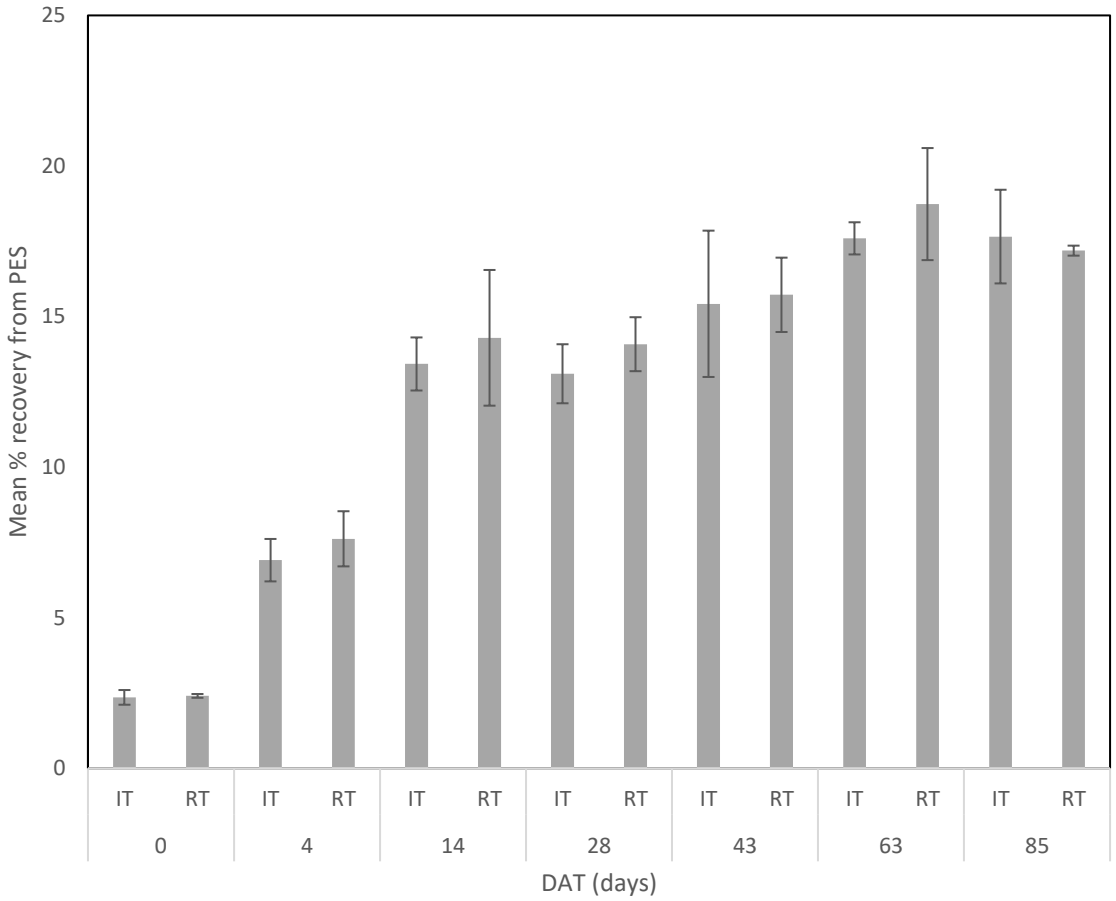


Figure 3.16 – Mean recovery (shown ± S.D.) of applied radioactivity recovered from PES for degradation of Simazine in soils under IT and RT

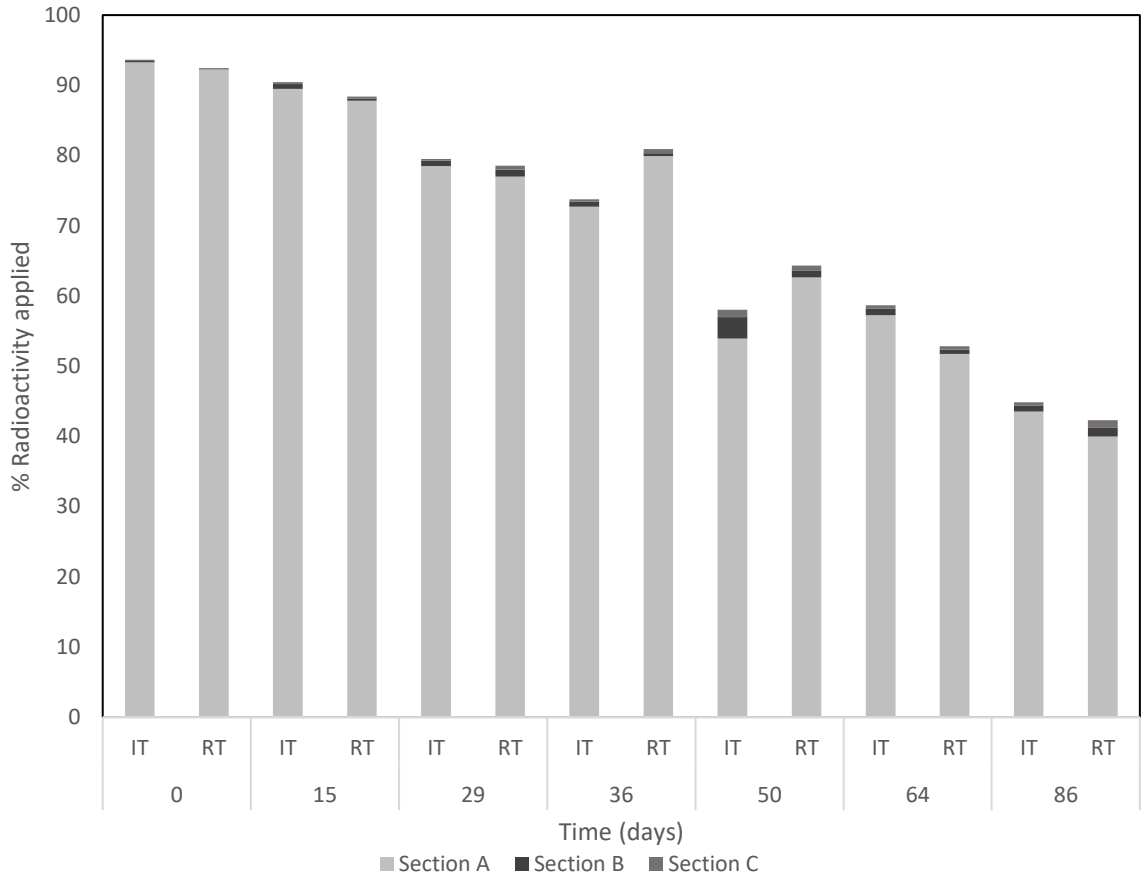


Figure 3.17 – Mean distribution of applied radioactivity per section combined extract for Simazine. Where Section A is the upper section of each core, Section B the middle, and Section C the lowest.

Each soil core is an individual and unique system within the experiment. Although sampled within a small area, the mass of the soil cores varied substantially, for IT between 401 and 556 g, and for RT 467 and 540 g. However, the mass of soil within each core has no relationship to the residual from the SFO modelled rate of degradation for either Mandipropamid (Figure 3.18 and 3.19) or Simazine (Figure 3.20 and 3.21). This suggests that variation in moisture content from core to core, which will have occurred due to each soil core of a tillage treatment being watered equally did not affect the rate of compound degradation.

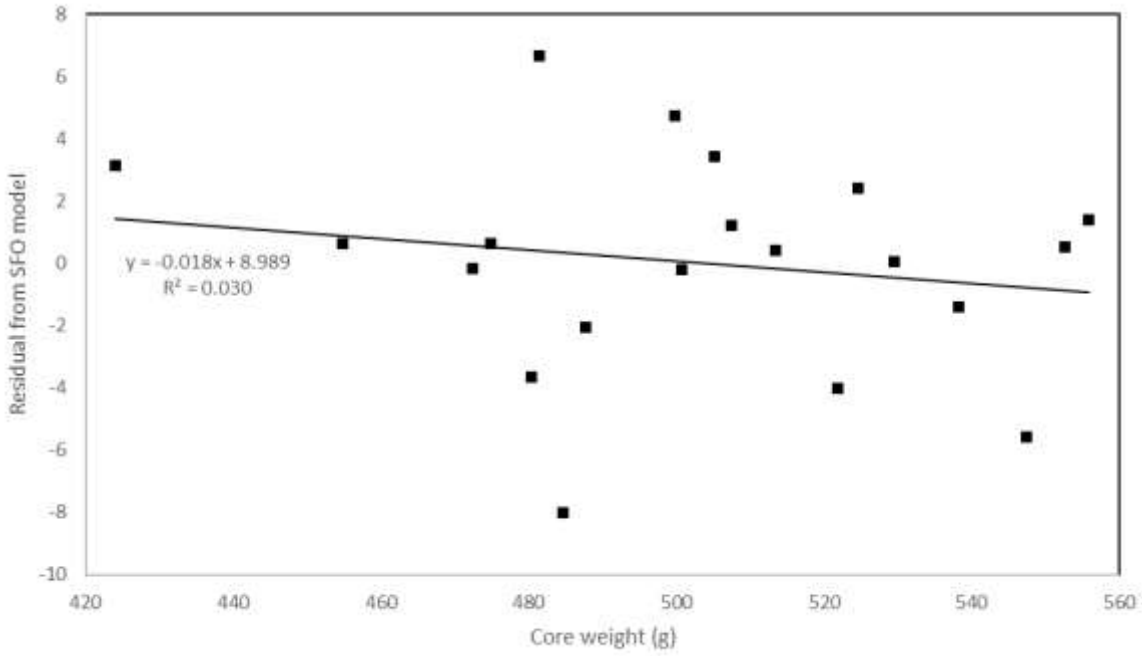


Figure 3.18 – Residual from modelled SFO degradation of Mandipropamid in IT soils relative to mass of soil in core

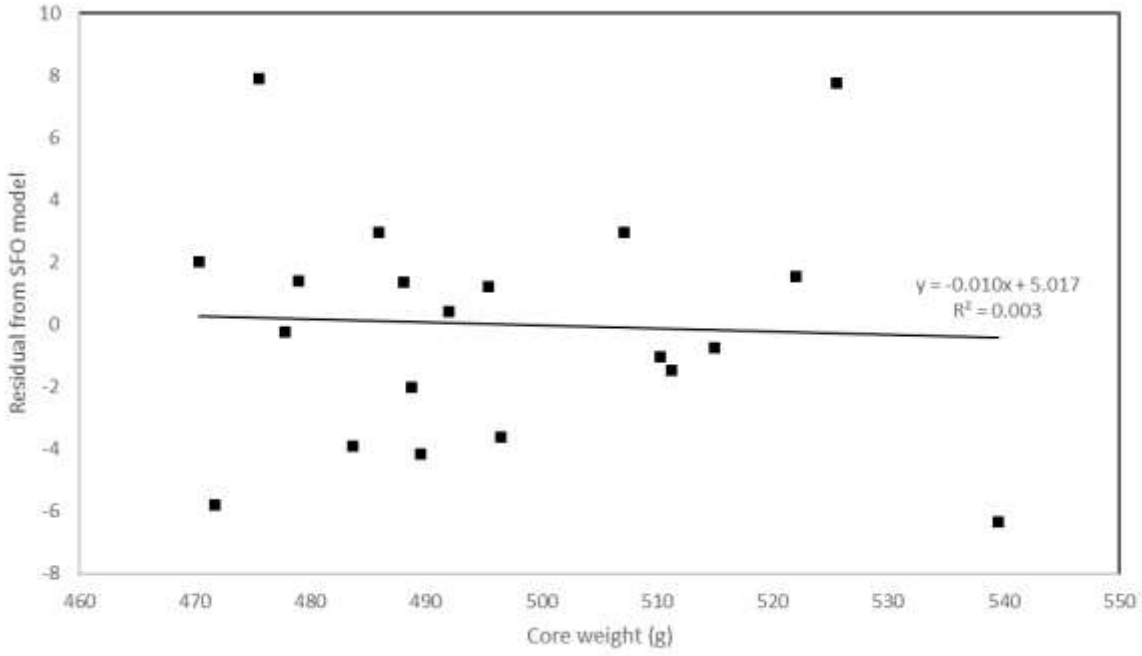


Figure 3.19 – Residual from modelled SFO degradation of Mandipropamid in RT soils relative to mass of soil in core

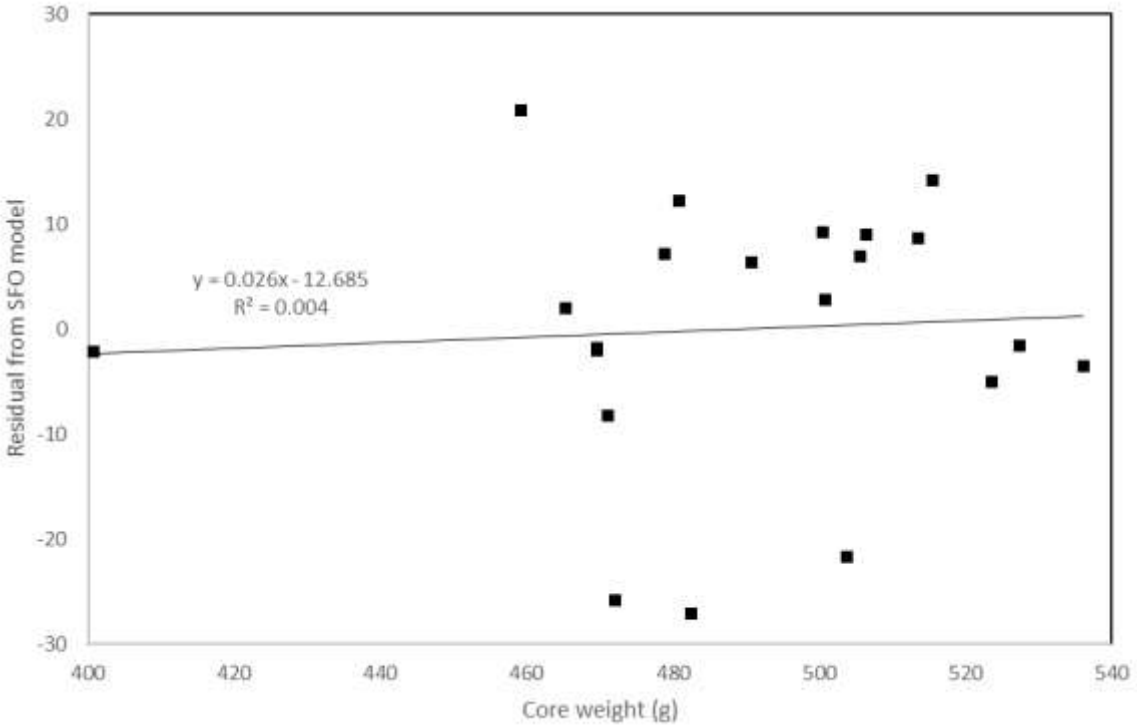


Figure 3.20 – Residual from modelled SFO degradation of Simazine in IT soils relative to mass of soil in core

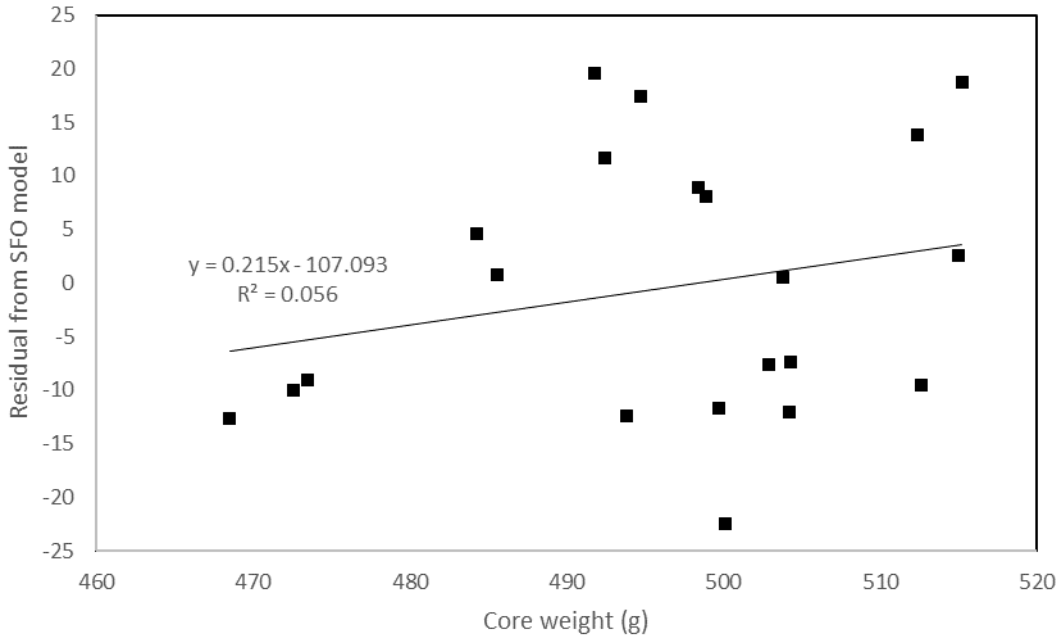


Figure 3.21 – Residual from modelled SFO degradation of Simazine in RT soils relative to mass of soil in core

3.4 Discussion

3.4.1 Degradation of Mandipropamid and Simazine

The rate of Mandipropamid degradation is substantially slower than as determined in previous studies. Regulatory OECD 307 based studies have reported the DT_{50} of Mandipropamid to range between 23.4 and 83.9 days (EFSA, 2012, Lewis et al., 2016) – however such studies are conducted with soils from pastures, not arable land. Field studies have found the rate of Mandipropamid degradation to be faster than lab-based studies with DT_{50} values of 5.6 – 29.2 days (EFSA, 2012, Lewis et al., 2016). The low rate of degradation of Mandipropamid in the intact core system is unexpected, the cores being expected to function as an intermediate tier of 'realism' between field soils (fast degradation) and OECD 307 studies (slow degradation) that has been reported previously. This may reflect differences in the composition of degraders between soils, or that Mandipropamid is a compound subject to degradation or sorption processes captured in field and OECD 307 studies, but not the present core system. Similarly, Dougan et al. (2013) report the degradation of Fomesafen to be slower in static core systems similar to those in the present study than OECD 307 studies. This difference was reduced by a dynamic moisture regime, given the variable control of moisture content through the present study (Figures 3.2 and 3.3), the observed slow rate of degradation may have been greater in a more tightly controlled system.

It is noted that the moisture control of study cores throughout the experiment – aiming to maintain a constant moisture content of 25% VWC – was poor (Figure 3.2). This is attributable to both poor experimental design – the sand moisture reserve failing to act as a sufficient or effective reservoir of water for the soil cores – and that being unable to access the experiment each weekend prevented close manual adjustment of moisture levels. The accuracy and precision of the soil moisture probes used in the present study is poor (Figure 3.3). These issues may be addressed by use of tension tables, peristaltic pumps, or weight-sensor controlled pumps to adjust moisture content automatically, or by closer manual access to control moisture content. Further, conducting the experiment in conditions where evaporation is reduced (the CT room having very high airflow for safety) may improve moisture control. However, Figures 3.18, 3.19, 3.20 and 3.21 show that the variable moisture content of the studied cores had no effect on the rate of degradation – cores of large range in mass (401-556g) receiving equal water to adjust for moisture, therefore having different moisture contents. Further work may have included normalisation of degradation rates based upon moisture and soil temperature – however given the variation between cores, the accuracy of any such adjustment is debatable.

The observed rate of Simazine degradation is consistent with previously reported data, where the DT_{50} of Simazine has been observed to vary between 27-102 days in soils (Lewis et al., 2016, Liao and Xie, 2008). Inoculated soils have been shown to more rapidly degrade Simazine (Liao and Xie, 2008) and Simazine degradation, like that of other triazine CPPs may be subject to accelerated degradation, and cross acclimatisation (Rouchaud et al., 2000).

The variance of degradation in Simazine is greater than that observed for Mandipropamid, and again is greater in soils under RT than IT. Variance through the duration of the experiment can be seen to vary for both IT and RT, being less at both the beginning (0 and 15 DAT) and end (86 DAT) than for time points in the middle of incubation. This may reflect the low degree of degradation that had occurred prior to 15 DAT, and a reduction in variation as the faster degrading systems have a lower availability of Simazine to degrade. See Figure 3.12.

Differences in the rate of degradation of compounds that may arise due to increased availability of compounds in IT soils relative to RT soils, from carbon accumulation are not observable. This is true both for effects that may have been immediately apparent – expected higher carbon content in RT soils reducing availability to degraders – and slower processes – such as aged sorption that reduce the availability of compounds to degraders as compounds diffuse into soil aggregates – a phenomenon that may be increased with the greater abundance and stability of soil macroaggregates in RT relative to IT (Suddaby et al., 2013, Suddaby et al., 2016, Commission, 2021).

The variation observed in the fate of Simazine may reflect either uneven application – identified as a leading cause of variation in Simazine degradation by Walker and Brown (1983) – or due to chemical and/or biological variance in the soil cores. Spatial variance in the degradation potential of soils has been repeatedly demonstrated, and is understood to be more apparent at smaller spatial scales (Walker et al., 2001, Bending et al., 2006, Dechesne et al., 2014). Causes of such spatial variation include pH and microbial variation.

The lack of substantial difference in the results may reflect the relatively short duration which the experimental plots had been under different tillage methods, a period of less than two years and two instances of tillage. More pronounced effects of cultivation on the environmental fate of crop protection products may occur in studies conducted with soils that have been managed for longer periods under differing systems – for instance, Haddaway et al. (2017) demonstrate reported SOC accumulation to be dependent on study duration.

In conclusion, no substantial difference was identified in the rate of degradation of either Mandipropamid or Simazine in soils under IT or RT. However, the results are suggestive of the more

heterogeneous effects of RT on arable soils creating areas of greater and lesser CPP degrading potential relative to arable land under IT. The proposed mechanism for this increased variation is that reducing tillage intensity (going from IT to RT (and potentially again to no tillage systems)) reduces the energy input (Morris et al., 2010) and therefore mixing of the soil (Hula and Novak, 2016). This reduced mixing is both vertical – stratification of crop residues and soil carbon at the soil surface and in top soils – and horizontal – reduced movement of material across a field, and reduced movement of material downhill, as that is encouraged by ploughing. Reduced mixing also reduces the rate of soil aggregate turnover, and increases macroaggregate abundance. A more diverse range of soil aggregates, pore spaces, and mineral and organic environments facilitated by reduced mixing may facilitate the development of distinct microbial communities, including variable presence of degraders and also more variance in non-microbial degradation processes. These more heterogenous soil environments may lead to more heterogenous rates of degradation within a sampled area.

Chapter 4: Microbial Community Response to Incubation and the Degrading Fraction

4.1 Introduction

4.1.1 Spatial variation in CPP degradation

Changing tillage methodology has the potential to alter the environmental fate of agrochemcials, by means including the interception of CPPs by crop residues, altered soil pore structures, and potentially more spatially distinct soil microbial communities (Alletto et al., 2010). Whilst in aggregate in Chapter 3, no substantial difference was observed in the rate of degradation in soils under IT or RT, greater variability in the rate of degradation and dissipation was apparent in soils under RT relative to IT (Figures 3.6, 3.7, 3.12, and 3.13).

At field scale, spatial variation in the degradation of Simazine may be most attributable to uneven application (Walker and Brown, 1983), however given the small degree of variation in recovered radioactivity at DAT 0 as detailed in Chapter 3, application or sample analysis variation is unlikely to be the cause of the observed differences in the rate of degradation between soil cores. The relative role of variation in biotic or abiotic processes varies by studied compound - Bending et al. (2006) reporting the majority of variation in isoproturon and azoxystrobin to be due to biotic process, but less than 7 % of variation in diflufenican may be attributed to biotic processes. Similarly Charnay et al. (2005) report spatial variation in atrazine and isoproturon to be related to biotic factors, and metamiron to sorption processes. Alteration of pH alone can strongly influence both compound availability to degradation processes, and the abundance of microbial degraders (Bending et al., 2003, Kah et al., 2007).

