Molecular fungal diversity and its ecological function in sand-dune soils

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Abstract

There are about 100,000 described fungal species, however, the diversity could be higher because conventional techniques do not allow identification of all groups of fungi and there are still unexplored geographical areas. High-throughput DNA sequencing methods provide the opportunity to resolve the diversity and distribution of mycelia in soil. Soils are the largest pool of terrestrial carbon and macromolecular materials, such as lignin and cellulose, form an important part of this soil carbon. Saprotrophs (decomposers) fungi degrade lignin and cellulose that is important to the global carbon cycle, although lignin is highly resistant to degradation if compared with cellulose.

In this work, we investigated the diversity of fungi in sand-dune soils and their involvement in the decomposition of lignin and cellulose.

The key findings of this work were:

- A comparison of sand-dune ecosystems from two reserves in the UK showed differences in the ion concentrations, pH and total organic carbon in soils, suggesting that there were different environmental conditions that could potentially affect the distribution/presence of microbial communities in soils, *e.g.* fungal communities.
- Fungi from field samples were identified using 454 pyrosequencing. The identified fungal species belong to groups with different ecologies, among which are wood-rotting fungi that are the main agents responsible for the lignin breakdown. The fungal communities were distributed differently across the different sand-dune ecosystems, sampling times and type of bait materials.
- Lignin and cellulose can be degraded in field samples over time. Lignin degradation was shown by the shifts in the [Ac/Al]S, [Ac/Al]G and [S/G] relative lignin decomposition state proxies, and cellulose degradation by the shifts in the [cellulose:cellulose+lignin] ratio. Cellulose degradation was faster than lignin, thus confirming previous studies.
- The degradation of both lignin and cellulose was different depending on the type of plant material, ecosystem/soil characteristics where the material was buried and fungal communities present on the bait materials.
- Lignin breakdown was most likely to be by white-rot fungi that were identified colonising the bait materials.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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The Author

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Chapter 1

Introduction

1.1. Diversity of fungi

Fungi are eukaryotic, heterotrophic and osmotrophic (absorbing nutrients) organisms whose cell wall contains chitin or chitosan (Ritz, 2005; Margulis and Chapman, 2009). Fungi are non-motile and reproduce both sexually and asexually *via* spores (Blackwell and Spatofora, 2004), most of them are terrestrial but there are a few species that are marine (Margulis and Chapman, 2009). They develop filaments called hyphae that are divided by cross walls (septa) into segments which contain several nuclei with chromosomes and remain interconnected making intercellular communication possible (Anderson *et al*., 1994; Carlile *et al*., 2001). The growth of these filaments is carried out by the deposition of cell wall material from a growing tip. This is called the hyphal growth-form, most fungi have this kind of growth, although unicellular forms are common in yeasts (Ritz, 2005; Margulis and Chapman, 2009). Growing tips expand and form a network called the mycelium which establishes and becomes capable of unlimited growth and persistence (Zak and Willig, 2004). As the mycelium expands, it occupies heterogeneous microenvironments and/or macroenvironments (Anderson *et al*., 1994).

Fungi belong to the Kingdom Fungi which is divided into ten phyla (Kirk *et al.*, 2008): the Chytridiomycota, the Monoblepharidiomycota, the Neocallimastigomycota, the Blastocladiomycota, the Microsporidia, the Zygomycota, the Entomophthorales, the Glomeromycota, the Ascomycota and the Basidiomycota (Kirk *et al.*, 2008; Blackwell, 2011). Within all the described phyla, a broad range of taxa, morphologies, ecologies and life history strategies can be recognised (Ritz, 2005). One can distinguish different morphologies within fungi, as the cultivated mushroom *Agaricus bisporus* (Basidiomycota) which consists of a sporocarp (fruiting body), where a large number of spores are produced that are borne on the gills below the cap (*e.g.* basidia in Basidiomycota), a stalk raises the sporocarp above the ground facilitating spore dispersal by air currents (Kendrick, 2000; Margulis and Chapman, 2009; Carlile *et al*., 2001). But, unicellular forms can also be recognised, as in yeasts which are characterized by their vegetative growth as unicells and by the formation of sexual

states that are not enclosed in sexual structures (*e.g.* Saccharomycetes, Ascomycota; Kurtzman, 1994; Kendrick, 2000; Carlile *et al.*, 2001, Margulis and Chapman, 2009). Regardless of their morphology, all fungi require access to obtain organic nutrients for their energy source and carbon intake for cellular synthesis. Fungi can acquire nutrients by growing as parasites (or pathogens) of another living organism, as symbionts in association with another organism or as saprotrophs of non-living materials (Blackwell and Spatafora, 2004; Deacon, 2006). As pathogens, they are detrimental to their host. *Geomyces destructans* (Ascomycota) is associated with White-nose syndrome, a cave-roosting bat disease that appears to cause either an increase in energy expenditure during bat hibernation or summer activity reduction, resulting in bat deaths leading to a rapid bat population decline (Dzal *et al*., 2011; Jonasson and Willis, 2011). The *Heterebasidion annosum* fungus (Basidiomycota) causes the conifer disease called 'root and butt rot' that causes conifer destruction which implies economic losses in the Northern Hemisphere because conifer wood is the basis of one of the broadest manufacturing industries in Europe. The economic loss in Europe because of *H. annosum* infection was estimated to be, in a year, *c.* 800 million Euros (Asiegbu *et al*., 2005; *et al*., 2009). In symbiotic relationships with either a green alga or a cyanobacterium, fungi form lichens (Galloway, 1995). Lichens are considered as an example of mutualism, where both species, the fungal partner (mycobiont) and the autotrophic partner (green alga or cyanobateria), are growing together and are forming a thallus that can endure some of the most inhospitable environments (Honegger, 1991). Lichens are sensitive to environmental disturbances, so they have been used as biomonitors, for example *Rhizocarpon geographicum* has been used to determine the atmospheric pollution in Amman City, Jordan (Jiries *et al*., 2008).

In most natural ecosystems, there are fungi associated with the roots of plants (mycorrhizas) which help to take up nutrients from soil, and the decomposition of plant litter by fungi (saprotrophs) is an essential part of the global carbon cycle. Mycorrhizas are symbiotic (mutualistic) associations between fungi and the root systems of the plants, which can be of vital importance for growth and persistence for the plant. The fine mycelial network of the fungus enables the increased uptake of mineral salts that occur at low levels in the soil. In return, the plant provides the fungus with metabolic energy in the form of photosynthetic products (Kendrick, 2000; van der Heijden and Kuyper, 2003; Deacon, 2006; Smith and Read, 2008). Saprotrophic fungi obtain their nutrients from dead organisms by degrading the dead organic material of various origins

and they have an essential functional importance in wood degradation, decomposition of herbaceous substrates of plants, in litter and humus layers. Their activity is essential for the continuation of life on Earth (Carlile *et al*, 2001; Deacon, 2006). The role of saprotrophs will be further explained in 1.4, since they are related to cellulose and lignin degradation.

The fungal life-history strategies allow them to grow anywhere, from temperate to tropical environments (Deacon, 2006). Arenz and Blanchette (2011) studied the abundance and diversity of soil fungi in Antartica. These authors described species from Ascomycota (*Antarctomyces psychrotrophicus*), Basidiomycota (*Cryptococcus gastricus*) and Zygomycota (*Mortierella alpina*) which are likely to be playing important roles as generalist decomposers in the Antarctic soil environment, but decomposers can also be found in habitats totally different from Antarctica habitat, as in coastal sand dunes.

Although it is known that the fungal kingdom has a great diversity of fungal ecologies, for example, The Dictionary of Fungi has reported about 100 000 species of described Fungi (Kirk *et al*., 2008), some authors have estimated that this number could be much larger as few habitats and regions have been studied intensively (Carlile *et al*., 2001; Hawksworth, 2001; Hyde, 2001; Blackwell, 2011). It is also probable that many fungi have not been yet recognized because they often differ only in tenuous characteristics (*e.g*. pigments, Margulis and Champan, 2009). Therefore, further approaches have been made in trying to estimate the real number of fungal species in the world (May, 1991; Hawksworth and Rossman, 1997; Hawksworth, 2001; Hyde, 2001; Blackwell, 2011). Some methods for the identification of fungal species are being improved, for example with the use of molecular techniques (Hawksworth, 2001; O'Brien *et al*., 2005; Blackwell, 2011). O'Brien *et al*. (2005) analysed the fungal content of forest soil and estimated that "the number of fungal species should be 3.5 to 5.1 million species", a much larger number of species than the number estimated in the last decade by Hawksworth (1991). The estimate of O'Brien *et al*. (2005) was based on the use of direct isolation of small-subunit and internal transcribed spacer (ITS) ribosomal ribonucleic acid (rRNA) genes by the technique of polymerase chain reaction (PCR) and high-throughput sequencing of cloned fragments. Furthermore, different geographic areas are being studied for the identification of the diversity of the mycota such as those areas that were underexplored, for example Hong Kong (Hyde, 2001), or where the

mycological diversity was little known such as coastal dunes in the Netherlands (Geml *et al*., 2014).

1.1.1 Fungal diversity in sand dunes

Sand dunes are agglomerations of sand grains, built into hills or ridges by the wind by means of gravity. Sand dunes are found in deserts, on coasts, and in some eroded and abandoned farm fields (Ranwell, 1972). Sand dunes are a very dynamic system, a very fragile environment, and are heavily used (*e.g*. recreation) which may cause the destruction of grass roots, after a while causing the destruction of the dune system. They have been classified as 'true dunes' formed without vegetation or as coastal dunes which are formed in conjunction with vegetation (Ranwell, 1972; Smith, 1999; Martinez *et al*., 2004a, Martinez *et al*., 2004b).

A number of sand dune types are recognised, depending on how they are formed, or stage of succession and vegetation type, including fore dunes, yellow dunes (mobile dunes), dune grassland, dune heath, dune slacks, dune scrub, fixed dunes and woodland (Fig. 1.1). For example, there are mobile 'yellow' (or sometimes 'white') dunes where the plant cover is patchy and the soil is mainly made by clean yellow or white sand. Marram grass (*Ammophila arenaria*) stabilizes the sand in this area at some distance below the surface because of its deep vertical root system and extensive horizontal root network (Ranwell, 1972; Ranwell and Boar, 1986; Holmes, 2007; Holden, 2007). Another type of sand dune is the dune slacks which are depressions within sand-dune system that are flooded during the rainy season, and that are very poor in nutrients and at the same time very rich in plant species (Grootjans *et. al*, 2004). In dune slacks, most species have to be adapted to wet and dry conditions because these parts of the dune system are temporary wetlands (Muñoz-Reinoso, 2001; Holden, 2007; Holmes, 2007).

Sand dune habitats are therefore highly heterogeneous and offer a wide variety of microhabitats which fungal species utilize different strategies to survive in, for example in yellow dunes (mobile dunes), few fungi are capable to colonise them and there are only specialist species which have a number of adaptations to enable them to survive in this difficult habitat, such as the development of a solid, subterranean aggregation of mycelium mixed with sand. This serves as a resource reservoir where moisture and nutrients are retained, as well as acting as a physical support (Rotheroe 1993). This first involves an association with a higher plant (typically *Ammophila arenaria*) for physical

support for basidiome production. Rotheroe (1993) suggested that the fungi have an important role in facilitating colonisation by higher plants and sustaining sand dune systems. Saprotrophy involving extremely limited resources in the youngest yellow dunes gives the way to greater specialisation and more subtle relationships making possible an increase of fungal species diversity in the older areas (Watling and Rotheroe, 1993). The semi-fixed 'grey'dunes, which is an area on the landward side of the mobile dunes, is characterised by the presence of saprotrophic fungi that are dependent on the variety of dead plant material. Semi-fixed dunes, dominated by semi-improved grassland, support several members of the fungal community associated with this habitat, including members of the *Clavariaceae* and genus *Hygrocybe.* In these areas *Salix repens* is a common colonizer and some mycorrhizal fungi can be found associated with it. Coprophilous fungi can also be found. The diversity of species within the dune slack may not be high, however large numbers of sporocarps (sexual structures) can be noticed and this area represents a high specialisation area (Watling and Rotheroe, 1993).

It should be emphasized that in the sand dunes, a large variety of macrofungi can be found, *e.g.* saprotrophs (including white or brown rot fungi, basidiomycetes) and also pathogens of plants and fungi (Mueller and Schmit, 2007) or animals (Dzal *et al*., 2011). Nevertheless, saprotrophism is a specialized fungal functional mode for nutrient gain in this habitat. Colonisation by saprotrophic fungi starts when there is dead plant material to decompose (Holden, 2007).

The range of nutritional modes and ecological strategies can be observed in the larger fungi in sand-dune habitats. Rotheroe (1993) described the larger fungi of Welsh sand dunes (Fig. 1.1). He found both saprotrophy and symbiosis as different nutritional and ecological modes in the macrofungal component at Whiteford Burrows sand dunes. At the early stage, no mycorrhizal fungi could be detected. He also found species from Agarics (e.g. *Coprinus ammophilae*; Basidiomycota). The species in the early stages demonstrated a high degree of adaptation and specialisation because they are restricted to stressful areas, the characteristics of which have been explained above. Saprotrophic fungi endemic to sand dunes appeared to have broader habitat preferences: they can be found in particular zones, but they can also occur often in semi-fixed dunes, dune grasslands and dune slacks. Symbiosis could be mainly encountered in older stages of the succession, from mobile yellow dunes until scrub, heaths and woodlands. The first important appearance of this kind of fungi was in the dune slacks, where the more

specialised ectomycorrhizal fungi dominated the flora. The main symbionts were creeping willow (*S. repens*) with fungal genera from *Cortinarius*, *Hebeloma* and *Inocybe*. In his study, Rotheroe found that members from the Agarics predominated in these British dunes, *e.g.* members from the families Agaricaceae, Coprinaceae, Hygrophoracea and Tricholomataceae.

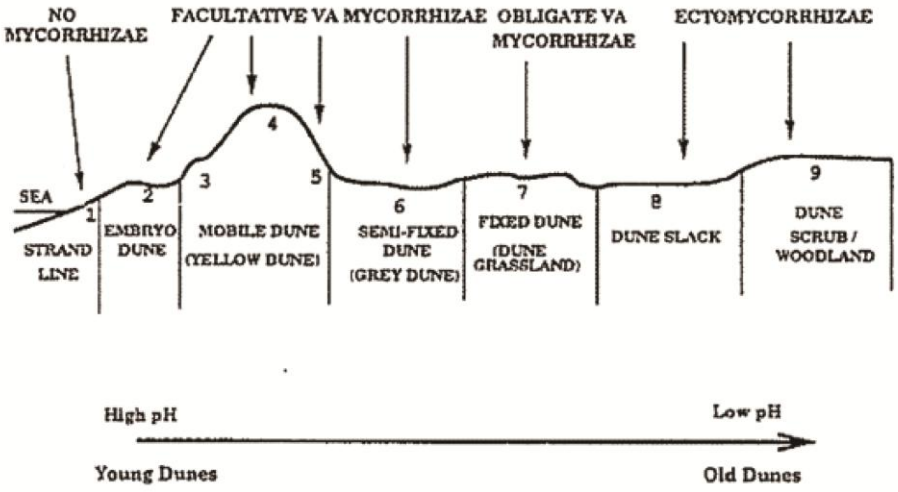


Figure 1.1. Stylised sand-dune transect at Whiteford Burrows, Wales as explained by Rotheroe (1993), showing the range of nutritional modes and ecological strategies followed by sand dune fungi.

Fig. 1.1 shows the importance of fungi which are considered to be essential to the health of coastal ecosystems and which play an important role in stabilizing the mobile sand and supporting higher plants in the different stages of dune development, thanks to their broad range of ecological function.

In the United Kingdom, sand dunes are of considerable extent and variety, and some are nationally important. They support a wide variety of species, from birds such as eider duck and terns, to rare mosses, lichens, fungi, and invertebrates (Holden, 2007), however, the mycological importance of the sand dune habitat in the UK has only been documented relatively sparsely with work being undertaken in Wales and Scotland (Brown, 1958; Rotheroe, 1993; Rotheroe *et al.*, 1987; Watling and Rotheroe 1989; Rhind and Jones, 1999; Leake *et al*., 2004).

1.2 Techniques to measure fungal diversity in soils

A comprehensive range of methods has been developed and applied to study the presence, abundance, activity, and distribution of fungi in soils. All these methods have advantages and disadvantages which are important to consider for the best performance of a particular research. These methods can be generally split into traditional techniques and more recent methods such as DNA-based techniques.

1.2.1 Traditional techniques

Soil fungal communities have traditionally been examined by collecting macroscopic sexual reproductive structures (sporocarps) that are produced above ground or by using isolation techniques to obtain pure cultures of fungal strains directly from soil (Rossman *et al*., 1998). However, these approaches have several limitations (Ritz, 2005; O'Brien *et al.*, 2005).

The most common method is isolation. Many fungi can be isolated from soils by traditional methods of dilution plating and enrichment growth on broad-scale or specific gel based media supplemented with a variety of nutrient sources, for example C-rich media such as Czapek-Dox medium (an agar gel based on 3% sucrose; Ritz, 2005). Deacon *et al*. (2006) stated that dilution plate count technique is 'meaningless' when trying to estimate the abundance of species, because colonies grow from hyphal fragments or spores in solution.

Another soil plating technique is the Warcup technique. Deacon *et al*. (2006) found that this technique allowed both a general view of the fungal community and cultures from soil to be obtained. They stated that this technique seems to be the most advantageous technique when assessing the frequency of fungal occurrence for each taxon.

Hyphal fragments or (larger) spores can be directly extracted from soils by micromanipulation, for example by elutriation and sieving. Elutriation is mainly used to separate light particles (>1μm) by a stream of gas or liquid. These lighter particles rise to the top because their velocities are lower than the velocity of the rising fluid (Clark *et al*., 1978).

However, these methods are likely to obtain only a subset of the whole community, because of their bias towards fast-growing species (Ritz, 2005).

Direct observation is another method for the assessment of fungal communities in soils. Hyphal length is stained using colored (*e.g.* aniline blue) stains to distinguish the hypha

from soil particles. So, hyphal measurement is directly done on thin films of scattered soil. Hyphal diameters can be measured as computerized image processing and analysis can be used to automate identification. It is necessary, however, to maintain a high degree of contrast between hyphae and the background, so it becomes difficult to identify fungi by these methods, since most hyphae do not have a clear distinct morphology (Ritz, 2005).
Sporocarp collection is another method for the assessment of fungal communities from soil, but this technique is restricted for detecting species that reproduce sexually by macroscopic sporocarps unless long-term studies are conducted (Straatsma *et al*., 2001, O'Brien *et al*., 2005).

Molecular analyses are also used to measure fungal communities in soils. They rely on biochemicals that are more or less specific to fungi. These methods avoid many issues associated with *in vitro* cultivation of fungi and offer a broad range of scales of resolution in quantitative analysis. Total fungal biomass in soils can be estimated by direct extraction and measurement of biomarker compounds such as chitin or ergosterol. Few compounds meet these criteria strictly, since fungi vary dramatically in their resource distribution and there is often a lack of correspondence between fungal community structure above ground and below ground (Gardes and Bruns, 1996; O'Brien *et al.*, 2005). Because of the difficulty in isolating and cultivating most soil fungi, molecular approaches have provided valuable methods for the assessment of their diversity. DNA-based approaches have transformed our understanding of microbial ecology, (O'Brien *et al*., 2005; Lindahl *et al*., 2007; Nilsson *et al*., 2011).

1.2.2 DNA-based methods

The characterisation of fungi has been revolutionized by the use molecular methods based on fungal-specific PCR amplification of the nuclear ribosomal internal transcribed spacer (ITS) (Gardes *et al*., 1991; Gardes and Bruns, 1993; Vralstad *et al*., 2002, O'Brien *et al*., 2005). PCR-based approaches are helping to overcome culture biases and limited morphological variation in mycorrhizal research (Taylor and Bruns, 1999; Bidartondo *et al*., 2004; McCormick *et al*., 2004; Taylor *et al*., 2008).
Identification of fungi using sequence-based methods have been carried out on different plant roots (*e.g.* Gordon and Gehring, 2011; Bahram *et al*., 2011), grass leaves

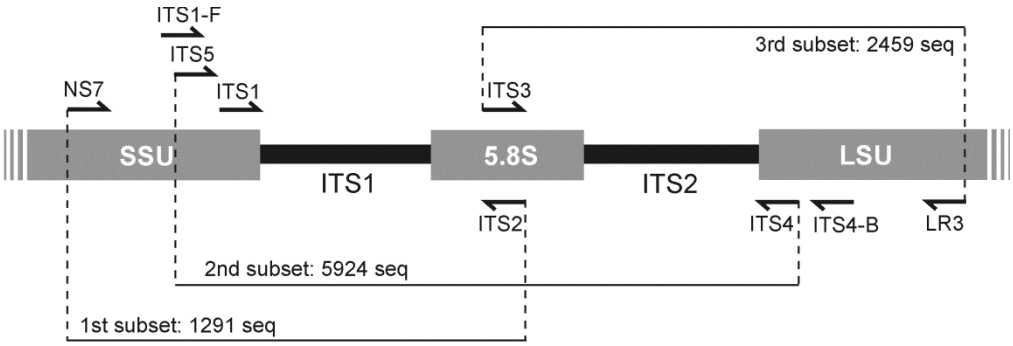
(Albrectsen *et al*., 2010), and soil (e.g. Lentendu *et al*., 2011). Many studies have been restricted to the use of methods based on fragment length polymorphisms (Arteau *et al*., 2010; Bouftass *et al*., 2010; Yu *et al*., 2009; Yang *et al*., 2008; Jung *et al*., 2008) or denaturing gradient gel electrophoresis (Hassan *et al.,* 2011; Knapp *et al*., 2011; Zhou *et al.,* 2011). These methods have the potential to facilitate ecological studies of soil communities and improve our understanding of fungal biodiversity (O'Brien *et al*., 2005).

