The decomposition of organic matter in soils by fungi

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**Total word count: 36,474**
Abstract

Macromolecular structures, such as lignin and cellulose, are important components of soil organic carbon, the major terrestrial global carbon pool. The degradation of these macromolecules, including lignin and cellulose, in plant-derived soil organic matter, is important to the global carbon cycle. In grasslands, saprotrophic (decomposer) fungi are major decomposers of such organic material.

Some of these compounds, such as lignin, are relatively resistant to decay by the microbial community if compared with other compound classes such as cellulose.

In this work we investigate the involvement of fungi in the decomposition of both lignin and cellulose and look to link the decomposition processes observed in the field to those observed in a laboratory-controlled environment.

The key findings of this work are:

- Field based experiments in both tropical and temperate environments indicated that lignin can be degraded completely, most likely by white-rot fungi, as shown by the shifts in the $[Ac/Al]_S$, $[Ac/Al]_G$ and $[S/G]$ relative lignin decomposition state proxies. The results confirm that even in a very low carbon environment, fungi are able to completely degrade lignin over time. However, lignin is degraded much faster in tropical environments.

- Culturing experiments showed that it was possible to isolate a number of fungi present on the degraded wheat straw collected in the field, especially soft-rot fungi. When used in microcosm experiments using a range of organic substrates, the relative lignin decomposition state proxies indicated that *Absidia cylindrospora* and *Trichoderma koningii* are not able to completely degrade lignin but preferentially degrade cellulose.

- Cellulose degradation rates are much higher than those of lignin in degraded field samples over time, confirming previous work.
Declaration

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The Author

The author graduated from The University of Manchester in 2008 with an upper second (2.1), Bachelor of Science in Environmental Science. Since 2008 the author has been engaged in the research reported in this thesis.
1.1. The importance of soil carbon in the carbon cycle

The carbon (C) cycle is defined as the steps in which carbon in the gas form of carbon dioxide (CO$_2$) is removed from the atmosphere by photosynthetic organisms stored in a range of different forms and ultimately returned to the atmosphere by respiration (Schimel, 1994). It comprises two major compartments i.e. the biosphere and geosphere.

The biosphere is the global “ecological” system integrating all living beings and their relationships and deals with all the organic carbon being produced. It begins with the fixation of CO$_2$ (production of carbohydrates) during photosynthesis by plants followed by the uptake by animals and ends with the recycling of carbon during decomposition of animal/plant material via respiration of CO$_2$ by fungi, algae and/or microbial decay (Brussaard & Juma, 1995; Field et al., 1998).

![Figure 1.1. A cartoon of the Global Carbon Cycle. Ten carbon pools in which carbon is stored (black writing with names and sizes of pools in gigatonnes of Carbon (Gt C, 1 Gt = 1x10$^9$ tonnes). Note: The process by which carbon is exchanged between these pools (fluxes in Gt C yr$^{-1}$) is indicated using purple arrows. One Gt C is equivalent to one Pg C (Petagram of carbon) [Illustration courtesy NASA Earth Science Enterprise]
Carbon cycling in the biosphere is relatively fast (days to years), as the amount of carbon taken up by photosynthesis and released back to the atmosphere by respiration each year is about 1,000 times greater than the amount of carbon that moves through the geosphere on an annual basis and therefore this pool of the C cycle is also known as the short carbon cycle (Schimel, 1994). A small percentage (in the past, approximately 0.1-1%) of organic matter (OM) in the biosphere escapes from remineralisation and enters the geosphere where it is stored for a much longer period of time (millions of years; Tegelaar et al., 1989). Therefore the geosphere plays a major role by acting as a long-term sink of CO$_2$ which ultimately leads to the formation of fossil fuels coal, gas and petroleum that can be converted to atmospheric C naturally, through oil seeps, or anthropogenically by human activities such as the burning of fossil fuels (Hedges and Keil, 1995). Within the biosphere, soils are one of the largest global C pools holding almost three times more C (1,580 Gt C) than that held in land plant biomass and twice as much as being present as CO$_2$ in the atmosphere (Fig. 1.1; Hall et al., 2003). The largest amount of this soil carbon can be found in the Arctic permafrost areas, containing approximately half of the global soil C (Tarnocai et al., 2009). Analysis of soil litter indicates that soil C is dominated by compound classes such as phenols, waxes, hemicellulose, sugars, lignin and cellulose that are all of plant origin. Of these, lignin accounts for approximately 14% and cellulose 75-80% in plant material such as straw (Table 1.1.; Harper and Lynch, 1981).

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<tr>
<th>Component</th>
<th>Leaf</th>
<th>Internode</th>
<th>Leaf base</th>
<th>Node core</th>
<th>Least significant difference ($P=0.05$)</th>
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<tr>
<td>Hot-water-solubles</td>
<td>14.6</td>
<td>7.2</td>
<td>18.9</td>
<td>13.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Lignin</td>
<td>15.3</td>
<td>14.2</td>
<td>14.1</td>
<td>16.7</td>
<td>1.2</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>32.4</td>
<td>33.8</td>
<td>34.2</td>
<td>32.7</td>
<td>NS</td>
</tr>
<tr>
<td>Cellulose</td>
<td>37.7</td>
<td>44.8</td>
<td>32.7</td>
<td>37.5</td>
<td>3.6</td>
</tr>
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</table>

Table 1.1. The chemical components of different parts of straw determined by gravimetric analysis. NS= not significant. The components were determined by sequential extraction which was followed by weighing. Our study is particularly interested in the degradation of lignin and cellulose by fungi. Note that the highest
percentages of these two macromolecules are found in the internode and node core (adaptation of Table 1 from Harper & Lynch 1981).

Although this contribution in plant material is relatively low, it remains important as it slows down leaf degradation and therefore lignin is assigned to the ‘slow pool’ of soil OM with mean residence times of 15 - 100 years (Paustian et al., 1992) and traditionally perceived as the rate-limiting step in biological C cycling (Martens, et al., 2002). The turnover time, i.e. in plant litter, is relatively slow for lignin which degrades at a rate of approximately 50% annually, whereas a significantly faster rate is observed for other compound classes (Fig. 1.2). For instance cellulose is degraded at a rate of 75% annually (Fig. 1.2; Minderman et al., 1968).

![Figure 1.2](image-url)

Figure 1.2. Figure showing the decomposition of various constituents (organic compounds) found in litter with their decomposition represented by a logarithmic function. The x- and y-axis represent the time in years and the % remaining of each constituent respectively. The number in front of the name of constituent indicates the percentage loss after 1 yr, the number after represents its percentage by weight of original litter (Minderman et al., 1968).
The resistance of lignin to many forms of microbial decay results in its selective enrichment, over that of the cellulosic and hemicellulosic components of wood, in albeit partially degraded forms in terrestrial ecosystems and sediments, for example soils and peats (Hatcher et al., 1982; Hedges et al., 1985; de Leeuw & Largeau, 1994). It is the most dominant constituent in woodland litter followed by the structural polysaccharides cellulose, hemicellulose and sugars respectively.

Given the current change in climate because of human enhanced global warming, a better understanding of the impacts of soil OM dynamics and the decomposition of these soil constituents is required. Particularly more information is required about the degradation of lignin and cellulose to understand climate enhanced changes in grasslands, especially tropical, environments (Connin et al., 1997; Hibbard et al., 2001; Archer et al., 2001; McCulley et al., 2004).

1.2. Characteristics of lignin and cellulose decomposition

Lignin is a polymeric material composed of phenylpropanoid units derived from three cinamyl alcohols (monolignols): p-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1.3). Sederoff et al. (1999) showed that common forms of intermonomeric links (arylglycerol-arylether bonds, carbon-carbon bonds and more complex coumaryl bondings) can be incorporated into lignin thus making its composition more complex than previously thought. It has an undefined (irregular) structure and can only be described by a potential structure such as that given by Alder, 1966, however (Fig. 1.3 a).

Lignin is an integral part of the secondary cell walls of plants, providing the plants with strength to stand upright and facilitate water transport (Swift et al., 1979). In addition lignin impedes the degradation of wall polysaccharides (cellulose and hemicellulose). Therefore, lignin acts as a major line of defence against pathogens, insects and other herbivores (Adler, 1966; Swift et al., 1979).

It is highly resistant to strong acid/base hydrolysis, supporting its status as a highly recalcitrant structure with resistance to depolymerisation (Dungait et al., 2008). Its large, heterogeneous, complex macromolecular structure makes lignin extremely difficult to breakdown and analyse directly. To allow for the analyses of these structures, they must be broken down into characteristic moieties that are much smaller,
gas chromatography-amenable and can therefore be analysed as in gas chromatography mass-spectrometry (GC-MS; Dungait et al., 2008).

Figure 1.3(A). Potential structure of lignin showing the common forms of intermonomeric links (arylglycerol-arylether bonds) such as those between (1) and (2), carbon-carbon bonds such as those between (8) and (9) and the more complex coumaryl bondings, such as those between (13) and (14) which make this heterogeneous branching molecule as complex as it is (Swift et al., 1979; Adler, 1966). 1.3(B). The three commonest monomeric forms of the phenylpropane form I: \( p \)-coumaryl alcohol, II: coniferyl alcohol and III: sinapyl alcohol (Adler, 1966).
The most commonly used methods to do this include pyrolysis gas chromatography mass-spectrometry (Py-GC-MS) and (CuO) copper oxidation. CuO oxidation involves the reaction of macromolecular structures with CuO in a closed environment allowing the determination of lignin chemistry (Nierop et al., 2005). It has been widely used, however, it is unable to determine the chemistry of tannins and demethylated lignin and has been shown to be primarily used in the analysis of lignin in organic poor soils and materials (Nierop et al., 2005; Nierop & Filley, 2008).

Gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis, also known as thermally assisted hydrolysis and methylation (THM) with tetramethylammonium hydroxide (TMAH), is a method better suited to analyse organic rich soils (i.e. grasslands) and materials (i.e. wheat straw) with mixed tannin/lignin chemistry (Filley et al, 1998; Filley et al, 2000; Nierop & Filley, 2008). It involves the breaking down of large macromolecular structures such as lignin into smaller volatile fragments/moieties by the use of heat in an inert atmosphere (usually helium or nitrogen).

![Figure 1.4. Photograph of a gas chromatograph mass-spectrometer (GC-MS) linked to the pyrolysis unit.](image)
These fragments are transferred (flushed using helium gas) onto the GC column, as they are now GC-amenable, i.e. these fragments are small enough to be moved through to the GC, and can be separated and identified/quantified using the MS (Fig.1.4). The moities analysed can be used to reconstruct the original lignin structure (Hoffmann & Stroobant, 2007). However, normal pyrolysis is often too destructive and valuable structural information such as the presence of carboxylic acids and alcohol functionalities can be lost (Fabbri et al., 1996). In gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis the functional groups react with TMAH as soon as they are formed/ liberated during the pyrolysis to be converted to their corresponding methylester and methoxyl groups which can be detected using GC-MS (Hoffmann & Stroobant, 2007; Fabbri et al., 1996). This is of interest because lignin contains a large number of functional groups that are converted to aldehyde groups upon fungal degradation. In order to determine whether these functional groups are fresh or degraded, the detection of these functional groups is essential and can be done using lignin proxies such as the acid/aldehyde and syringyl/guaiacyl ratios (Filley et al., 2000).

One of the setbacks of using TMAH enhanced THM is that no distinction can be made between alcohol and methoxyl groups present in the macromolecular structures. Hydrolysable tannins (i.e. 1,2,3,6-tertagalloylgucose; Fig 1.5) have a completely different structure compared with lignin but considering that they have the same basic aromatic structures and the fact that all alcohol groups formed will be converted into methoxyl groups means, that ultimately the methylated aromatic products formed will be the same as those originating from lignin (Fig. 1.5; Filley et al., 2000; Mason et al., 2009). To be able to distinguish between the presence of the original lignin, altered/degraded lignin and hydrolysable tannin TMAH, enhanced THM using $^{13}\text{C}$ labelled TMAH is developed (Filley et al., 2006; Mason et al., 2009). The relative amount of $^{13}\text{C}$ incorporated can be detected using GC-MS and is directly related to the amount of free moieties present and can thus be used to distinguish between lignin, altered lignin and non-lignin phenol input (e.g. tannins) in wood.

Cellulose is found in plants as microfibrils (2-20 nm diameter and 100-4000 nm long), forming a significant proportion of cell walls (cellulose fibrils constitute 40-60% of cell walls) making it the most abundant macromolecular compound on Earth (Eriksson, 2000).
1981; Paul and Clark, 1996). It is a chemical compound (a β-D-glucan) built from glucose molecules that are covalently bonded together in long chains (Fig. 1.6).

Figure 1.5. Illustration of how multiple sources lead to the same methylated aromatic structure upon TMAH thermochemolysis. Specifically methyl-3,4,5-trimethoxy benzoate is shown derived from three potential sources (unaltered native lignin, hydrolyzable tannins, highly altered lignin; Filley et al., 2006).
This chain is unbranched and contains for 3,000 to 5,000 glucose units. Each alternate at
the glucose subunit of the cellulose molecule is flipped over and the water molecule has
been removed, leaving an oxygen molecule between each ring (Eriksson, 1981). Comparable
with lignin, one of its functions is to strengthen the cell walls but also to
allow water and solutes in and out of the cell allowing the cell to hold water (Swift et
al., 1979). Some fibrous cellulose products can hold a considerable amount of water in
their pores even though water is unable to penetrate through crystalline cellulose
(Eriksson, 1981). Cellulose (38–50%), hemicellulose (17–32%), and lignin (15–30%)
all form a matrix known as lignocellulose. This matrix constitutes of cross-linked
polysaccharide networks, glycosylated proteins, and lignin (Adler, 1966; Swift et al.,
1979). Comparable with lignin, cellulose can also be analysed by gas chromatography-
mass spectrometry (GC-MS) with TMAH thermochemolysis yielding distinctive
products such as levoglucosan (Gauthier, 2003).

1.3. The role of fungi in lignocellulose decomposition
When plant material decays, decomposition is usually initiated by generalist primary
colonisers involving a diverse community of fungi, bacteria and other microorganisms
which utilise simple sugars, oligosaccharides and other low molecular weight
compounds (Frankland, 1992). After this initial flush of microbial activity, specialist
secondary colonisers, notably the basidiomycetes, which are less competitive than the micro-fungi in exploiting labile resources (Frankland, 1992), effect the decomposition of more recalcitrant plant polymers such as lignocellulose complexes. However, the presence and roles of decomposer fungi, in particular basidiomycetes, in nutrient cycling, plant succession, soil stabilization and other ecosystem processes are often under-recognized (Hassouna et al., 1984; de Boer et al., 2005). Fungi belonging to the basidiomycete group of fungi are mainly responsible for white rot and brown rot decay (Eriksson, 1981; Schwarze, 2007). Brown rot fungi are predominantly associated with gymnosperms and primarily decompose cellulose and the hemicelluloses in wood (Carlile et al., 2001; Eriksson, 1981). However decomposition of lignin is limited and they are therefore known as lignin modifiers (Carlile et al., 2001). Little is known about the capacity of brown rot fungi to completely decompose lignin, apart from the reported presence of ligninolytic enzymes in brown rot fungi (Goñi et al., 1993; Schwarze & Baum 2000). These fungi are capable of rapidly degrading the middle layer of cell walls but not the lignin-rich middle lamellae and the inner secondary cell wall layers (Schwarze et al., 2004). Cellulose and hemicellulose are decomposed at different stages, with hydrogen peroxide formed in a pre-cellulolytic phase (easily penetrating into the cell wall) overcoming the lignocellulose matrix by oxidative depolymerisation. Therefore, diffusion of large molecules of the cellulose-degrading enzymes into the cell wall tends to be delayed when the lignin content is high (Schwarze, 2007). White rot fungi, like soft and brown rots, have the ability to degrade lignin and other structural polysaccharides in woods completely. However, the rates in which these fungi decompose lignin and cellulose vary depending on the conditions within the wood and the species of fungi involved (Schwarze, 2007). White rot fungi, such as basidiomycetes, also have the ability to produce extracellular enzymes which oxidize phenolic compounds related to lignin such as fungal laccases which oxidize aldehydes, and acids or alcohols by catalysing the break down of the Caryl-Calkyl bond (Eriksson, 1981). The order in which these organisms attack different litter and wood components varies. However, they normally degrade cellulose, hemicellulose and lignin at the same time (simultaneous rottung; Cadisch & Giller, 1997; Schwarze, 2007). Approximately 8,500 described species of basidiomycetes are lignocellulose-degrading saprotrophs (Hibbett & Thorn, 2001) with about half of these occurring in soil and on fallen plant litter. At least 4,500 species of soil-inhabiting basidiomycetes form mutual associations
known as ectomycorrhizas with roots of vascular plants (Hibbett & Thorn, 2001), therefore approximately 4,000 described species of basidiomycetes are terrestrial. Basidiomycete fungi, either as saprotrophs or the fungal component of ectomycorrhizas, are difficult to isolate from soil and to grow in culture, therefore diversity estimates have usually been based on surveys of their ephemeral fruiting bodies ‘mushrooms’ (Figs 1.7 and 1.8; Kwok et al., 1990; Watling, 1995). In brown rot cellulose and hemicelluloses are broken down in the wood substrate, but decomposition of lignin is limited.

The basidiomycete *Agaricus bisporus* (Lange) Imbach is regarded as the model fungus for the adaptation, persistence, and growth in the humic-rich environments, where nutrition is not readily available to primary degrading fungi (Burton et al., 1997; Fermor and Wood, 1981). The ability to use humic proteins gives the fungus an advantage over other saprobes in this complex substrate. *A. bisporus* is a very poor competitor on fresh non-degraded plant wastes but competes well on partially decomposed plant litter on forest floors and grassland soils rich in humic substrates. The machinery used by this fungus to exploit the diverse mixture of nutrient resources is poorly understood, however these genetic and enzymatic mechanisms will be useful in further defining the critical role that basidiomycetes play in contributing to soil structure and carbon sequestration (Morin et al., 2012).

Soft rots differ from both brown and white rots in their pattern of development, which involves a process of hyphal tunnelling inside the lignified cell walls. Soft rot has been described as distinct from brown- and white rot forms of wood decay normally attributed to lignolytic basidiomycetes and some of the larger ascomycetes (Savory, 1954; Schwarze, 2007). Soft rots are chemically more similar to brown rots than white rots since cellulose and hemicellulose is decomposed while lignin decomposition is limited (Eriksson et al., 1990; Schwarze, 2007). Another common feature of brown and soft rot is the demethylation of methxyl groups (Eaton 2000; Schwarze, 2007).

However, the terms brown rot, white rot and soft rot may not be obsolete, but rigid definitions for fungi that are placed into these categories maybe less appropriate than thought previously (Eaton 2000; Schwarze, 2007).

Little is known of basidiomycete species diversity in grassland and agricultural ecosystems outside Europe. European grasslands contain 4 to 135 species per plot (which ranged from 1 m² to >25 ha) in unfertilised grasslands, 16 to 29 species in fertilised grasslands and 2 to 21 in weedy communities (Arnolds, 1992). There have
been few previous intensive studies of basidiomycete diversity in grassland and agricultural, and sand dune soils such as Thorn et al. (1996) and Lynch & Thorn (2006), Brown (1958). The recognition and further characterization of this diversity will have value to soil ecology, sustainable agriculture and fungal conservation.

All strong white rotters produce manganese-oxidizing peroxidases as the key enzymes of ligninolysis whereas lignin peroxidase activity is not detectable in the wood extracts (Liers et al., 2011). The activities of two peroxidases (aromatic peroxygenase and a manganese-independent peroxidase of the DyP-type) were detected in the culture extracts of *Agrocybe aegerita* and *Auricularia auricula-judae*, respectively (Liers et al., 2010). The activity of classic peroxidases correlated to some extent with the removal of wood components and the release of small water-soluble fragments characterized by aromatic constituents. In contrast, laccase activity correlated with the formation of high-molecular mass fragments. The differences observed in the degradation patterns allow to distinguish the rot types caused by basidiomycetes and ascomycetes and may be suitable for following the effects of oxidative key enzymes (ligninolytic peroxidases versus laccases) during wood decay (Liers et al. 2011).

![Reproductive part of fungus](image1)

**Figure 1.7.** Photograph of basidiomycete fruit bodies growing on straw in the field. This is the spore-bearing, reproductive part of the fungus (photo taken by C.H. Robinson, 1994).
Figure 1.8. Photograph of basidiomycete mycelium in agar culture growing from 2 mm² pieces of straw cut out from longer ones (photo taken by C.H. Robinson, 1994).