Variability in pesticide degradation tends to increase as the studied volume of soil decreases (Dechesne et al., 2014). Gonod et al. (2003) report greater variability in CPP degrading potential in soil aggregates of 2-3 mm than aggregates of 4-6 mm. Degradation potential of soil aggregates may depend upon both the microbiome of the aggregate, and available nutrients – principally C – to degraders within each aggregate (Gonod et al., 2003). Higher variability in the distribution of soil carbon within the soil profile due to a lower intensity of tillage could lead to increased variability compound degradation.

4.1.2 Microbial degradation of Simazine

The degradation of Simazine, and other s-triazine herbicides is well studied within scientific literature, see for example Kaufman et al. (1965), Wackett (2002), Kodama et al. (2001), Caracciolo et al. (2005), Govantes et al. (2009), Rehan et al. (2016), (Scherr et al., 2017). Comparatively Mandipropamid degradation – with few recent exceptions (Han et al., 2021) – is relatively poorly detailed within the literature. Consequently, analysis of fraction of the soil microbiome associated with CPP degradation focused on degradation of Simazine.

A possible biodegradation pathway for Simazine is proposed in Figure 4.1, adapted from Hershberger (1998), Govantes et al. (2009), and Sagarkar et al. (2013). Biodegradation of Simazine may occur by several pathways, producing a range of intermediary metabolites, leading to a common metabolite, cyanuric acid, which is further metabolised to the point of mineralisation (Hershberger, 1998, Govantes et al., 2009, Sagarkar et al., 2013).

Genes associated with s-triazine degradation are known to be widespread amongst a multitude of soil bacteria (de Souza et al., 1998b). At least some of the associated genes – *atzA, atzB,* and *atzC* – are known to be borne by self-transmissible plasmids (de Souza et al., 1998a, Wackett, 2002). Mineralisation of s-triazine herbicides is most commonly achieved by a cohort of bacteria each carrying one or more relevant genes, as opposed to a single bacteria (Govantes et al., 2009).

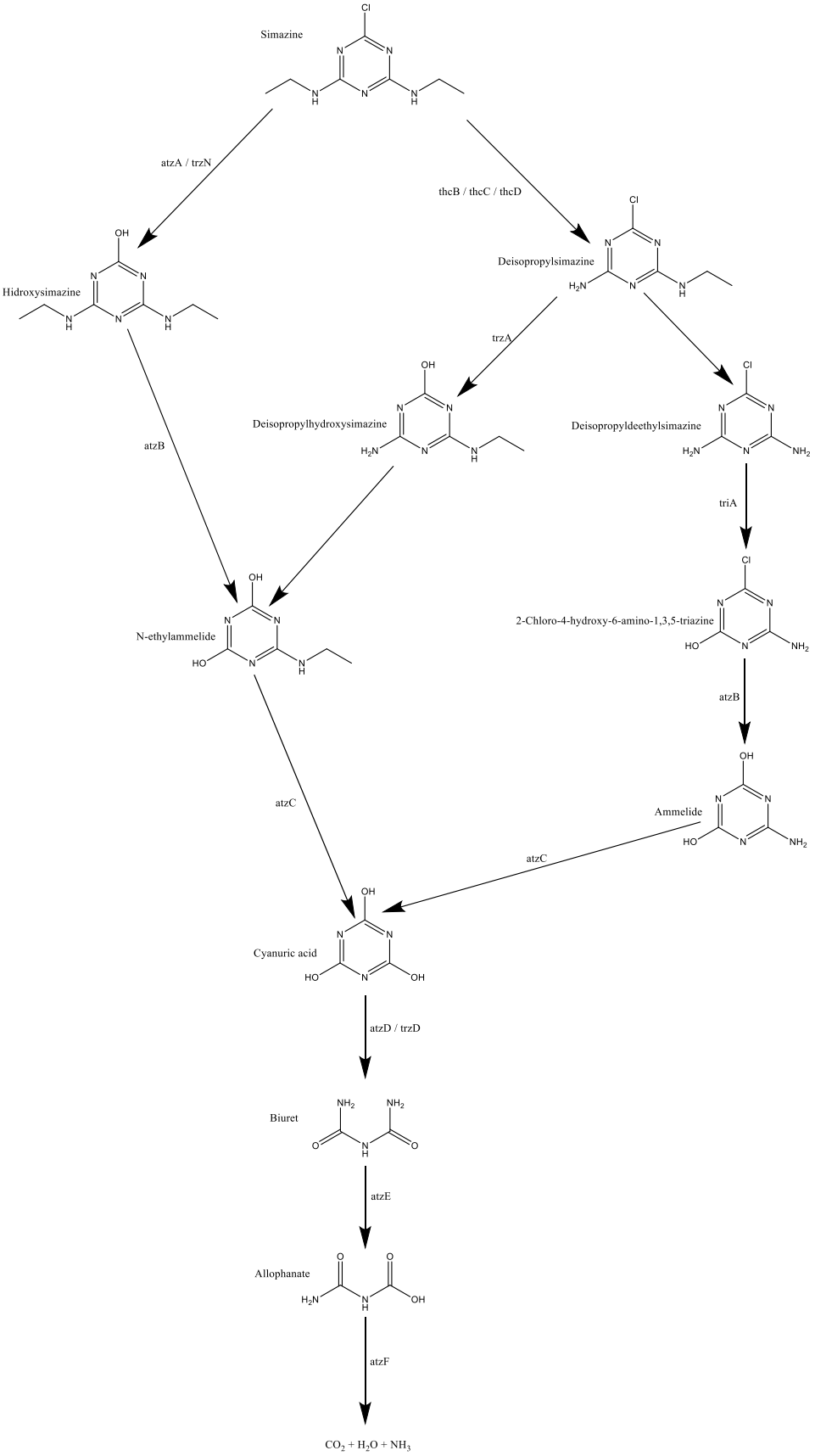


Figure 4.1 – Proposed degradation pathway of Simazine and associated functional genes. Adapted from Hershberger (1998), Govantes et al. (2009) and Sagarkar et al. (2013).

4.1.3 Incubation conditions and the soil microbiome

Sales of CPPs in most markets are subject to extensive control from regulators (for example EFSA in the EU, CRD in the UK, and EPA in the USA), who permit the sale and use of active ingredients and pesticide formulations (Commission, 2013a, Commission, 2013b). Regulators require detailed dossiers which include information on the safety of compounds to humans, wildlife and the environment. Central to the safety profile of a CPP is the persistence of a compound and its metabolites. Persistence of these compounds may be assessed both in laboratory and field studies (Commission, 2013a, Commission, 2013b). Laboratory assessment of compound persistence in soils is usually centred on tests conducted to the OECD 307 standard (OECD, 2002). The incubation of soils under standardised conditions – moisture at pF2 (where pF is defined as the log of cm water column, see OECD (2002)), in the dark and at 20 °C are common to these standard regulatory tests and in some higher tier – or more realistic – experiments (including Chapter 3) (OECD, 2002, Dougan et al., 2013). Both incubation conditions, and soil processing – including sieving and moisture control – conducted in OECD 307 studies can alter the microbial community of study substrates (Miranda, 2019). Miranda (2019) identified the soil processing and incubation conditions of OECD 307 to cause unpredictable stochastic shifts in microbial community, depletion of available nutrients, and a retardation of metabolism.

Conducting degradation experiments under standardised conditions allows simpler comparison and modelling of compound behaviours in the environment, with OECD 307 intended to capture the microbial degradation of compounds (OECD, 2002). However, the current standardised testing is insufficient to accurately characterise total degradation of compounds by the microbial community, in particular failing to capture the role of phototrophs in the degradation of CPPs (Thomas and Hand, 2011, Davies et al., 2013a, Davies et al., 2013b).

Moisture cycling of microbial communities, such as occurred in Chapter 3 (see Figure 3.2), can alter microbial communities. Patterns of drying and rewetting soils can alter soil microbiome structure, with communities that are frequently exposed to drought growing up to 40 % more rapidly following rewetting (de Nijs et al., 2019). de Vries et al. (2018) identify that soil fungal communities are more resilient to drought conditions than bacterial communities. Bacteria with the highest relative abundance were most strongly affected (reduced in abundance) by drought (de Vries et al., 2018).

4.1.4 Aims and objectives

The present study sought to establish if the functional genes considered responsible for the degradation of Simazine were more heterogeneously distributed in soils under RT than IT, either by way of presence/absence, abundance, or genetically. Further, the present study investigated if the community composition of the soil microbiome was more heterogeneous in soil cores collected from RT soils than IT soils, and to establish any effect of incubation conditions on soil microbial communities.

4.2 Methodology

4.2.1 Field Site

Soil cores were collected from the RT and IT strips of Warren field of the SoilBioHedge experiment, detailed in Chapter 2 and by Holden et al. (2019), and sampled at the same time, and by the same techniques as detailed in Chapter 3.

4.2.2 Incubation of soil cores

Soil cores for studying the soil microbiome throughout the compound degradation experiment detailed in Chapter 3 were collected and incubated under identical conditions (excluding the application of compound (due to constraints required by the sponsor)) to those in the degradation study. 12 soil cores for each tillage type (IT and RT) were incubated, and sampled in triplicate at 0, 30, 56 and 87 DAT.

At sampling soil was removed from the metal cores, loosely homogenised by hand, placed in a polyurethane bag and flattened. Samples were then rapidly frozen by being placed in a -80 °C freezer. Samples were stored at -80 °C at Jealott's Hill International Research Centre before being shipped with dry ice to the University of York. Samples were again stored at -80 °C in York. Samples were halved for analysis, half being freeze dried (analysis presented within this study) and half remaining at -80 °C.

4.2.3 Molecular methods for functional genes

In triplicate – as a single sampling was considered insufficient for a presence/absence assessment of functional genes – DNA was extracted as detailed in Chapter 2 for each sample. Of the functional genes identified in Figure 1, PCR primers and conditions were identified in the literature for the following genes – atzA, atzB, atzC, atzD, atzE, atzF, trzD and trzN. PCR was attempted using the primers as detailed in Table 4.1. Reaction conditions were varied extensively, including; multiple DNA polymerases, annealing temperatures, extension periods, cycle number, and primer, template, DNA polymerase, BSA, $MgCl_2$ and dNTP concentrations. Repeat PCRs using the nested product of previous reactions were conducted. Success of PCR reactions assessed on basis of bands of desired product length identified by gel electrophoresis, agarose gels containing SYBRSAFE (Invitrogen) at 1-10 µl per 100 ml of gel, excitation by blue light, loading dye 6 X Loading Dye (#R0611, Thermo Scientific).

All attempts to amplify functional genes expected to facilitate Simazine degradation were unsuccessful, the reasons for which are discussed below, in section 4.4.1.

Table 4.1 – Primers for functional genes for Simazine degradation

Functional Gene	Primer	Reference
atzA	CCATGTGAACCAGATCCT	de Souza et al. (1998b)
	TGAAGCGTCCACATTACC	
atzB	TCACCGGGGATGTCGCGGGC	
	CTCTCCCGCATGGCATCGGG	
atzC	GCTCACATGCAGGTACTCCA	
	GTACCATATCACCGTTTGCCA	
atzD	TCCCACCTGACATCACAAAC	Devers et al. (2004)
	GGGTCTCGAGGTTTGATTG	
atzE	GAGCCTCTGTCCGTAGATCG	
	GATGGCGTGTACCGTTTACC	
atzF	ACCAGCCCTTGAATCATCAG	
	TATTGTCCCGATACCCAACG	
trzD	CACTGCACCATCTTCACC	Fruchey et al. (2003)
	GTTACGAAC CTCACCGTC	
trzN	CACCAGCACCTGTACGAAGG	Mulbry et al. (2002)
	GATTCGAACCATTCCAAACG	

4.2.4 Molecular methods for community composition

DNA was extracted, amplified and sequenced according to the protocols detailed in Chapter 2.

4.2.5 Soil property characterisation

General soil properties of the sampled cores are the same as those detailed in Chapter 3.

Soil pH on a per core basis was assessed in triplicate by subsampling (10 g per measurement) frozen soils, allowing to thaw with 25 ml of Soil Sample Preparation Solution (Hanna Instruments HI-7051L). Samples were thoroughly mixed by shaking, and then left to stand for 5 minutes. pH measurements were taken using a HI-991001 pH meter (Hanna Instruments) and a HI1292D probe (Hanna Instruments) following manufacturer's instructions. pH meter calibrated with pH 4 and pH 7 buffers (ECBU4TBC and ECBU7BTC (Thermo Scientific)).

4.2.6 Data Analysis – Bioinformatics

Amplicon sequence data for community composition was analysed according to the protocols detailed in Chapter 2.

Table 4.2 details the normalisation rates of the data, and the number of samples retained.

Table 4.2 – Data on normalization and retention of samples

	Normalization			Total Samples	Samples retained		
Study	Bacteria	Eukaryotes	Fungi		Bacteria	Eukaryotes	Fungi
JHC	22522	29325	9492	24	24	24	24

4.2.7 Data Analysis – Statistics

Statistical analysis of data was conducted according to the protocols detailed in Chapter 2 Note assumptions on the dispersion of data for PERMANOVA were breached for mean core pH in bacteria, eukaryotes and fungi, and for time (DAT) for eukaryotes (See Chapter 2).

4.3 Results

4.3.1 Functional genes for Simazine Degradation

All attempts to amplify genes associated with the degradation of Simazine were unsuccessful.

4.3.2 Community composition of soil cores through incubation

Tables 4.3 and 4.4 detail mean ASV richness and Simpsons Index (respectively) per sample at each sampling date, for IT and RT. RT samples contained on average a higher number of unique bacterial and fungal ASVs, with IT samples containing on average a greater number of unique eukaryote ASVs.

Table 4.3 – Mean ASV richness per sample by date of sampling and tillage treatment

	Inversion				Reduced			
DAT (days)	0	30	56	87	0	30	56	87
Bacteria	842	783	576	715	662	677	855	767
Eukaryotes	553	477	517	489	480	518	516	425
Fungi	53	54	68	89	88	80	49	77

Table 4.4 – Mean Simpsons Index per sample by date of sampling and tillage treatment

	Inversion				Reduced			
DAT (days)	0	30	56	87	0	30	56	87
Bacteria	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Eukaryotes	0.98	0.98	0.97	0.99	0.97	0.98	0.98	0.98
Fungi	0.90	0.91	0.91	0.92	0.92	0.91	0.88	0.90

For bacteria, tillage type and length of incubation had a significant effect on community composition (PERMANOVA, DF=1 F=2.3 P<0.002, DF=1 F=1.9 P<0.006). Core pH was not significant (DF=1, F=1.1, P>0.3), and accounted for 4.2 % of variance as percentage of total sum of squares. Treatment accounted for 7.7. % of variance, and length of incubation 9.2 %, with residuals totalling 79 %. Figure 4.2 shows the DCA ordinations of bacterial community composition grouped by tillage and sampling point (time). There is no clear temporal shift or direction within the data in Figure 4.2B, however this may be explained due to each core being a discreet unit sample once, and therefore the data being non-continuous. Median distances from the centroid of the group for treatment

reveal bacterial communities within RT soils to be more variable in composition than those under IT (Figure 4.3), with this greater dissimilarity between RT cores also being apparent in Figure 4.2. Figures 4.4 and 4.5 show relative abundance of taxonomic groups of bacteria grouped by treatment and sampling respectively, aggregated by treatment, the relative abundance of the ten most frequently observed taxonomic groups is highly similar (Figure 4.4), and after 30 days of incubation, these abundances maintain a broadly steady state (Figure 4.5). The change from 0 DAT to 30 DAT is due to an increase in the relative abundance of the most common taxa, with the 'other' category declining between 0 and 30 DAT.

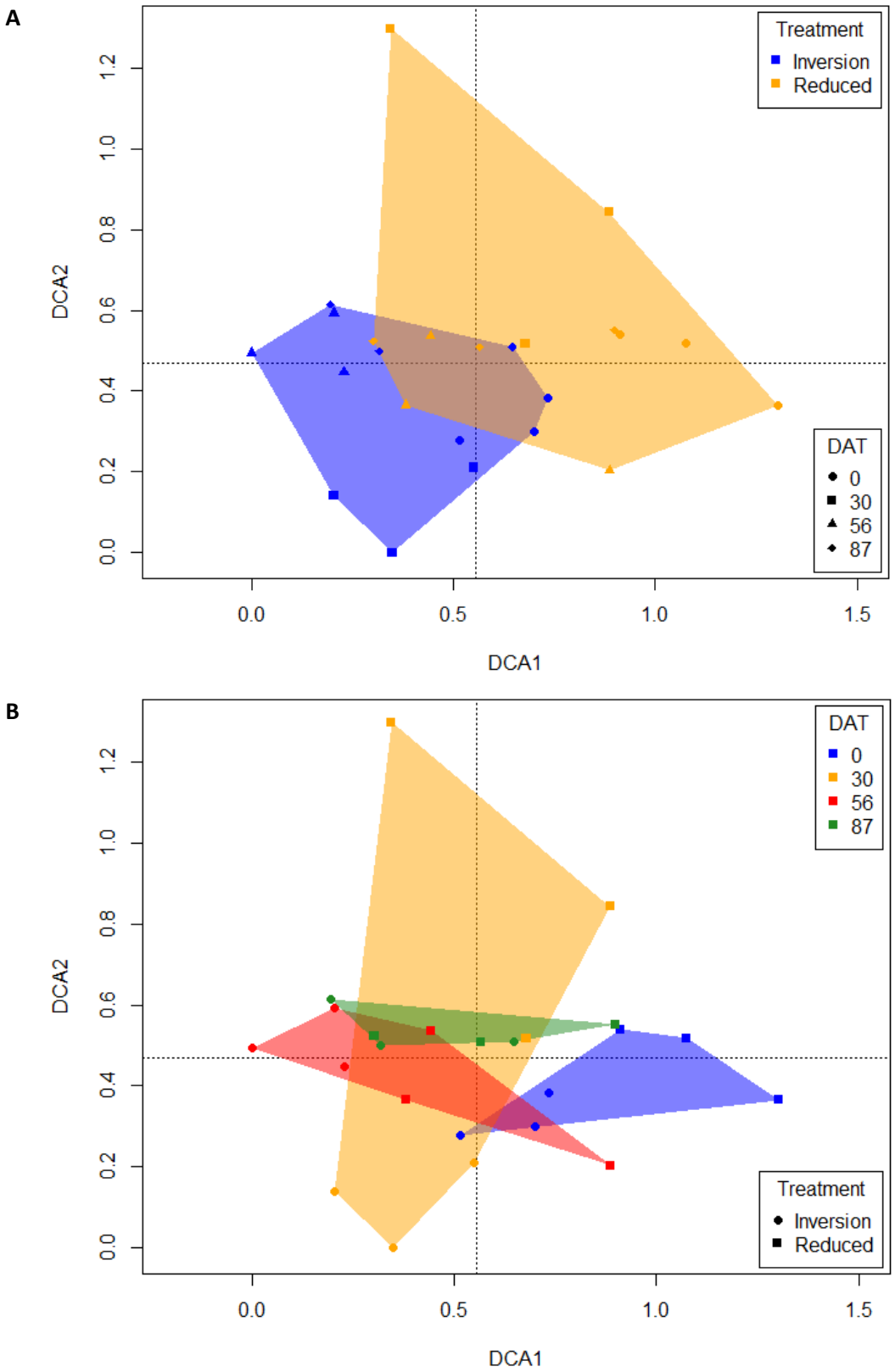


Figure 4.2 – DCA sample scores (axis 1 and 2) for bacterial community from IT and RT soils. (A) Coloured hulls show the range of all samples from IT (blue), and RT (yellow). (B) Coloured hulls show different lengths of incubation – 0 DAT (blue), 30 DAT (yellow), 56 DAT (red) and 87 DAT (green).

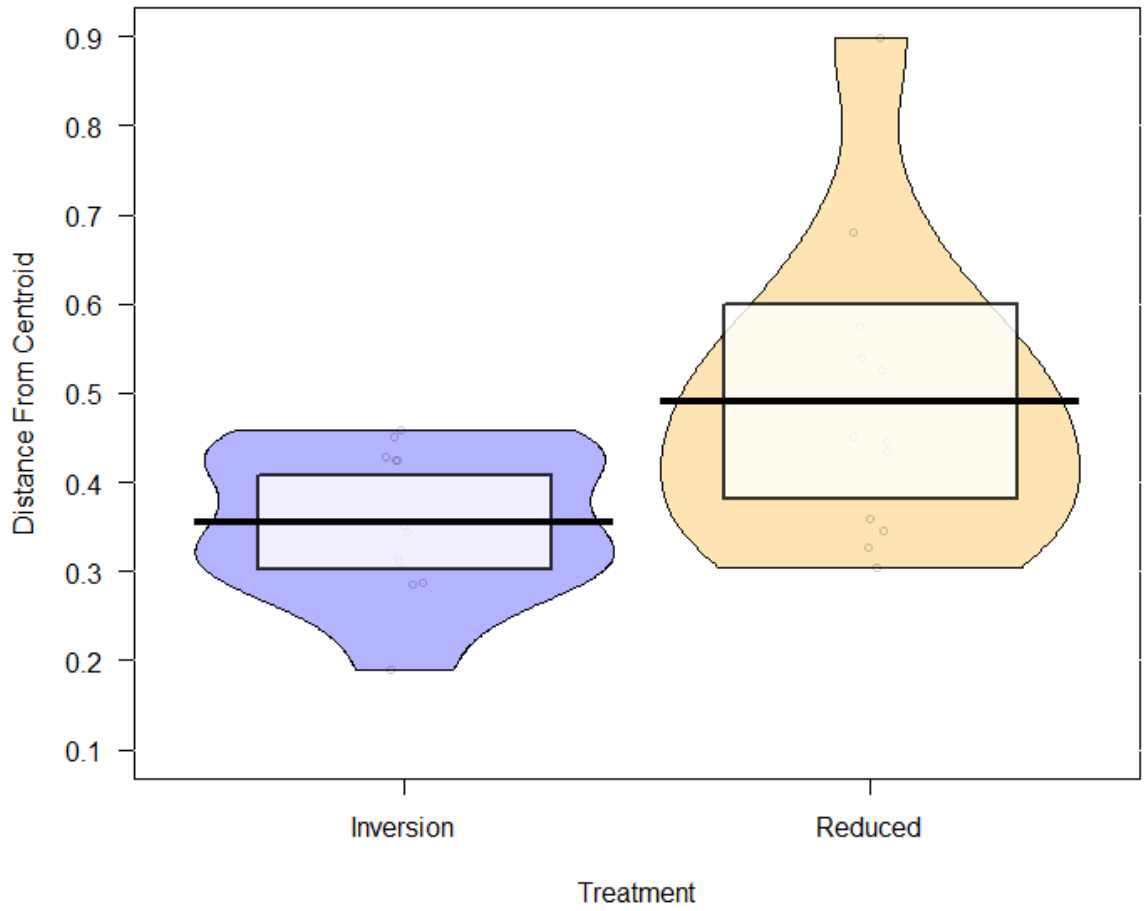


Figure 4.3 – Distance from median sample score (across all 4 DCA axis) for bacterial composition in soil cores from IT (blue) and RT (yellow) treatments.

