Changes in Soil Microbial Community and Function During OECD307 Degradation Studies

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Abstract

Global manufacturers use OECD guidelines to safety test Crop Protection Products (CPPs). Of these the first test is OECD307 whereby microbial degradation rates are observed in bottled soil samples. OECD307 is unreflective of in-field degradation as guidelines require standardised processing of the soil and specifies a potential storage regime. These processing steps have previously been shown to alter the microbial community and function. This thesis aims to elucidate the impact of these processes on the soil microbial community within an OECD307 test.

In Chapter 2 amplicon fingerprinting revealed that processing alters the composition but not the structure of the soil fungal community, and that the season of sampling and site from which soil is sampled has a more significant impact on the resulting fungal community due to fact it determines the starting community.

In Chapter 3 amplicon fingerprinting and nutrient measurements revealed that a depletion in organic carbon is a key driver of the change in the bacterial community, and a depletion in organic carbon and nitrogen is a key driver of the change in fungal community.

In Chapter 4 LC-MS-MS metabolomics revealed a significant change in the microbial metabolic fingerprint during incubation. A reduction in markers of carbon sources, biosynthesis, and respiration indicated an overall reduction in metabolic activity of the microbial community.

In the General Discussion a mechanistic interpretation of the data is detailed. Briefly, homogenisation of the soil during processing causes the mixing of previously distinct microbial communities and nutrient pools. This causes a stochastic shift in community and a rapid depletion of the shared nutrient pool. This in turn causes a retardation in metabolism. This will alter the degradation rate of a CPP, potentially accounting for the unreliability of degradation data. Further work involving an integrated multi-omics approach to validate this model is also detailed here.

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Author's Declaration

I, Jean-Pascal Miranda, 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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1 Chapter 1: General Introduction

Safety testing is a major aspect of the research and design process of crop protection products. Test guidelines utilised by both the US Environmental Protection Agency and European Commission are developed by the Organisation for Economic Co-Operation and Development (OECD). One such test, OECD307, constitutes the primary test a product must pass before further testing. This test examines microbial breakdown of a product in soil, however soil must be processed before use in the test in order to conform to OECD guidelines. Test results are often difficult to replicate (unpublished data, Syngenta), and this has been linked to the possible changes in the microbiome caused by processing.

This body of work presents experiments undertaken to examine the changes in microbial community and function caused by soil processing, and utilises the data obtained to infer how it may impact the degradation rates observed within OECD307 tests.

1.1 OECD Outline and Guidelines

Persistence of Crop Protection Products (CPPs), such as glyphosate and metalaxyl, in the environment can potentially be harmful to both natural and agricultural ecosystems (Freemark and Boutin, 1995). CPP manufacturers therefore have to perform extensive testing to assess the degradation rates of their products before licensing for sale (EPA, 2019; HSE, 2019) . Most major manufacturers follow guidelines developed by the OECD. The OECD Guidelines for the Testing of Chemicals is a collection of internationally agreed methods for regulatory safety testing, which examine the environmental fate and behaviour of compounds which are candidates for licensing (OECD, 2019).

The Guideline tests are tiered with laboratory tests constituting the initial step before modified laboratory tests, semi-field, and field studies. One of the initial tests, OECD 307 (see Appendix), examines aerobic and anaerobic transformation in soil. This test consists of the application of a candidate compound to a sample soil

and monitoring the breakdown of the compound using ^{14}C labelling to create a mass balance.

Mass balance experiments use ^{14}C radiolabelling to trace the metabolites, products, and breakdown pathways of a parent compound. By utilising liquid chromatography coupled with mass spectroscopy (LC-MS) it is possible to detect any ^{14}C labelled molecules extracted from the soil sample. Quantifying the different ^{14}C metabolites creates a "balance" which accounts for all mass deriving from the parent compound, which reveals the ratios of their respective concentrations. This allows the inference of transformation pathways. This experimental approach gives a mechanistic overview of how the parent compound behaves in the soil (Mendes *et al.*, 2017).

The test is carried out at 20°C in the dark with moistened air flushed through the incubation vessel, usually a 500ml Duran bottle, for a period of no longer than 120 days. Further study of transformation products may allow a test to continue for up to 6 months or, in rare cases, 12 months. Tests are conducted in the dark to prevent growth of plant material and minimise photolysis in order to focus solely on microbial and chemical breakdown.

OECD307 guidelines specify soil storage and processing regimes. Processing regime guidance specifies air-drying, sieving, and moisture adjustment. Freshly sampled soil, after a 2-28 day pre-incubation to allow seed germination to facilitate plant removal, must be air dried until it can pass through a 2mm aperture sieve. Then after sieving it must be moisture readjusted up to its PF2 (100% field capacity, usually 20-25% w/w). Storage guidelines allow for processed soil to be stored for up to three months including any time spent in pre-incubation. This accounts for the possibility of fresh sampling being unfeasible due to weather conditions, long distances to field sites, or importation of soil. The guidelines specify that four soil types must be tested for each compound, which can necessitate the import or transportation of soil across continental distances (OECD, 2002). These processing and storage regimes have the potential to impact the degradation capabilities of the soil microbial community.

1.2 Mechanisms of Degradation

A CPP may be broken down via a variety of mechanisms depending on its physical and chemical properties. These include photolysis, hydrolysis and microbial activity (Kah, Beulke and Brown, 2007). Photolysis involves the abiotic absorption of photons causing excitation of the CPP molecule, inducing either specific or non-specific reactions which results in the oxidisation (and thus loss of function) of the molecule's functional groups. This process is poorly understood due to confounding factors such as soil adsorption and light attenuation in the soil (Katagi, 2004). Hydrolysis of a CPP is a mechanism of breakdown involving the reaction of a water molecule via specific catalysis by a proton or a hydroxide present in the aquatic environment (Katagi, 2002). Phytolysis, or phytoremediation, is the use of plants to degrade a compound. However this is the most limited approach as plants lack the complete set of catabolic pathways for the mineralisation of CPPs (Laura *et al.*, 2013).

Degradation by microbial metabolism is the only process by which a CPP can be completely mineralised into CO_2 over a timespan acceptable to regulators (Pesaro *et al.*, 2004). Microbial communities degrade CPPs either through direct use as nutrient source, or via co-metabolism (Grant and Betts, 2004).

There are two key factors that govern the microbial breakdown of CPPs; the bioavailability of the compound and the microbial community structure and composition. The availability of a CPP to a potential degrading organism is determined by its adsorption coefficient which is dependent on the physiochemical properties of both the CPP and of the soil (Trabue *et al.*, 2006). Adsorption of CPPs are determined by many factors, including moisture, organic matter content, composition and pH of the soil (Shelton and Parkin, 1991), as well as the solubility, size, logP, and pKa of the CPP (Gevao, Semple and Jones, 2000).The adsorption of non-ionic CPPs is also highly dependent on the soil organic matter content, and higher adsorption can inhibit breakdown rates (Ahmad, Nelson and Kookana, 2006).

The diversity and function of the soil microbial community plays a crucial role in CPP breakdown (Freemark and Boutin, 1995)(Trabue *et al.*, 2006). Within these communities only a few species may be active at any one time, the remainder being

dormant due to the environmental conditions such as the nutrient availability or abundance of competitor species (Stenström, Svensson and Johansson, 2001). Microbial community biomass and activity can be affected by changes to soil structure and other environmental conditions, including tilling, changing of crops, and manure spreading (Stenberg *et al.*, 1998; Magid *et al.*, 1999; Pesaro *et al.*, 2004; Hartmann *et al.*, 2006; Sun *et al.*, 2011; Souza *et al.*, 2013).

Previous studies have shown that elements of soil processing alters the physical soil structure, which may not only alter CPP adsorption, but also the structure or composition of the resident microbial communities (Shelton and Parkin, 1991; Pesaro *et al.*, 2003, 2004; Trabue *et al.*, 2006). Changes to the soil structure can alter community activity via species mixing from niche disruption and changes to nutrient availability. This shifts certain species from dormancy to dominance and vice versa. In the context of soil processing this is particularly important, as the drying, sieving, homogenisation and re-wetting of samples has the potential to impact the community of degrading consortia. Initial studies at Syngenta (unpublished data) and elsewhere (Trabue *et al.*, 2006) have found that mineralisation rates can be greater in unprocessed soil cores compared to soils processed as per OECD guidelines, suggesting current methods of degradation testing are likely unrepresentative of the microbial processes in the field.

Complete breakdown pathways are yet to be fully elucidated for most CPPs. The persistence of certain CPPs may be due to Cl atoms or sulphite groups within their structure, as enzymes evolved to break down such groups are absent from most microbes (Singh, 2008). However it has been observed that communities without the enzymatic apparatus for the breakdown of these CPPs can develop degradation activity after exposure (Aelion, Swindoll and Pfaender, 1987). This could be potentially due to a rise in dominance of a CPP-degrading species within the community, a mutation of existing enzymes (McAllister, Lee and Trevors, 1996), horizontal gene transfer (Springael, Kreps and Mergeay, 1993), or novel use of pre-existing pathways (Copley, 2000).

Single species are rarely responsible for the complete mineralisation of a CPP, instead it is usually a consortium of species, with groups of species acting on one or

more steps within the metabolic pathway (Janssen *et al.*, 2005). Within in-vitro studies bacterial species have been found to degrade CPPs at a higher rate than fungi, but it is postulated that some consortia may require fungi for rapid degradation (Levanon, 1993).

Fungal activity may be a rate-determining step during breakdown in the field. This potential dependency could be linked to fungi utilising different metabolic routes to bacteria (Levanon, 1993; Rønhede *et al.*, 2005), which may alter the chemical structure of CPPs to better facilitate bacterial breakdown. However some fungal orders are known to be efficient at CPP breakdown. Certain orders of Basidiomycota involved in white rot are efficient at the breakdown of aromatic pesticides, likely due to high peroxidase activity (Bending, Friloux and Walker, 2002). Fungal transformation and breakdown of CPPs may be driven more by detoxification mechanisms than direct utilisation as a nutrient source, which is more common in bacteria (Sutherland, 1992; Rønhede *et al.*, 2005).

Theories about the effects of altered microbial biodiversity on CPP breakdown are present in the literature. Dougan et al propose that a net reduction in degradation rate observed in some studies following processing may be due to a reduction in biodiversity and degrading consortia (Dougan, C. Hand, L. Nichols, C. Oliver, 2013). Disruption of soil aggregates may destroy fungal hyphal networks (Tisdall, 1994), impacting fitness and potentially diversity. Bacteria are less resistant (the ability to withstand an environmental disturbance) than fungi and so may also be impacted by processing, although the faster rate of bacterial growth may increase their resilience (the rate of recovery post-disturbance).(Pimm 1984) (de Vries *et al.*, 2012)(Orwin, Wardle and Greenfield, 2006; de Vries *et al.*, 2012). However, functional redundancy, whereby multiple taxa have the same function, can mean the functional output of a community may not alter after environmental disturbance (Allison and Martiny, 2008). The high abundance and genetic diversity (bolstered by rapid adaptation such as horizontal gene transfer) would suggest that function is not sensitive to the loss of select species (Meyer, 1994; Finlay, Maberly and Cooper, 1997).

Dougan et al's model posits three types of CPP active ingredient briefly described here. Type A compounds are degraded by pathways present in a wide range of soil microbes and persist in surviving microbes following processing therefore degradation rate is unaffected by processing. Type B compounds are degraded by pathways which are only present in a narrower section of the microbiome which may potentially be lost during processing, thus making degradation sensitive to processing. Type C compounds are largely resistant to microbial degradation and degradation rate remains low after processing. According to this model OECD307 is an appropriate study for compounds belonging to Types A and C, but will provide inaccurate data for Type B compounds. However, this model assumes overall metabolic activity will remain at constant after processing. The OECD307 test is a closed and dark system where it is possible for the microbial community to begin to deplete labile carbon and other nutrient sources without photosynthesis to restore them. This could result in an overall lower metabolic rate, and so degradation rate, regardless of the abundance of breakdown pathways.

1.3 Soil Diversity

Assessing the diversity, function, and state of a soil microbial community presents a significant technical challenge. Soil microbial diversity constitutes the largest biodiversity on the planet with previous estimates suggesting up to 4×10^6 taxa can be present in a tonne of soil, and yet it remains largely uncharacterised (Torsvik and Øvreås, 2002; Mocali and Benedetti, 2010; Eisenhauer *et al.*, 2017). This diversity appears paradoxical as competition theory dictates that only the most competitive species should remain. However the heterogeneous structure of soil and limited pore connectivity allows for such high diversity (Carson *et al.*, 2010). Low pore connectivity can decrease microbial motility and substrate diffusion by up to 4 orders of magnitude (Long and Or, 2009). This could create spatially distinct environments within these poorly connected soil pores, allowing for the co-existence of otherwise competing species, driving up the diversity of the environment.

Assessing the extent of this diversity using traditional methods is unfeasible as the majority soil microbes are unculturable under lab conditions. Novel methodologies such as the ichip (Nichols *et al.*, 2010) and microfluidics (Aleklett *et al.*, 2018) provide new platforms to culture microbes which have previously been difficult to isolate outside of native soils, but do not allow the broad characterisation of total microbes within a given sample.

Ichip, short for isolation chip, is a technique utilising a chip made up of hundreds of miniature diffusion chambers. Each of these chambers are inoculated with a single microbial cell by dipping the chip into a suspension of cells created by serially diluting the desired environmental matrix (such as soil or seawater) in an agar medium. The chip is then placed back into the source matrix where the suspended cells can access their natural growth components via diffusion. This allows for the cultivation of microbes which are usually incapable of growth under lab conditions. (Nichols et al 2010)

In contrast, microfluidics aims to culture soil microbes by artificially replicating the three-dimensional microscale soil environment both physically and chemically. In order to fabricate these microscopic habitats at the scale of soil particles a photolithography method is used, which etches the desired surface pattern with UV light onto a silicone wafer. This approach allows the spatio-temporal heterogeneity of soil to be replicated in lab conditions with full optical access, a property absent from real soils. This allows the culturing and observation of species which are previously unable to be cultured in solid media. (Aleklett et al 2018)

Both ichip and microfluidics present new opportunities to be able to study microbes in both real and simulated soil environments, but do not allow for the capture of the total diversity. Ichip may exclude rare or low abundance species during the dilution step, and microfluidics primarily is a tool to culture constructed communities as opposed to observing natural communities.

The most predominant technique for assessing microbial diversity in previous years is that of ARISA. However, molecular fingerprinting is a powerful tool which can be used to overcome the limitations of these methods (Mocali and Benedetti, 2010).

Composition and diversity of microbial communities can be determined by extracting DNA from soil samples and using PCR to amplify marker regions on the genome, commonly ribosomal DNA (such as 16S or ITS), which are used to identify and quantify the members of the microbial community (Buée *et al.*, 2009; Lumini *et al.*, 2010; Huffnagle and Noverr, 2013).Below the constituent steps of a fingerprinting approach are detailed, along with their caveats.

Sampling of soil to get an accurate view of the massively varied structures within is a challenge due to the random spread of nutrient, moisture and mineral gradients (Carson *et al.*, 2010; Mocali and Benedetti, 2010). Fine scale heterogeneity can occur (Certini, Campbell and Edwards, 2004), making it difficult to capture the entire microbial community during sampling regimes.

Sample storage is crucial to the accuracy of downstream analysis as many storage methods have inherent biases. Inactivation of the microbial community is necessary to prevent growth and contamination of the initial community. Rapid freezing is widely considered to be best-practice but is not always achievable in-field (Hugerth and Andersson, 2017). In addition care must be taken not to repeatedly freeze-thaw samples, which has been demonstrated to cryodamage DNA (Todorova *et al.*, 2012). For samples requiring extensive transportation or long-term storage freeze-drying has been shown to sufficiently preserve DNA (Weißbecker, Buscot and Wubet, 2017).

1.4 DNA Extraction and PCR

The extraction of DNA from sample soils can also present problems with accuracy and downstream analyses. DNA extraction methods often co-purify PCR inhibitors, whilst also biasing against dormant or inactive microbes which can yield smaller DNA concentrations (Mocali and Benedetti, 2010). Disruption of cell walls must be sufficient to allow release of the cell's DNA in order to avoid under-representation of those organisms. Mechanical bead-beating can achieve this, in addition to breaking up soil particles containing cells, and has been shown to achieve higher DNA yields (Pollock *et al.*, 2018).

The region of interest in the extracted DNA must be amplified before sequencing prep using PCR. This typically will be the ribosomal ITS, 18S, or 16S regions for fungal, eukaryotic, and bacterial data respectively (Buée *et al.*, 2009)(Huffnagle and Noverr, 2013)(Lumini *et al.*, 2010). The ribosomal region is of interest as the exons are well conserved across species whilst the introns vary in length and composition. This facilitates the design of universal PCR primers to bind onto the exons and amplify the introns for sequencing as a proxy for species identity (forming operational taxonomic units, hereby OTUs. However, PCR can potentially add biases to the sample as not all sequences will necessarily be equally amplified. Errors during PCR are difficult to quantify as an incorrectly amplified sequence may still be sequenced with complete accuracy and have an associated high quality score (Schirmer *et al.*, 2015). The use of high-fidelity polymerases can mitigate potential copy errors, which in Taq polymerase I can be up to 2×10^{-4} errors per base per doubling event (Ling *et al.*, 1991). Stringent filtering during data analysis can mitigate false reads in a data set. The likelihood of any two errors being identical are low, as are the subsequent clustering of PCR error reads into OTUs (see below) as low count molecules are unlikely to be captured by sequencing depth. Further to this, any OTU arising from a group of PCR error reads will be unlikely to match to existing taxonomic references and will be discarded later in the pipeline.

Most DNA extraction methods will co-purify negatively charged inhibitor molecules which can inhibit downstream PCR (Wang *et al.*, 2017). These molecules can be inorganic, such as calcium ions, but the majority are organic in nature, such as phenols, humic acids, and tannic acids (Schrader *et al.*, 2012). The two main solutions to this problem are to attempt to sequester the inhibitors using positively charged molecules such as BLOTTO or to dilute the inhibitors (De Boer *et al.*, 1995; Wang *et al.*, 2017). A variation of the latter is to perform a nested or semi-nested PCR, whereby a secondary PCR is carried out using the product of the first as template (Blake and Weimer, 1997). The ratio of template to inhibitor is much lower in a secondary PCR, allowing greater amplification.

1.5 Illumina Sequencing

Illumina Next Generation Sequencing is a flow-cell based sequencing by synthesis approach which utilises fluorescently labelled dNTPs. Libraries are prepared by ligating index and adapter oligos to DNA sequences. The inner surface of each glass flow-cell is lined with both forward and reverse oligos complementary to the library adapters. An Illumina sequencing run is as follows. The prepared library is loaded into the flow-cell by the denaturation of the dsDNA into ssDNA which bind to the surface oligos and are immobilised. The surface oligo acts as a primer to synthesise the library sequence before another round of denaturation where the initial library molecule is washed away. Using isothermal bridge amplification, the copied fragments are replicated to form a "cluster" of identical sequences bound to the flow-cell. This bridge amplification consists of the unbound end of the copied strand hybridising with the opposite complementary surface oligo before polymerases generate the complementary strand resulting in a double stranded "bridge" structure. This molecule is denatured resulting in two single stranded copies attached to the cell. For the forward read the reverse strands are cleaved and washed away, and for the reverse read vice versa after another amplification. The sequencing process consists of cycles of fluorescently labelled dNTPs washing over the cell with a fluorescent microscope observing the emission after dNTP incorporation onto the template molecules following excitation by a light source. The wavelength and intensity of the emission allows a base call to be determined. Millions of molecules can be bound to the cell at any one time and the parallel sequencing of these DNA strands can be simultaneous. Illumina MiSeq can achieve 25 million reads per run of both forward and reverse sequences of up to 300bp for a relatively low cost, and as such has become the preferred platform for sequencing amplicon libraries.

Illumina has a several inbuilt biases which must be considered. Read quality may degrade towards the end of each strand as incorporation errors accumulate, either a strand failing to incorporate a base and lagging or a base incorporating early, causing the signal to noise ratio of each cluster to decrease. Shorter strands may produce denser clusters and more intense emission signals, biasing the sequencing

towards these sequences. This may be accounted for by adding a lower ratio of shorter strands when utilising multiple known strand lengths on a single flow-cell. Sequences may also be undetectable because of false clustering which can occur when emissions from differing strands using the same PCR primer set are detected. To avoid this randomised bases can be added during library prep ahead of the PCR primer (Buermans and den Dunnen, 2014).

Considerable variation amongst technical replicates has been observed in Illumina run. (Wen *et al.*, 2017). Whilst these uncertainties present a challenge in interpreting Illumina MiSeq data, the reproducibility of the technique is sufficient. Wen et al observed OTU overlap between technical replicates can be up to 85% and increased to 95% when sequencing depth is increased. Higher diversity coverage was found when replicates were sequenced in separate runs at the expense of OTU overlap. Despite this variation the reproducibility was enough to diagnose treatment effect.

The swapping of indices (sample-switching) during library prep can result in sequences being falsely attached to a sample they did not originate from and is very challenging to control for. This issue has been estimated to affect up to 2% of reads (Hugerth and Andersson, 2017).

1.6 Clustering Sequences

Once sequence reads are obtained, they must be processed and clustered into operational taxonomic units. Commonly reads are sorted by primer set and index before being quality filtered and, if possible, merged with their respective paired end reads to increase confidence. Sequences are then subject to OTU clustering, sometimes referred to as “picking”, usually based on percentage similarity (commonly either 97% or 99%). Three approaches to OTU picking exist, closed reference, open reference, and de novo. Briefly, closed reference compares sequences to a set database and picks OTU identity based on best match, with failed matches being discarded. Open reference is a similar approach with failed matches then passing on to a de novo picking. De novo approaches match sequences to a “centroid” sequence assigned from the dataset and using a similarity cut-off creates clusters of matched sequences, with each cluster forming

an OTU. Chimeric sequences are commonly filtered out before OTU picking. A number of tools exist for these processes, including Usearch (Edgar, 2010), MOTHUR (Schloss *et al.*, 2009), and QIIME (Caporaso *et al.*, 2010). Taxonomy may be assigned to OTUs by BLAST alignment against a database such as SILVA (Quast *et al.*, 2013) or UNITE (Abarenkov *et al.*, 2010).

OTU picking also has the possibility of introducing further bias into the dataset. The commonly used 97% similarity cut-off is arbitrary, designed to ensure sequences are derived from the same species. However differing taxa, especially prokaryotes, may have a much smaller difference in sequence but still originate from distinct species (Gevers *et al.*, 2005), making a 99% cut-off more widely used when observing bacteria.

Due to the nature of the clustering algorithms described above the replicability of OTU-picking is also a concern. The order in which centroids are picked may alter the abundance and identity of OTUs, and different methods can produce different clustering patterns even when the number of OTUs produced are comparable. As such the biological relevance of OTUs are often called into question (Schmidt, Matias Rodrigues and von Mering, 2015), although the delineation between prokaryotic species itself is a topic of controversy amongst microbiologists (Gevers *et al.*, 2005). Novel clustering methods such as Swarm (Mahé *et al.*, 2015) do not rely on centroid sequences in order to overcome this bias at the expense of an increased computational complexity. OTUs arising from erroneous sequencing artifacts are typically controlled for by eliminating singleton sequences before OTU picking, as any two errors are unlikely to be the same.

OTU over-splitting often occurs, which creates an inflation in the number of OTUs reported (Mysara *et al.*, 2017). Once taxonomy is assigned this can result in multiple OTUs with the same taxonomic label. Choosing whether to collapse these OTUs together provides another source of uncertainty, especially in cases where the label is only a higher taxonomic level. Ideally a manual phylogenetic analysis should be utilised to determine which OTUs to collapse together, however in analyses with thousands of OTUs this can be unfeasible and instead collapsing identically labelled OTUs may be the most efficient solution. Phylogenetic analysis is

subject to observer subjectivity which must be taken into account when reporting OTUs, however Bayesian classifier algorithms are approaching a greater and greater level of accuracy in their generated phylogeny (Edgar, 2018). Provided measures as detailed above are taken to mitigate the potential errors surrounding the OTU clustering of Illumina MiSeq data, the technique remains a useful tool for elucidating microbial diversity and assessing treatment effects on the community.

1.7 Thesis Aims

The OECD307 guidelines provide a framework for the majority of degradation studies conducted across industry. The soil processing regimes, including sieving and drying-rewetting cycles, and storage regimes potentially may alter the degradation rate of compounds via alterations to the microbial community. As evidenced above, sieving, drying-rewetting, and storage aspects of the regimes will impact on the composition, diversity, and metabolic capacity of the microbial community. The extent of these changes within the OECD307 bottle tests are currently uncharacterised.