The internal transcribed spacers (ITS) that separate the small-subunit (SSU) and large-subunit (LSU) rRNAs in eukaryotes evolve at a faster rate and have been used to identify fungi to genus and often to species level (Fig. 1.2; Taylor and Bruns). Therefore, taxon-specific primers for fungi have been developed for the amplification of the ITS region that have provided a useful knowledge of species identification, revealing, for example, the presence of unexpected taxa (*e.g.* compared with basidiocarp data) and that basidiomycetes are two to three times less abundant (relative to total fungal abundance) in prairie grassland soil than woodland (O'Brien *et al*., 2005; Lynch and Thorn, 2006). One of the most recent and detailed study in coastal dunes (Geml *et al*., 2014) identified 600 to 971 operational taxonomical units (OTUs) from 10 different sand-dune sites in a protected area in the Netherlands, using next generation sequencing and ITS as DNA barcode. Molecular identification of fungi by DNA barcoding has relatively recently provided new insights into the diversity and ecology of many different groups of fungi and it is becoming an essential and integrated part of fungal ecology research (O'Brien *et al.,* 2005; Bellemain *et al*., 2010; Kelly *et al*., 2011; Botnen *et al*., 2013; Geml *et al*., 2014). Fungal molecular identification has allowed the study of fungal species richness and abundance at a high rate and more reliably than conventional surveys (*e.g.* direct observations), because it is carried out not only by means of sporocarps , but also by means of their mycelial stage (Zinger *et al*, 2009; Bellemain *et al*., 2010). The Fungal Barcoding Consortium proposed the ITS region of nuclear DNA (nrDNA) as the primary DNA barcoding marker for the identification of fungal taxa (Schoch *et al*., 2012)

The ITS region includes the ITS1 and ITS2 regions which are separated by the 5.8S gene. The ITS region is situated between the SSU and the LSU rRNAs in eukaryotes (Fig. 1.2). Variation among individual rDNA repeats has been observed within both the ITS and IGS regions, because of its higher degree of variation if compared with the SSU and LSU regions of rDNA. That is why the ITS region has proved to be useful for

molecular systematics at the species level, and within species to identify geographic races (Gardes and Bruns, 1993). Most research groups commonly use the standard ITS1+ITS4 primers, however, several taxon-specific primers have been described to allow selective amplification of fungal sequences (O'Brien *et al*, 2005, Bellemain *et al*., 2010). The rDNA fragments, including 18S, 25/28S and the internal transcribed spacer (ITS) region surrounding the 5.8S, have been used as markers to assess fungal diversity in soils (O'Brien *et al*., 2005; Manter and Vivanco, 2007; Toberman *et al*., 2008; Robinson *et al*., 2009, Bellemain *et al*., 2010).

(a)



(b)

Primer	Author	Primer sequence	Position
Forward primers			
NS7	[19]	GAGGCAATAACAGGTCTGTGATGC	1403-1426
ITS1-F	[18]	CTTGGTCATTTAGAGGAAGTAA	1723-1744
ITS5	[19]	GGAAGTAAAAGTCGTAACAAGG	1737-1758
ITS1	[19]	TCCGTAGGTGAACCTGCGG	1761-1779
ITS3	[19]	GCATCGATGAAGAACGCAGC	2024-2045
Reverse primers			
ITS2	[19]	GCTGCGTTCTTCATCGATGC	2024-2043
ITS4	[19]	TCCTCCGCTTATTGATATGC	2390-2409
ITS4-B	[18]	CAGGAGACTTGTACACGGTCCAG	2526-2548
LR3	[13]	CCGTGTTTCAAGACGGG	3029-3045

Figure 1.2. The position of the ITS region and the most utilized ITS primers in fungi. a) Relative position of the primers, design of the subsets and number of sequences in each subset. b) Primer sequences, references and position of the primer sequence. Authors: (13) Vylgalys and Gonzalez, 1990; (18) Gardes and Bruns, 1993; (19) White *et al*., 1990 (Picture taken from Bellemain *et al*., 2010).

Particularly, the use of the relatively new next generation sequencing (NGS) technique has helped in overcoming the problem of working with environmental DNA (Margulies

et al., 2005). Environmental DNA (eDNA) is the genetic material found, for example, in soils, water or air, and it is mixture of DNA from different organisms because it is not the DNA extracted from a specific individual organism or their parts (Ogram *et al*., 1987). Traditionally, the Sanger-sequencing method (Sanger *et al*., 1977) has been the technique most commonly used in DNA-based studies, however, it is inadequate for processing complex environmental samples because these samples contain mixtures of DNA from hundreds or thousands of organisms or this eDNA could also be degraded (*e.g*. DNA from fossil environmental samples), and the Sanger technique requires *a priori* the identification of the organism (or their parts), whose DNA will be sequenced (Shokralla *et al*., 2012). Next generation sequencing technologies have the ability to read DNA from multiple templates in parallel, therefore allowing the recovering of DNA sequences from thousands of organisms that are present in an environmental bulk and sample (Shokralla *et al*., 2012).

Using NGS, it has been possible to determine, for example, animal diet through the analysis of coprolites (fossilized faeces) of the cave hyena (Bon *et al*., 2012) or gut contents of the Sea cucumber (Gao *et al*, 2014), and to infer the health of an ecosystem by analyzing its biodiversity (Hajibabaei *et al*., 2011). In fungal studies, the use of NGS has allowed the analysis of the fungal communities in *Salix repens* beds in a protected sand-dune area which allowed the identification of red listed species (Geml *et al*., 2014), to determine an unexpected high diversity of soils fungi in Breuil-Chenue forest in Burgundy, France (Buée *et al*., 2009), to show the seasonal dynamics in soil fungi in an oak forest at Xaverovský Háj Natural Reserve, Czech Republic (Voříšková *et al*., 2014) and to assess the fungal paleodiversity of ancient DNA from arctic permafrost in northern Sakha Republic and southern Chukotka, Russia (Bellemain *et al*., 2013).

One of the first sequencers for carrying out NGS studies was the Roche 454 genome sequencer that was introduced in 2005 (Margulies *et al*., 2005). The basic principle is illustrated in Fig. 1.3 and explained here as in Margulies *et al*. (2005). (1) Library Preparation**:** Before carrying the amplification of the extracted DNA, the primers that will be used during the polymerase chain reaction (PCR) have to be in the form: Adapter+Key+Multiplex identifiers(MID)+Primer where the 'Adapter" is a 26-base-pair sequence that is recognised by the sequencer when the amplicons are loaded into, thus each adapter is carrying a DNA library. (2) One fragment = one bead: The DNA

libraries are mixed with DNA capture beads, each bead will carry a unique single-stranded library fragment. (3) Bead emulsion: The beads are emulsified with amplification reagents and mixed in a water-in-oil mixture to trap individual beads in amplification microreactors. (4) Emulsion PCR Amplification: The entire emulsion is amplified, which is called 'emulsion PCR amplifcation' (emPCR), in parallel (all the emulsified beads) to yield millions of clonally copies of each single library fragment on each bead. Then the emulsion is broken while the amplified fragments remain bound to their specific beads. (5) Sequencing, one bead = one read: The beads are loaded onto the PicoTiterPlate (PTP) device, only one bead per well, and the PTP device is loaded in the instrument for sequencing (sequencer). Individual nucleotides are moved smoothly in sequence across the wells. (6) Pyrosequencing chemistry**:** there is an incorporation of a nucleotide complementary to the template strand which results in a chemiluminescent light signal that is recorded by a camera which is contained into the sequencer. Each DNA Capture Bead will contain millions of copies of a single clonal fragment. (7) Data processing and analysis: The sequencer is linked to a computer that has installed specialized software for data analysis. The software uses the signal intensity that is emitted during each (nucleotide) incorporation event at each well position for determining the sequence of all reads in parallel. The resulting sequenced reads are analysed using bioinformatics software, such as Qiime (Carporaso *et al.*, 2010), for de novo assembly, mapping and amplicon variant detection.

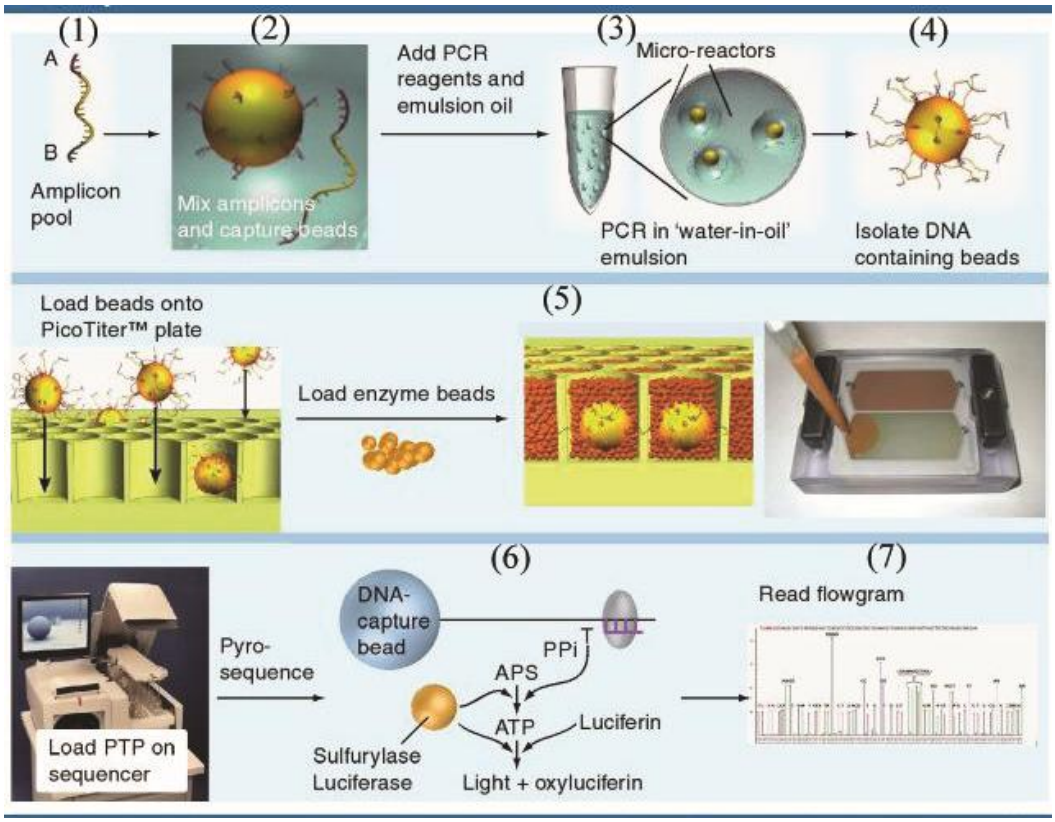


Figure 1.3. Schematic representation of the basic principle of the 454 Roche pyrosequencing (Margulies *et al.*, 2005). See text for an explanation to the workflow. Picture modified from http://imihumangenomproject.blogspot.co.uk/2012/12/454-life-sciences.html.

DNA based methods provide a better assessment of fungal communities than traditional based methods, because they do not discriminate against non-culturable and slow growing taxa (Jany and Barbier, 2008). For example, sequence comparisons with databases may enable identification of non-sporulating taxa, including basidiomycetes (Pinruan *et al.*, 2010), which have an important role in the environment as degraders of plant materials (Weber and Webster, 2007). However, fungal diversity and its ecological function are "poorly understood, even though fungi are an essential group in Earth processes, and they have shown high levels of diversity (Blackwell, 2011). The use of new molecular techniques is clarifying fungal species description and identification (O'Brien *et al.*, 2005), which remains necessary for the understanding of the roles of fungal species in nature (Blackwell, 2011).

1.3 Fungal role in the environment

Fungi play a vital role in global ecosystem functioning. Their ability to break down major plant components, particularly lignin and cellulose, is the basis of their organic recycling role (Krivtsov *et al.*, 2006). Carbon and minerals on Earth are continuously being accumulated, replaced, distributed and captured; this is called nutrient cycling which is a primary process for the maintenance of ecosystems. The role of fungi in nutrient cycling is fundamental, as they capture, accumulate, release and recycle nutrients (*e.g.* carbon, phosphorus, nitrogen). For example, soil fungi take and retain soil nutrients, prevent leaching and make nutrients available for plant intake (Tlalka *et al*., 2008). Even though fungi are mainly involved in cycling C, P and N, they have also roles in most of the other soil element cycles, thanks to their mycelia which capture and release nutrients after death and lysis or attack by pathogens and grazers (Park, 1976; Galloway, 1995). In the nitrogen cycle, the process by which nitrogen is converted between its various chemical forms, fungi convert the organic nitrogen (into ammonium NH_4^+), after a plant or animal dies, or after an animal discharges waste. This process is called ammonification or mineralization (Fig. 1.4). The nitrogen availability can affect, for example the rate of decomposition and primary production, that is why the nitrogen cycle is of particular interest to ecologists. The global nitrogen cycle has been dramatically altered by human activities such as fossil fuel combustion, use of artificial nitrogen fertilizers, and release of nitrogen in wastewater (Long and Hall, 1987; Meyer, 1993).

On the other hand in ecosystem carbon cycling, basidiomycetes (Basidiomycota) are particularly of great importance. They are the main decomposers in N-limited soils (Boddy and Watkinson, 1995) where the largest amount of terrestrial carbon is sequestered (Post *et al*., 1982). About 8,500 of Basidiomycota are lignocellulose-degrading saprotrophs, approximately half of them can be found in soils and on plant remains (Hibbett and Thorn, 2001). Saprotrophs are, among all the decomposer fungi, the main agents responsible for breaking down of organic matter (Lynch and Thorn, 2006).

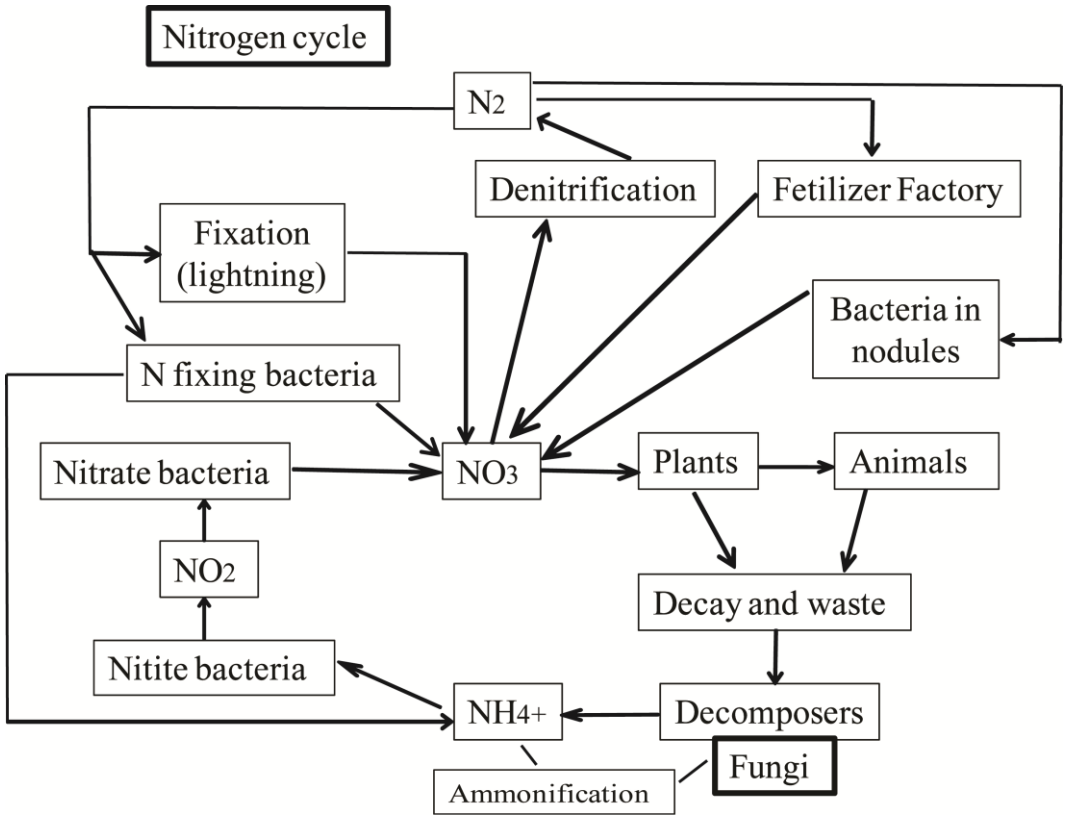


Figure 1.4. Schematic representation of the flow of nitrogen through the environment, showing the fungal role (decomposers) in ammonification (mineralization) process (Meyer, 1993).

The breaking down of dead organic matter such as occurs in soil, litter, dung, and wood is the way that fungi obtain energy. Fungi capture, thanks to their mycelial growth, scattered organic resources which are essentially composed of cell wall plant polysaccharides (e.g. cellulose, lignin), cell wall polysaccharides of fungi (chitin) and other biopolymers (e.g. starch and proteins; Baldrian, 2008). To gain access to utilize these organic compounds, fungi produce extracellular enzymes (e.g. lignin-modifying enzymes) which enable them to break down the resistant compounds of plant litter (Lynch and Thorn, 2006). Some examples of basidiomycete decomposers are the wood-rotting cellulolytic fungi (*e.g. Armillaria sp.*) which have developed complex systems of non-enzymatic cellulose cleavage based on the production of reactive oxygen species (Stoytchev and Nerud, 2000), and fungi from the genus *Trametes* which produce ligninolytic enzymes (*i.e.* manganese and lignin peroxidases) that enable them to degrade the most substantial components of wood (Tomsovsky and Homolka, 2003). Wood and cereal straws are mainly composed of cellulose, hemicellulose and lignin (Fig. 1.5): lignin is the most complex compound (Antongiovanni and Sargentini, 1991;

Deacon, 2006). Up until now, the only organisms known to decompose lignin completely are white-rot fungi (Kirk and Farrell, 1987; Deacon, 2006), specifically white-rot fungi that produce peroxidases (PDOs) together with enzymes (cellubiohydrolases) that attack crystalline cellulose as the fungus *Phanerochaete chrysosporium* (Rilley et al., 2014) They are the most numerous among all the wood decaying fungi (Deacon, 2006), and there are also lignin modifiers: brown-rot and soft-rot fungi (Crawford and Crawford, 1980; Berg and McClaugherty, 2003). Kirk and Farrell (1987) stated that the complete degradation of lignin is *via* an 'enzymatic combustion', carried mainly out by enzymes: lignin peroxidase, manganese peroxidase, H_2O_2 generating enzymes and laccases (Deacon, 2006). Nevertheless, lignin chemical structure is not homogenous within plant tissues (McCarthy and Islam, 2000) which has made it difficult to identify clearly all the enzymes involved in the degradation of plant material, and therefore the role of fungi as decomposers of different lignins of plant tissues (Horwath, 2005).

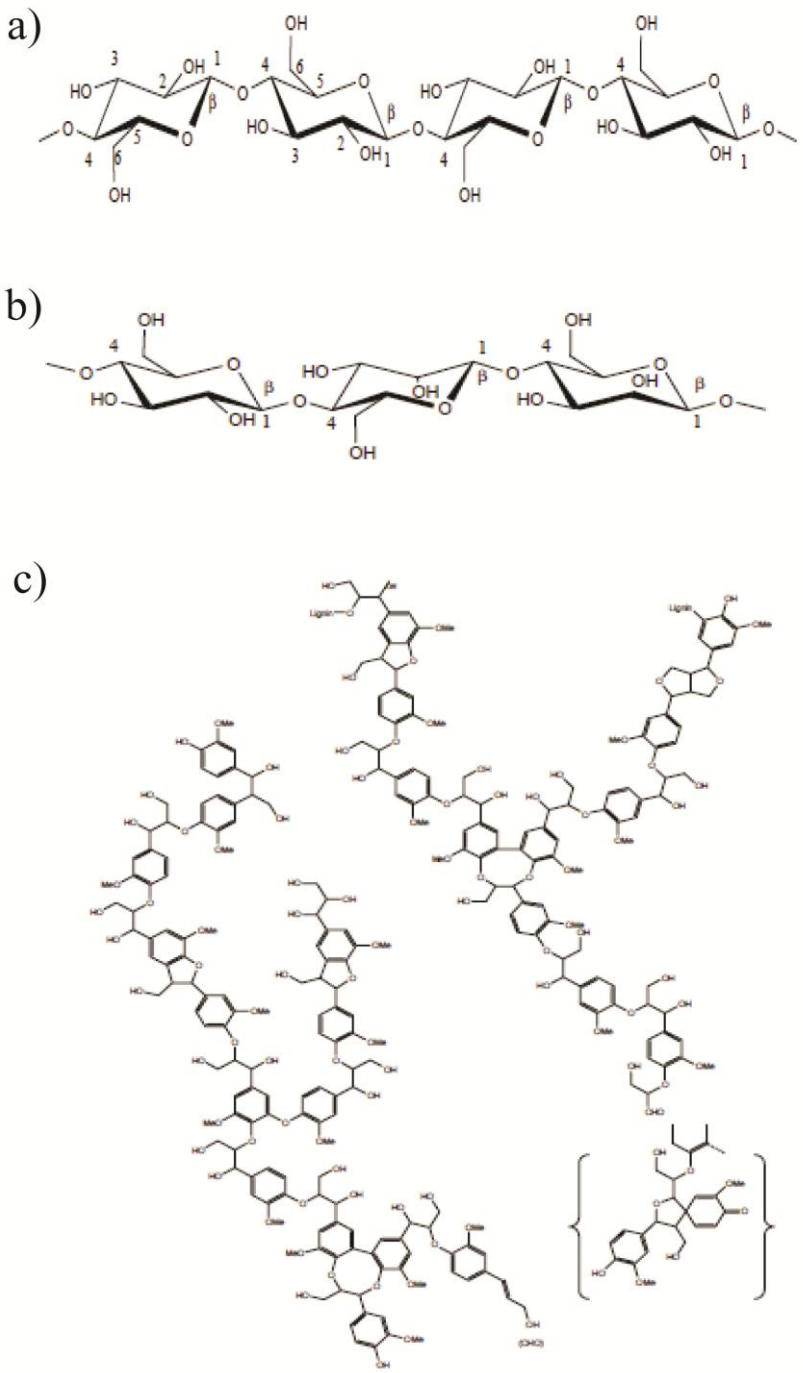


Figure.1.5. Schematic structures of a) cellulose, b) glucomannan hemicelluloses and c) softwood lignin showing the complex structure of lignin when compared with cellulose and hemicelluloses. Pictures taken from Laine (2005).