1.4. Laboratory and field based studies on the decomposition of lignin

Lignin degradation has been studied in controlled environments (e.g. Chefetz et al., 2000; Dungait et al., 2008; Filley et al., 2006; Hatcher and Minard, 1996; Mason et al., 2009; Nierop & Filley, 2008; Robertson et al., 2008) and in the field (Bowen & Harper, 1989; Harper & Lynch, 1981; Robinson et al., 1994). Robertson et al. (2008) specifically looked at the commonly cultivated grass, wheat (*Triticum aestivum*) straw under solid-state cultivation (a process occurring in the near-absence of free water, using the carbon compounds in the wheat straw as a carbon source) conditions to compare the efficiency of three different analytical techniques used to monitor lignin decomposition patterns by white-rot fungi. Robertson et al. (2008) were interested in understanding how the products of lignin decomposition changed during stages of the fungal life cycle, as a way of understanding the dynamics of lignin-derived carbon in soils. They found that lignin was degraded at a much faster rate than previously, however, a finding which was comparable with Dungait et al., (2008), who undertook a study to test the hypothesis that lignin from plant residues is not as resistant to fungal decomposition as thought in previous studies, and therefore not as stable as traditionally perceived (Rasse et al., 2006; Robertson et al., 2008). Exact breakdown mechanisms in
laboratory-based conditions are still unclear. However, field-based studies are really required to better understand the degradation of lignin. In the field Harper and Lynch (1981) showed that ~80% of the straw that was incorporated into soil was decomposed during a year in the field. This suggests that soil micro-organisms, such as the white rot basidiomycete fungi (e.g. *Pleurotus ostreatus*), were able to decompose straw. The use of straw (75-80% cellulose and hemicellulose, 14% lignin and 1-3% phenolic acids) is therefore a good representative organic material when studying the determining factors (e.g. different fungal species, greater temperatures) influencing the decomposition of lignocellulose. The ability of fungi to decompose lignocellulose is likely to be an important characteristic for fungi to be active in straw decay (Bowen & Harper, 1990). The lignin in straw is degraded by a number of different species of fungi, specifically basidiomycetes which have been isolated from wheat straw that was decomposed in cereal fields (Agosin et al., 1985; Zadrazil, 1985; Warcup, 1959). However, little is known about the ability of isolates to decompose the major components of straw (Bowen & Harper, 1990). The little that was known was covered in a study determining the resistance of major parts (leaves, stem and nodes) of straw to colonisation by micro-organisms in relation to the chemical composition of these parts to straw (Table 1.1 and Fig. 1.9; Harper & Lynch, 1981; Robinson et al., 1994).

![Figure 1.9](image_url)

Figure 1.9. Photograph of pure cultures of a basidiomycete as mycelium growing on sterile straw (Robinson et al., 1994). This is the vegetative part of the fungus consisting of a mass of branching threadlike hyphae (photos taken by C.H. Robinson, 1994).
The fungal species *Fusarium culmorum*, *Trichoderma* spp. and *Chaetomium globosum* appeared to be the most frequently isolated cellulolytic fungi found when wheat straw was inoculated with arable soil in the laboratory, and are therefore regarded as being particularly suited to straw decay (Domsch et al., 1980; Harper & Lynch, 1985).

Other successful decomposers are *Penicillium* spp. which showed the ability to grow during the cellulolytic stages of straw decay indicating that this fungus must depend on the products of cellulases produced by other organisms (Harper & Lynch, 1985).

However, straw decay is also dependent on other soil conditions such as the pH, temperature and availability of water and nutrients. These factors affect the ability of organisms to compete with each other, therefore determining the rate and pattern of straw decay in soils (Harper & Lynch, 1985; Harper & Lynch, 1981).

Studies involving the inoculation of fungi into litter to determine the main competitors in the field are relatively rare, particularly those focused on the function of inoculated fungi in comparison to already present field/litter fungi. Our study will not involve the inoculation of any fungi into soil in the field, however, it will involve the burial of organic matter (wheat straw internodes) into the field. This will narrow our focus to lignocellulose, as the internode is the most lignin-rich part of straw, and to the fungi involved in the decomposition of this type of organic matter.

In addition, there have been numerous studies on the fungal communities involved in the decomposition of wheat straw (Robinson et al., 1994; Bowen & Harper 1990; Bowen & Harper, 1989). The fungi involved have been identified from isolations of specific fungi on different media. This begins to illustrate the importance of different fungal communities involved in the decomposition of various forms of organic matter in soils. Hence, there is a need to study ligninocellulose decomposition, particularly the roles of specific fungal species such as basidiomycetes, involved in the breakdown of the macromolecular structures lignin and cellulose.
1.5. Techniques used to detect the fungal community in organic matter in soil

Using traditional techniques, the closest one can come to quantifying the abundance of fungal species in OM is to bring into the laboratory specific fragments of organic matter which are serially washed to remove ‘contaminant’ surface spores (after Harley & Waid, 1955) and plated into defined media (e.g. Robinson et al., 1994). Estimates of percentage frequency of occurrence can be obtained by relating the number of observations, or isolations, of each species to unit amounts of the substratum (organic matter). This technique has the obvious problem that fungi which cannot grow (e.g. arbuscular mycorrizal fungi) will not be isolated. Much has been about problems of competition between fungi within the plated particle affecting fungal outgrowth (e.g. Bååth, 1988). Isolation frequencies from fragment plating that favours mycelial outgrowth, rather than spore germination, are preferable to dilution plate counts of fungal colonies. As dilution plate colonies arise from hyphal fragments and spores, they are meaningless in terms of either abundance or biomass of a species. Heavily sporulating species of decomposer microfungi (e.g. Penicillium) are typically isolated by such dilution plate methods.

Many different approaches have been taken to study the structure of the fungal community. It is probably unknown what proportion of the saprotrophic fungal community is unculturable, but to circumvent the inability to quantify non-culturable fungi, traditional culture-based techniques are being reinforced with molecular methods that study either nucleic acids or some cellular components (e.g. phospholipid fatty acids; PLFA).

The resulting view of the fungal community is affected greatly by the methodology used. When comparing the fungi detected in natural organic matter by culturing techniques with direct DNA analysis of the OM, generally, different groups of fungi have been detected by the two methods (e.g. Borneman & Hartin, 2000; Vainio & Hantula, 2000). In attempting to characterise the structure of a particular fungal community, it is essential to cross-reference the information obtained from several different techniques, which each may have inherent biases.

In 1957, Warcup isolated fungi from a wheat field soil, by preparing soil and dilution plates which were prepared with Czapek Dox + yeast agar medium. The dilution plates were prepared at a dilution of 1:5000, with 5 g of soil being added to sterile water to give an initial 1:50 dilution. This was then raised in one step to give 1:5000. He maintained these cultures on the Dox + yeast agar medium unless their growth was
unsatisfactory, in which case they would be grown on other media such as malt extract. He found that even though he was able to isolate a number of different fungal species including those from the genera *Mucor* and *Penicillium*, in comparison to the ‘hyphal picking’ method he used, it was observed that a large group of fungi were neglected by this method (Warcup, 1957). Eggins and Pugh (1962) used a culturing technique based on Warcup’s, to allow for the comparison of their plates with Warcup’s. They discovered that early colonizers, known as ‘sugar fungi’ were fast growing and unless methods for particular groups of fungi were adopted, these colonizers would take over routine fungal investigations (Eggins & Pugh, 1962). In 1984, Harper and Lynch also isolated microbial populations involved in cellulolysis and from decomposing straw. They, however, ensured that the straw was serially washed in sterile solution to remove surface populations and reduce competition for the cellulolytic fungi they were interested in isolating. They also plated their straw on a wider range of agar media including malt and onto agars containing glucose which served as a richer source of energy for the fungi. Therefore, they were able to isolate a more diverse fungal population using these media, especially of cellulolytic fungi (Harper & Lynch, 1984).

This isolation method of fungi from decomposing wheat straw, using serial washing, was widely adopted. Studies include those carried out by Bowen & Harper (1989) who prepared an agar medium that they hoped would be a particularly relevant source for cellulose and lignin decomposing fungi. This was initially based on Warcup’s soil plates which were prepared with Czapek Dox + yeast agar. This modified version contained (all in g l\(^{-1}\)): sodium nitrate (NaNO\(_3\)) 0.5, di-potassium hydrogen orthophosphate (K\(_2\)HPO\(_4\)) 0.5, potassium chloride (KCl) 0.5, ball milled cellulose 10.0 and kraft lignin which was dissolved in 0.5 M NaOH (lignin agar medium) and added after autoclaving (Bowen & Harper, 1990).

This was further improved on by Robinson *et al.* (1994) who modified the initial Dox + agar medium into the presently used modified Czapek Dox agar medium, which contains an addition of yeast extract (0.5 g l\(^{-1}\)) and 1:15,000 Rose Bengal. Alternatively, they used lignin agar medium (Bowen & Harper, 1989) as above. The nutritionally rich agar medium, Czapek Dox + yeast agar medium, was used to isolate initially the fungi growing on the wheat straw. In our project, it is known to be suitable to isolate a wide range of fungi, including basidiomycete fungi (Bowen & Harper 1989; Robinson *et al.*, 1994).
Selective media for the isolation of wood-rotting basidiomycetes were developed by Russell (1956), Kuhlman and Hendrix (1962) and Tsao (1970). However, strongly cellulolytic \textit{Trichoderma} spp. were still able to grow on these media therefore, the search for a more appropriate medium which primarily isolated basidiomycetes continued (Hunt & Cobb, 1971). Combinations of the active ingredients benomyl (a fungicide) and dichloran (a fungal growth inhibitor), and phenol were found to be very successful in isolating this group of fungi and inhibiting the growth of all other fungi including \textit{Trichoderma} spp. (Hunt & Cobb, 1971).

The particle washing technique to remove surface populations, including ascomycetous and zygomycetous molds, still proved to be successful when isolating cellulolytic or basidiomycete fungi (Harper & Lynch, 1984; Thorn et al., 1996). A medium developed by Thorn et al. (1996) was based on Hunt & Cobb’s improved medium, and in addition contained lignin and guaiacol. The lignin encouraged the selection of ligninolytic fungi and the guaiacol acted as an indicator of lignin modifying enzymes such as laccases and peroxidises. This medium also contained a basal medium which contained ingredients including di-potassium hydrogen orthophosphate (K$_2$HPO$_4$) 0.5 g, potassium chloride (KCl) 0.02 g, calcium nitrate (Ca(NO$_3$)$_2$) 0.05 g, malt extract and others listed in Appendix A. This medium was then autoclaved and cooled to approximately 55°C, when a number of other ingredients, including the antibiotic Streptomycin sulphate, were added aseptically (Thorn et al., 1996).

\textbf{1.6. Scope, aims and objectives}

Based on the previous sections, it is clear that there is a vast amount of information about the roles that different carbon pools play within the global carbon cycle, including the importance of soils (the largest store of terrestrial organic carbon). However, the degradation of organic matter which largely determines soil properties, particularly the role of macromolecules in organic matter such as lignin and cellulose, is understudied. The process by which lignin, for instance is broken down into cinnamic and precursor coumaric acids is unclear, but fungi are known to be involved (Filley, 2000; Rasse et al., 2006; Robertson et al., 2008).

As explained in section 1.3, specific fungi degrade these macromolecules in a different way. Soft rot fungi (e.g. ascomycete \textit{Trichoderma} spp. and zygomycete \textit{Absidia} spp. and \textit{Kretzschmaria deusta}) primarily decompose cellulose and modify lignin in woods and brown rot fungi are capable of decomposing cellulose and hemicellulose (Schwarze,
2007). However soft rot fungi have a limited capability of degrading lignin as they preferentially decompose carbohydrates and are able to modify lignin (Schwarze et al., 2007). Presently, white rot fungi, from the phylum Basidiomycota are the only fungi known to be capable of completely mineralising the highly resistant lignin compound (Kirk & Farrell, 1987). However, there is limited information on the mechanisms by which these fungi do this (Mason et al., 2009).

In general, there have been few reports about how specific fungal species might be involved in the decomposition of woody material, including degradation patterns of preferential and simultaneous rot (Schwarze et al., 2007). There remains limited information on the mechanisms by which these fungi are able to decompose lignin in wood and particularly straw (Robinson et al., 1994). Also, the implications that this breakdown may have on the soil carbon pool, and ultimately on the global carbon cycle remain poorly understood. In addition, the effects of different environmental conditions in soils, such as soil temperature and moisture contents, which may enhance these degradation processes, are also still unclear.

In this thesis the following hypotheses will be tested:

1. The fungal community dominated by the *Trichoderma* species present in the sand dunes at Ainsdale National Nature Reserve at depth is able to decay winter wheat (*Triticum aestivum* var. Swatham) straw (including lignin and cellulose) over time.

2. It is possible to culture a select few fungal species, from winter wheat (*Triticum aestivum* var. Swatham) straw, including *Trichoderma* and *Absidia* species from sand dune field samples.

3. Lignin degradation under tropical conditions (Kenya) is much faster if compared with temperate conditions (UK). This is most likely to be because of a combination of the increase in temperature and/or rainfall.

4. Although fungi are capable of degrading lignin in wheat straw under both laboratory and field conditions, the rate of lignin degradation is always lower when compared with the degradation of cellulose present in the wheat straw.
The aims of this thesis are to:

1. Characterise the diversity of saprotrophic basidiomycete, ascomycete and zygomycete mycelia in representative carbon-poor systems of sand dunes and grasslands. To investigate how lignin and cellulose are degraded and determine the influence of these mycelia on soil organic matter.

2. To use two different types of fungi, *Absidia cylindrospora* (putative lignin modifier) and *Trichoderma koningii*, (putative cellulose decomposer), which were isolated from field samples in March 2009, from Ainsdale National Nature Reserve, that had been buried in sandy soil since 2005 (*A. cylindrospora, T. koningii:* see chapter 2, section 2.3.1) or were brought from a named culture collection (*C. utriformis*), and establish them onto PDA (potato dextrose agar medium) in Petri dishes in the laboratory. These fungi were selected because they were frequently isolated (*A. cylindrospora* and *T. koningii*) and because basidiomata of *C. utriformis* were frequently observed in the field, together with their capability to degrade different carbon substrates in plant biomass. To use these fungi to set up a long term microcosm experiment (300 days) to determine the effects of different fungal species on various types of natural substrata containing lignin and cellulose, i.e. *Triticum aestivum* var. Swatham (wheat straw), *Hordeum vulgare* (barley straw), *Crataegus monogyna* (hawthorn wood chips) and pure cellulose. Fungal growth will be monitored using ergosterol as a fungal biomarker.

3. To test whether the method modified from Robertson et al. (2008) can be used when low amounts of starting material are used.

4. To compare lignin degradation of wheat straw, using acid/aldehyde and syringyl/guaiacyl ratios, in a tropical climate (Kenya) and in more temperate conditions (UK).

5. To compare the rates of cellulose and lignin degradation occurring with the same analytical technique, gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis.

To achieve these aims a number of objectives have been identified and these are:

- To collect wheat straw buried for 46 months at a sand dune site in the UK (Ainsdale Nature Reserve).
- To obtain a general overview of the fungal community present on the wheat straw at this field site.
- To isolate and culture the fungal species colonising the organic matter on these straw samples.
- To determine the breakdown products of lignin/cellulose composition from the straw samples and compare these with the original straw material placed in the field in May 2005.
- To use the isolated fungal cultures to set up a range of laboratory based microcosm experiments using a range of plant substrata such as straw and pure cellulose.
- To repeat the field experiment as set out in the UK in relation to a Kenyan grassland site (in Embu District).
- To investigate the impact, if any, that soil characteristics may have on decomposition of organic matter in the Kenyan compared with the UK field site.
- To determine the extent of cellulose decomposition in the Kenyan soils.
- To correlate the results of the Kenyan and UK field experiments to understand better the differences in the degradation process of wheat straw in temperate compared to tropical environments.

1.7. Approach and thesis structure

The thesis consists of a general introduction (Chapter 1) followed by three chapters written in a scientific paper format, a conclusions chapter, and three appendices summarizing the details of the methods utilized and conference contributions. Each of the chapters have either been published (Chapter 2) or will be submitted in the near future (Chapter 3 and Chapter 4).

The first part of the thesis (Chapter 2) deals with lignin breakdown products analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis, in initial wheat (Triticum aestivum var. Swatham) straw samples compared with those in samples which had been buried as a “model” resource for 46 months in a sand-dune grassland at Ainsdale National Nature Reserve, Lancashire, UK. This study revealed that lignin oxidation occurred in the straw over the 46 month period, as there were general increases in the [Ac/Al]_S and [Ac/Al]_G ratios and a clear decrease in the [S/G] ratio. These data provided tentative support for the theory that white rot basidiomycete
fungi may be involved in the degradation of lignin in grasslands. Chapter 3 deals with fungal degradation of cellulose and modification of lignin by soft rot-fungi. This is monitored using ergosterol as a biomarker to quantify fungal biomass, and gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis to determine how different fungal species decompose different plant substrata. The 300 day microcosm experiment showed that ergosterol could be detected at low concentrations and that soft rot fungi, namely Absidia cylindrospora and Trichoderma koningii, isolated from the field experiment in Chapter 2 was efficient at utilizing cellulose more than lignin in all four carbon sources tested. Chapter 4 deals with a comparative study between a temperate (Ainsdale, UK) (Chapter 2) and a tropical (Embu, Kenya) field study. This study revealed that there was greater modification of lignin, shown by differences in the [Ac/Al]$_S$ and [Ac/Al]$_G$ and [S/G] ratios, in the tropical soils than the temperate soils. Substrate utilisation, in particular that of cellulose occurred over a significantly shorter period of time at the Kenyan compared with the UK field site.

Only scientific papers for which the author is the lead author have been included in this thesis. For all the chapters, the author was responsible for writing the papers and undertaking the majority of the laboratory data reported, unless this was otherwise stated elsewhere. Details of conference presentations have also been given in Appendix C.

1.8. Author’s and co-authors’s contributions to each paper

- Paper 1 (Chapter 2) - Author - the principal investigator, collecting the samples, analysing the samples for fungal culturing and organic geochemical content, writing of manuscript. Clare H. Robinson - burial of samples 46 months prior to study; Christopher Boothman - performing PCR analysis, Alastair D. Bewsher – obtaining water extracted cations and anions in soil samples; Clare H. Robinson and Bart E. van Dongen - total conceptual guidance and manuscript review.

- Paper 2 (Chapter 3) – Author - the principal investigator, setting up microcosms, performing organic geochemical analysis, analysing and interpreting data, writing of manuscript. Pippa-Jane Mason - assisting in setup of microcosms,
Clare H. Robinson and Bart E. van Dongen - full conceptual guidance and manuscript review.

- Paper 3 (Chapter 4) - Author - the principal investigator, setup of field experiment, collection of samples, performing organic geochemical analysis, analysing and interpreting data, writing of manuscript. Stephen W. Njoka - providing access to field site at the Kenya Agricultural Research Institute (KARI) in Embu; Clare H. Robinson and Bart E. van Dongen - complete conceptual guidance and manuscript review.

1.9. References


The Global Carbon Cycle- Illustration courtesy NASA Earth Science Enterprise retrieved from Dr Ian Bull’s webpage found at http://www.chm.bris.ac.uk/ogu/people/bull.htm accessed on 1/07/09.


Chapter 2

Paper 1. Decomposition of lignin in wheat straw in a sand-dune grassland

This chapter is partly published and contains the following short communication:

Chapter 3

Paper 2. A comparison between two different fungi and their ability to degrade lignin and cellulose in various substrata

This chapter contains the following paper which is in preparation to be submitted to the Journal of Organic Geochemistry:
Rachel N.T.M. Kabuyah, Pippa- Jane Mason, Clare H. Robinson and Bart E. van Dongen. A comparison between two different fungi and their ability to degrade lignin and cellulose in various substrata.

Chapter 4

Paper 3. Decomposition of wheat straw in a Kenyan grassland site

This chapter contains the following paper which is in preparation to be submitted to Journal of Organic Geochemistry:
Rachel N. M. Kabuyah, Bart E. van Dongen and Stephen W. Njoka, Clare H. Robinson. Decomposition of wheat straw in a Kenyan grassland site.
Chapter 2

Paper 1. Fungal decomposition of wheat straw in a sand-dune grassland.

Abstract
The degradation of organic macromolecules, including lignin, in plant-derived soil organic matter, is important to the global carbon cycle. In grasslands, saprotrophic (decomposer) fungi are major decomposers of such organic material. The aim of this study was to characterise lignin degradation, particularly with respect to lignin oxidation typical of white-rot basidiomycete fungi. Lignin breakdown products, analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis, in initial wheat (Triticum aestivum var. Swatham) straw samples were compared with those in samples which had been buried as a “model” resource for 46 months in a sand-dune grassland at Ainsdale National Nature Reserve, Lancashire, UK. Our results showed that lignin oxidation occurred in the straw over the 46 month period, as there were general increases in the [Ac/Al]S and [Ac/Al]G ratios and a clear decrease in the [S/G] ratio. These data provide tentative support for the theory that white-rot basidiomycete fungi are involved in the degradation of lignin in grasslands.