Previous studies have established a change in bacterial biodiversity using ARISA techniques. ARISA (Automated rRNA intergenic spacer analysis) is a more coarse technique than amplicon sequencing involving the gel electrophoresis of amplified PCR product to separate out different length spacer regions. This allows for the characterisation of the microbial community in terms of numbers of species present, however this only provides coarse biodiversity metrics without taxonomic identity. No current studies exist examining changes in the metabolic potential of soils within OECD307. The physical drivers of these changes also remain largely uncharacterised

This thesis aims to elucidate the changes in the microbial community during OECD307 bottle tests by exploring the following research questions:

- How does the microbial community alter in structure and composition during OECD307 tests? (Chapter 2)
- Which environmental factors drive the change in the microbial community? (Chapter 3)

- How does the metabolic function of the community change during OECD307 tests? (Chapter 4)

From this data a model of the microbial changes and the subsequent effects on degradation rate of CPPs will be synthesised. (Chapter 5)

2 Chapter 2: Effects of Soil Processing and OECD307 Incubation on Soil Fungal Community

2.1 Introduction

Crop protection products (CPPs), alternatively “plant protection products”, are defined as any product with herbicidal, fungicidal, or pesticidal properties. The persistence of CPPs after application is potentially hazardous to the environment and human health (Pogăcean and Gavrilescu, 2009). As such extensive safety testing of a new product is required prior to licensing for sale. Regulatory bodies worldwide including the US Environmental Protection Agency and European Commission use the OECD (Organisation for Economic Co-operation and Development) guidelines for safety testing.

The OECD guidelines examine the environmental fate and ecotoxicology of a product in a series of tiered tests, ranging from lab experiments through to larger scale field studies. These tests monitor photolysis, phytolysis, hydrolysis, and microbial breakdown. Monitoring microbial degradation of product is crucial as it is the only process by which the CPP can be completely mineralised to CO_2 and removed from the environment over an acceptable timespan (Pesaro *et al.*, 2004), either by direct use as a nutrient source, or via co-metabolism (Grant and Betts, 2004).

As detailed in Chapter 1, first test in the tiered system is OECD307, or “Closed Bottle Test”. A bottled soil sample is treated with the test compound and then incubated under constant temperature and moisture in the dark. To measure aerobic breakdown the test system is constantly flushed through with moistened air to ensure aerobic conditions and to prevent the soil from drying out. To measure anaerobic breakdown the system is instead flushed with inert gas, usually nitrogen or argon. Sub-samples are taken at time intervals to monitor the breakdown of the compound by measuring concentrations of parent compound and breakdown products. The use of ^{14}C-labelled product allows establishment of a mass balance to estimate degradation rates, though non-labelled compounds can

be tested with sufficiently sensitive analytical methods. These tests last a maximum of 120 days (OECD, 2002).

The OECD guidelines also specify how soil must be prepared before use in degradation studies. Soil to be used in the test, either sandy loam, silty loam, loam, or loamy sand with 0.5 – 2.5% organic carbon content, is collected from the top 20cm layer before vegetation, soil fauna, and stones are removed using a 2mm sieve. Some light air drying may be necessary before the soil can easily pass through the sieve. After sieving soil can be stored for a maximum of 3 months before use to allow for its transportation and for potentially irregular sampling regimes, which could be due to unfeasibility of sampling due to weather conditions or distant field sites. Guidelines specify that soil cannot be sampled during, or immediately following, a period of frost, drought, or flooding. In addition to this the OECD guidelines require testing on four different soil types, which can necessitate the transport of soil (OECD, 2002).

However microbial breakdown observed in OECD307 tests are not representative of that in the field, as processing soil in this way alters both the structure of the soil and the resident microbial community. Schimel *et al.* (1999) posited that drying-rewetting could lower functional diversity and decrease decomposition rates, and later work by other groups have explored this hypothesis. Pesaro *et al.* (2004) demonstrated drying and re-wetting soil has a significant impact on the microbial community, reducing biomass by up to 51% and degradation rates by up a factor of 5.4. (Bapiri, Bååth and Rousk, 2010) found that drying-rewetting decreased bacterial growth rate, elevating the fungi:bacteria ratio, while Dinh *et al.* (2016) found drying-rewetting causes a microbial community composition dependent mobilisation of phosphorus. Trabue *et al.* (2006) found that storage and bottling of soil also had a significant effect with an observed change in community composition and a reduction in degradation rate in both storage and flow-through bottles, in addition to a reduction in biomass within the bottle system. Also, (Fierer and Schimel, 2003) found a 2 to 6 day pulse of CO_2 in dry soils after re-wetting, indicating a possible change in metabolism.

The disruption to soil via sieving has also been shown to impact the microbial community. (B. Thomson *et al.*, 2010) discovered that both dried and fresh soil sieving significantly altered microbial community composition, with drying prior to sieving having a greater effect size. The disruption of nutrient-rich aggregates, whereby fine sieving breaks up spatial heterogeneity and brings microbes into contact with the released substrate was considered to be the best explanation of the change in community. This is consistent with the evidence showing that drying-rewetting causes a reduction in intra-aggregate carbon (Denef *et al.*, 2001).

The OECD guidelines do not take into account microbial differences across sampling sites or sampling seasons. (Štursová *et al.*, 2016) found that microbial communities within forests were spatially heterogeneous, especially fungal communities which had a greater beta diversity than bacterial communities. (Lauber *et al.*, 2008) discovered that the structure of both bacterial and fungal communities is significantly influenced by variation of edaphic properties between land-use types, with variation in soil pH and nutrient content being the primary driver of community composition of bacteria and fungi respectively. (Stevenson, Hunter and Rhodes, 2014) demonstrated that whole microbial communities respectively form distinct seasonal assemblages, with moisture and temperature acting as driving climatic variables in addition to seasonal variation in pH and particulate carbon (either from litter input or root exudates). Further to this (Voříšková *et al.*, 2014) discovered that litter decomposition was a key factor in determining the seasonal assemblage of soil fungi, in addition to finding an increase of enzymatic activity during the winter season. Similarly Dumbrell et al (2011) found mycorrhizal fungi to form seasonal assemblages correlated to the seasonal variation in host-plant carbon (Dumbrell *et al.*, 2011). The described effects of each component of soil processing taken with the variability of the soil community across site and season could explain the difficulty in reproducing data for OECD307 studies (unpublished data, Syngenta).

Although the role fungi play in CPP degradation is currently not well understood it is thought they may carry out processes crucial to CPP breakdown. Fungi are key degraders in the environment and excrete a host of catalytic enzymes, some of

which may be useful for CPP degradation. For example, white rot fungi, characterised by their lignin degrading potential, have been shown to be efficient in the breakdown of certain compounds; this is likely due to their excretion of lignin degrading peroxidases which may target aromatic groups on a CPP molecule (Bending, Friloux and Walker, 2002). Other work on the globally distributed genus *Mortierella* has shown its degradation activity on the insecticide endosulfan, which increased with the addition of wheat bran to test soils, indicating a link between organic matter availability and fungal degradation (Kataoka, Takagi and Sakakibara, 2011).

It is possible these functions allow fungi to catalyse steps in the breakdown pathways of CPPs. A single species is rarely responsible for the complete mineralisation of a CPP; instead it is usually a consortium of species with groups of species acting on one or more steps within the metabolic pathway (Janssen *et al.*, 2005). Levanon (1993) found in soils treated with selective inhibitors that bacteria had a greater degradation rate of a test compound than fungi, but bacteria and fungi together had a greater rate than bacteria alone, indicating fungi may be performing rate-limiting steps such as the mineralisation of alkyl-amino side chains. These separate modes of action between fungi and bacteria could be linked to fungi utilising different metabolic routes to bacteria Rønhede *et al.* (2005), which may alter the chemical structure of CPPs to better facilitate bacterial breakdown. Further evidence for the increased efficiency of fungal-bacterial consortia is shown by Ellegaard-Jensen *et al.* (2014), who demonstrated the same effect as Levanon with artificial communities in microcosm rather than using inhibitors. Fungal activity may therefore be a rate-determining step during breakdown in the field.

Previous studies by Talbot et al 2015 demonstrated that community sequencing methods that quantify functional guilds can predict the enzymatic activity of a soil (Talbot *et al.*, 2015). By testing enzymes produced from 48 different model species in microcosm experiments they found the activity correlated with a given taxa's functional guild, more so than it did with that taxa's respective phylogeny. Thusly this functional guild approach allows for an overview of functional changes using existing amplicon data without the need for further molecular analysis.

The fungal community may also be a potential driver for the bacterial community. de Boer *et al.* (2005) posited that the fungal community may shape the bacterial community in the soil, perhaps in a similar manner to the mechanisms described above. Many bacteria rely on fungal exudates and grow along hyphae, the fungi effectively creating bacterial niches.

The most abundant organic compound on the planet is cellulose, which provides a key energy source for microorganisms (Boer *et al.*, 2005).Fungi are primary degraders of cellulose, with many bacterial taxa lacking the complete set of enzymes needed to fully access the cellulose molecules. This is because cellulose is often contained within a matrix of hemicellulose and lignin, which are heavily recalcitrant due to their crystalline structure inhibiting enzymatic breakdown. Lignin decomposition, such as that exhibited by white rot fungi, is not widespread amongst microbial taxa, although filamentous fungi and actinomycetes can access the cellulose through the matrix directly by penetrating the lignin layer with fine hyphae (Lynd *et al.*, 2002). As such many bacterial rely upon fungal exudates for carbon sources, and fungus-associated species have been previously characterised (Tsukamoto, Murata and Shirata, 2002).

This means any changes in the fungal community could potentially cascade into changes in bacterial community. In addition to this (Knudsen *et al.*, 2013) found that, within an artificial community of *Mortierella* fungi and CPP degrading *Aminobacter* bacteria placed in sand columns, the translocation of both CPP and Aminobacter was facilitated by fungal hyphae. This demonstrated that the fungal community may be essential for the dispersal of the bacterial community and the distribution of compound to degrading bacteria. These observations of fungal community effects emphasise the importance of the fungal community in not only determining potential niches, and therefore total microbial community, but also efficiency of CPP breakdown.

Based on the information above I hypothesise that there will be a reduction in biodiversity of the soil fungal community following an OECD307 test. In this study the impact of soil processing on the fungal community across different sites and seasons is assessed. Soil samples were taken from three field sites, two pastureland

sites and an agricultural field, over three seasons in 2015 and processed according to the OECD307 guidelines before being stored for six weeks and then bottled for a month. Subsamples were taken prior to processing and after bottle incubation, and DNA was extracted from each time point. Extracted DNA underwent PCR amplification of the ITS ribosomal DNA fingerprints (Buée *et al.*, 2009) (Huffnagle and Noverr, 2013) (Lumini *et al.*, 2010). These amplicons were used to elucidate the diversity and taxonomy of the soil fungal communities via Illumina MiSeq amplicon sequencing. By grouping the Operational Taxonomic Units (OTUs) obtained from the amplicons into Functional Guilds any possible changes in microbial function were assessed.

2.2 Methods

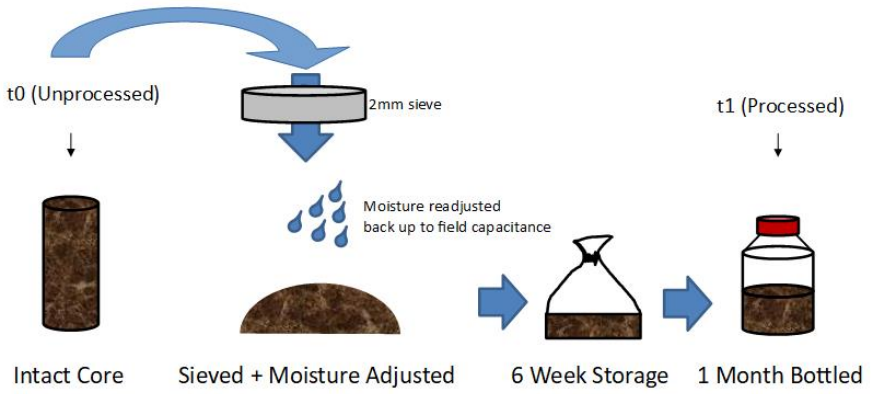

Figure 1: Sampling schematic for OECD307 fungal community analysis (t0 and t1 indicate when subsamples were taken)

2.2.1 Sampling

Cores were taken at seasonal intervals during April/May 2015, August 2015, and November 2015 from 18 Acres near Jealott's Hill (Bracknell, Area A: 51.454453, -0.720672, Area B: 51.454388, 0.720630), University of Leeds Farm (Tadcaster, Area A (Rabbit field): 53.871684, -1.330853, Area B (Poplar field): 53.872840, -1.331129), and Walmgate Stray (York, Area A: 53.947520, -1.059897, Area B: 53.947104, -1.059850).

18 Acres and Walmgate stray are permanent pastures and the fields at University of Leeds Farm are arable land.

Cores were 15cm in length, and 3.5cm in diameter, with three cores taken from each area. Cores were briefly stored in a 4°C cold room for up to 5 days prior to processing to allow for sampling across all sites.

2.2.2 Processing

The cores were processed according to OECD 307 (OECD 2002) guidelines and a 2.5 g subsample of each underwent DNA extraction to provide baseline data for each sample.

Cores were air-dried for between 20 and 30 minutes to facilitate sieving, and then sieved through a 2mm aperture sieve. Sieved soil was then adjusted to its respective PF2 (field capacity) value. Field capacity was determined by Syngenta's external contract labs, measured by saturating a sample soil core, allowing it to drain until excess drainage stops, and then determining percentage moisture content by weighing before and after oven drying. After re-wetting soils were stored in plastic bags (sealed with a small opening of approx. 1cm diameter to allow air flow) at 20°C in the dark for six weeks.

After storage the soil was subjected to OECD307 standard bottle test conditions: one month storage in a 250ml Duran bottle at 20°C with an air flow cap which allowed moistened air to be flushed through the bottle at a rate sufficient to cause an attached gas trap to bubble once per second.

The incubation periods of six weeks storage and one month bottle test were chosen as an industry average incubation time. (Unpublished data, Syngenta)

2.2.3 DNA extraction and PCR

DNA extraction from both the processed soil and the intact cores was carried out using the MoBio Powersoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) using 0.25g of dried sample (oven dried for ~2 days at 70°C).

Extracted DNA was subjected to a semi-nested PCR using GoTaq Green Master polymerase (Promega), using NS31 (Simon, Lalonde and Bruns, 1992) forward and

ITS4 (White *et al.*, 1990) reverse primers for the primary reaction (PCR conditions: 5 min at 95°C; 30 cycles of 0.5 min at 95°C, 0.5 min at 58°C, 2 min at 72°C; and 5 min at 72°C). Secondary PCR was carried out using ITS1F (Gardes and Bruns, 1993) and ITS4 modified at the 5' end with Illumina sequencing adapters in addition to a 13 base random sequence to prevent false clustering during sequencing (PCR conditions: 5 min at 95°C; 20 cycles of 0.5 min at 95°C, 0.5 min at 58°C, 2 min at 72°C; and 5 min at 72°C). The final products were cleaned up using GenElute™ PCR Clean-Up (Sigma-Aldrich, St Louis, MO, USA).

2.2.4 Sequencing

To prepare for sequencing, amplicons tagged with the Illumina adapter sequence were then subject to a final round of amplification to add unique barcode sequences. 8 cycles of PCR amplificiation were performed using NEBNext Q5 Polymerase 2X mastermix (NEB) and Illumina Nextera XT index primers. Amplicons were purified using 0.9 X AMPure XP beads and eluted into low-EDTA TE buffer before quantitation and pooling at approximately equimolar ratios. Samples were diluted to 4nM before denaturing with 0.2 N NaOH ready for loading at approximately 12 pM, with a 20 % PhiX library spike in (Illumina; for added sequence variety to prevent false clustering). Amplicon pools were sequenced on an Illumina MiSeq with a 600 cycle kit, and the fastq generation workflow in Illumina's BaseSpace Sequence Hub (Illumina, 2019).

2.2.5 Sequence Analysis

Using USEARCH10 (Edgar, 2010), the resulting sequences were stripped of the 13 randomised bases with the fastx_truncate command before forward and reverse reads were merged using the fastq_mergepairs command. Primers were trimmed from the sequence using cutadapt (Martin, 2011) and dereplicated using the USEARCH command fastx_uniques . The amplicon sequences were clustered using the command cluster_otus to 97% similarity, with a minimum size of 2 in order to filter out potentially erroneous singleton reads..This generated operational taxonomic units (OTUs) for each soil sample based on the alignment of the ITS regions. An OTU table was generated using the otutab command, and normalised using otutab_norm. These OTUs were also BLAST (Altschul *et al.*, 1990) aligned with

the UNITE (Abarenkov *et al.*, 2010) fungal ITS database to assign taxonomic information to the OTUs. Afterwards any non-fungal OTUs (as determined by the BLAST alignment) were filtered out before collapsing any OTUs with identical taxonomy into a single OTU. OTUs were then sorted into functional guilds using the FunGuild script (Nguyen *et al.*, 2015). Functional guild abundance pre- and post-treatment was then compared using paired t-tests. Although in practice fungi rarely perform only a single function within an environment, classification into guilds allows an approximate observation of possible soil function without conducting separate functional studies.

USEARCH's alpha diversity scripts were used to rarefy the samples and calculate Simpson's index for each sample to investigate any significant differences between the community structures of processed and non-processed samples (Morris *et al.*, 2014).

Bray-Curtis matrices were also calculated for NMDS ordination, and PERMANOVA was implemented using the adonis command in Vegan (Oksanen *et al.*, 2016) to determine any significant difference caused by treatment, season, and sampling site. PERMANOVA is a non-parametric multivariate ANOVA that can use any distance measure appropriate to the dataset without the need for normal distribution.

2.3 Results

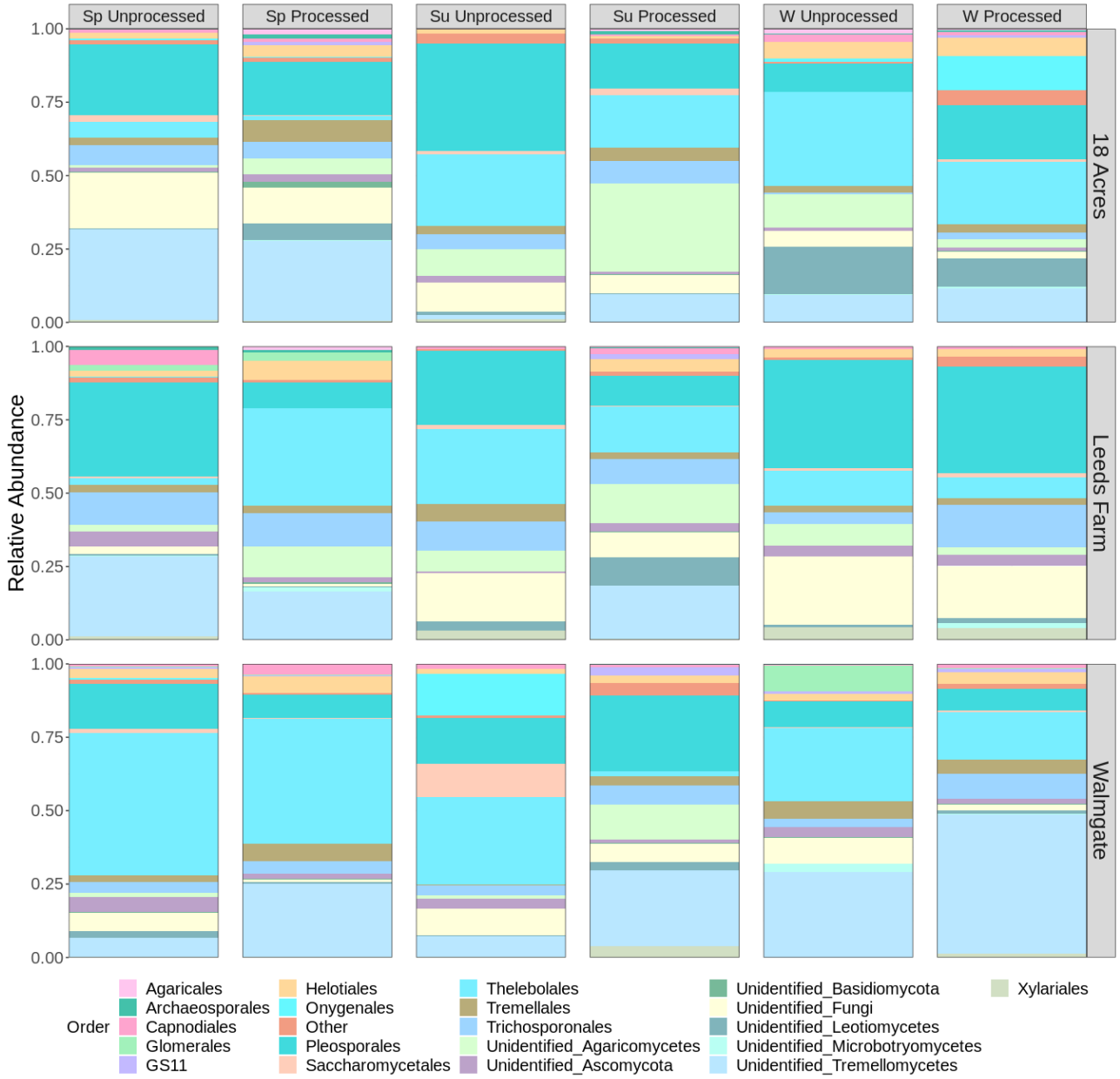

Figure 2: Stacked taxa charts of the Fungal community at each condition, summarised by order level taxonomy. Charts are organised by Treatment, Season, and Site. (n=6 for each plot)

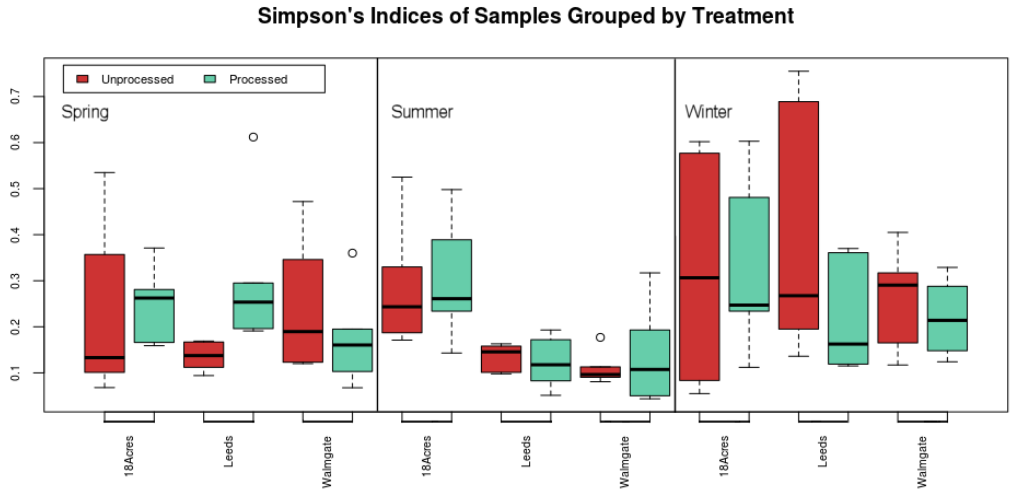

Figure 3: Box-plots of Simpson's Indices for Unprocessed and Processed samples grouped by site and season.Bars are coloured by Processing treatment. Dark line represents median, box represents upper and lower quartiles, bars represent minimum and maximum values, dots designate outliers (N=6 for each condition). Paired t-tests revealed no significant difference between Unprocessed and Processed samples.

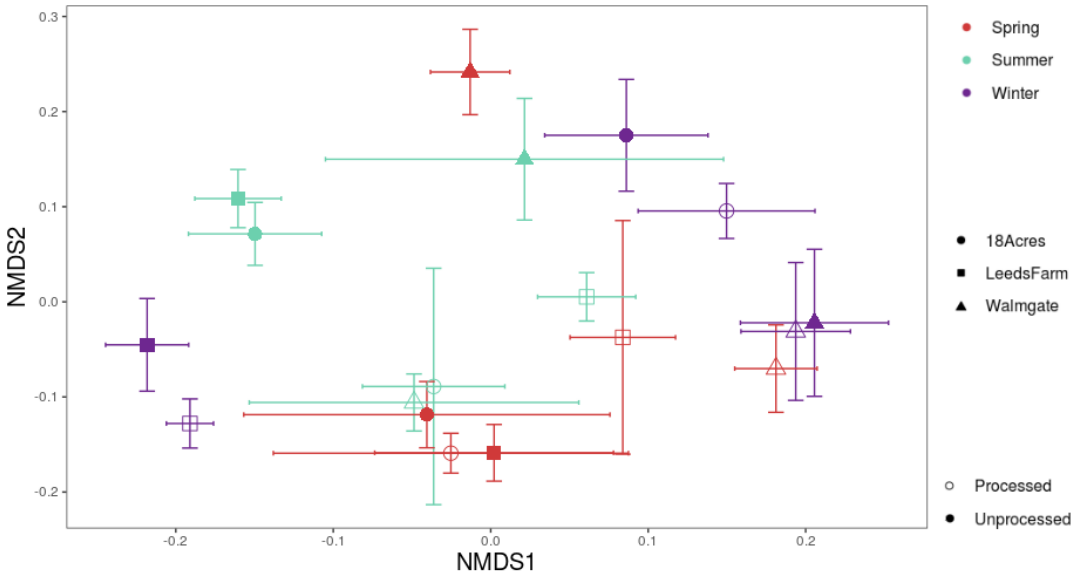

Figure 4: Mean NMDS of Bray-Curtis Dissimilarity values for each condition in the study.(N=6 for each condition). Each condition is coloured by season, and shape indicates site. Open and closed points represent Processed and Unprocessed samples respectively.