Lignin is the second most abundant plant polymer on Earth, its degradation is of general significance for the global carbon cycle (Deacon, 2006). Tissues of many plants are composed of storage carbohydrates (*e.g.* starch) and structural polymers e.g.

lignin (Dickinson, 2000). Lignin function is to give vascular plants the rigidity needed to stand upright and to protect cellulose and hemicelluloses from attack by other organisms. In this way they form a strong and durable cell wall (Hammel, 1997). Plant lignins are classified in three broad classes: softwood (gymnosperms) hardwood (angiosperms) and grass lignins. One of the main differences among them is the composition of a chemical compound present in plants, which is a fundamental part for the biosynthesis of lignin: the monolignol. For example, softwood lignin is mainly composed of guaiacyl and, hardwood lignin is mainly composed of guaiacyl and syringyl. Hardwood and grass lignins have been less studied, even though they exhibit variance between species (Browning, 1963; Maijala, 2000). The transformation of lignin is mainly carried out by fungi from Basidiomycota and Ascomycota (Kirk and Farrell, 1987; Martinez *et al*., 2005; Sinsabaugh, 2010), but it is not a homogenous process, it has showed differences among the three main fungi groups of decomposers: white-rot, brown-rot and soft-rot fungi (Crawford and Crawford 1980; Berg and McClaugherty, 2003). That is the reason why the transformation of lignin is still an important topic to assess. For example, Liers *et al*. (2011) carried out a microcosm experiment with four different wood rotters, three basidiomycetes: *Phlebia radiata* (‘classic wood white rot’), *Agrocybe aegerita* (‘nonspecific wood rot’) and *Stropharia rugosoannulata* (‘white rot of leaf litter’), and an ascomycete: *Xylaria polymorpha* (‘soft rot of wood’). They found essential enzymes in ligninolysis (manganese-oxidizing peroxidases) produced by ‘all strong white rotters’ (basidiomycetes) but these enzymes were not produced by ascomycetes.

In general, white rot fungi invade the lumens of wood cells, where they secrete enzymes that degrade lignin and the other wood components, in order to gain access to cellulose and hemicelluloses that are enclosed in the lignin matrix (Hammel, 1997). Studies from electron-microscopic features of wood decay by white-rot fungi revealed that lignin is degraded at some distance from the hyphae and is removed progressively from the lumens toward the middle lamella (Kirk and Farrell, 1987). During its mineralization by white-rot fungi, lignin undergoes a number of oxidative changes, including aromatic ring cleavage. Some studies have suggested that a progressive depolymerization occurs and releases a wide array of low-molecular weight fragments (Berg and McClaugherty, 2003). Hatakka (2001; cited by Berg and McClaugherty, 2003) suggested that lignin mineralization is fungal species-specific and dependent on the ecological niche, because there have been found differences in lignin degradation by

Ganoderma lucidum. This fungus produces different enzymes when it is in different media. In a medium with poplar wood, it produced Mn peroxidase but not in a medium with pine wood. Brown-rot fungi only modify lignin by demethylation. They are phylogenetically related to the white-rot fungi, so they share similar characteristics and they are considered to have similar degradation (D'Souza *et al*., 1999). They invade the lumens of wood cells, where they secrete enzymes that decompose and remove the polysaccharides, leaving behind a brown, modified lignin residue. Studies show that the lignin undergoes limited aromatic hydroxylation and ring cleavage, but that the major effect is demethylation of aromatic methoxyl groups. Brown-rot fungi are different from white-rot fungi because the breaking down of both lignin and cellulose starts with the penetration of diffusible small molecules and most of them do not produce Mn peroxidases (except for example *Polyporus ostreiformis;* Dey *et. al,* 1994*)* as white-rot fungi produce (Kirk and Farrell, 1987; Berg and McClaugherty, 2003). Soft rot fungi are mainly members of the Ascomycota. Some of them are able to degrade lignin by attacking the secondary plant cell wall and decreasing the amount of acid-insoluble material (called Klason lignin) in angiosperm wood. For example, *Daldinia concentrica* is able to degrade nonphenolic structures in lignin, similar to white-rot basiomycetes (Shary *et al*., 2007). It oxidizes the syringyl lignins present in hardwoods, but it is not able to oxidase the guaiacyl present in softwood lignin (Obst, 1982). As it has been shown, lignin degradation is a heterogeneous process which depends on the lignin structure and the fungi responsible for breaking it down. Current research considers members of the Basidiomycota as the main lignin decomposers, but the pathway they follow for lignin breakdown still remains ambiguous *i.e.* all the enzymes they secrete in the process; and it also remains unclear if other groups of fungi are also responsible for the complete breakdown of lignin.

In addition, bacteria might also be involved in lignin degradation (Chen *et al*., 2012; Zeng *et al*., 2013; Mathews *et al*., 2014, 2015). For example, *Novosphingobium sp*. B-7 was capable of secreting MnP and Lac using kraft liginin as sole carbon source, it broke down kraft lignin and produced alcohols and monomer benzene compounds (Chen *et al*., 2012). *Streptomyces viridosporus* T7A was able to remove lignin and hemicelluloses in wheat straw, as indicated by the modification of carbonyl and methoxyl groups in the lignin structure and deduction of guaiacyl units (Zeng et al., 2013). *Paenibacillus glucanolyticus*, a facultative anaerobic microorganism, was able to degrade cellulose, hemicellulose, and lignin (Mathews et al., 2014).

Cellulose is the most abundant plant polymer on Earth, during its hydrolysis, products such as soluble sugars and organic acids, that are essential sources of energy and carbon for microbial communities, are released (Leschine, 1995). It is built by glucose molecule units (3,000 to 5,000) that are bonded together by covalent bonds (Fig. 1.5; Kögel-Knaber, 2002). Cellulose is found in plant cell walls forming microfibrils (together with lignin and hemicelluloses) where it gives strength to the cell wall and allows circulation of water and solutes in and out of the cell, and water storage in the cell (Raven *et al*., 2005; Kögel-Knaber, 2002). Soft-rot fungi decay cellulose and hemicelluloses by moving 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 hemicelluloses, respectively: for example, cellulases break down bonds that results in the formation of short chain cellulose molecules which are more easily decayed (Carlile, 2001). Fungi such as *Fusarium culmorum, Trichoderma sp*. and *Chaetomium* are cellulolytic species that produce cellulases during the degradation of plant materials (Domsch *et al*., 1980; Harper and Lynch, 1985).

1.4 Techniques to assess the role of fungi as lignin and cellulose modifiers and saprotrophs

Fungi are able to degrade the lignocellulose component (LCS) of plant materials. However, this LCS is highly resistant to degradation, particularly because of the lignin structure (Kögel-Knaber, 2002). Lignin depolymerisation and its direct analysis are challenging because lignin is a large, complex, heterogenous polymer and it is highly resistant to strong acid/base hydrolyisis. To be able to analyse lignin structures, they must be broken down into smaller moieties ((Dungait *et al.*, 2008). Therefore, methods for the study and characterization of lignocellulose structures have been widely used, such as the copper (CuO) oxidation and gas chromatography-mass spectrometry (GC-MS) with tetramethylammonium hydroxide (TMAH) thermochemolysis. In CuO oxidation, there is a reaction of the macromolecular structures of lignin with the addition of CuO in a closed environment, thus allowing the determination of lignin chemistry (Nierop *et al*., 2005). However, during CuO oxidation, it is not possible to determine the chemistry of tannins and demethylated lignin (Nierop *et al*., 2005).

Gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis, also known as thermally assisted hydrolysis and methylation (THM) with TMAH, is a method well suited to analyse materials, e.g. barley straw and hawthorn wood, that have a mixture of tannin/lignin chemistry (Filley *et al*, 1999; Filley *et al*, 2006). During GC-MS with TMAH thermochemolysis, firstly the samples are mixed with TMAH to allow for the functional groups to react with the TMAH as soon as they are liberated during pyrolyis, for converting their corresponding methylester and methoxyl groups which will be detected using GCMS (Hoffmann and Stroobant, 2007). Then, a pyrolysis unit is used for breaking down large polymers into smaller fragments (moieties) through heating (May *et al*., 1977). The resulting smaller volatile fragments from the pyrolysis are transferred (using helium gas) into the column of a gas chromatograph to separate such fragments/moieties, and a mass spectrometer gives the information for identifying/quantifying such separated fragments (Fig. 1.6; Message, 1984). Finally, the moieties are analysed with specialised software to reconstruct the original structure of the polymers (Hoffmann and Stroobant, 2007).

The role of the TMAH is of interest because lignin contains a large number of functional groups that are converted to aldehyde groups upon fungal degradation. To be able to determine whether these functional groups are from the original or degraded materials, the detection of these functional groups is essential and can be done using lignin proxies such as the acid/aldehyde and syringyl/guaiacyl ratios (Vane *et al*., 2001; 2005; Kabuyah *et al*., 2012). However, the use of TMAH does not allow distinction between alcohol and methoxyl groups that are present in macromolecular structures such as in lignin and tannins (Vane *et al*., 2005).

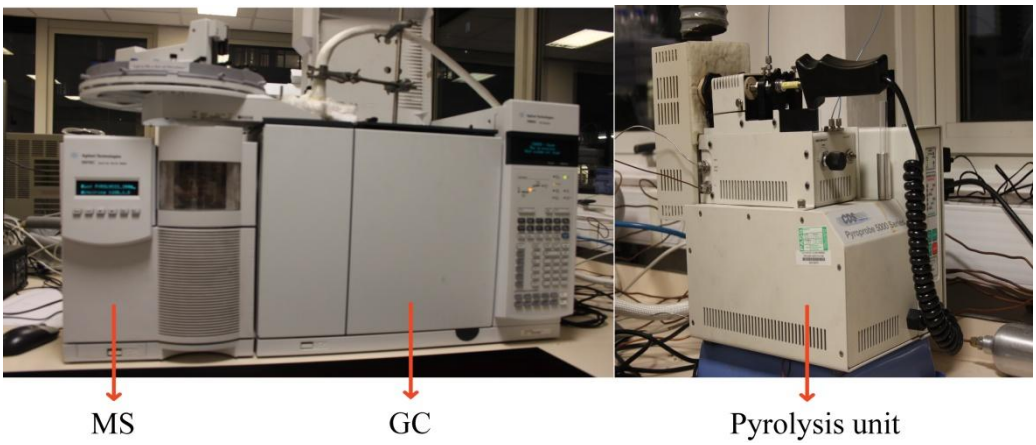


Figure 1.6. Photograph of a gas chromatograph mass-spectrometer (GC-MS) linked to a pyrolysis unit.

Even though hydrolysable tannins (*i.e.* 1,2,3,6-tertagalloylglucose; Fig 1.7) and lignin have different structures, they have the same basic aromatic structures. This is meaningful to highlight because all alcohol groups formed will be converted into methoxyl groups, but all the methylated aromatic products formed will be the same as those originating from lignin, therefore it is not possible to differentiate those from tannins or lignin (Fig. 1.7; Filley *et al*., 2006; Mason *et al*., 2009). To overcome this, enhanced THM using ^{13}C labelled TMAH is used (Filley *et al*., 2006; Mason *et al.*, 2009). The relative amount of ^{13}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 can also be analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis yielding distinctive products such as levoglucosan or furaldehyde (Gauthier *et al*, 2003, Kabuyah *et al*., 2012).

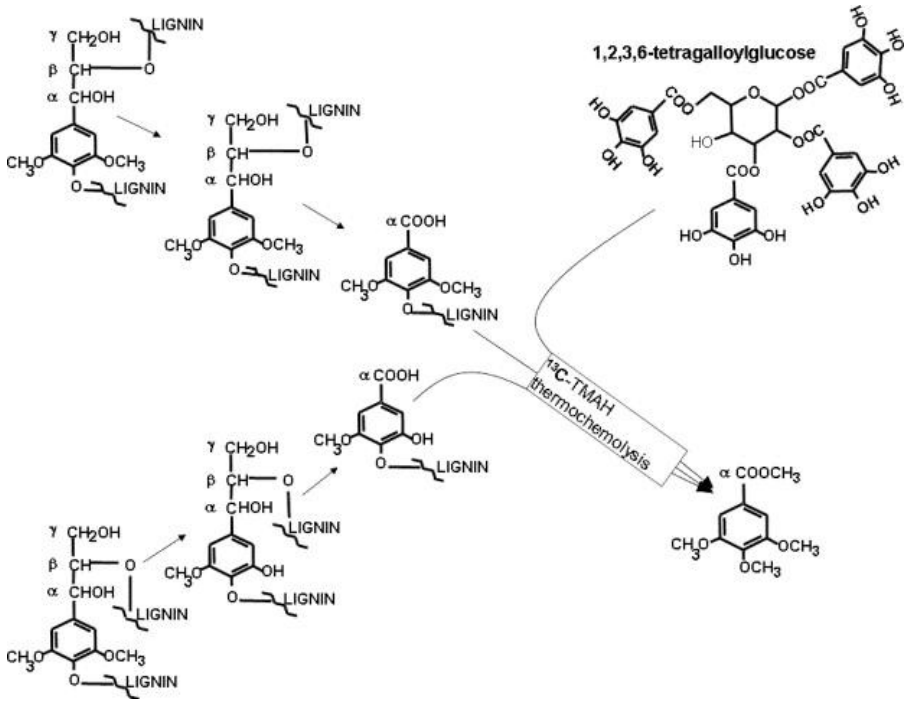


Figure 1.7. Illustration of how multiple sources lead to the same methylated structure upon TMAH themochemolysis. Specifically methyl-3,4,5-trimethoxy benzoate is shown derived from three potential sources (e.g. unaltered native lignin and hydrolysable tannins). Picture taken from Filley *et al*., 2006.

1.5 Aims and objectives of the thesis

Based on previous sections, it is clear that fungi play a vital role in ecosystem functioning. However, there are some challenges in the study of both their diversity and specific roles in the ecosystems. Firstly, it is unknown the real number of fungi on Earth which ultimately leads to a lack of knowledge about the roles of fungi in specific ecosystems (*e.g.* in sand dunes) or in unexplored areas. Therefore, there has been a need of an accurate estimate of fungal diversity which has led to the development of diverse methods and techniques for their identification. Even though traditional techniques, *e.g.* culturing, have helped in the estimation of fungal diversity, they have shown some bias against non-culturable or low sporulating fungi or it has been difficult to identify fungi by means of their sexual structures because they are sometimes similar. That is why relatively new DNA-based techniques are better suited for the identification of "difficult" samples such as environmental samples, which are a mixture of hundreds or thousands of different organisms, or samples with degraded DNA (*e.g.* from fossils). One of these DNA-based techniques is pyrosequencing which allows the assessment of a mixture of DNA without knowing *a priori* the taxonomic group of the organisms. In the case of fungi, this technique has allowed the assessment of the fungal diversity from, for example, permafrost samples (Bellemain *et al.*, 2013), boreal and alpine soils (Davey *et al.*, 2013) and coastal sand dunes (Geml *et al.*, 2014).

Secondly, it is known that fungal are involved in the degradation of organic matter in soils, such as in the degradation of lignin and cellulose. Soils are the largest reservoirs of terrestrial organic carbon and the degradation of organic matter largely determines soil properties. Therefore, it becomes essential to determine the process by which macromolecules such as lignin and cellulose are broken down. In general, there have been few laboratory-based reports about how fungi might be involved in the decomposition of woody materials. But it still remains unclear the mechanism of fungal degradation of woody materials in soils, such as of straws (but see Robinson *et al.*, 1994 and Kabuyah *et al.*, 2012). Also, the implications that fungal degradation may have on the soil carbon pool, at different periods of time and/or across different ecosystems which could imply the effects of different environmental conditions (*e.g.* changes in pH), remains ambiguous.

Therefore, in this thesis the following hypotheses will be tested:

1. The fungal community present in bait materials, *i.e. Crataegus monogyna* (hawthorn) wood and *Hordeum vulgare* L. (barley) straw, buried in sand-dune soils will fluctuate depending on the bait material types, the amount of time they remained buried, and the depth and ecosystem types at which the baits were buried.
2. The fungal community in the ecosystems where soils are well differentiated (*e.g.* fixed dunes) will be more diverse if compared with the fungal community in soils little/not differentiated (*e.g.* mobile dunes).
3. The extent of the degradation of the bait materials will be higher in well differentiated soils and at later sampling times, due to the richness in fungal communities at those soils and/or the time that the material was available to the fungal communities.
4. The degradation of different types of bait materials, barley straw and hawthorn wood, will be different because of their chemical properties.
5. Although fungi are capable of degrading lignin and cellulose in woody materials, the rate of lignin degradation will be lower if compared with the degradation of cellulose in woody materials.

The aims of this thesis are to:

1. Characterise the diversity of fungal mycelia colonising different types of bait materials buried across different sand-dune soils using 454 pyrosequencing.
2. Investigate how lignin and cellulose are degraded by these mycelia and compare the rates of lignin and cellulose degradation of the bait materials using the same analytical technique: gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis.
3. Compare lignin and cellulose degradation of barley straw and hawthorn wood, using acid/aldehyde, syringyl/guaiacyl and cellulose to lignin ratios

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

1. To assess the geochemical composition of the different sand-dune soils where the experiments will take place.

2. To bury different types of bait materials, *Hordeum vulgare* L. (barley straw) and *Crataegus monogyna* (hawthorn) wood, across different sand-dune ecosystems of two English National Nature Reserves, and at different depths from the soil surface.
3. To collect the samples randomly 3, 6 or 12 months after their burial (May/June 2012) in the field.
4. To amplify the fungal DNA of all the recovered samples and to select the samples that show the best DNA amplification for their sequencing using 454 pyrosequencing, for obtaining an overview of the fungal diversity at the sand-dune sites and at different sampling times.
5. To compare the chemical composition of barley straw and hawthorn wood using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis.
6. To determine the breakdown products of lignin/cellulose composition of the degraded straw and wood samples and compare these with the original materials buried in the field in May/June 2012.
7. To determine and compare the extent of lignin and cellulose decomposition of the different types of bait materials in the different sand-dune soils and from different sampling times.

1.6 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 two appendices summarizing the details of the methods utilized and conference contributions. Each of the chapters will be submitted in the near future **(Chapter 2, Chapter 3 and Chapter 4).**

The first part of the thesis **(Chapter 2)** deals with the geochemichal characterization/comparison of soils from different sand-dune ecosystems in two sand dunes National Nature Reserves in the UK, *i.e.* Sandscale Haws and Ainsdale Sand Dunes, to obtain insights into the status of the soil before the field experiment took place. This study revealed differences in the distribution of ions, *e.g.* the greatest ion concentrations were found in the dune slacks at Sandscale Haws and in the fixed dunes at Ainsdale. There were also some similarities in the geochemical characteristics of

soils, *e.g.* the absence of phosphate in all mobile dune soils or the same pattern in soil pH (*i.e.* alkaline *versus* more acidic sites). **Chapter 3** deals with assessment of the diversity of fungal communities colonising buried bait materials barley (*Hordeum vulgare*) straw and hawthorn (*Crataegus sp.*) wood. Bait materials were buried at defined depths, at three sand-dune ecosystems of two UK sand-dune reserves, and collected at different sampling times to characterise the fungal mycelia colonising the materials by 454 pyrosequencing. A great richness of fungi was detected, including 275 Operational Taxonomical Units (OTUs) from which only 92 OTUs had >97% ITS1 sequence similarity. These OTUs corresponded to different fungal ecologies (*e.g.* wood-rotting fungi) and were distributed differently across the ecosystems and material types in Sandscale Haws, and ecosystems and sampling time in Ainsdale. **Chapter 4** deals with lignin and cellulose breakdown products analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis, in the original *Hordeum vulgare* L. (barley) straw and *Crataegus monogyna* (hawthorn) wood samples compared with those in samples which had been buried as a "model" resource for 3, 6 or 12 months in different sand-dune ecosystems, at defined depths from the soil surface, at Sandscale Haws and Ainsdale National Nature Reserves, UK. This study revealed that lignin oxidation occurred in both bait materials during the period, as there were general increases in the $[Ac/Al]_S$ and $[Ac/Al]_G$ ratios. There was also a clear decrease in the [S/G] ratio in the recovered barley straw samples, however, this [S/G] ratio increased in the retrieved hawthorn wood samples probably because of tannins. These data provided tentative support for the theory that white-rot basidiomycete fungi may be involved in the degradation of lignin in sand-dune soils, the presence of this group of fungi was confirmed in **Chapter 3**.

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 fieldwork and laboratory data reported, unless this was otherwise stated elsewhere. Details of conference presentations have also been given in Appendix B.