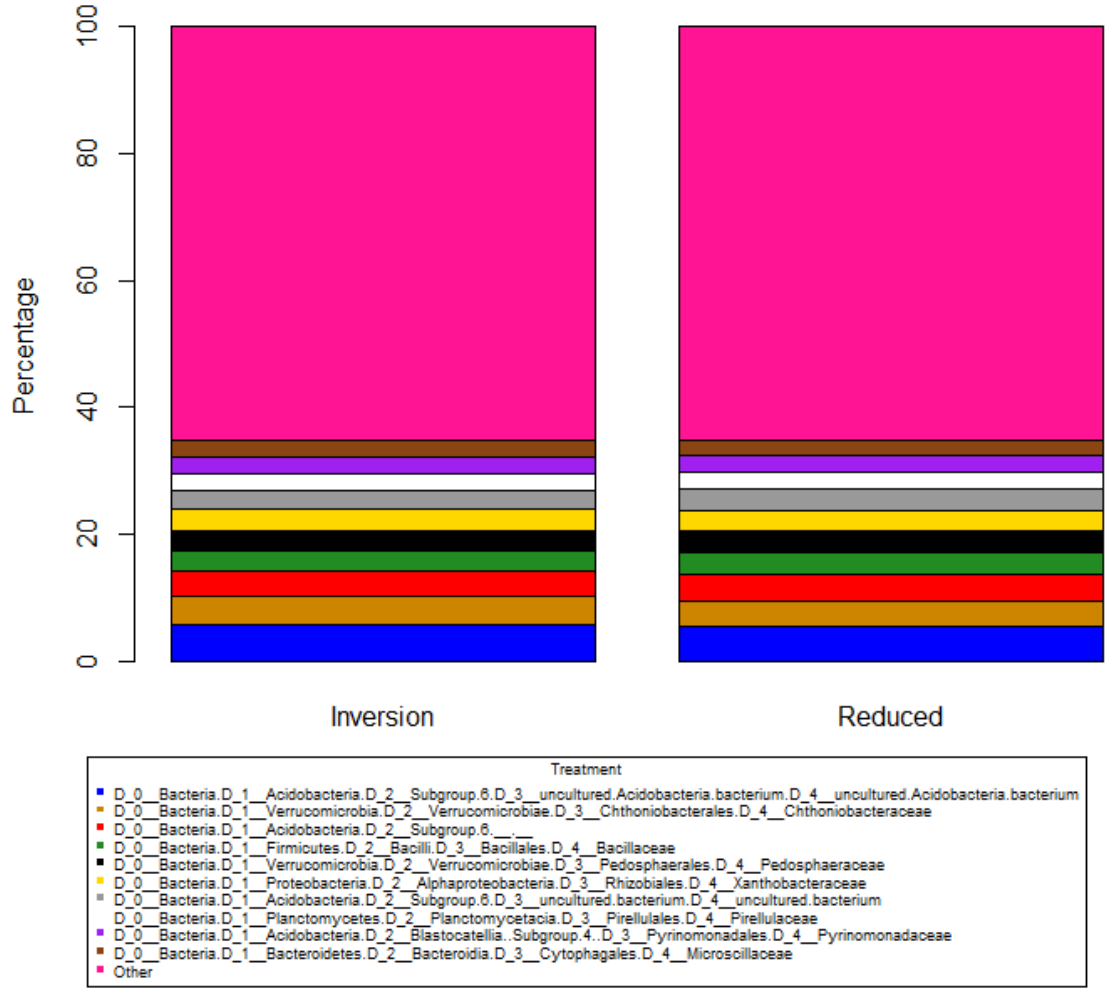


Figure 4.4 – Relative abundance of soil bacterial taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by tillage method.

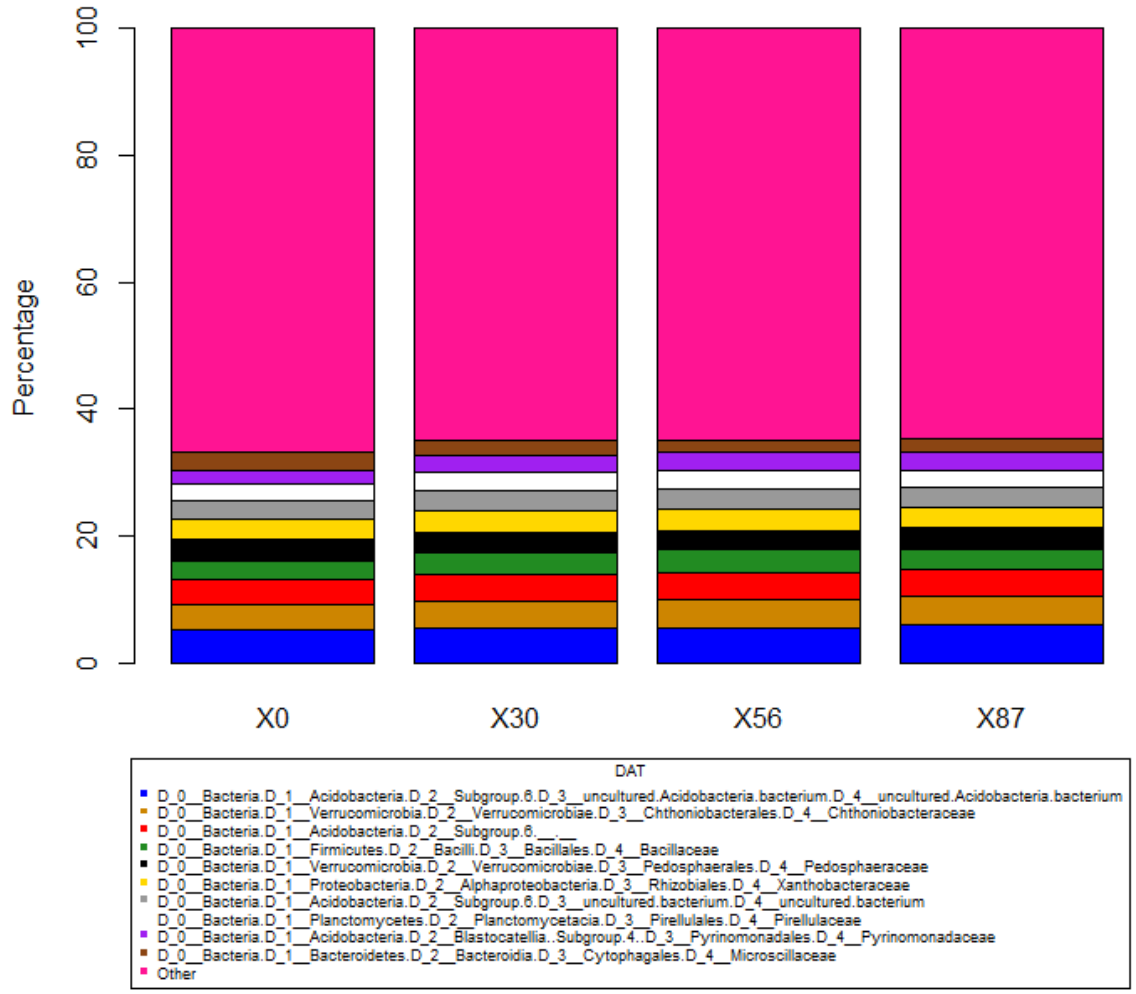


Figure 4.5 – Relative abundance of soil bacterial taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by incubation period.

For total soil eukaryotes, PERMANOVA did not identify incubation period, tillage method or soil pH as a significant determinant of community composition (DF=1 F=0.63 P>0.95, DF=1 F=0.58 P>0.95, DF=1 F=0.79 P>0.80) accounting for 2.9 %, 2.6 % and 3.6 % of variance respectively. Figure 4.6 shows the DCA ordinations of eukaryotes community composition grouped by tillage and sampling point, consistent with the PERMANOVA results, it can be seen that the communities show little dissimilarity (hulls highly overlapping) between tillage type (Figure 4.6A). At the longest incubation time (87 DAT), dissimilarity has reduced from previous timepoints, which could be suggestive incubation conditions favouring a particular final state - ASV richness having declined from 0 DAT to 87 DAT (Table 4.3). Figures 4.7 and 4.8 show relative abundance of taxonomic groups of eukaryotes grouped by treatment and sampling respectively, consistent with PERMANOVA results, there are no clear differences in community composition from tillage method or incubation

period, although the relative abundance of the most abundant taxa is declining from 30 DAT to 87 DAT (Figure 4.8).

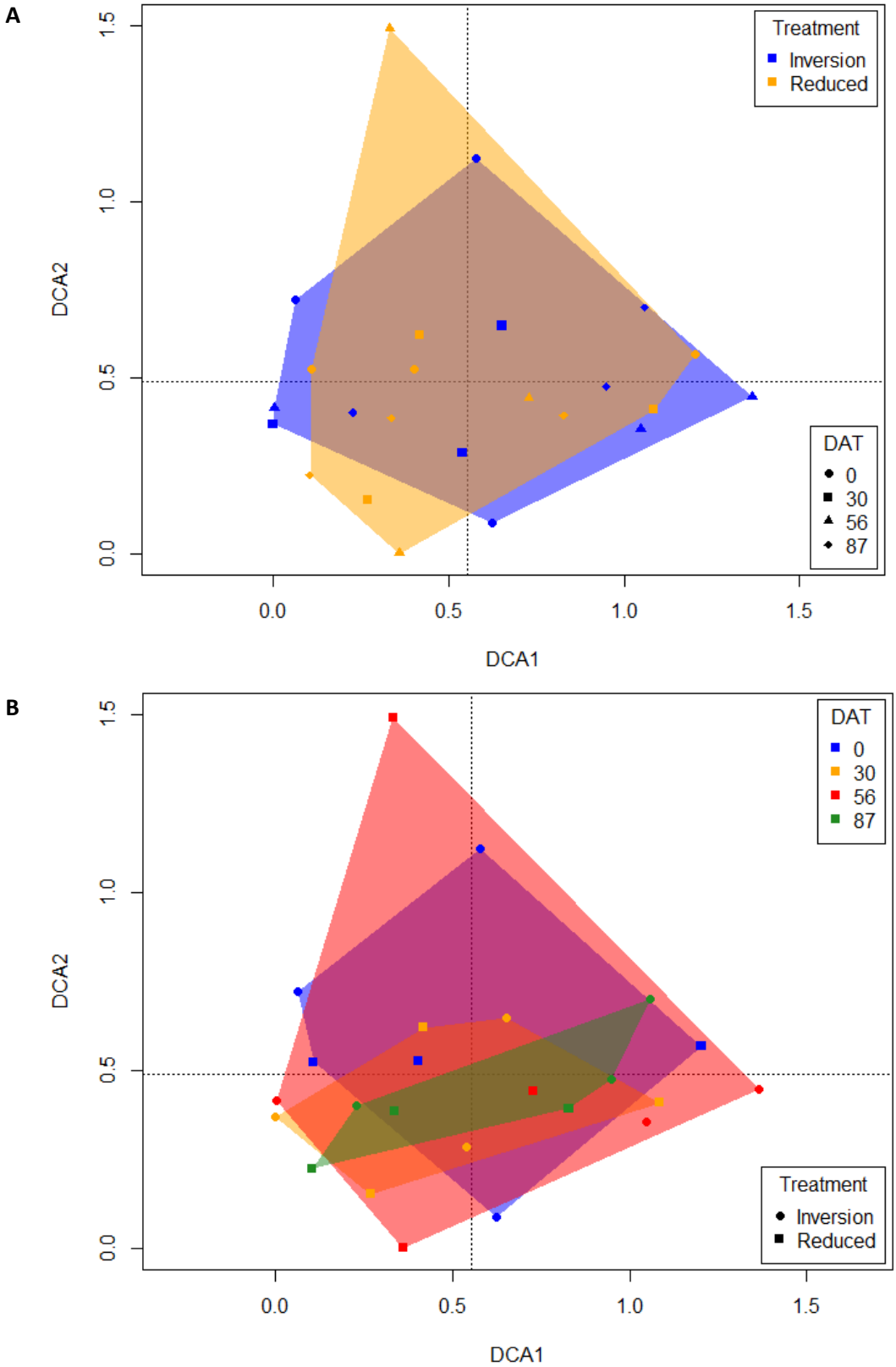


Figure 4.6 – DCA sample scores (axis 1 and 2) for eukaryotic community from IT and RT soils. (A) Coloured hulls show the range of all samples from IT (blue), and RT (yellow). (B) Coloured hulls show different lengths of incubation – 0 DAT (blue), 30 DAT (yellow), 56 DAT (red) and 87 DAT (green).

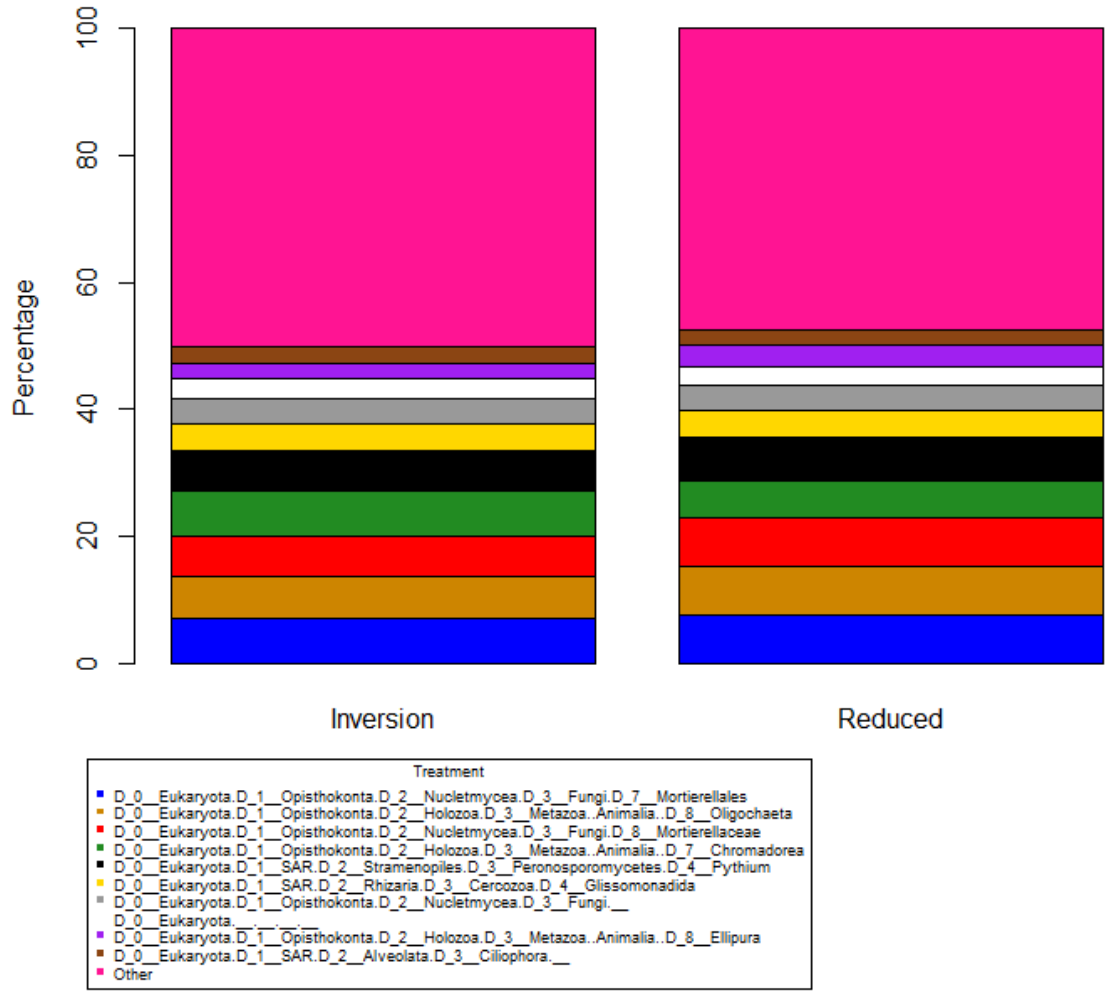


Figure 4.7 – Relative abundance of soil eukaryotes taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by tillage method.

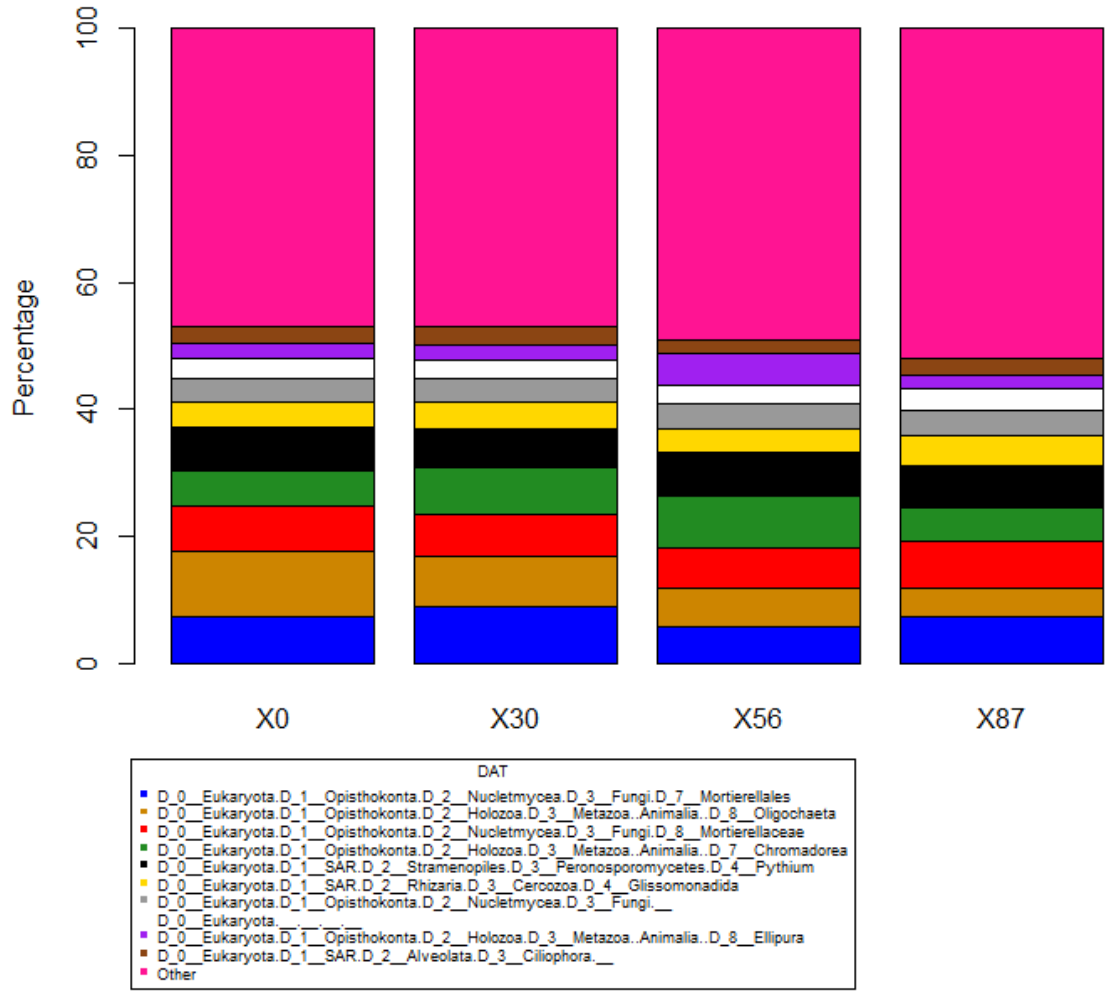


Figure 4.8 – Relative abundance of soil eukaryote taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by incubation period.

For soil fungi, PERMANOVA did not identify incubation period, tillage method or soil pH as a significant determinant of community composition (DF=1 F=1.6 P>0.08, DF=1 F=1.5 P>0.11, DF=1 F=1.2 P>0.29) accounting for 6.7 %, 6.1 % and 4.8 % of variance respectively. Figure 4.9 shows the DCA ordinations of fungal community composition grouped by tillage and sampling point. Fungal communities from RT soils can be observed to be more variable than those under IT (Figure 4.9A). Consistent with PERMANOVA results, there is no clear trend or gradient in community dissimilarity through time (Figure 4.9B), and while the reduced dissimilarity between cores at 87 DAT may represent a convergence as a consequence of incubation, this may also be an artifact of the sampled units. Figures 4.10 and 4.11 show relative abundance of taxonomic groups of fungi grouped by treatment and sampling respectively, and these are broadly consistent both between treatments, and incubation length. Notable is the significantly smaller proportion of relative abundance that may

attributed to ‘other’ (not the 10 most abundant taxonomic groupings) in these incubated samples relative to ‘fresh’ environmental samples (Figure 2.30).

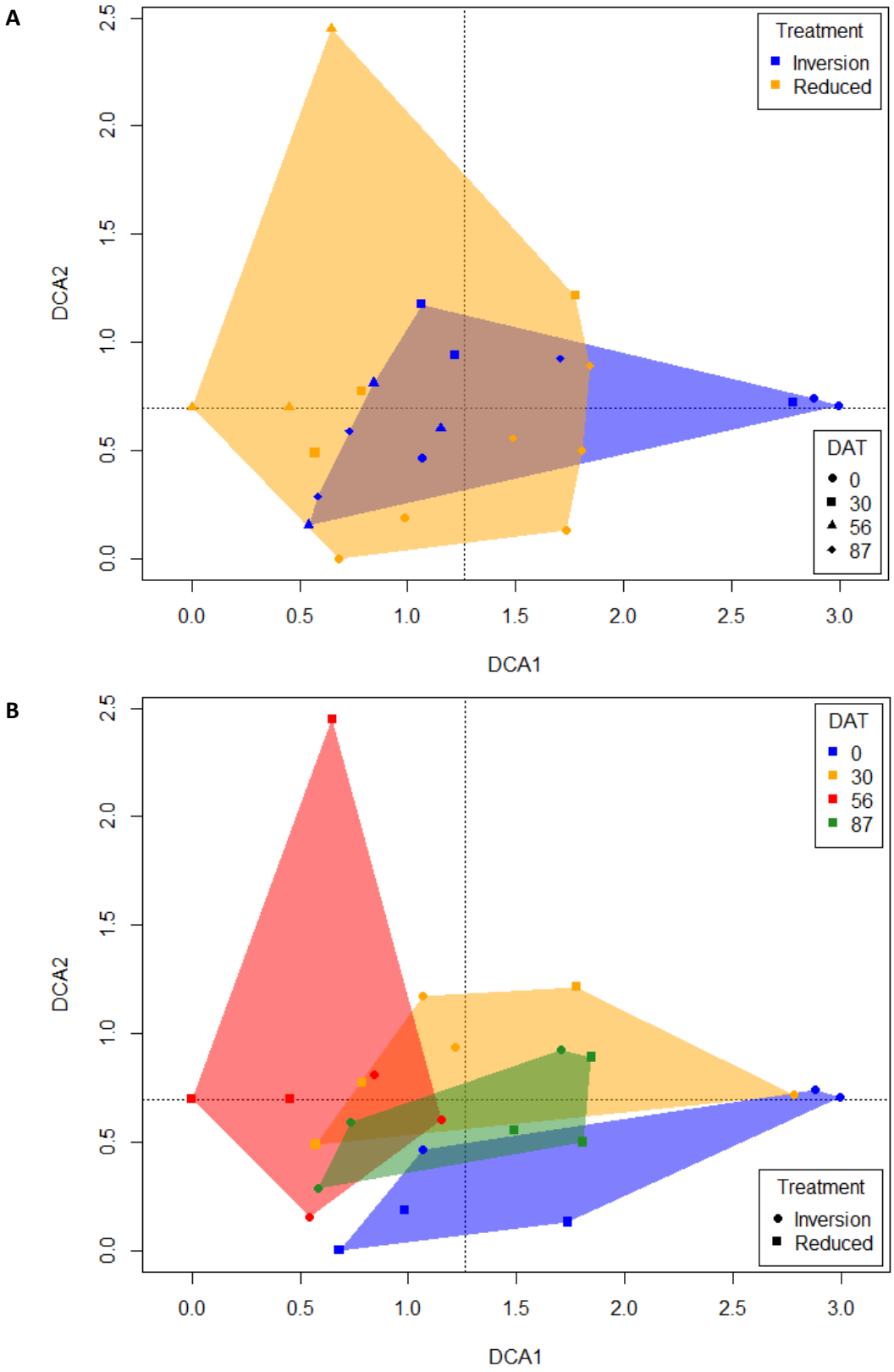


Figure 4.9 – DCA sample scores (axis 1 and 2) for fungal community from IT and RT soils. (A) Coloured hulls show the range of all samples from IT (blue), and RT (yellow). (B) Coloured hulls show different lengths of incubation – 0 DAT (blue), 30 DAT (yellow), 56 DAT (red) and 87 DAT (green).