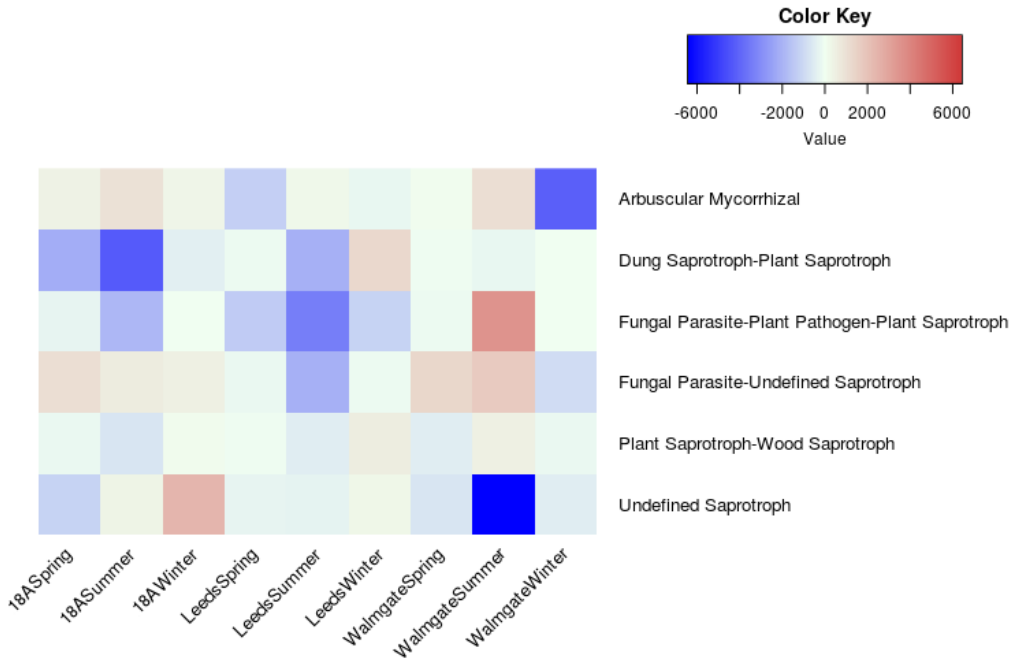

Figure 3: Functional guild response matrix of each condition in the study. Values are differences in absolute abundance. Red colour indicates an increase in abundance of a functional guild within that condition after processing, blue colour indicates a decrease, off-white indicates no change (n=6 for each treatment).

A total of 11,534,558 reads were obtained. USEARCH normalisation scripts were used to rarefy the samples down to 60200 reads each, equal to the sample with the 104th highest reads (out of 117 samples). A total of 134 OTUs were obtained after filtering steps. Paired T-tests revealed that treatment had no significant effect on alpha diversity (Figure 3) regardless of site or season. There was no significant difference between the Processed and Unprocessed samples. In Figure 4 samples separate by season, with spring and summer clustered together separate to winter. Samples also appear to be separated by site, with samples more strongly clustering within site. This change in beta diversity with no change in alpha diversity indicates community composition has altered but structure has not. Processing does not appear to drive differences in any one direction, suggesting it does not cause a predictable shift in community. PERMANOVA revealed that Season, Location, and Processing all have significant effects (DF= 2 F=2.37 F= 2.36 p=0.001, DF=2 F=3.01 $p<0.05$, & DF=1 F=3.97 $p<0.05$ respectively). Guild data in Figure 5 does not show any one guild consistently increasing or decreasing after processing.

2.4 Discussion

Alpha diversity remains the same across treatments . As Simpson's Index is a measure of dominance this indicates that there is no change in the community structure after processing (Boer *et al.*, 2005; Hanson *et al.*, 2008; Moll *et al.*, 2016).

All samples displayed an overdominance pattern in their respective rank abundances. The pattern was preserved even after processing with the highest ranked species much higher in abundance than the second or third highest ranked species, with distribution quickly tailing off in rare species. This pattern is frequently observed when examining microbial community abundances (Dumbrell *et al.*, 2010; Gannes *et al.*, 2016) and it is partially explained by niche apportionment theory, supported by observations that only a small proportion of the microbial community is actively growing within the soil at a given time (Stenström, Svensson and Johansson, 2001). Niche apportionment theory posits that a niche is divided among species by deciding factors such as fitness, adaptation, and stochastic access, and then species abundance is determined by that niche division (Tokeshi and Schmid, 2002). In context of the fungal species here, it is likely only the few dominant species has access to the nutrient pool, and the rarer species are dormant.

Whilst the structure of the community, that is to say the richness and evenness, was not significantly altered after processing, the composition significantly varied. Both site and season were major driving factors of the changes in community composition. In Figure 4 the treatments cluster by site along the x-axis and by season on the y-axis, with spring and summer samples clustering together separate from winter. The three different sites varied by soil type and land use, and microbial diversity has been shown to be altered by both soil type and differing plant cover (Garbeva, Veen and Elsas, 2004; Lauber *et al.*, 2008). Seasonality can determine the amount of soil organic matter and overall nutrient content, which also impacts the microbial community heavily (Koranda *et al.*, 2013; Siles and Margesin, 2017). Studies have demonstrated that soil carbon is highest during autumn and winter (likely due to litter deposition) and lower after activity pulses during spring and summer, correlating to a change in microbial community (Bardgett *et al.*, 1999; Siles and Margesin, 2017). Koranda et al (2013) discovered that fungi in winter soils

have a greater carbon degrading ability, demonstrating that seasonality can also alter function. In the results presented here, the separation of winter communities from spring and summer is consistent with these findings (Koranda *et al.*, 2013).

Processing also has a significant impact on community composition. Sieving, drying, rewetting (Pesaro *et al.*, 2004; Iovieno and Bååth, 2008; B Thomson *et al.*, 2010) all alter microbial community, but this effect does not appear to be as strong when compared to the effects of site or season. This is due to the composition of the end point community being dependent on that of the starting community, which is determined as above by site and season, observable by the processed samples clustering closer to their matching unprocessed points in Figure 4. This is a similar observation to that made by (Choi *et al.*, 2017), who demonstrated that soil disturbance creates a successional niche differentiation and the community does not restore to its original composition pre-disturbance.

The changes due to processing were neither uniform nor predictable. No convergence effect, whereby treatment biased the community into similar assemblages, was observed. The compositions of the endpoint communities were not consistently biased towards any single composition or guild. Instead each treatment combination evolved in its own way after processing, as demonstrated by the lack of clustering of processed samples in Figure 4, with the identity of the most dominant species varying greatly between processed samples. In most cases the direction of travel is inconsistent as well, suggesting each bottle is its own independently evolving microcosm.

No convergence effect was observed in the guild data either; no single functional guild increased or decreased across all site/season combinations. This inconsistency across treatments demonstrates not only the unpredictability of community composition post-processing, but also microbial function. This dataset further reduces confidence in the accuracy of OECD307 as a microbial degradation test system.

A potential mechanism for the community shifts and microcosm effect is described here. Micro-scale determinants are responsible for the high biodiversity observed in

the soil environment, including soil particle size, hyphal networks, and nutrient hotspots (Vos *et al.*, 2013). Hanson et al (2008) demonstrated fungal richness alters in response to different carbon sources. These carbon sources can vary in the spatially heterogeneous soil environment, including root exudates, different crystallinities of cellulose, and hyphal mass (de Boer *et al.*, 2005)all of which create different carbon niches (Boer *et al.*, 2005; Hanson *et al.*, 2008). As soil depth increases the root content varies, as does labile carbon and diversity (Moll *et al.*, 2016).

Upon sieving these features are largely disrupted and homogenised, rapidly altering the soil matrix environment. Existing nutrient pools, such as the carbon pools described above, are disrupted and homogenised, whilst spatially distinct microbial communities are brought together. The fungal taxa that rises to dominance after the disturbance event does not appear to be reliant on fitness in relation to its ability to access the nutrient pools, but instead there appears to be a stochastic process whereby a community-level factor such as dispersal could determine which taxa can access the nutrient pool first, and therefore exploit the pool first. Only taxa already present in the sample have the potential to access the new pool, and therefore the variation in community between samples collected at different sites and seasons is another key driver in the observed diversity between endpoint samples. The majority of the soil microbial community accessing a single pool instead of distinct nutrient niches could result in a rapid depletion of that pool and therefore a reduced carrying capacity, potentially explaining the observed change in alpha diversity in some treatments. This combination of neutral and niche theory dynamics (Dumbrell *et al.*, 2010) could result in the varied communities evolving from the same starting community as observed in this study.

2.5 Conclusions

The soil fungal community present within OECD307 degradation studies is significantly altered by the processing regimes as outlined in the OECD guidelines. Both the sampling site soil is collected from and the season in which it is collected determines the starting fungal community. The processing required by the OECD

guidelines does not seem to significantly alter the fungal community structure; however, the community composition is significantly changed.

This change is partially stochastic, resulting in an unpredictable change in taxa and functional guild abundance. These observations further reduce confidence on the accuracy and efficacy of the OECD307 test and could help explain the difficulty in its repeatability. Without a clear biasing towards a certain community or functional guild composition, the processing will result in each OECD307 bottle test being unique in its microbiology, and thusly each CPP degradation result will be difficult to accurately compare.

As discussed in the next chapter, to further test the hypothesised mechanism of nutrient pool disruption a second experiment was conducted observing the concentrations of multiple nutrients over an increased number of time points over the course of an OECD307 test, in addition to the observation of the microbial community to further characterise the response of the total microbial community.

3 Chapter 3: Organic Carbon Depletion Drives the Soil Microbial Community Shift in OECD307 Tests

3.1 Introduction

The soil processing regimes during OECD307 tests have a significant impact on the soil fungal community which is stochastic in nature (Chapter 2). A primary mechanistic driver for the community changes observed in Chapter 2 is likely to be nutrient dynamics resulting from the physical changes to the soil structure. Many changes in soil characteristics affect microbial community composition and structure, such as soil pH (Liao et al 2016), aggregate size (Li et al 2015), and nutrient concentration (Hou et al 2017). These characteristics are described here.

Micro-scale heterogeneity in soil structure is thought to be responsible for the large microbial biodiversity found in soils (Carson *et al.*, 2010), in addition to the density, quantity, and heterogeneity of nutrients. (Falconer *et al.*, 2015) used X-ray CT scanning to model the distribution of organic matter in relation to pore space within the soil matrix and found that the micro-scale distribution of organic matter can cause a substantial variability in respiration. This contrasts with previous models of soil organic matter decomposition which assume linear relationships between decomposition and carbon pools, without accounting for the microbial physiology, distribution, or structural heterogeneity (Falconer *et al.*, 2015). During sieving/drying-rewetting the destruction of these micro-scale environments may disrupt these nutrient dynamics by altering the structure of the soil matrix.

Disruption to the spatial distribution of microbes and soil nutrients could potentially rapidly alter nutrient pool accessibility (Franklin and Mills, 2007). Drying-rewetting could also further mobilise dissolved nutrients, and spur rapid uptake by previously dormant members of the microbial community. Hassink (1992) and Van Gestel et al (1993) posited that soil drying increases microbial sporulation, whilst subsequent rewetting returns spores to active states (Hassink, 1992; Van Gestel, Merckx and

Vlassak, 1993). Germination and growth to utilise any increased available substrate could then cause a rapid burst in respiration and nutrient uptake. (Iovieno and Bååth, 2008) found a burst in respiration upon rewetting soils, with the CO_2 flux over 10 times higher than control soil, which supports the theory of rapid nutrient use following rewetting. The addition of glucose to soils during the drying-rewetting cycle did not cause any significant increase in respiration when compared to drying and rewetting without glucose, suggesting that drying-rewetting causes a saturating amount of labile carbon to be available to the microbial community.

Further to this (B Thomson *et al.*, 2010) found no net difference in CO_2 efflux between sieved and intact soil, but found drying-rewetting to cause a significant increase in respiration in addition to a community shift, measured using terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997), indicating that physical disruption of the soil matrix without a drying-rewetting cycle may not be enough to impact the microbial community rapidly, potentially as much of the community would be in a dormant and therefore more stable state. In addition to altering the community composition, wet sieving when compared to dry sieving has been found to alter the abundance of certain functional genes as demonstrated by (Blaud *et al.*, 2017), who found little difference in functional gene diversity between dry-sieved soil fragment sizes but a significant effect of wet-sieving on the gene abundances for nitrification and denitrification genes.

The observed pulse in carbon utilisation following rewetting could result from either a release of carbon from the soil matrix, or from microbial sources. (Denef *et al.*, 2001) found drying-rewetting reduced the number of water-stable microaggregates within soil macroaggregates, in tandem with releasing intra-aggregate particulate organic matter-carbon, which reduces overall C concentrations within any newly formed macroaggregates. This supports a model whereby organic carbon is released from aggregate formations during sieving, and then dissolved and flushed from the matrix during rewetting. In a contrasting study

(Fierer and Schimel, 2003) found rewetting of dry soil results in a pulse of CO_2 for 2 to 6 days and found using labelled glucose that this is a result of the mineralisation of biomass C, suggesting that the carbon utilised during the respiration pulse is microbial in origin instead (Iovieno and Bååth, 2008) posit that this C could result either from the lysis of cells that have died as a result of the environmental stress of drying-rewetting, or from the release of cytoplasmic molecules due to the change in osmotic potential.

Another key determinant of community structure, diversity, and function is soil pH. Soil pH was found to be the most constant predictor of bacterial community by (Kaiser *et al.*, 2016) after assessing environmental factors and the bacterial community of 300 soils from forests and grasslands. Crucially, pH is a key predictor of soil organic carbon stocks (Liao, Wu and Zhu, 2016) in addition to other edaphic properties. (Tsiknia *et al.*, 2014) linked the spatial diversity of microbial communities to the spatial variation in pH and organic carbon, with some microbial groups strongly correlated together, suggesting pH and organic carbon to be a predictor of syntrophic groups. (Anderson *et al.*, 2018) found that the concentrates of dissolved organic C, N, P and S all increased as pH increased, and so an increase in pH may cause an increase in nutrients available to the microbial community.

However, the effect of sieving/drying-wetting on soil pH is not completely understood. A previous study (Haynes and Swift, 1989) demonstrated that soil pH after rewetting is largely dependent on the initial pH of the soil in the field. Without any inputs during sieving, it is unlikely sieving will significantly impact soil pH in OECD307 tests.

The composition of the soil microbial community is in turn determined by altered nutrient availabilities. Certain soil bacteria taxa were found to correlate significantly to soil carbon by (Hou *et al.*, 2017), who also found that soil bacteria community structure as a whole was largely influenced by the addition of soil organic matter. Microbial biomass was found to be correlated with organic carbon and nitrogen in a

comparison of afforested and agricultural land (Zheng *et al.*, 2016). The ratio of Bacteria:Fungi has also been shown to be significantly correlated with the organic matter content of soil (García-Orenes *et al.*, 2013). Higher C:N ratios tend to favour fungi (Sinsabaugh *et al.*, 2013), likely due to their greater suite of enzymes capable of acquiring and stabilising carbon (Strickland and Rousk, 2010). However there still remains a lack of understanding of the physiological and genetic mechanisms which underpin these differences (Malik *et al.*, 2016).

Temperature is a major determinant of the rate of soil microbial respiration, though is unlikely to be a key driver of change in microbial community or nutrient uptake in OECD307 tests. The optimum temperature at which a community achieves maximum respiration will vary based on the origin of the soil. (Liu *et al.*, 2018) found that optimum temperature increased significantly with increasing latitudes, suggesting that substrate supply is more important to microbial carbon dynamics than climate adaptation, and (Tang *et al.*, 2018) posited that climate and substrate properties of native soil may play a more significant role in driving mineralisation than the microbial community as the latter is affected by, and adapts to, the former. Tang et al., 2018 also found no consistent pattern of thermal adaptation in test communities, whilst (Auffret *et al.*, 2016) reported that no significant differences were found in biomass nor enzymatic activity during temperature changes when substrate depletion is controlled for. The OECD307 test system is at a constant 20°C throughout storage and bottle incubation in order to mimic soil temperature in temperate climates (OECD, 2002), much lower than the 38.5-46°C optimum temperature for respiration reported by (Liu *et al.*, 2018), and thus unlikely to be a major factor in the nutrient dynamics of the OECD307 test compared to physical disruptions to the soil.

In Chapter 2 a mechanism by which processing may cause a change in microbial community composition was posited. Briefly, physical changes to the soil induced by sieving and drying-rewetting will disrupt the spatial distribution of both microbes

and nutrient pools, resulting in a shared pool between species which were previously spatially distinct. Stochastic access to the pool and a rapid depletion of nutrients due to a spike in microbial activity leads to an unpredictable shift in dominance. Therefore, it is the nutrient pool that drives the observed community shift.

Given the OECD307 test system is conducted in the dark, which prevents the photosynthesis of phototrophs, carbon cannot be fixed in the system. I therefore hypothesise that the primary nutrient depletion driving the change in community is that of organic carbon. To test this hypothesis, and to examine at which point this depletion takes place, a single season repeat of the experiment conducted in Chapter 2 was carried out with multiple nutrient measurements in addition to both fungal and bacterial community microbial profiling. Additional sampling points post-sieving, and post-storage were added in order to elucidate at which stage any nutrient depletion or shift in microbial community is taking place. Finally, storage was extended to 3 months, the maximum period permitted under the OECD307 guidelines, in order to ensure any possible depletion effects could take effect.

3.2 Methods

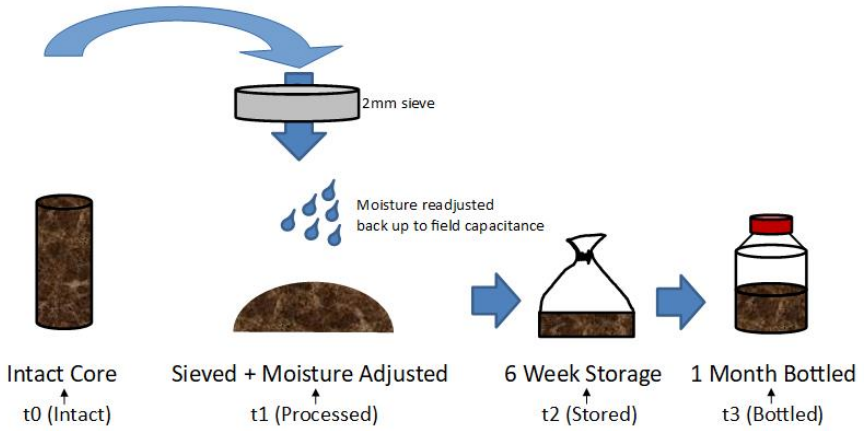

Figure 4: Sampling schematic of OECD307 Nutrient and Community Analysis (t0, t1, t2, and t3 indicate when subsamples were taken)

3.2.1 Sampling

During September 2016 cores were taken from the same sites as in Experiment 1 . Cores were 15cm in length, and 3.5cm in diameter, with three cores taken from each area.

3.2.2 Processing

Cores were processed according to OECD 307 guidelines as in Experiment 1 and 8 grams of soil were subsampled at the following intervals across the processing regime; before sieving (“intact”), before storage (“sieved”), before bottling (“stored”), and after the complete incubation (“bottled”). Storage time was increased to the full 3 months allowed by OECD guidelines in order to observe the greatest possible changes.

3.2.3 DNA extraction/PCR

DNA extraction was carried out using the same methodology as in Chapter 2 (2.2.3)

Extracted DNA was subjected to a semi-nested PCR using GoTaq Green Master polymerase (Promega), and PCR was carried out using 515f and 806rmod primers (Caporaso *et al.*, 2011), a standard use bacterial primer set for illumina amplicon sequencing (Apprill *et al.*, 2015), to obtain bacterial 16S sequences (PCR conditions for bacterial primers: 5 min at 95°C; 20 cycles of 0.5 min at 95°C, 0.5 min at 53°C, 2 min at 72°C; and 5 min at 72°C). NS31 (Simon, Lalonde and Bruns, 1992) forward and ITS4 (White *et al.*, 1990) reverse primers were used for the ITS primary reaction (PCR conditions: 5 min at 95°C; 30 cycles of 0.5 min at 95°C, 0.5 min at 58°C, 2 min at 72°C; and 5 min at 72°C). Secondary PCR was carried out using ITS1F (Gardes and Bruns, 1993) and ITS4 to obtain fungal ITS sequences (PCR conditions for fungal primers : 5 min at 95°C; 20 cycles of 0.5 min at 95°C, 0.5 min at 58°C, 2 min at 72°C; and 5 min at 72°C). 515f, 806rmod, ITS1F and ITS4 primers were modified at the 5' end with Illumina sequencing adapters in addition to a 13 base random sequence to prevent false clustering during sequencing. PCR product was purified using AMPure XP (Beckman). Fungal and bacteria amplicons were pooled in a 3:1 ratio and sent for Nextera barcoding and MiSeq Illumina sequencing at the University of York, UK, in The Department of Biology Technology Facility. This 3:1 ratio was required to mitigate the sequencing bias that Illumina Mi-seq has towards shorter reads.

3.2.4 Sequencing

To prepare for sequencing, amplicons tagged with the Illumina adapter sequence were then subject to a final round of amplification to add unique barcode sequences. 8 cycles of PCR amplificiation were performed using NEBNext Q5 Polymerase 2X mastermix (NEB) and Illumina Nextera XT index primers. Amplicons were purified using 0.9 X AMPure XP beads and eluted into low TE buffer before quantitation and pooling at approximately equimolar ratios. Samples were diluted to 4nM before denaturing with 0.2 N NaOH ready for loading at approximately 12 pM, with a 20 % PhiX library spike in (Illumina; for added sequence variety to prevent false clustering). Amplicon pools were sequenced on

an Illumina MiSeq with a 600 cycle kit, and the fastq generation workflow in Illumina's BaseSpace Sequence Hub. Sequencing Prep was carried out using the same methodology as in chapter 2 (2.2.4).

3.2.5 Nutrient Determination

To measure pH a suspension of 5 grams of soil with 25 ml of water was shaken on an orbital shaker for 15 min and rested for 45 min prior to pH analysis using a Sentek pH electrode (Reference method: ISO 10390).

To measure Total Nitrogen, Carbon, Organic Carbon and Inorganic Carbon (TN, TC, TOC, TIC) a combustion method was carried out using a Carlo Erba CN analyser (Flash1112 series). Samples were ball milled for until homogenised. Approximately 30 mg of milled soil was weighed in tin capsules using a 6 decimal balance and then pressed into a pellet before being analysed simultaneously for total carbon and total nitrogen. 50 mg of soil was weighed in a silver capsule and placed in a furnace at 500°C for 2 hours to remove the organic carbon and finally analysed for the inorganic carbon fraction by treating the soil with hydrochloric acid. The organic carbon fraction was calculated by difference between Total carbon and inorganic carbon (Reference method ISO 10694 & 13878).

Analyses were carried out by the Forestry Commission's Soil Analysis service(Commission, 2019).

3.2.6 Sequence Analysis

16S and ITS reads were separated by respective primer sets using cutadapt (Martin, 2011), and then each were separately OTU clustered using the pipeline detailed in chapter 2 (2.2.5).

These OTUs were also BLAST (Altschul *et al.*, 1990)aligned with the UNITE fungal ITS database (Abarenkov *et al.*, 2010) to assign taxonomic information to the ITS OTUs and the SILVA bacterial 16S database (Quast *et al.*, 2013) to assign taxonomic information to the 16S OTUs. OTUs were manually quality filtered to remove non-

bacterial non-fungal contaminants and to collapse OTUs with identical taxonomy together.

Samples were rarefied using USEARCH normalisation commands to allow for ecological analyses. Simpson's indices of ITS and 16S OTUs were calculated to assess shifts in alpha diversity. Bray-Curtis matrices were calculated for NMDS ordination and PERMANOVA was implemented using the adonis command in Vegan (Oksanen *et al.*, 2016) to determine any significant effects of during the time-course. Significantly correlated environmental variables were plotted onto the NMDS using the envfit command in Vegan to determine which were predictor variables.