2.1. Introduction
Soils contain the largest store of terrestrial organic carbon at approximately 1,580 Gt C, which is two to three times more carbon than that held in terrestrial plant biomass (e.g. Schimel, 1995). The degradation of organic matter in soil, however, which largely determines soil properties, is understudied. For instance, the process by which lignin is broken down into cinnamic and precursor coumaric acids is unclear but fungi are known to be involved. Even so, lignin is highly resistant to degradation because of its structural complexity. Nonetheless, there are specific organisms, fungi belonging to the phylum Basidiomycota, which are known to mineralise lignin completely (Kirk and Farrell, 1987). The extent to which “model” organic matter (wheat straw here), is degraded in non-agricultural soils in the field is not clearly understood, particularly in a soil that is relatively low in native organic matter (sand dunes) and likely to have a relatively simple fungal community (Brown, 1958). Wheat straw is a type of organic matter which has previously been used to determine the effect of fungi on the decomposition of lignin and cellulose (e.g. Bowen and Harper, 1989; Robinson et al., 1994). It is an ideal material to use because of its composition, consisting of 14% lignin, 75-80% cellulose and hemicellulose and 1-3% phenolic acids (Harper and Lynch, 1981). There remains limited information under field conditions about the mechanisms by which fungi are able to decompose lignin in grass (but see Gramss, 1997) and particularly in straw (e.g.
Robinson et al., 1994). Additionally, more widely, according to Grinhut et al. (2007), the precise role of saprotrophic fungi in organic matter degradation and turnover, and the role of individual fungal species in decomposition, remain major questions. Thus, the aims of this study were: (1) to obtain a general overview of the fungal community which could be isolated from wheat straw buried, as a “model” resource, for 46 months at this field site, (2) to determine the potential substrate utilisation of this community in relation to the degradation of organic macromolecules in buried wheat straw, particularly lignin and cellulose, and (3) to characterise lignin degradation, particularly with respect to lignin oxidation typical of white-rot basidiomycete fungi, by comparing the lignin breakdown products in the initial straw samples with those in the decomposed straw samples collected 46 months later. To achieve aim (3), we used gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Studies concerning the use of this analytical method on field samples to determine the fungal decomposition of wheat straw, appear rare. Instead, previous work with gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis and fungal decomposition of wheat straw has been largely focused on laboratory based experiments, using pure cultures of fungi and without incubation in a soil matrix.

2.2. Materials and Methods

2.2.1. Field sampling

The study site was at Ainsdale National Nature Reserve, Lancashire, UK (SD295109) in sand-dune grassland in the fixed, grey dune zone (Brown, 1958) (Fig.2.1). Typically, the surface A horizon of this inceptisol contains brown humus to ca. 7 cm in depth, and the lower horizon contains grey sand. The limit of plant rooting occurs at approximately 11 cm depth. There are relatively low amounts of native soil organic matter at the site: in the upper soil horizon at 5 cm depth there was 6.65% organic matter (standard error of ± 2.19%, n = 5), and in the lower soil horizon at a depth of 11 cm there was 0.74% organic matter (standard error of ± 0.08%, n = 5). A pH value of 6 was observed for all ten soil samples. Concentrations of soil anions and cations in each of the two horizons are shown in Table 1. Forty, bait bags were prepared and buried on 14th May 2005. Each bait bag was 15 x 20 mm, made of 50 μm mesh and contained five internodes of winter wheat straw *Triticum aestivum* var. Swatham. These internodes had been split longitudinally and cut to size 5 x 15 mm, with each bag attached to an orange nylon string and metal tag to facilitate collection. The mesh reduced plant root colonisation
and access to soil fauna, but still allowed for microbial colonisation, increasing the likelihood of basidiomycete fungi reaching the straw and therefore utilising the refractory lignin compound (present as lignocellulose).

Figure 2.1. Map of the UK, pointing out Sefton’s Natural coast, Southport and opposite: map showing study site (with grid reference SD 295 109 GB) in the Ainsdale National Nature Reserve, Southport, Lancashire, UK.

The samples were buried in each of the two clearly defined horizons outlined above, at depths of 5 and 11 cm from the soil surface, along a transect. The surface organic horizon was at 5 cm depth while the limit of rooting depth and ‘grey’ sand horizon was at 11 cm depth. Along the transect, there were five bait bags in each upper and lower horizon placed at specific distances (0.5 m, 2.5 m, 4.5 m, 6.5 m, and 8.5 m) from the point of origin. On March 1st 2009, nominally 10 bait bags were collected, although the bag along the transect at 4.5 m distance, 11 cm depth, could not be located and was considered to be lost. This meant that 9 bait bags were collected in total, five from the upper horizon and four from the lower horizon, which were essentially treated as replicate bags within each horizon (i.e. horizontal distances of the samples from the point of origin were not used subsequently in the analyses). Bait bag samples were taken from both depths, as were soil samples (ranging from 1.6 to 4.9 g in weight) for
chemical analyses (Table 2.1 and loss on ignition and pH as above), taken immediately adjacent to the location of each bag, from each depth. Each bag, and each separate soil sample, was carefully folded into individually pre-furnaced, labelled aluminium envelopes to prevent contamination of the straw and soil for future analyses. These envelopes were then placed in Ziploc bags to prevent the straw from drying out, and stored in a 10°C cold room until processing (carried out within 48 hours).
Table 2.1. Concentrations (mg g⁻¹) of water-extracted cations and anions in soil samples adjacent to each bait bag sample, collected on March 1st 2009 from Ainsdale sand-dunes.

<table>
<thead>
<tr>
<th>Soil horizon</th>
<th>Upper A horizon (n = 5)</th>
<th>Lower grey sand horizon (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺</td>
<td>14.2 ± 5.6</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Na⁺</td>
<td>98.8 ± 29.0</td>
<td>48.0 ± 15.9</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>9.28 ± 2.9</td>
<td>2.95 ± 0.41</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>3.40 ± 0.98</td>
<td>1.52 ± 0.30</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>1.25 ± 0.67</td>
<td>0.28 ± 0.43</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>0.18 ± 1.41</td>
<td>0.04 ± 0.09</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>46.4 ± 21.3</td>
<td>2.95 ± 1.04</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>12.6 ± 3.4</td>
<td>2.40 ± 0.18</td>
</tr>
</tbody>
</table>

2.2.2. Wheat straw washing and plating

Each bait bag, containing a variable number of pieces of straw (initially 5 x 15 mm when first placed in soil), was opened individually using sterile scissors and placed on a white tile. Each piece of straw was divided into two equal pieces using a sterile scalpel. One half was used to plate out onto the two agar media (later described below) and the other half was stored at -20°C for future macromolecular analyses.

For plating, this resulted in approximately ten 2 mm² fragments from each bait bag. These fragments were vigorously shaken end-to-end by hand in autoclaved McCartney bottles containing sterile de-ionised water for 5 minutes. The suspension was then poured through a sterile laboratory test sieve (150 μm mesh size). Each piece was picked up with sterile forceps and blotted onto sterile glass microfibre filter paper (47 mm circles).

Five fragments from each bait bag were individually pushed into a Petri dish of lignin-guaiacol-benomyl agar medium (LGBA; Thorn et al., 1996—see Appendix A) and a further five fragments into a Petri dish of modified Czapek-Dox agar medium (after
Warcup, 1957; the modifications were K$_2$HPO$_4$ (1 g l$^{-1}$), yeast extract (0.5 g l$^{-1}$), glucose (30 g l$^{-1}$) and Rose Bengal (6.7 x 10$^{-5}$ g l$^{-1}$)). The lignin-guaiacol-benomyl agar medium was developed by Thorn et al. (1996); the inclusion of lignin was to encourage selection of ligninolytic fungi and the guaiacol acted as an indicator of lignin modifying enzymes such as laccases and peroxidases. The relatively nutrient-rich modified Czapek-Dox agar medium (Appendix A) was used because it is known to provide suitable growth conditions for a wide range of fungi (Robinson et al., 1994), including basidiomycete fungi (Bowen & Harper, 1989).

2.2.3. Isolation of fungi

Once the straw was plated out, the Petri dishes were packed in sterile plastic sleeves and stored in a 10°C cold room for 48 hours before any isolations were made. During this time, plates were scanned for any sign of fungal growth around each piece of straw. These areas (approximately 2 mm$^2$) were then carefully cut out using sterile needles and isolated onto malt extract agar medium, and stored at room temperature for further screening. The screening process was carried out every day for the first week where fast growing spreading fungi (areas of approximately 2-15 mm$^2$) were cut out and removed completely, once 2 mm$^2$ had been sub-cultured onto malt extract agar medium. This was then repeated every 48 hours. Sub-culturing was longer for fungi growing on the LGBA medium (about 3 weeks before any sub-culturing was done). The percentage frequency of colonisation was calculated using the total number of fragments colonised by a fungus, i.e. if one straw fragment from the five plated fragments was colonised, the percentage colonisation was calculated at 20% (1/5=20).

2.2.4. DNA analysis to identify pure cultures of fungi isolated

DNA was extracted from fungal mycelium, growing on potato dextrose agar medium, using a PowerSoil DNA Isolation Kit (PowerSoil DNA Isolation Kit, MO BIO Laboratories INC, Solana Beach, CA, USA) with the following modifications. Since the fungal mycelium was growing on agar medium, ~0.5 cm$^3$ samples were excised from the agar plates using a sterile spatula and these were transferred directly to the bead beating tubes provided with the PowerSoil kit. A fragment of the internal transcribed spacer region was amplified with the eukaryotic specific primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). PCR reactions were performed in thin-walled tubes using a BioRad iCycler (BioRad, Hemel Hempstead, Herts, UK). PCR
reactions were performed in 50 μl volumes, containing 2 μl of extracted DNA with the reaction mix containing 5 μl each of 10X PCR reaction buffer and 25 mM MgCl2, 1 μl of 10 μM dNTP mix, 0.5 μl of each primer (25 μM working stock), 0.5 μl Sigma Taq DNA polymerase, and 35.5 μl purified deionised H2O. The PCR amplification protocol used with the ITS1F and ITS4 primers was: initial denaturation at 94°C for 1 min, melting at 94°C for 35 s, annealing at 52°C for 1 min, elongation at 72°C for 1 min; 35 cycles, followed by a final extension step at 72°C for 5 min. Purity of the amplified products was determined by electrophoresis in Tris-acetate-EDTA (TAE) gel. DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, Hemel Hempstead, Herts, UK). Samples were prepared for sequencing by purification using the QIAquick purification kit (QIAGEN, UK). Purified samples were analyzed using the Nanodrop 1000 spectrometer to determine the quantity of DNA and the purity of the samples. Nucleotide sequences were determined by the dideoxynucleotide method (Sanger et al., 1977). An ABI Prism BigDye Terminator Cycle Sequencing Kit was used in combination with an ABI Prism 877 Integrated Thermal Cycler and ABI Prism 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). Sequences (typically 500-700 base pairs in length) were analysed against the NCBI (USA) database using BLAST program packages and matched to known ITS fungal sequences.

2.2.5. On-line thermochemolysis

Samples, approximately 0.1 mg, were analysed by normal flash gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Prior to analysis, 4 μl of the hydrocarbon 5α-androstane internal standard (100 μl of a 0.256 mg ml−1 solution in dichloromethane) and 5 μl tetramethylammonium hydroxide (TMAH) were added to each sample. To avoid problems because of different TMH conditions (e.g. temperature and/or time; Klingberg et al., 2005) all samples were analysed under the same conditions. Samples were pyrolysed using a chemical data system (CDS) 5200 series pyroprobe pyrolysis unit by heating at 600°C for 10 seconds to fragment macromolecular organic components. Fragments were analysed using an Agilent 7890A fitted with HP-5 fused capillary column (J+W Scientific; 5% diphenyl-dimethylpolysiloxane; 30 m length, 0.32 m internal diameter, 0.25 μm film thickness) coupled to an Agilent 5975 MSD single quadrupole mass spectrometer operating in electron ionisation (EI) mode (scanning a range of m/z 50 to 600 at 2.7 scans second−1;
ionisation energy 70 eV). The pyrolysis transfer line and injector temperatures were set at 350 °C, the heated interface at 300 °C, the EI source at 230°C and the MS quadrupole at 150°C. Helium was used as the carrier gas and the compounds were introduced in split mode (split ratio 40:1). The oven was programmed from 40°C (held for 2 min) to 220°C at 2.5°C min⁻¹, held at this temperature for 1 min before being heated to 300°C at 20°C min⁻¹ and held at this temperature for 11 min. Compounds were identified by comparison of spectra with those reported in the literature (Vane et al., 2001). The naming of the THM products follows the conventions established previously (Clifford et al., 1995; Hatcher et al., 1995). Because of the limited amount of sample available, as a result of decay in the field, it was only possible to analyse the samples in duplicate for the majority of the bait bags (from the upper horizon at 5 cm, sampling points 0.5, 4.5, 6.5, 8.5 m and from the lower horizon at 11 cm, sampling points 0.5, 2.5, 6.5 and 8.5 m). In case of the sample from the upper horizon at 2.5 m there was only enough material available for a single analysis.

2.2.6. Lignin parameters and nomenclature
The acid/aldehyde \([\text{AcAl}]_S\) parameter is a relative decomposition state proxy for the syringyl lignin monomer which was measured using the peak areas of 3,4,5-trimethoxybenzoic acid, methyl ester (S6) and 3,4,5-trimethoxybenzaldehyde (S4; Fig. 2.2; Vane et al., 2001). The acid/aldehyde \([\text{Ac/Al}]_G\) parameter, which is also a relative decomposition state proxy for the guaiacyl lignin monomer was measured using peak areas of 3,4-dimethoxybenzoic acid, methyl ester (G6) and 3,4- dimethoxybenzaldehyde (G4). The syringyl/guaiacyl ratio \(S/G\) in THM products from wheat straw buried at Ainsdale sand dunes describes a relative intensity ratio of TMAH thermochemolysis products.
It was calculated by dividing the sum of the peak areas from syringyl derivatives (S4+S5+S6) by the sum of the peak areas from their guaiacyl counterparts (G4+G5+G6; Mason et al., 2009). Because of the limited amount of material available, as explained above, only mean ratios of duplicate analyses, for \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and \(S/G\) could be calculated for each sample point. In addition, for comparison, the original pre-burial wheat straws were cut, weighed (ca. 0.1 mg) and analysed in a similar manner to those collected upon degradation. These analyses were run 5 times and the average ratios obtained were used as a baseline for determining the extent of fungal degradation of the wheat straw samples collected from Ainsdale after burial in soil for 46 months.

2.3. Results

2.3.1. Saprotrophic fungi isolated from wheat straw internodes

Visual degradation of the wheat straw was observed after 46 months (Fig. 2.3). Thirteen fungal species isolated from these straw internodes, representative of groupings made by culture morphology, were identified by DNA analysis. Using information from Domsch et al. (1980), the species’ potential substrate utilisation was assigned (however,
it is likely that each species in Fig. 2.4 can use a range of substrates, and it is possible that aspects of resource quality, other than its chemical composition, were responsible for the observed patterns of colonisation; Robinson et al., 1994).

The spectra of fungi isolated were largely different between the two different agar media used. Some species were isolated on one medium alone (Fig. 2.4): for example, *Trichoderma koningii* (*Hypocrea koningii*; cellulose degrader) was the most frequently occurring fungus isolated on the modified Czapek-Dox agar medium (present on 38% of particles plated from straw at 5 cm depth and on 47% from straw at 11 cm depth), whereas this species was not isolated from any of the particles cultured on the LGBA medium (Fig. 2.4).

Similarly, *Penicillium canescens* (weakly cellulolytic) was isolated on modified Czapek-Dox agar medium alone and *Absidia cylindrospora* (lignin modifier) was isolated on LGBA alone (Fig. 2.4).

Figure 2.3. Images of the contents of bait bag samples, initially comprising five wheat straw internodes (5 x 15 mm), placed in nylon mesh bags, prior (A) and 46 months after burial (B).
Figure 2.4. Pie charts showing distribution of fungi isolated on Czapek Dox agar and lignin guaiacol-benomyl agar (LGBA) media from wheat straw collected from the upper (5 cm depth) and lower (11 cm depth) soil horizons. Samples were collected along a transect; 5 samples collected at 0.5, 2.5, 4.5, 6.5, 8.5 m (plating out 25 straw particles) from the upper soil horizon and 4 samples collected at 0.5, 2.5, 6.5, 8.5 m (plating out 20 straw particles) from the lower soil horizon. All samples collected after 46 months burial at Ainsdale sand dunes.

Several species, however, were isolated on both modified Czapek-Dox agar medium and lignin-guaiacol-benomyl-agar medium (LGBA), although their isolation frequencies were different between the two media. For example, *Absidia glauca* (degrades pectin, subsequently termed pectinolytic; there are no reports of lignin or cellulose decomposition in Domsch et al., 1980 for this species, but there is no information included whether such tests have ever been carried out) was the most
frequently isolated species on the LGBA medium (present on 55% of particles plated from straw at 5 cm depth and on 38% from straw at 11 cm depth; Fig. 2.4), and *A. glauca* was also found on modified Czapek Dox agar medium (present on 12% of particles plated from straw at 5 cm depth; Fig. 2.4). Even though the species spectra were different between two agar media, generally, on each type of agar medium separately, the same species of fungi were isolated with same functional capabilities from the two different depths i.e. mainly cellulose-degrading fungi on the modified Czapek-Dox agar medium and mainly pectinolytic and lignin modifiers on the LGBA. More detail about this statement follows: (i) from straw particles at 5 cm depth, cellulolytic fungi were the most frequently isolated “functional group” (72%) on modified Czapek-Dox agar medium, followed by equal frequencies of weakly cellulolytic and pectinolytic fungi (each group at 12%; Fig. 2.5). At 11 cm depth, the order of the “functional groups” of fungi isolated fungi on modified Czapek-Dox agar medium was as follows: cellulolytic (70%), equal frequencies of weakly cellulolytic and lignin modifying fungi (each group at 12%; Fig. 2.5) and (ii) from straw particles at the same 5 cm depth as above, on LGBA medium, high frequencies of pectinolytic fungi (55%) and lignin modifying fungi (36%) were isolated (Fig. 2.5). Relatively equal frequencies of pectinolytic (38%) and lignin modifying fungi (39%) were isolated on the LGBA medium at 11 cm depth, followed by lower frequencies of cellulolytic (15%) fungi (Fig. 2.5).

No basidiomycetes, often the most effective lignocellulose decomposers in soil organic matter, were found at either depth or on either medium.

2.3.2. Lignin analyses

To determine lignin degradation of the *T. aestivum* straw, the samples collected 46 months after burial in Ainsdale sand dunes were analysed by TMAH thermochemolysis. Similar to previous studies (Filley et al., 2000), a suite of THM products was identified (Fig. 2.1). The \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and \(S/G\) ratios in the recovered material ranged from 3.2 to 10.8, 0.38 to 1.76 and 0.67 to 2.49, respectively (Fig. 2.6). For comparison, the values of the original straw material were 3.3±0.81, 0.43±0.15 and 3.3±0.33 for the same ratios, respectively. Thus, over the 46 months of decay, \([\text{Ac/Al}]_S\) values generally increased, \([\text{Ac/Al}]_G\) values generally increased, and \(S/G\) values were generally lower than the original values. After 46 months of decay, there were no clear differences for
each type of ratio between straw samples collected from the two different horizons (Fig. 2.6).

2.4. Discussion
2.4.1. Identity and “function” of fungal isolates

In spite of the large number of isolates obtained from the buried winter wheat straw (*Triticum aestivum* var. Swatham) collected on March 1st 2009 from Ainsdale sand dunes in both the 5 and 11 cm horizons, there were several noticeable differences between the fungal species cultured on the two different agar media (Fig. 2.4). The fungal species isolated were separated according to their potential functional capabilities i.e. which particular carbon substrate each species was capable of utilising. More cellulose-decomposing fungi were isolated on the modified Czapek Dox agar medium compared with on the LGBA medium, with 72% compared with 9% at 5 cm depth (Fig. 2.5). Similarly, at a depth of 11 cm there was 70% of these fungi observed on the Czapek Dox medium compared with 15% on the LGBA medium (Fig. 2.5).