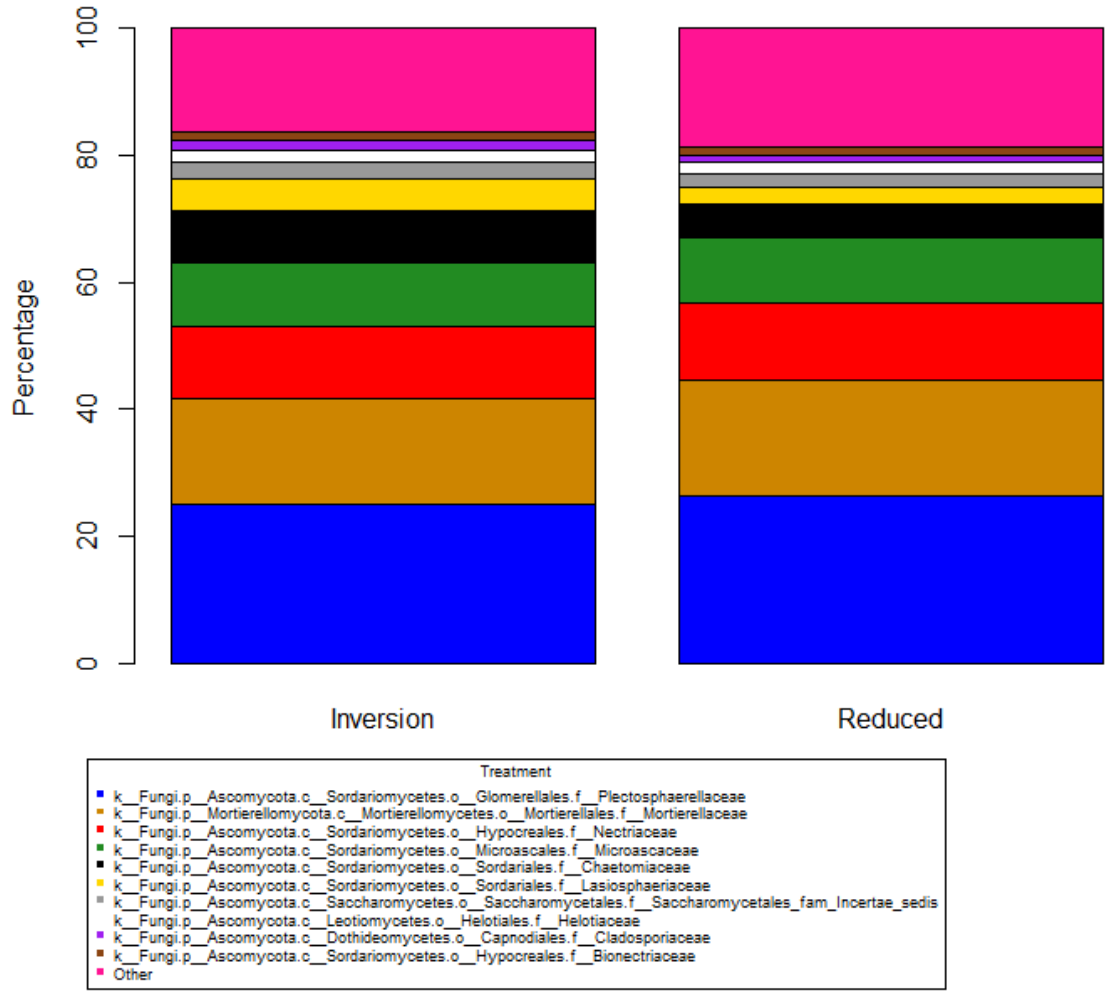


Figure 4.10 – Relative abundance of soil fungal taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by tillage method.

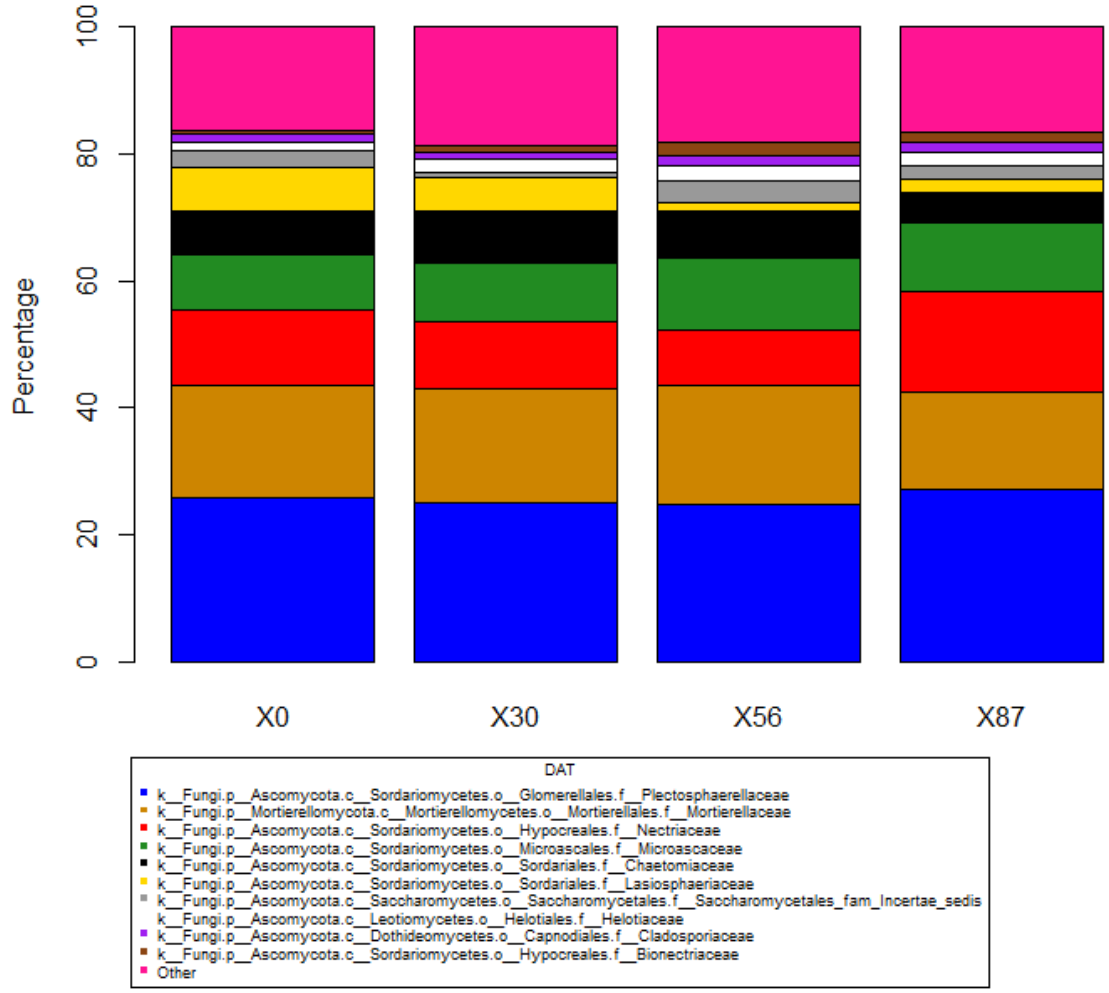


Figure 4.11 – Relative abundance of soil fungal taxonomic groupings – 10 most common taxonomic groupings and 'others' – in incubated soil cores from IT and RT plots grouped by incubation period.

4.4 Discussion

4.4.1 Functional Genes

All attempts to amplify functional genes expected to facilitate Simazine degradation were unsuccessful. There are a number of reasons that may explain this; (i) that trialled range of PCR conditions were insufficient (ii) that the trialled primers designed are too specific to account for the genomic variation of the studied genes (iii) that the genes responsible for Simazine degradation are very rare, and the conducted analysis was not sufficiently sensitive to identify their presence and (iv) that Simazine degradation in the study soils was dominated by genes which were not attempted to be amplified – potentially *thcB, thcC, thcD, trzA,* and *triA* – or other unknown genes.

Possibility (i) cannot be discounted, and experiments seeking to demonstrate the presence/absence of genes would benefit from positive controls. However, the range of PCR conditions trialled was extensive, and based upon published and previously successful methods (de Souza et al., 1998b, Mulbry et al., 2002, Fruchey et al., 2003, Devers et al., 2004, Yale et al., 2017).

Considering possibility (ii) it can be noted that the primers used to identify functional genes are much more specific than those used for community composition (see Chapter 2), containing no degenerate bases, and target genes with a specialised function. de Souza et al. (1998b) report s-triazine degrading genes to be highly conserved (>99 % sequence similarity) between a diverse range of bacteria. Available information on sequence variants for designing primers targeting a greater number of sequence variants of target genes is limited, but a potential route to further studies on the distribution of s-triazine degrading genes.

Possibility (iii) would limit a study conducted with redesigned primers and ideal reaction conditions. Genes for s-triazine degradation are mobile, and widespread (de Souza et al., 1998b, Shapir et al., 2007). However, at very low abundance, PCR is not a sensitive enough technique to detect genes. Consequently, to detect gene presence, for analysis of presence/absence and sequence diversity, use of an enrichment technique would be beneficial. In an enrichment experiment, an s-triazine would be applied to the study soils and incubated, prior to DNA extraction. If the samples contain genes associated with s-triazine degradation, these would be expected to increase in abundance and potential detectability. Such an approach would be similar to that demonstrated by Yale et al. (2017) who identified an increased percentage of the bacterial microbial community containing the *trzN* gene following application of atrazine to soils. Alternate experimental approaches to identifying the presence/absence of these genes may include whole soil metagenome sequencing.

Possibility (iv) is improbable, with the microbial degradation of Simazine – and other s-triazine compounds – being well described within the literature – see Kodama et al. (2001), Wackett (2002), Caracciolo et al. (2005), Govantes et al. (2009), Rehan et al. (2016), (Scherr et al., 2017) and others. The formation of some possible Simazine metabolites – deisopropylsimazine, deisopropylhydroxysimazine, deisopropyldeethylsimazine, and 2-chloro-4-hydroxy-6-amino-1,3,5-triazine – could be metabolised by genes that PCR amplification was not attempted for – *thcB, thcC, thcD, trzA,* and *triA* – however the loss of mass balance as detailed in Chapter 3 is suggestive of mineralisation to CO_2 or formation of volatile compounds, which by the proposed degradation pathway (Figure 4.1) would involve the studied genes *atzD, atzE, atzF and trzD* (Govantes et al., 2009, Sagarkar et al., 2013).

4.4.2 Community composition

RT samples contained on average a higher number of unique bacterial and fungal ASVs, with IT samples containing on average a greater number of unique eukaryote ASVs (Table 4.3). There is no notable temporal change decrease in the richness of any group – increases in abundance are considered to represent an inter-sample noise (n=3) (Table 4.3). Diversity as measured by Simpson's Index was generally consistent for each taxonomic grouping throughout the experiment.

PERMANOVA revealed the length of incubation to have had a significant effect on bacterial community composition. These differences are visualised in Figure 4.2B – little overlap between DAT 0 samples and those with longer incubation periods, which are generally consistent. More pronounced differences due to incubation conditions may have been identified by sampling additional cores at the time soil cores were collected, or in the period between fieldwork and the start of degradation experiment – 21 days. The delay between sampling and application would ideally have been shorter, however was dictated by farming conditions – the final harvest of the crop strips having been conducted prior to sampling – and the availability of radiolabelled compounds – delivered to Jealott's Hill 20 September 2017. Miranda (2019) identifies differences in the soil metabolome within the first 7 days of storage for soils prepared for OECD 307 testing, however the relative role of sieving vs incubation conditions consistent with the present study are indeterminable. PERMANOVA did not identify significant shifts in soil eukaryote or fungal community composition attributable to the period of incubation – again this may be due to shifts between sampling and DAT 0, may reflect no changes in community composition from incubation conditions, or may be attributable to relic DNA obscuring temporal processes (Carini et al., 2016). ASV richness and diversity show no clear trend relative to incubation condition for any studied taxonomic group (Tables 4.3 and 4.4). The relative abundances of taxonomic groupings are generally consistent

throughout the studied period of incubation, start-to-end, with a suggestion (although n=6, split 3-3 of IT to RT per sampling) that the 10 most common bacterial taxonomic groupings increasing in relative abundance with a greater period of incubation (Figure 4.5), and the most common eukaryotic groupings decreasing in relative abundance (Figure 4.8). Fungal communities are dominated by *Ascomycota*, and the 10 most common taxonomic groupings account for >80 % of the measured community composition (Figures 4.10 and 4.11), whilst the 10 most abundant groupings of eukaryotes and bacteria account for <55 % and <35 % respectively (Figures 4.4, 4.5, 4.7, and 4.8). In further studies, the use of non-disruptive/non-destructive sampling techniques to study functional changes in soil processes, such as sampling the soil volatilome (Redeker et al., 2018), would allow for repeat sampling to identify functional changes through time of heterogenous soil samples.

In the present study, tillage type was found to significantly alter bacterial community composition, but not that of total eukaryotes or fungi (PERMANOVA results), ordinations of DCA analysis (Figures 4.2A, 4.6A, and 4.9A) are consistent with these results. Treatment account for 7.7 % of variance (as total sum of squares) for bacteria, 2.6 % for eukaryotes and 6.1 % for fungi. RT tillage was found to lead to an increase in the spatial (horizontal) variance of bacterial community composition (Figure 4.3), but not for total eukaryotes and soil fungi (data not shown). These results are consistent with those observed in Chapter 3 of CPP degradation being more spatially variable in RT soils than those under IT, with Simazine degradation (and its variability) being controlled by the presence of s-triazine associated genes, within the bacterial metagenome (Kaufman et al., 1965, Wackett, 2002, Govantes et al., 2009).

Core pH was not found to have a significant effect on community composition of soil bacteria, eukaryotes, or fungi (PERMANOVA results). Mean pH for IT soils was 7.1, and 7.2 for RT soils. While soil pH is a key determinant of the availability of ionisable CPPs such as Simazine (Kah et al., 2007), within the narrow range of soil pH (6.99-7.24) present within this study, availability of Simazine to degradation processes between cores is unlikely to have been substantial. Degradation of atrazine – an s-triazine herbicide – in soils within this pH range is typically due to growth linked metabolism, as opposed to cometabolic metabolism (Houot et al., 2000, Bending et al., 2006). The narrow range of pH within the present study may also account for the low variability (as mean sum of squares) associated with soil pH – 4.2 %, 3.6 %, and 4.8% for bacteria, eukaryotes and fungi respectively. Whereas soil pH – usually over larger ranges – is often determined as a key driver of soil microbial diversity (Rousk et al., 2010, Zhalnina et al., 2015, Wang et al., 2019), and specifically the abundance of CPP degrading cohorts (Walker et al., 2001). Further, in contrast to Rousk et al. (2010) soil fungi were identified to be more strongly influenced by soil pH than soil bacteria.

Whilst cores for analysis of the microbial analysis were sampled and maintained under identical conditions to those detailed in Chapter 3, they differ in the lack of application of a compound (Mandipropamid or Simazine) and the corresponding solvent. This difference was a requirement of sponsor support of the study. However, despite this difference, the study of these cores has potential benefits – both in facilitating the attempted amplification of functional genes (see 4.4.1), and in understanding the effects of incubation on soil microbial communities in soil cores. The incubation conditions of the study – darkness, temperature controlled to 20 °C, moisture control, and the lack of plants – are substantially removed from field conditions, and equalise conditions between tillage treatments. Understanding if the equalisation of conditions drove convergence of microbial community composition, and therefore similar rates of degradation and dissipation is of value. Further, as core studies of similar designs may be considered to compliment or replace OECD 307 studies due to concerns over the design and validity of the OECD 307 guidelines (OECD, 2002, Dougan et al., 2013, Miranda, 2019), assessing whether core designed studies can support more stable, and environmentally relevant microbial communities has significant scientific value.

4.4.3 Conclusion

The present study failed to identify the presence of Simazine degrading genes, and to assess their abundance or diversity. Analysis of the composition of key soil microbiome components – bacteria, eukaryotes and fungi revealed incubation conditions of the degradation to alter bacteria community composition, but not eukaryotes and fungi. Assessment of the effects of tillage on the soil microbiome should be considered based upon analysis in Chapter 2 and 5, with the limited sampling and incubation conditions limiting conclusions that can be drawn on this issue from this study. However the higher variability of soil bacterial communities in soils under RT than IT are consistent with the hypothesised causes of CPP degradation variability in Chapter 3 – more variable bacterial communities in soils under RT than IT may lead to more variable rates of CPP degradation.

Chapter 5: Tillage Intensity and Soil Depth

5.1 Introduction

5.1.1 Soil microbial communities and depth

Baas Becking (1934) stated that 'everything is everywhere, but, the environment selects' (de Wit and Bouvier, 2006). However such a niche model relies upon unlimited dispersion of species or genes (Fondi et al., 2016), for which Baas Becking (1934) suggests dispersion of microbes by atmospheric transport (de Wit and Bouvier, 2006). However, dispersal within soil matrices is limited, and community composition may in part be considered to be a function of limited dispersion and stochastic processes (Dumbrell et al., 2010). Consequently, soil community composition is neither a true fit of niche or neutral ecological models.

IT tillage mixes the upper soil (generally 0-30 cm) of ploughed fields, both inverting soil in the vertical, and translocating soil horizontally across the field (De Alba, 2003, Hula and Novak, 2016). While RT techniques can relocate soil particles across a field, some RT techniques, e.g. disc tillage, may translocate particles only 33 – 50 % as far as IT, and with significantly less vertical mixing (Hula and Novak, 2016). Such translocation of soil will provide a means of dispersal for microorganisms and genes. The degree of mixing and the depth to which RT disturbs the soil profile varies dependent upon the exact techniques employed (Arvidsson, 1998, Derpsch et al., 2014, Townsend et al., 2016).

Many of the most significant determinants of soil microbiome composition, including organic carbon content, nutrient availability and soil moisture, are strongly correlated to depth in the soil profile (Fierer, 2017). Consequently, vertical sampling within the soil profile may yield bacterial communities within tens of centimetres of each other that are as dissimilar as samples collected from horizontal distances measured in kilometres (Chu et al., 2016). Microbial biomass and oxidative enzyme activity declines with depth (Hsiao et al., 2018).

Within RT tillage systems, soil organic carbon content becomes increasingly stratified (Haddaway et al., 2017). This change is likely a direct consequence of reduced mixing as tillage intensity decreases (Hula and Novak, 2016), and therefore crop residues are not incorporated throughout as large a depth in the soil profile. Changes in the total carbon, and carbon to nitrogen ratio within soils were identified by Chu et al. (2016) as the best predictors of soil microbial community distribution. Similarly, Fierer et al. (2003) find that soil microbial communities are shaped by increasing resource limitations at greater soil depths. Consequently, it may be predicted that

increased soil depths may favour greater relative abundances of *actinomycetes*, and reduce the relative abundance of soil fungi (Fierer et al., 2003). How soil fungal communities respond to reduced resource availability at depth, traded off against reductions in mechanical disturbance from a reduction in tillage intensity are unknown (Helgason et al., 1998, Holland, 2004). Contrastingly, Hsiao et al. (2018) report carbon not to be limiting to subsurface soil microbial communities, but rather nitrogen and phosphorus availability.

In addition to resource availability due to tillage of arable fields, the role of plants in providing resources to microbial communities, should not be overlooked. Root systems exudates, in addition to symbiotic or parasitic interactions between plants and the microbial community may increase resource availability deep within soil profiles. The relative resource abundance within the rhizosphere may increase heterogeneity within the soil profile, and be more notable at resource poor depths (Johnson et al., 2002, Kamel et al., 2016, Fan et al., 2018). Such inputs may arise both from crops, and potentially unevenly within agricultural landscapes, with field boundaries potentially supporting more diverse arbuscular mycorrhizal (AM) fungal communities (Holden et al., 2019), potentially with greater rooting depths or distances.

In addition to resource availability, soil moisture varies with depth in the soil profile, and can act as a significant determinant of soil microbial composition (Fierer, 2017, Griffin et al., 2020, Hao et al., 2021). Increased depth within the soil profile, and the frequency and duration within which soil is saturated can influence soil microbial communities (Griffin et al., 2020, Hao et al., 2021), with greater saturation tending to decrease bacterial diversity. RT may be associated with greater water holding capacity and rain infiltration due to improved pore structure, as a consequence of greater soil macroaggregate stability (Holland, 2004). Reducing the probability of saturation by improved water holding capacity may therefore be predicted to increase soil microbial community diversity, however such effects may be small relative to other determinants of a fields hydrological properties – topology, underlying lithology, and drainage systems (Brady and Weil, 2012).

Higher intensity of tillage is associated with a higher metabolic quotient – increased respiration per unit biomass – than conservation tillage techniques (Zuber and Villamil, 2016). This may be attributable to the higher availability of crop residues to the microbial community, both by incorporation of all residues to within the soil profile, and reduced protection of these resources via incorporation into soil aggregates (Holland, 2004, Six et al., 2000, Six et al., 2004). Further, disturbance, or poorly aggregated soils may induced greater glomalin production by soil fungi (Rillig and Mummey, 2006).

5.1.2 Aims and objectives

The present study aims to identify changes in the composition of the soil microbiome, considering bacteria, eukaryotes and fungi, subject to different tillage techniques – IT and RT, and how tillage method may alter the spatial (both horizontal and vertical) variability of soil microbial communities. It was hypothesised that a reduction in tillage intensity would lead to an increased stratification of soil microbial communities in the otherwise ploughed, heavily mixed, soil layer. It was further hypothesised that a reduction in tillage intensity would facilitate an increase the horizontal heterogeneity of communities with depth as more stable and differentiated conditions become established.

5.2 Methodology

5.2.1 Field Site and Sampling

The field experiments of the MycoRhizaSoil project were conducted at the University of Leeds farm, England (53.874, -1.323) (Leake et al., 2014, Leake et al., 2015). Holden et al. (2019) detail mean annual precipitation to be 674 mm, mean annual temperature of 9.2 C, and the soils to be loamy calcerous brown earth (0.5-0.9 m in depth) above limestone. The present study draws soils from the MycoRhizaSoil study field, 'Quarry' – Figure 5.1 farm layout, Figure 5.2 field layout.