3.3 Results

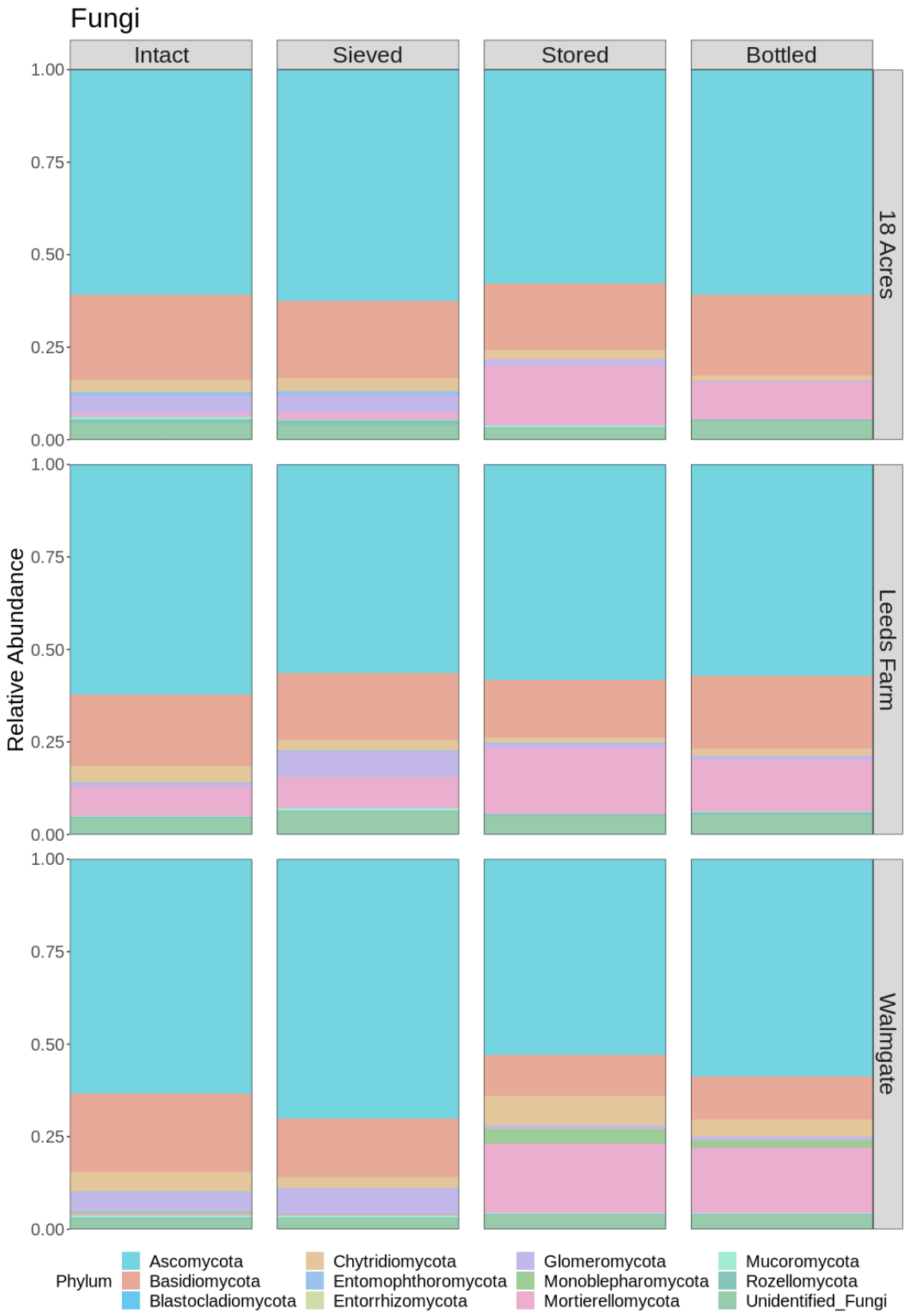

Figure 5: Stacked taxa charts of the fungal community at each condition, summarised by phylum level taxonomy. Charts are organised by Time point and Site. (n=6 for each plot).

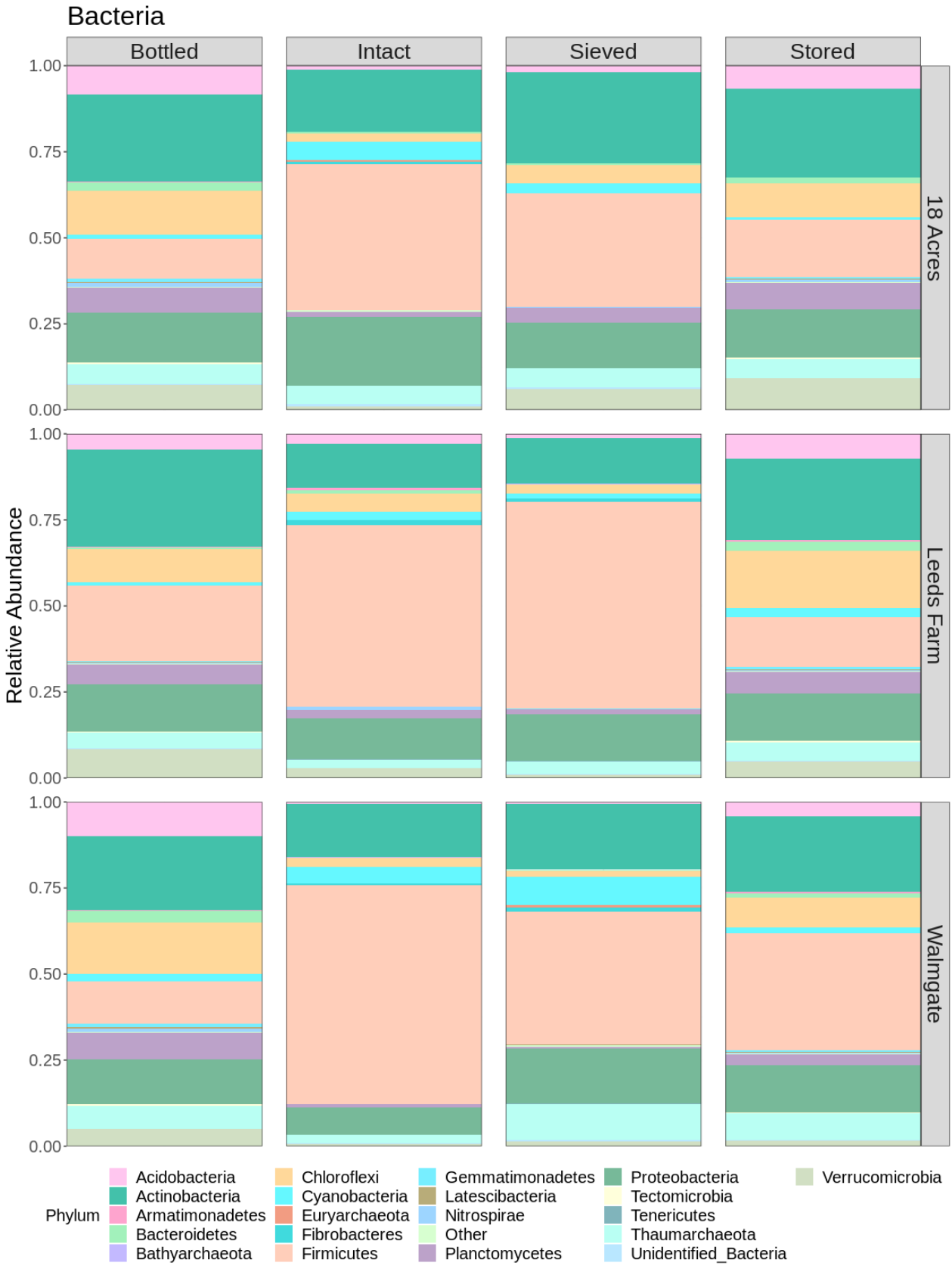

Figure 6: Stacked taxa charts of the bacterial community at each condition, summarised by phylum level taxonomy. Charts are organised by Time point and Site. (n=6 for each plot).

Time point	ITS OTU Richness			16S OTU Richness		
	18	L	W	18	L	W
t0 Intact	159	153	161	559	395	485
t1 Sieved	146	174	179	600	455	511
t2 Stored	195	522	768	661	723	685
t3 Bottled	238	215	224	709	595	718

Table 1: Mean ITS and 16S OTU richness observed at each site and subsampling time point (n=6 for each condition)

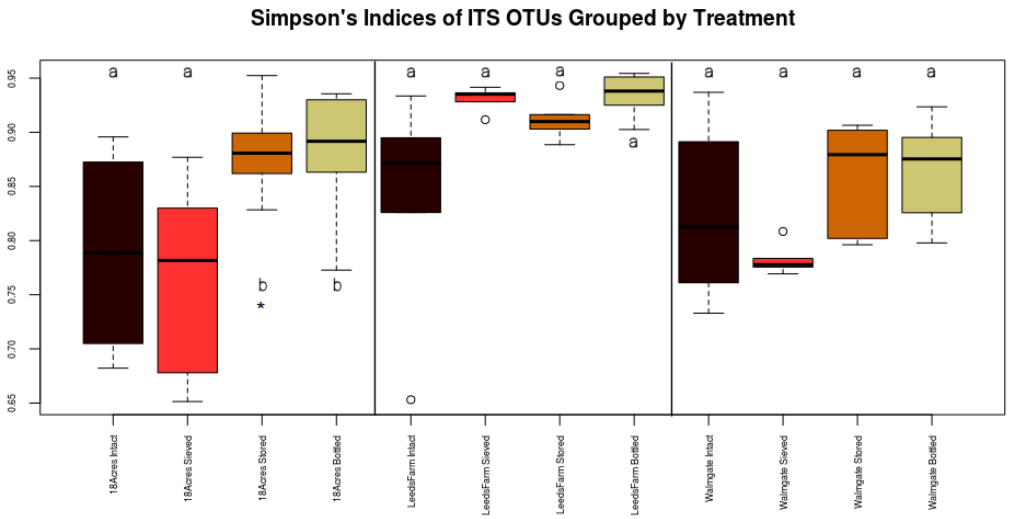

*Figure 7: Box plots of the Simpson's Indices of fungal OTUs of samples grouped by sampling site and time point. Colour indicates time point. Compact letter displays indicate significance grouping (according to post-hox ANOVA testing, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001). Dark line represents median, box represents upper and lower quartiles, bars represent minimum and maximum values, dots designate outliers (N=6 for each condition)*

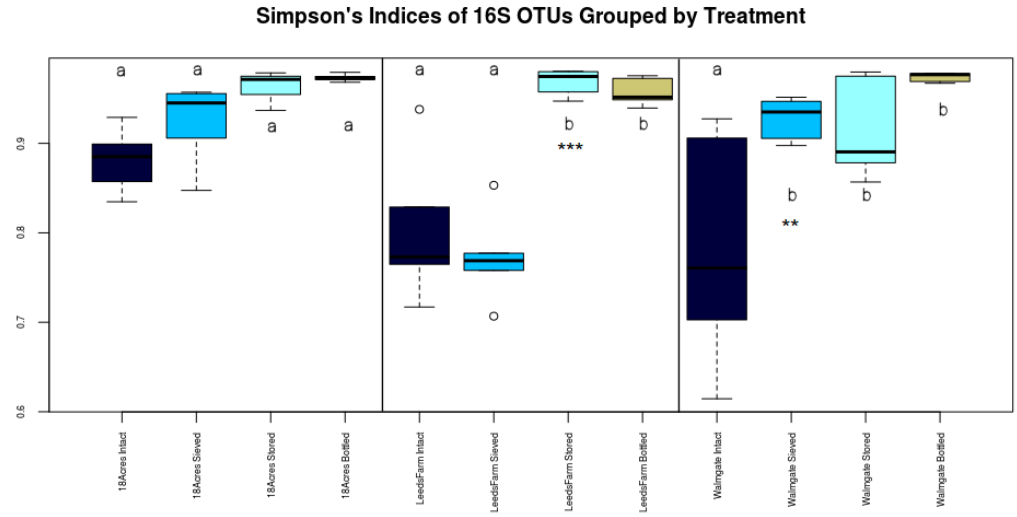

*Figure 8: Box plots of the Simpson's Indices of bacterial OTUs of samples grouped by sampling site and time point. Colour indicates time point. Compact letter displays indicate significance grouping (according to post-hox ANOVA testing, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001). Dark line represents median, box represents upper and lower quartiles, bars represent minimum and maximum values, dots designate outliers (N=6 for each condition)*

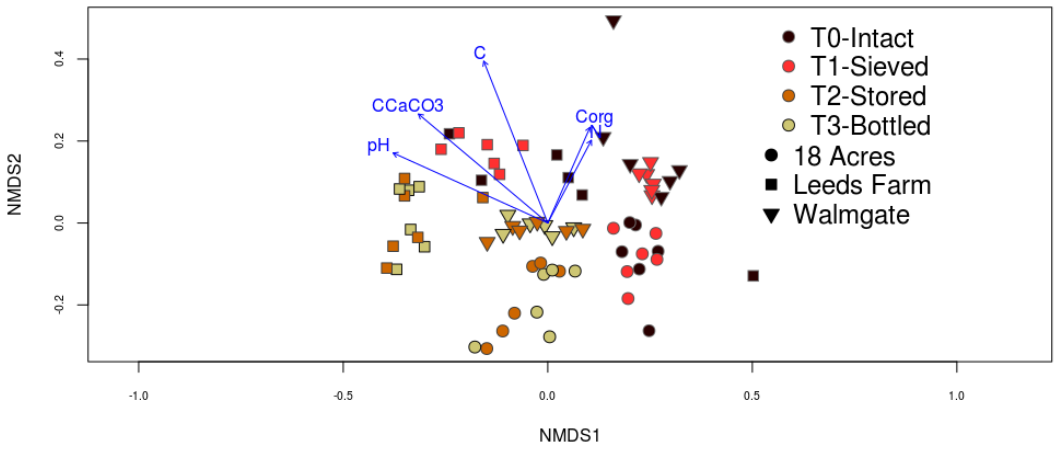

Figure 9: NMDS of Bray-Curtis Dissimilarity values of the fungal community. Arrows indicate driving environmental variables as calculated using envfit function of vegan. Each condition is coloured by time-point, and shape indicates site. (N=6 for each condition)

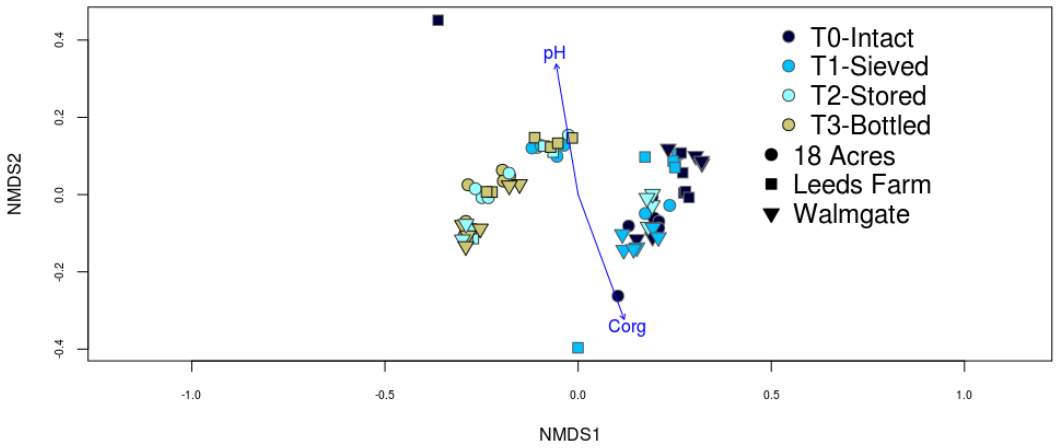

Figure 10: NMDS of Bray-Curtis Dissimilarity values of the bacterial community. Arrows indicate driving environmental variables as calculated using envfit function of vegan. Each condition is coloured by time-point, and shape indicates site. (N=6 for each condition).

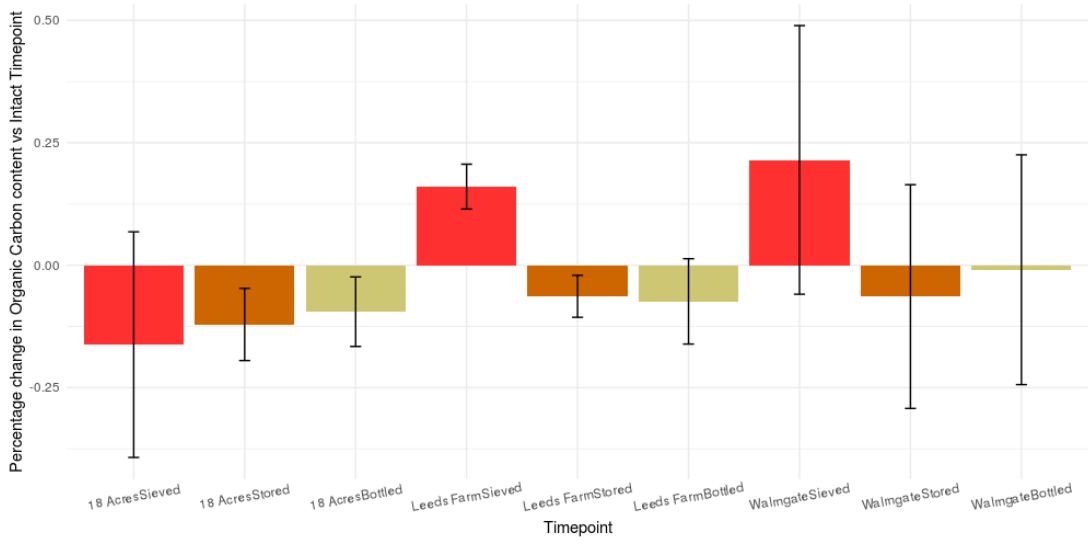

Figure 11: Bar plots of percentage changes in concentrations of organic carbon at each time point compared to Intact time point, grouped by site. Colour indicates time-point, (n=6 for each condition).

	pH H2O	N	C	C(CaCO3)	C(Org)
18 Acres Intact	5.85	0.26	2.46	0.00	2.46
18 Acres Sieved	5.84	0.24	2.30	0.00	2.30
18 Acres Stored	6.10	0.23	2.34	0.00	2.34
18 Acres Bottled	5.99	0.24	2.36	0.00	2.36
Leeds Farm Intact	5.65	0.40	4.14	0.00	4.14
Leeds Farm Sieved	7.18	0.24	5.01	2.50	2.51
Leeds Farm Stored	7.69	0.22	4.83	2.55	2.28
Leeds Farm Bottled	7.73	0.22	4.79	2.52	2.27
Walmgate Intact	5.65	0.40	4.14	0.00	4.14
Walmgate Sieved	5.57	0.43	4.35	0.00	4.35
Walmgate Stored	5.44	0.40	4.07	0.00	4.07
Walmgate Bottled	5.29	0.41	4.13	0.00	4.13

Table 2: Mean pH and nutrient content in % of total mass of each condition (n=6)

A total of 5838208reads were obtained. Core diversity analysis scripts were used to rarefy the samples down to 75000 reads each, equal to the sample with the 53rd highest reads (out of 73 samples). A total of 355 Fungal OTUs and 864 Bacterial were obtained after filtering steps. One-way ANOVA revealed a significant increase in fungal Simpson's Index (Figure 5) between sieved and stored time points for 18 Acres ($DF= 3$, $f= 4.25$, $P<0.05$), a significant increase in bacterial Simpson's Index between intact and sieved time points in Walmgate ($DF=3$, $f=9.27$, $p<0.01$) and

between sieved and stored in Leeds (df=3, F=29.9, p<0.001). PERMANOVA (Oksanen *et al.*, 2016) revealed organic carbon, nitrogen, inorganic carbon, and pH having a significant effect on the fungal community (DF=1 F=8.11 P<0.001, DF=1 F=7.37 P<0.001, DF=1 F=15.62 P<0.001, and DF=1 F=18.91 P=0.001 respectively) (Figure 11). PERMANOVA (Oksanen *et al.*, 2016) revealed organic carbon and pH having a significant effect on the bacterial community (DF= 1 F=2.64 P<0.05 and DF=1 F=2.28P<0.05 respectively) (Figure 12).

3.4 Discussion

In contrast to Chapter 2's results the alpha diversity increases after storage, markedly so in the Leeds 16S samples (Figures 9 + 10). This is likely linked to the decrease in carbon (Figure 13 & Table 2), as a depletion of nutrients has been found to increase evenness. (Zhou *et al.*, 2002) found that sub-surface soils also have a lower carbon content and a more uniform alpha diversity distribution when compared to more carbon rich soils, which would confirm carbon concentration is a key driver of the alpha diversity in the OECD307 system. This assumption is further confirmed by the increasing number of OTUs present over the time-course (Table 1). As the OECD307 test system is a closed system throughout the time-course the appearance of biologically "true" OTUs cannot occur from an addition to the system. Rather, an increase in community evenness will allow rare species to reach abundances above the detection threshold of the PCR/Clustering method (Zhou *et al.*, 2002), resulting in an increase of reported OTUs.

PERMANOVA analysis revealed that organic carbon concentration has a significant effect on the community composition of both fungi (Figure 11) and bacteria (Figure 12), and that nitrogen has a significant effect on the bacteria community (Figure 12). Distinct clustering can be observed in both NMDS (Figures 11 + 12), with Intact and Sieved time points and Stored and Bottled time points clustering together respectively. This was expected as sieving time point sub-sampling was at maximum 2 hours after intact time point sub-sampling, which is an insufficient timeframe for any changes in the microbial community to occur. The clustering of stored and bottled time point samples indicates the microbial community undergoes major changes during storage and remains stable during bottle incubation. This may be

due to an absence of any further major environmental disturbances, the soil does not undergo any further changes in structure but instead is transferred to another vessel maintained at similar temperature, moisture, and lighting conditions. Organic carbon (Figure 13) and nitrogen decrease across the time course, suggesting that the alteration in community is driven by a depletion effect. The correlation of nitrogen to fungal community but not bacterial community may be due to stoichiometric composition, however this is contradictory to the findings of (Strickland and Rousk, 2010) who found fungi have a higher C:N ratio than bacteria. Another possible explanation is the nitrogen fixing capability of many bacteria taxa.

Organic carbon concentration (Figure 13) is reduced during processing in Leeds and Walmgate sites, and is also reduced during storage in all sites and gradually increases during bottle incubation. In 18 Acres samples, processing increased the amount of observable carbon. This was potentially released from the disrupted soil matrix but not yet utilised by the community. Given Thomson et al's (2010) observation that glucose addition to re-wetted soil does not significantly alter the net CO_2 efflux, and therefore respiration, it may be that saturating carbon may be released from either the soil matrix or cell lysis during sieving and drying-rewetting and immediately utilised (B. Thomson *et al.*, 2010).

The gradual increase in organic carbon may consist of an increase in necromass or a utilisation of microbial biomass as primary carbon sources, matching the increase in microbial biomass utilisation following rewetting as demonstrated by Fierer and Schimel (2003). This could indicate a starvation effect following the immediate reduction in carbon, whereby microbes are either scavenging carbon from their own biomolecules, or the breakdown and consumption of other dormant microbes.

Inorganic carbon and pH appear to be predictors of differences between field sites (Figures 11 + 12), with higher pH levels observed in Leeds Farm samples. Agricultural land frequently is treated with lime to mitigate soil acidity and facilitate uptake of plant nutrients in order to increase yield and could be a potential cause for this observed higher pH, as well as differences in underlying geology and soil type. In addition, a higher soil pH would facilitate the formation of inorganic carbon

in the form of carbonates (Guo *et al.*, 2016), which would explain the co-correlation of pH and inorganic carbon in the NMDS.

This study has omitted reporting Bacteria:Fungi ratios. The number and abundance of OTUs is a low confidence metric due to the relative nature of OTU clustering without an internal standard during sequencing or biomass measurements to report. In addition, PCR is a not an absolute measure (see Chapter 1).

Firmicutes and *Actinobacteria* were dominant in the bacterial community throughout the time-course. Both phyla were dominant in the initial Intact time point and remained dominant throughout, potentially due to high saprotrophic capabilities buffering against the depletion of labile carbon. *Actinobacteria* were reported as one of the 2% of bacteria taxa that account for over half of all soil bacterial communities observed on Earth by (Delgado-Baquerizo *et al.*, 2018), and thus were expected to be present in high abundance in this study system. Many *Actinobacteria* behave in a similar manner to fungi, growing a network of mycelia and performing saprotrophic roles, which would allow them to rapidly adapt to the depleting carbon pool and retain dominance. *Firmicutes* have been observed to endosporulate during stress events, including starvation (Bueche *et al.*, 2013), and may have utilised this strategy here to maintain high abundance. A caveat with amplicon fingerprinting is that it does not discriminate between active and sporulated forms, and therefore the functional capacity of *Firmicutes* in this system cannot be determined. Photosynthetic *Cyanobacteria*, found in Intact samples, decreased in abundance in later time points, likely due to the absence of light in the test system. This adds confidence to the above assertion that no new carbon is being fixed by phototrophs in the OECD307 test system.