*T. koningii* and another fungus from the *Trichoderma* genus, are potentially strong cellulose degraders. These ‘soft rot’ fungi preferentially decompose carbohydrates and have a limited capability to decompose lignin (Schwarze, 2007). They are able to split the glycosidic linkage in cellulose via the production of a number of enzymes, including α-glucosidase and amylase. In addition to this, *Penicillium canescens* is a weakly cellulolytic fungus as it is not known to attack cellulose fibres, however it can attack methylcellulose which is a chemical compound derived from cellulose (Domsch et al., 1980). It was only isolated on the more general agar medium, Czapek Dox, and was equally observed in isolations made from both depths (12%). *Absidia cylindrospora* was the only known lignin modifier isolated from the field samples, found primarily on the lignin rich LGBA medium (with 36% from 5 cm depth and 39% at 11 cm depth; Fig. 2.4). This was expected as the LGBA medium is known to be a particularly relevant source of carbon and nutrients for lignin decomposing fungi. Nonetheless, we were unable to isolate true lignin decomposing fungi from any of the straw samples collected on March 1st 2009 (please see below for discussion concerning the importance of basidiomycetes in lignin breakdown). *Absidia glauca* is from the same fungal genus as *Absidia cylindrospora*. There have been, however, no reports to indicate whether or not *Absidia glauca* is cellulolytic or ligninolytic, although it can degrade pectin (Domsch et al. 1980). It was therefore grouped as a pectinolytic
fungus. *Absidia glauca* and *Mucor hiemalis* have been isolated previously from dune soils. Similarly, *Trichoderma* species, *e.g.* *T. viride*, have also been isolated from dunes (Brown, 1958), although we isolated *T. koningii* which appears not to have been isolated from a sand dune system before (Brown, 1958; Domsch et al., 1980). Lignin modifying *Absidia cylindrospora* has been frequently isolated from grasslands, however, there are no reports of this species being isolated from dune soils. This species is the most frequently isolated from the *Absidia* genus and closely resembles *A. spinosa* which has been isolated from sand dunes (Domsch et al., 1980; Brown, 1958). Typically, fungi from the *Penicillium* genus are commonly isolated from sand dune soils (Brown, 1958), and *P. canescens* has been isolated from dune systems (Domsch et al., 1980). From our study, using these two media, we can deduce that, when we sampled after 46 months, the culturable fungal community on the *Triticum aestivum* var. Swatham straw buried in sand-dune soil was dominated by *Trichoderma* and *Absidia* species and mainly cellulolytic, pectinolytic and lignin-modifying fungi. There were no great differences in species spectra or potential “function” between the two horizons at which the straw was buried, despite clear physical and chemical differences between the two horizons (Table 2.1 and described in Section 2.1 above). Basidiomycete fungi, the only organisms capable of completely mineralising the recalcitrant lignin compound in soils (Kirk and Farrell, 1987), develop a pathway (phenylpropanoid-acetate pathway) whereby lignin is broken down to cinnamic and precursor coumaric acids (Killops and Killops, 2005). Basidiomycetes were not isolated here, although basidiomata of *Calvatia utriformis* were common at the site in late summer, suggesting the mycelia of this species were present in the soil. Thorn et al. (1996) were able to isolate basidiomycetes from soil using agar media (LGBA), and using culture morphology and identification of basidiomata, managed to identify 67 basidiomycete isolates, from the 111 they initially thought were basidiomycetes (Thorn et al., 1996). However, more usually, this group of fungi is particularly difficult to isolate from soil (Thorn et al., 1996). Therefore, it was not unexpected that we were unable to isolate these fungi directly from mycelium growing in straw. We were, however, able to determine the extent of lignin degradation typically caused by basidiomycete fungi by analysing the wheat straw samples, using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis.
Figure 2.5. Pie charts showing fungal frequency of isolation and their primary enzymatic capabilities (after Domsch et al., 1980) from the upper (5 cm depth) and lower (11 cm depth) soil horizons on both Czapek Dox agar and lignin-guaiacol-benomyl agar (LGBA) media. Samples were collected along a transect; 5 samples collected at 0.5, 2.5, 4.5, 6.5, 8.5 m (plating out 25 straw particles) from the upper soil horizon and 4 samples collected at 0.5, 2.5, 6.5, 8.5 m (plating out 20 straw particles) from the lower soil horizon. All samples collected after 46 months burial at Ainsdale sand dunes.

2.4.2. Lignin degradation
A comparison in the distributions of a suite of unlabelled TMAH thermochemolysis products (Fig. 2.1; Fig. 2.6) from initial and buried wheat straw (Fig. 2.1) was used to indicate lignin oxidation. The measurement of these ratios has been used previously to highlight lignin side chain oxidation. Our results clearly showed that lignin oxidation, particularly of the syringyl moieties, occurred over the 46 month period, as there was a
general increase in the [Ac/Al]$_S$ ratio and a general decrease in the [S/G] ratio (Robertson et al., 2008). Comparison of thermochemolysis products from initial and decayed wheat straw showed a relative increase in S6 compared with S4 counterparts (Fig. 2.6). Therefore the increased [Ac/Al]$_S$ values indicated that the fungi had induced oxidative cleavage of the Cα-Cβ bonds at the Cα position and then the aldehyde was oxidized to a carboxylic acid (Tien & Kirk, 1983; Robert and Chen, 1989). These Cα-Cβ bonds are located on the aliphatic side chain in a similar manner to those attacked by many white-rot fungi such as *Phanerochaete chrysosporium* and *Trametes (Coriolus)* *versicolor* (Robert and Chen, 1989; Vane et al., 2001). The changes in this [Ac/Al]$_S$ parameter can arise from changes in concentration of acid and aldehyde. S6 and G6 may also be generated as TMAH reaction products from undecayed lignin, however, the amounts formed are lower than those produced by fungal decay (Hatcher et al., 1995).

The levels of these compounds observed during TMAH treatment were such that, in the initial wheat straw sample, the lignin levels were always lower than in the degraded lignin suggesting that the relative amounts of aromatic acids and aldehydes could be indicative of fungal degradation. By contrast to the [Ac/Al]$_S$ ratio, comparison of the [Ac/Al]$_G$ values indicated that there was a smaller difference between the guaiacyl ratio in the original wheat straw and the degraded material (Fig. 2.6), suggesting a minor effect on the guaiacyl moieties. Even so, correlating both [Ac/Al] proxies indicated a substantial difference between the initial wheat straw and the recovered material (Fig. 2.7), supporting the decay/alteration of lignin over time. Previous studies, however, have similarly reported a larger increase in the value for [Ac/Al]$_G$ compared with that of [Ac/Al]$_S$ of wheat straw decayed for 80 days by the white-rot fungi *Pleurotus eryngii* and *P. chrysosporium* (Camarero et al., 1997). A suggested explanation for our results here, where [Ac/Al]$_S$ of wheat straw substantially increased after 46 months of decomposition compared to [Ac/Al]$_G$, is that syringyl units may be much more susceptible to degradation, since they are: (i) less condensed and have a lower redox potential than guaiacyl units (Kirk and Farrell, 1987) and (ii) syringyl in grass forages, such as wheat straw, is located mainly in secondary cell walls, which are more easily decomposed than primary cell walls (Grabber, 2005), and at advanced stages of decay by soft-rot fungi, this secondary wall is broken down, whereas the guaiacyl-rich middle lamella persists (Schwarze, 2007). Similarly, Camarero et al. (1997) found that the amount of etherified residues of syringic acids was very low in undecayed straw, but that the molar abundances of these acid residues increased strongly during fungal
degradation of lignin, attaining 8% of total lignin-derived compounds recovered after pyrolysis of straw treated with *Pleurotus eryngii* (after 50% lignin degradation).

Figure 2.6. (A) 3, 4, 5-trimethoxybenzoic acid, methyl ester (S6) to 3, 4, 5-trimethoxybenzaldehyde (S4) Acid/aldehyde ratio ([Ac/Al]S), (B) 3, 4-dimethoxybenzoic acid, methyl ester (G6) to 3, 4-dimethoxybenzaldehyde (G4), [Ac/Al]G and (C) the relative amounts of syringyl derivatives (S4+S5+S6) to guaiacyl counterparts (G4+G5+G6), [S/G] (Mason et al., 2009) in THM products from wheat straw buried at Ainsdale sand dunes collected after 46 months. The rectangle dashed box indicates the original ratios of the buried wheat straw based on 5 measurements. O = upper soil horizon (5 cm depth); ▲ = lower soil horizon (11 cm depth).
Although [Ac/Al] ratios have been used previously as relative decomposition state proxies, caution needs to be exercised when acid/aldehyde ratios are used. This is because a potential contribution of 3,4,5-trimethoxybenzoic acid from hydrolysable tannins to S6 may be possible. However, wheat straw contains no, or only very low amounts, of condensed hydrolysable tannins (Harper and Lynch, 1981; Robertson et al., 2008) meaning that no interference with the [Ac/Al]S ratio by tannins is expected. The syringyl/guaiacyl ratio [S/G] describes the ratio of methylated syringyl derivatives to guaiacyl derivatives and is generally used as an indicator of lignin biodegradation for angiosperm woods, agricultural crops and composts (Vane et al., 2001). All the samples collected after 46 months showed decreased S/G ratios compared with the values of the original straw (Fig. 5). This suggests a higher oxidative reactivity of syringyl moieties relative to guaiacyl derivatives during decay at Ainsdale sand dunes over the 46-month burial period further supporting the differences observed between the relative decrease in [Ac/Al] proxies. In addition, previous lab-based experiments showed that [S/G] ratios tend to decrease when lignin is decayed by white-rot fungi (Pal et al., 1995; Vane et al., 2001), supporting the involvement of these fungi in the decay processes occurring at the
Ainsdale field-site. No clear difference for each type of ratio between the two different horizons could be observed, supporting the earlier mycological observations.

In the current study, regarding the relative importance of non-basidiomycete fungi in decomposing lignin in wheat straw, we classified *Absidia cylindrospora* as a lignin modifier because it has been shown to produce phenoloxidases and to have the ability to utilise lignosulphonate, humic and fulvic acids (Domsch et al., 1980). Even so, according to de Boer et al. (2005), such fungi are believed not to be major decomposers of lignin in natural environments. Similarly, there are reports elsewhere of soil anamorphic (asexual) ascomycetes (e.g. *Fusarium oxysporum*, *F. proliferatum*, *F. solani*, *Penicillium chrysogenum*, *P. janczewskii*) having the potential to transform and degrade lignin (Rodriguez et al. 1996; Regalado et al. 1997; Deacon et al. 2006), although again this is thought to be confined to the initial stages of fungal metabolism.

It is important to note that possible bacterial colonisers of straw at the field-site could have the potential to degrade lignin (e.g. Antai & Crawford, 1981; Kellner et al., 2008) and cellulose (e.g. Harper and Lynch, 1981). Abiotic degradation of lignin in the straw by photodegradation with UV-B (Gehrke et al. 1995) is highly unlikely however, because penetration of UV-B is low through plant cover and soil to the depths at which the wheat straw was buried.

2.5. Conclusions

In winter wheat straw *Triticum aestivum* var. Swatham buried in sand-dune soil, the fungi isolated were mainly putative cellulose and pectin decomposers and lignin modifiers. No basidiomycetes, expected to be responsible for enzymatic combustion of lignin, were isolated even though white-rot basidiomycetes were likely to have been present at some stage of straw decomposition in the soil, through evidence of lignin breakdown obtained by thermochemolysis. There was preferential decomposition of syringyl compared with guaiacyl units, and lignin decay occurred via oxidative Cα-Cβ bond cleavage, suggesting white-rot fungi of the Basidiomycota had been present, even though not isolated by us at this stage of decay. Gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis more normally used for analysing chemical changes in laboratory-controlled studies of fungal decay of wheat straw, has been demonstrated as being very useful to follow fungal decomposition in samples collected from the field. The degradation of macromolecular structures, in particular lignin, is of significant importance to the global carbon cycle. Given that only limited amounts of
field-based data are available currently, the results of our study provide cautious support to the theory that white-rot fungi are involved in the degradation of this highly resistant carbon source in grasslands (e.g. Thorn et al., 1996; Gramms, 1997; Deacon et al., 2006; Lynch and Thorn, 2006; Robinson et al., 2009), even in carbon-poor ecosystems.

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2.6. References


Chapter 3

Paper 2. A comparison between two different fungi and their ability to degrade lignin and cellulose in various substrata.

This chapter contains the following paper which is in preparation to be submitted to the *Journal of Organic Geochemistry*

Rachel N.T.M. Kabuyah, Pippa Jane Mason, Clare H. Robinson and Bart E. van Dongen. A comparison between two different fungi and their ability to degrade lignin and cellulose in various substrata.
Abstract

Plant biomass is the greatest single important component of the global carbon cycle. Within plant biomass there are compound classes, such as lignin, that are relatively resistant to decay by the microbial/fungal community present if compared with other compound classes, such as cellulose. White rot fungi are generally believed to be the only organisms to completely degrade lignin. However, recent analyses of fungi obtained from field samples in which clear lignin degradation was observed indicated an absence of these fungi and an abundance of soft-rot fungi. It remains unclear if these soft-rot fungi are capable of degrading lignin completely. In the present microcosm study, these isolated fungi were inoculated into organic substrates including wheat, barley straw, hawthorn wood chips and cellulose to test if these can decompose lignin and/or cellulose. A combination of ergosterol and lignin and cellulose analyses using gas chromatography-mass spectrometry (GC-MS) with TMAH thermodemolysis indicated that these fungi are able to degrade cellulose possibly occurring in conjunction with a slower rate of lignin modification but are not capable to degrade lignin completely. This highlights that although these fungi were isolated from wheat straw material in which clear lignin degradation was observed, they were unlikely to be directly involved in this process, indicating that other fungi which were not culturable were causing the degradation. This highlights the need for the use of other microbial techniques such as DNA analysis in order to obtain a more reliable representation of the fungal community involved in cellulose/lignin degradation.

3.1. Introduction

Soil organic matter (SOM) is one of largest carbon (C) reservoirs, storing 1,580 Gt C, often in rapid exchange with atmospheric CO₂, holding for instance three times more carbon than that held in land plant biomass (Hall et al., 2003; Schimel, 1994). SOM holds important nutrients (e.g. N, P) needed for both plants and microorganisms. It is made up of unrecognisable (partly-decayed) plant residues, soil microorganisms, soil fauna and the by-products of decomposition, producing humic substances in a process called humification (Adler 1966; Swift et al., 1979). However, the role of fungi in the degradation of organic matter remains understudied. Within plant biomass, there are three main macromolecule components that make up the cell wall; hemicellulose, cellulose and lignin, all present in varying amounts. Cellulose is the most abundant plant compound, accounting for a significant 40-60% of plant
biomass (Paul and Clark, 1996). Its structure is composed of several glucose molecules linked together by β (1-4) linkages forming an unbranched D-glucan chain. The chains form a crystalline lattice that is held together by hydrogen bonds, forming cellulose microfibrils (Swift et al., 1979). These microfibrils are a combination of elongated elementary fibrils that form rope like structures (Frey-Wyssling, 1989). The function of cellulose is to strengthen the cell wall and to allow water to flow in and out of the cell.

Lignin is an integral part of the secondary walls of plants, providing plants with strength to stand upright and facilitate water transport. It is found in the trachea elements and sclerenchyma of terrestrial plants. The structure is based on a phenyl propanoid unit with an aromatic ring and a 3-carbon (3-C) side chain (Frey-Wyssling, 1989). The complex structure of lignin causes its recalcitrance to chemical decay (Hedges et al., 1985).

Many organisms can break down plant matter; these include bacteria, algae, protozoa and fungi. Both bacteria and fungi can degrade cellulose, although it is thought that fungi are the most effective (Beguin, 1990). However, it is known that fungi are the only organisms capable of decaying completely the most resilient component of plant biomass, lignin (Eriksson, 1981; Schwarze et al., 2000; Webster and Weber, 2007). Thus fungi are fundamental to the soil C cycle, because of their ability to mineralise organic carbon. There are different stages of plant matter decay: firstly the rapid degradation of readily decomposable material such as sugars, cellulose, followed by the degradation of the more resistant lignin material (Frankland, 1992).

The hyphae of fungi are very important to the degradation of lignin and cellulose. These hyphae release enzymes (e.g. glycosidases such as amylase and phenol oxidase and peroxidase enzymes; Domsch et al., 1980) which penetrate and degrade the cell wall (Swift et al., 1979). Soft-rot are known to be some of the key decomposers in wood decay.

Soft-rot fungi decay (hemi) cellulose but there is much debate as to whether they are capable of only modifying the structure of lignin or degrading it completely (Eriksson et al., 1990; Schwarze 2007). It is generally thought that soft-rot fungi can only modify the structure of lignin (Eriksson et al., 1990; Schwarze 2007). These fungi move into the cell wall through pits or bore holes of adjacent cells, which allows them to begin attacking the middle lamella (Webster and Weber, 2007). The hyphae release the enzymes cellulase and hemicellulase which degrade cellulose and hemicellulose respectively. In the case of cellulase, cellulase causes the breakdown of bonds resulting
in the formation of short chain cellulose molecules, which are more easily decayed (Cowling, 1961; Carlie, 2004). Soft-rot fungi leave only a matrix of lignin, causing the wood to shrink and lose its strength. It is for this reason that the wood forms cubes and eventually crumbles to a brown powder. It remains to be seen if soft-rot fungi are capable of completely degrading lignin.

White-rot is a kind of wood decay that is principally caused by basidiomycetes and ascomycetes (Webster and Weber, 2007). These fungi are known to produce polyphenol oxidising enzymes such as laccase, lignin peroxidase and manganese peroxidase, which cause the degradation of lignin (Schwarze, 2000). The hyphae release reactive free radicals of oxygen that react with lignin which break down the covalent bonds and release phenolic compounds (Carlie, 2004). The order in which lignin and cellulose are decayed differs greatly depending on the fungi and the structure of the cell wall. There are two important forms of white-rot. One describes the simultaneous decay of both lignin and cellulose and the other is selective delignification, where there is a preference over which component is decayed first (Blanchette, 1984; Advaskaveg, 1986; Rayner & Boddy, 1988; Schwarze et al., 2000). Selective delignification breaks down lignin more rapidly than cellulose, removing the protective layer. Once this has protective layer has been decomposed, fungi can easily degrade the rest of the cell wall (Eriksson et al., 1990). The degradation of lignin does not provide enough energy to support fungal growth and the further degradation of the cell wall. Consequently, nitrogen, from the soil is needed to allow the fungi to have enough energy to decay lignin and cellulose.

After decay, a white fibrous residue (known as white-rot) is left on the wood.

Early methods of quantifying fungal growth in wood decay (Mille-Lindblom et al., 2004; Lau et al., 2006) have been conducted through the counting of cultivated colonies. The results from such techniques can be misleading and cause over- or under-estimations and are largely influenced by the analyser’s subjectivity (Domsch et al., 1980; Stahl et al., 1995; Pasanen et al., 1999; Lau et al., 2006; Gors et al., 2007). Ergosterol has been highlighted as a useful biomarker for fungal growth (Robertson et al., 2008). It is an important and abundant membrane sterol found mainly in filamentous fungi. It is also found in some microalgae, protozoa and yeasts. However, it is not a sterol found in vascular plants (Zhao et al., 2005; Robertson et al. 2008). Despite the increasing use of ergosterol as a fungal biomarker, its use as such has caused a large degree of controversy. In a series of papers, the usefulness of ergosterol as an appropriate biomarker for fungal biomass was discussed. Zhao et al. (2005) suggested
that it could only be used as an indicator of the presence of fungal biomass in soil, rather than the quantity. However this finding was criticised by Young et al. (2006), who argued that the data presented in the paper by Zhao et al. (2005) did in fact support the use of ergosterol as a reliable indicator of living fungal biomass, going on to say that it can be used as a robust and relatively inexpensive technique. Robertson et al. (2008) and Stahl and Parkin (1996) both conducted studies, showing this to be the case by using ergosterol as a biomarker to quantify fungal biomass. This current study is focused around techniques and principles as stated in Robertson et al. (2008).

Many different approaches, including those discussed above, have been taken to study the structure of the fungal community. It is probably unknown what proportion of saprotrophic fungi is unculturable, but to circumvent the inability to quantify non-culturable fungi, traditional culture-based techniques are being re-inforced with molecular methods that study either nucleic acids or some cellular components (e.g. phospholipid fatty acids; PLFA; Borneman & Hartin, 2000). Chitin, the main component of the cell walls of fungi has recently emerged as a significant player in the activation and attenuation of immune responses to fungi and other chitin-containing parasites (Lenardon et al., 2010). Therefore, in addition to ergosterol, PLFAs and chitin can also be used as biomass proxies.

Based on the current information about the fungal degradation of lignin and cellulose, the general aim of the present study is to increase further our understanding of the role that different fungi, particularly soft-rot fungi, play in the degradation of lignocellulose. More specifically, to test if different types of fungi can degrade lignin and/or cellulose under laboratory conditions. A long term microcosm experiment (300 days) was set up to determine the effects of different fungal species on various types of natural substrata containing lignin and cellulose, i.e. *Triticum aestivum* var. Swatham (wheat straw), *Hordeum vulgare* (barley straw), *Crataegus monogyna* (hawthorn wood chips) and pure cellulose. For this the different fungi were first cultured/grown on potato dextrose agar medium (PDA) and barley. Fungal growth was monitored using ergosterol as a fungal biomarker but since, the microcosms were set up using relatively low amounts of material, the method by Robertson et al. (2008) was modified and tested to enable it to deal with these low concentrations.
3.2. Methods

3.2.1. Culturing of the fungi

Three different types of fungi were selected: Soft-rot fungi *Absidia cylindrospora* (putative lignin modifier), *Trichoderma koningii* (putative cellulose decomposer; Domsch et al., 1980; Harper & Lynch, 1985; Schwarze et al., 2007) and *Calvatia utriformis* (putative lignin decomposer). The first two were isolated from field samples collected in Ainsdale National Nature Reserve, (Kabuyah et al., 2012; Chapter 2, section 2.3.1) while the *Calvatia utriformis* (*C. utriformis*), was isolated from a named culture collection in Utrecht University, Netherlands. These fungi were selected because they were frequently isolated (*A. cylindrospora* and *T. koningii*) and because basidiomata of *C. utriformis* were frequently observed in the field, together with their capability to degrade different carbon substrates in plant biomass. Sub-cultures of the fungi grown on potato dextrose agar medium (PDA from Oxoid Limited, Basingstoke, UK) for inoculation of the barley fragments were followed visually. Two out of the three subcultures, *Absidia cylindrospora* and *Trichoderma koningii*, formed pure cultures, however *Calvatia utriformis* was contaminated at the initial sub-culture on PDA (Fig. 3.1). This meant that agar discs of this fungus could not be transferred onto the barley fragments. The initial sub-culture of *Calvatia utriformis* was re-grown three times. Unfortunately, it was not possible to obtain a suitable pure culture of *Calvatia utriformis* and we were forced to continue running the microcosm experiment without it.