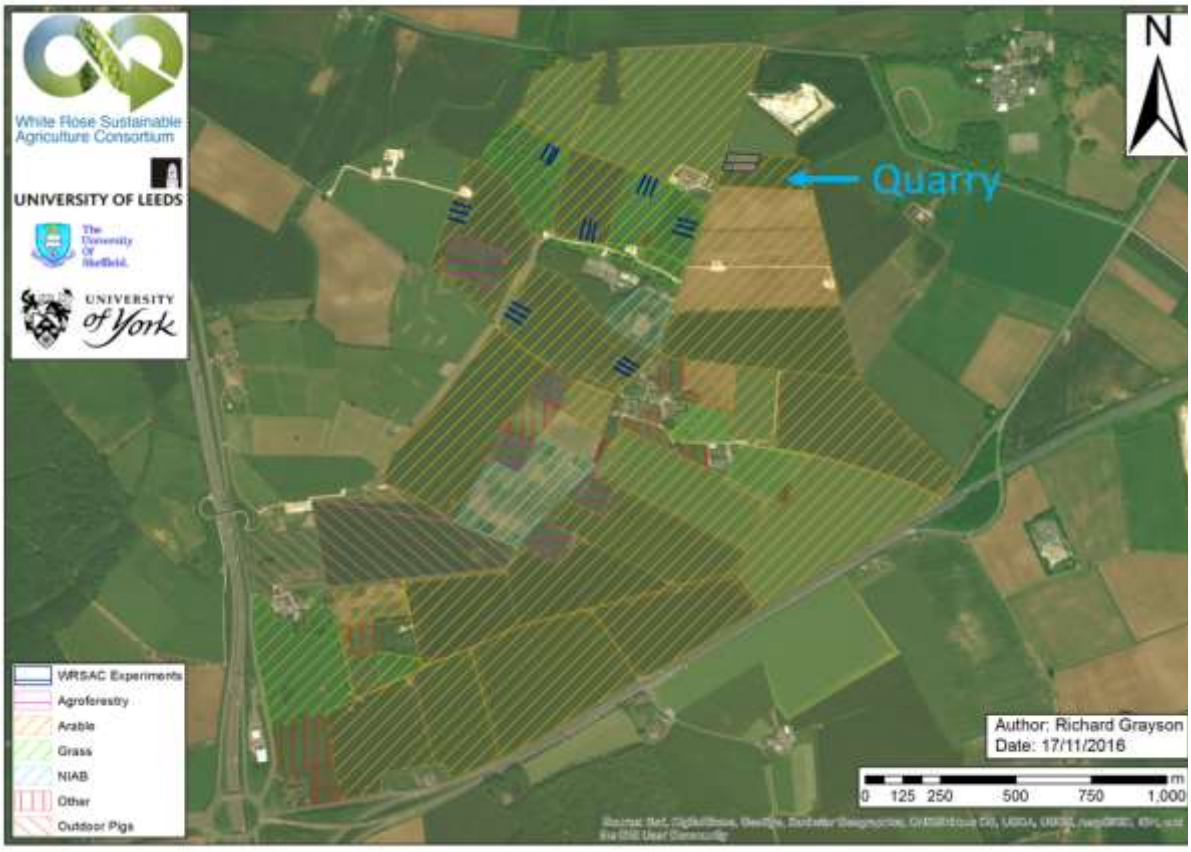


Figure 5.1 – University of Leeds farm layout. Adapted from Grayson (2016). MycoRhizaSoil field plots located in North-West corner of Quarry field.

Quarry field has been an arable cultivated field from at least 2002, subject to IT.

Experimental logs maintained by WRSAC technician (Lappage, 2015, Lappage, 2016, Lappage, 2017, Lappage, 2018) were referred to confirm details of field experiments. Briefly, the MycoRhizaSoil field study comprised of 124 wheat plots, each 1.2 m x 1.7 m (layout Figure 2). Field plots were established in Quarry field in 2014, with a crop of peas grown in the season 2014-2015. Two field plots under IT and RT were established, one of each treated with a mycorrhiza inoculant, and the others with only a clay carrier from the inoculant. A total of 7 wheat lines – 6 study and 1 in

guard plots – were grown over three crop cycles (2015-16, 2016-17 and 2017-18). Field plots were divided into four blocks (A-D), and 31 rows. IT conducted with either a 2 or 5 furrow reversible plough, RT with either a Lemken Heliodor or Sumo Trio, plots sown with a Oyjord plot drill.

	RT								IT								RT								IT						
	Row 1	Row 2	Row 3	Row 4	Row 5	Row 6	Row 7	Row 8	Row 9	Row 10	Row 11	Row 12	Row 13	Row 14	Row 15	Row 16	Row 17	Row 18	Row 19	Row 20	Row 21	Row 22	Row 23	Row 24	Row 25	Row 26	Row 27	Row 28	Row 29	Row 30	Row 31
Block A	Wheat 6	Wheat 5	Wheat 1	Wheat 4	Wheat 2	Wheat 3	Wheat 6	Wheat 7	Wheat 6	Wheat 3	Wheat 1	Wheat 2	Wheat 5	Wheat 4	Wheat 7	Wheat 7	Wheat 1	Wheat 3	Wheat 6	Wheat 2	Wheat 5	Wheat 4	Wheat 7	Wheat 7	Wheat 4	Wheat 1	Wheat 3	Wheat 2	Wheat 5	Wheat 6	Wheat 7
Block B	Wheat 4	Wheat 1	Wheat 3	Wheat 5	Wheat 2	Wheat 6	Wheat 4	Wheat 7	Wheat 4	Wheat 1	Wheat 2	Wheat 6	Wheat 5	Wheat 3	Wheat 7	Wheat 7	Wheat 1	Wheat 5	Wheat 6	Wheat 3	Wheat 4	Wheat 2	Wheat 7	Wheat 7	Wheat 1	Wheat 2	Wheat 6	Wheat 4	Wheat 5	Wheat 3	Wheat 7
Block C	Wheat 4	Wheat 1	Wheat 5	Wheat 6	Wheat 3	Wheat 2	Wheat 4	Wheat 7	Wheat 6	Wheat 3	Wheat 2	Wheat 4	Wheat 5	Wheat 1	Wheat 7	Wheat 7	Wheat 6	Wheat 3	Wheat 1	Wheat 4	Wheat 5	Wheat 2	Wheat 7	Wheat 7	Wheat 3	Wheat 6	Wheat 2	Wheat 5	Wheat 4	Wheat 1	Wheat 7
Block D	Wheat 3	Wheat 4	Wheat 5	Wheat 2	Wheat 1	Wheat 6	Wheat 3	Wheat 7	Wheat 5	Wheat 6	Wheat 2	Wheat 4	Wheat 1	Wheat 3	Wheat 7	Wheat 7	Wheat 5	Wheat 3	Wheat 4	Wheat 1	Wheat 2	Wheat 6	Wheat 7	Wheat 7	Wheat 5	Wheat 6	Wheat 1	Wheat 3	Wheat 4	Wheat 2	Wheat 7
	AM inoculant								**Carrier, no AM**																**AM inoculant**						

Key			
	Crop included in present study		Crop not included in present study
	No inoculum or carrier		Carrier only
	Live inoculum		

Figure 5.2 - Field layout of MycoRhizaSoil experiment within Quarry field. (AM = Arbuscular Mycorrhiza)

Sampling was conducted on seven occasions; March, May, and July 2016, March, and June 2017, April and June 2018. In March and May 2016, sampling was conducted as soil cores taken at 10 cm intervals in depth from 0 – 40 cm, resulting in four cores (0-10, 10-20, 20-30, 30-40 cm) per plot. In July 2016, sampling was conducted as soil cores taken at 10 cm intervals in depth from 10 – 50 cm, resulting in four cores (10-20, 20-30, 30-40, and 40-50 cm) per plot. In March and June 2017, and April and June 2018 sampling was conducted as cores taken from 10-20 cm in depth.

Samples were stored at – 20 °C prior to freeze drying. Batches of samples were freeze dried for approximately 48 hours, until they were visibly dry, crumbled easily, and felt cool – not cold. Dried samples were stored at approximately 20 °C.

For the present study, samples collected from two wheat lines, Wheat 1 and Wheat 2, were analysed.

5.2.2 Molecular Methods

DNA was extracted, amplified and sequenced according to the protocols detailed in Chapter 2.

5.2.3 Data Analysis – Bioinformatics

Amplicon sequence data for community composition was analysed according to the protocols detailed in Chapter 2.

Table 5.1 details the normalisation rates of the data, and the number of samples retained.

Table 5.1 – Data on normalization and retention of samples

	Normalization			Total Samples	Samples retained		
Study	Bacteria	Eukaryotes	Fungi		Bacteria	Eukaryotes	Fungi
MRS	13792	9414	16153	284	283	258	283

5.2.4 Data Analysis – Statistics

Statistical analysis of data was conducted according to the protocols detailed in Chapter 2. Note assumptions on the dispersion of data for PERMANOVA were breached for sampling date and depth for bacteria, eukaryotes and fungi (See Chapter 2).

5.3 Results

5.3.1 Analysis of cultivated soils

For bacteria, PERMANOVA revealed significant effects of treatment, depth, sampling, row and block on community composition (DF=1 F=1.92 P<0.05, DF=1 F=61.2 P<0.001, DF=1 F=4.42 P<0.001, DF=8 F=1.35 P<0.02, DF=3 F=1.75 P<0.001). Wheat variety did not have a significant effect on bacterial community composition (DF=1, F=0.90, P>0.4). Depth is the most determinant variable of bacterial community composition accounting for 17 % variance (as percentage of total sum of squares), with treatment, sampling, row, block and wheat variety accounting for 0.5 %, 7.2 %, 2.9 %, 1.7 %, 0.2% respectively (residuals 71 %, all to 2 sf.). Figures 5.3A and 5.3B show the DCA ordinations of the bacterial community composition grouped by treatment and depth respectively – the sizeable effect of depth, and the slight effect of treatment can be observed. The effect of depth is evident both as in increased variability at ≥ 30 cm of depth, and in the suggestion of a gradient with increasing depth. Median distances from the centroid of the group for treatment, grouped by depth reveal the much greater variability in community composition in soils deeper than the reach of ploughs, and RT communities are more variable than IT at depths between 0 and 0.4 m (Figure 5.4). Figures 5.5 and 5.6 show relative abundance of taxonomic groups of bacteria grouped by treatment and depth respectively. Despite the PERMANOVA result that treatment has a significant difference on ASV composition, no clear difference can be observed in the relative abundance of the most abundant taxonomic groups (Figure 5.5), this may be due to the changes being within the 'other' taxonomic groups, or an effect of assessing the data at different resolutions (ASVs as opposed to grouped taxa). At depths greater than 30 cm from the soil surface, substantial differences can be observed relative to shallower soil samples – a reduction in the relative abundance of *Bacillaceae,* and an increase in *Actinobacteria.* Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 5.7, sample diversity is universally high, with no universal trend in richness or diversity as a function of treatment.

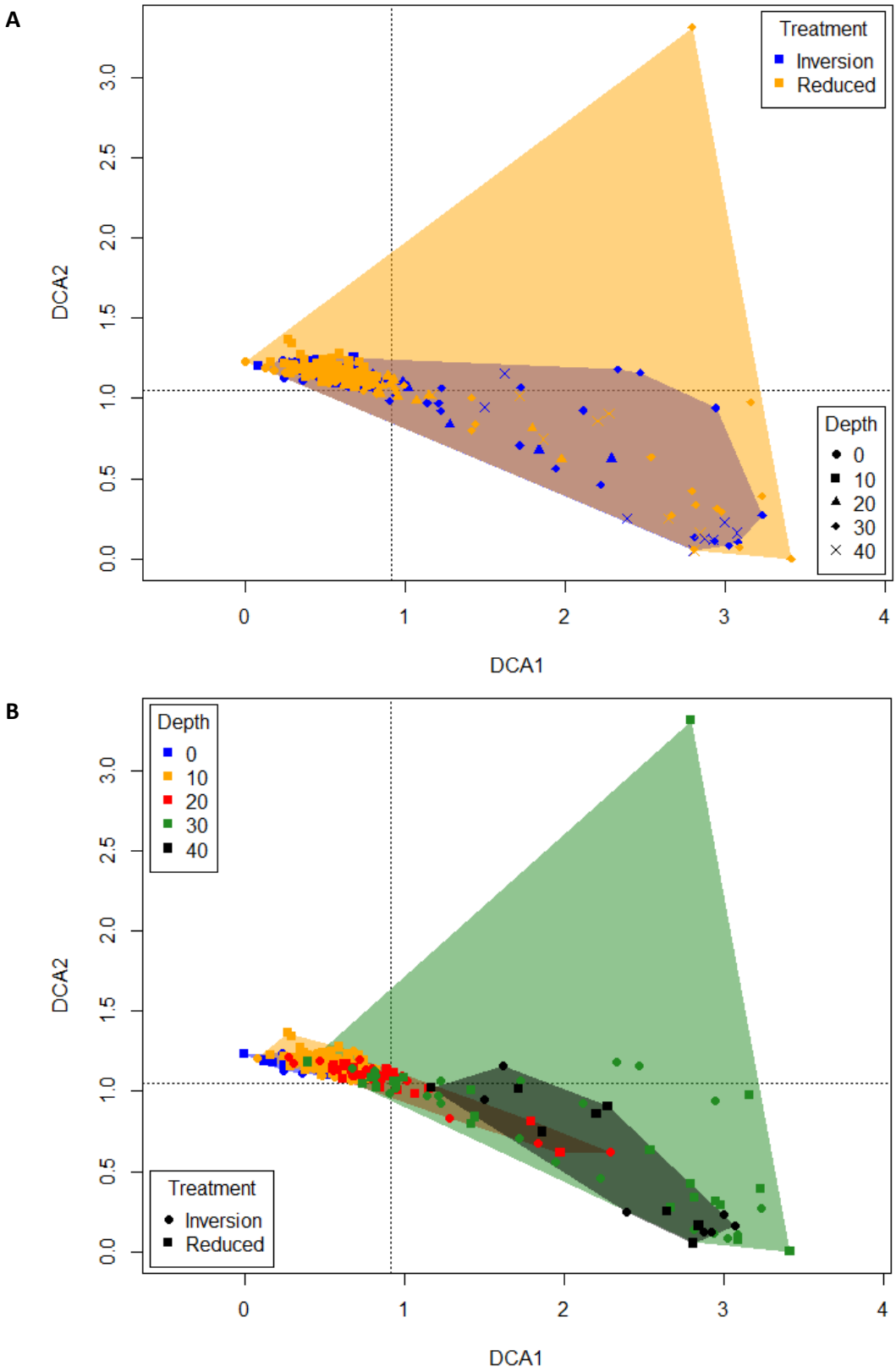


Figure 5.3 – DCA sample scores (axis 1 and 2) for bacterial community composition. (A) Coloured hulls show the range of all samples from IT (blue) and RT (yellow). (B) Coloured hulls show the range of all samples from 0-10 cm (blue), 10-20 cm (yellow), 20-30 cm (red), 30-40 cm (green) and 40-50 cm (black).

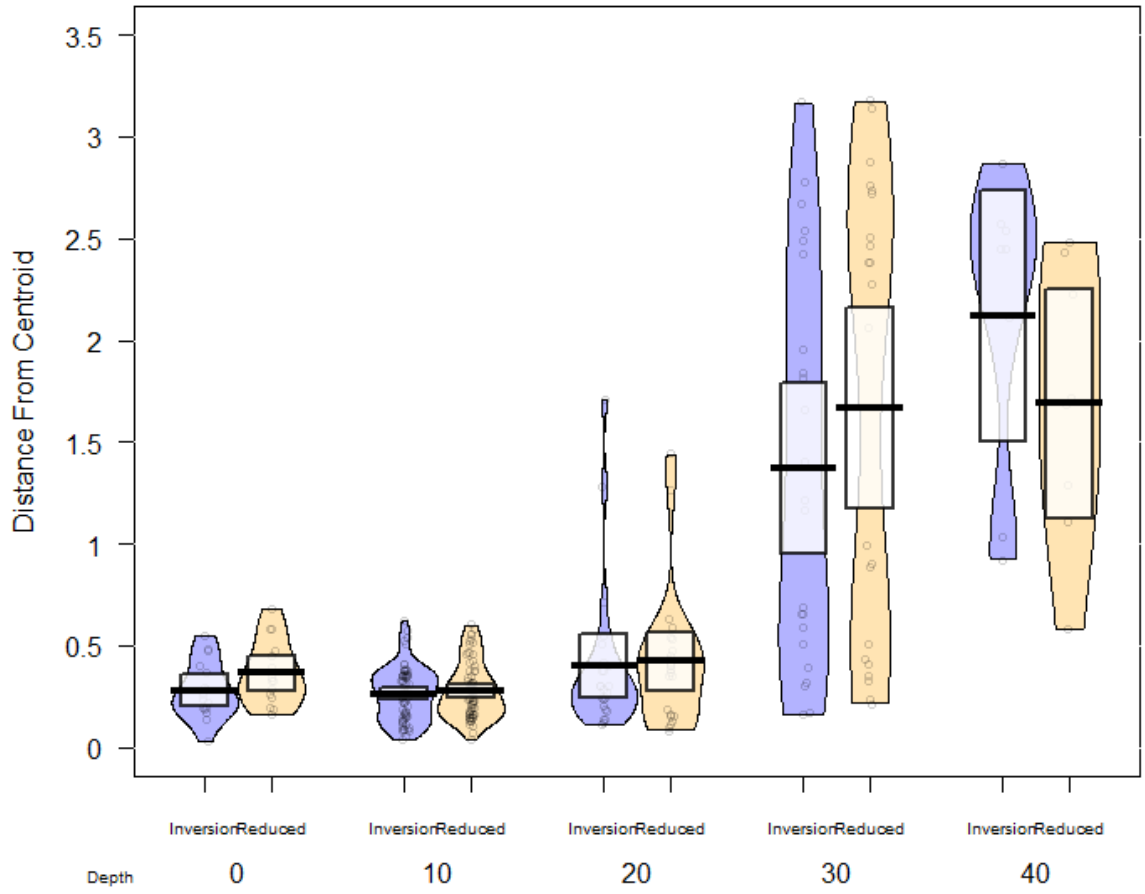


Figure 5.4 – Distance from median sample scores (across all 4 DCA axis) for bacterial community composition in soils under IT (blue) and RT (yellow), split by depth.

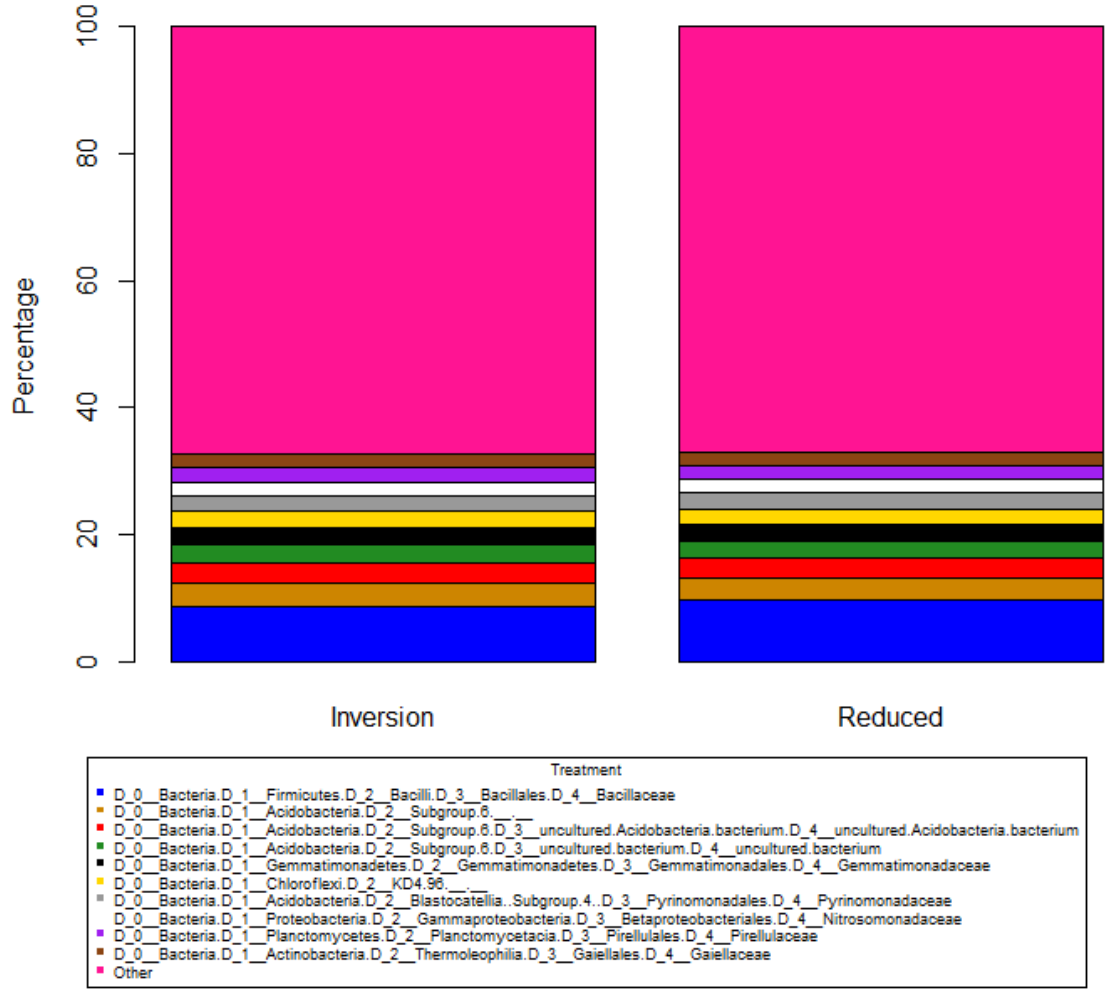


Figure 5.5 – Bacterial community composition. Grouped by treatment.

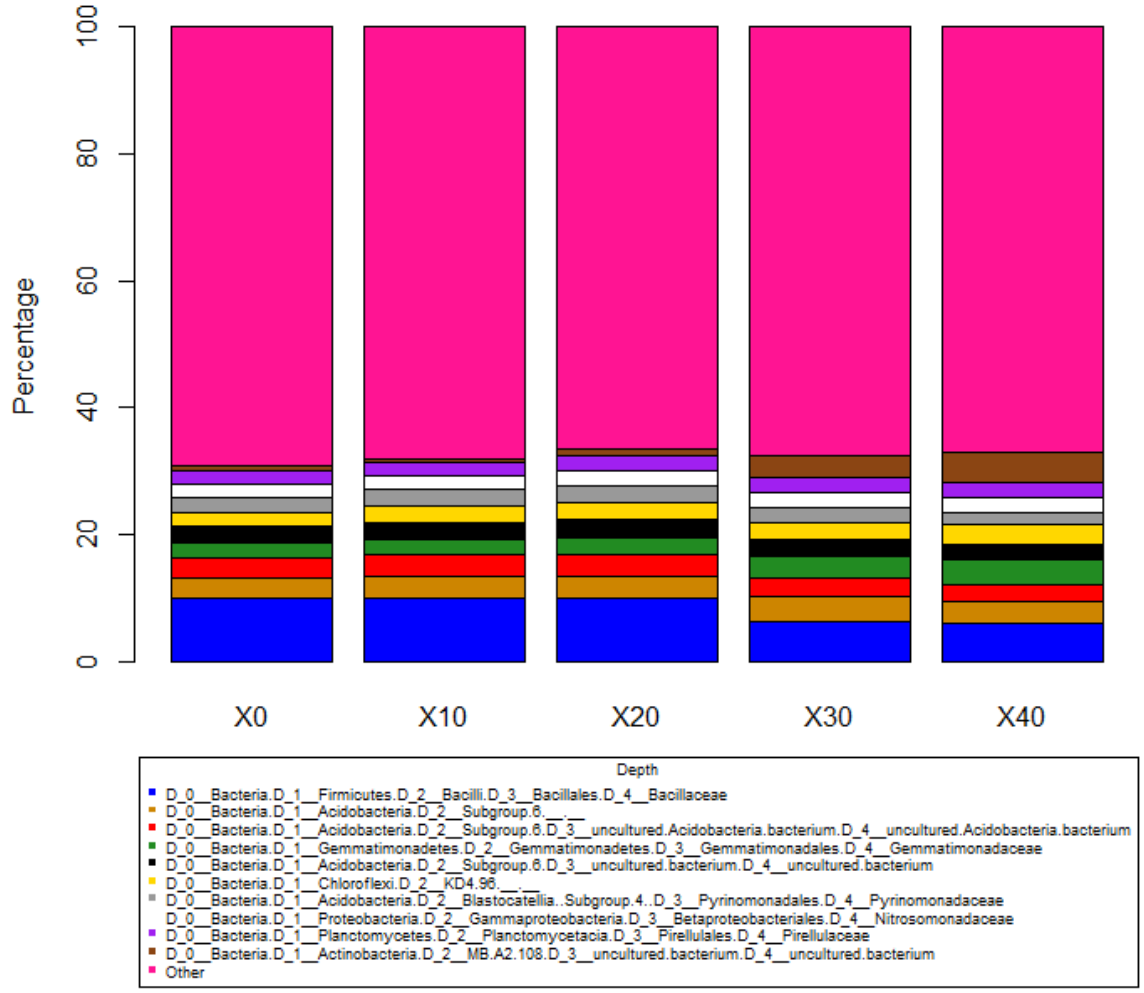


Figure 5.6 – Bacterial community composition. Grouped by depth.