Members of Basidiomycota and Ascomycota were dominant in the fungal community in time points preceding storage. An expected decrease in abundance of *Glomeromycota* was observed after storage. Given the absence of active plants to colonise and provide host-carbon it is likely these populations were unable to adapt to the soil disturbance.

The results observed in Chapter 2 demonstrated that these community shifts are highly influenced by sampling site and sampling season, therefore this single season observation of community shift in this chapter must be considered within the context of that framework. Similar trends were observed across all sites in this study, confirming the findings in Chapter 2 that seasonality is the major driver of initial, and therefore endpoint, community composition.

3.5 Conclusions

The findings of this study support the mechanism posited in Chapter 2. Briefly, sieving/drying-rewetting causes the homogenisation of the spatially distinct nutrient pools and microbial communities of the soil matrix. This releases carbon stored within pores into a combined nutrient pool, which is stochastically accessed by the microbial community, causing a shift in dominance. This pool, in contrast to carbon stored in distinct niches, is depleted rapidly during storage. This depletion potentially shifts the community towards saprotrophs and microbes with starvation strategies, such as sporulation, indicating that storage could be a critical step in determining both the microbial community and the metabolic capability of the community during a standard OECD307 test run.

The depletion of nitrogen and especially organic carbon is driving the microbial community change within the OECD307 closed bottle test, altering both alpha diversity and community composition. The impact on CPP degradation caused by a shift in community is difficult to determine as taxonomy is a poor predictor of function. In addition, a reduced concentration in labile carbon could potentially cause a decrease in respiration rates, and therefore a reduction in ATP available for metabolic activity, independent of taxonomy. Metabolomic fingerprinting is a powerful sequence-independent tool which provides snapshots of total metabolite concentrations and can be used to infer the metabolism of a given biological sample. As discussed in the next chapter, in order to elucidate the impact the depletion of carbon has on the metabolic capability of the microbial community a third bottle test experiment was set up and sub-samples were subject to a novel metabolomics pipeline developed at Syngenta.

4 Chapter 4: Metabolomic Analysis of Soil Microbial Metabolism in OECD307 Tests

4.1 Introduction

In Chapter 3 depletion in organic carbon was found to be a key driver of the change in the soil microbial community during OECD307 tests. A shift in community may alter the abundance of functional genes, but taxonomy is a poor indicator of functional genes (Louca *et al.*, 2018). In addition to this, carbon depletion may alter microbial function independent of a community's functional genes by altering survival strategies toward nutrient scavenging (Fontaine *et al.*, 2011). Metabolomics, as detailed further below, is the study of small molecule metabolites, allowing the study of biological metabolism independent of the biological sample's gene set (Aldridge and Rhee, 2014). This chapter will present results of a targeted metabolomic analysis of OECD307, examining metabolites from select pathways and classes to elucidate the change in microbial function caused by carbon depletion.

Nutrient depletion has previously been shown to change microbial function by altering extracellular enzyme production. The quantity, diversity, and composition of available nutrients all affect enzyme activity, with each enzyme having its own unique control mechanisms (Hernández and Hobbie, 2010). (Hernández and Hobbie, 2010)Olander and Vitousek (2000) found that chitinase activity is increased at N-limited sites, and in a later study Allison and Vitousek (2005) found that many extracellular enzymes increase their activity when their substrate is present as complex molecules but not in simple forms. Conversely an abundance of labile carbon can also cause an increase in nutrient scavenging, such as the priming effect demonstrated by Fontaine *et al* (2011) whereby fungi increase the decomposition of recalcitrant SOM in the presence of labile carbon.

Investigating microbial function is crucial for a more complete understanding of microbial dynamics as taxonomy alone is a poor indicator of metabolic capability. Communities with differing taxonomic compositions can have conserved functional gene content, for example gut microbiomes can vary greatly between hosts whilst

the community gene content is strongly preserved (Louca *et al.*, 2018). Large scale ecosystem functioning may also be independent of community composition, with many generalist species being adapted to a range of conditions (Langenheder and Székely, 2011), although certain processes where the functional genes are held only by a smaller number of specialised species may be affected by a change in community (Koranda *et al.*, 2013). In addition, bacteria show a high degree of horizontal gene transfer, rendering taxonomy-function relationships difficult to predict (Young, 2001). Therefore taxonomy alone is insufficient to determine community function.

Alternatively, the determination of metabolic activity directly from RNA methods without directly observing the CO_2 efflux or a similar direct metric of respiration/activity is possible, but presents a significant technical challenge. Analysing rRNA using metatranscriptomics or via qPCR has been previously used as a common method of determining which members of the community are active within an environment. However, Blazewicz *et al.* (2013) demonstrated that the relationship between rRNA, growth, and activity are complex and potentially confounding as not all species share the same growth strategies and so growth and non-growth activities may not be completely distinct when observed using this methodology. Instead, observing metabolites produced by these activities may allow the direct inference of the types of metabolism taking place independent of taxonomy.

Metabolomics is a sequence-independent method of detecting a wide range of small molecule metabolites of a biological system. Recent advances in both technology and methodologies in mass spectrometry and NMR have allowed for measurement of thousands of metabolites simultaneously (Patti, Yanes and Siuzdak, 2012). Whilst metagenomics and metatranscriptomics can reveal gene content and its expression together to describe a community's functional capacity, metabolomics can demonstrate functional activity directly by measuring the

abundance of metabolite products. Metabolomics, unlike genomic or proteomic approaches, does not require fully annotated sequences (Jones *et al.*, 2013) and metabolites are largely universal across microbial species, with molecules such as the carboxylic acid intermediates of the TCA cycle being found in all known organisms despite the vast diversity of genes, mRNAs and proteins found in nature, likely due to the shared evolutionary ancestry of all lifeforms (Smith and Morowitz, 2004; Peng, Li and Peng, 2015).

Metabolomics utilises three primary methods; nuclear magnetic resonance (NMR), liquid chromatography coupled with mass spectrometry (LC-MS), and gas chromatography coupled with mass spectrometry (GC-MS) (Aldridge and Rhee, 2014). Briefly, NMR based metabolomics utilises the magnetic resonance of each distinct proton within a sample to determine the chemical shift relative to a standard compound (Dona *et al.*, 2016). LC-MS and GC-MS utilise liquid and gas chromatography respectively to separate compounds before they enter a coupled mass spectrometer to determine their characteristic mass to charge ratio and retention time (Zhou *et al.*, 2012).

There are numerous challenges associated with metabolomics studies. No single methodology has the capacity to universally capture all metabolites due to the range of sizes, chemical properties, and complexity of small molecules within biological samples. Limitations of each method are briefly described here. NMR has a lower sensitivity, up to 100 to 1000 fold lower than MS methods, and it can be computationally limited when identifying a complex mixture of metabolites. LC-MS has a much greater sensitivity than NMR, but it is unreliable when detecting volatile chemicals due to lower sensitivity, needs specialised chromatographic methods in order to detect compounds of different polarities, and has a limited capacity for structural identification and quantitation (Riekeberg and Powers, 2017). GC-MS often requires derivatisation of compounds prior to mass spectrometry and

therefore prior knowledge of the properties of the metabolite molecules (Aldridge and Rhee, 2014).

Many challenges of metabolomics arise from properties of metabolites. Many metabolites have very short half-lives (ATP has an approximate half-life of between 1 and 60s) (Tang, 2011) and are susceptible to enzymatic activity after sample harvesting. A quenching step is required to inactivate metabolic activity and capture an accurate snapshot of the metabolome. Standard quenching methods involve rapid cooling of samples using cold (-48°C) methanol or liquid N_2 (Hiller *et al.*, 2007) to rapidly decrease the temperature-dependent activity of enzymes.

Another key challenge is sufficiently extracting metabolites from samples. Bead-beating frozen samples has been found to have the most efficient yield of metabolites when compared to sonication and freeze-thaw cycles (Sasidharan *et al.*, 2012). Solvent choice is critical as it will determine the class of chemicals retrieved (Zhou *et al.*, 2012). Methanol and methanol/ethanol mix have become solvents of choice thanks to their high metabolite coverage; however, their wide selectivity means that lower abundance metabolites may not be detected. Therefore consideration must be given to the potential biological relevance of which compounds can be retrieved (Sitnikov, Monnin and Vuckovic, 2016). Another major consideration is the compatibility of solvents with analytical instruments.

LC-MS has become a popular platform for metabolomics because of its sensitivity and high throughput (Bowen and Northen, 2010; Aretz and Meierhofer, 2016). Enhanced structural elucidation can be achieved using tandem mass spectrometry (MS/MS), whereby compounds separated by weight and fragmented by a primary mass spectrometer can be identified by those fragments on a second mass spectrometer. Use of rapid polarity switching can greatly increase coverage by acquiring scan data in both positive and negative modes simultaneously, allowing a much greater set of all key metabolites to be captured (Yuan *et al.*, 2012).

Variability in LC-MS data can arise from multiple factors, including biological variation, sample preparation, and instrument conditions. This variation takes the form of drifting retention times, intensity values, and m/z values (Zhou *et al.*, 2012). Intensity drift can be due to the variability in the stability of a metabolite, while retention times may drift due to slight differences in the matrix of a sample, which in turn may be due to legacy effects of extraction methods (Sitnikov, Monnin and Vuckovic, 2016; Riekeberg and Powers, 2017; Gertsman and Barshop, 2018). The m/z values of metabolites may drift during mass spectrometry due to adducts forming between two ionised metabolites, or between a metabolite and the mobile phase solvent (Draper *et al.*, 2009). This can result in a compound giving multiple m/z values, or the same compound giving multiple m/z values.

In order to monitor variation, it is recommended throughout the LC-MS run to periodically analyse aliquots of a homogeneously mixed pool of equal amounts of multiple samples which form a quality control (QC). This allows the evaluation of any analytical variation and elimination of any potentially unreliable sample runs (De Vos *et al.*, 2007). In addition to this the inclusion in each sample of an internal standard composed of isotopic compounds of known concentrations will allow further evaluation of any drift in retention times and the quantification of the observed peaks.

The identification of metabolites from their spectra is another primary challenge of metabolomics. The high number of chemicals and their varied classes makes it difficult for general rules to predict fragmentation. (Zhou *et al.*, 2012). Comparison of m/z values to a pre-existing database is currently the most common method of compound identification (Go, 2010), but this does not allow unambiguous discrimination between compounds with similar molecular weights (Kind and Fiehn, 2006), or isomers with the same elemental composition. MS-MS can be used to confirm the identity of a compound by further fragmentation and detection

following initial detection, resulting in a characteristic fragmentation pattern (Zhang *et al.*, 2019).

The analysis of metabolomic data presents a considerable statistical challenge. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) is a popular and powerful statistical model frequently used to determine if two groups are statistically separate. A potential pitfall of OPLS-DA is the over-fitting of data to the model, which could create false conclusions based on unreliable statistics. This is usually overcome by diagnosing group effect using a Principal Components Analysis (PCA), termed "unsupervised" data, before testing those groups in an OPLS-DA, termed "supervised" data. Unsupervised methods are used to elucidate patterns and trends in the data, whereas supervised methods are used to then classify the potential driving metabolites behind those patterns. This more rigorous approach will validate the models tested and provide higher confidence results. (Wheelock and Wheelock, 2013)

Metabolomics approaches have been used to effectively elucidate metabolic activities of soil microbial communities. A study by Swenson *et al.* (2018) compared metabolomes of biological soil crusts to find specific correlations between certain metabolites and bacterial taxa, linking environmental chemistry to the composition of the microbial community. A previous study by Swenson *et al.* (2015) compared the metabolome of lab-grown *Pseudomonas stutzerii* to that of a soil extract and found that less than 27% of metabolites freed from cell lysis were recoverable from the soil, which suggests that many may be microbially inaccessible due to strongly binding to soil particles and providing a potential mechanism for the accumulation of microbially-derived SOM. Metabolomics can also be used to assess metabolic responses to stresses, for example nicotine exposure triggers a complex disruption to catabolism and osmoregulation pathways in *Pseudomonas* (Ye *et al.*, 2012).

In this study a LC-MS-MS metabolomics approach was used to assess changes in function between intact soil and soil treated to OECD guidelines, as well as assess the impact of a storage regime on the microbial metabolism. In order to examine what changes in metabolism the OECD processing caused, a number of time course samples across a standard OECD bottle test were taken and put through a novel LC-MS-MS setup at Syngenta's Jealott's Hill R&D site. This approached utilised a broad library of standards to examine a broad range of metabolites to observe biosynthesis, nutrient uptake, TCA cycle and environmental responses.

From a library of standards available at Syngenta's Jealott's Hill laboratories 51 key metabolites were selected in order to examine potentially affected pathways and functions.

4.2 Metabolite selection rationale

The rationale for metabolite selection is described here, grouped by metabolite class.

4.2.1 Carbon Sources

One of the key findings of Chapter 3 was that a reduction in organic carbon drives the shift in community. Therefore, it is important to monitor labile carbon sources in order to examine any correlated metabolic shifts.

Common carbon sources available to microbes in the soil environment include glucose, sucrose, and fructose (Gunina and Kuzyakov, 2015). Abundance of Glucose-6-Phosphate (G6P), the most common form of intracellular glucose, is an indicator of ATP activity (Schwartz, 2007) and has been used in previous metabolic studies to indicate nutrient mineralisation (Heuck, Weig and Spohn, 2015). Additionally, Barra et al found G6P to be required for the use of trehalose (detailed below) as an osmoprotectant (Barra *et al.*, 2003).

Mannitol is the most common starch present in fungi (Dulermo *et al.*, 2009), used as a storage or translocated carbohydrate (Witteveen and Visser, 1995). Of particular interest to this study is its essential role in spore germination under starvation conditions (Meena *et al.*, 2015).

Sorbitol, in addition to its utilisation as a carbon source (Pérez-Ramos *et al.*, 2017), is also an intermediate in fructose, mannose, and galactose metabolism (Kanehisa, 2019).

COMPOUND	FUNCTION
Glucose	sugar
Sucrose	sugar
fructose	Plant-derived sugar
G6P	phosphorylated glucose, most intracellular glucose is in this form
Mannitol	carbon storage molecule
Sorbitol	fructose, mannose, galactose metabolism

4.2.2 TCA Cycle Intermediates

The Tricarboxylic Acid (TCA) Cycle, also called the Krebs cycle, is a key biochemical reaction pathway which is found across all aerobic organisms (Berg, Tymoczko and Stryer, 2002). The cycle consumes acetate (in the form of Acetyl-CoA) and water molecules to reduce NAD+ molecules, which feed directly into the electron transport pathway, which produces ATP. Thusly the TCA cycle is crucial to any ATP-mediated activity within the cell, and is considered as central metabolism (Winder, Dunn and Goodacre, 2011). An increase or decrease in TCA cycle activity would result in an increase or decrease of overall microbial activity.

TCA Intermediates have been previously used as a measure of carbon metabolism in metabolomic studies (Zhao *et al.*, 2019).

In this study six TCA intermediates were targeted, in addition to Acetyl-L-carnitine, which is utilised in shuttling Acetyl-CoA into the mitochondria in eukaryotes (Hynes *et al.*, 2011).

COMPOUND	FUNCTION
Acetyl-L-carnitine	facilitates movement of Acetyl CoA into matrices of mitochondria
Malic acid	intermediate in the TCA cycle
Fumaric acid	intermediate in the TCA cycle
Fumaric_acid	intermediate in the TCA cycle
ketoglutaric_acid	intermediate in the TCA cycle
Pyruvic_acid	intermediate in the TCA cycle
2-Oxoglutaric acid	intermediate in the TCA cycle

4.2.3 DNA/Protein Molecules

Previous studies have used quantitative metabolomics targeting the concentrations of amino acids and their precursor molecules in order to infer biosynthetic flux (Carnicer *et al.*, 2012), and in a similar manner DNA nucleotide concentrations have been used to infer DNA and RNA biosynthesis (Lane and Fan, 2015), processes which are necessary for DNA replication and RNA transcription, both growth related processes.

In this study a range of amino acids and nucleobases/nucleosides were targeted, in addition to related intermediate metabolites which arise from biosynthesis or catabolism.

The amino acids Alanine, Aspartic Acid, Glutamic Acid (and its anion Glutamate), Isoleucine, Leucine, Methionine, Norleuicine, Phenylalanine, Proline, Serine, Threonine, Typtophan, and Tyrosine were all available from the standard library and targeted. Due to availability issues at the time of study, other proteinogenic amino acids were not included. The primary nucleobases, Adenine, Cytosine,

Thymine, Guanine, and Uracil were targeted, in addition to the nucleosides Thymidine, Inosine, Adenosine, Cytidine, and Deoxyuridine.

N-Acetyl-Glutamate and N-acetylornithine are intermediates in the arginine biosynthesis pathway (Fernández-Murga and Rubio, 2008), and citrulline is a catabolite of arginine (Fang *et al.*, 2018).

Hypoxanthine, Xanthine, and Xanthosine are intermediates in purine biosynthesis and salvage pathways (Kilstrup *et al.*, 2005), while 2-Isopropylmalic Acid is an intermediate in Leucine biosynthesis (Calvo, Kalyanpur and Stevens, 1962). Aminoadipate is a key intermediate in fungal lysine biosynthesis pathways (Fazius *et al.*, 2012).

COMPOUND	FUNCTION
Alanine	amino acid
Aspartic_Acid	amino acid
Glutamate	amino acid
Glutamic_Acid	amino acid
Isoleucine	amino acid
Leucine	amino acid
Methionine	amino acid
Norleucine	amino acid
Phenylalanine	amino acid
Proline	amino acid
Serine	amino acid
Threonine	amino acid
Tryptophan	amino acid

Tyrosine	amino acid
Adenine	nucleobase
Cytosine	nucleobase
Guanine	nucleobase
Thymine	nucleobase
Uracil	rna nucleobase
Thymidine	nucleoside
Inosine	nucleoside
Adenosine	nucleoside
Cytidine	nucleoside
Deoxyuridine	nucleoside
N-acetyl_glutamate	precursor to arginine
N-Acetylornithine	precursor to arginine
Citrulline	product of arginine catabolism
Hypoxanthine	intermediate in purine metabolism
Xanthine	product of purine metabolism
Xanthosine	intermediate in purine metabolism
2-Isopropylmalic Acid	Intermediate in leucine metabolism
Aminoadipate	lysine synthesis/breakdown

4.2.4 Cyclic AMP pathway

Cyclic AMP (cAMP) is a cell signalling molecule that plays a role in many pathways including the lac operon (Schmitz, 1981) and biofilm formation (McDonough and Rodriguez, 2012). These functions are of interest to the study as they indicate microbial adaptation to starvation and environmental stress (Kalivoda *et al.*, 2013).

Biofilms are a survival strategy in nutrient limited environments which also can mediate antimicrobial tolerance (Fux *et al.*, 2005). Cyclic AMP concentrations are inversely proportional to glucose concentration, activating pathways when the most catalisable substrate is absent (i.e. as the cell enters a starvation state) (Ullman and Danchin, 1980; Green *et al.*, 2014).

Targeting cAMP in addition to the carbon sources listed above will allow further insight into the metabolic state of the community, with an increase suggesting a shift of metabolism into starvation strategies.

COMPOUND	FUNCTION
Cyclic AMP	cell signalling molecule which mediates several starvation responses

4.2.5 Defence

Starvation strategies often include responses to mitigate environmental stress, such as acid and oxidative stress (Watson, Clements and Foster, 1998), and competition mediated stresses such as antibiotic stress (Nguyen *et al.*, 2011). Filamentous fungi secrete antibiotic compounds as effectors against microbial competitors (Künzler, 2018). Osmoprotectants such trehalose can aid against osmotic stress, but also double as a nutrient source to protect from nutrient starvation (Zevenhuizen, 1992). Targeting this class of metabolites will reveal any increase in microbial defence mechanisms.

Benzoic acid is a fungistatic compound which is produced by lactic acid bacteria, primarily via the degradation of phenylalanine, auto-oxidation of benzaldehyde, or fatty acid catabolism (Hertweck *et al.*, 2001; Yu *et al.*, 2016).

Glutamic Acid, detailed above, is repeated here in the context of potential defence utilisation. In *E.coli* the acid activated glutaminase YbaS converts glutamine into glutamic acid and releases gaseous ammonia. This neutralises free protons, raising

the pH and protecting from intracellular acid stress (Lu *et al.*, 2013; Pennacchietti *et al.*, 2018).

Ergothioneine is an amino acid synthesised by bacteria of the order Actinomycetales (Genghof and Damme, 1964) and the fungi of the divisions Ascomycota and Basidiomycota (Genghof, 1970). It is commonly found within mitochondria, suggesting a specific role in antioxidant protection (Paul and Snyder, 2010).

Betaine is a well characterised bacterial osmoprotectant (Cairney, Booth and Higgins, 1985; Sleator and Hill, 2002; Wargo, 2013), while trehalose is a major osmoprotectant found across most organisms (Iturriaga, Suárez and Nova-Franco, 2009).

Trehalose also functions as a carbon storage molecule in fungi (Lillie and Pringle, 1980), with additional roles in oxidative stress protection (Wiemken, 1990), cell wall formation (Ngamskulrungroj *et al.*, 2009) and glycolysis (Perfect *et al.*, 2017).

Y-Aminobutyric Acid (GABA) in a non-protein amino acid involved in spore germination and resisting acid stress (Foerster and Foerster, 1973; Dhakal, Bajpai and Baek, 2012).

COMPOUND	FUNCTION
Benzoic acid	Fungistatic
Glutamic_Acid	protects against acid stress
Ergothioneine	compound protects against oxidative stress
Betaine	osmoregulator
Trehalose	osmoregulator
Y-Aminobutyric Acid (GABA)	Involved in spore germination and acid stress

The hypotheses for this study are as follows:

- The depletion of organic carbon observed in Chapter 3 will be mirrored by a reduction in carbon source metabolites with a subsequent decrease in carbon-dependent TCA metabolites, and an increase in cAMP, as the system enters starvation.
- A reduction in DNA/Protein metabolites will be observed due to reduced energetic input from a lowered TCA cycle., resulting in lowered biosynthesis
- Environmental stress and nutrient depletion during OECD307 processing will result in an increase in defence related metabolites.
- Storage treatment will exaggerate the metabolic shifts detailed above.

4.3 Methods

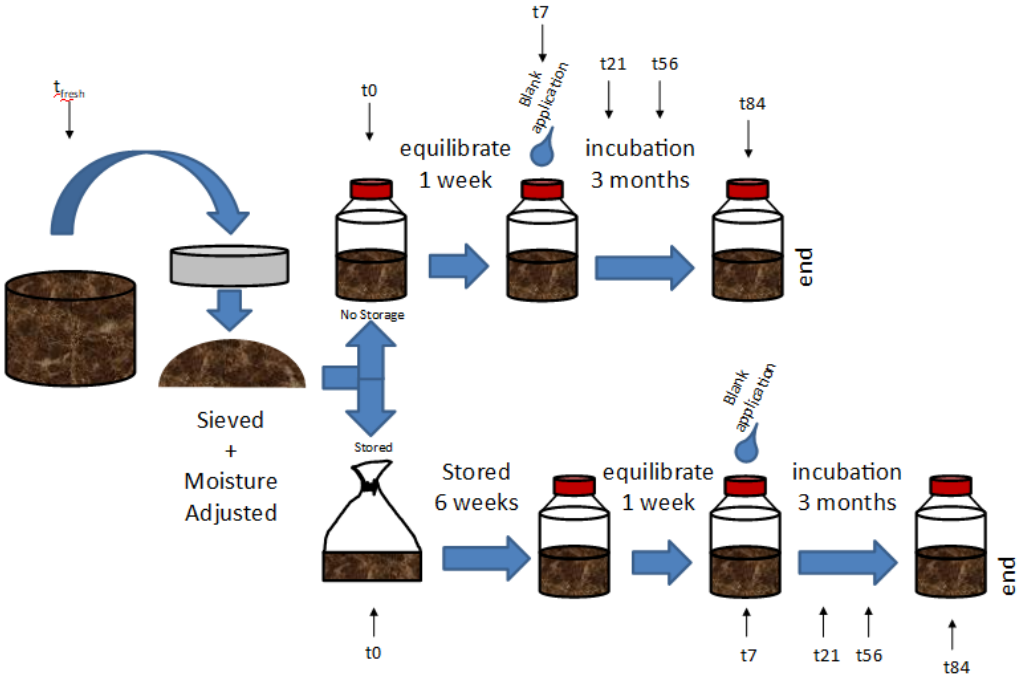

Figure 12:Sampling schematic for OECD307 metabolomic analysis (t0, t7, t21, t56, and t84 indicate when subsampling took place). Blank application is 4.0ml dH2O:0.3ml Methanol (as detailed in methods).