1.7. Author's and co-authors's contributions to each paper

- Paper 1 (Chapter 2) - *Author* - the principal investigator, collecting the samples, preparing and analysing the samples for geochemical content, analysing and interpreting data, writing of manuscript. *Alastair D. Bewsher* – obtaining

concentrations of 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 the field experiment, collecting samples, performing DNA extraction and amplification, preparing samples for 454 pyrosequencing, analysing and interpreting data, writing of manuscript. *Clare H. Robinson* – setting up the field experiment and collecting samples; *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. *Clare H. Robinson* - setting up the field experiment and collecting samples; *Clare H. Robinson* and *Bart E. van Dongen* - complete conceptual guidance and manuscript review.

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Chapter 2

Paper 1. Geochemical characterisation of soils from Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves

This chapter contains the following paper which is in preparation to be submitted to the *European Journal of Soil Science*

Irma González González, Bart E. van Dongen and Clare H. Robinson. Geochemical characterisation of soils from Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves

Paper 1. Geochemical characterisation of soils from Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves

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Abstract

Soil nutrient availability in relation to the distribution and diversity of living organisms has been studied for plants and microorganisms. Hence, it has been recognized that there is a link between, for example, microbial and plant diversity which is also linked to geochemical characteristics of soils. The influence of living organisms, such as variation and abundance of soil organisms, is also a key determinant of nutrient release. Geochemical characteristics of soils from different sand-dune ecosystems (*i.e.* mobile dunes, dune slacks and fixed dunes) at Sandscale Haws and Ainsdale Sand Dunes National Nature Reserve have been assessed such as ion concentrations, pH and total organic carbon (loss-on-ignition). There were differences in the ion concentrations within and between reserves: the highest concentration of ions were at the dune slacks in Sandscale Haws and the fixed dunes in Ainsdale. Some ions had the highest concentration at particular ecosystems, such as calcium and ammonium ions in the fixed dunes in Ainsdale, potassium in the dunes slacks in Sandscale and phosphate in the fixed dunes also in Sandscale. There were also some similarities in the geochemical characteristics of soils such as the absence of phosphate in all mobile dune soils, the very low nitrate concentration and the high sodium concentration in all fixed dunes ecosystems, and the same pattern in both soil pH (*i.e.* alkaline *versus* acidic sites) and LOI suggesting that similar soil processes in both reserves could be occurring.

2.1. Introduction

The physical and chemical characteristics of soils play an important role in the establishment of biological communities (Torsvik and Øvreås, 2008; MaciaVicente *et al*., 2012). For example, the predominance of cyanobacteria in dry mountains of the Himalaya is due to a combination of different factors such as high soil pH and unstable soil substrate (Reháková *et al*., 2010), soil pH appears to control the distribution patterns of bacterial diversity in different ecosystem of North and South America (Fierer and Jackson, 2006), and the spatial variability in nutrients, organic matter inputs and soil temperature may control bacterial functionality in an oak-pine forest (Clark *et al*., 2009).

Although fungi have developed different life strategies to exploit a great variety of habitats, there are essential elements that are needed for fungal growth. Nitrogen, for instance, is essential for the synthesis of cell components such as nucleic acids and chitin; many fungi are able to utilize nitrate which is common in soils as a nitrogen source (Carlile *et al*., 2004).

Jones and Jennings (1964) studied the effect of cations on the growth of fungi in different media: they showed that fungal vegetative growth was inhibited by sodium, although the concentration varied from species to species. However, the addition of calcium ions reduced the extent of this inhibition in some species of fungi, *e.g Dendryphiella salina.* They also showed that *Penicillium notatum* required calcium for sporulation but the need of this cation was reduced as the concentration of sodium nitrate decreased.

Non-essential elements for fungal growth can sometimes partially replace the requirement for an essential element: provision of sodium may reduce the amount of potassium needed. However, an organic form of carbon is always needed, as all fungi are heterotrophic, which is obtained in the form of, for example, glucose, starch, cellulose or lignin. Fungi also must be prepared to cope with changes in external pH to a greater extent than do animal or plant cells (Ingold and Hudson, 1995; Deacon, 2006). Geml *et al.* (2014) showed that fungal richness in protected coastal communities in the Netherlands was negatively correlated with higher soil pH.

Fungi are able to modify the concentration of ions in their environment as they capture, accumulate, release and recycle nutrients (*e.g* carbon, phosphorus, nitrogen). For example, fungi convert organic nitrogen into ammonium (NH_4^+), after a plant or animal dies, or an animal excretes waste. This process, which occurs during the nitrogen cycle,

is called ammonification or mineralization. Additionally, soil fungi take and retain soil nutrients, prevent leaching and make nutrients available for plant intake (Tlalka et al., 2008), because of their mycelia which capture and release nutrients after death and lysis or attack by pathogens and grazers (Galloway, 1995).

Dune soils are generally poor in organic and inorganic nutrients (van der Meulen *et al*., 2004). However, the low level of nutrients allows a great diversity of plant species since it restricts the growth of tall plants and allows many smaller plants to establish (van der Meulen *et al*., 2004). Coastal dunes are highly vulnerable ecosystems because of their position at the border between land and sea that makes them attractive to many other functions than nature conservation alone (*e.g* recreation, van der Meulen *et al*., 2004). In sand dunes with sand comprised mainly of sea-shell fragments and other calcareous materials, species-rich grassland is often colonised by *Salix repens* (creeping willow) which commonly forms ectomycorrhizas with fungi (Geml *et al.*, 2014). In sand dunes made of silica, the calcium carbonate has leached from the surface, so this results in acidic conditions that allow the dominance of plants such as *Calluna vulgaris* (heather, Agate and Brooks, 1986).This plant forms a symbiosis with ericoid mycorrhizal (ErM) fungi to overcome nutrient limitation through fungal mobilization of nutrients complexed in recalcitrant organic matter (Vohník *et al*., 2012). However, sodium chloride (salt) concentrations, water and organic matter contents of the sand are probably the most important factors that influence the distribution of fungi in a dune system (Brown, 1958). Therefore, as the geochemical composition of soils is relevant for the composition of fungal communities in any given study area, the aim of this study is to assess/compare the geochemical composition of soils from different ecosystems in two sand dunes National Nature Reserves in the UK, *i.e.* Sandscale Haws and Ainsdale Sand Dunes to know the status of the soil before the field experiment takes place.

2.2 Methods

2.2.1 Study sites

The first study site was at Sandscale Haws National Nature Reserve (National Grid Reference SD200756; Fig. 1A & B, Appendix A), which comprises areas of different sand-dune successional ecosystems, such as dune slacks, grassland and freshwater

marsh and small areas of woodland and scrub with few disturbances. It is one of most botanically rich coastal sites in the UK where over 600 species of plant species have been recorded (Skelcher, 2012).

The second site was at Ainsdale Sand Dunes National Natural Reserve (Grid Reference: SD292110, Fig. 1A & C, Appendix A). This reserve protects important plant and animal biodiversity in England. The most typical sand dune ecosystems are well characterized: embryonic and mobile dunes, humid dune slacks, fixed dune grasslands and dune heath (Skelcher, 2009).

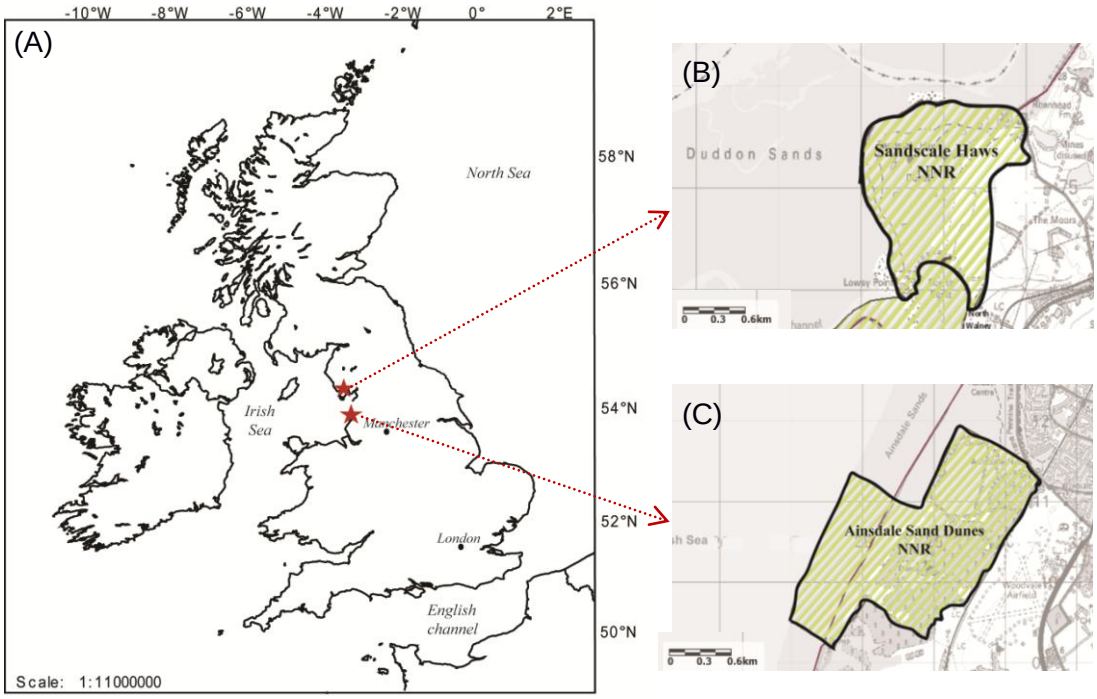

Figure 2.1. (A) Map of the United Kingdom pointing out the study sites. (B) Sandscale Haws and (C) Ainsdale Sand Dunes National Nature Reserves (NNR).

Three different ecosystems within each reserve were chosen for the experimental set up. The choice of the different ecosystems is based on the research about fungal succession in sand dunes made by Brown (1958) who described a pattern in ecological succession using classical techniques of fungal isolation on agar media.

Special notes about these ecosystems at Sandscale Haws NNR (Skelcher, 2012; Appendix A):

a) *Mobile dunes* in Sandscale Haws are found in the A*mmophila arenaria* mobile dune community. The soil profile is undifferentiated with accreting mobile dunes (personal

observation). There is no active management to allow natural processes of accretion to occur. This area is classified as SD6 *Ammophila arenaria* mobile dune community under the National Vegetation Classification (NVC) for the United Kingdom.

b) *Dune grassland / slack* in Sandscale Haws is located within the *Salix repens – Calliergon cuspidatum* dune slack community. This area is grazed by cattle, sheep and rabbits. No scrub control has taken place in this area in the past five years. This area is classified as SD16 *Salix repens – Holcus lanatus* dune-slack community or SD15 *Salix repens – Calliergon cuspidatum* dune-slack community under the NVC.

c) T*he fixed dune* is located within the *Salix repens – Holcus lanatus* dune slack community. This area is grazed by cattle, rabbits and sheep. No control of the scrub has taken place in the past five years. This area is classified as SD16.

Special notes about these ecosystems at Ainsdale Sand Dunes NNR (Skelcher, 2009):

a) *Mobile dunes* have had scrub species cleared from them in the past and more than 100 years ago the dunes were built up artificially high by the use of sand trap fencing. The dune will have rolled inland over the top of a slack because of coastal erosion processes, perhaps as recently as the last 20 years, so a new dune has been made from an eroding old dune. This area is classified as SD6 *Ammophila arenaria* mobile dune community under the NVC for the UK.

b) *Dune grassland / slack* has been mowed on several occasions and been grazed with sheep every winter since early 1990s. There were some *Salix repens* patches (personal observation). This area corresponds to the SD16 *Salix repens- Holcus lanatus* dune slack community under the NVC for the UK.

c) The fixed dune is located within a *Betula* (Birch) woodland that was used to store water up until 1965 and has had no management since then apart from grazing in winter by cows over the last 4 years.

In addition, two soil depths were chosen for the experimental set up. The selection of the depths 5 and 11 cm depends on the horizons' delimitations and characteristics which allow the presence of fungal communities. The 5 cm depth is the limit of the organic horizon. Bardgett (2005) pointed out that this horizon is biologically important since we can find decomposing litter with different biological communities associated which can be more or less active (e.g. beneath a grassland soil, fungal communities can be driving the litter decomposition rather than bacteria). The limit of rooting depth can be found

around 11 cm and, for example, in sand alkaline dunes it is the limit of the "grey" sand horizon in semi-fixed "grey" dunes (Brown, 1958).

2.2.2 *Experimental set up and field sampling*

Nine 20 x 45 cm plots were set up across three different ecosystems, *i.e*. mobile dunes, dune grassland/slacks and fixed dunes, within both reserves. Six 20 x 45 cm plots were set up at Sandscale Haws NNR, on the 16th and 22nd of May 2012, two plots in each of three different ecosystems (Table 2.1). Three 20 x 45 cm plots were set out at Ainsdale Sand Dunes NNR, on the 7th of June 2012. These plots were chosen for the field experiment where mesh bags filled with bait material were buried for assessing fungal diversity (Chapter 3) and degradation of bait materials (Chapter 4). The choice of three different ecosystems is based on the research about ecological fungal community composition in sand dunes made by Brown (1958).

Triplicate soil samples were randomly collected within each of the nine plots (triplicates because three is the minimum number of replicates in order to be able to obtain a statistical valid mean and standard error). Each of the nine soil collections were made with a pre-furnaced spatula which was washed with ultrapurified water between each sampling. The soils were placed separately into pre-furnaced glass jars and stored in a coolbox containing frozen coolpacks in the field, and finally stored in the freezer at -20 ºC in the laboratory until needed for geochemical analyses.

2.2.3 Ion chromatography analysis

Twenty-seven soil samples (4.9 – 14.8 g dry weight): 18 soil samples from Sandscale Haws and 9 soil samples from Ainsdale Sand Dunes NNR, were oven-dried at 60ºC overnight, then transferred into clean tubes and weighed on an analytical balance. Bidistilled water, 24 ml, was added to each tube and the tubes were placed into the ultrasonic extraction equipment for half an hour. The solutions in the tubes were left to stand overnight. Finally, the supernatant was filtered into clean tubes with a syringe through 0.2 µm polypropylene filters and the pH was measured using an automatic pH meter which was calibrated with both pH 4 and pH 7 buffers.

Cations were analysed by ion chromatography on a Dionex DX120 Ion Chromatograph and anions were analysed on a Metrohm 761 Compact Ion Chromatograph.

2.2.4 Loss on ignition analysis

Approximately 1 g of soil sample was placed into pre-weighed porcelain crucibles, heated at 105°C for 1 h and placed into a desiccator for cooling for 20 min. After the weight was recorded, the crucibles containing the sample were heated at 1000°C for 1 h, cooled in a desiccator for 30 min and finally weighed again. The loss on ignition is calculated as a percentage of the original weight using the weight loss between 105°C and 1000°C.

2.3 Results

Ion chromatography analyses and loss-on-ignition as a measure of total organic carbon were carried out. Results of ion chromatography analyses are shown in Table 2.2. Our results showed that there were differences in the geochemical composition of soils within and between reserves. There were also patterns in nutrient distribution both, between and within the two different reserves (Fig. 2.2-2.6). These results showed the differences in composition between the reserves which are in accordance with the chemical and physical composition of Sandscale Haws as lime-rich dunes (Nelson, 1975) and Ainsdale, as calcareous sand dunes (Smith *et al.*, 2011). Nevertheless, living organisms can modify this composition by releasing, circulating or decomposing nutrients.

In mobile dunes, the concentration of Cl^- and Na^+ was higher than other ions. Ca^{2+} concentration was similar across the three mobile dune sites, this could suggest that sand dunes are made from sea-shells ($CaCO_3$). The concentration of the K^+ ion was higher than other ions in fixed dunes in Sandscale Haws and in dune grassland/slack, fixed dunes in Ainsdale. According to our results in general in Ainsdale sand dunes NNR, there was a marked pattern in the ion concentration distributions across succession in vegetation. The ion concentrations increased from the nearest ecosystem to the sea (*i.e.* mobile dunes) to the farthest one (*i.e.* fixed dunes; Fig. 2.6).

Soils samples from the fixed dunes at Ainsdale sand dunes NNR showed the highest percentage weight lost on ignition (21.29 %; Table 2.2, Fig. 2.7b). A visual examination of the samples before being assessed for LOI showed that all the fixed dunes contained a high amount of roots and were more “compact” soils compared with sandy soils from the mobile dunes (Fig. 2.7a).

Table 2.1 Soil samples collected in May and June 2012 from Sandscale Haws and Ainsdale Sand dunes National Nature Reserves.

Sample	Location (Ecosystem)	Type of soil	Dominant vegetation	Type of buried bait material
Sandscale Haws National Nature Reserve				
S1	Mobile dunes	Yellow sand	Sea lyme grass *(Leymus arenarius)*	Barley straw
S2	Mobile dunes	Yellow sand	Sea lyme grass *(L. arenarius)*	Hawthorn wood
S3	Dune grassland/slack	Grey sand	Marram grass *(A. arenaria)*	Barley straw
S4	Dune grassland/slack	Grey sand	Marram grass *(A. arenaria)*	Hawthorn wood
S5	Fixed dunes	Brown humus/grey sand	*C*ommon hawthorn (*Crataegus monogyna)*	Barley straw
S6	Fixed dunes	Brown humus/grey sand	*C*ommon hawthorn (*C. monogyna)*	Hawthorn wood
Ainsdale National Nature Reserve				
A1	Mobile dunes	Yellow sand	Marram grass *(Ammophila arenaria)*	Barley straw
A2	Dune grassland/slack	Grey sand/Brown humus	Creeping willow *(Salix repens)*	Barley straw
A3	Fixed dunes	Brown humus/grey sand	Birch *(Betula spp)*	Barley straw

Table 2.2 Concentrations (µg g^{-1}) of water extracted cations and anions in soil samples collected in May and June 2012 from Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves.

Sites	**Sandscale Haws NNR**						**Ainsdale Sand Dunes NNR**		
Sample ID[a]	S_1	S_2	S_3	S_4	S_5	S_6	A_1	A_2	A_3
Cations									
NH_4^+	0.53 ± 0.1	0.52 ± 0.07	76.8 ± 93.6	8.53 ± 6.8	13.7 ± 7.3	10 ± 9.8	0.21 ± 0.01	5.72 + 3.42	58.5 ± 53.3
Na^+	28.4 ± 6.6	10.1 ± 11.6	39.7 ± 28.9	14.8 ± 7.8	62.6 ± 16.7	80.5 ± 53.6	2.6 ± 1.1	43.1 ± 10.5	63.6 ± 21.6
Ca^{2+}	19.4 ± 2.4	19.9 ± 3.8	70.9 ± 81.1	17.4 ± 6.3	20.7 ± 9.2	10.9 ± 7.7	20.3 ± 3.0	20.9 ± 5.4	113.7 ± 61.2
Mg^{2+}	3.8 ± 0.51	2.9 ± 0.4	27.1 ± 30.7	7.3 ± 2.5	7.6 + 3.1	4.6 + 3.8	1.7 ± 0.4	4.5 ± 1.4	18.9 ± 9.4
K^+	6.1 ± 0.48	6.7 ± 1.6	169.9 ± 133.7	47.3 + 36.5	18.7 ± 6.03	34.7 ± 37.5	4.8 ± 0.88	11.1 ± 1.06	154.2 ± 87.1
Anions									
Cl^-	49.7 ± 9.8	6.4 ± 8.6	42.9 ± 40.3	19.9 ± 15.2	15.4 ± 5.9	37.9± 39.0	1.9 ± 2.4	6.7 ± 1.4	62.5 ± 24.0
NO_3^-	0.58± 0.11	0.7 ± 0.4	*bd*	*bd*	*bd*	1.9 ± 0.8	0.4 ± 0.04	3.4 ± 2.3	*bd*
PO_4^{3-}	*Bd*	*bd*	162.1 ± 210.6	*bd*	23.1 ± 10.4	58.1 ± 63.7	*bd*	6.7 ± 2.0	25.3 ± 12.6
SO_4^{2-}	11.9 ± 2.6	2.3 ± 2.3	33.3 ± 20.7	13.3 ± 10.9	9.4 ± 4.8	15.3 ± 9.5	0.82 + 0.5	5.6 ± 1.5	40.7 ± 19.1
Loss-on-ignition (%)	1.41 ± 0.07	1.74 ± 0.24	9.25 ± 5.3	2.09±1.19	2.31 ± 0.43	7.72 ± 6.45	3.19 ± 0.26	7.35 ± 1.39	21.3 ± 2.74
pH (average value and min. and max for 3 samples)	7.2 (7.13-7.26)	7.4 (7.36-7.49)	6.5 (6.13-6.86)	6.3 (6.18-6.62)	6.4 (5.53-6.91)	6 (5.7-6.2)	7 (6.85-7.19)	6.6 (6.47-6.75)	6.2 (6.04-6.42)

[a]For sample ID see Table 2.1

bd: Below detection limit

S_n: Mean of three (± S. E.) soil samples collected from the same ecosystem in Sandscale Haws National Nature Reserve.