Both *A. cylindrospora* and *T. koningii* initial sub-cultures grew well and were used to inoculate the barley fragments. It was possible to see the fungal growth for both species, highlighted in red in Fig. 3.2, on the barley straw fragments. The controls (i.e. barley straw fragments not inoculated with fungi, Fig. 3.2) did not show any obvious fungal colonisation.
Figure 3.1. Photograph of contaminated culture (left) and clean, pure culture (right) of *C. utriformis*.

Figure 3.2. Petri dishes with fungi on barley straw fragments (a) with *Absidia cylindrospora*, (b) with *Trichoderma koningii* growing and (c) barley straw fragments with no fungi growing on them used as a control. Red squares show visible growth of the fungus.
Barley fragments, rather than intact straws, were needed to increase the surface area of organic matter. This was done to increase the likelihood of colonization of the plant substrata by the inoculated fungi. These fragments were prepared by hand-blending barley straw, using a Braun Aromatic KSM2 coffee-grinder for 60 seconds and placing 0.8 g of the fragments into 55 mm Petri dishes. Uninoculated barley fragments were used as a laboratory control. The inoculated barley fragments in the 55 mm Petri dishes were then incubated for 6 weeks in darkness at 15°C, until the majority of the barley fragments were colonised. The vials were hydrated using Gilson pipettes with 400-600 μl of sterile water and left to soak overnight. They were then inoculated with 2 fragments of colonised barley straw from single species of fungi using sterile flamed forceps and needle and remained in the 15°C incubator until sampling time.

To ensure that all the samples were sterile before inoculation all the 3.5 ml glass vials containing various organic matter and Petri dishes filled with barley straw fragments, were sent to be gamma-irradiated in heat sealed Ziploc bags at 25-35 kGy (kilograys) by the company Isotron in Swindon. From the original cultures, grown on PDA (Appendix A) and species identity confirmed by DNA analysis, 4.5 mm diameter discs of agar were transferred on to sterile barley fragments and incubated for up to 300 days (Fig.3.3).

3.2.2. Microcosm experiment

Fungal growth was carried out on four plant substrata Triticum aestivum var. Swatham (wheat) straw, Hordeum vulgare (barley straw), Crataegus monogyna (hawthorn) and pure cellulose. The straws of each species were washed in tap water and oven dried overnight at 40°C. The ears, nodes and leaves were removed and the internodes collected for use. The wheat straw internodes, barley straw internodes and hawthorn wood chips were ground up separately using a ball mill, for 15 minutes. Ground aliquots (0.2 g) of each carbon source were placed into 3.5 ml vials, with individual vials used for each sampling point (Robertson et al., 2008). Each glass vial (3.5 ml) was set up using a pre-furnaced spatula and a pair of forceps with 0.2 g of homogenised organic material and 2 fragments of colonised barley straw. These were all hydrated with 400-600 μl of sterile water at the start of microcosm experiment before being placed into the 15°C incubator until they were removed at specific sampling times. In total, thirty-six vials were set up per species and at each sampling time (T1 at 5 days, T2
at 90 days and T3 at 300 days after incubation) three vials for each of the plant substrata were removed, and the contents homogenised, freeze dried and analysed as described below. Controls with no fungi added to the plant substrata were included and sampled at the same time as those samples inoculated with fungi.

Figure 3.3. Flow chart showing the methodology. T1 is at 5 days, T2 at 90 days and T3 at 300 days of incubation at 15 °C. Pre-conditions were that all samples were gamma-irradiated including all the samples vials with various plant substrata and all the barley
fragments used. Cultures grown on Potato Dextrose Agar (PDA) medium were subject to DNA identity tests to ensure that the correct fungi were isolated. Blanks were run through the GC/MS after every batch of 10 samples to ensure there was no contamination in the laboratory.

3.2.3. Ergosterol extraction

The extraction of ergosterol was modified using the methods originally defined by Robertson et al. (2008), Stahl and Parkin (1996) and Eash et al. (1996; Appendix B). To summarise, 50 mg aliquots of the homogenized, colonised plant substrata (e. g. wheat straw colonised by T. koningii from the original vials that were inoculated) were weighed into 7 ml screw topped vials. Distilled water (DCM extracted); 0.4 ml 15 MΩcm, an internal standard (5α-androstan-3β-ol; 20 μl; 0.52 mg/ml in dichloromethane), methanol (2 ml) and 0.8 ml sodium hydroxide in ethanol (0.8 g solid sodium hydroxide in 20 ml 95% v/v ethanol/water) were added. The vials were ultrasonically agitated for 5 minutes in an ultrasonic bath, heated for 2 h at 85°C, and cooled immediately by immersion in cold water. The water layers were washed three times with hexane and the combined hexane layers were blown down under a stream of dry nitrogen and dried over sodium sulphate. The extracts were dissolved in 30-50 μl N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, purum 99%) and heated (70 °C; 60 min) to convert the alcohols into their trimethylsilyl ethers. The derivatised extracts were analysed using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Blanks were run to ensure that no contamination was introduced during the extraction and derivatisation procedure. GC/MS was performed using an Agilent 7890A GC, equipped with an Agilent 7683B auto-sampler and programmable temperature vaporization (PTV; 280°C) inlet, interfaced to an Agilent 5975C MSD mass spectrometer operating with electron ionisation at 70 eV. The samples were analysed in simultaneous scan (scanning from m/z 50 to 650 at 2.7 scans second⁻¹) and sim mode (take m/z values 333, 337 and 363) with a solvent delay of 25 minutes. The heated interface temperature was set at 280°C with the mass source temperature at 230°C and the MS quadrapole at 150°C. Analyses were performed using an HP-5 MS column (J+W Scientific; 5% diphenyl-dimethylpolysiloxane; length 30 m, I.D. 250 μm, film thickness 0.25 μm) capillary column. The samples were run at constant pressure (12.942 psi) with helium as the carrier gas. The oven temperature was programmed
from 70°C to 130°C with 20°C min⁻¹, then to 300°C with 4°C min⁻¹, where it was kept for 10 minutes.

A conversion factor is needed to get a true value for the concentration of ergosterol. For this one sample with clear peaks in both the sim and scan mode was run 5 times to get an average area for ergosterol and the internal standard. A conversion factor was calculated for each replicate for both the internal standard and ergosterol areas, and a mean and standard error for the three replicates was taken.

There were large numbers of mass fragments present; however only a few were unique to ergosterol and not found in the other sterols present in the biomass. The key mass fragments of the mass spectrum m/z 363 and 337 (as seen in Fig. 3.4) were not observed in the other sterols, which makes them clear to identify in sim mode (Fig. 3.5). This is the same for the internal standard, where 333 is a unique m/z value. Blank extractions confirmed that no ergosterol was introduced during the handling of the samples. In order to calculate a concentration of ergosterol from the sim mode, a conversion factor from the sim/scan mode was needed. The conversion factors, obtained by comparison of scan and sim data for five samples, were comparable, 53.5 ± 1.7 for ergosterol and 11.2 ± 0.1 for the internal standard.
Figure 3.4. Mass spectra showing $m/z$ values for (A) Ergosterol and (B) Internal standard (5α-androstan-3β-ol). See figure 3.6. for structures of sterols.
Figure 3.5. Chromatograms showing the peaks for ergosterol and the internal standard (5α-androstan-3β-ol) in scan and sim mode. II denotes ergosterol-TMS and IS the internal standard. Full names and other sterols can be found in Figure 3.6.

3.2.6. Lignin and cellulose analysis

Aliquots (approximately 0.1 mg) were analysed in triplicate using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Prior to analysis, 4 μl of the hydrocarbon 5α-androstane internal standard (100 μl of a 0.256 mg ml⁻¹ solution in
dichloromethane) and 5 μl tetramethylammonium hydroxide (TMAH) were added to each sample. To avoid problems because of different TMH conditions (e.g. temperature and/or time; Klingberg et al., 2005) all samples were analysed under the same conditions. Samples were pyrolysed using a chemical data system (CDS) 5200 series pyroprobe pyrolysis unit by heating at 600°C for 10 seconds to fragment macromolecular organic components. Fragments were analysed using an Agilent 7890A fitted with HP-5 fused capillary column (J+W Scientific; 5% diphenyl-dimethylpolysiloxane; 30 m length, 0.32 m internal diameter, 0.25 μm film thickness) coupled to an Agilent 5975 MSD single quadrupole mass spectrometer operating in electron ionisation (EI) mode (scanning a range of m/z 50 to 600 at 2.7 scans second-1; ionisation energy 70 eV). The pyrolysis transfer line and injector temperatures were set at 350°C, the heated interface at 300°C, the EI source at 230°C and the MS quadrupole at 150°C. Helium was used as the carrier gas and the compounds were introduced in split mode (split ratio 40:1). The oven was programmed from 40°C (held for 2 min) to 220°C at 2.5°C min⁻¹, held at this temperature for 1 min before being heated to 300°C at 20°C min⁻¹ and held at this temperature for 11 min. Compounds were identified by comparison of spectra with those reported in the literature (Vane et al., 2001). The naming of the THM products follows the conventions established previously (Clifford et al., 1995; Hatcher et al., 1995). Because of the limited amount of sample available, as a result of decay in the field, it was only possible to analyse the samples in duplicate for the majority of the bait bags (from the upper horizon at 5 cm, sampling points 0.5, 4.5, 6.5, 8.5 m and from the lower horizon at 11 cm, sampling points 0.5, 2.5, 6.5 and 8.5 m). In case of the sample from the upper horizon at 2.5 m there was only enough material available for a single analysis.

The lignin acid/aldehyde [AcAl]₅ parameter is a relative decomposition state proxy for the syringyl lignin monomer which was measured using the peak areas of 3,4,5-trimethoxybenzoic acid, methyl ester (S6) and 3,4,5-trimethoxybenzaldehyde (S4; Fig. 3.1; Vane et al., 2001). The acid/aldehyde [Ac/Al]₅ parameter, which is also a relative decomposition state proxy for the guaiacyl lignin monomer was measured using peak areas of 3,4-dimethoxybenzoic acid, methyl ester (G6) and 3,4- dimethoxybenzaldehyde (G4). The syringyl/guaiacyl ratio (S/G) in THM products from wheat straw buried at Ainsdale sand dunes describes a relative intensity ratio of TMAH thermochemolysis products. It was calculated by dividing the sum of the peak areas from syringyl derivatives (S4+S5+S6) by the sum of the peak areas from their guaiacyl counterparts
(G4+G5+G6; Mason et al., 2009; Fig.3.8). The same [Ac/Al]s, [Ac/Al]G and [S/G] ratios were used in this study as those used in the Ainsdale field study for comparison purposes. These analyses were run 5 times and the average ratios obtained were used as a baseline for determining the extent of fungal degradation of the wheat straw samples collected from the Ainsdale field site after burial in soil for 46 months.

To determine the relative contribution of cellulose present, common cellulose markers (moieties) seen in all the chromatograms were identified according to Gauthier et al (2003). Six abundant moieties (Guaiacol, 1,4-Dimethoxybenzene, 2,6-Dimethoxytoluene, 4-Methoxybenzaldehyde, 3,5-Dimethoxyphenol and 1,2,3-Trimethoxy-5-methylbenzene) were selected and the relative peak areas were determined. To calculate the relative amounts of cellulose to lignin within the initial plant substrata and after the 300 day microcosm experiment the [cellulose:cellulose+lignin] ratios were determined using the sum of these six selected cellulose markers and six lignin markers (G4, G5, G6, S4, S5 and S6).

3.3. Results

3.3.1. Ergosterol analysis

GC-MS analysis indicated the presence of a range of sterols, including ergosterol (II, Figs 3.5 and 3.6) in all samples analysed with sitosterol (IV) being the most abundant homologue. Other sterols present included cholesterol (I, Fig. 3.6), stigmasterol (III, Fig. 3.6) and, campesterol (V, Fig. 3.6).

Quantification indicated that the ergosterol amounts varied amongst the four different homogenised carbon substrata used in this study and over time (Table 3.1). Focusing on the ergosterol concentration of fungi on Triticum aestivum var. Swatham, T. koningii over the total incubation period showed an initial increase in the amount of ergosterol present from 0.7 ± 0.4 to 29.3 ± 27.2 µg g⁻¹ followed by a significant decrease between 90 and 300+ days to 2.0 ± 0.1 µg g⁻¹ (Table 3.1, Fig. 3.7a).

A. cylindrospora started with a low concentration of ergosterol with 0.2 ± 0.1 µg g⁻¹ at 5 days, followed by a clear increase to 22.1± 6.1 µg g⁻¹ and then a drop to 4.2 ± 1.2 µg g⁻¹ at the end of the microcosm experiment. The same trend with an initial concentration of 0.7 ± 0.5 µg g⁻¹ followed by a steep increase to 42.2 ± 21 µg g⁻¹ and drastic drop to 6.3 ± 4 µg g⁻¹ at the end of the microcosm experiment was observed for wheat straw which was not inoculated with any fungi.
Absidia cylindrospora and T. koningii growing on Hordeum vulgare (barley straw) displayed the same trend as when growing on the wheat straw samples: a sharp increase during the first 90 days of incubation (from 0.6 ± 0.1 to 10.3 ± 10.3 µg g⁻¹ and 1.1 ± 0.1 to 11.0 ± 9.7 µg g⁻¹ for A. cylindrospora and T. koningii respectively. Towards the last days of incubation steady decreases in ergosterol amounts were detected (Table 3.1, Fig. 3.7b). The controls followed this trend with an equally sharp increase from 0.5 ± 0.1 to 15.7 ± 12.9 µg g⁻¹ within the first 90 days and a sharp decrease to 3.7 µg g⁻¹ at the end of the experiment.

![Figure 3.6](image.png)

Figure 3.6. Structures of sterols. IS-androstanol, I-cholesterol, II- ergosterol, III-stigmasterol, IV-sitosterol and V- campesterol (Barajas-Aceves et al., 2002).

Crataegus monogyna (hawthorn) wood chips inoculated with the fungus T. koningii showed relatively high amounts of ergosterol at the end of the 300 day incubation period with average levels of 5.0 ± 0.4 µg g⁻¹. Markedly low amounts were observed for hawthorn that was not inoculated with fungi. A. cylindrospora showed ergosterol levels that increased sharply from non-detectable to average levels of 30.9 ± 15.8 µg g⁻¹ during the first 90 days, followed by an equally sharp decrease (to 0.8 ± 0.1 µg g⁻¹) towards the end of the incubation period (Table 3.1, Fig. 3.7c).
Amounts of ergosterol for fungi colonising the pure cellulose substrate were notably different from those for fungi colonising the other three plant substrata. Ergosterol in the *T. koningii* treatment was not detectable at the start of the incubation period. However, ergosterol increased more than threefold throughout the remaining incubation period (to an average concentration of 27.1 ± 9.2 µg g⁻¹). A time lag was observed, followed by a gradual increase, for cellulose inoculated with *A. cylindrospora* (Table 3.1, Fig.3.7d). Minimal levels of ergosterol were noted for samples with no inoculated fungi throughout the incubation period (levels did not exceed 1.0 µg g⁻¹).
Table 3.1. Ergosterol concentrations (µg g\(^{-1}\)) across three time points (5, 90 and 300 days with means and standard errors) for the different fungi cultured on varying plant substrata. Mean concentrations were taken from three replicate samples. Note: BDL indicates that the ergosterol amounts were below detection limits.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Fungi</th>
<th>Ergosterol Concentration (µg g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>A. cylindrospora</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>T. koningii</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>No fungi</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Barley straw</td>
<td>A. cylindrospora</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>T. koningii</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>No fungi</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Hawthorn</td>
<td>A. cylindrospora</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>T. koningii</td>
<td>0.1 ± 0</td>
</tr>
<tr>
<td></td>
<td>No fungi</td>
<td>BDL</td>
</tr>
<tr>
<td>Cellulose</td>
<td>A. cylindrospora</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>T. koningii</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>No fungi</td>
<td>BDL</td>
</tr>
</tbody>
</table>
Figure 3.7. Graphs showing the ergosterol amounts (mg ergosterol/g freeze-dried solids) measured during cultivation of *Absidia cylindrospora* and *Trichoderma koningii* over the 300 day period on (a) wheat straw, (b) barley straw, (c) hawthorn wood chips and (d) pure cellulose.
3.3.2. Lignin and cellulose analysis

Gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis analysis produces chromatograms with numerous peaks representing a suite of THM products. Six lignin products (G4, G5, G6, S4, S5, S6) were chosen for this study and peak areas of these were calculated to obtain acid aldehyde ratios \([\text{Ac/Al}]_S\) and \([\text{Ac/Al}]_G\) and the \([S/G]\) ratio. A typical partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from a control sample (not inoculated with any fungi) with pure cellulose is shown in Figure 3.8 as an example and peak assignments of these cellulose and lignin products are listed in Table 3.2. Although values varied, no significant increase or decrease was observed for the lignin proxies \([\text{Ac/Al}]_G\), \([\text{Ac/Al}]_S\) and \([S/G]\) ratios in wheat straw, barley straw and hawthorn wood chips inoculated with both *A. cylindrospora* and *T. koningii* (Fig.3.9).

![Figure 3.8](image)

Figure 3.8. Partial chromatogram of the total ion current (TIC) for the TMAH thermochemolysis products from a control sample (not inoculated with any fungi) with pure cellulose. Peak assignments are listed in Table 2. The peak labels in blue are the six cellulose markers and red show the six lignin makers that were selected to calculate the [cellulose:cellulose+lignin] and IS denotes the internal standard 5α-androstane (Clifford et al., 1995; Gauthier et al., 2003; Vane et al., 2001).
Table 3.2. List of TMAH thermochemolysis products (Vane et al., 2001; Gauthier et al. 2003). Labels 1-18 are characteristic cellulose markers and IS denoted the Internal standard used in this microcosm experiment. G denotes a breakdown product of guaiacyl and S denotes a breakdown product of syringyl.

<table>
<thead>
<tr>
<th>Label</th>
<th>Assignment</th>
<th>Characteristic ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Furaldehyde</td>
<td>96, 95, 39</td>
</tr>
<tr>
<td>2</td>
<td>Butyroacetone</td>
<td>42, 41, 28</td>
</tr>
<tr>
<td>3</td>
<td>2-Methoxytoluene</td>
<td>122, 107, 77</td>
</tr>
<tr>
<td>4</td>
<td>3-methyl-1,2,4-cyclopentanetrione</td>
<td>126, 83, 56</td>
</tr>
<tr>
<td>5</td>
<td>Guaiacol</td>
<td>109, 124, 81</td>
</tr>
<tr>
<td>6</td>
<td>1,4-Dimethoxybenzene</td>
<td>123, 138, 95</td>
</tr>
<tr>
<td>7</td>
<td>3-Methoxycatechol</td>
<td>140, 125, 97</td>
</tr>
<tr>
<td>8</td>
<td>2,6-Dimethoxytoluene</td>
<td>152, 121, 77</td>
</tr>
<tr>
<td>9</td>
<td>4-Methoxybenzaldehyde</td>
<td>135, 136, 77</td>
</tr>
<tr>
<td>10</td>
<td>1,4-Dimethoxy-2-methylbenzene</td>
<td>137, 152, 109</td>
</tr>
<tr>
<td>11</td>
<td>3,5-Dimethoxyphenol</td>
<td>154, 125, 94</td>
</tr>
<tr>
<td>12</td>
<td>1,2,3-Trimethoxybenzene</td>
<td>168, 153, 110</td>
</tr>
<tr>
<td>13</td>
<td>1,2,3-Trimethoxy-5-methylbenzene</td>
<td>182, 167, 139</td>
</tr>
<tr>
<td>14</td>
<td>Dimethoxybenzene (a)</td>
<td>138, 109, 78</td>
</tr>
<tr>
<td>15</td>
<td>Dimethoxybenzene (b)</td>
<td>138, 109, 78</td>
</tr>
<tr>
<td>16</td>
<td>Dimethyl-naphthalene (a)</td>
<td>156, 141, 125</td>
</tr>
<tr>
<td>17</td>
<td>Dimethyl-naphthalene (b)</td>
<td>156, 141, 125</td>
</tr>
<tr>
<td>18</td>
<td>Levoglucosan</td>
<td>60, 57, 73</td>
</tr>
<tr>
<td>IS</td>
<td>Androstane, (5α)-</td>
<td>245, 260, 95</td>
</tr>
<tr>
<td>G4</td>
<td>3,4-Dimethoxybenzaldehyde</td>
<td>151, 165, 166</td>
</tr>
<tr>
<td>G5</td>
<td>3,4-Dimethoxyacetophenone</td>
<td>137, 165, 180</td>
</tr>
<tr>
<td>G6</td>
<td>Methyl 3,4-dimethoxybenzoate</td>
<td>165, 181, 196</td>
</tr>
<tr>
<td>S4</td>
<td>3,4,5-Trimethoxybenzaldehyde</td>
<td>125, 181, 196</td>
</tr>
<tr>
<td>S5</td>
<td>3,4,5-Trimethoxyacetophenone</td>
<td>195, 210, 139</td>
</tr>
<tr>
<td>S6</td>
<td>Methyl 3,4,5-trimethoxybenzoate</td>
<td>226, 211, 195</td>
</tr>
</tbody>
</table>
Figure 3.9. (a), (b), (c) Mean ± S.E., n = 3, 3, 4, 5-trimethoxybenzoic acid, methyl ester (S6) to 3, 4, 5-trimethoxybenzaldehyde (S4) Acid/aldehyde ratio ([Ac/Al]S), (d), (e), (f) Mean ± S.E., n = 3, 3, 4-dimethoxybenzoic acid, methyl ester (G6) to 3, 4-dimethoxybenzaldehyde (G4), [Ac/Al]G and (g), (h), (i) the mean ± S.E., n = 3, relative amounts of syringyl derivatives (S4+S5+S6) to guaiacyl counterparts (G4+G5+G6), [S/G] (Mason et al., 2009) in THM products from the 300 day microcosm experiment of fungi inoculated with wheat straw, barley straw and hawthorn wood chips. The two different fungi used in the microcosm experiment are denoted in the key on the bottom left of this figure. Three sampling points were used at T1-5 days, T2-90 days and T3-300 days.