Sampling Time point	Time point Details	Total Days Incubated in Bottle
tFresh	before soil was processed	N/A
t0	as the soil first enters the bottle (for no storage treatment) OR when soil first enters storage (for storage treatment)	0
t7	as the blank application is first added (end of equilibration)	7
t21	end of first month incubation	21
t56	end of second month incubation	56
t84	end of third month incubation	84

Table 3: Subsampling time point details

4.3.1 Sampling

In October 2017 appox. 5kg of soil was sampled from Jealott's Hill (Bracknell: 51.454453, -0.720672). A single sample site was chosen due to time and budgetary constraints. Sample prep was time intensive and preparing samples from multiple sites would have potentially caused the metabolite content of samples to drift and degrade before the LC-MS run. A "Fresh" subsample was taken before the soil was processed according to OECD 307 guidelines (see Chapter 2). A portion of the soil (hereby "bottled") was immediately placed into six bottles (200g dry weight per bottle) and left to equilibrate for 1 week before application of a blank treatment solution (4.0ml dH_2O:0.3ml Methanol), and then incubated according to OECD guidelines for 3 months. The remainder of the soil (hereby "stored") is put into storage according to OECD guidelines for 6 weeks before then undergoing the same bottling, equilibration, application, and incubation procedure as the immediately bottled soil (See Figure 14 and Table 3).

The Bottled course was sampled as the soil was first bottled, after the blank application was added, then after each month of incubation. The Stored course was sampled as the soil was first stored, after the blank application was added following bottling and incubation, then after each month of incubation. At each timepoint for both Bottled and Stored treatments 5g of soil was sampled.

4.3.2 Sample Extraction

1.5g of soil samples were weighed into a 15 ml falcon tube along with FastPrep™ lysing matrix E and three 0.5cm diameter ceramic balls. They were extracted with 4.5ml water for 30 seconds on a Fastprep at speed 5 for 30 seconds. To separate the supernatant from the solid material, the samples were centrifuged at 8000 rpm (approx 10000 RCF) for 10 mins at 4°C. The pellet was extracted once more and the supernatants combined and filtered through a GxF/GHP 0.45µm, 25mm diameter syringe filter. The supernatant was evaporated to dryness using Genevac EZ-2(aqueous method, 40°C), and the sample was reconstituted with 400µl AcN:water (5:95) and mixed well. A final filtration using a Spartan RC 0.3 µm syringe filter was performed and the sample was spiked with 10µl of an internal standard (consisting of equal concentrations of the isotopic compounds d6-ABA, d3-Glutamic Acid, and d-5 Phenylalanine) prior to analysis. A 40µl aliquot of all test samples were pooled to create a quality control (QC) sample prior to spiking with 10µl internal standard.

4.3.3 Chromatography and Detection

The method for detecting metabolites in soil extracts used an Agilent 1290 LC coupled to a high resolution-accurate mass spectrometer (Q-Exactive™, Thermo). For chromatography, a gradient separation on a C18 coupled to a HILIC column was used. This set up allowed better separation of compounds from a diverse class of polar compounds in a single run of 50 minutes. Further details on the column conditions, mass spectrometer settings, and other variables are at present protected by and confidential to Syngenta.

For detection, the mass spectrometer separated the compounds according to their masses. The samples were analysed in both positive and negative polarity. The Q-Exactive™ can perform several experiments, offering structural information on any ionisable compounds detected above the limit of detection. Full scan (mass range 70 – 1050 m/z) data provided accurate masses to discriminate between isomeric compounds. Fragmentation data was collected from an all ion fragmentations and data dependent acquisitions experiment for structural elucidation.

Samples were randomised in order to minimise the effects of experimental variation during data processing. A six point calibration standard, consisting of the targeted metabolites detailed above, was prepared (0.05 µg/ml,0.5 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml) and injected in the beginning and end of sequence. Multiple injections of the QC sample and a calibration standard (5 ug/ml) were introduced at specific intervals to ensure the quality of the data. The quantified results of the QC sample and standard were compared and a relative standard deviation (or RSD), a metric of the variance observed in each reading, was calculated for determination of instrument precision as part of a process to assess if the response of the instrument has varied over the run or between runs.

4.3.4 Data Processing

For targeted analysis, compounds in the soil extracts were quantified by external standard calibration using Xcalibur™ (Thermofisher, 2018). For untargeted analysis, peak picking and peak annotation used Compound Discoverer (CD), where the fragmentation spectra, retention time and molecular formula of unknowns were used to match with those of standard peaks from an in-house mzVault library and online mzcloud (https://www.mzcloud.org/) spectral database. The fully quantified targeted data and the untargeted data with only peak areas from the CD output were pooled into the same spreadsheet output for multivariate statistical analysis.

4.3.5 Analysis

Multivariate statistics were performed on the entire data set using SIMCA (Umetrics, 2019). The PCA (unsupervised) was performed to visually assess overall trends/outliers. The OPLS-DA (supervised) was used to find discriminating metabolites between different sample groups. The OPLS-DA model was validated

with PERMANOVA cross validation (CV-ANOVA) tests. In order to elucidate which metabolites are driving the differences between fresh and Bottled t1, fresh and Stored t1, Bottled t0 and t84, and Stored t0 and t84, a V plot of OPLS-DA loadings was used to select differentiating metabolites that significantly influence the metabolome (according to PERMANOVA cross validation). The differentiating metabolites were ranked according to the value from multiplication of the p(corr) and the VIP (variable influence on projection) output from OPLS-DA. The larger the p(corr) value, the more confidence in differentiating metabolites as being significant. VIP was used as the parameter to measure the degree of influence of the metabolites. Metabolites with the 25% highest and 25% lowest p(corr)*VIP values were plotted to visualise the driving changes in metabolite concentrations. Only targeted metabolites with matching standards and therefore high confidence in abundance were selected to observe significant changes.

4.4 Results

Principal Components Analysis (Figure 15) (unsupervised data) of all compounds (both targeted and untargeted) demonstrated a separation of samples by time point with a clear gradient effect.

CV-ANOVA revealed a significant difference in the metabolic fingerprints between Bottled t0 and Bottled t84 (DF=5, F=6.43, $p<0.05$) and between Stored t0 and Stored t84 (DF=5, F=6.73, $p<0.05$). OPLS-DA failed to generate a model between tFresh and Bottled t7, and between tFresh and Stored t7, likely because the metabolite fingerprints were too different to compare within the same model.

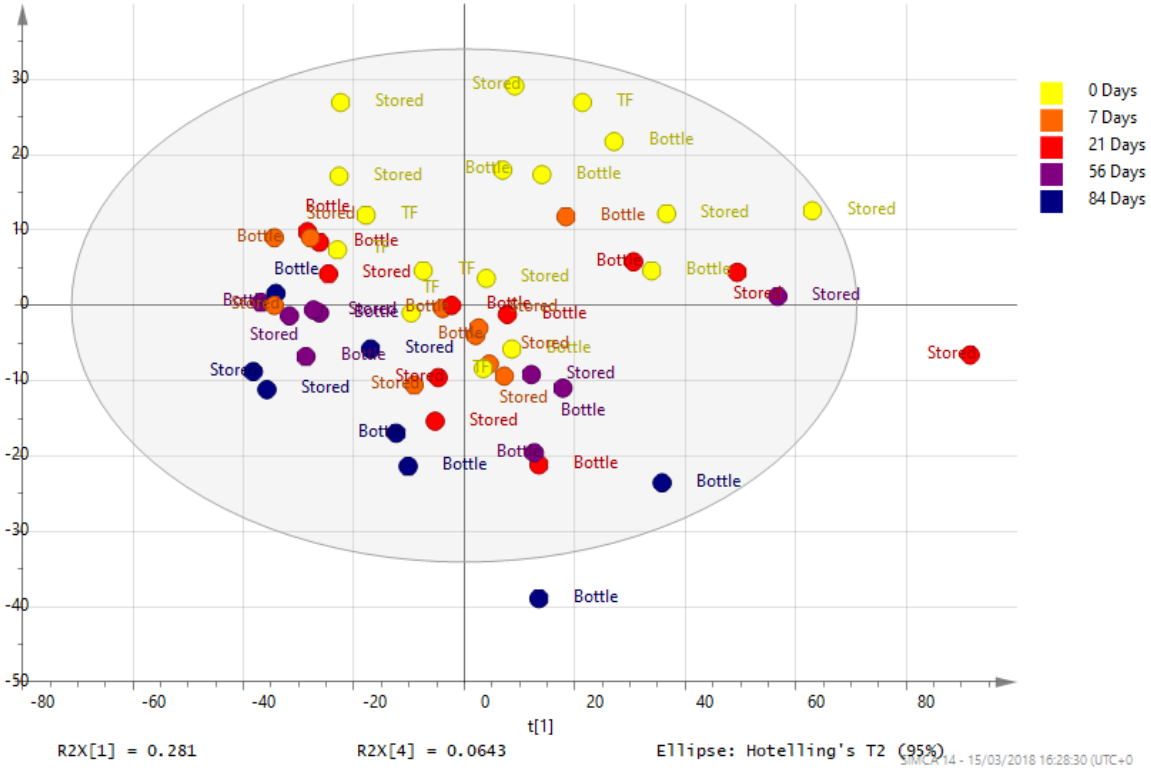

Figure 13: Principal Components Analysis of all samples, coloured by Incubation Time point. Each dot represents a single sample. A gradual change in metabolite fingerprint is visible across a time point gradient. Two outlier samples sit outside of the 95% confidence interval indicated by the Hotelling's Eclipse.

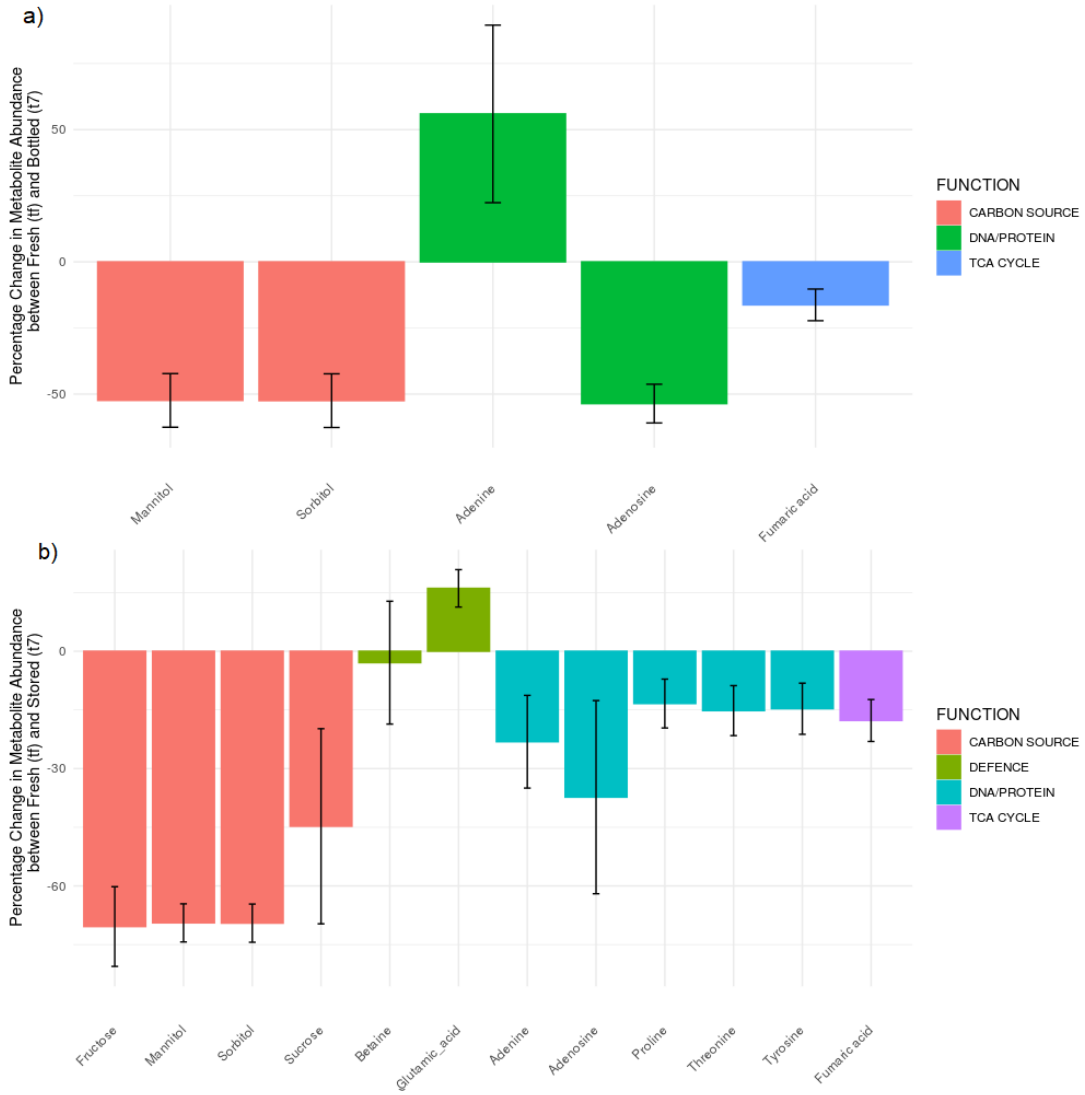

Figure 14: Significant changes in abundance of metabolites between Fresh samples and 7 Day time point for Bottled (16a) and Stored (16b) treatments as revealed by CV-ANOVA. Only metabolites appearing in the upper and lower 25% of p(corr)/VIP values from the tFresh vs t7 VPLOTS were plotted.

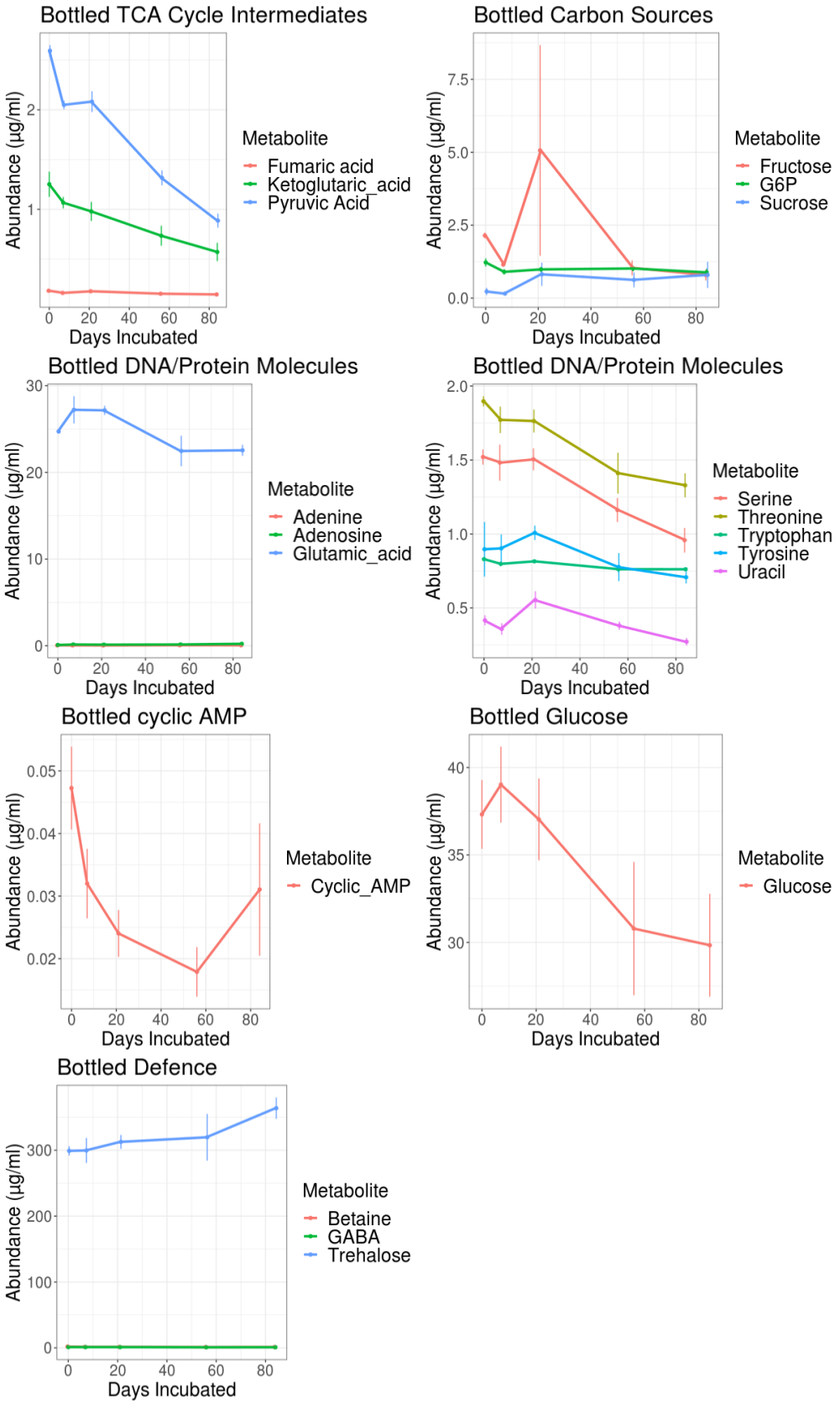

Figure 15: Changes in abundance of metabolites between 0 Days and 84 Days time point for Bottled treatment. Only metabolites appearing in the upper and lower 25% of p(corr)/VIP values from the t0 vs t84 VPLOTS were plotted. Metabolites are grouped into targeted pathways or class of molecule.

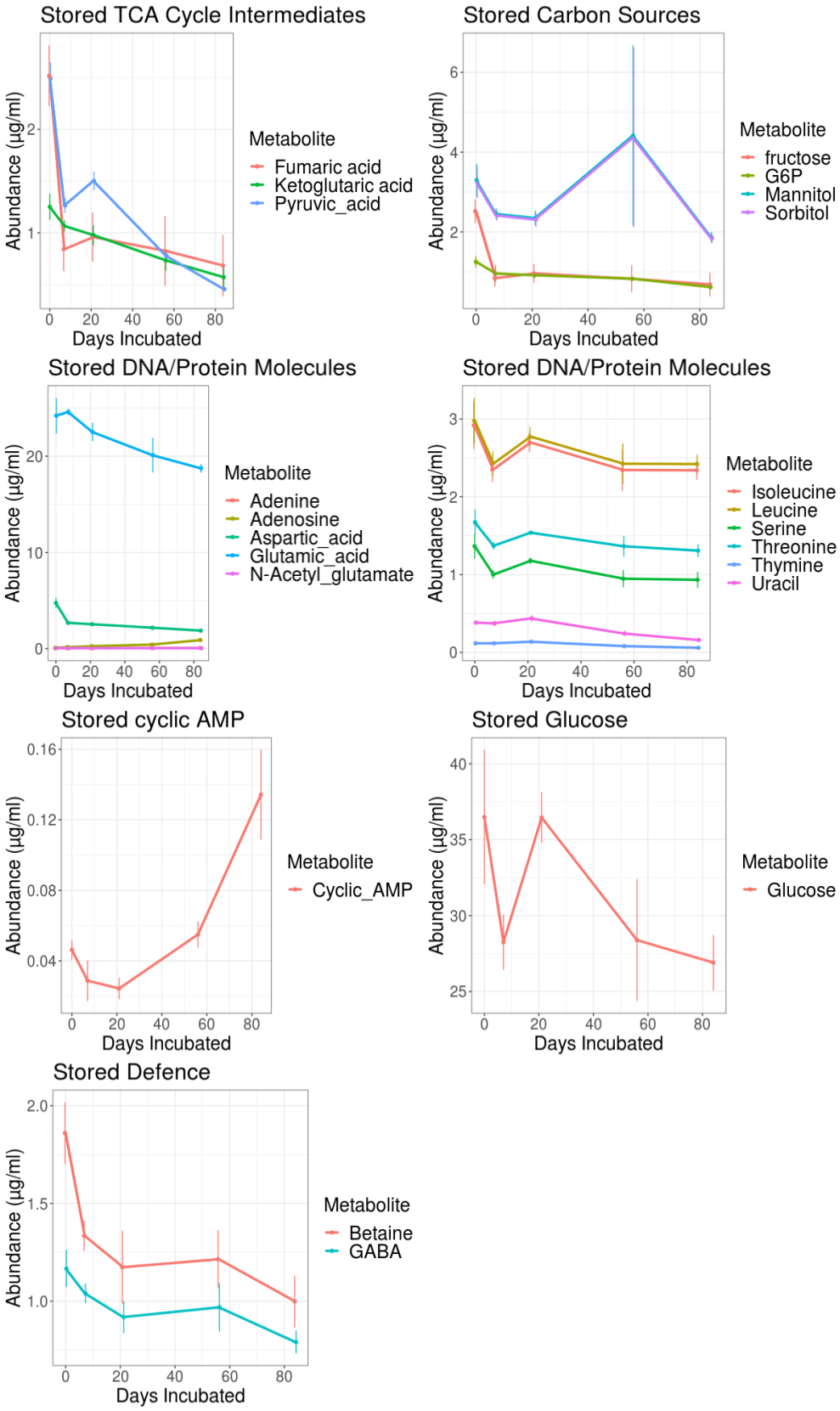

Figure 16: Changes in abundance of metabolites between 0 Days and 84 Days time point for Stored treatment. Only metabolites appearing in the upper and lower 25% of p(corr)/VIP values from the t0 vs t84 VPLOTS were plotted. Metabolites are grouped into targeted pathways or class of molecule

4.5 Discussion

4.5.1 Effect of Storage

Briefly, as the OPLS-DA failed to construct a model between both tFresh/Stored t7 and tFresh/Bottled t7 it is not possible to attach a p value and thusly a true confidence to the change, however this difference suggests that the initial equilibration following OECD307 processing alters the metabolic fingerprint of the community.

A greater number of targeted metabolites were observed within the 25% p(corr)*VIP limits in the storage treatment compared to the bottle treatment, confirming the hypothesis that storage treatment exaggerates the metabolic shift caused by OECD307 processing. Notable changes in the targeted pathways and classes (Figure 16) are discussed here.

4.5.1.1 Carbon/cAMP

The observed reduction in carbon sources mirrors the carbon reduction observed in Chapter 3. A reduction in mannitol and sorbitol in both Stored and Bottled treatments and a reduction in fructose and sucrose in stored treatment was observed (Figure 16). Microbes such as *Lactobacillus plantarum* are known to utilise mannitol and sorbitol in environments with few other sources (Yang *et al.*, 2018). It is possible that in Stored treatment the selective mannitol and sorbitol sugars are depleted and the microbes able to utilise them begin to move onto utilising fructose and sucrose as a carbon source, further reducing the total abundance of fructose and sucrose. It is also worth noting that sorbitol is an intermediate in fructose, mannose, and galactose metabolism (Kanehisa, 2019), and so this reduction could indicate that fewer carbon sources are being accessed or metabolised by the community.

4.5.1.2 TCA

Fumaric acid was reduced in abundance in both Stored and Bottled treatment (figure 16), indicating a lowered rate of TCA cycle reactions. Coupled with the depletion of carbon sources, this suggests the community may be entering a starvation state. The percentage decrease in Fumaric acid is greater in stored

treatment, consistent with the hypothesis that storage treatment further exaggerates the metabolic shift.

4.5.1.3 DNA

The reduction in concentration of DNA bases and amino acids, with a greater reduction in concentration in the storage treatment (Figure 16), could potentially be due to either a slower rate of biosynthesis or from being utilised as a nutrient source. This corroborates with the findings of (Middelboe, Borch and Kirchman, 1995) whereby dissolved amino acids constituted up to 78% of total N uptake in N-limited culture, and up to 62% in C-limited cultures, whilst contributing up to 10% of C uptake in both. The increased reduction in storage treatment is likely a consequence of prolonged incubation and therefore further nutrient uptake over time.

The increase in Adenine in the Bottled treatment and the decrease in Stored treatment is consistent with the initial spike in the adenine pool during early nutrient starvation of *Beneckea natrigens* followed by a large decrease observed by Nazly et al (1979). This release was theorised to be the utilisation of adenine as a nutrient source, likely as a nitrogen source (Nazly, Carter and Knowles, 1980).

4.5.1.4 Defence

There was no driving change in defence metabolites in the bottled treatment, however a decrease in betaine and an increase in glutamic acid was observed in the storage treatment (Figure 16). This is indicative of increased stresses on the community in the storage treatment. A sharp increase in intracellularly stored glutamic acid has previously been observed in response to osmotic stress (Botsford and Lewis, 1990). Given the considerable standard error associated with the betaine bar on Figure 11, it is not possible to assign a high confidence in this metabolic shift. However, it has been suggested by Wargo (2013) that betaine may, in a similar manner to trehalose, act as a nutrient storage molecule, its high N:C ratio making it a suitable source of nitrogen.

4.5.2 Effect of Incubation

As hypothesised, processing during OECD307 bottle tests has a significant impact on the metabolic fingerprint of the soil microbial community. The initial shifts observed between tFresh and t7 continue in a similar manner across the time-course. (Figures 17 and 18)

4.5.2.1 Carbon and cAMP

Glucose concentration reduces in both treatments, and correlates with a spike in cyclic AMP (figures 12 and 13). Cyclic AMP is a second messenger molecule which up-regulates the expression of catabolic enzymes. In turn intracellular cAMP levels are suppressed by glucose (Perlman and Pastan, 1971). The observed increase in cAMP, coupled with the correlated decrease in glucose, would indicate microbial cells are in a starvation state and are actively scavenging secondary carbon sources (Görke and Stülke, 2008). The delayed cAMP spike observed in the bottled treatment may be due to the methanol in the blank application at t7 following the equilibration period providing an additional labile carbon source, delaying the depletion of glucose reserves. This delay was likely not observed in storage treatment due to already depleted carbon stocks.