A_n: Mean of three (± S. E.) soil samples collected from the same ecosystem in Ainsdale Sand dunes National Nature Reserve

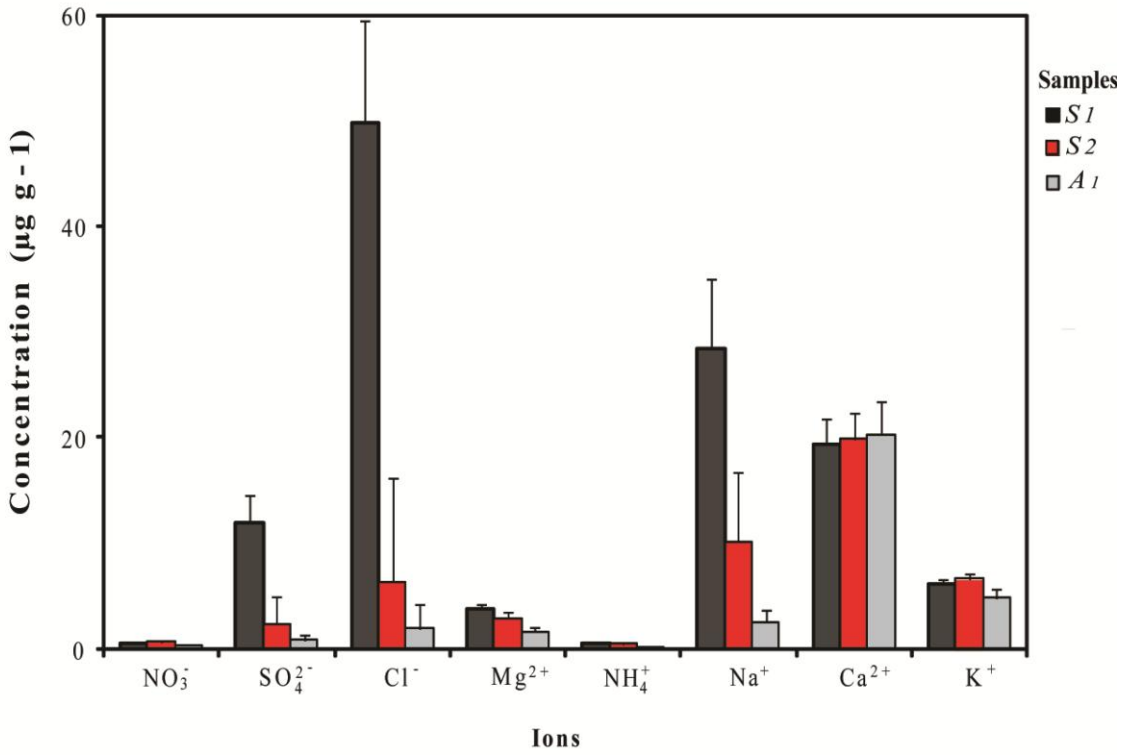

Figure 2.2. Distribution of ions in mobile dunes. For Sample ID, see Table 2.1.

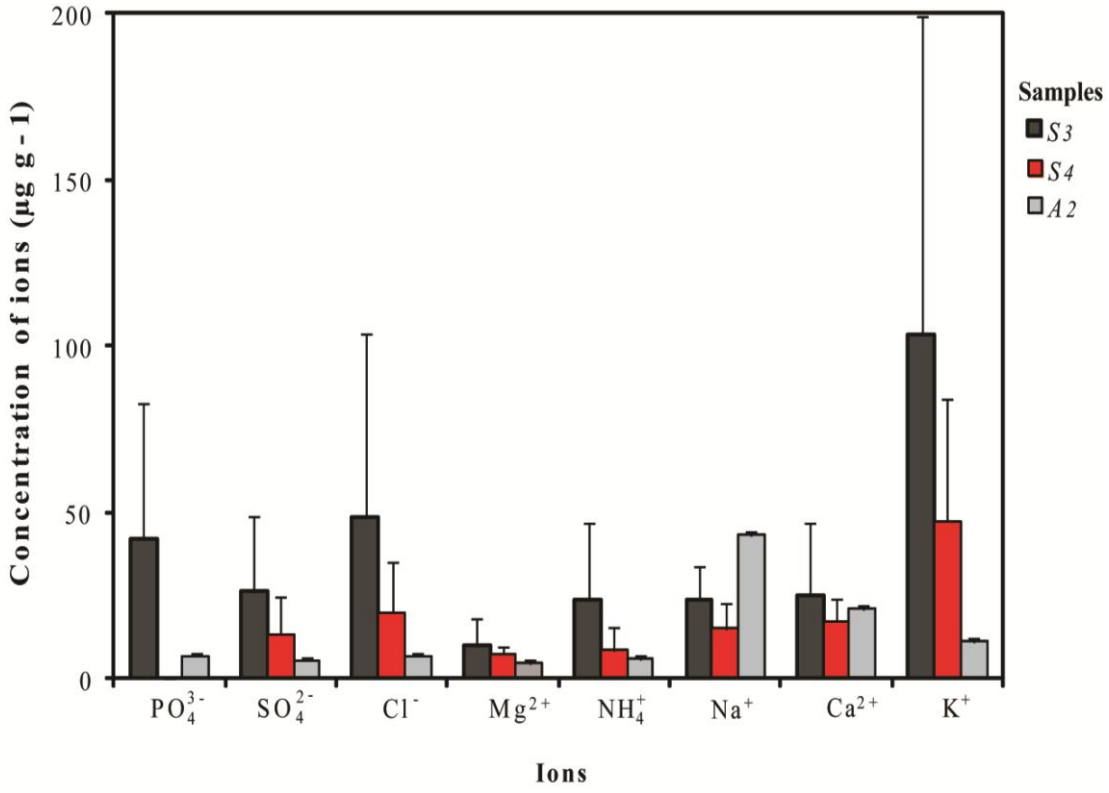

Figure 2.3. Distribution of ions in dune grassland/slack. For sample ID see Table 2.1

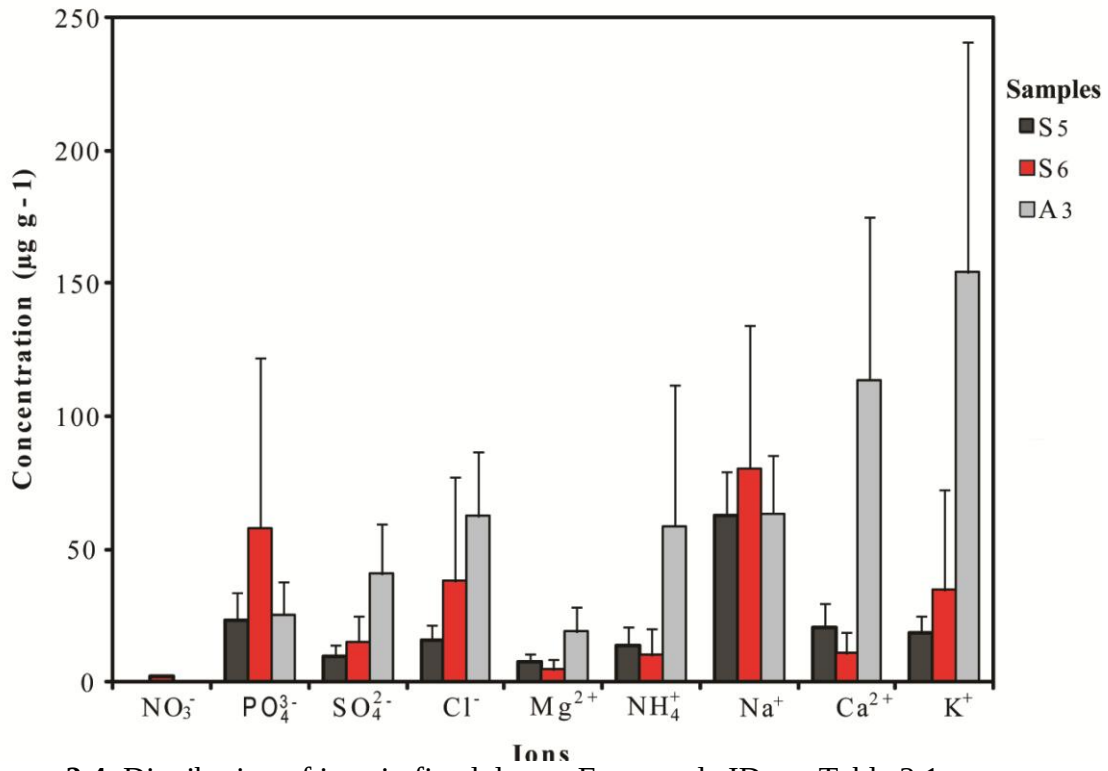

Figure 2.4. Distribution of ions in fixed dunes. For sample ID see Table 2.1.

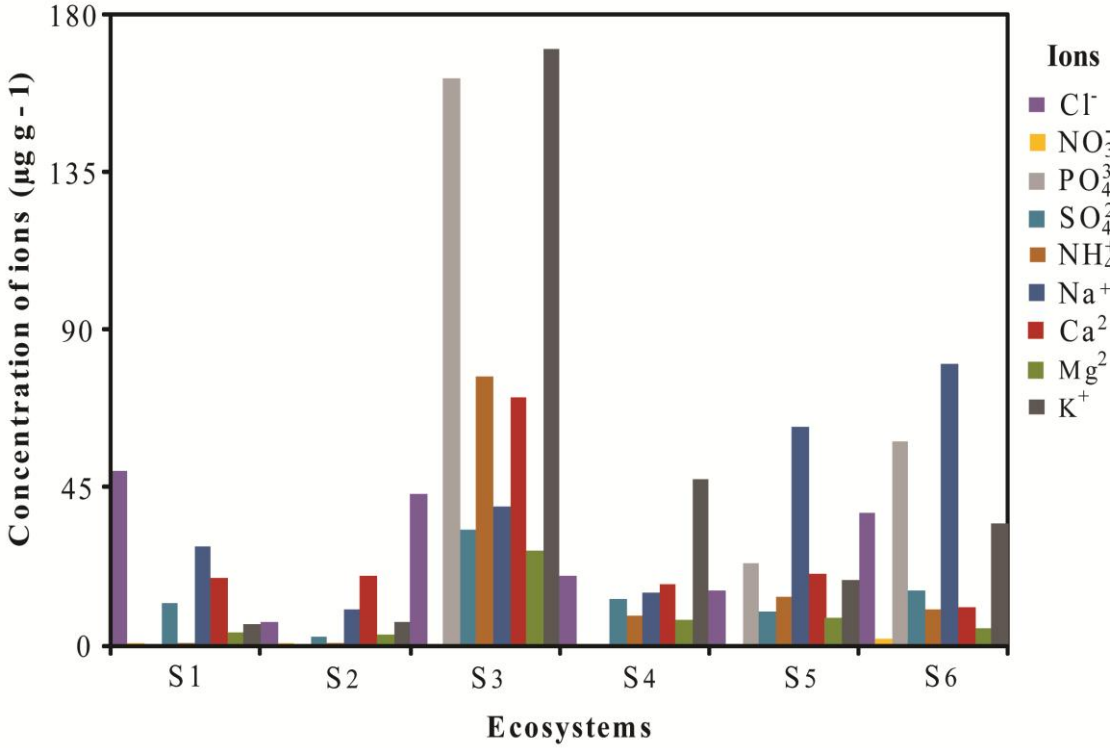

Figure 2.5. Distribution of ions in Sandscale Haws NNR across different ecosystems: mobile dunes (S1 and S2), dune slacks (S3 and S4) and fixed dunes (S5 and S6).

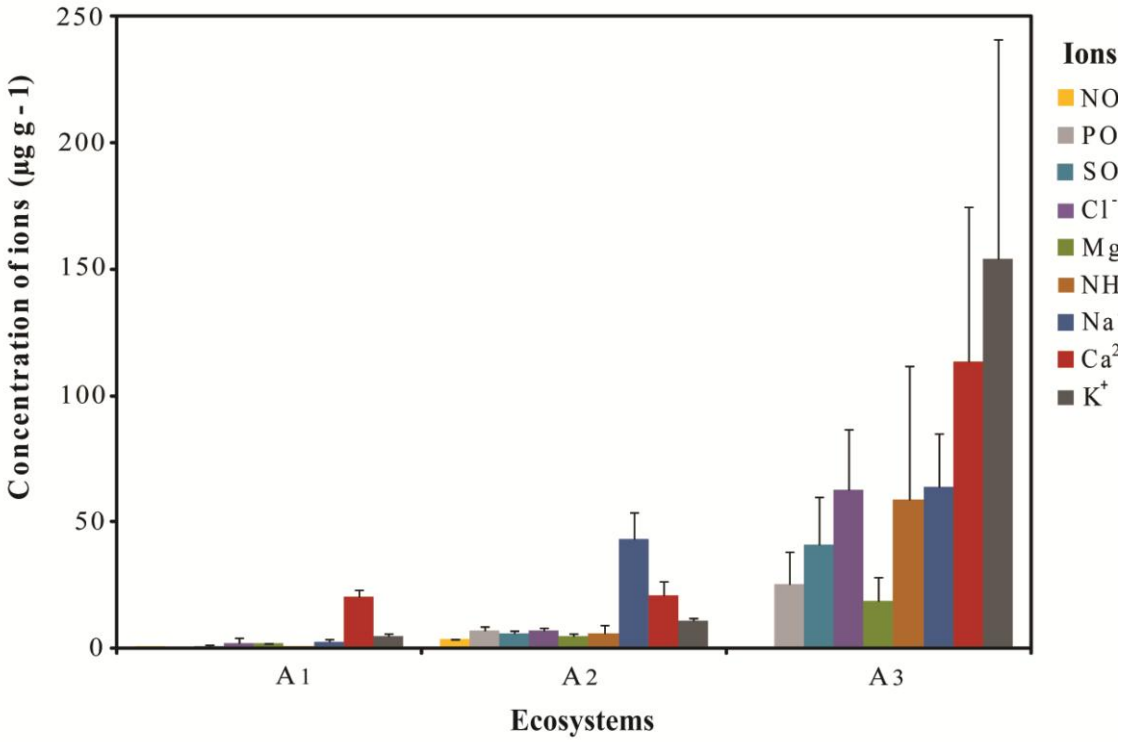

Figure 2.6. Distribution of ions in Ainsdale sand dunes NNR across different ecosystems: mobile dunes (A1), dune slacks (A2) and fixed dunes (A3).

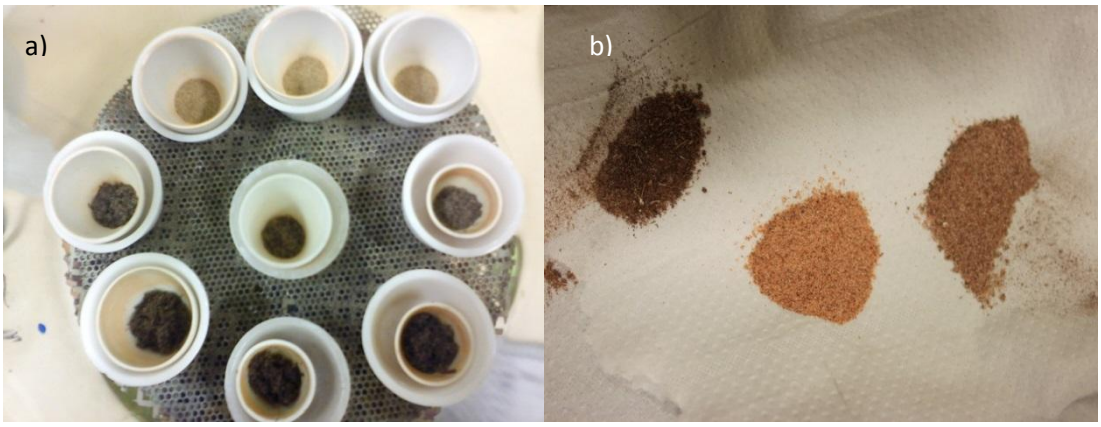

Figure 2.7. Soil samples from Sandscale Haws and Ainsdale sand dunes NNR. a) Soils prior to being treated for LOI analysis, top row: mobile dunes (S1, S2 and A3); middle row: dune slacks (S3, S4 and A2); bottom row: Fixed dunes (S5, S6 and A3). b) Soils after treating for LOI analysis, left: fixed dunes (S6), middle: mobile dunes (S4), right: dune slacks (A3).

2.4. Discussion

Concentration of ions across sand-dune soils

Sand dune soils are poor in organic matter and nutrients, including nitrogen, phosphate and potassium (Ranwell and Boar, 1986), particularly, in freshly blown sand from the beach (Willis, 1985). Kabuyah *et al*. (2012) determined the concentrations of water-extracted ions in samples from a sand-dune grassland site at Ainsdale Sand Dunes NNR. Our results from a similar site at the same reserve are different, as the concentrations of $NH_4^{+,}$ Na^+, PO_4^{3-} and SO_4^{2-} were smaller than reported in that study, by contrast , Ca^{2+}, Mg^{2+}, Cl^- and NO_3^- were higher. These differences could be explained by the fact that our soil samples were collected at a different time to the samples analysed in Kabuyah et al. (2012) which were collected in March 1st 2009, and not exactly from the same sand-dune site.

Soluble nutrients that are leached downwards by rain in a dune system enrich the water table, so, while the dunes become depleted of nutrients the slacks become richer (Ranwell and Boar, 1986). This could be true in Sandscale Haws because most of the ions from dune-slack soils showed generally higher concentrations if compared with soils from the other sites, and this area was particularly flooded during the autumn and winter 2012 - 2013. By contrast, at Ainsdale, soils from the fixed dune showed higher concentrations of ions probably due to the effect of grazing and manure as the fixed dunes are grazed by cows which can alter the soil composition (Shen *et al*., 2011).

A study in the dune system of Braunton Burrows, North Devon Coast, UK, showed that there were generally low levels of several major nutrients, *i.e.* nitrogen, phosphorus, potassium and magnesium, however, magnesium was highest in the foredunes which is the closest ecosystems to the sea and decreased further inland (Willis, 1985). Our results are different to that study as the concentration of magnesium is higher in all dune slacks and fixed dunes sites than in the mobile dunes (Fig. 2.5 & 2.6; Table 2.2) which was the closest ecosystem to the sea in our study.

In our study, the concentration of potassium was particularly high in the fixed dunes and in the dune slacks if compared with the concentration of potassium in the mobile dunes. K^+ is necessary for plant metabolic processes, thus, the high concentration of this ion could show the stability of the vegetation in these sites, as a deficiency of the ion could

result in poor plant growth and colonization. Moreover, the presence of this ion could tell us about the soil moisture and age because readily leached potassium is scarce in the older drier parts of the dune system (Willis, 1985).

Chloride concentration is appreciable in the foredunes, but at low levels elsewhere (Willis, 1985). At Ainsdale the chloride concentration was extremely high in the fixed dunes if compared with the mobile dunes, while in Sandscale the concentrations of this ion did not follow a pattern. These results are surprising as the mobile dunes is the ecosystem that is closest to the sea, therefore it should be expected to measure a higher concentration of chloride at this ecosystem as it is more in contact to seawater which contains high levels of chloride (Tsang, 1997).

Nevertheless, the concentrations of nutrients in sand-dune soils might be altered by human disturbance and trampling, grazing and manure deposition (Wallis, 1985). Human disturbance lowers levels of major nutrients (N, C, P, K and Ca, Ranwell and Boar ,1986), while manure adds important nutrients to the soil, such as nitrogen because of its high ammonia content, and also phosphorus, potassium and other nutrients (Whalen *et al*., 2000; Shen *et al*., 2011).The study sites are open to the public and are grazed by sheep, cows and rabbits, therefore, the concentration of ions might be a result of a mixture of natural, geological and anthropogenic factors.

pH assessment in different sand-dune ecosystems

Sand dune soils present wide variations in their $CaCO_3$ content and this variation is reflected in a variation in pH across the dune system (Ranwell and Boar, 1986). A variation in pH was observed in the assessed soils in our study (Table 2.2).

All the assessed mobile-dune soils had alkaline soils which could be attributed to the fact that these ecosystems are closer to the foreshore where the deposit of calcium carbonate from shells is higher (Wallis, 1985). Moreover, the fresh sand that is being deposited is commonly alkaline, calcareous and low in organic matter (James, 1993).

By contrast, all soils from dune slack/grasslands and from fixed dunes, in both study sites, were more acidic (Table 2.2). An explanation for this is the role of rain in nutrient leaching. Rain leaches soluble Ca and other nutrients, but organic matter is added to the soil and results in an increase in moisture holding capacity, and plant cover increases as

a result. Thus, the soil always tends to become more acidic with time (Ranwell and Boar, 1986) and with distance from the sea. The distance from the sea is an important factor which contributes to soil pH, and that is also related to soil age. pH of the organic topsoil (A horizon) decreases significantly with distance from the sea (James, 1993). In sand of the South Haven Peninsula, Dorset, UK, leaching resulted in decreases of carbonate content that led to build-up of organic carbon from progressive plant colonization and growth associated with the loss of carbonate, therefore, leading to acidification (Willis, 1985). In the current study, during one field sampling (January 2013) at both sites, the area in the dune slacks (Sandscale Haws and Ainsdale) and in the fixed dunes (Sandscale Haws) where the plots were placed were flooded, thus, indicating that rain could have indeed leached nutrients from the soils. Kabuyah *et al*. (2012) reported the pH of a dune grassland site which was slightly lower than the pH reported here for a similar dune slack/grassland site at the same reserve, (Ainsdale Sand Dunes NNR. However, both the pH value (6) measured by Kabuyah *et al*. (2012) and the pH value (6.6) measured in our study correspond to slightly acidic/nearly neutral soils. Some other sand-dune grassland soils have been classified as acidic soils (pH from 5.32 to 6.39; Geml *et al.* 2014). A pH value close to acidic could be due to the vegetation that characterises these areas which plays a relevant role in the stabilisation of dune soil or, in the case of our study areas, they are grazing areas where manure is commonly observed and manure lowers the pH (Shen *et al*., 2011).