For comparison between cellulose and lignin degradation rates the abundance of the different moieties was determined using the [cellulose:cellulose+lignin] ratio (see section 3.26). The [cellulose:cellulose+lignin] ratios for the pure cellulose samples decreased slightly during the 300 day microcosm experiment from 0.93 to 0.76 (not shown). In contrast, in winter wheat straw *Triticum aestivum* var. Swatham, the original value of the [cellulose:cellulose+lignin] decreased from 0.8 to 0.3, giving a value of 0.26 for wheat straw inoculated with *T. koningii* and 0.36 for wheat straw inoculated with *A. cylindrospora* (Fig. 3.10). A similar original ratio of 0.83 was observed for *Hordeum vulgare* (barley straw) with a decrease to 0.33 for barley straw inoculated with *T. koningii* and 0.51 for *A. cylindrospora* over 300 days of incubation (Fig. 3.10). *Crataegus monogyna* (hawthorn) wood chips gave a ratio of 0.85 at the start of the incubation period. *T. koningii* produced a ratio of 0.34 and *A. cylindrospora* a ratio of 0.49 (Fig. 3.10).
3.4. Discussion

3.4.1. Ergosterol as marker for fungal growth

There has been much debate on the use of ergosterol to quantify fungal biomass in growth experiments. Zhao et al. (2005) argued that ergosterol only represents part of the picture and that other methods need to be used simultaneously to measure fungal biomass accurately. They suggested that ergosterol concentrations vary depending on the fungal species and other factors such as age and temperature of incubation.

However, Robertson et al. (2008) and Stahl and Parkin (1996) both conducted studies showing that ergosterol can be used as a marker to quantify fungal growth. The results
from present study indicate that when a pure substrate was present that can be used by the fungi, such as cellulose, significant shifts in ergosterol concentration can be observed over time in line with these previous studies. This was, for instance, especially well demonstrated by *T. koningii* growing on pure cellulose where ergosterol concentrations increased, being below detection limit after the first 5 days to approximately 27.1 µg g⁻¹ at 300 days (Table 3.1, Fig. 3.7d). Because of the relatively large size of the experiment and the relatively small-scale of the resources available, some modifications were made to the method in order to downsize it, when compared with the Robertson et al. (2008) experiments. Less original material was used: 50 mg here compared with 250 mg used in the Robertson et al. (2008) study. This was because of the small amounts of the original material available. Consequently, there was a lower biomarker signal for all the samples analysed. It was for this reason that the GC-MS was run in scan/sim mode lowering the detection limit, to extenuate the peaks and obtain a better signal to noise ratio. This is clearly shown in our results as a concentration of ergosterol was detected after 5 days, whereas Robertson et al. (2008) believed that there was no significant fungal growth before 21 days of incubation, when grown on wheat straw. They define significant fungal growth as concentrations above 100 µg g⁻¹. The cellulose experiments highlight that this is not a problem when pure substrates are used.

In contrast, the results of the experiments using the plant substrata were more complicated. The results from the ergosterol analysis showed that there were large standard errors on the replicates especially for wheat straw at 5 days (*A. cylindrospora* with 0.2 ± 0.1 µg g⁻¹ *T. koningii* with 0.7 ± 0.4 µg g⁻¹ and the control sample with 0.7 ± 0.5 µg g⁻¹) and barley straw at 90 days (10.3 ± 10.3 µg g⁻¹). This was most likely caused by the fact that the plant substrata used were all sourced from the field, with little control over the growth conditions. Despite gamma-irradiating the samples before hand, designed to kill any living fungi and microorganism, the initial large differences observed between replicates of the same treatment will most likely be caused by the differences in the absence of uniformity in the starting material. Robertson et al. (2008) and Stahl and Parkin (1996) already highlighted this as a possible limitation in the use of ergosterol as a biomarker for fungal growth and our results confirm this. In addition, it is possible that partial fungal degradation had already occurred on the source material prior to its arrival in the lab. Although these fungi will have been killed during the gamma-irradiation, the ergosterol produced would not have been destroyed and as such
could cause a larger error between triplicate analyses. To reduce this potential error source material was rigorously homogenised using a ball mill, which was seen as suitable and sufficient for the starting material used in our study. However, homogenisation of solids can be difficult compared with that of liquids, therefore increasing the variability in the replicate samples.

However, even taking the errors in to consideration, the results indicated a substantial increase in ergosterol over the first 90 days confirming the growth of both *A. cylindrospora* and *T. koningii* in all four plant substrata (Fig.3.7). Between 90 and 300 days, there was a substantial drop in ergosterol concentrations indicating that that the fungi were no longer growing, most likely since they are not able to degrade the organic material present/left at that stage.

3.4.2. Degradation of organic carbon sources present

There are two major carbon sources present that the fungi can use during these microcosm experiments: lignin and cellulose. Comparison of the [cellulose:cellulose+lignin] ratios indicated a substantial drop during the 300 day microcosm experiments for both *T. koningii* and *A. cylindrospora* (Fig.3.10). This showed that cellulose was preferentially degraded, in line with previous studies that have shown that cellulose is generally faster degraded if compared with lignin (Carlile et al., 2001; Dungait et al., 2008). A relatively small drop for [cellulose:cellulose+lignin] ratio was also observed when pure cellulose was used but this is most likely due to the use of barley straw during the inoculation procedure.

*T. koningii* and *A. cylindrospora* are both soft-rot fungi and as such are assumed to be able to degrade cellulose and modify lignin, by ligninolytic enzymes, but not completely degrade the lignin (Carlile et al., 2001; Goñi et al., 1993; Schwarze & Baum 2000). No substantial shift in any of the relative lignin decomposition state proxies [Ac/Al]_S, [Ac/Al]_G, and relative intensity ratio [S/G] were observed when both fungi were used confirming that these fungi were indeed unable to completely decompose lignin. This suggests that if the ‘bioavailable’ cellulose was completely degraded during the microcosm experiments, the growth rate of the fungi slowed down/stopped reducing the production of ergosterol. This could explain the drops in ergosterol observed between the 90 and 300 days during this experiment (Fig 3.7).

These results indicate that although both fungi were isolated from wheat straw material in which clear lignin degradation was observed (Kabuyah et al., 2012) and were actually
two of the most abundant fungi present (see chapter 2, section 2.3.1) they were perhaps not directly involved in the initial degradation of this lignin. This confirms that other fungi, most likely basidiomycetes (Eriksson, 1981; Frankland, 1992) are degrading the lignin in situ. It further highlights, that the results of culturing experiments could be misleading since the key fungi might not be culturable. This suggests that other microbial techniques such as direct DNA analysis are needed to get a reliable representation of the fungal community involved in cellulose/lignin degradation.

3.5. Conclusions
Microcosm experiments using a range of soft-rot fungi collected from the field, inoculated into organic substrates including wheat, barley straw, hawthorn wood chips and cellulose indicated that these fungi were able to decompose cellulose, possibly occurring in conjunction with a slower rate of lignin modification, but are not capable to degrade lignin completely. This is confirmed by a combination of ergosterol and lignin and cellulose analyses using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis.

Although these fungi were isolated from wheat straw material in which clear lignin degradation was observed they were perhaps not directly involved in the initial degradation of this lignin, indicating that other fungi which were not culturable were involved in this degradation. This highlights the need for the use of other microbial techniques such as direct DNA analysis in order to get a more reliable representation of the fungal community involved in cellulose/lignin degradation.

Acknowledgments
We are extremely grateful to Mr & Mrs Kabuyah for sponsoring this study and to Drs Jack Fisher and John Cornick who kindly provided the wheat and barley straw.
3.6. References


Chapter 4


This chapter contains the following paper which is in preparation to be submitted to the Journal of Organic Geochemistry

Rachel N. M. Kabuyah, Bart E. van Dongen and Stephen W. Njoka, Clare H. Robinson. Decomposition of wheat straw in a tropical grassland soil.
Abstract

Macromolecular structures such as lignin and cellulose are important components of soil organic carbon, the major terrestrial global carbon pool. However, the involvement of fungi in the decomposition of both lignin and cellulose remains unclear, particularly in tropical environments. The extent to which cellulose and lignin in wheat (*Triticum aestivum* var. Swatham) straw buried in a tropical grassland soil was modified was examined using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Shifts in the [Ac/Al]$_S$, [Ac/Al]$_G$ and [S/G] lignin decomposition state proxies indicate substantial degradation probably induced by white-rot fungi over a 12 month period, but comparison of presence/absence of lignin and cellulose moieties indicates that the cellulose was degraded substantially faster than lignin. The results of the present study, when compared with those obtained from a more temperate area (Ainsdale, UK), provide further support for the theory that higher rates of decomposition of lignin and cellulose may be observed in tropical/wetter areas if compared with temperate/drier areas. With the current global warming, the temperatures as well as the annual precipitation in these temperate regions are likely to increase. Given the higher relative decomposition rates of organic matter in soil in tropical environments, this climate change could potentially lead to higher decomposition rates in these regions, which could have major consequences for the global carbon cycle.

4.1. Introduction

Soil is the largest store of terrestrial organic carbon, holding 1,580 Gt of carbon (Schimel, 1993). In particular the decomposition of organic matter, which greatly determines soil properties, needs to be understood better. The breakdown of the complex cell wall component lignin is of great importance, as lignin accounts for an estimated 30% of organic carbon in the biosphere (Boerjan et al., 2003). Its decomposition is known to involve fungi, in particular, white-rot basidiomycete fungi which are the only organisms known to mineralise lignin completely (Kirk and Farrell, 1987), however the degradation process which produces cinnamic and precursor coumaric acids remains unclear. The decomposition of cellulose is similarly significant as the cellulose compound built of glucose molecules makes up the most abundant macromolecular compound on Earth, comprising a significant proportion of cell walls (Erikssson, 1981). Its decomposition is vital to microorganisms such as fungi as it provides them with a reliable source of energy and nutrition. Previous lignin
experiments have shown that lignin can be degraded (Filley et al., 2000; Mason et al., 2009; Robertson et al., 2008) in temperate climates, however far less is known about degradation of lignin in tropical environments where higher temperatures and increase average rainfall may create more favourable conditions for this degradation. Therefore, wheat straw, which has been suggested as a model resource due to its composition to study the effect of fungi on decomposition (Harper and Lynch, 1981; Kabuyah et al., 2012) has been used here again to determine the decomposition of lignin and cellulose at a grassland site in Kenya, East Africa.

Previously, many field studies conducted in Kenya have focused on soil fertility with regards to farming practices in this region, with the hope of improving crop yields and thus economic benefits (Mucheru-Muna et al., 2010). In particular there appear to be no comparative studies to date between temperate and topical grasslands specifically about fungal decay of organic matter. Many studies have been based on intercropping, alleviation of soil fertility constraints and particularly nitrogen availability and fixation (Cadisch & Giller, 1997; Nambiar et al., 1983) with regards to tropical African soils. They, however, have mainly been based on field sites in west (Rhodes et al., 1996) and southern Africa (Ncube et al., 2007) giving us another reason to look more closely at soil and plant substrata decomposition in Eastern African soils. Again, the resistance of lignin to decomposition prompted us to investigate to what extent a change in climate (tropical versus temperate climate) would have on the decomposition of the same winter wheat straw (Triticum aestivum var. Swatham) which had been buried in temperate UK soils (Kabuyah et al., 2012).

Pyrolysis GC-MS in the presence of tetramethylammonium hydroxide (TMAH) has been used as a method to characterise lignin degradation (Kabuyah et al., 2012). It involves the use of a thermal “hammer” which breaks down large, heterogeneous macromolecules such as lignin and cellulose into products. This allows them then to be transported to the gas chromatogram (as they are now GC-amenable) where organics are separated, and onto the mass spectrometer where compounds are identified and quantified.

As Embu is located on the foothills of Mt Kenya (the highest mountain in Kenya), the soils in this area are volcanic and very rich in nutrients, a fact which is in contrast to the sand dunes found in Ainsdale (Kabuyah et al., 2012) where the soils are nutrient-poor.
For this reason, as well as the ease of accessibility to the Kenya Agricultural Research Institute (KARI), we chose the field site described below for our comparative study. Thus, the aim of this study was to characterise lignin degradation, particularly with respect to lignin oxidation typical of white-rot basidiomycete fungi, in a tropical African grassland environment. This will be accomplished by comparing lignin breakdown products in the initial straw with those in the decomposed straw samples, collected 12 months later, using tetramethylammonium hydroxide (TMAH) thermochemolysis. The results will be compared with those from a recent field study carried out in Ainsdale sand dunes, UK (Kabuyah et al., 2012), which would allow us to understand better the environmental factors such as soil type and temperature on the decomposition of lignin/organic matter.

The aims of this study are to (i) repeat the field experiment as set out in the UK in relation to a Kenyan grassland site (in Embu District), (ii) investigate the impact, if any, that soil characteristics may have on decomposition of organic matter in the Kenyan compared with the UK field site, (iii) determine the extent of cellulose decomposition in the Kenyan soils, (iv) correlate the results of the Kenyan and UK field experiments to understand better the differences in the degradation process of wheat straw in temperate compared to tropical environments.

4.2. Methods

4.2.1. Site description and field sampling

The study site was at the Kenya Agricultural Research Institute (KARI) in Embu district, Kenya. This site is located in the central highlands of Kenya and is found within the Eastern Province of the country (Fig. 4.1). It occupies a total area of 708 square kilometres. It lies 3 km north of Embu town and is located on latitude 0°30’S and longitude 37° 27’E at an elevation of 1480 m above sea level (Jaetzold et al., 2006) and is found on the southeastern slopes of Mount Kenya that is the most prominent physical feature found in the region. The rainfall pattern in this area is bimodal with two distinct rainy seasons: a long rain season between March and June and a short rain season between October and December. The total amount of precipitation in this area averages between 1000 to 1600 mm per year, but varies with altitude. Temperatures range from a minimum of 12°C in July to maximum of 27.1°C in March (Jaetzold et al., 2006). Typically the soils in this area are mainly Typic Palehhumult (humic Nitisols according to Mucheru-muna et al., 2010) derived from basic volcanic rocks. These soils
are found in warm temperate zones and support vegetation. They are deep, highly weathered with friable clay texture and moderate to high inherent fertility.

Forty bait bags were prepared with each bait bag being 15 x 20 mm, made of 50 μm mesh and containing five internodes of winter wheat straw *Triticum aestivum* var. Swatham. These internodes had been split longitudinally and cut to size 5 x 15 mm, with each bag attached to an orange nylon string and metal tag to facilitate collection (Fig.2). The mesh size reduced plant root colonisation and access to soil fauna, but still allowed microbial colonisation, increasing the likelihood of basidiomycete fungi reaching the straw and therefore utilising the refractory lignin compound (present as lignocellulose). The samples were buried on the 28th of April 2010 at depths of 5 and 11 cm from the soil surface, along a transect. No clear soil horizons were observed at the study site but the same soil depths were maintained as in Chapter 2 for comparative purposes between this and the previous UK study (Fig.4.2 top photo; Kabuyah et al., 2012). Along the transect, there were five bait bags in each upper and lower horizon placed at specific distances (0.5 m, 2.5 m, 4.5 m, 6.5 m, and 8.5 m) from the point of origin.

On April 30th 2011, 12 months after burial to allow for a rainy season giving us a justifiable account of the decomposition pattern over a year, nominally 10 bait bags were to be collected. However, the bags along the transect at 0.5, 4.5 and 8.5 m distance, 5 cm depth, could not be retrieved and the area within the soil where these samples were buried only consisted of bait bag remains, none of which could be used adequately for analysis. This meant that 7 bait bags were collected in total, three from the upper horizon and four from the lower horizon, which were essentially treated as replicate bags within each horizon (i.e. horizontal distances of the samples from the point of origin were not used subsequently in the analyses). Bait bag samples were taken from both depths, placed in envelopes, then into Ziploc bags to prevent the straw from drying out. Soil samples (ranging from 4.8 to 6.2 g in weight) for chemical analyses, were taken immediately adjacent to the location of each bag from 5 and 11 cm depths. Both bait bag and soil samples were carefully folded into individually pre-furnaced, labelled aluminium envelopes to prevent contamination.
Figure 4.1. Map of study area in Embu district (indicated by the star) at the Kenya Agricultural Research Institute (KARI; latitude 0°30’S and longitude 37° 27’E) in central Kenya, East Africa.
Figure 4.2. (A) Photograph of bait bag samples at two chosen depths of 5 and 11 cm below the soil surface (KARI in Embu, Kenya). Bait bags were attached to tent pegs (to ease the process of maintaining the depth at which they were initially buried) .Bait bag samples were made of nylon mesh bags (15 x 20 mm) containing 5 x 15 mm pieces of winter wheat straw (*Triticum aestivum* var. Swatham) (B) Original straw before burial in soil, (C) 12 months later, (D) completely decomposed bait bag sample 15 months after burial at the Embu, Kenya field site.

All samples were then stored in a -20 °C freezer before transportation to the UK in a cool box where they were again stored in a -20°C freezer, and were thawed before
analysis. In addition, an attempt to collect ten bait bag samples was made in the same way 15 months from initial burial on the 20th of July 2011 which were not found.

4.2.2. Soil analyses
The soil samples were oven-dried at 40°C overnight to remove the excess water and ion chromatography of water extracts was carried out the next day. This was done by weighing 5 g of soil from each of the 10 samples and diluting in 15 ml of de-ionised (18.2 MΩ H₂O) water to make a slurry. The samples, in individually labelled plastic vials, were then filtered using a 13 mm Pall filter (0.2 micron, 10 nm diameter to remove bacteria). Blanks were run first, then all ten slurry samples were analysed. Each soil sample and blank was placed in an ultrasonic bath for 30 minutes before analysis. A colorimeter was first calibrated with a cuvette containing the control solution of deionised water. It was used to measure the ammonium in our soil samples by measuring the absorbance of a specific wavelength of light (420 nm). Extractable NH₄⁺, Cl⁻, NO₃⁻, PO₄³⁻, SO₄²⁻ were obtained using water extractions, and in these water extracts cations, Ca²⁺, Mg²⁺, Na⁺ were obtained. High performance liquid chromatography was used to separate the compounds that were dissolved in the soil solutions. These compounds were separated by injecting 600 μl of each sample mixture onto the column. The different components of the mixture then passed through the column at different rates because of the differences in their partitioning behaviour between the mobile, liquid phase and the stationary phase. Soil pH was measured using indicator paper. The Total Organic Carbon (TOC) of all ten soil samples was calculated using the loss-on-ignition technique where empty crucibles were initially weighed then soil samples were weighed and separately placed into the crucibles. These were then placed into a furnace at 110 °C for one hour, weighed after heating and placed back into the furnace at 1000 °C and weighed again. The percentage weight loss (water loss) was then calculated at each stage to determine the TOC.

4.2.3. On-line thermochemolysis
Samples, approximately 1 mg, were analysed by normal flash gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Prior to analysis, 4 μl of the hydrocarbon 5α-androstane internal standard (100 μl of a 0.256 mg ml⁻¹ solution in dichloromethane) and 5 μl tetramethylammonium hydroxide (TMAH) were added to each sample. To avoid problems caused by difference in thermally assisted hydrolysis
and methylation (TMH) conditions (e.g. temperature and/or time; Klingberg et al., 2005) all samples were analysed under the same conditions. Samples were pyrolysed using a chemical data system (CDS) 5200 series pyroprobe pyrolysis unit by heating at 600 °C for 10 seconds to fragment macromolecular organic components. Fragments were analysed using an Agilent 7890A fitted with HP-5 fused capillary column (J+W Scientific; 5% diphenyl-dimethylpolysiloxane; 30 m length, 0.32 m internal diameter, 0.25 μm film thickness) coupled to an Agilent 5975 MSD single quadrupole mass spectrometer operating in electron ionisation (EI) mode (scanning a range of m/z 50 to 600 at 2.7 scans second-1206; ionisation energy 70 eV). The pyrolysis transfer line and injector temperatures were set at 350 °C, the heated interface at 300 °C, the EI source at 230 °C and the MS quadrupole at 150 °C. Helium was used as the carrier gas and the compounds were introduced in split mode (split ratio 40:1). The oven was programmed from 40°C (held for 2 min) to 220 °C at 2.5 °C min⁻¹, held at this temperature for 1 min before being heated to 300 °C at 20 °C min⁻¹ and held at this temperature for 11 minutes. Compounds were identified by comparison of spectra with those reported in the literature (Vane et al., 2001). The naming of the THM products follows the conventions established previously (Clifford et al., 1995; Hatcher et al., 1995).