G6P, the most common intracellular form of glucose, consistently decreases in storage treatment. The spike in mannitol observed at t56 in stored treatment could be explained by its utilisation by some fungal species to quench reactive oxygen species in the environment (Meena *et al.*, 2015), consistent with this study's observation of increased stress responses. Taken together this dataset demonstrates that carbon reserves deplete over the course of an OECD307 bottle test, impacting the response of the soil microbial community.

4.5.2.2 TCA

Further decreases in TCA intermediates were observed across the time-course in both treatments. Brauer et al (2006) also noted a similar marked decrease in TCA intermediates across a starvation time-course. This contrasts with the CO_2 pulses observed by (B. Thomson *et al.*, 2010), although it is possible microbial metabolism immediately after soil processing was briefly increased in a similar manner before rapid depletion caused the reduction observed here. A key limitation whilst

interpreting this data is that the metabolites in the TCA cycle cannot be matched to an organism of origin. Whilst the data implies an overall reduction in total respiration as time goes on, it does not take into account whether the community as a whole has reduced respiration evenly, or if select taxa are still respiring at the same rate but in lower abundance.

4.5.2.3 DNA/Proteins

All observed DNA and protein related molecules consistently reduced in both treatments. Brauer *et al.* (2006) observed that amino acids, biosynthetic intermediates, and TCA intermediates all correlated in their reduction during a starvation time-course, and a similar effect is observed in this study. This can be related to protein catabolism as a result of carbon starvation, and amino acid catabolism as a result of nitrogen starvation (Kuroda, 2001).

4.5.2.4 Defence

Betaine continues to decrease in the stored treatment. Betaine's mode of action is to build up within the cell to maintain intercellular:intracellular osmotic potential. Water content increases post re-wetting, and so a gradual reduction in betaine may be due to the adaptation of microbes to the altered osmotic potential of the soil environment. Conversely, another osmoprotectant, trehalose, increased during the bottled treatment. Trehalose may be used as a carbon storage molecule in fungi in addition to possible use as an osmoregulator (Bago *et al.*, 1999). Therefore, this increase may reflect a survival strategy by the fungal community. A lack of increased trehalose in storage treatment may be linked to the further depletion of carbon sources relative to the bottled treatment, causing the fungal community to deplete its trehalose reserves as a nutrient source. The consistent reduction in GABA may be due to the downregulation of the energetically demanding enzymes involved in GABA production, which has previously been observed after the onset of starvation in (Wu and Janetopoulos, 2013).

4.5.3 Microbial Activity Through Time Course

These changes in the metabolic fingerprints would suggest an overall reduction in microbial activity. The reduction in carbon source metabolites and TCA cycle intermediates implies a drop in respiration and energy available to the community.

As *de novo* biosynthesis is often de-activated early on in starvation as a nutrient deprivation response (Brauer *et al.*, 2006), a decrease in DNA/Protein molecules was observed. Finally, the increases in cAMP and defence metabolites show that the remaining energy is put towards nutrient scavenging and adapting to environmental stress.

The impact this metabolic shift could have on the OECD307 test likely depends on the class of CPP being tested. The decrease in metabolism would cause a decrease in the degradation of CPPs with non-metabolite structures, for example CPPs with phenol groups. When accessing complex recalcitrant nutrient substrates microbes can potentially exhibit what is called the priming effect, whereby freely available labile carbon is utilised to undergo the energetically expensive break down of the substrate (Fontaine *et al.*, 2011; Chen *et al.*, 2014). In low carbon systems this effect is not observed, and therefore it is unlikely to occur in the OECD307 test system, increasing the likelihood of a test CPP persisting or having a low degradation rate unless an entirely abiotic chemical breakdown pathway is possible.

However, if a candidate CPP is a simple organic molecule it is possible for its rate to increase. As the microbial community is in a starvation state it is possible any such molecule may be scavenged and metabolised as a nutrient source, increasing the degradation rate observed within the test system. Although these trends were observed to a greater degree in the stored treatment, they were still observable within the bottled treatment. This suggests that, whilst storage may exacerbate the change in function, the pre-processing metabolism of the soil community is not preserved even without a storage step.

These observed changes in the metabolite fingerprints have allowed the characterisation of pathways and classes of metabolite relating to primary metabolism and their potential impact on CPP degradation, however these findings do not explicitly demonstrate the functions demonstrated here. Metabolomics provides an informed view of the metabolic state of a community, however many metabolites are found in multiple metabolic pathways, and as such it is difficult to

determine which of these pathways may be active at the time of sampling. In order to fully describe the changes in function with a higher degree of confidence an integrated multi-omics approach is needed. Targeted metatranscriptomics or qPCR would allow the direct observation of gene expression of enzymes involved in nutrient acquisition and biosynthesis, whilst observing the metagenome would reveal which genetic pathways are preserved after the community shift caused by processing (see Chapter 2).

4.6 Conclusions

This study confirms that the soil microbial metabolism is altered over the course of the OECD307 bottle test. Depletion of carbon source molecules mirrors the depletion in organic carbon observed in Chapter 3. This depletion is a likely driver behind the other changes in metabolism observed, including a decrease in defence molecules such as trehalose and a reduction in amino acid/DNA base concentrations, both of which may be utilised as nutrient sources. The reduction in both amino acid/DNA base and TCA intermediate concentrations suggests a lowered rate of biosynthesis and respiration, indicating an overall reduction in metabolism. Taken together with cyclic AMP increases this suggests that cells do not have sufficient labile carbon and have entered a low metabolism starvation state. Storage treatment causes a greater change in microbial function when compared to that of fresh soils, however bottled treatment also demonstrated a shift in metabolite fingerprint across the time-course.

Therefore, the degradation rate of a given CPP in this context may vary depending on its chemical nature. A simple organic molecule may be catabolised for nutrient content by the community, whilst a more complex aromatic CPP may persist due to the energetic cost of chemical transformation of such a molecule. This model of microbial metabolism could account for the variability observed in degradation rates within OECD307 tests.

5 Chapter 5: General Discussion

This study aimed to assess the changes in the soil microbial community and function caused by the soil processing and incubation during OECD307 bottle tests. Here, the findings of the study are discussed in context of the OCED307 test and the proposed model describing a mechanism by which these changes occur is fully synthesised. Finally, further work which would verify this model is proposed.

5.1 Findings

The results of Chapter 2 demonstrated that the fungal community is significantly changed by the OECD307 bottle test system. The shift in community composition was unpredictable, however only species present within the starting soils could be present in the endpoint soils. These starting communities in turn are determined by the edaphic properties and climate of their point of origin, making the location from which they are sampled and the season in which they are sampled the major factors in determining the endpoint community. In turn the soil processing steps of sieving/drying-rewetting create a stochastic shift in the starting community, which makes it difficult to predict which of the starting taxa will become dominant in the resulting assemblage. According to the FunGuild data no single functional guild consistently increased or decreased in abundance across all treatments. This suggests that the functional capability of the endpoint community is also unpredictable, although using taxonomy to predict function is an unreliable methodology. The metabolomics approach in Chapter 4 was a more direct examination of which pathways and metabolite classes are impacted, revealing a reduction in metabolic activity in respiration pathways and a shift towards adaptations to environmental stresses.

This shift in community was hypothesised to be due to a depletion of nutrients in tandem with a dispersal effect on the spatial distribution of the microbial community caused by the disruption to the soil. Results from Chapter 3 confirm

that the bacterial community also are significantly impacted by the OECD307 processing and incubation. Depletion of organic carbon was found to be a major driver of the shift in community of both bacteria and fungi, with depletion of nitrogen an accompanying driver of the fungal community. Further sampling points indicated the community shift happens during storage and remains stable during bottle incubation, likely due to a lack of further environmental insult. A persistence in the dominance of *Actinobacteria* and *Firmicutes* in the bacterial community, however as the data in Chapter 2 demonstrates this may be a consequence of the effects of sampling season, and a different time-course effect could be observed in a different season.

This depletion of nutrients was hypothesised to also impact the metabolism and function of the community. Results from Chapter 4 show an increased in cAMP and a decrease in metabolites involved in the TCA cycle, indicating that members of the community have depleted their preferential carbon source and are in a starvation state, whilst respiration is at a reduced rate. The starvation state hypothesis is further evidenced by the reduction in carbon source molecules and the reduction in amino acids and DNA bases associated with biosynthesis. A lower biosynthesis rate would lead to fewer catalytic enzymes being produced to degrade a CPP. This would suggest that, potentially independent of taxonomy, the depletion effect is reducing metabolic activity. A reduction in overall activity would also suggest that the potential degradation rates of a CPP would be decreased, although this could depend on scavenging strategy (as detailed in Chapter 4).

5.2 Community Change Model

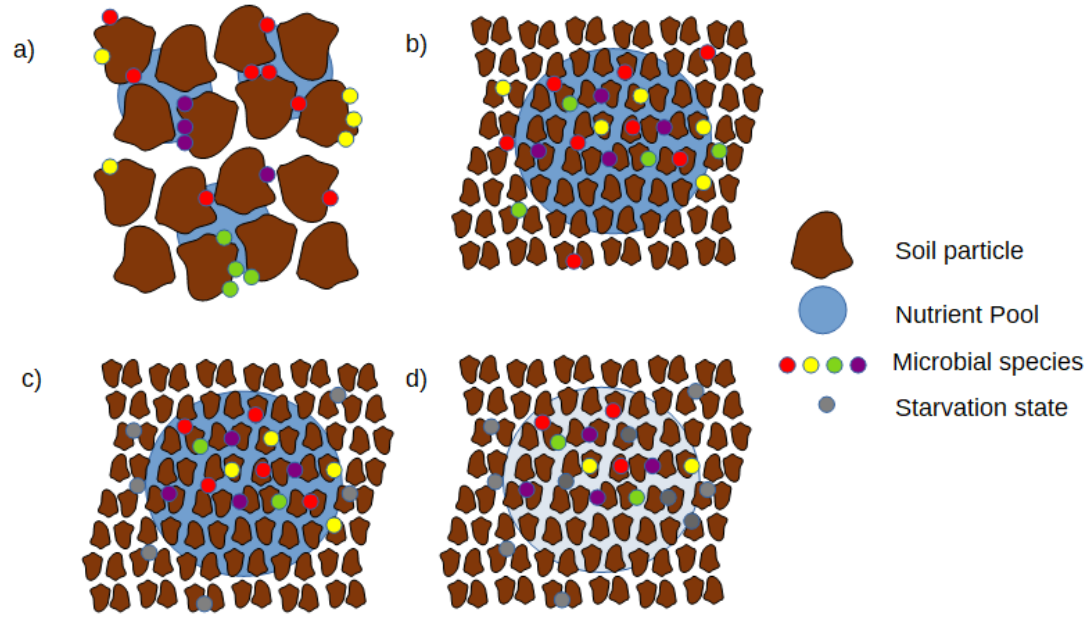

Figure 17: Cartoon illustrating community change model. A) Pre-processing, microbial communities and nutrient pools are spatially distinct. B) Post-processing the community and nutrient pools are homogenised. C) Species with immediate access to the pool remain active, whilst the others begin to enter a starvation state D) After incubation the depletion of the shared nutrient pool the remaining species begin to enter the starvation state.

The soil microbial community prior to processing is significantly influenced by the properties of the environment the soil is harvested from. Both nutrient distribution and the microbial community are spatially heterogeneous. Air drying prior to sieving may increase sporulation and increase the number of dormant cells within the soil (Pesaro et al 2004). Sieving disrupts the structure of macroaggregates and causes microbes and nutrient-rich micro-aggregates to be homogenised (Denef et al 2001). The rewetting after sieving mobilises both the microbial community and the nutrient pools, creating a less heterogeneous pool (Dinh et al 2016). The microbes and nutrients are no longer separated across microaggregate structures and the pre-existing niche structure is destroyed. The microbial community immediately accesses the nutrient pool, with community-level factors such as dispersal determining which species may access it first. Those which access the pool first gain a resource advantage over the remainder of the community and rise to dominance. This shared pool is rapidly depleted, resulting in a pulse of activity. During storage this nutrient pool, specifically organic carbon and nitrogen, begins to deplete

further, resulting in an increase in alpha diversity as the dominant species, in this model those species which accessed the pool first, no longer have a resource advantage and therefore no growth advantage. The reduction in organic carbon shifts metabolism towards nutrient scavenging, for example metabolising amino acids to obtain nitrogen and carbon.

This metabolic shift is likely independent of taxonomy and will alter the way a CPP behaves in the bottle test environment. Given that many CPPs are complex molecules with many phenol groups (such as Azoxystrobin, Paclobutrazol, Fomesafen) which have complicated degradation pathways due to the potential number and position of substitution groups, in addition to commonly conferring toxicity to the compound (Krastanov, Alexieva and Yemendzhiev, 2013). The energetically demanding and complex multi-step degradation process will likely be retarded in a carbon-limiting environment. This could be due to either the reduced ATP to supply to enzymatic reactions, or the overall reduced rate of biosynthesis to produce the enzymatic machinery required for each transformation step. If a CPP is instead composed of an aliphatic molecule it is possible the molecule will be scavenged at a greater rate by enzymes excreted by the community during starvation state.

5.3 Limitations of This Study

Before detailing the further work required to examine this model, the limitations of some aspects of this study must be addressed.

Although the results presented in this thesis illustrate the mechanisms by which the soil community alters during OECD307 bottle tests, each experiment has been limited by experimental caveats primarily due to cost and practical feasibility. Chapter 2 examined fungal communities across site and season but had a limited number of time points and no accompanying environmental metadata. Inversely, Chapter 3 examined nutrient metadata, in addition to fungal and bacterial community data, across a greater number of time points within the OECD307 test but was limited in scope to a single season. Finally, Chapter 4 examined metabolite

fingerprints using a high throughput LC-MSMS method with an even greater number of time points, but was limited to a single site and season.

The data in Chapter 2 reveals that seasonal and geographical variation significantly impacts the community composition of OECD307 tests, and so the results of the single season experiments must be considered with this variability considered. The trends in community shifts and nutrient depletion observed in Chapter 3 may occur at a different rate in different seasons. (Wuest, 2014) found a variation in organic carbon of up to 29% in soils sampled monthly from the same location over a 39 month period. Therefore the dynamics of the depletion observed in Chapter 3 may be significantly affected by temporal variations in organic carbon stocks, which in turn may alter the community shifts observed in Chapters 2 and 3, in addition to rate of the metabolic fingerprint shifts observed in Chapter 4. Initial carbon stocks, and microbial communities, are also highly dependent on land management, and so the site of origin of the test soil will also impact the depletion dynamics.

This study has used overall changes in metabolite fingerprint to infer the potential CPP degrading function, rather than directly observing CPP breakdown. No experiments in this study have applied a CPP to the soil in order to solely examine the changes to community and function caused by soil processing and subsequent incubation. However, exposure to CPPs has been demonstrated to alter the microbial community (Muturi *et al.*, 2017),and so this effect must be considered in future studies.

CPP degradation data was not collected primarily due to budgetary constraints. The findings presented within this thesis however still present a novel contribution towards the understanding of the microbiology of OECD307. Whilst addition of degradation study data would have further elucidated the direct impact of the community changes described in this thesis on the efficacy of the OECD307 bottle test, it would have also presented another layer of confounding variables. The mechanisms of CPP degradation are diverse and are in part dependent on chemical properties of the CPP (Gevao, Semple and Jones, 2000), and even a single CPP may

have multiple mechanisms of degradation. For example the CPP aztrazine has multiple degradation pathways, each of which has its own optimum pH and condition (Solomon, Kumar and Satheeja Santhi, 2013). A focus on CPP degradation data would have shifted the focus of this body of work towards how processing alters the breakdown of specific CPPs, rather than the efficacy of OECD307 as a whole.

In order to account for the diversity of CPP properties and degradation pathways an extensive array of CPPs would have had to be tested, the budget for which, in addition to the sequencing costs, was unavailable during this work. Despite this caveat, this body of work still presents a clear model by which a given CPP degradation in the OECD307 test system could be affected. This thesis has sought to understand the underlying processes which occur during community shift post OECD307 processing, and how these processes may predict degradation behaviour.

Finally, one final variable which was not accounted for in this study is the effect of soil mass on the shifts. There is the possibility that the volume of soil used in the test is simply too small to sustain natural diversity and metabolic function. Additionally macroecological factors such as dispersal can significantly alter the composition of a community across distance gradients within a given soil (Albright and Martiny, 2018). This could constitute a further area of study by itself, although the focus of this body of work was to examine the default OECD307 conditions and so the effect of varying soil mass was outwith the remit of this thesis.

5.4 Further Work/Case Study

A multi-season, multi-site bottle test experiment, such as in Chapter 2, with multiple time points across the incubation, such as in Chapters 3 and 4, using a model CPP with a well characterised breakdown pathway, such as glyphosate, would help verify the model proposed above.

By applying radiolabelled glyphosate, the breakdown rate, specifically half-life (DT50), can be determined, allowing the comparison of DT50s across site and season. Sufficient repeat samples within treatment can also elucidate any

microcosm effect, whereby samples originating from the same initial soil may experience community shifts independent of each other as observed in Chapter 2.

Microbial breakdown of glyphosate via the C-P lyase pathway has been well characterised, with the protein phnJ being the critical enzyme within the catalytic complex, and 5-phospho-α-d-ribosyl 1-diphosphate (PRPP) the key by-product molecule unique to the pathway (Hove-Jensen, Zechel and Jochimsen, 2014).

Examination of the phnJ gene utilising a targeted metagenomics and metatranscriptomics approach will reveal the effects of site, season, processing, and incubation on the presence and expression of the C-P lyase pathway. Addition of a PRPP standard to a metabolomics analysis will quantify the amount of PRPP produced by the C-P lyase pathway in order to directly examine activity in tandem with the DT50 data. This will confirm whether the hypothesised reduction in CPP activity proposed above takes place.

Nutrient metadata collected from each sample will allow the comparison of depletion effects across site and season treatments, allowing the confirmation of organic carbon depletion as the driving factor in each treatment, specifically to link it to degradation activity.

Finally, amplicon fingerprinting of the fungal and bacterial communities paired with emerging integrated network analysis methodologies (Aguiar-Pulido *et al.*, 2016) can demonstrate the relationships between the breakdown processes observed, environmental variables, the metagenome, and specific taxa, potentially linking specific genes and OTUs with the degradation activity.

5.5 Concluding Statements

The OECD307 bottle test is designed as one of the initial tests in a series of studies to test the degradation rates of CPPs, focusing on microbial breakdown in the soil in order to understand the persistence of that CPP in the field. This study has found that the processing specified in OECD guidelines significantly alters both the composition of the microbial community and the metabolic activity of that community. In practice this calls into question the accuracy and validity of OECD307 results. The variability in degradation rates observed and the difficulty in matching

it to larger-scale experiments (unpublished data, Syngenta) likely stems from this microbial shift, meaning that OECD307 data is not reflective of in-field degradation and is a poor measure of degradation rate. Further work in this area will help the understanding of how this shift impacts the degradation rates of specific CPPs.

Appendix 1: OCED 307

OECD/OCDE 307

Adopted:
24th April 2002

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Aerobic and Anaerobic Transformation in Soil

INTRODUCTION

1. This Test Guideline is based on existing guidelines (1)(2)(3)(4)(5)(6)(7)(8)(9). The method described in this Guideline is designed for evaluating aerobic and anaerobic transformation of chemicals in soil. The experiments are performed to determine (i) the rate of transformation of the test substance, and (ii) the nature and rates of formation and decline of transformation products to which plants and soil organisms may be exposed. Such studies are required for chemicals which are directly applied to soil or which are likely to reach the soil environment. The results of such laboratory studies can also be used to develop sampling and analysis protocols for related field studies.

2. Aerobic and anaerobic studies with one soil type are generally sufficient for the evaluation of transformation pathways (8)(10)(11). Rates of transformation should be determined in at least three additional soils (8)(10).

3. An OECD Workshop on soil and sediment selection, held at Belgirate, Italy in 1995 (10) agreed, in particular, on the number and types of soils for use in this test. The types of soils tested should be representative of the environmental conditions where use or release will occur. For example, chemicals that may be released in subtropical to tropical climates should be tested with Ferrasols or Nitosols (FAO system). The Workshop also made recommendations relating to collection, handling and storage of soil samples, based on the ISO Guidance (12). The use of paddy (rice) soils is also considered in this Guideline.

PRINCIPLE OF THE TEST

4. Soil samples are treated with the test substance and incubated in the dark in biometer-type flasks or in flow-through systems under controlled laboratory conditions (at constant temperature and soil moisture). After appropriate time intervals, soil samples are extracted and analysed for the parent substance and for transformation products. Volatile products are also collected for analysis using appropriate adsorption devices. Using ^{14}C-labelled material, the various mineralisation rates of the test substance can be measured by trapping evolved $^{14}CO_2$ and a mass balance, including the formation of soil bound residues, can be established.

APPLICABILITY OF THE TEST

5. The method is applicable to all chemical substances (non-labelled or radiolabelled) for which an analytical method with sufficient accuracy and sensitivity is available. It is applicable to slightly volatile, non-volatile, water-soluble or water-insoluble compounds. The test should not be applied to chemicals which are highly volatile from soil (e.g. fumigants, organic solvents) and thus cannot be kept in soil under the experimental conditions of this test.

INFORMATION ON THE TEST SUBSTANCE

6. Non-labelled or labelled test substance can be used to measure the rate of transformation. Labelled material is required for studying the pathway of transformation and for establishing a mass balance. ^{14}C-labelling is recommended but the use of other isotopes, such as ^{13}C, ^{15}N, ^{3}H, ^{32}P, may also be informative. As far as possible, the label should be positioned in the most stable part(s) of the molecule[1]. The purity of the test substance should be at least 95 %.

7. Before carrying out a test on aerobic and anaerobic transformation in soil, the following information on the test substance should be available:

(a) solubility in water [OECD Guideline 105] (13);
(b) solubility in organic solvents;
(c) vapour pressure [OECD Guideline 104] (13) and Henry's law constant;
(d) n-octanol/water partition coefficient [OECD Guidelines 107 and 117] (13);
(e) chemical stability in dark (hydrolysis) [OECD Guideline 111] (13);
(f) pK_a if a molecule is liable to protonation or deprotonation [OECD Guideline 112] (13).

8. Other useful information may include data on toxicity of the test substance to soil microorganisms [OECD Guidelines 216 and 217] (13).

9. Analytical methods (including extraction and clean-up methods) for quantification and identification of the test substance and its transformation products should be available.

REFERENCE SUBSTANCES

10. Reference substances should be used for the characterisation and/or identification of transformation products by spectroscopic and chromatographic methods.

DEFINITIONS

11. See Annex 1.

QUALITY CRITERIA

Recovery

12. Extraction and analysis of, at least, duplicate soil samples immediately after the addition of the test substance gives a first indication of the repeatability of the analytical method and of the uniformity of the application procedure for the test substance. Recoveries for later stages of the experiments are given by the respective mass balances. Recoveries should range from 90% to 110% for labelled chemicals (8) and from 70% to 110% for non-labelled chemicals (3).

[1] For example, if the test substance contains one ring, labelling on this ring is required; if the test substance contains two or more rings, separate studies may be needed to evaluate the fate of each labelled ring and to obtain suitable information on formation of transformation products.

Repeatability and sensitivity of analytical method

13. Repeatability of the analytical method (excluding the initial extraction efficiency) to quantify test substance and transformation products can be checked by duplicate analysis of the same extract of the soil, incubated long enough for formation of transformation products.

14. The limit of detection (LOD) of the analytical method for the test substance and for the transformation products should be at least 0.01 $mg \cdot kg^{-1}$ soil (as test substance) or 1% of applied dose whichever is lower. The limit of quantification (LOQ) should also be specified.

Accuracy of transformation data

15. Regression analysis of the concentrations of the test substance as a function of time gives the appropriate information on the reliability of the transformation curve and allows the calculation of the confidence limits for half-lives (in the case of pseudo first order kinetics) or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values.

DESCRIPTION OF THE TEST METHOD

Equipment and chemical reagents

16. Incubation systems consist of static closed systems or suitable flow-through systems (7)(14). Examples of suitable flow-through soil incubation apparatus and biometer-type flask are shown in Figures 1 and 2, respectively. Both types of incubation systems have advantages and limitations (7)(14).