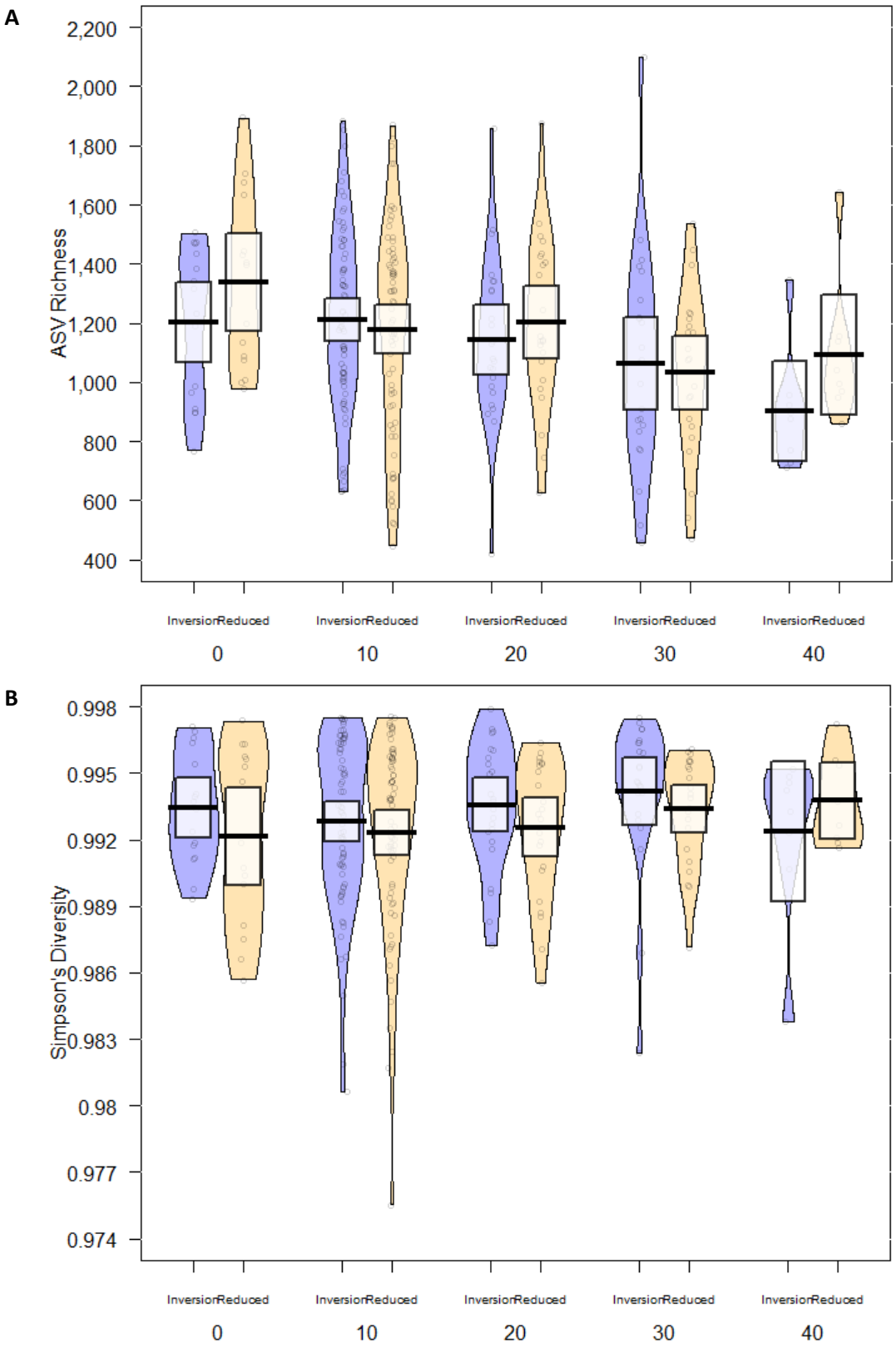


Figure 5.7 – (A) Bacterial ASV richness per sample. (B) Bacterial ASV diversity per sample.

For eukaryotes, PERMANOVA revealed significant effects of treatment, depth, sampling, row and block on community composition (DF=1 F=1.97 P<0.02, DF=1 F=11.3 P<0.001, DF=1 F=4.36 P<0.001, DF=8 F=1.39 P<0.01, DF=3 F=1.48 P<0.02). Wheat variety did not have a significant effect on eukaryote community composition (DF=1, F=0.89, P>0.5). Sampling is the most determinant variable of eukaryote community composition accounting for 8.9 % variance (as percentage of total sum of squares), with treatment, depth, row, block and wheat variety accounting for 0.7 %, 3.9 %, 3.8 %, 1.5 %, 0.3% respectively (residuals 81 %, all to 2 sf.). Figures 5.8A and 5.8B show the DCA ordinations of the eukaryote community composition grouped by treatment and depth respectively – the sizeable effect of depth (increased variability at depths ≥30 cm), and the slight effect of treatment (increase variability with a reduction in tillage intensity) can be observed. Median distances from the centroid of the group for treatment, grouped by depth reveal the much greater variability in community composition in soils deeper than the reach of ploughs, and RT communities are more variable than IT at depths between 0.1 and 0.4 m (Figure 5.9). Figures 5.10 and 5.11 show relative abundance of taxonomic groups of eukaryotes grouped by treatment and depth respectively. Aggregated relative abundances for samples separated by treatment do not a significant treatment effects (Figure 5.10), as was assessed by PERMANOVA – this may be a consequence of analysis conducted at different resolutions (ASVs vs taxonomic groupings). With increased depth, the general trend is an increased abundance of *Poales* (excluding 20-30 cm in depth), while there is a notable reduction in *Embryophyta* at depths ≥30 cm (Figure 5.11). Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 5.12. Within the ploughed layer (<30 cm in depth) of IT plots, eukaryotic diversity is less than the corresponding depths of RT (Figure 5.12), but this is slight, and there is no clear trend in ASV richness at these depths for treatment. At depths ≥30 cm in depth, there are on average slight reductions in ASV richness, and diversity (Figure 5.12).

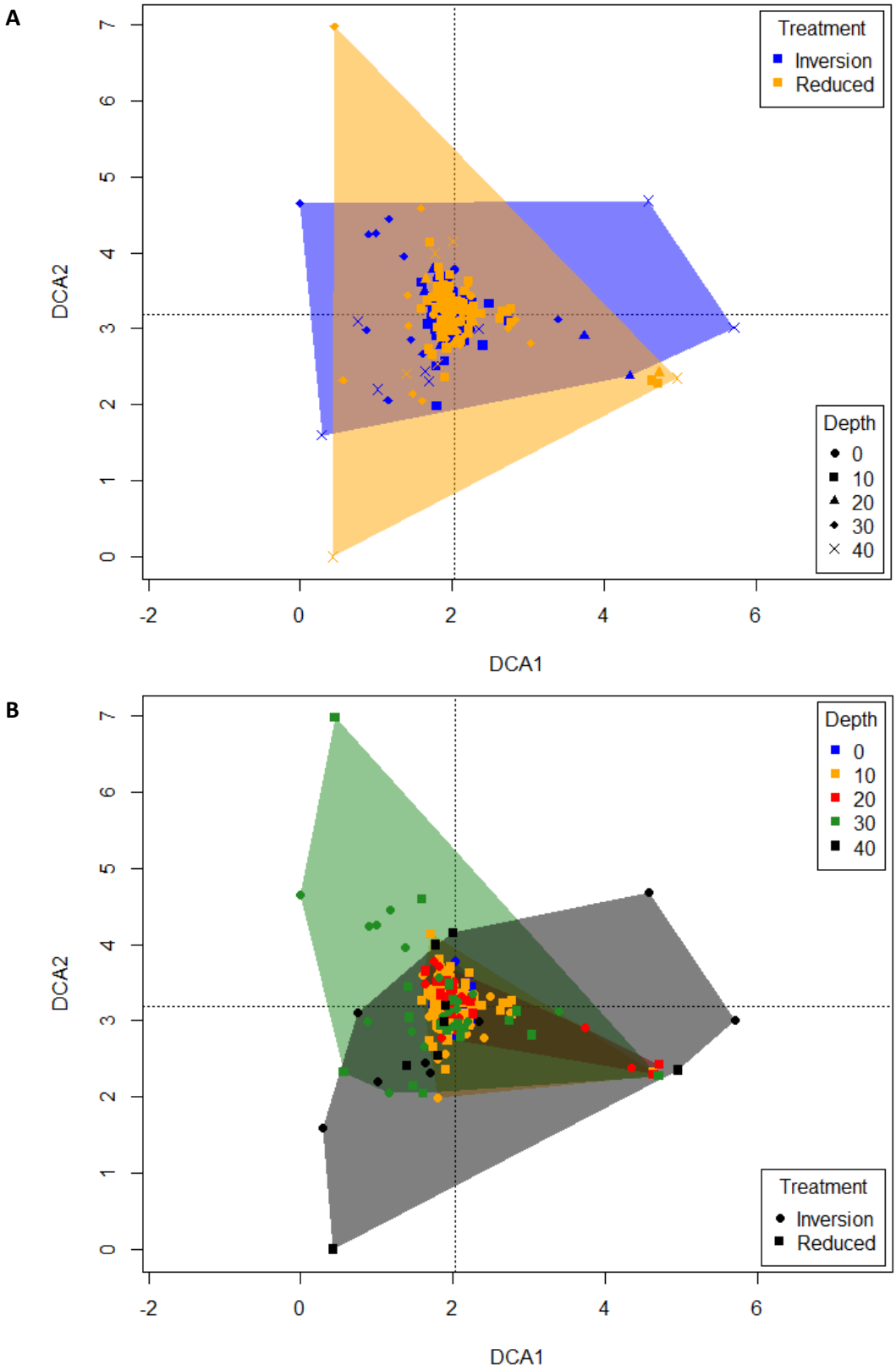


Figure 5.8 – DCA sample scores (axis 1 and 2) for eukaryotic community composition. (A) Coloured hulls show the range of all samples from IT (blue) and RT (yellow). (B) Coloured hulls show the range of all samples from 0-10 cm (blue), 10-20 cm (yellow), 20-30 cm (red), 30-40 cm (green) and 40-50 cm (black).

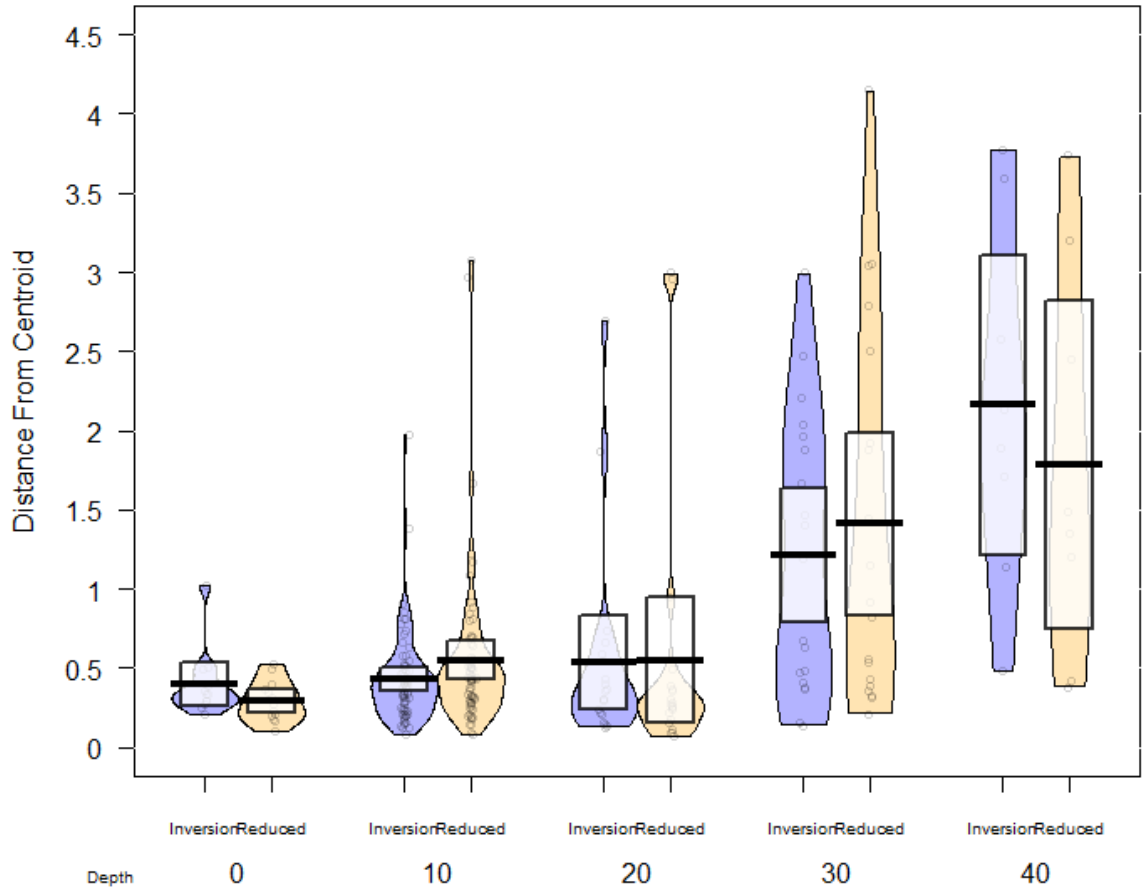


Figure 5.9 – Distance from median sample scores (across all 4 DCA axis) for eukaryotic community composition in soils under IT (blue) and RT (yellow), split by depth.

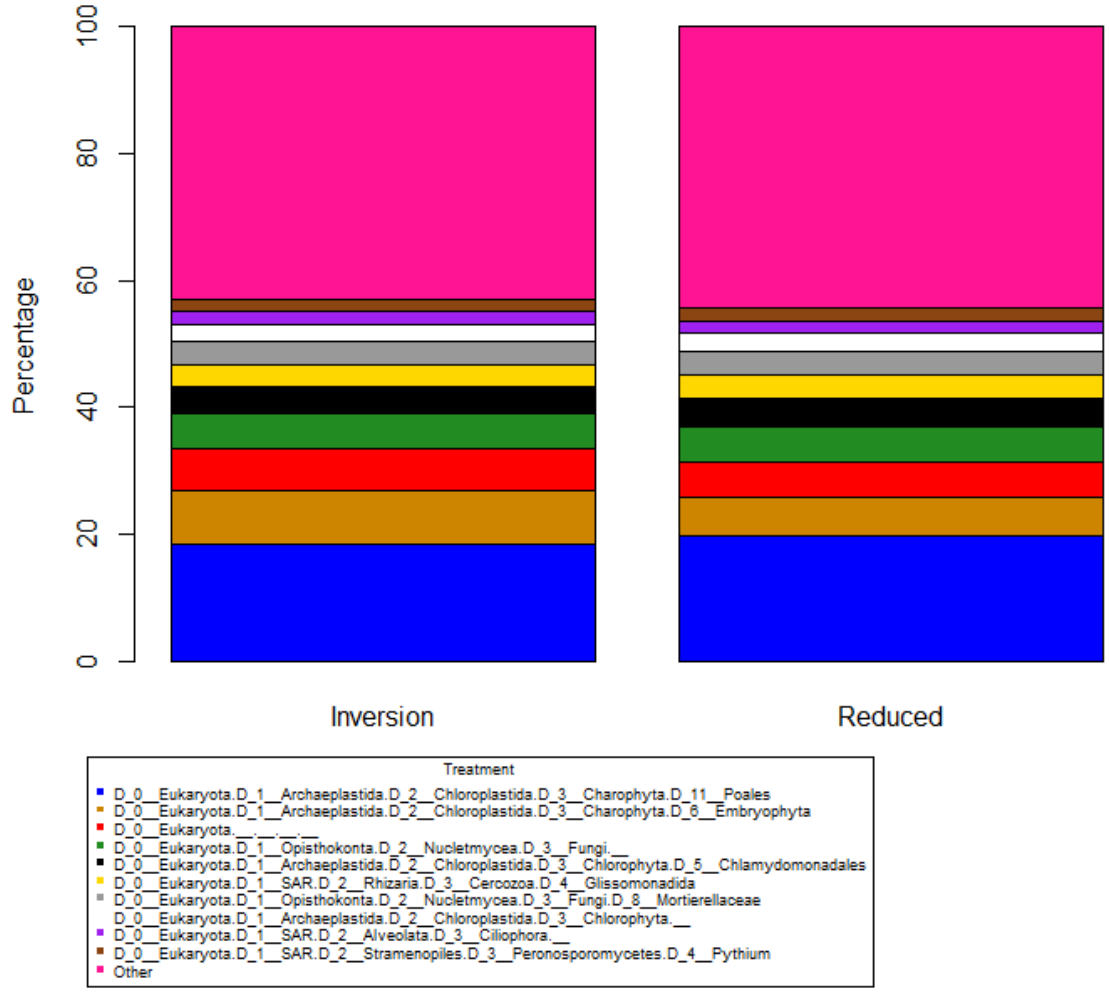


Figure 5.10 – Eukaryotic community composition. Grouped by treatment.

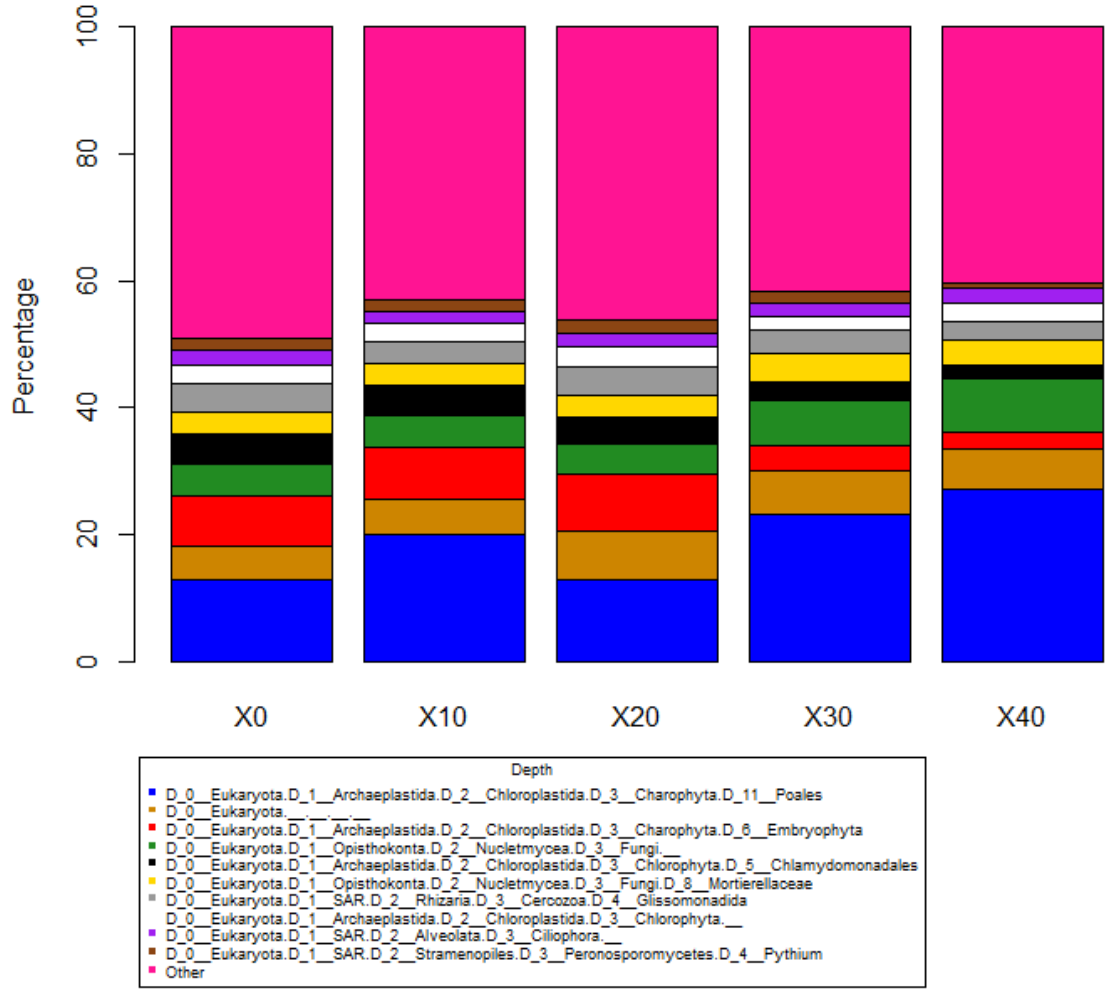


Figure 5.11 – Eukaryotic community composition. Grouped by depth.

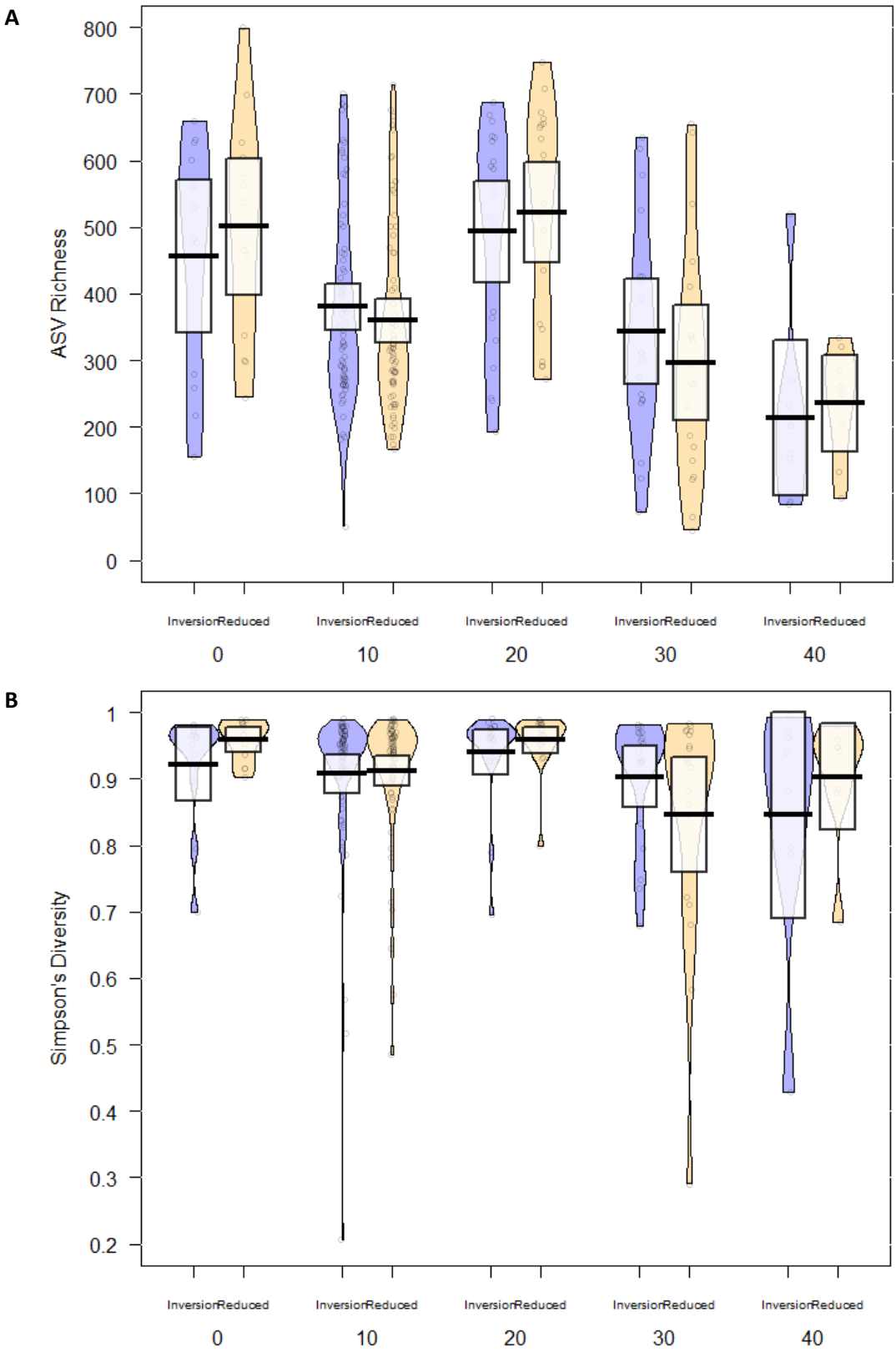


Figure 5.12 – (A) Eukaryote richness per sample. (B) Eukaryote diversity per sample.