Loss on ignition as a measure of total organic carbon

The assessment of loss-on-ignition (LOI) of soils showed a pattern that has been commonly explained before: soils on inland parts of the dune system characteristically have higher losses on ignition than freshly blown sand near the shoreline sand (Wallis, 1985). Brown (1958) was able to determine the organic matter content (% LOI) in soils from different UK sand dune sites. She found that the percentage of LOI in mobile yellow dunes was lower if compared with the LOI of soils from other sites of the dune system. Our results are in line with previous studies as the mobile-dune ecosystems had indeed the lowest amount of LOI if compared with the dune slacks and fixed dunes, in both reserves. Moroever, the fixed dune site at Ainsdale showed the highest LOI (21.3 ± 2.7, Table 2.2) if compared with the other ecosystems at Ainsdale and at Sansdcale which could be explained by the fact that these soils were indeed from the furthest ecosystem

to the sea and they had high amount of roots and were more "compact" soils if compared with the other ecosystems. This means that they had a higher increase of organic matter than the other ecosystems, thus suggesting that these soils could be more developed (Willis, 1985).

Brown (1958) was able to determine the organic matter content (%loss-on- ignition) in soils from different sand dune sites. For the alkaline sites, she found that the percentage of loss-on-ignition in mobile "yellow" dunes 0.8-1.5%. Our results are different from those she has previously reported in similar soils as the LOI of soils from mobile dunes which are more alkaline sites is much higher (1.41 – 3.9 %, Table 2.2). A recent study (Kabuyah *et al,*. 2012) at Ainsdale showed that a sand-dune grassland site had relatively low amounts of native organic matter; our results are comparable to this study as the amount of organic matter that was measured in this study (7.35 $\pm$ 1.39) was close to the value they measured (6.65% $\pm$ 2.19).

2.5. Conclusions

Sand-dune soils from different English reserves showed differences and similarities in their geochemical composition. The main differences between both reserves was in the distribution of ions concentration within reserve, the richest ecosystem regarding ion concentration was the dune slacks in Sandscale Haws, by contrast, the fixed dunes were the richest ecosystem in Ainsdale. There were also differences in the concentration of some specific ions such as calcium and ammonium that showed the highest concentration in the fixed dunes in Ainsdale, or potassium which had the highest concentration in the dunes slacks in Sandscale or phosphate that showed the highest concentration in the fixed dunes in Sandscale Haws. These differences could allow particular characteristics to the ecosystems assessed but also to each of the reserves.

Even though there were differences found in both reserves, some similarities such as the absence of phosphate in all mobile dunes soils, the very low nitrate concentration, the high sodium concentration in all fixed dunes ecosystem and the same pattern in both soil pH (*i.e.* alkaline *versus* more acidic sites) and LOI suggest that there could be similar soil processes occurring in both reserves.

These patterns in ion concentration and distribution that showed geochemical differences in soil characteristics could be, in future, related to the presence of different

organisms *(e.g.* different species of fungi) and their ecologies (*e.g*. as saprotrophs or autotrophs), and to geological and anthropogenic factors. Hence, the results of our study will provide valuable and recent information about the geochemical conditions of sand dune sites at the two reserves. This information will lead to a better understanding of processes such as nutrient cycling (*e.g.* carbon cycling), organic matter decomposition, erosion and vegetation shifting in sand dune ecosystems, because the richness of plant and animal life of dune systems depends strongly on the diversity of environmental conditions which may range from dry to wet, from alkaline to acidic, and from fertile to infertile (Willis, 1985).

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Chapter 3

Paper 2. Diversity of fungal communities colonising bait materials in sand-dune ecosystems

This chapter contains the following paper which is in preparation to be submitted to the *Soil Biology and Biochemistry Journal*

Irma González González, Bart E. van Dongen and Clare H. Robinson. Diversity of fungal communities colonising bait materials in sand-dune ecosystems

Chapter 3

Paper 2. Diversity of fungal communities colonising bait materials in sand-dune ecosystems

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Abstract

Fungi are key players in global ecosystem functioning, particularly as recyclers of major plant components of organic matter. O'Brien *et al*. (2005) estimated that "the number of fungal species should be 3.5 to 5.1 million species", based on using environmental DNA. However, fungal mycelia are difficult to identify by conventional techniques of sporocarp surveys and culturing as these approaches are severely limited, since they cause discrimination against non-culturable and slow-growing taxa. High-throughput DNA sequencing methods provide the opportunity to resolve the diversity and distribution of mycelia in soil.

The diversity of fungal communities colonising buried bait materials barley (*Hordeum vulgare*) straw and hawthorn (*Crataegus sp.*) wood was assessed. The bait materials were buried at defined depths, at three sand-dune ecosystems of two UK sand dunes reserves, and retrieved at different sampling times (3, 6 or 12 months after burial), to characterise the fungal mycelia colonising the materials by 454 pyrosequencing. A great richness of fungi was detected, including 275 Operational Taxonomical Units (OTUs) from which only 92 OTUs had ≥97% ITS1 sequence similarity. The communities were dominated by Ascomycota and Basidiomycota, but Glomeromycota and Zygomycota were also detected, although in low numbers. There were differences in the species richness for the different bait materials as the hawthorn wood samples showed the highest number of observed OTUs (67 OTUs after rarefaction) if compared with barley

straw samples. The different ecosystems in both reserves affected the fungal community composition as the fungal communities of the mobile 'alkaline' dunes clustered apart from the communities of the 'more acidic' dune slacks and fixed dunes at Sandscale Haws, and the communities of the dune slacks and of the fixed dunes were separated at Ainsdale, as shown in the NMDS and DCA analyses. However, the community composition depending on the sampling time differed only in Ainsdale, and the burial depth did not structure the fungal communities in either of the reserves. Fungal communities with different ecologies were identified colonising both bait materials and across the different sand-dune soils, such as ectomycorrhizal fungi and saprotrophs, among which wood-rotting fungi were identified.

3.1 Introduction

Fungi are key players in nutrient cycling: they capture, accumulate, release and recycle carbon and nutrients (*e.g.* phosphorus, nitrogen). Fungi break down dead plant materials, aided by soil fauna and bacteria, and they are able to modify and decompose the most resistant plant polymers such as lignin (Crawford, 1980; Berg and McClaugherty 2003; Watling, 2003; Deacon, 2006). The decomposition of lignin, the second most abundant plant polymer on Earth, is of great significance for the global carbon cycle (Deacon, 2006). Thus, the assessment of the diversity of fungal communities that are meaningful for ecosystem functioning, such as fungi that are able to degrade and modify resistant polymers, appears necessary (Krivtsov *et al.*, 2006). However, the number of total fungal species remains unclear until now (O'Brien et al., 2005; Blackwell, 2011) since it is probable that many fungi have not been yet recognized because they often differ only slightly in characteristics (*e.g.* pigments, Margulis and Champan, 2009) or because there are unexplored geographical areas (Hyde, 2001).

Traditional studies for the identification of fungi such as collection of sexual reproductive structures (e.g. sporocarps visible to the naked eye) or culturing of fungal isolates directly from soil (Rossman et al., 1998) have several limitations. It is difficult to identify fungal sexual reproductive structures that are produced above-ground because they are often inconspicuous, or they are conspicuous but only last a short period of time (Carlile *et al*., 2001: Deacon, 2006; Webster and Weber, 2007). In addition, culturing discriminates against non-culturable and slow growing taxa (Ritz,

2005; O'Brien *et a*l., 2005; Jany and Barbier, 2008). Thus, a comprehensive range of methods has been developed and applied to study the presence, abundance, activity, and distribution of fungi in soils. In particular, relatively new molecular techniques have provided valuable methods for clarifying fungal species description and identification which remain necessary for understanding the roles of fungi in the natural environment (O'Brien *et al.,* 2005; Blackwell, 2011). Fungal molecular PCR-based identification has allowed the study of abundance and species richness of fungi and it is more reliable than traditional surveys because it is carried out by means of both their sexual structures and their mycelial stage (Zinger *et al*., 2009; Bellemain *et al*., 2010). For example, PCR-based approaches have revealed that basidiomycetes are two to three times less abundant (relative to total fungal abundance) in prairie grassland soil than woodland (O'Brien *et al.,* 2005; Lynch and Thorn, 2006). The most detailed PCR-based study in grasslands (Lynch and Thorn, 2006) identified almost 300 basidiomycete species in adjacent pasture and arable plots, with up to 9 species in some 10 g soil samples. Next generation sequencing has provided new insights into fungal diversity, for example, land use practice influenced the species composition and distribution patterns of Basidiomycota in cultivated (71 operational taxonomic units recovered) and fallow (69 operational taxonomic units recovered) fields in Copenhagen, Denmark in which the most abundant operational taxonomic units (OTUs) in the cultivated field were *Coprinellus sp* and *Panaeolus foenisecii*, whereas the most abundant OTU in the fallow field was an unknown basidiomycete similar to the "sister clade to Volvariella" (Kjøller and Rosendahl, 2014). In another study, next-generation sequencing was used to characterize fungal communities from nine pea fields in Denmark, in which pea roots showed different degrees of root rot disease, from 145 to 200 OTUs in each soil sample were obtained and the fungal communities were dominated by Ascomycota and Basidiomycota (Xu *et al*., 2012).

The preferred DNA barcoding marker for the identification of mixed environmental samples is the internal transcribed spacers (ITS) that separate the small sub-unit (SSU) and large-subunit (LSU) rRNAs in eukaryotes. This ITS region is the official primary barcoding marker for fungi (Bellemain *et al.,* 2010, Schoch *et al*., 2012). The use of this region has transformed our understanding of fungal ecology, as it can be used to identify fungi, to genus and species level, that are present in different habitats (Gardes *et al*., 1991; Gardes and Bruns, 1993; Vrålstad *et al.,* 2002; O'Brien *et al*., 2005; Taylor

and McCormick, 2008; Lindahl *et al*., 2007; Nilsson *et al*., 2011; Toju *et al*., 2012, Botnen *et al*., 2013; Geml *et al*., 2014).

Fungi colonise habitats and/or resources depending on their ability to adapt to the physical, chemical and ecological conditions of the habitat and on their ability to assimilate the available nutrients from different resources (Brown, 1958; Pugh, 1962; Harper and Lynch, 1985; Robinson *et al*., 1994; McGuire *et al.,* 2011). For instance, in a sand-dune system, few fungi are able to colonise the mobile 'yellow' dunes where there are only specialist species which have a number of adaptations, such as the development of a solid, subterranean aggregation of mycelium mixed with sand for the retention of moisture and nutrients that enable them to survive in this difficult habitat (Rotheroe, 1993). In addition, the fungal colonisation of a new resource in soils, such as straw, is dependent on the stages of the decomposition of the resource (Robinson *et al*., 1994). The occurrence of micro-organisms is dependent on the resource quality because different components of the material become available at different time during its decomposition (Harper and Lynch, 1985; Robinson *et al*., 1994). The amount of soil organic matter and soil pH also affect the frequency of fungal species in soils (Brown, 1958).

Soil pH has been shown to affect the microbial community composition in soils (Rousk *et al*., 2009). In forest soils, the biomass of some bacterial and fungal communities increased with increasing pH (Bååth and Anderson, 2003) and, in coastal dunes a comparison among alkaline *versus* acidic soils showed that these soils held different fungal communities: *Cadophora sp.* and *Exophiala sp.* were found predominantly in the alkaline sites and saprotrophs such as *Crytococcus sp*., *Fusarium sp.* and *Mortierella sp.* were found exclusively in acidic soils (Geml *et al*., 2014).

Fungi are mainly involved in cycling C, P and N, however, they also have roles in most of the other soil element cycles. Therefore, it is essential to assess the fungal species richness and to determine their ecological function in "poorly-known" sites to get insights into the role of fungi in the stability and productivity of such areas, as in sand-dunes ecosystems. Sand dunes are a very dynamic system, a very fragile environment which undergo heavy use (*e.g*. recreation) that may cause the destruction of plant roots, after a while causing the destruction of the dune system (Ranwell, 1972; Smith, 1999; Martinez *et al.,* 2004a; Martinez *et al*., 2004b). Although sand dunes have, in general,

low levels of soil nutrients (Brooks *et al*., 1986), in the United Kingdom it has been recognised that sand dunes support a wide variety of species, such as rare mosses, lichens and fungi (Holden, 2007). However, there is still relatively little information about the mycological richness in British sand-dune ecosystems (Brown, 1958; Rotheroe, 1993; Rotheroe *et al*., 1987; Watling and Rotheroe, 1989; Rhind and Jones, 1999; Leake *et al*., 2004). Therefore, the aims of this study were (i) to characterise the fungal diversity, particularly of basidiomycetes, colonising bait materials in sand-dune ecosystems, (ii) to compare the fungal community composition within and between sites and among different sampling times, (iii) to relate the fungal community composition to some soil chemical characteristics, *i.e.* soil pH and loss-on ignition, and (iv) to identify the ecological function of the fungi detected.

3.2 Methods

3.2.1 Study sites

The first study site was at Sandscale Haws National Nature Reserve (National Grid Reference SD200756; Fig. 1A & B, Appendix A), which comprises areas of different sand-dune successional ecosystems, such as dune slacks, grassland and freshwater marsh and small areas of woodland and scrub with few disturbances. It is one of most botanically rich coastal sites in the UK where over 600 species of plant species have been recorded (Skelcher, 2012).

The second site was at Ainsdale Sand Dunes National Natural Reserve (Grid Reference: SD292110, Fig. 1A & C, Appendix A). This reserve protects important plant and animal biodiversity in England. The most typical sand dune ecosystems are well characterized: embryonic and mobile dunes, humid dune slacks, fixed dune grasslands and dune heath (Skelcher, 2009).

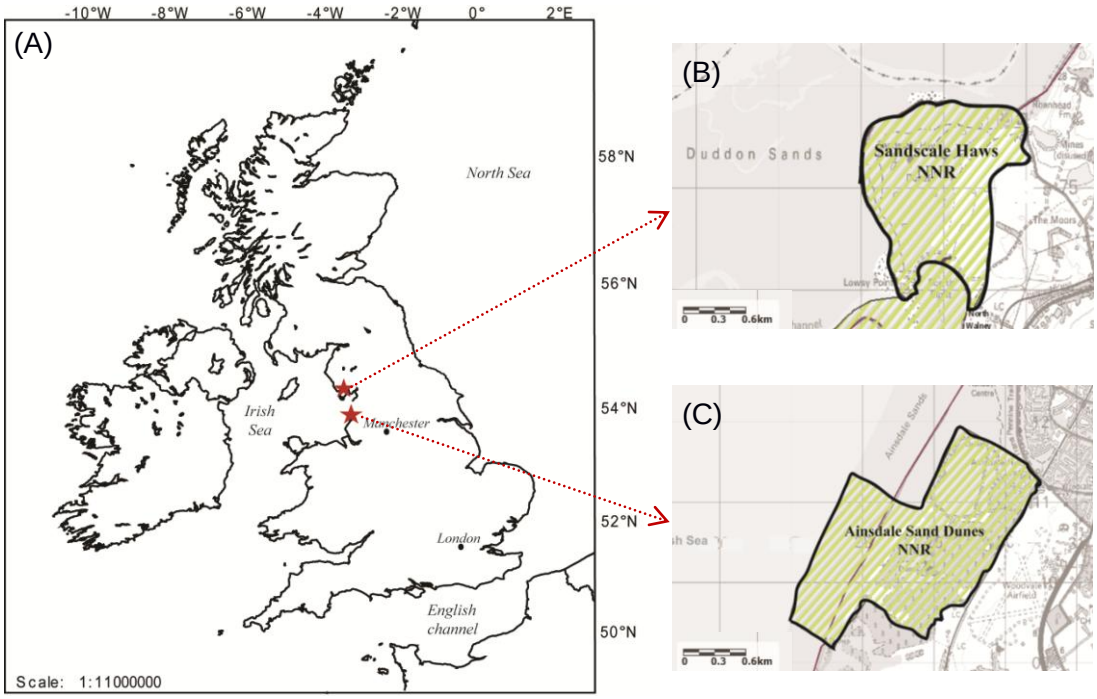

Figure 3.1. (A) Map of the United Kingdom pointing out the study sites. (B) Sandscale Haws and (C) Ainsdale Sand Dunes National Nature Reserves (NNR).

Three different ecosystems within each reserve were chosen for the experimental set up. The choice of the different ecosystems is based on the research about fungal succession in sand dunes made by Brown (1958) who described a pattern in ecological succession using classical techniques of fungal isolation on agar media.

Special notes about these ecosystems at Sandscale Haws NNR (Skelcher, 2012, Appendix A):

a) *Mobile dunes* in Sandscale Haws are found in the A*mmophila arenaria* mobile dune community. The soil profile is undifferentiated with accreting mobile dunes (personal observation). There is no active management to allow natural processes of accretion to occur. This area is classified as SD6 *Ammophila arenaria* mobile dune community under the National Vegetation Classification (NVC) for the United Kingdom.

b) *Dune grassland / slack* in Sandscale Haws is located within the *Salix repens – Calliergon cuspidatum* dune slack community. This area is grazed by cattle, sheep and rabbits. No scrub control has taken place in this area in the past five years. This area is classified as SD16 *Salix repens – Holcus lanatus* dune-slack community or SD15 *Salix repens – Calliergon cuspidatum* dune-slack community under the NVC.

c) T*he fixed dune* is located within the *Salix repens – Holcus lanatus* dune slack community. This area is grazed by cattle, rabbits and sheep. No control of the scrub has taken place in the past five years. This area is classified as SD16.

Special notes about these ecosystems at Ainsdale Sand Dunes NNR (Skelcher, 2009):

a) *Mobile dunes* have had scrub species cleared from them in the past and more than 100 years ago the dunes were built up artificially high by the use of sand trap fencing. The dune will have rolled inland over the top of a slack because of coastal erosion processes, perhaps as recently as the last 20 years, so a new dune has been made from an eroding old dune. This area is classified as SD6 *Ammophila arenaria* mobile dune community under NVC for the UK.

b) *Dune grassland / slack* has been mowed on several occasions and been grazed with sheep every winter since early 1990s. There were some *Salix repens* patches (personal observation). This area corresponds to the SD16 *Salix repens- Holcus lanatus* dune slack community under the NVC for the UK.

c) The fixed dune is located within a *Betula* (Birch) woodland that was used to store water up until 1965 and has had no management since then apart from grazing in winter by cows over the last 4 years.

In addition, two soil depths were chosen for the experimental set up. The selection of the depths 5 and 11 cm depends on the horizons' delimitations and characteristics which allow the presence of fungal communities. The 5 cm depth is the limit of the organic horizon. Bardgett (2005) pointed out that this horizon is biologically important since we can find decomposing litter with different biological communities associated which can be more or less active (e.g. beneath a grassland soil, fungal communities can be driving the litter decomposition rather than bacteria). The limit of rooting depth can be found around 11 cm and, for example, in sand alkaline dunes it is the limit of the "grey" sand horizon in semi-fixed "grey" dunes (Brown, 1958).

3.2.2 Experimental set up and field sampling

Hordeum vulgare L. (barley) straw internodes (0.08 to 0.10 g) or *Crataegus monogyna* (hawthorn) wood curls (0.10 g) were placed in 3 x 4 cm 50 µm nylon mesh bags (Figure 4.2). This size of bait bag is proposed to minimize the environmental impact on the ecosystem: the mesh size was large enough to allow colonisation by fungi but small enough to avoid the colonization of fauna and plant roots. Each bag was attached to a nylon string, metal numbered tag and a metal tent peg to make bait bag collection as straightforward as possible. The *Hordeum vulgare* and *Crataegus monogyna* baits were unsterilized to maintain their natural chemical composition. Thus, the plant bait materials were naturally pre-colonised by fungi.

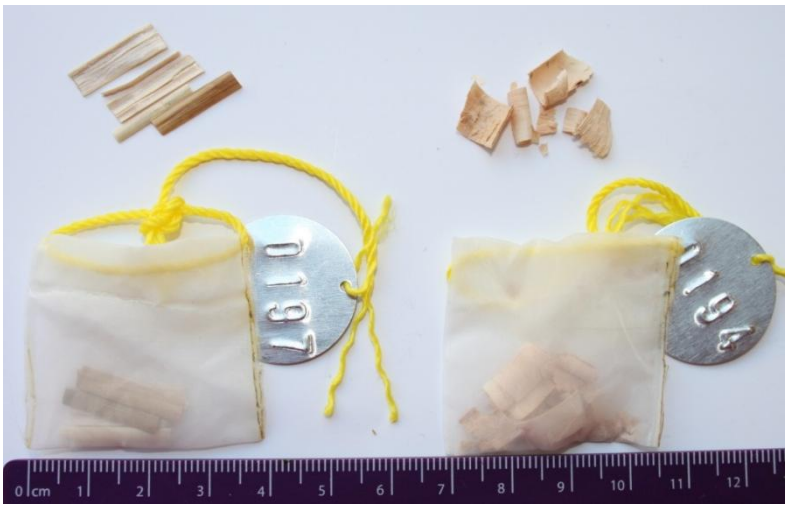

Figure 3.2. *Hordeum vulgare L.* (barley) straw internodes (top left) and *Crataegus monogyna* (hawthorn) wood curls (top right) prior to burial. Mesh bags (3 x 4 cm) filled with bait materials, *H. vulgare L.* (barley) straw (bottom left) and *C. monogyna* (hawthorn) wood (bottom right). Photo Irma González.

Nine 20 x 45 cm plots were set up across three different ecosystems, *i.e.* mobile dunes, dune grassland/slacks and fixed dunes, within both reserves. Six 20 x 45 cm plots were set up at Sandscale Haws NNR, on the 16th and 22nd of May 2012, two plots in each of three different ecosystems. Each plot within an ecosystem corresponded to a different bait material burial, *i.e.* either *Hordeum vulgare L.* (barley) straw or *Crataegus monogyna* (hawthorn) wood. Three 20 x 45 cm plots were set out at Ainsdale Sand Dunes NNR, on the 7th of June 2012, for the burial of *Hordeum vulgare* straw, one plot

in each of three different ecosystems. One hundred and sixty-two mesh bags were buried in total, 108 mesh bags at Sandscale Haws NNR and 54 mesh bags at Ainsdale Sand Dunes NNR, at depths of 5 and 11 cm below the soil surface evenly spread across the different ecosystems (Table S1).