17. Standard laboratory equipment is required and especially the following:

- Analytical instruments such as GLC, HPLC, TLC-equipment, including the appropriate detection systems for analysing radiolabelled or non-labelled substances or inverse isotopes dilution method;
- Instruments for identification purposes (e.g. MS, GC-MS, HPLC-MS, NMR, etc.);
- Liquid scintillation counter;
- Oxidiser for combustion of radioactive material;
- Centrifuge;
- Extraction apparatus (for example, centrifuge tubes for cold extraction and Soxhlet apparatus for continuous extraction under reflux);
- Instrumentation for concentrating solutions and extracts (e.g. rotating evaporator);
- Water bath;
- Mechanical mixing device (e.g. kneading machine, rotating mixer);

18. Chemical reagents used include, for example:

NaOH, analytical grade, 2 mol • dm^{-3}, or other appropriate base (e.g. KOH, ethanolamine);
H_2SO_4, analytical grade, 0.05 mol • dm^{-3};
Ethylene glycol, analytical grade;
Solid absorption materials such as soda lime and polyurethane plugs;
Organic solvents, analytical grade, such as acetone, methanol, etc.;
Scintillation liquid.

Test substance application

19. For addition to and distribution in soil, the test substance can be dissolved in water (deionised or distilled) or, when necessary, in minimum amounts of acetone or other organic solvents (6) in which the test substance is sufficiently soluble and stable. However, the amount of solvent selected should not have a significant influence on soil microbial activity (see paragraphs 8 and 44). The use of solvents which inhibit microbial activity, such as chloroform, dichloromethane and other halogenated solvents, should be avoided.

20. The test substance can also be added as a solid, e.g. mixed in quartz sand (6) or in a small sub-sample of the test soil which has been air-dried and sterilised. If the test substance is added using a solvent the solvent should be allowed to evaporate before the spiked sub-sample is added to the original non-sterile soil sample.

21. For general chemicals, whose major route of entry into soil is through sewage sludge/farming application, the test substance should be first added to sludge which is then introduced into the soil sample (see paragraph 41).

22. The use of formulated products is not routinely recommended. However, e.g. for poorly soluble test substances, the use of formulated material may be an appropriate alternative.

Soils

Soil selection

23. To determine the transformation pathway, a representative soil can be used; a sandy loam or silty loam or loam or loamy sand [according to FAO and USDA classification (15)] with a pH of 5.5-8.0, an organic carbon content of 0.5 - 2.5% and a microbial biomass of at least 1% of total organic carbon is recommended (10).

24. For transformation rate studies at least three additional soils should be used representing a range of relevant soils. Those soils should vary in their organic carbon content, pH, clay content and microbial biomass (10).

25. All soils should be characterised, at least, for texture (% sand, % silt, % clay) [according to FAO and USDA classification (15)], pH, cation exchange capacity, organic carbon, bulk density, water retention characteristic[2] and microbial biomass (for aerobic studies only). Additional information on soil properties may be useful in interpreting the results. For determination of the soil characteristics the methods recommended in references (16)(17)(18)(19)(20) can be used. Microbial biomass should be determined by using the substrate-induced respiration (SIR) method (21)(22) or alternative methods (17).

Collection, handling, and storage of soils

26. Detailed information on the history of the field site from where the test soil is collected should be available. Details include exact location, vegetation cover, treatments with chemicals, treatments with organic and inorganic fertilisers, additions of biological materials or other contamination. If soils have

[2] Water retention characteristic of a soil can be measured as field capacity, as water holding capacity or as water suction tension (pF). For explanations see Annex 2. It should be reported in the test report whether water retention characteristics and bulk density of soils were determined in undisturbed field samples or in disturbed (processed) samples.

been treated with the test substance or its structural analogues within the previous four years, these should not be used for transformation studies (10)(12).

27. The soil should be freshly collected from the field (from the A horizon or top 20 cm layer) with a soil water content which facilitates sieving. For soils other than those from paddy fields, sampling should be avoided during or immediately following long periods (> 30 days) of drought, freezing or flooding (12). Samples should be transported in a manner which minimises changes in soil water content and should be kept in the dark with free access of air, as much as possible. A loosely-tied polyethylene bag is generally adequate for this purpose.

28. The soil should be processed as soon as possible after sampling. Vegetation, larger soil fauna and stones should be removed prior to passing the soil through a 2 mm sieve which removes small stones, fauna and plant debris. Extensive drying and crushing of the soil before sieving should be avoided (12).

29. When sampling in the field is difficult in winter (soil frozen or covered by layers of snow), it may be taken from a batch of soil stored in the greenhouse under plant cover (e.g. grass or grass-clover mixtures). Studies with soils freshly collected from the field are strongly preferred, but if the collected and processed soil has to be stored prior to the start of the study storage conditions must be adequate and for a limited time only (4 ± 2°C for a maximum of three months) to maintain microbial activity[3]. Detailed instructions on collection, handling and storage of soils to be used for biotransformation experiments can be found in (8)(10)(12)(23)(24).

30. Before the processed soil is used for this test, it should be pre-incubated to allow germination and removal of seeds, and to re-establish equilibrium of microbial metabolism following the change from sampling or storage conditions to incubation conditions. A pre-incubation period between 2 and 28 days approximating the temperature and moisture conditions of the actual test is generally adequate (12). Storage and pre-incubation time together should not exceed three months.

PERFORMANCE OF THE TEST

Test conditions

Test temperature

31. During the whole test period, the soils should be incubated in the dark at a constant temperature representative of the climatic conditions where use or release will occur. A temperature of 20 ± 2 °C is recommended for all test substances which may reach the soil in temperate climates. The temperature should be monitored.

32. For chemicals applied or released in colder climates (e.g. in northern countries, during autumn/winter periods), additional soil samples should be incubated but at a lower temperature (e.g. 10 ± 2 °C).

[3] Recent research results indicate that soils from temperate zones can also be stored at –20°C for more than three months (25)(26) without significant losses of microbial activity.

Moisture content

33. For transformation tests under aerobic conditions, the soil moisture content[4] should be adjusted to and maintained at a pF of between 2.0 and 2.5 (3). The soil moisture content is expressed as mass of water per mass of dry soil and should be regularly controlled (e.g. in 2 week intervals) by weighing of the incubation flasks and water losses compensated by adding water (preferably sterile-filtered tap water). Care should be given to prevent or minimise losses of test substance and/or transformation products by volatilisation and/or photodegradation (if any) during moisture addition.

34. For transformation tests under anaerobic and paddy conditions, the soil is water-saturated by flooding.

Aerobic incubation conditions

35. In the flow-through systems, aerobic conditions will be maintained by intermittent flushing or by continuously ventilating with humidified air. In the biometer flasks, exchange of air is maintained by diffusion.

Sterile aerobic conditions

36. To obtain information on the relevance of abiotic transformation of a test substance, soil samples may be sterilised (for sterilisation methods see references 13 and 26), treated with sterile test substance (e.g. addition of solution through a sterile filter) and aerated with humidified sterile air as described in paragraph 35. For paddy soils, soil and water should be sterilised and the incubation should be carried out as described in paragraph 38.

Anaerobic incubation conditions

37. To establish and maintain anaerobic conditions, the soil treated with the test substance and incubated under aerobic conditions for 30 days or one half-life or DT_{50} (whichever is shorter) is then water-logged (1-3 cm water layer) and the incubation system flushed with an inert gas (e.g. nitrogen or argon)[5]. The test system must allow for measurements such as pH, oxygen concentration and redox potential and include trapping devices for volatile products. The biometer-type system must be closed to avoid entrance of air by diffusion.

Paddy incubation conditions

38. To study transformation in paddy rice soils, the soil is flooded with a water layer of about 1-5 cm and the test substance applied to the water phase (9). A soil depth of at least 5 cm is recommended. The

[4] The soil should neither be too wet nor too dry to maintain adequate aeration and nutrition of soil microflora. Moisture contents recommended for optimal microbial growth range from 40-60% water holding capacity (WHC) and from 0.1-0.33 bar (6). The latter range is equivalent to a pF-range of 2.0 – 2.5. Typical moisture contents of various soil types are given in Annex 3.

[5] Aerobic conditions are dominant in surface soils and even in sub-surface soils as shown in an EU sponsored research project [K. Takagi et al. (1992). Microbial diversity and activity in subsoils: Methods, field site, seasonal variation in subsoil temperatures and oxygen contents. Proc. Internat. Symp. Environ. Aspects Pesticides Microbiol., 270-277, 17-21 August 1992, Sigtuna, Sweden]. Anaerobic conditions may occur only occasionally during flooding of soils after heavy rainfalls or when paddy conditions are established in rice fields.

system is ventilated with air as under aerobic conditions. pH, oxygen concentration and redox potential of the aqueous layer should be monitored and reported. A pre-incubation period of at least two weeks is necessary before commencing transformation studies (see paragraph 30).

Test duration

39. The rate and pathway studies should normally not exceed 120 days[6] (3)(6)(8), because thereafter a decrease of the soil microbial activity with time would be expected in an artificial laboratory system isolated from natural replenishment. Where necessary to characterise the decline of the test substance and the formation and decline of major transformation products, studies can be continued for longer periods (e.g. 6 or 12 months) (8). Longer incubation periods should be justified in the test report and accompanied by biomass measurements during and at the end of these periods.

Treatment and application of test substance

40. About 50 to 200 g of soil (dry weight basis) are placed into each incubation flask (see Figures 1 and 2 in Annex 4) and the soil treated with the test substance by one of the methods described in paragraphs 19-22. When organic solvents are used for the application of the test substance, they should be removed from soil by evaporation. Then the soil is thoroughly mixed with a spatula and/or by shaking of the flask. If the study is conducted under paddy field conditions, soil and water should be thoroughly mixed after application of the test substance. Small aliquots (e.g. 1 g) of the treated soils should be analysed for the test substance to check for uniform distribution. For alternative method, see paragraph 42.

41. The treatment rate should correspond to the highest application rate of a crop protection product recommended in the use instructions and uniform incorporation to an appropriate depth in the field (e.g. top 10 cm layer[7] of soil). For example, for chemicals foliarly or soil applied without incorporation, the appropriate depth for computing how much chemical should be added to each flask is 2.5 cm. For soil incorporated chemicals, the appropriate depth is the incorporation depth specified in the use instructions**.** For general chemicals, the application rate should be estimated based on the most relevant route of entry; for example, when the major route of entry in soil is through sewage sludge, the chemical should be dosed into the sludge at a concentration that reflects the expected sludge concentration and the amount of sludge added to the soil should reflect normal sludge loading to agricultural soils. If this concentration is not high enough to identify major transformation products, incubation of separate soil samples containing higher rates may be helpful, but excessive rates influencing soil microbial functions should be avoided (see paragraphs 8 and 19).

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[6] Aerobic studies might be terminated much before 120 days provided that ultimate transformation pathway and ultimate mineralisation are clearly reached at that time. Termination of the test is possible after 120 days, or when at least 90% of the test substance is transformed, but only if at least 5% CO_2 is formed.

[7] Calculation of the initial concentration on an area basis using the following equation:

$$C_{soil}[mg/kg_{soil}] = \frac{A[kg/ha] \bullet 10^6[mg/kg]}{l[m] \bullet 10^4[m^2/ha] \bullet d[kg_{soil}/m^3]}$$

C_{soil} = Initial concentration in soil [mg • kg^{-1}]

A = Application rate [kg • ha^{-1}]; 1 = thickness of field soil layer [m]; d = dry bulk density of soil [kg • m^{-3}].

As a rule of thumb, an application rate of 1 kg • ha^{-1} results in a soil concentration of approximately 1 mg • kg^{-1} in a 10 cm layer (assuming a bulk density of 1 g • cm^{-3}).

42. Alternatively, a larger batch (i.e. 1 to 2 kg) of soil can be treated with the test substance, carefully mixed in an appropriate mixing machine and then transferred in small portions of 50 to 200 g into the incubation flasks (for example with the use of sample splitters). Small aliquots (e.g. 1 g) of the treated soil batch should be analysed for the test substance to check for uniform distribution. Such a procedure is preferred since it allows for more uniform distribution of the test substance into the soil.

43. Also untreated soil samples are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements during and at the end of the studies.

44. When the test substance is applied to the soil dissolved in organic solvent(s), soil samples treated with the same amount of solvent(s) are incubated under the same conditions (aerobic) as the samples treated with the test substance. These samples are used for biomass measurements initially, during and at the end of the studies to check for effects of the solvent(s) on microbial biomass.

45. The flasks containing the treated soil are either attached to the flow-through system described in Figure 1 or closed with the absorption column shown in Figure 2 (see Annex 4).

Sampling and measurements

46. Duplicate incubation flasks are removed at appropriate time intervals and the soil samples extracted with appropriate solvents of different polarity and analysed for the test substance and/or transformation products. A well-designed study includes sufficient flasks so that two flasks are sacrificed at each sampling event. Also, absorption solutions or solid absorption materials are removed at various time intervals (7-day intervals during the first month and after one month in 14-day intervals) during and at the end of incubation of each soil sample and analysed for volatile products. Besides a soil sample taken directly after application (0-day sample) at least 5 additional sampling points should be included. Time intervals should be chosen in such a way that pattern of decline of the test substance and patterns of formation and decline of transformation products can be established (e.g. 0, 1, 3, 7 days; 2, 3 weeks; 1, 2, 3 months, etc.).

47. When using ^{14}C-labelled test substance, non-extractable radioactivity will be quantified by combustion and a mass balance will be calculated for each sampling interval.

48. In the case of anaerobic and paddy incubation, the soil and water phases are analysed together for test substance and transformation products or separated by filtration or centrifugation before extraction and analysis.

Optional tests

49. Aerobic, non-sterile studies at additional temperatures and soil moistures may be useful for the estimation of the influence of temperature and soil moisture on the rates of transformation of a test substance and/or its transformation products in soil.

50. A further characterisation of non-extractable radioactivity can be attempted using, for example, supercritical fluid extraction.

DATA AND REPORTING

Treatment of results

51. The amounts of test substance, transformation products, volatile substances (in % only), and non-extractable should be given as % of applied initial amount and**,** where appropriate, as $mg \cdot kg^{-1}$ soil (based on soil dry weight) for each sampling interval. A mass balance should be given in percentage of the applied initial amount for each sampling interval. A graphical presentation of the test substance concentrations against time will allow an estimation of its transformation half-life or $DT_{50.}$ Major transformation products should be identified and their concentrations should also be plotted against time to show their rates of formation and decline. A major transformation product is any product representing ≥ 10% of applied dose at any time during the study.

52. The volatile products trapped give some indication of the volatility potential of a test substance and its transformation products from soil.

53. More accurate determinations of half-lives or DT_{50} values and, if appropriate, DT_{75} and DT_{90} values should be obtained by applying appropriate kinetic model calculations. The half-life and DT_{50} values should be reported together with the description of the model used, the order of kinetics and the determination coefficient (r^2). First order kinetics is favoured unless $r^2 < 0.7$. If appropriate, the calculations should also be applied to the major transformation products. Examples of appropriate models are described in references 28 to 32.

54. In the case of rate studies carried out at various temperatures, the transformation rates should be described as a function of temperature within the experimental temperature range using the Arrhenius relationship of the form:

$$k = A \bullet e^{-B/T} \quad \text{or} \quad \ln k = \ln A - \frac{B}{T},$$

where ln A and B are regression constants from the intercept and slope, respectively, of a best fit line generated from linearly regressing ln k against 1/T, k is the rate constant at temperature T and T is the temperature in Kelvin. Care should be given to the limited temperature range in which the Arrehenius relationship will be valid in case transformation is governed by microbial action.

Test report

55. The report must include:

Test substance:

- common name, chemical name, CAS number, structural formula (indicating position of label(s) when radiolabelled material is used) and relevant physical-chemical properties (see paragraph 7);
- purity (impurities) of test substance;
- radiochemical purity of labelled chemical and specific activity (where appropriate);

Reference substances:

- chemical name and structure of reference substances used for the characterisation and/or identification of transformation products;

Test soils:

- details of collection site;
- date and procedure of soil sampling;
- properties of soils, such as pH, organic carbon content, texture (% sand, % silt, % clay), cation exchange capacity, bulk density, water retention characteristic, and microbial biomass;
- length of soil storage and storage conditions (if stored);

Test conditions:

- dates of the performance of the studies;
- amount of test substance applied;
- solvents used and method of application for the test substance;
- weight of soil treated initially and sampled at each interval for analysis;
- description of the incubation system used;
- air flow rates (for flow-through systems only);
- temperature of experimental set-up;
- soil moisture content during incubation;
- microbial biomass initially, during and at the end of the aerobic studies;
- pH, oxygen concentration and redox potential initially, during and at the end of the anaerobic and paddy studies;
- method(s) of extraction;
- methods for quantification and identification of the test substance and transformation products in soil and absorption materials;
- number of replicates and number of controls.

Results:

- result of microbial activity determination;
- repeatability and sensitivity of the analytical methods used;
- rates of recovery (% values for a valid study are given in paragraph 12);
- tables of results expressed as % of applied initial dose and, where appropriate, as $mg \cdot kg^{-1}$ soil (on a dry weight basis);
- mass balance during and at the end of the studies;
- characterisation of non-extractable (bound) radioactivity or residues in soil;
- quantification of released CO_2 and other volatile compounds;
- plots of soil concentrations versus time for the test substance and, where appropriate, for major transformation products;
- half-life or DT_{50} , DT_{75} and DT_{90} for the test substance and, where appropriate, for major transformation products including confidence limits;
- estimation of abiotic degradation rate under sterile conditions;
- an assessment of transformation kinetics for the test substance and, where appropriate, for major transformation products;
- proposed pathways of transformation, where appropriate;
- discussion and interpretation of results;

- raw data (i.e. sample chromatograms, sample calculations of transformation rates and means used to identify transformation products).

Interpretation and evaluation of results

56. Although the studies are carried out in an artificial laboratory system, the results will allow estimation of the rate of transformation of the test substance and also of rate of formation and decline of transformation products under field conditions (33)(34).

57. A study of the transformation pathway of a test substance provides information on the way in which the applied substance is structurally changed in the soil by chemical and microbial reactions.

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ANNEX 1

DEFINITIONS

Test substance: any substance, whether the parent compound or relevant transformation products.

Transformation products: all substances resulting from biotic or abiotic transformation reactions of the test substance including CO_2 and products that are in bound residues.

Bound residues: "Bound residues" represent compounds in soil, plant or animal, which persist in the matrix in the form of the parent substance or its metabolite(s)/transformation products after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix. The nature of the bond can be clarified in part by matrix-altering extraction methods and sophisticated analytical techniques. To date, for example, covalent ionic and sorptive bonds, as well as entrapments, have been identified in this way. In general, the formation of bound residues reduces the bioaccessibility and the bioavailability significantly (1) [modified from IUPAC 1984 (2)].

Aerobic transformation: reactions occurring in the presence of molecular oxygen (3).

Anaerobic transformation: reactions occurring under exclusion of molecular oxygen(3).

Soil is a mixture of mineral and organic chemical constituents, the latter containing compounds of high carbon and nitrogen content and of high molecular weights, animated by small (mostly micro-) organisms. Soil may be handled in two states:

(a) undisturbed, as it has developed with time, in characteristic layers of a variety of soil types;
(b) disturbed, as it is usually found in arable fields or as occurs when samples are taken by digging and used in this guideline (3).

Mineralisation is the complete degradation of an organic compound to CO_2 and H_2O under aerobic conditions, and CH_4, CO_2 and H_2O under anaerobic conditions. In the context of this guideline, when ^{14}C-labelled compound is used, mineralisation means extensive degradation during which a labelled carbon atom is oxidised with release of the appropriate amount of $^{14}CO_2$ (3).

Half-life, $t_{0.5,}$ is the time taken for 50% transformation of a test substance when the transformation can be described by first-order kinetics; it is independent of the concentration.

DT_{50} (Disappearance Time 50) is the time within which the concentration of the test substance is reduced by 50%; it is different from the half-life $t_{0.5}$ when transformation does not follow first order kinetics.

DT_{75} (Disappearance Time 75) is the time within which the concentration of the test substance is reduced by 75%.

DT_{90} (Disappearance Time 90) is the time within which the concentration of the test substance is reduced by 90%.

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ANNEX 2

WATER TENSION, FIELD CAPACITY (FC) AND WATER HOLDING CAPACITY (WHC)(1)

Height of Water Column [cm]	pF[(a)]	bar[(b)]	Remarks
10^7	7	10^4	Dry Soil
$1.6 \cdot 10^4$	4.2	16	Wilting point
10^4	4	10	
10^3	3	1	
$6 \cdot 10^2$	2.8	0.6	
$3.3 \cdot 10^2$	2.5	0.33[(c)]	
10^2	2	0.1	Range of
60	1.8	0.06	Field capacity[(d)]
33	1.5	0.033	
10	1	0.01	WHC (approximation)
1	0	0.001	Water saturated soil

(a) pF = log of cm water column.
(b) 1 bar = 10^5 Pa.
(c) Corresponds to an approximate water content of 10% in sand, 35% in loam and 45% in clay.
(d) Field capacity is not constant but varies with soil type between pF 1.5 and 2.5.

Water tension is measured in cm water column or in bar . Due to the large range of suction tension it is expressed simply as pF value which is equivalent to the logarithm of cm water column.

Field capacity is defined as the amount of water which can be stored against gravity by a natural soil 2 days after a longer raining period or after sufficient irrigation. It is determined in undisturbed soil in situ in the field. The measurement is thus not applicable to disturbed laboratory soil samples. FC values determined in disturbed soils may show great systematic variances.

Water holding capacity (WHC) is determined in the laboratory with undisturbed and disturbed soil by saturating a soil column with water by capillary transport. It is particularly useful for disturbed soils and can be up to 30 % greater than field capacity (1). It is also experimentally easier to determine than reliable FC-values.

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ANNEX 3

SOIL MOISTURE CONTENTS (g water per 100 g dry soil) OF VARIOUS SOIL TYPES FROM VARIOUS COUNTRIES

Soil Type	Country	Soil Moisture Content at		
		WHC[1]	pF = 1.8	pF = 2.5
Sand	Germany	28.7	8.8	3.9
Loamy sand	Germany	50.4	17.9	12.1
Loamy sand	Switzerland	44.0	35.3	9.2
Silt loam	Switzerland	72.8	56.6	28.4
Clay loam	Brazil	69.7	38.4	27.3
Clay loam	Japan	74.4	57.8	31.4
Sandy loam	Japan	82.4	59.2	36.0
Silt loam	USA	47.2	33.2	18.8
Sandy loam	USA	40.4	25.2	13.3

[1] Water Holding Capacity

ANNEX 4

Figure 1

Example of a flow-through apparatus to study transformation of chemicals in soil (1)(2)

1: needle valve

2: gas washing bottle containing water

3: ultramembrane (sterile conditions only), pore size 0.2 µm

4: soil metabolism flask (water-logged only for anaerobic and paddy conditions;)

5: ethylene glycol trap for organic volatile compounds

6: sulphuric acid trap for alkaline volatile compounds

7, 8: sodium hydroxide trap for CO_2 & other acidic volatiles

9: flow meter.

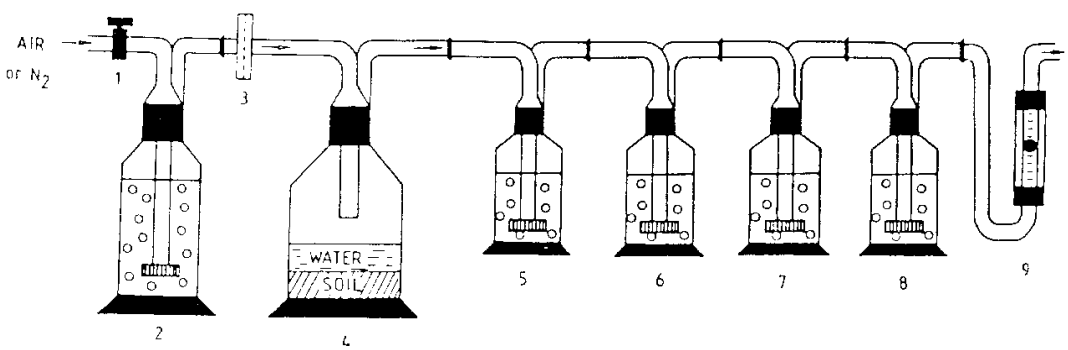
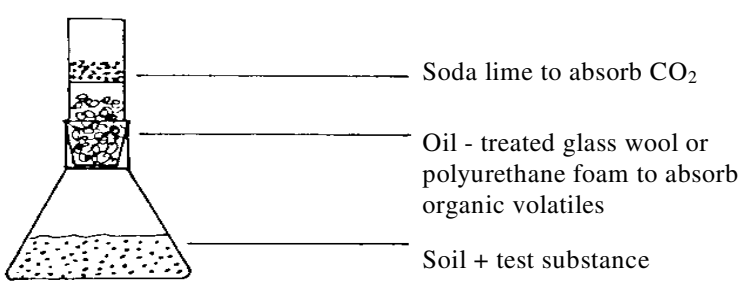


Figure 2

Example of a biometer-type flask for studying the transformation of chemicals in soil (3)



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