For fungi, PERMANOVA revealed significant effects of treatment, depth, sampling, row and block on community composition (DF=1 F=5.86 P<0.001, DF=1 F=31.6 P<0.001, DF=1 F=5.43 P<0.001, DF=8 F=1.35 P<0.02, DF=3 F=1.75 P<0.001). Wheat variety did not have a significant effect on fungal community composition (DF=1, F=0.74, P>0.7). Depth is the most determinant variable of fungal community composition accounting for 9.3 % variance (as percentage of total sum of squares), with treatment, sampling, row, block and wheat variety accounting for 1.7 %, 9.1 %, 3.1 %, 1.5 %, 0.2 % respectively (residuals 71 %, all to 2 sf.). Figures 5.13A and 5.13B show the DCA ordinations of the fungal community composition grouped by treatment and depth respectively – the sizeable effect of depth (a gradient along DCA1 axis, with one or more of the lowest scoring (in DCA1) sample of each successive depth being a greater value than the proceeding (shallower) depth), and the slight effect (greater variability) of treatment can be observed. Median distances from the centroid of the group for treatment, grouped by depth reveal the greater variability in fungal community composition in soils deeper than the reach of ploughs, and RT communities are more variable than IT at depths between 0 and 0.4 m (Figure 5.14). Figures 5.15 and 5.16 show relative abundance of taxonomic groups of fungi grouped by treatment and depth respectively. With respect to treatment, IT relative to RT favours a higher relative abundance of *Plectosphaerellaceae* and *Lasiosphaeriaceae*, with a lower relative abundance of *Didymellaceae* (Figure 5.15). With respect to depth, collectively, the ten most abundant taxonomic groupings tend to decline with increasing depth – particularly *Plectosphaerellaceae*, *Didymellaceae* and *Nectriaceae*, whilst the relative abundance of *Geminibasidiaceae* increases substantially at depths which are expected not to be have been historically ploughed (≥30 cm depth) (Figure 5.16). Per sample ASV richness and diversity (as Simpsons Index) is shown in Figure 5.17. Sample fungal ASV diversity at 0-10 cm in depth is lower in RT soils than IT soils, but at other depths consistent (Figure 5.17B), conversely ASV richness is greater in RT soils in all depths 0-50 cm, excluding 30-40 cm.

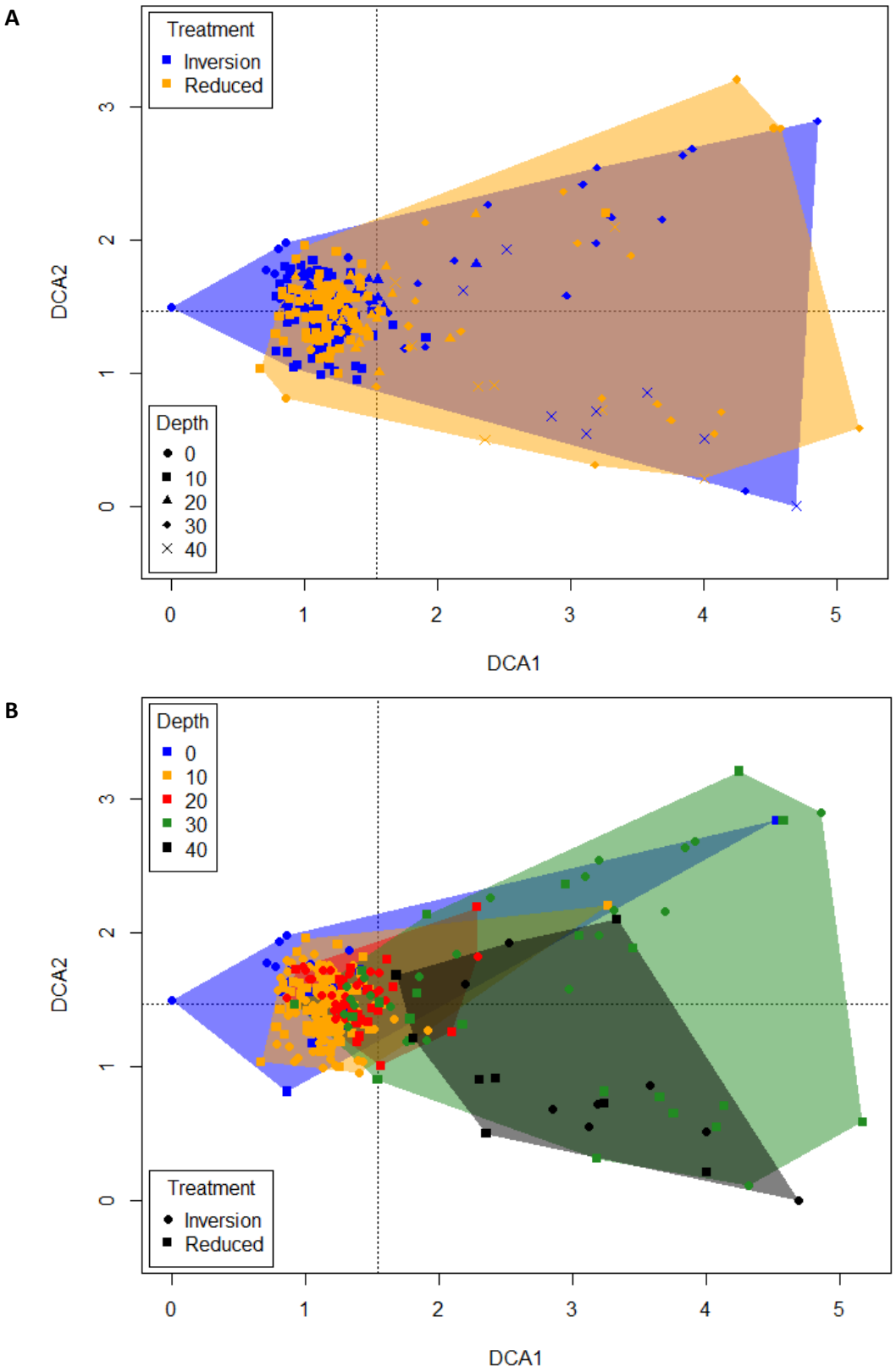


Figure 5.13 – DCA sample scores (axis 1 and 2) for fungal community composition. (A) Coloured hulls show the range of all samples from IT (blue) and RT (yellow). (B) Coloured hulls show the range of all samples from 0-10 cm (blue), 10-20 cm (yellow), 20-30 cm (red), 30-40 cm (green) and 40-50 cm (black).

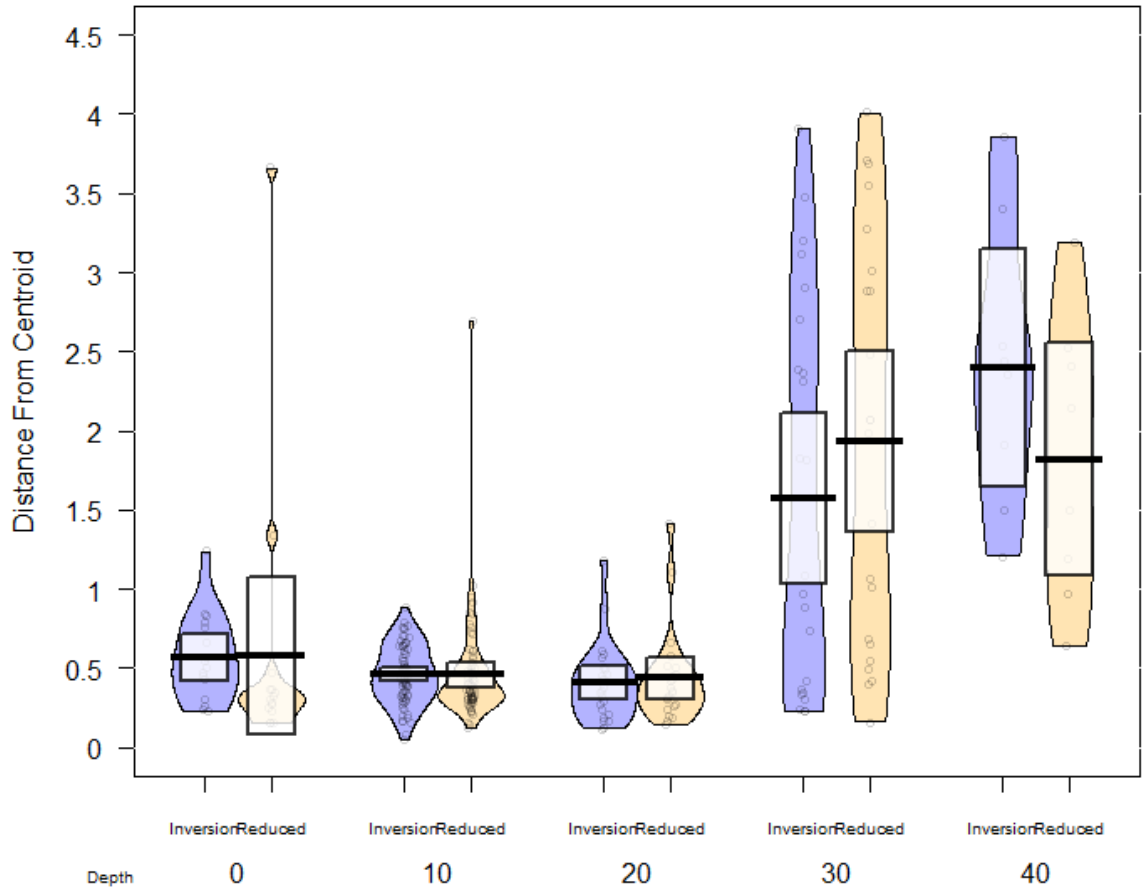


Figure 5.14 – Distance from median sample scores (across all 4 DCA axis) for fungal community composition in soils under IT (blue) and RT (yellow), split by depth.

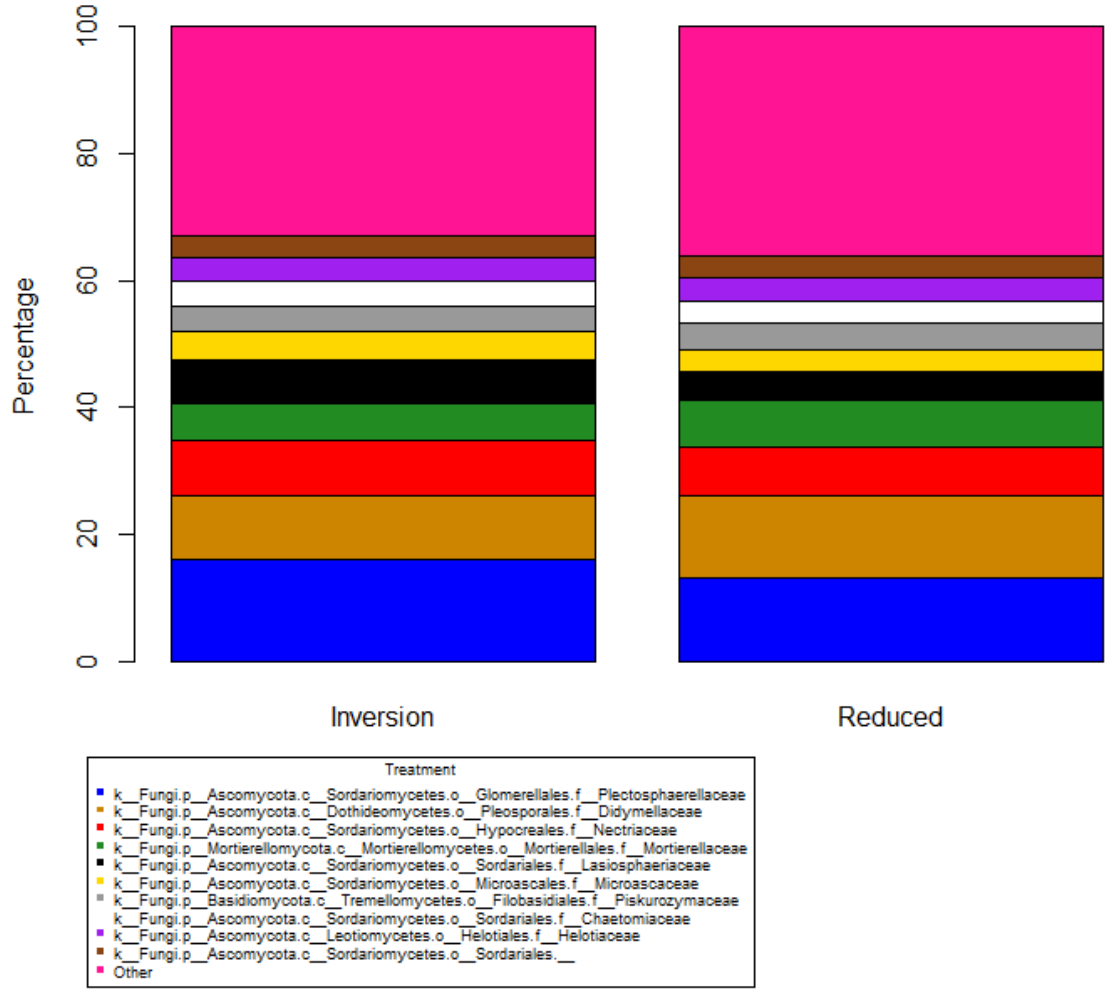


Figure 5.15 – Fungal community composition. Grouped by treatment.

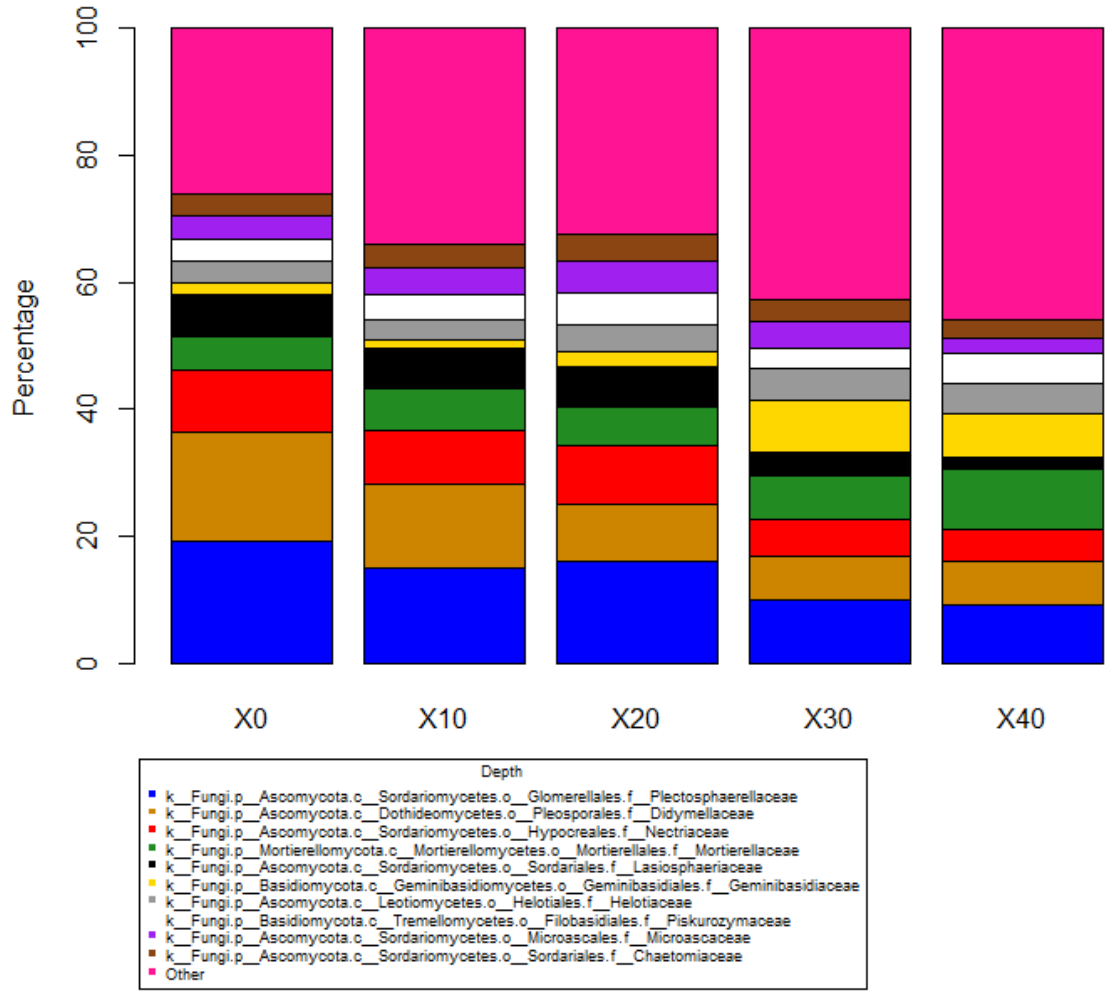


Figure 5.16 – Fungal community composition. Grouped by depth.

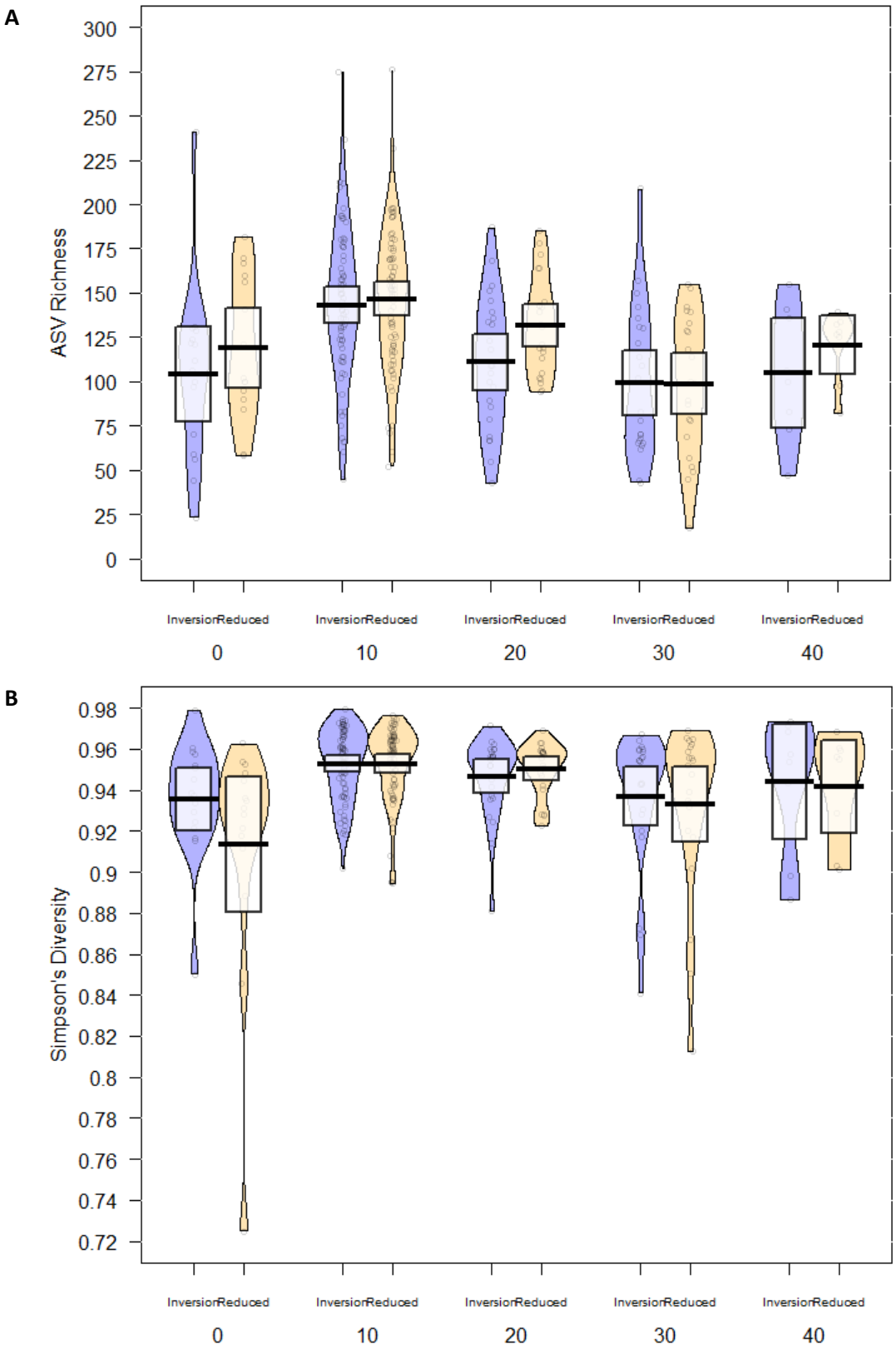


Figure 5.17 – (A) Fungal ASV richness per sample. (B) Fungal ASV diversity per sample.

5.4 Discussion

5.4.1 Effects of tillage method on the soil microbiome

Quarry field has a long history of being cultivated, and had been subject to IT for more than a decade before the field experiment was established. Consequently, the treatments as described within the present study represent the continuation of IT, and a divergence to lower intensity tillage techniques (RT). This transitional phase is beneficial for understanding the transition that many fields are undergoing as RT becomes more common, and limiting relative to longer term experiments to investigate differences between IT and RT.

Nevertheless, reducing the intensity of tillage significantly alters soil microbial community composition, including bacteria ($P<0.05$), eukaryotes ($P<0.02$) and fungi ($P<0.001$) (PERMANOVA results). The relative differentiating impact of tillage type was slight (see Figures 5.3A, 5.8A, and 5.13A), explaining 0.5 %, 0.7 %, and 1.7 % of variance (as percentage of total of sum of squares) for bacteria, eukaryotes and fungi respectively. ASV richness and diversity (Simpsons Index) are similar between treatments for all studied components of the soil microbiome (Figures 5.7, 5.12 and 5.17). These results, consistent diversity but with changes in community composition, are consistent with Boscutti et al. (2015), who identified the same effect in Carabids and flora. Amongst *Charophyta*, IT communities contained a higher relative abundance of *Embryophyta*, with RT samples containing higher relative abundances of *Poales*, *Chlamydomonadales*, and other unidentified *Charophyta*. Amongst fungi, IT saw higher relative abundances of *Plectosphaerellaceae*, *Lasiosphaeriaceae*, and *Mircoascaceae*, and RT higher relative abundances of *Didymellaceae* and *Mortierellaceae*. All studied sections of the soil microbiome were more variable in composition under RT than IT.

The present study further expands the evidence base of fungi being more greatly affected by tillage intensity than bacteria. Higher intensity of tillage is associated with a higher relative abundance of *Glomerellales* and *Sordariales*, and a reduction in the relative abundance of *Pleosporales*. Gosling et al. (2014) report increase tillage intensity to more negatively impact upon the presence of *Glomerellales* spp. A reduction in tillage intensity did not increase the diversity of fungi within the sampled soils, which may have been expected to benefit hyphal species (Helgason et al., 1998).