Additionally, triplicate soil samples were randomly collected within each of nine plots (triplicate because three is the minimum number of replicates in order to be able to obtain a statistical valid mean and standard error) at the time of the burial of the bait materials (May/June 2012). Each of the nine soil collections was made with a pre-furnaced spatula which was washed with ultrapurified water between each sampling. The soils were placed separately into pre-furnaced glass jars and stored in a coolbox containing frozen coolpacks in the field, transported to the laboratory and stored in the freezer at -20 ºC until needed for geochemical analyses.

The bait bags were retrieved 3, 6 or 12 months after burial in the field, to allow for different times giving us account of the differential decomposition pattern and fungal colonization over a year. However, not all bags could be retrieved as the areas where the plots were placed were either flooded or the sand accretion was too high which made it impossible to retrieve all bags. One hundred and forty-two bait bags were collected in total, from the majority ecosystems and from all depths, but not from all the different sampling times (Table S1). Bags from the same ecosystem, sampling time and depth were essentially treated as replicate bags. For collection, each bait bag was placed separately in a pre-furnaced foil packet, then placed inside a Ziploc bag to prevent the material from drying out and to prevent contamination, stored in a coolbox containing frozen coolpacks in the field, transported to the laboratory and stored in the fridge at -20 ºC in the laboratory until needed for DNA extraction and amplification.

3.2.3. Soil analysis

pH measurements

Twenty-seven soil samples (4.9 – 14.8 g dry weight) were collected from the reserves at the plots where, and on the dates when, the bait materials were buried, 18 soil samples from Sandscale Haws and 9 soil samples from Ainsdale Sand Dunes NNR. Soils were oven-dried at 60ºC overnight, then transferred into clean tubes and weighed on an analytical balance. Bidistilled water, 24 ml, was added to each tube and the tubes were

placed into the ultrasonic extraction equipment for half an hour. The solutions in the tubes were left to stand overnight. Finally, the supernatant was filtered into clean tubes with a syringe through 0.2 µm polypropylene filters and the pH was measured using an automatic pH meter which was calibrated with both pH 4 and pH 7 buffers.

Loss-on-ignition analysis

Approximately 1 g of each soil sample was placed into a pre-weighed porcelain crucible, heated at 105°C for 1 h and placed into a desiccator for cooling for 20 min. After the weight was recorded, the crucibles containing the sample were heated at 1000°C for 1 h, cooled in a desiccator for 30 min and weighed again. The loss on ignition is calculated as a percentage of the original weight using the weight loss between 105°C and 1000°C.

3.2.4 DNA extraction and sequencing

From the total of 142 retrieved bags, only 29 samples were selected for DNA extraction and sequencing. The selection of the bags was based on the quality of the DNA isolation and PCR products, and ensuring that all ecosystems and sampling times were represented (Table 3.1).

DNA was isolated from 18 field-recovered *H. vulgare L.* (barley) straw bait samples and 11 *C. monogyna* (hawthorn) wood bait samples using the DNeasy Plan Mini Kit (Qiagen, Sample and Assay Technologies), following the kit manufacturer's instructions (Appendix B). Each sample was ground separately with a ceramic mortar and pestle which were washed up with Virkon and cleaned up with ethanol (70%) before use. A maximum of 20 mg wet sample was used, as recommended by the manufacturer.

Amplification of the internal transcribed spacer (ITS) region 1of the nuclear ribosomal DNA was performed using a pair of barcoded (fusion) primers which were HPLC-purified, for unidirectional pyrosequencing and to allow post-sequencing differentiation of sequences. The forward fusion primer included an A-key adaptor sequence ('Lib-L' Primer A sequence, key: *TCAG;* Roche GS Junior System Titanium Chemistry) and a 10 bp multiplex identifier sequence (MID1-30, except MID15; see Table 2.1, Technical

bulletin 005-2009, Roche Diagnostics Corp., Basel, Switzerland) in front of the fungal specific primer ITS1f (Gardes and Bruns, 1993), resulting as forward fusion primer A*key*-MID-ITS1f (5'-ccatctcatccctgcgtgtctccgac*TCAG*-MID-CTTGGTCATTTAGAGGAAGTAA-3'). The reverse fusion primer included a B-key adaptor sequence ('Lib-L' Primer B sequence, key: *TCAG;* Roche GS Junior System Titanium Chemistry*)* in front of the ITS2 primer (White et al. 1990), resulting as reverse fusion primer B*key*-ITS2 (5'- ctatcccctgtgtgccttggcagtc*TCAG* GCTGCGTTCTTCATCGATGC-3').

All 29 extracts were amplified separately on a BioRad iCycler (BioRad, Hemel Hempstead, and Herts, UK). PCR reactions were performed using the Fast Start High Fidelity PCR system (Roche Diagnostics Corp., Basel, Switzerland) in 50 µl volumes containing 2 µl of extracted DNA, 40 µl sterile purified water, 5 µl of 10x EX buffer, 1 µl of dNTP mixture, 0.8 µl of each primer (10 µM working stock) and 0.4 µl High Fidelity enzyme blend. The PCR cycle was as follows: an initial denaturation step at 94ºC for 1 min, then 35 cycles of 94ºC for 35 sec (melting), 52ºC for 1 min (annealing) and 72ºC for 1 min 30 sec (extension); followed by a final extension at 72ºC for 10 min. A negative PCR control was included where the template DNA was replaced with sterile H_2O to determine any contamination. These negative controls remained free of PCR amplicons. After the programme was finished the samples were stored frozen (-20ºC) until further analysis.

The quality of the amplified PCR products was confirmed by 1% agarose gel electrophoresis (0.5X TAE buffer). The DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, Hemel Hempstead, and Herts, UK).

Before carrying out pyrosequencing, PCR products were purified in order to remove any impurities (salts, nucleotides or enzymes) which could inhibit the pyrosequencing reactions. Gel extraction enables the purification and selection of specific fragment sizes by excising the desired DNA band from a gel. Therefore, 50µl of each sample was loaded on a 2% TAE agarose gel for good separation of DNA bands, some samples had to be run in duplicate as they showed weak bands on the gel. The DNA was stained with ethidium bromide and viewed under short-wave UV light using a BioRad Geldoc 2000 system (BioRad, Hemel Hempstead, and Herts, UK). The appropriate fragment from the

agarose gel (ca. 300- to 400 bp) was excised with a clean, sharp blade and DNA was gel-extracted and purified using the Qiaquick gel extraction kit (Qiagen) following the manufacturer's instructions. The DNA concentration after gel extraction and purification was determined using a NanoDrop 2000 spectrophotometer from Thermo Scientific (Saveen Werner ApS, Denmark). The purified PCR product was stored frozen (-20 ºC) until needed. The purified amplicons were sequenced at the Sequencing Facilities of The University of Manchester (Manchester, UK) using the 454 GS Junior Titanium System (454 Life Sciences,Roche Diagnotics, Basel, Switzerland) which yields reads lengths of ~400 bp.

Table 3.1 Codes for samples used in fungal DNA analysis based on sampling location of buried bait materials in Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves: ecosystem type, depth of burial and sampling time.

Sample ID[a]	Ecosystem	Depth of burial (cm)	Sampling time (months)
Sandscale Haws National Nature Reserve			
Barley straw			
SBM11_3	Mobile dunes	11	3
SBM11_6	Mobile dunes	11	6
SBM11_12	Mobile dunes	11	12
SBD5_12	Dune slacks	5	12
SBD11_3	Dune slacks	11	3
SBD11_6	Dune slacks	11	6
SBF5_3	Fixed dunes	5	3
SBF5_3	Fixed dunes	5	3
SBF5_12	Fixed dunes	5	12
SBF11_12	Fixed dunes	11	12
Hawthorn wood			
SHM5_3	Mobile dunes	5	3
SHM5_6	Mobile dunes	5	6
SHM11_12	Mobile dunes	11	12
SHD5_3	Dune slacks	5	3
SHD5_6	Dune slacks	5	6
SHF5_3	Fixed dunes	5	3
SHF5_6	Fixed dunes	5	6
SHF5_12	Fixed dunes	5	12
SHF11_3	Fixed dunes	11	3
SHF11_6	Fixed dunes	11	6
SHF11_12	Fixed dunes	11	12
Ainsdale Sand Dunes National Nature Reserve			
Barley straw			
ABD11_3	Dune slacks	11	3
ABD11_12	Dune slacks	11	12
ABF5_3	Fixed dunes	5	3
ABF5_6	Fixed dunes	5	6
ABF5_12	Fixed dunes	5	12
ABF11_3	Fixed dunes	11	3
ABF11_6	Fixed dunes	11	6
ABF11_12	Fixed dunes	11	12

[a]ID key: S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the bait material barley straw or hawthorn wood, M/D/F corresponds to the different sand-dune ecosystems where the bait materials were buried

mobile dunes or dune slacks or fixed dunes, 5/11 is depth of burial of the bait material, 5 or 11 cm and 3/6/12 is the sampling time in months. For example: SBM11_3 corresponds to a sample buried in Sandscale Haws (S), the buried bait material was barley straw (B) which was buried in the mobile dunes (M), at 11 cm depth and was retrieved 3 months later. Samples collected at 3 months were collected in September 2012, samples collected at 6 months were collected in January 2013 and samples collected at 12 months were collected in June 2013.

3.2.5 Bioinformatics

Functional groups classification

All sequence reads were quality-filtered, denoised and clustered using Qiime v. 1.8.0 (Caporaso *et al*., 2010). Reads with lengths < 200 bp and > 450 bp, average Phred quality < 25, or mismatches in the tags, were discarded. Sequences with homopolymers exceeding 10 bp were removed from the data. No ambiguous bases (N) were allowed. Sequences meeting the requirements were retained in the data set and were denoised using Denoiser v. 0.91 (Reeder and Knight, 2010) as implemented in Qiime v 1.8.0 (Caporaso *et al*. 2010). Denoised sequences were checked for chimeras using the USEARCH algorithm (Edgar *et al*., 2011) against the UNITE database (5th, May 2014), chimera checking was implemented using both the novo and reference based methods. Chimeric sequences obtained after this step were excluded from the data set. Those sequences that met the requirements were clustered into operational taxonomic units (OTUs) using USEARCH (Edgar *et al*., 2011), the most abundant sequence in each cluster was designated as the representative sequence which was aligned using default parameters in Qiime v 1.8.0 (Caporaso *et al*., 2010); both analyses were carried out using the reference UNITE database at a 97 % similarity threshold (5th, May 2014). As suggested by Tedersoo *et al*. (2010), those OTUs represented by a single sequence were discarded from the data set as they could represent PCR or pyrosequencing artefacts. Each representative sequence was subjected to taxonomic classification using BLASTn search against NCBI-nt database (April 2014). Taxonomy was assigned at the level of genus or species when the blast hit had an e-value close to zero, particularly for those sequences matching Basidiomycota fungi, otherwise it was assigned at higher taxonomic levels, as follows: if the top hit was either not taxonomically consistent, matched an unclassified or uncultured fungus, the next 10 hits were screened for

selecting a match; sequences with a best BLASTn match to a nonfungal sequence, or <70% similarity and/or 50% coverage to a fungal sequence were removed, as implemented by Davey *et al*. (2013).
Thus, a read abundance matrix of samples versus OTUs was obtained where each OTU is associated to a certain number of samples (OTU frequency per sample): a value > 0 represents the number of read sequences of the OTU in the sample, therefore meaning that the OTU was identified on the sample, a value = 0 (no read sequence) means that the OTU was not present in the sample.

Community similarity analyses

Samples had uneven numbers of sequences (ranging from 56 to 17,149 sequences per sample), therefore the read abundance matrix of samples versus OTUs was rarefied to 100 read sequences per sample and rarefaction curves were constructed. Accumulation curves for each ecosystem were also constructed following the approach in Ugland *et al*. (2003) using the rarefied OTU matrix which is a method for calculating exact species-accumulation curves that does not depend on an underlying distribution of individuals among species and gives exact fits to data. All these analyses were made using the rarefaction function in the Vegan package v. 2.0.10 (Oksanen *et al*., 2013) of the statistical environment R v.3.0.3 (http://www.R.project.org/).

Pairwise numbers of shared species between samples were calculated with EstimateS *v*. 9.00 (Colwell, 2013). Extrapolated total species richness values were calculated using Chao's method, first and second Jackknife estimates with the specpool function in the R Vegan package v. 2.0.10 (Oksanen *et al*., 2013).

The non-rarefied OTU matrix was transformed using the Hellinger equation before further analyses, and all samples with <100 reads were omitted from the OTU matrix. The Hellinger transformation is commonly used to account for 'blind sampling' and numerous rare taxa and absences and it helps homogenizing variation due to blind sampling among species abundances (Legendre and Legendre, 1998), as implemented by Botnen *et al*. (2013) who followed suggestions by Ramette (2007). In our study, this transformation was done because there were large numbers of absences (zeros, as the OTUs were not present in all the samples) in the final matrix and the sampling was carried out randomly. The transformed OTU matrix was used to perform two types of

ordination in order to display overall community similarities and investigate the effects of various factors on fungal community structure. The two methods provided similar results, which together with the absence of visual artefacts on the ordinations, confirmed that reliable gradient structure was found (Økland, 1990). These ordinations were performed with the Vegan and MASS packages of R (Oksanen *et al*., 2013). Firstly, a Global Nonmetric Multidimensional Scaling (GNMDS; Kruskal, 1964a, b) was performed following settings by Botnen *et al*. (2013) using the function *metaMDS*, as follows: distance measure – Bray–Curtis, maximum iterations – 200, dimensions – 2, initial configurations – 100. Detrended correspondence analysis (DCA; Hill, 1979; Hill and Gauch, 1980) was implemented using the function *decorana* with default settings. Ordinations were screened for outliers and checked for possible artefacts such as the arch-effect in the GNMDS and the tongue-effect in the DCA (Økland, 1990; Økland and Eilertsen, 1993). Correlation tests between the axes in the two ordinations were conducted, using Kendall's tau coefficient with the function *Kendall* in R, and were used as an indicator that reliable gradients were recovered. The effect of material, ecosystem, depth and sampling time were assessed on fungal assemblage structure, by analyzing permutational multivariate analyses of variance. These analyses were carried out with the R *vegan* package v. 2.0.10 (Oksanen *et al*., 2013) using the *adonis* function with the average Bray-Curtis dissimilarity index and 999 permutations.

Ecological function of fungi

The ecological functions of fungi were determined, based on the literature, for those OTUs that showed > 97% ITS sequence similarity to fully identified fungi (Peay *et al*., 2008) at the level of genus and species.

3.3 Results

pH and loss-on-ignition across ecosystems

The pH values of the soil samples were different between the mobile dunes and the other two ecosystems, *i.e.* dune slacks and fixed dunes. The mobile dunes had an alkaline pH and by contrast, the pH in the dune slacks and in the fixed dunes was more acidic (Table 3.2). Soil samples from the fixed dunes at Ainsdale Sand Dunes NNR

showed the highest percentage weight loss on ignition (21.29 %; Table 3.2). A visual examination of the samples before being assessed for LOI showed that all the fixed dunes contained a high amount of roots and were more "compact" soils compared with sandy soils from the mobile dunes which had the lowest LOI (1.41-1.74 %, Table 3.2).

Table 3.2 pH and loss-on-ignition values for soil samples from sand-dune ecosystems.

Sample ID[b]	**Ecosystem**	**pH** (average value and min. and max. for 3 samples)	**Loss-on-ignition (%)**
Sandscale Haws National Nature Reserve			
S1	Mobile dunes	7.2 (7.13-7.26)	1.41 ± 0.07
S2	Mobile dunes	7.4 (7.36-7.49)	1.74 ± 0.24
S3	Dune slack	6.5 (6.13-6.86)	9.25 ± 5.3
S4	Dune slack	6.3 (6.18-6.62)	2.09±1.19
S5	Fixed dunes	6.4 (5.53-6.91)	2.31 ± 0.43
S6	Fixed dunes	6 (5.7-6.2)	7.72 ± 6.45
Ainsdale Sand Dunes National Nature Reserve			
A1	Mobile dunes	7 (6.85-7.19)	3.19 ± 0.26
A2	Dune slack	6.6 (6.47-6.75)	7.35 ± 1.39
A3	Fixed dunes	6.2 (6.04-6.42)	21.3 ± 2.74

[b]**Sample ID:**
Sn: Mean of three soil samples collected from the same ecosystem in Sandscale Haws National Nature Reserve.
An: Mean of three soil samples collected from the same ecosystem in Ainsdale Sand Dunes National Nature Reserve.

Fungal DNA sequences characteristics

From the 29 samples (Table 3.1), 147,350 ITS1 raw reads were obtained. After applying quality filter and denoising, 142,192 (96.5 %) reads were retained for further analysis with a range of 56 to 17149 sequence reads per sample, on average 5077.1 reads per sample. The read sequences were clustered into 444 OTUs. After removal of singletons (78, 17.5 % of OTUs), non-target organisms (2, 0.45% of OTUs: *Ammophila arenaria* and *Calluna vulgaris*), chimeras (40, 9% of OTUs) and OTUs with unreliable BLAST matches (49, 11%) from the data set, a final OTU matrix of 275 OTUs remained (Table S1).

The average observed OTUs per sample was 27.2, with a minimum of 4 OTUs on sample SHM5_3, and a maximum of 94 OTUs on sample SHF5_6 (Table 3.3). A same OTU was colonising different number of bait samples, on on average 2.9 bait samples: some OTUs were retrieved from only 1 bait sample, by contrast other OTUs were present in up to 19 bait samples (Table 3.6 & Table S2). On average, the number of shared OTUs among all samples was 5.2 ranging from 0 to 45.

Four samples had the highest number of OTUs: SHF5_12, ABD11_3, SHF11_12 and SBF5_12. Although there was not a clear relation between the number of OTUs and the ecosystem as most of the samples (21samples) had similar number of OTUs, *i.e.* less than 30. There was not clear differences among the number of OTUs retrieved based on the depth of burial either (Table 3.3).

The number of OTUs changed depending on the time that the material remained buried in the field. In Sandscale Haws twelve-month samples appeared to have on average a greater number of OTUs if compared with samples buried for 3 or 6 months. By contrast in Ainsdale, the samples buried for 3 or 6 months had on average a greater number of OTUs if compared with the samples buried for 12 months (Table 3.3).

Table 3.3 Overview of the taxonomic composition of fungal communities found on buried bait materials, barley straw and hawthorn wood. The taxonomic overview of the fungal communities found on each bait material is represented as number of operational taxonomic units (OTUs). For sample ID, see Table 3.1.

Sample ID	# Ascomycota OTUs	# Basidiomycota OTUs	# Glomeromycota OTUs	# Zygomycota OTUs	Total OTUs
Total OTUs	191	62	15	7	275
% of each phylum	69.5	22.5	5.5	2.5	
% of total reads	93.2	6.2	0.3	0.4	
SBM11_3	19	1	0	1	21
SBM11_12	12	1	0	0	13
SBM11_6	8	1	0	0	9
SBD11_6	26	2	2	1	31
SBD5_12	14	2	1	2	19
SBD11_3	10	1	0	0	11
SBF5_12	50	9	3	1	63
SBF5_3a	31	2	0	2	35
SBF5_3b	20	6	1	1	28
SBF11_12	13	1	0	2	16
SHM11_12	8	14	1	0	23
SHM5_6	8	1	0	0	9
SHM5_3	3	1	0	0	4
SHD5_6	24	2	1	1	28
SHD5_3	25	0	0	0	25
SHF5_12	69	9	11	5	94
SHF11_12	57	8	5	3	73
SHF5_6	17	1	0	0	18
SHF11_3	9	1	0	1	11
SHF11_6	5	2	1	0	8
SHF5_3	5	2	0	0	7
ABD11_3	55	13	6	2	76
ABD11_12	36	7	2	2	47
ABF5_3	33	3	0	3	39
ABF11_12	24	1	2	1	28
ABF5_12	16	2	1	1	20
ABF11_6	11	1	0	1	13
ABF11_3	8	1	1		10
ABF5_6	7	2	0	0	9

Taxonomic diversity of fungi across sand-dune sites

The total 275 OTUs corresponded to four fungal phyla, the most dominant phylum was the Ascomycota (69.5 % OTUs, 93.2 % reads), followed by the Basidiomycota (22.5% % OTUs, 6.2 % reads). A small proportion was assigned to the Glomeromycota (5.5 % OTUs, 0.3 % reads) and Zygomycota (2.5%, 0.4 % reads). All phyla were present in both sites and throughout all ecosystems, most of the samples were colonised by the four retrieved fungal phyla, Ascomycota, Basidiomycota, Glomeromycota and Zygomycota: only sample SHD5_3 was colonising by only one phylum (Table 3.3).

OTUs representing 38 orders of fungi were detected. The dominant order was the Sordariales which belongs to the Ascomycota phylum which accounted for 30 (10%) of the OTUS, however, most of the fungal orders (30) had less than 10 OTUs. Dominant Basidiomycota orders were the Tremellales (30 OTUs), followed by the Agaricales (11 OTUs). The order Polyporales was also identified which contains white-rot fungi (Fig. 3.3).

The most common OTU was present in 19 samples and was assigned to the *Lasiosphaeriaceae* which has a pairwise similarity of 98%, however, it had low query coverage of 80% (GenBank Accession No EU754958.1). The second most abundant OTU was *Exophiala sp*. (100% coverage and 98% similarity, GenBank Accession No. AB701673.1) which belongs to the Chaetothyriales and was present in 14 bait samples. *Glomus* and *Cryptococcus* were the fungal genera most commonly recovered accounting for 10 of the OTUS. *Mortierella sp* and *Schizothecium* accounted for 7 OTUs each (Table S2).