4.2. Lignin parameters and nomenclature

The acid/aldehyde [AcAl]₆ parameter is a relative decomposition state proxy for the syringyl lignin monomer which was measured using the peak areas of 3,4,5-trimethoxybenzoic acid, methyl ester (S₆) and 3,4,5-trimethoxybenzaldehyde (S₄; Fig.4.3; Vane et al., 2001). The acid/aldehyde [Ac/Al]₀ parameter, which is also a relative decomposition state proxy for the guaiacyl lignin monomer, was measured using peak areas of 3,4-dimethoxybenzoic acid, methyl ester (G₆) and 3,4-dimethoxybenzaldehyde (G₄). The syringyl/guaiacyl ratio (S/G) in THM products from wheat straw buried at Ainsdale sand dunes (Kabuyah et al., 2012) describes a relative intensity ratio of TMAH thermochemolysis products. It was calculated by dividing the sum of the peak areas from syringyl derivatives (S₄+S₅+S₆) by the sum of the peak areas from their guaiacyl counterparts (G₄+G₅+G₆; Mason et al., 2009). Mean ratios of duplicate analyses, for [Ac/Al]₆, [Ac/Al]₀ and S/G could be calculated for each sample point. In addition, for comparison, the original pre-burial wheat straws were cut, weighed (ca. 0.1 mg) and analysed in a similar manner to those collected upon degradation. These analyses, which were the same as those used in the Ainsdale UK
study (for comparative purposes) were run 5 times and the average ratios obtained were used as a baseline for determining the extent of fungal degradation of the wheat straw samples collected from Embu after burial in soil for 12 months. Cellulose analysis in the samples was carried out by selecting six common markers (products) seen in all the chromatograms. Peak areas of the following cellulose products (according to Gauthier et al., 2003) were measured; (i) Guaiacol, (ii) 1,4-Dimethoxybenzene, (iii) 2,6-Dimethoxytoluene, (iv) 4-Methoxybenzaldehyde, (v) 3,5-Dimethoxyphenol and (vi) 1,2,3-Trimethoxy-5-methylbenzene. Ratios were determined to quantify the change in cellulose levels during the 300 day microcosm experiment by using the sum of the six selected cellulose markers and six lignin markers to calculate the [cellulose:cellulose+lignin] ratio for us to compare the relative amounts of cellulose to lignin used.

4.3. Results

4.3.1. Soil characterisation

The soils had a reddish brown to dark brown colour and a clay loam texture composed of sand, silt, humus and clay at both upper and lower soil horizons (Fig. 2a). Concentrations (mean ± S.E., n =3) of soil cations were 107 ± 6 and 94.1 ± 7.7 µg g⁻¹ for ammonium (NH₄⁺), 48.1 ± 4 and 42.3 ± 0.9 µg g⁻¹ for sodium (Na⁺), 128 ±14 and 110 ± 10 µg g⁻¹ for calcium (Ca²⁺), 46.1 ± 4.94 and 41.3 ± 3.8 µg g⁻¹ for magnesium (Mg²⁺) in soil samples collected from the upper and lower soil depths respectively. Soil anions had concentrations of 45.6 ± 4.9 and 43.2 ± 6.4 µg g⁻¹ for chloride (Cl⁻), 54.9 ± 3.7 and 62.6 ± 5.2 µg g⁻¹ for nitrate (NO₃⁻), 184 ± 13 and 161 ± 17 µg g⁻¹ for sulfate (SO₄²⁻) in soil samples collected from the upper and lower soil depths respectively, however concentrations of phosphate (PO₄³⁻) were <0.02 µg g⁻¹ which was below the detection limit at both soil horizons (Table 4.1). A pH value of 4 (acidic) was observed for all the ten soil samples. There are relative high amounts of native soil organic carbon at the site, as illustrated by the total organic carbon (TOC) which ranged from 11.9% ± 0.6% (n=5) in the lower soil horizon at 11 cm depth to 13.0% ± 0.9% (n=5) in the upper horizon at 5 cm depth (Table 4.1).
Table 4.1. Concentrations (in $\mu$g g$^{-1}$) of water-extracted cations and anions and Total Organic Carbon (TOC) in soil samples adjacent to each bait bag sample collected on April 30th 2011 from Embu field site. Note: All samples for phosphate ($\text{PO}_4^{3-}$) produced values of <0.02 $\mu$g g$^{-1}$ and were classified as below detection limit (BDL).

<table>
<thead>
<tr>
<th></th>
<th>Upper soil depth ($n=5$)</th>
<th>Lower soil depth ($n=5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NH}_4^+$</td>
<td>107 ± 6</td>
<td>94.1 ± 7.7</td>
</tr>
<tr>
<td>$\text{Na}^+$</td>
<td>48.1 ± 4</td>
<td>42.3 ± 0.9</td>
</tr>
<tr>
<td>$\text{Ca}^{2+}$</td>
<td>128 ±14</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>$\text{Mg}^{2+}$</td>
<td>46.1 ± 4.94</td>
<td>41.3 ± 3.8</td>
</tr>
<tr>
<td>$\text{Cl}^-$</td>
<td>45.6 ± 4.9</td>
<td>43.2 ± 6.4</td>
</tr>
<tr>
<td>$\text{NO}_3^-$</td>
<td>54.9 ± 3.7</td>
<td>62.6 ± 5.2</td>
</tr>
<tr>
<td>$\text{PO}_4^{3-}$</td>
<td>BDL (&lt;0.02)</td>
<td>BDL (&lt;0.02)</td>
</tr>
<tr>
<td>$\text{SO}_4^{2-}$</td>
<td>184 ± 13</td>
<td>161 ± 17</td>
</tr>
<tr>
<td>TOC</td>
<td>13.0% ± 0.9%</td>
<td>11.9% ± 0.6%</td>
</tr>
</tbody>
</table>
4.3.2 Lignin and cellulose analyses

To determine lignin degradation of the *T. aestivum* straw, the samples prior to being buried, and those that were collected 12 months after burial in Embu, were analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. Similar to previous studies (Filley et al., 2000; 2006; Gauthier et al., 2003; Mason et al., 2009; Robertson et al., 2008) a suite of THM products was identified (Fig. 4.3, 4.4, Table 4.2). The original pyrolysate clearly showed typical lignin (G4, G5, G6, S4, S5, S6, P18, G14, G15, G18, S14, S15) and cellulose (1-18) products as described in Table 4.2. In contrast, analyses of samples collected 12 months after burial produced clear peaks characteristic of lignin products whereas cellulose moieties were all below the detection limit and therefore not quantifiable. The original wheat straw material prior to burial produced \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and S/G ratios of 3.3 ± 0.8, 0.4 ± 0.15 and 3.3 ± 0.3 respectively. In the recovered material, the range of values for the same ratios were 2.9 to 21.7, 0.29 to 8.13 and 0.31 to 0.69 respectively with average ratios of 10.1 ± 1.67 for the \([\text{Ac/Al}]_S\), 2.45 ± 0.69 for the \([\text{Ac/Al}]_G\), and 0.47 ± 0.05 for the S/G ratio (Fig. 4.3). Therefore over the 12 months of decomposition, \([\text{Ac/Al}]_S\) and \([\text{Ac/Al}]_G\) values clearly increased with one or two values falling within the original straw ratio boundary, and S/G values were distinctly lower than the original values (Fig.4.3). The [cellulose:cellulose+lignin] ratio for the original wheat straw material was 0.85 ± 0.012, but could not be detected after 12 months as it gave a ratio of 0.

Because of the limited amount of sample available, as a result of rapid decay in the field, it was only possible to analyse the samples in duplicate for the majority of the bait bags (from the upper horizon at 5 cm, sampling points 2.5, 4.5, 6.5 m and from the lower horizon at 11 cm, sampling points 0.5, 2.5, 6.5 and 8.5 m). In the case of the sample from the lower horizon at 0.5 m there was only enough material available for a single analysis. The samples collected 15 months from initial burial on the 20th of July 2011 were degraded to a point where the bags were torn open, as a result of extensive degradation, and their contents lost within the soil, therefore we could not obtain samples to validate a third sampling point (Fig. 4.2c).
Figure 4.3. (A) Partial ion chromatogram from the original wheat straw in a bait bag sample before burial on 28th April 2010 (original straw analyses) with labelled lignin (red) and cellulose (blue) products and (B) partial ion chromatogram from a bait bag sample collected 12 months after burial on April 30th 2011 with only lignin products labelled. IS = internal standard and peak assignments of characteristic TMAH thermochemolysis products is listed in Table 4.2.
Table 4.2. List of TMAH thermochemolysis products (Vane et al., 2001; Gauthier et al. 2003). Labels 1-18 are characteristic cellulose markers and IS denoted the Internal standard 5α-androstane. G denotes a breakdown product of guaiacyl and S denotes a breakdown product of syringyl.

<table>
<thead>
<tr>
<th>Label</th>
<th>Assignment</th>
<th>Characteristic ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Furaldehyde</td>
<td>96,95,39</td>
</tr>
<tr>
<td>2</td>
<td>Butyrolactone</td>
<td>42,41,28</td>
</tr>
<tr>
<td>3</td>
<td>2-Methoxytoluene</td>
<td>122,107,77</td>
</tr>
<tr>
<td>4</td>
<td>3-methyl-1,2,4-cyclopentanetrione</td>
<td>126,83,56</td>
</tr>
<tr>
<td>5</td>
<td>Guaiacol</td>
<td>109,124,81</td>
</tr>
<tr>
<td>6</td>
<td>1,4-Dimethoxybenzene</td>
<td>123,138,95</td>
</tr>
<tr>
<td>7</td>
<td>3-Methoxycatechol</td>
<td>140,125,97</td>
</tr>
<tr>
<td>8</td>
<td>2,6-Dimethoxytoluene</td>
<td>152,121,77</td>
</tr>
<tr>
<td>9</td>
<td>4-Methoxybenzaldehyde</td>
<td>135,136,77</td>
</tr>
<tr>
<td>10</td>
<td>1,4-Dimethoxy-2-methylbenzene</td>
<td>137,152,109</td>
</tr>
<tr>
<td>11</td>
<td>3,5-Dimethoxyphenol</td>
<td>154,125,94</td>
</tr>
<tr>
<td>12</td>
<td>1,2,3-Trimethoxybenzene</td>
<td>168, 153,110</td>
</tr>
<tr>
<td>13</td>
<td>1,2,3-Trimethoxy-5-methylbenzene</td>
<td>182,167,139</td>
</tr>
<tr>
<td>14 (B)</td>
<td>Dimethoxybenzene (a)</td>
<td>138,109,78</td>
</tr>
<tr>
<td>15 (C)</td>
<td>Dimethoxybenzene (b)</td>
<td>138,109,78</td>
</tr>
<tr>
<td>16 (D)</td>
<td>Dimethyl-naphthalene (a)</td>
<td>156,141,125</td>
</tr>
<tr>
<td>17 (E)</td>
<td>Dimethyl-naphthalene (b)</td>
<td>156,141,125</td>
</tr>
<tr>
<td>18 (H)</td>
<td>Levoglucosan</td>
<td>60,57,73</td>
</tr>
<tr>
<td>IS</td>
<td>Androstane, (5α)-</td>
<td>245,260,95</td>
</tr>
<tr>
<td>G4</td>
<td>3,4-Dimethoxybenzaldehyde</td>
<td>151,165,166</td>
</tr>
<tr>
<td>G5</td>
<td>3,4-Dimethoxyacetophenone</td>
<td>137,165,180</td>
</tr>
<tr>
<td>G6</td>
<td>Methyl 3,4-dimethoxybenzoate</td>
<td>165,181,196</td>
</tr>
<tr>
<td>S4</td>
<td>3,4,5-Trimethoxybenzaldehyde</td>
<td>125,181,196</td>
</tr>
<tr>
<td>S5</td>
<td>3,4,5-Trimethoxyacetophenone</td>
<td>195,210,139</td>
</tr>
<tr>
<td>S6</td>
<td>Methyl 3,4,5-trimethoxybenzoate</td>
<td>226,211,195</td>
</tr>
<tr>
<td>P18</td>
<td>trans-3-(4-Methoxyphenyl)-3-propenoate</td>
<td>161, 192, 133</td>
</tr>
</tbody>
</table>
4.4. Discussion

4.4.1. Lignin and cellulose degradation

Similar to other studies using THM with TMAH to investigate lignin decomposition by fungi, a suite of THM products were identified and their distributions changed over the 12 months during which the bait bags, containing the wheat straw, were buried in the soil (Vane et al., 2001; Filley et al., 2000; Kabuyah et al., 2012). All lignin ratios previously used to monitor lignin degradation such as the [Ac/Al]$_S$, [Ac/Al]$_G$ and [S/G] ratios (Dungait et al., 2008; Filley et al., 2006; Kabuyah et al., 2012; Mason et al., 2009; Nierop & Filley, 2008; Robertson et al., 2008) showed a substantial shift between T0 and T1. The increase in the [Ac/Al]$_S$ and [Ac/Al]$_G$ observed in this study (Fig.4.4) is indicative of lignin oxidation in syringyl and guaiacyl moieties respectively in line with previous observations in microcosm experiments (Mason et al., 2009; Robertson et al., 2008) and field studies such as the Ainsdale UK field study (Kabuyah et al., 2012, Chapter 2). The greater increase in the [Ac/Al]$_S$ is most likely because syringyl units are often assumed to be much more susceptible to degradation than guaiacyl (Kirk and Farrell, 1987; Schwarze, 2007).

The [S/G] showed a substantial decrease between T0 and T1. This ratio has been reported to decrease when lignin is decayed by white-rot fungi (Pal et al., 1995; Vane et al., 2001) indicating that white-rot fungi were indeed most likely present in Embu soils and involved in decomposition of the organic matter present, although the specific fungal species involved are not identified.
Figure 4.4. (A) 3, 4, 5-trimethoxybenzoic acid, methyl ester (S6) to 3, 4, 5-trimethoxybenzaldehyde (S4) acid/aldehyde ratio ([Ac/Al]₆), (B) 3, 4-dimethoxybenzoic acid, methyl ester (G6) to 3, 4- dimethoxybenzaldehyde (G4), [Ac/Al]₆ and (C) the relative amounts of syringyl derivatives (S₄+S₅+S₆) to guaiacyl counterparts (G₄+G₅+G₆), [S/G] (Mason et al., 2009) in THM products from wheat straw buried at the Embu field site in Kenya collected after 12 months in April 30th 2011. The rectangle dashed box indicates the original ratios of the buried wheat straw based on 5 measurements. O = upper soil horizon (5 cm depth); ▲= lower soil horizon (11 cm depth).
It has previously been shown that lignin is degraded slower than cellulose by the degradation rates of cellulose and lignin annually, where 20% of cellulose in plant litter remains after a year whereas 75% of lignin in the same plant litter remains after a year (Kabuyah et al., 2012; Minderman et al., 1968). In this present study, besides low levels of lignin being detected from the remains of the bait bag samples after 12 months, only trace amounts of cellulose were present in the retrieved samples as the cellulose peaks were generally below detection limit causing the [cellulose:cellulose+lignin] to drop from 0.85 ± 0.012 to 0. The fact that cellulose was no longer detectable confirms the slower degradation rate of lignin compared with cellulose and is in line with previous studies (Carlile et al., 2001; Dungait et al., 2008). In order to be better be able to determine lignin versus cellulose degradation rates a more frequent sampling regime will be required.

Only 7 samples could be retrieved a year later as the rest had been completely decomposed and we were unable to locate the straw remains within the disintegrated mesh bags. The cellulose products labelled 1-18 (Fig.4.3) and described in Table 4.2 were all below the detection limit and therefore were not quantifiable. However the six selected lignin products G4, G5, G6, S4, S5, S6 were quantifiable in these same seven samples collected 12 months after burial. This was expected and indicates that although the degradation of all organic matter was relatively fast that of cellulose was even faster. A possible explanation for this may be that cellulose in the wheat straw was bio-available in greater amounts before the bait bag samples were buried in the soil and was completely broken down and quickly used as a source of energy and nutrition by the fungi present in the soil at the beginning of the 12 month burial period, and can therefore no longer be detected at the end of the 12 months. We can deduce this from the [cellulose:cellulose+lignin] ratio which was 0.85 ± 0.012 in the original straw material and was not quantifiable in the retrieved straw material (also see Chapter 3, section 3.4.2).

4.4.2. A comparison of lignin degradation in a temperate and tropical environment

Considering that exactly the same organic material (winter wheat straw Triticum aestivum var. Swatham) and the same method of preparation, storage and analysis of soil, bait bag samples and their contents were used in a previous study performed at the Ainsdale site (UK; Kabuyah et al., 2012), as in the present study, a comparison of the
relative lignin decomposition state proxies, \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and relative intensity ratio \([S/G]\) is possible. Compared with the Ainsdale study in the UK, where both the \([\text{Ac/Al}]_S\) and \([\text{Ac/Al}]_G\) increased over the 4 year field study, the results in present study show a similar trend.

In Ainsdale the ranges of \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and \([S/G]\) were 3.2 -10.8, 0.38-1.76 and 0.67-2.49 respectively, whereas at the Embu field site the ranges for the same ratios were 2.9-21.7, 0.29-8.13 (both substantially wider than those observed in UK) and an \([S/G]\) ratio with values from 0.31-0.69 which was a smaller range than that seen at Ainsdale. This may be because fewer samples (seven compared to nine) were retrieved from the Embu site. In contrast to the Ainsdale study (Kabuyah et al., 2012), where it took several years to observe clear visual degradation of the wheat straw, it only took 12 months for this to occur in our Embu field site. When we returned to collect a second batch of samples 15 months later (on July 30th 2011), the bait bag contents had been completely decomposed with only remains of the bags left (Fig. 4.2), therefore we were not able to salvage any samples for analysis.

Typically the soils are mainly Typic Palehumults which are well drained soils with an enrichment of organic carbon, often producing a dark colour (see Fig. 4.2). These are particularly found in the mountainous areas of low latitudes such as our field site located at the foot of Mt. Kenya, 1480 m above sea level (Jaetzold et al., 2006). Higher levels of \(\text{NH}_4^+\), \(\text{Ca}^{2+}\) and \(\text{SO}_4^{2-}\) were observed at both soil layers compared to those at the UK site (Kabuyah et al., 2012; Table 4.1). Furthermore, there are higher amounts of native soil organic matter observed in present study: in the upper soil horizon at 5 cm depth there was 13% total organic carbon (TOC) (standard error of 0.92, \(n=5\)) and in the lower horizon at a depth of 11 cm there was 11.9% TOC (standard error 0.57, \(n=5\)). This may be because the fact that Ainsdale soils are carbon-poor, being derived from sand, whereas Embu soils are rich in organic carbon. This supports earlier observations where we see similar changes in decomposition of wheat straw in the shorter 12 month field study in Kenya as in a 46 month period at Ainsdale in the UK. Increased temperature and rainfall, particularly during the long rains between March and June at this Kenyan field site may have caused this substantial increase in the rate of lignin and cellulose degradation.

This high decomposition rate of lignin and cellulose, as a result of increased temperatures in temperate and tropical regions due to current global warming, may change the soil dynamics in the soil carbon pool and potentially, in the long term, have
implications on the carbon cycling within this global carbon pool (Connin et al., 1997; Hibbard et al., 2001; Archer et al., 2001; McCulley et al., 2004). It is clear that cellulose is degraded at a considerably faster rate than lignin. However, setting up this field experiment again with the same number of bait bag samples containing the same starting material of wheat straw, and conducting sampling and analysis regularly (i.e. at 3 month intervals) may greatly aid us in determining decomposition rates allowing us to conduct a clearer comparative study of fungal decomposition of wheat straw between temperate and tropical soils.

4.5. Conclusions
Field based experiments in a tropical environment indicated that lignin was degraded, potentially by white-rot fungi, as indicated by the shifts in the [Ac/Al]s, [Ac/Al]c and [S/G] relative lignin decomposition state proxies. Comparison of presence/absence of lignin and cellulose moieties indicated that the cellulose was degraded substantially faster over the 12 month period, confirming earlier observations. The results of the present study, when compared with those obtained from a more temperate area (Ainsdale, UK), provide further support for the theory that higher rates of decomposition of lignin and cellulose may be observed in tropical/wetter areas if compared with temperate/drier areas.