However, disturbance of filamentous bacteria (*Actinobacteria*) potentially as a legacy effect of prolonged ploughing of the field is apparent, with their relative abundance increase at depths of greater than 30 cm (Figure 5.7). Amongst the total eukaryotic fraction of the soil microbiome, higher intensity of tillage is associated with a higher relative abundance of *Charophyta*, with relatively more

Charophyta Embryophyta to Charophyta Poales. This may be due to greater incorporation of plant residues throughout the soil profile.

5.4.2 The soil microbiome varies with depth

Depth was found to account for the greatest variance in bacterial and fungal community composition (17 % and 9.3 % respectively) and second (to time of sampling) for total eukaryotic community composition (3.9 %). The relative impact of depth can be observed in the DCA results, with bacteria and fungi – slightly – separating by depth along axis 1 (Figures 5.3B and 5.13B), whilst for eukaryotes community dissimilarity radiates in all axis from a relatively central shallow zone (Figure 5.8B).

For all studied segments of the soil microbiome, variance in community composition increased with depth (Figures 5.4, 5.9 and 5.14). The increase in horizontal heterogeneity in community composition is not uniform with depth, but mostly abrupt, separating shallow (0-10, 20-30 and 20-30 cm depth) and deep (30-40, and 40-50 cm) samples. Deeper samples contain on average fewer unique ASVs (Figures 5.7A, 5.12A and 5.17A) than shallower samples, and for eukaryotes each community is on average less diverse in deeper soils (Figure 5.16). A limitation of this analysis is the disproportionate number of samples per depth, with 10-20 cm samples collected at 7 timepoints; 20-30, and 30-40 cm at three timepoints; 0-10 at 2 timepoints; and 40-50 cm at 1 timepoint. This suggests that soil communities at greater depths are more variable than those in the upper soils, even when accounting for seasonal variation in upper soils.

The sharp transition in horizontal heterogeneity with depth between the upper and lower samples could be explained by the field history of the site, which has been subject to IT since as early as 2002. This prolonged period of IT may have homogenised the upper soil, with material translocated both vertically and horizontally across the field (De Alba, 2003, Yamaguchi et al., 2012, Hula and Novak, 2016). The relative abundance of some soil taxa are strongly separated at the transition at 30 cm in depth; in bacteria decreased abundances of *Firmicutes* and higher abundances of *Actinobacteria* below 30 cm (Figure 5.6), and for fungi decreased abundances of *Lasiosphaeriaceae* and increased abundance of *Geminibasidiaceae* below 30 cm (Figure 5.16). Conversely some taxa can be observed to taper in abundance irrespective of the 30 cm transition for example *Didymellaceae* (fungi, decreasing with depth (Figure 5.16)), or have similar relative abundances throughout the soil profile – *Ciliophora* (eukaryote (Figure 5.11)).

The differentiation of community composition with depth may be attributable not only to mixing caused by tillage, but also compaction of the plough-pan underneath the cultivated soil

(Arvidsson, 1998, Li et al., 2019), physicochemical differences in soil dynamics with depth, and the influence of vegetation via the rhizosphere on bulk soils. Soil temperatures at depths greater than 30 cm may remain near constant throughout a day, while the temperature of upper soils may fluctuate significantly (Gulser and Ekberli, 2004). Soil moisture is a significant determinant of soil bacterial diversity (Griffin et al., 2020), with both deeper and more frequently saturated soils being less diverse. However, within the present study bacterial diversity did not decline with depth (Figure 5.7).

The hypothesised increase in stratification of soil microbial community composition under RT, expected with a decrease in mixing is not supported within the presented data. This may be attributable to the limited number of cultivations conducted prior to the sampling across the depth of the soil profile (twice), the reduction in intensity of the RT techniques within the present being relatively close in intensity to IT, or the hypothesis being wrong.

5.4.3 Shortcomings

As detailed in Chapter 2, the presence of relic DNA may mask changes in community composition (Carini et al., 2016), and the same criticisms of a lack of quantitative data can be applied to the present study.

Chapter 6: General Discussion

6.1 Discussion

With respect to thesis aim 1 (identify changes in the composition of soil microbial communities under different tillage intensities): In Chapter 2, a two-year conversion of pasture fields to crop strips under IT and RT, tillage intensity was found to significantly alter the community composition of soil eukaryotes. In a longer-term study (four instances of variable tillage intensities), as detailed in Chapter 5, tillage intensity was found to significantly alter the community composition of soil bacteria, eukaryotes, and fungi. Further, Chapter 5 clearly details that upper soils subject to tillage, both in the duration of the experiment and previously, contain substantially more homogenous bacterial, eukaryotic and fungal communities than deeper non-tilled soils. With limited (2 samplings after 1, and 3 samplings after 2 instances of tillage) data (see Figures 5.4B, 5.9B and 5.13B) suggest that as hypothesised, a reduction of tillage intensity may lead to a more variable community composition throughout the soil profile, and that this is greater at greater depths. Thus, chapter 5 can be considered to also address thesis aim 6 (identify how tillage intensity alters the spatial variability of soil microbial communities).

These finds are generally consistent with the hypothesis of the present work, and with the scientific literature, that tillage intensity alters the soil microbiome (Helgason et al., 1998, Yin et al., 2010, Zuber and Villamil, 2016, Wang et al., 2017, Somenahally et al., 2018). The present thesis does so across broad taxonomic groupings at a higher resolution than many preceding works, due to the application of high throughput amplicon sequencing/metabarcoding techniques. The present work details how tillage shapes the variability in soil communities, in both the horizontal and vertical. It could be concluded that a legacy effect of ploughing of Quarry field has led to a vertical divide with previously ploughed soils substantially more homogenous than those (deeper) that are unploughed. Horizontally, reduced tillage intensity can be expected to increase the heterogeneity of soil, soil microbial communities, and their functions, but that in aggregate these effects may be minor.

As detailed in Chapter 3, the rate of degradation of Mandipropamid and Simazine were similar in soils under IT and RT, but the rate of degradation was more variable at a lower intensity of tillage. Thus, thesis aim 3 (identify differences in the rate of degradation of CPP in soils at different tillage intensities) can be considered to have been addressed. This finding suggests whilst sampling regimes for studies may be need to be adapted to account for increased heterogeneity as RT becomes increasingly common, at landscape or catchments scales the effects of RT with respect to CPP environmental fate may be minimal. In Chapter 4, it was discovered that the soil cores from RT

tillage hosted more variable soil bacterial communities than IT soils. Combining these findings, we can conclude that tillage can alter the distribution of soil bacterial communities, that this alteration may extend to the distribution of important functional genes that might otherwise be more widely distributed to soil bacterial communities via self-transmissible plasmids (de Souza et al., 1998a), and that tillage intensity can alter, at least at small scales, the environmental fate of crop protection products.

With respect to thesis aim 5 (identify the effects of the incubation of soil for degradation studies on soil microbial communities), as detailed in thesis section 4.4.2, PERMANOVA analysis of the bacterial, eukaryotic, and fungal communities of the incubated soils, found there only to be a significant difference in bacterial community composition. However, such conclusions are limited, both due to each core being discreet units sampled destructively at each timepoint, and the potential for temporal effects to be masked by relic-DNA (Carini et al., 2016, Hannula et al., 2019).

Determination of universal rules for predicting the environmental fate of CPPs in soils is challenging due to the broad array of compound characteristics, variable microbial degradation potential, and soil physicochemical characteristics, that determine the availability to, and rate of degradation processes (Kah et al., 2007, Alletto et al., 2010). Altering tillage intensity can alter many key soil properties and microbial communities, and in some combinations of soil and CPP, compound persistence may increase, remain constant, or decrease. The present study can conclude that for Mandipropamid and Simazine, degradation rates are near consistent at differing tillage intensities. However, the present study can suggest that the decreased mixing in RT systems may lead to greater spatial variability in CPP fate. The relevance of such small-scale variation in the rate of degradation may be valuable to inform the sampling strategies of future studies, even if only mean or aggregated values from studies are then subsequently used for environmental risk assessment.

In Chapter 4, failure to amplify genes associated with the degradation of s-triazine herbicides is detailed, and possible reasons for this are discussed. Of these possible reasons, low abundance of the genes (or at least a subset thereof) for which amplification was attempted may be considered the most probable. It can be concluded that thesis aim 4 (identify if tillage intensity alters the distribution of genes associated with CPP degradation within the soils) was not achieved. It has been suggested that studies of microbial biogeography should be focussed upon genes, and not species or taxa (Nesbo et al., 2006, O'Malley, 2008). This is important, as it suggests that PCR amplification and by extension metabarcoding are not sufficiently sensitive tools to study the rarest fractions of the soil microbiome. In the future, use of shotgun-metagenome sequencing may be an effective way of assessing relatively rare but functionally important genes within soil communities.

Additionally, as detailed in Chapter 2, the present work identifies, for the first time, that soils under hedgerows host distinct communities of soil bacteria and eukaryotes, and expands Holden et al. (2019) findings for distinct fungal communities to bulk soil fungal communities, Holden et al. (2019) detailing DNA extraction from roots, but not soil.

This finding suggests a need to conserve hedgerows, and the distinct communities, as these may act as reservoirs of functionally valuable species or genes, that may otherwise not be found within agricultural landscapes (Holden et al., 2019). Recovery of earthworm populations following a reduction in management intensity is dependent upon recruitment from infield populations, and not from field boundary populations (Prendergast-Miller et al., 2021). However, the greater variability of infield communities closer to field boundaries (see Figures 2.6, 2.14, 2.19, 2.19, 2.24 and 2.29) may be suggestive of dispersal from field boundary communities. Findings regarding the potential dispersal from field boundaries are valuable with respect to thesis aim 2 (contextualise the composition of soil microbial communities in arable soils relative to other soils in an agricultural landscape (from pastures, field margins, and hedgerows)). This suggests a need for future sampling of pasture or tilled soils for CPP studies (either laboratory OECD 307, or terrestrial field dissipation studies) to sample at greater distances from field boundaries than may currently be permissible, to more be representative of the majority of field conditions (abiotic) and communities (biotic).

Elements of both a reduction in tillage intensity, and the protection of hedgerows feature within DEFRA (2021b) Sustainable Farming Incentive (SFI) scheme. Under the SFI scheme, farmers within England will be able (the scheme will be open to all farmers from 2024) to enter agreements on a per field basis, to receive payments for conducting sustainable activities (DEFRA, 2021b, DEFRA, 2021c). DEFRA (2021a) guidance on RT and NT farming includes provision for the use of ploughing every three years, the effects of which may limit both biodiversity and SOC gains under the scheme, as ploughing may disrupt fungal networks, and increase soil aggregate turnover (Helgason et al., 1998, Sheehy et al., 2015, Six et al., 2004). As concluded from Chapter 2, hedgerows host distinct soil microbial communities, and whilst DEFRA (2021b) guidance describes some benefits of hedgerow protection, this should be extended to reflect these distinct communities.

In conclusion, the present study has demonstrated how tillage intensity alters the composition of key soil microbial communities, and that this effect may be observed even within short term (2 tillage events) experiments. Whilst it was observed that a reduction in tillage intensity may increase the heterogeneity in the rate of degradation of crop protection products, that aggregated amongst samples this effect is not significant.

6.2 Recommendations for future work

The following are specific recommendations to develop the work presented here and to evolve the questions and issues raised:

1. Application of predictive functional profiling tools, may be applied to the data detailed in Chapters 2, 4 and 5. For the 16S datasets, this may involve use of either PICRUST Langille et al. (2013) and/or Tax4Fun (Asshauer et al., 2015). Reviewing these two tools, Koo et al. (2017) recommends the use of both tools concurrently for a more comprehensive analysis. For fungal data, FUNGuild (Nguyen et al., 2016) provides a tool for functional profiling based upon the guild system. Such techniques would expand upon the presented findings to; understand the impact of tillage on soil function (where applied to Chapters 2 and 5); expand the understanding of incubation conditions on soil function (Chapter 4, and thesis aim 5); and as an alternative technique in that may address the distribution of functional genes associated with CPP degradation.
2. Application of cooccurrence network analysis approaches such as those described by Cobo-Diaz et al. (2019), would allow for exploration of associations between ASVs or taxa identified between samples and datasets, and may identify key nodal subjects, and/or symbiotic or antagonistic relationships between subjects. Were such symbiotic and/or antagonistic relationships to be identified, these may facilitate understanding if particular groups (taxonomic, or if the above point were to be completed, functional) are more sensitive to changes in tillage techniques, and therefore longer-term community changes might be predicted from short term experiments, as detailed in Chapters 2 and 5.

The following are general recommendations for future studies that aim to assess the role of tillage in shaping the soil microbiome, and in determining the environmental fate of CPPs:

1. The use of long-term field sites, in which different tillage intensities have been applied consistently to plots for five or more years prior to sampling. Such plots would be beneficial in establishing the conditions within stable agriculturally managed soils, and not transitional conditions as detailed within the present work.
2. A greater sampling effort of field studies on the effect of depth on the composition, abundance and function of soil microbial communities under different tillage intensities. As described in Chapter 5, it appears that tillage through the translocation of abiotic and biotic materials creates a homogenised zone in the upper section of soil profiles. Had greater sampling been conducted through the depth of the soil profiles of the SoilBioHedge experiment (as described in Chapter 2, and by Holden et al. (2019)), it is possible that this mixing effect would have been observed as pasture plots transitioned to tilled (IT and RT

plots). Further, the legacy and potential recovery of Warren field (known to be previously tilled), relative to Paddock and Valley field may have been addressable. Additionally, while near surface (ca. <15 cm in depth) soils under hedgerows were found to highly distinct from infield communities (Chapter 2), whether this distinctness holds at greater depths in the soil profile is unknown.

3. Studies to address how the environmental fate of CPPs is altered by different tillage intensities should address the occurrence of metabolites, which may be formed and/or degrade at different rates depending upon specific combinations of tillage and soil type. Such an analysis would address a weakness of Chapter 3, where only the rate of degradation of the parent compound was assessed. Were different formation fractions of metabolites found to be formed in soils that had been tilled differently, this would evidence that tillage intensity alters the route of degradation of CPPs, which in turn would imply alteration of the distribution of the necessary functional genes within agricultural landscapes.
4. Field dissipation studies of CPPs applied to plots under different tillage intensities may identify more accurately alterations in the rates of degradation, dissipation and leaching of compounds, more accurately capturing differences in soil conditions. An intermediary stage may include the conduct of lysimeter studies. The use of field trials avoids the potential influence of laboratory incubation conditions, soil sampling, and soil processing techniques, that may influence the findings of laboratory studies, such as detailed in Chapter 3. As detailed in Chapter 4, incubation conditions may have led to significant differences in soil bacterial community composition, thereby potentially lessening or preventing a divergence in the rate of compound degradation in the two soil types.
5. Studies may adopt metagenomic approaches to identify the distribution of functional genes within the soil profile. Such studies may investigate the abundance of CPP degrading genes directly, or should these remain elusive, may imply from the distribution of other functional genes, for example nitrate reducing, how tillage intensity shapes the variability of gene distribution within agricultural soils. Application of such techniques may overcome the significant challenges encountered in the attempted amplification of Simazine degrading genes, as discussed in detail in thesis section 4.4.1.
6. Assess how the microbial community of crop residues left on the soil surface by reduced tillage techniques differ from the soil biological crust of adjacent soils, both compositionally and functionally. Inclusion of crop residues in degradation studies, such as on the surface of soil cores should be investigated. Inclusion of crop residues within degradation studies would encapsulate one of defining features of RT soils relative to IT (≥30% of the crop

surface being covered in crop residues) (Holland, 2004). Consequently, the interception of CPPs, and potential contribution for degradation of CPPs from a potentially distinct community should be accurately characterised across a range of compounds.

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Appendix 1

Appendix 1 Table 1 details applications of CPPs to SBH study fields between 2009 and the 9th of March 2017 (Table 1). Data summarised from data held by WRSAC (2017).

Table A1.1 – Active ingredients applied to each of the pasture fields with crop strips from 2009 to the 9th March 2017

	Field		
Year	Sub Paddock	Valley Field	Warren Paddock
2009	-	-	Chlormequat Chlorothalonil Cyproconazole Diflufenican Epoxiconazole Fenpropimorph Flufenacet Fluroxypyr Iodosulfuron-methyl-sodium Kresoxim-methyl Mesosulfuron-methyl Prothioconazole Tebuconazole
2010	Clopyralid Fluroxypyr Triclopyr	Clopyralid Fluroxypyr Triclopyr	Carbendazim Chlormequat Chlorothalonil Clomazone Cypermethrin Cyproconazole Epoxiconazole Florasulam Fluazifop-P-butyl Fluroxypyr Flusilazole Glyphosate Lambda-cyhalothrin Metaldehyde Metazachlor Metconazole Metsulfuron-methyl Prochloraz Propaquizafop Propiconazole Prothioconazole Pyraclostrobin Pyroxsulam Quinmerac Tebuconazole Tribenuron-methyl
2011	Clopyralid Fluroxypyr Triclopyr		Clopyralid Glyphosate Lambda-cyhalothrin Prothioconazole
2012	-	Clopyralid Fluroxypyr Triclopyr	Clopyralid Fluroxypyr Triclopyr
2013	-	Clopyralid Fluroxypyr Triclopyr	-
2014	-	-	-
2015	Glyphosate	Glyphosate	Glyphosate
2016	Glyphosate **KNOWN UNKNOWNS***	Glyphosate	Glyphosate
2017	Diflufenican Iodosulfuron-methyl-sodium Mecoprop-p Mesosulfuron-methyl	Diflufenican Iodosulfuron-methyl-sodium Mecoprop-p Mesosulfuron-methyl	Diflufenican Iodosulfuron-methyl-sodium Mecoprop-p Mesosulfuron-methyl

*Field notes record CPP application within this field, but records detailing the active ingredients used in these treatments are not available.

Appendix 2

Appendix 2 Table 1 summarises data on soil properties from SoilBioHedge fields Paddock, Valley and Warren, generated by Grayson (2020) – data has been summarised, arithmetic means and standard deviations calculated.

Table A2.1 – Soil property data for Paddock, Valley and Warren fields. Data is arithmetic mean and standard deviation.

Field	Treatment	Depth cm	pH		Conductivity		Nitrate mg l^{-1}		Ammonia mg l^{-1}		Phosphate mg l^{-1}		DOC mg l^{-1}		DIC mg l^{-1}	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Paddock	Hedge	5	5.58	1.20	0.89	0.30	256.23	158.42	0.99	1.73	0.50	0.61	51.65	14.66	16.30	34.30
		40	5.64	1.29	0.66	0.26	156.30	117.03	0.30	0.36	0.06	0.09	20.45	13.43	16.15	29.04
		All	5.60	1.21	0.80	0.30	216.26	149.62	0.71	1.39	0.32	0.52	41.67	20.41	16.25	31.89
	Pasture	5	6.03	0.67	0.31	0.26	84.49	86.32	0.28	0.42	0.13	0.17	15.17	4.07	12.33	27.63
		40	5.31	2.08	0.50	0.19	177.92	109.36	1.42	2.99	0.08	0.07	11.05	3.91	4.28	4.00
		All	5.67	1.54	0.40	0.24	133.67	107.65	0.88	2.21	0.11	0.13	13.27	4.39	8.62	20.14
	Inversion	5	7.08	0.43	0.56	0.16	124.20	89.20	0.12	0.09	0.12	0.18	21.91	2.89	28.24	25.47
		40	6.97	0.47	0.56	0.22	232.90	156.50	0.13	0.09	0.11	0.13	19.95	7.93	2.40	0.92
		All	7.01	0.43	0.56	0.19	186.31	139.16	0.13	0.09	0.11	0.15	20.73	6.23	12.74	19.87
	Reduced	5	6.17	0.69	0.34	0.14	113.33	86.28	0.08	0.08	0.04	0.04	14.01	5.43	9.61	20.44
		40	7.65	NA	0.83	0.28	362.19	181.97	0.13	0.10	0.05	0.06	3.48	NA	77.99	NA
		All	6.33	0.81	0.54	0.30	215.66	173.48	0.10	0.09	0.05	0.05	12.84	6.17	17.21	29.75
Valley	Hedge	5	7.70	0.34	0.85	0.12	182.27	262.16	0.20	0.14	2.34	2.98	48.88	9.60	45.10	5.22
		40	8.33	0.71	0.89	0.31	144.19	132.43	0.19	0.24	0.21	0.32	20.03	5.78	50.04	3.24
		All	7.87	0.53	0.87	0.24	162.04	201.16	0.20	0.20	1.21	2.29	42.09	15.32	46.26	5.20
	Pasture	5	7.68	0.56	0.63	0.11	52.98	52.88	2.69	4.06	1.81	2.47	18.38	8.66	67.10	11.60
		40	7.73	0.31	0.65	0.07	49.51	23.41	0.14	0.11	0.12	0.28	18.69	4.45	62.98	5.99
		All	7.70	0.45	0.64	0.09	51.24	39.71	1.42	3.07	0.97	1.91	18.51	6.94	65.38	9.53
	Inversion	5	8.01	1.27	0.73	0.25	187.61	154.64	0.14	0.15	3.50	1.57	33.03	14.24	43.63	14.34
		40	7.14	NA	0.65	0.12	99.93	61.79	0.22	0.17	0.14	0.16	NA	NA	NA	NA
		All	7.86	1.19	0.69	0.19	143.77	121.93	0.18	0.16	1.82	2.05	33.03	14.24	43.63	14.34
	Reduced	5	8.57	0.75	0.50	0.14	64.72	59.93	1.31	3.21	0.40	0.52	46.59	41.04	48.59	6.10
		40	7.91	0.24	0.63	0.11	103.23	75.10	0.40	0.36	0.42	0.38	30.65	11.05	63.26	20.10
		All	8.27	0.65	0.57	0.14	85.11	69.17	0.83	2.19	0.41	0.44	37.73	27.63	56.74	16.61

Table A2.1 Continued

Field	Treatment	Depth cm	pH		Conductivity		Nitrate mg l^{-1}		Ammonia mg l^{-1}		Phosphate mg l^{-1}		DOC mg l^{-1}		DIC mg l^{-1}	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Warren	Hedge	5	7.59	0.98	0.81	0.19	77.41	134.90	0.58	1.73	1.50	1.42	48.45	15.46	47.98	22.66
		40	7.50	0.94	0.76	0.09	89.03	96.15	0.27	0.33	0.17	0.26	36.60	26.14	48.55	18.10
		All	7.56	0.94	0.78	0.15	83.22	115.50	0.43	1.23	0.83	1.21	43.44	21.04	48.22	20.46
	Pasture	5	7.16	1.04	0.59	0.08	64.66	69.23	0.19	0.22	0.14	0.16	12.24	3.85	57.11	28.53
		40	7.34	0.60	0.63	0.15	78.08	106.78	0.41	0.92	0.20	0.43	18.55	17.63	63.19	44.30
		All	7.23	0.87	0.61	0.11	70.41	83.74	0.28	0.60	0.16	0.29	14.76	11.07	59.87	34.67
	Inversion	5	7.39	0.31	0.62	0.06	88.11	111.79	0.24	0.38	0.25	0.54	12.55	2.75	65.68	9.47
		40	7.72	0.45	0.62	0.15	114.03	140.32	0.28	0.44	0.19	0.36	NA	NA	74.57	NA
		All	7.49	0.36	0.62	0.10	99.22	120.33	0.26	0.39	0.22	0.45	12.55	2.75	66.80	9.31
	Reduced	5	7.23	0.49	0.57	0.13	55.21	49.45	0.08	0.08	0.17	0.29	9.52	4.06	60.21	28.02
		40	7.50	0.17	0.79	0.11	106.21	176.60	0.09	0.07	0.06	0.06	9.16	8.57	86.66	13.98
		All	7.37	0.38	0.68	0.16	80.71	128.51	0.08	0.07	0.12	0.21	9.34	6.44	73.43	25.31