Acknowledgements
We are extremely grateful to Mr & Mrs Kabuyah for sponsoring this study. Drs Jack Fisher and John Cornick kindly provided the wheat straw and Dr Njoka provided access to the Kenya Agricultural Research Institute in Embu, Kenya.
4.6. References


Chapter 5: Conclusions and future work

5.1. Conclusions

Soil including organic matter, is the largest terrestrial component of the global carbon cycle, indicating that the mechanisms controlling carbon distribution from this pool will have a direct impact on carbon cycling. Within soil organic matter there are compound classes, such as lignin, that are relatively resistant to decay by the microbial/fungal community present if compared with other compound classes, such as cellulose.

The main objective of this work was to determine lignin and cellulose degradation in field experiments and compare these with the results of laboratory based microcosm experiments to determine to what extent fungi can degrade these important macromolecular components.

In summary, the results of this research showed that:

- Field-based experiments in both tropical (Embu, Kenya) and temperate (Ainsdale, UK) soils indicated that lignin can be degraded completely, most likely by white-rot fungi, as shown by the shifts in the [Ac/Al]s, [Ac/Al]c and [S/G] relative lignin decomposition state proxies as determined by Py-GC-MS in the presence of tetramethylammonium hydroxide. The results confirm that even in a very low carbon environment such as the sand dunes in Ainsdale, fungi are able to completely degrade lignin over time. However, the comparison with a tropical environment indicates that this degradation is much faster in the latter. This is most likely due to a combination of higher temperatures and/or increased precipitation.

- Culturing experiments showed that it was possible to isolate a number of fungi present on the degraded wheat straw collected in the field, especially soft-rot fungi. When used in microcosm experiments using a range of organic substrates, the relative lignin decomposition state proxies indicated that Absidia cylindrospora and Trichoderma koningii are not able to completely degrade lignin but preferentially degrade cellulose. This suggests that white-rot fungi, although not culturable from the field samples, actually caused the lignin degradation indicating that culturing experiments can be misleading if not confirmed by DNA analysis.
Based on the comparison of relative amounts of cellulose and lignin moieties in degraded field samples over time, we can deduce that although lignin is degraded, the cellulose decomposition rates are much higher confirming previous work.

5.2. Future work

Therefore the following suggestions for future research have been made:

1. To possibly confirm the presence of white-rot fungi in the temperate and tropical soils by conducting DNA analysis of fungi present on the wheat straw collected to determine better the effect that these fungi may have on degradation rates.

2. Setting up additional field experiments again with the same number of bait bag samples containing the same starting material of wheat straw and conducting sampling and analysis regularly (i.e. at 3 month intervals), which would help to determine further decomposition rates at the Embu field site and allow us to conduct a clearer comparative study of fungal decomposition of wheat straw between temperate and tropical soils including the effect of wet versus dry season degradation.

3. Conduct the same microcosm experiment, including white-rot fungi, at different temperatures, keeping all other aspects constant to facilitate a better understanding of the direct effect that temperature has on the decomposition of wheat straw, especially lignin, by fungi.

4. More interlinking of the results from our Ainsdale and Kenyan studies by having a closer look at the differences in soil characteristics (as described above), including moisture content of the soils and temperature logging at both study sites to further investigate other factors determining degradation rates in these two contrasting environments. Considering that the factors that could affect lignin decomposition rates in situ are relatively complex, the results from the Ainsdale and Kenyan studies can currently not be properly compared. Specifically, differences in soil characterisation such as concentrations of water extracted ions, pH, total organic carbon and the bio-availability of this organic carbon and not solely moisture content and temperature could have an (in) direct effect on the lignin degradation/alteration rates.

5. Additional study sites, both in the UK and Kenya could be set-up to further study variability between these different environments.
Appendices

Appendix (A): Culture media and formulae

All media were made using distilled (de-ionised) water (suppliers of chemicals were Sigma Aldrich and Oxoid).

Each agar media plate was filled with approximately 20 ml of medium poured into sterile 90 mm, non-vented Petri dishes. These were stored in a 10°C cold room until they were used.

The three individual agar media used were all autoclaved at 121°C for 15 minutes.

Modified Czapek-Dox agar medium (mCDA) with di-potassium hydrogen orthophosphate, yeast extract, glucose and Rose Bengal (Warcup, 1957).

This nutritionally rich agar medium was used to identify initially the fungi growing on the wheat straw. It is known to provide a hospitable environment, and reduce bacterial contamination on the isolation plates, for a wide range of fungi, including basidiomycete fungi.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>30.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>3.0</td>
</tr>
<tr>
<td>di-potassium hydrogen orthophosphate (K₂HPO₄)</td>
<td>1.0</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.01</td>
</tr>
<tr>
<td>Agar technical (No.3)</td>
<td>15.0</td>
</tr>
<tr>
<td>Rose Bengal (stock solution)</td>
<td>20.0 ml l⁻¹</td>
</tr>
</tbody>
</table>

The stock solution of Rose Bengal was made with 0.8375 g in 250 ml of distilled water. There were 6.7 x 10⁻⁵ g ml⁻¹ of Rose Bengal in 1 l of medium.

Lignin-guaiacol-benomyl agar medium (LGBA; Thorn et al., 1996).

This agar medium was used for the purpose of specifically isolating basidiomycete fungi.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>962.6 ml</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
NH₄NO₃ 0.1 g  
KCl 0.1 g  
FeSO₄·7H₂O 0.02 g  
Ca(NO₃)₂·4H₂O 0.05 g  
Malt extract 2.0 g  
Agar technical (No.3) 15 g

After autoclaving and cooling to 55 °C, the following were added with sterile equipment:

1M KOH 5 ml (5.6 g in 100 ml sterile water)  
Guaiacol 0.4 ml  
Lignin alkali (1.0 g suspended and partially dissolved in 10 ml 1,3 Dioxane)

*Chlortetracycline-HCL 60 mg  
*Streptomycin sulphate 30 mg  
*Penicillin G 30 mg  
*Benlate solution 2 ml

* from stock solutions:  

<table>
<thead>
<tr>
<th></th>
<th>Volume of solution used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlortetracyline-HCL</td>
<td>10 ml 0.6 g in 100 ml sterile water</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>10 ml 0.3 g in 100 ml sterile water</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10 ml 0.3 g in 100 ml sterile water</td>
</tr>
<tr>
<td>Benomyl (To make benlate solution use)</td>
<td>2 ml 0.2 g of benomyl in 50 ml acetone &amp; 50 ml 70% ethanol</td>
</tr>
</tbody>
</table>

**Malt extract agar medium**

This was used a general agar medium for the initial isolation of fungi found growing on the mCDA and LGBA agar media, making it the most used agar medium. Fifty grams of malt extract agar were dissolved in 1 l of distilled water.

Malt extract agar 50 g  
Distilled water 1000 ml
Appendix (B): Sample preparation method for ergosterol extraction

- Make 3 cultures of each of the 3 chosen fungi (*T. koningii, A. cylindrospora, C. utriformis*) onto PDA in 90 mm Petri dishes.

- For each inoculum microcosm, transfer a 4.5 mm diameter disc (using cork borer) of agar medium onto sterile barley fragments (0.8 g to fill up a 55 mm Petri dish) from an advancing margin.

- Wash wheat straw and barley in tap water and oven dry at 40 °C in oven overnight.

- Remove ears, nodes and leaves of barley and wheat straw and only use internodes

- Grind up (in ball mill for 15 minutes) wheat straw/ wood (hawthorn) chips/ barley. 72 vials per carbon source, 6 sampling points, therefore total number of vials to prepare is 432.

- Place 0.2 g (ground up, wheat straw/hawthorn wood chips/ pure cellulose) into pre-furnaced 3.5 ml vials with solid lids, using separate vials for each time point in the series (Robertson *et al.*, 2008), should fill 1/3 of each vial with material.

- Wheat straw, wood chips, barley, pure cellulose will be gamma-irradiated at 25-35 kGy (kilograys) by Isotron in Swindon to kill microbes *i.e.* sterilise them.

*Note 1:* Barley fragments which have been hand blended (using Braun Aromatic KSM2) for 60 seconds should also be gamma-irradiated in heat sealed zip loc bags.

Pre-microcosms

- Pre-microcosms, which contain irradiated hand blended barley in 55 mm Petri dishes will be set up in triplicate (total of 3 replicates for each of 3 fungal species and 3 blanks)

- Incubate these Petri dishes for 6 weeks in darkness at 15 °C, until a majority of the carbon source is colonised. This is to ensure the inoculum grows before inoculating the vials.

- Use Gilson pipettes to add drops of sterile water to maintain regular hydration of the samples from the time of incubation to the start of inoculation.

*Note 2:* Analyse the same amount (make 5 replicates of 0.2 g in 3.5 ml vial) of all four carbon sources before gamma-irradiation/ any form of sterilisation for a point of reference to determine what the effect, if any, of irradiation on carbon sources.
- Soak each vial in 400-600 μl (case of 3.5 ml vials) of sterile distilled water overnight in closed vial

- Cap the vials with matching irradiated solid lids

- Inoculated with 2 fragments, using sterile, flamed forceps, of the following fungi:
  1. *Absidia cylindrospora*
  3. *Trichoderma koningii*

3. No fungi ‘blank’- to determine if there is any abiotic breakdown of lignin and monitor contamination that may affect the results produced.

- Remove vials for analysis of Ergosterol and lignin/cellulose analysis (measure amounts produces using GC/MS) during fungi cultivation at the following times:

- At each sampling time, remove three samples for each carbon source, freeze dry them, take half of each sample for ergosterol analysis and other half for lignin analysis and place into separate sterilised (pre-furnaced) vials. Store vials in freezer when analysis is complete.

**Ergosterol extraction**

1. Take sample containing water, fungi and medium (cellulose, wheat straw, barley or hawthorn) at 15 °C.

2. Take vial and freeze at -20°C.

3. Freeze dry to lose water

4. Grind up to homogenize then take a sub-sample

5. Extract sub sample containing residue and extract
   - Weigh in homogenized, freeze dried samples (50 mg) into 7 ml screw topped vials (leave out this step when preparing a blank)
   - Hydrate with 0.4 ml 15M Ωcm distilled water (DCM extracted)
   - Add an aliquot (20 μl) of a 5α-androstan-3β-ol internal standard (0.52 mg/ml in dichloromethane) added to each 7 ml vial.
- 2 ml of methanol and 0.8 ml sodium hydroxide in ethanol (0.8 g solid sodium hydroxide in 20 ml 95% v/v ethanol/water) were added to each sample. 19 ml ethanol/1 ml water.

- Ultrasonically agitate samples for 5 min in an ultrasonic bath

- Vials heated for 2 h at 85 °C in block heater and cooled immediately by immersion in cold water

- n-Hexane (3x 1 ml) was added to each vial and vials were shaken for 2 minutes in shaker and left to sit for approximately 5 minutes before separation

- The hexane layer was transferred to a 3 ml vial and reduced in volume under a steam of dry nitrogen (do not blow down the sample completely)

- The hexane layer was run through a sodium sulphate column into another 3 ml vial.

- Blow down under a stream of nitrogen

- Add 30-50 μl of BSTFA, heat at 80 °C for 1 hour and blow down excess of BSTFA.

- Add 50 μl of Hexane to 3 ml vial and transfer to autosampler vial using a syringe

- Inject into GC-MS

- Two replicates were analysed for the three time points 5 days, 90 days and 300 days

Making of sodium hydroxide solution

The sodium hydroxide solution was made by adding 1 ml of water then 19 ml of ethanol to 0.8 g of solid sodium hydroxide pellets.
Appendix (C): Publication and conference contributions

Publication:

Conference contributions:


Decomposition of lignin in wheat straw in a sand-dune grassland

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Soft rot
Soil organic matter
Substrate utilisation
Syringyl
TMAH thermochemolysis
Triticum aestivum
Wheat straw
White rot

ABSTRACT

The degradation of organic macromolecules, including lignin, in plant-derived soil organic matter, is important to the global carbon cycle. In grasslands, saprotrophic (decomposer) fungi are major decomposers of such organic material. The aim of this study was to characterise lignin degradation, particularly with respect to lignin oxidation typical of white-rot basidiomycete fungi. Lignin breakdown products, analysed by gas chromatography–mass spectrometry (GC–MS) with TMAH thermochemolysis, in initial wheat (Triticum aestivum var. Swatham) straw samples were compared with those in samples which had been buried as a “model” resource for 46 months in a sand-dune grassland at Ainsdale National Nature Reserve, Lancashire, UK.

Our results showed that lignin oxidation occurred in the straw over the 46 month period, as there were general increases in the [Ac/Al]S and [Ac/Al]G ratios and a clear decrease in the [S/G] ratio. These data provide tentative support for the theory that white-rot basidiomycete fungi are involved in the degradation of lignin in grasslands.

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Soils contain the largest store of terrestrial organic carbon at approximately 1580 Gt C, which is two to three times more carbon than that held in terrestrial plant biomass (e.g. Schimel, 1995). Although lignin is highly resistant to degradation because of its structural complexity, fungi belonging to the phylum Basidiomycota are known to degrade lignin extensively (Kirk and Farrell, 1987).

The extent to which “model” organic matter (wheat straw here), is degraded in non-agricultural soils in the field is not clearly understood, particularly in a soil that is relatively low in native organic matter (sand-dune grassland) and may have a relatively simple fungal community (Brown, 1958). Wheat straw is an organic resource which has previously been used to determine the effect of fungi on the decomposition of lignin (e.g. Bowen and Harper, 1989; Robinson et al., 1994). Typically, it consists of 14% lignin, 75–80% cellulose and hemicellulose and 1–3% phenolic acids (Harper and Lynch, 1981). There remains limited information under field conditions about the mechanisms by which fungi are able to decompose lignin in grass (but see Gramss, 1997) and particularly in straw (e.g. Robinson et al., 1994). Additionally, more widely, according to Grinhut et al. (2007), the precise role of saprotrophic fungi in organic matter degradation and turnover remains a major question.

Thus, the aim of this study was to characterise lignin degradation, particularly with respect to lignin oxidation typical of white-rot basidiomycete fungi, by comparing the lignin breakdown products in the initial straw samples with those in the decomposed straw samples collected 46 months later. To achieve this aim, we used thermally assisted hydrolysis and methylation with...
The study site was at Ainsdale National Nature Reserve, Lancashire, UK (UK grid reference SD295109) in sand-dune grassland in the fixed ‘grey’ dune zone (Brown, 1958). Typically, the surface A horizon of this Inceptisol contains brown humus to ca. 7 cm in depth, and the lower horizon contains grey sand. The limit of plant rooting occurs at approximately 11 cm depth. There are relatively low amounts of native soil organic matter at the site: in the upper soil horizon at 5 cm depth there was 6.65% organic matter (standard error of ±2.19%, n = 5), and in the lower soil horizon at a depth of 11 cm there was 0.74% organic matter (standard error of ±0.08%, n = 5). A pH value of 6 was observed for all ten soil samples. Concentrations of soil anions and cations in each of the two horizons are shown in Table 1.

Forty ‘bait’ bags were prepared and buried on 14th May 2005. Each bait bag was 15 × 20 mm, made of 50 μm mesh and contained five internodes of winter wheat straw Triticum aestivum var. Swatham. These internodes had been split longitudinally and cut to size 5 × 15 mm, with each bag attached to a nylon string and metal tag to facilitate collection. The mesh reduced plant root colonisation and access to soil fauna, but still allowed for microbial colonisation, increasing the likelihood of basidiomycete fungi reaching the straw and therefore utilising lignin (present as lignocellulose).

The samples were buried in each of the two clearly defined horizons outlined above, at depths of 5 and 11 cm from the soil surface, along a transect. The surface organic horizon was at 5 cm depth while the limit of rooting depth and ‘grey’ sand horizon was at 11 cm depth. Along the transect, there were five bait bags in each upper and lower horizon placed at specific distances (0.5 m, 2.5 m, 4.5 m, 6.5 m, and 8.5 m) from the start of the transect.

On March 1st 2009, 9 bait bags were collected in total, five from the upper horizon and four from the lower horizon, which were essentially treated as replicate bags within each horizon (i.e. horizontal distances of the samples from the point of origin were not used subsequently in the analyses). Soil samples (ranging from 1.6 to 4.9 g in weight) for chemical analyses (Table 1 and loss on ignition and pH as above) were taken immediately adjacent to the location of each bag. Each bag, and each separate soil sample, was carefully folded into individually pre-furnaced, labelled aluminium envelopes to prevent contamination of the straw and soil for future analyses. These envelopes were then placed in Ziploc bags and stored in a 10 °C cold room until processing (carried out within 48 h). Each bait bag, containing a variable number of pieces of straw (initially 5 × 15 mm when first placed in soil), was opened individually and wheat straw was stored at −20 °C for future analyses of lignin breakdown products.

Samples, approximately 0.1 mg, were analysed by GC–MS with TMAH thermochemolysis (please see Supplementary data for details). Because of the limited amount of sample available, as a result of decay in the field, it was only possible to analyse the
Visual degradation of the wheat straw was observed after 46 months (Figs. 1 and 2). Similar to previous studies (e.g. Filley et al., 2000), a suite of THM products was identified (Fig. 1). Our results showed that lignin oxidation occurred over the 46-month period. The \([\text{Ac/Al}]_S\), \([\text{Ac/Al}]_G\) and S/G ratios in the recovered material ranged from 3.2 to 10.8, 0.38 to 1.76 and 0.67 to 2.49, respectively (Fig. 3). For comparison, the values of the original straw material were 3.3 ± 0.81, 0.43 ± 0.15 and 3.3 ± 0.33 for the same ratios, respectively. Thus, over the 46 months of decay, \([\text{Ac/Al}]_S\) values generally increased, \([\text{Ac/Al}]_G\) values generally increased, and S/G values were generally lower than the original values. After 46 months of decay, there were no clear differences for each type of ratio between straw samples collected from the two different horizons (Fig. 3).

The increase in both syringyl and guaiacyl to aldehyde ratios (Fig. 3) indicated that oxidative cleavage of the C6–C6 bonds at the C6 position had taken place and that the aldehyde was oxidized to a carboxylic acid (Tien and Kirk, 1983; Robert and Chen, 1989; Vane et al., 2001; Robertson et al., 2008). These C6–C6 bonds are located on the aliphatic side chain in a similar manner to those attacked by many white-rot fungi such as *Phanerochaete chrysosporium*, *Pleurotus eryngii*, *Trametes* (*Coriolus*) versicolor and *Agaricus bisporus* (Robert and Chen, 1989; Camarero et al., 1997; Vane et al., 2001).

Syringyl units are often assumed to be much more susceptible to degradation than guaiacyl, since syringyl units are: (i) less condensed and have a lower redox potential than guaiacyl units (Kirk and Farrell, 1987) and (ii) syringyl in grass forages, such as wheat straw, is located mainly in secondary cell walls, which are more easily decomposed than primary cell walls (Grabber, 2005), and at advanced stages of decay by soft-rot fungi, this secondary wall is broken down, whereas the guaiacyl-rich middle lamella persists (Schwarze, 2007).

Previous lab-based experiments showed that S/G ratios tends to decrease when lignin is decayed by white-rot fungi (Pal et al., 1995; Vane et al., 2001), supporting the involvement of these fungi in the decay processes occurring at the Ainsdale field-site. No clear difference for each type of ratio between the two different horizons could be observed, suggesting that lignin oxidation occurred in both horizons.

Regarding the relative importance of non-basidiomycete fungi in decomposing lignin in wheat straw, there are reports elsewhere of soil anamorphic (asexual) ascomycetes (e.g. *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium solani*, *Penicillium chrysogenum*, *Penicillium janczewskii*) having the potential to transform and degrade lignin (Rodriguez et al., 1996; Regalado et al., 1997; Deacon et al., 2006), although this is thought to be confined to the initial stages of fungal metabolism. According to de Boer et al. (2005), such fungi are believed not to be major decomposers of lignin in natural environments. It is important to note that possible bacterial colonisers of straw at the field-site could have the potential to degrade lignin (e.g. Antai and Crawford, 1981; Kellner et al., 2008). Thus, some caution should be exercised when trying to attribute lignin breakdown patterns to particular groups of microbes (e.g. Kirk and Farrell, 1987; Vane et al., 2005). Abiotic degradation of lignin in the straw by photodegradation with UV-B (Gehrke et al., 1995) is highly unlikely however, because penetration of UV-B is low through plant cover and soil to the depths at which the wheat straw was buried.

In winter wheat straw *T. aestivum* var. Swatham buried in sand-dune soil, decomposition of syringyl and guaiacyl units occurred, and lignin decay took place via oxidative C6–C6 bond cleavage, suggesting white-rot fungi of the Basidiomycota may have been present. GC–MS with TMAH thermochemolysis, more normally used for analysing chemical changes in laboratory-controlled...
studies of fungal decay of wheat straw, has been demonstrated as being useful to follow potential fungal decomposition in samples collected from the field.

The degradation of biomacromolecules in soil, particularly lignin, is important to the global carbon cycle. Given that only limited amounts of field-based data are currently available, our results provide tentative support for the theory that white-rot fungi are involved in the degradation of lignin in grasslands (e.g. Thorn et al., 1996; Gramss, 1997; Deacon et al., 2006; Lynch and Thorn, 2006; Robinson et al., 2009), even in carbon-poor soils.

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Appendix. Supplementary information

Supplementary information associated with this article can be found, in the online version, at doi:10.1016/j.soilbio.2011.10.014.

References