Saprotrophic fungi that belong to the phylum Basidiomycota were also identified, *i.e. Melanoleuca cinereifolia, Phlebiella christiansenii* and *Phanerochaete sp.* (Table S2). *Melanoleuca cinereifolia* was identified on SHM5_6, *Phlebiella christiansenii* colonized ABD11_3 sample and *Phanerochaete sp.* was recovered from SHF5_6 and SHF11_12 samples. A fifth of the OTUs were unclassified at genus level (52 OTUs, Table S2).

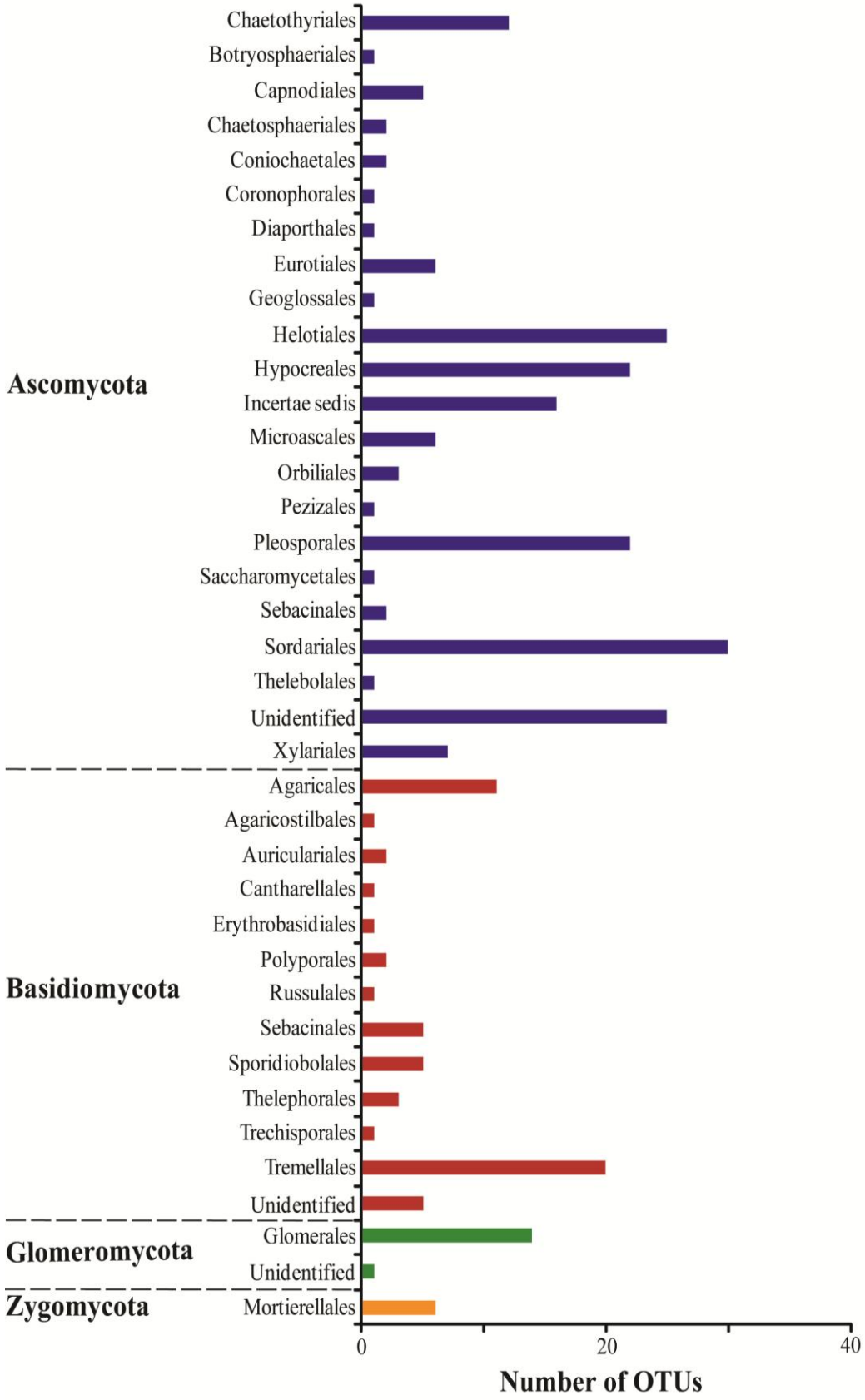

Figure3.3. Number of OTUs per fungal orders recovered from bait samples buried in Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves, across different sand-dune ecosystems: mobile dunes, dune slacks and fixed dunes.

Fungal richness across bait materials

After rarefaction of the matrix down to 100 sequences per sample, 120 OTUs in 26 samples were retained. Fungal accumulation curves of OTUs richness *versus* sampling effort for the different bait materials showed that the total fungal diversity in the study sites was not recovered. At this depth of sequencing, fungal diversity per bait material differed; the accumulation curves did not reach an asymptote but they started to level off. In addition, the rarefaction curves indicates that the species accumulation in barley samples from Sandscale Haws is slower than in hawthorn samples in the same site and barley samples in Ainsdale Sand Dunes (Fig. 3.4). Estimates of total OTU richness associated to each different bait material ranged from 67 to 228 in hawthorn wood buried and 53 to 95 in barley straw buried in Sandscale Haws, and 51 to 104 in barley straw buried in Ainsdale Sand Dunes (Fig. 3.5).

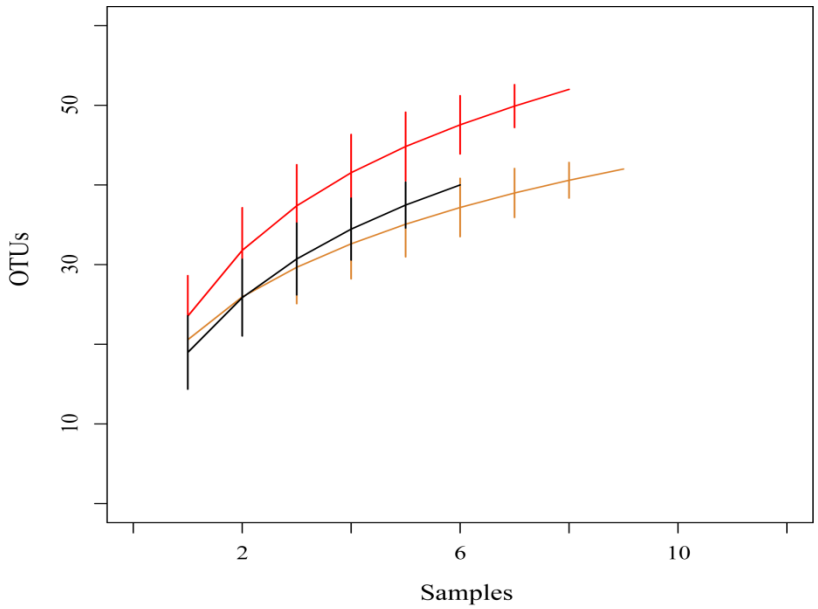

Figure3.4. Accumulation curves of sampling effort against OTU richness for the different bait materials, hawthorn wood Sandscale Haws: red, barley straw Sandscale Haws: orange, barley straw Ainsdale sand dunes: black. Using the rarefied OTU table.

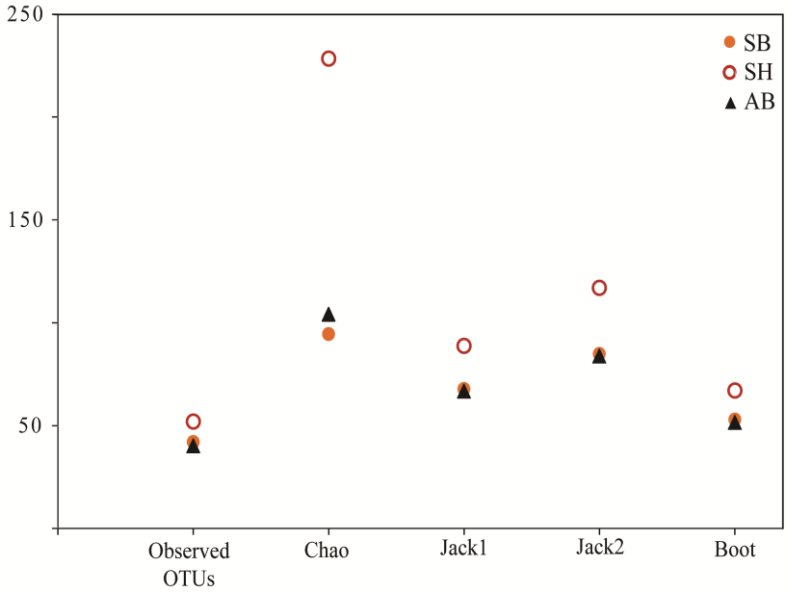

Figure3.5. Observed and estimated total number of OTUs per bait material using four different estimators Chao (Chao, 1987), 1st-order Jackknife, 2nd-order Jackknife and Bootstrap (Smith and van Belle, 1984). S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the type of buried bait material: barley straw or hawthorn wood. For example: SB corresponds to barley straw samples buried in Sandscale Haws.

Community composition

Both the GNMDS and the DCA showed that fungi followed a distribution pattern. In Sandscale Haws, this pattern was strongly related to the ecosystem where the bait material was buried, and it was less but significantly related to bait material type that fungi were colonising. In Ainsdale Sand Dunes, both the ecosystem and the period of time that the bait material remained buried influenced the distribution of fungal communities (Fig. 3.6).

In Sandscale Haws, fungal communities strongly clustered in two; one cluster corresponded to fungi from samples buried in the mobile dunes and a second group contained those fungi from samples buried either in the dune slacks or fixed dunes. Moreover, fungal communities in the mobile dunes clustered based on the bait material type they colonised (Fig. 3.6A&B). In Ainsdale, the variables that shaped the distribution of fungal communities were the ecosystem and the time that the material remained buried in the field (Fig. 3.6C&D)

The significance of the results found in the GNMDS and DCA are supported in the permutational analysis of variance (Table 3.4). Permutational multivariate analysis of variance confirmed that the ecosystem was a significant factor influencing the composition of fungal communities in both reserves. Material type was also a significant variable only in Sandscale Haws and the time that samples remained buried in the field was only a significant variable in Ainsdale. Interaction among all the variables did not have any affect in structuring the fungal community composition (Table 3.4).

Correlation tests between the axes in the two ordinations showed that reliable gradients were recovered (Table 3.5). The structure of the ordinations was corroborated by the strong and significant correlation of the DCA and GNMDS in Sandscale Haws (first axes: Kendall's tau = -0.8, second axes: Kendall's tau = -0.69) and in Ainsdale Sand Dunes (first axes: Kendall's tau = 0.93, second axes no significant correlation).

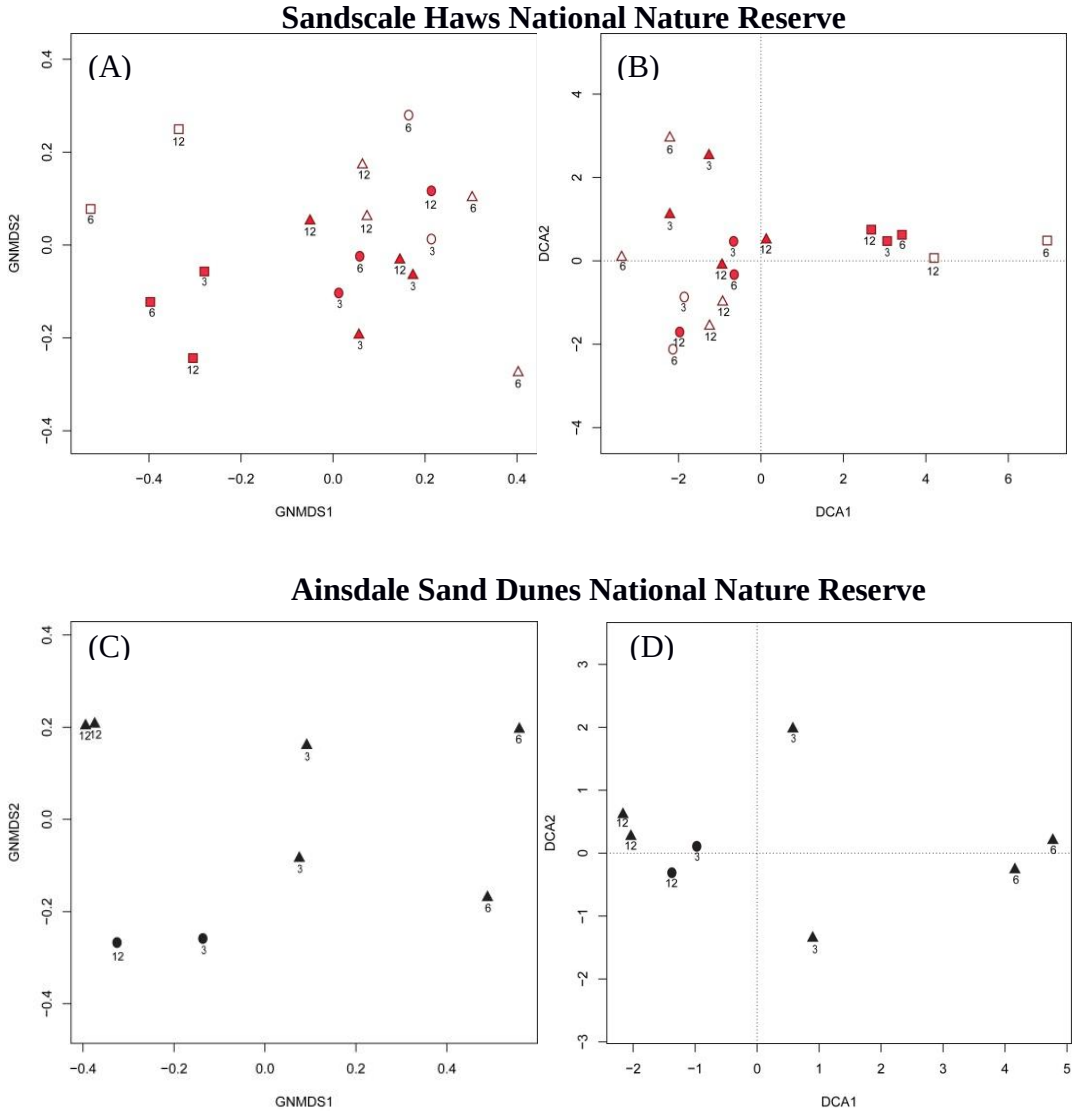

Figure3.6. (A & C) Global nonmetric multidimensional scaling (GNMDS) and (B & D) Detrended Correspondence Analysis (DCA) ordinations based on Hellinger transformed abundance data of the operational taxonomic units. The different symbols represent the two different bait materials (barley straw: filled symbols and hawthorn wood: open symbols) which were buried in different ecosystems (mobile dunes: squares, dunes slacks: circles and fixed dunes: triangles). Numbers below each symbol represent the time that the material remained buried in the field (in months).

Table 3.4 Permutational multivariate analysis of the compositional dissimilarity between fungal assemblages at two National Nature Reserves .

	Sandscale Haws NNR			Ainsdale sand dunes NNR		
	Df	***Sq***	***F.Model***	***Df***	***Sq***	***F.Model***
Material	1	0.6	**1.56***	ND	ND	ND
Ecosystem	2	1.66	**2.30*****	1	0.63	**2.31***
Depth	2	0.62	0.85	2	0.74	1.37
Time	2	0.7	0.95	2	0.98	**1.8***
MaterialxEcosystem	2	0.7	0.82	ND	ND	ND
MatrialxDepth	1	0.51	1.21	ND	ND	ND
EcosystemxDepth	2	0.8	0.95	2	0.91	1.22
MaterialxTime	2	0.67	0.79	ND	ND	ND
EcosystemxTime	1	0.21	0.5	1	0.33	1.33
DepthxTime	1	0.35	0.82	1	0.24	0.56
Residuals	1	0.42	0.06	2	0.54	0.19

p=0 ***, p <0.001 **, p<0.01 *; ND: not determined as only one type of bait material was buried in Ainsdale Sand Dunes.

Table 3.5 Kendall's Tau correlation tests between GNMDS and DCA axes for GNMDS ordinations run in two dimensions. Significant correlation between axes ($p<0.05$) is indicated by bold font.

		DCA1		DCA2	
		Kendall's Tau	**p-value**	**Kendall's Tau**	**p-value**
Sandscale Haws	**GNMDS1**	-0.8	**3.80E-06**	-0.2	0.4
	GNMDS2	-0.1	0.7	-0.7	**8.17E-05**
Ainsdale sand dunes	**GNMDS1**	0.9	**0.002**	-0.1	0.7
	GNMDS2	0.01	0.9	-0.4	0.2

Fungal ecologies across the different sand-dune ecosystems

From the total of 275 OTUs that were identified (Table S2), only 92 showed $\geq$ 97% ITS1 sequence similarity to fully identified fungi in the Genbank database (Table 3.6), therefore only the OTUs that were identified to the species level were used to describe the ecologies of fungi that colonized the bait materials across different sand-dune ecosystems (Tables 3.6 and S2). The fungal species identified from the bait materials,

barley straw and hawthorn wood, have different ecologies and some of them fluctuated depending on both the ecosystem where they were buried and the sampling times at which the bait materials were retrieved (Table 3.6 and 3.7). In the mobile dunes at Sandscale Haws, saprotrophs and animal and plant pathogens were identified on barley straw that remained buried for three, six and twelve months, but only coprophilous fungi were found on the same material that remained buried for three months. In the dune slacks, saprotrophs, coprophiles and plant parasites were retrieved from samples buried for three or twelve months, and root endophytes and animal and plant pathogens on samples buried for six months. In the fixed dunes, the identified fungal species that were colonising barley straw were saprotrophs, coprophiles or plant parasites and were retrieved from samples buried for three and twelve months. It was not possible to retrieve samples from the six-month sampling as the site was flooded (Table 3.7).

The fungal species identified from hawthorn wood curls that remained buried in the mobile dunes for three, six and twelve months were saprotrophs, plant pathogens and/or plant parasites, and only one arbuscular mycorrhiza from samples buried for twelve months. In the dune slacks, fungal mycelia from hawthorn wood buried for three months belonged to saprotrophs, coprophiles and plant pathogens. Arbuscular mycorrhizas (AM) were only identified on samples buried for six months. It was not possible to retrieve hawthorn wood samples buried for twelve months in the dune slacks as the samples were not found. In the fixed dunes, saprotrophs, coprophiles, plant parasites, animal and plant pathogens were retrieved from all the sampling times, however, plant symbionts were only present on samples buried for six months, and AM and plant endophytes on samples buried for twelve months. Wood-decay fungi were retrieved from hawthorn wood samples: the brown-rot fungus *Pilidium concavum* and the white-rot fungus *Phanerochaete sp.* The brown-rot fungus was retrieved from a sample buried for 12 months (SHF5_12) and the white-rot fungus from samples buried for six (SHF5_6) and for twelve months (SHF11_12; Table 3.7).

In Ainsdale Sand Dunes, the ecologies of fungal species that were identified from barley straw samples also appear to be contrasting across the different ecosystems and sampling times. In the dune slacks, saprotrophs, plant endophytes, ectomycorhizas (ECM) and AM were identified from samples buried for three and twelve months, however, a brown-rot fungus (*Pilidium concavum*) was only identified on a sample from twelve-month sampling (ABD11_12). It was not possible to retrieve samples from the

six-month sampling because the site was flooded. Saprotrophs, coprophiles, animal and plant pathogens were characterized colonising barley straw buried in the fixed dunes at all the sampling times and AM only from samples buried for three and twelve months, and ECM from samples buried for three months (Table 3.7).

Table 3.6 Ecological strategies of fungal species retrieved from bait materials buried in sand-dune ecosystems, at Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves. List of OTUs that showed ≥ 97% ITS1 sequence similarity to fully identified fungi with the best match (Genbank), frequency (number of samples where the OTU was present), ecological strategy and references.

OTU ID	Classification	Frequency	Ecological strategy	References
Sandscale Haws National Nature Reserve				
Barley straw				
140	*Cryptococcus sp.*	3	Saprotrophs and plant parasites	Domsch *et al*., 1980
161	*Isaria farinosa*	2	Entomopathogenic	Zimmerman, 2008
168	*Massarina rubi*	1	Saprotroph	Zhang *et al.*, 2009
49	*Microdochium sp.*	5	Saprotrophs and plant parasites	Domsch *et al*., 1980
226	*Sarocladium sp.*	1	Plant pathogens	Summerbell *et al*., 2011
115	*Bipolaris eleusines*	5	Plant pathogen	Zhang *et al*., 2014
172	*Leptodontidium orchidicola*	3	Root endophyte	Fernando and Currah, 1996
131	*Pochonia suchlasporia*	7	Pathogenic to nematodes	Kirk *et al*., 2008
264	*Trichoderma asperellum*	2	Plant pathogen	de los Santos-Villalbosa *et al*., 2013
279	*Doratomyces sp.*	1	Sapotrophs and coprophilous	Domsch *et al*., 1980
316	*Schizothecium aloides*	1	Coprophiles	Doveri, 2011
130	*Tetracladium sp.*	5	Saprotrophs	Domsch *et al*., 1980
200	*Heteroconium sp.*	4	Saprotrophs and coprophilous	Domsch *et al*., 1980
122	*Schizothecium glutinans*	2	Saprotrophs and plant parasites	Domsch *et al*., 1980
51	*Fusarium acuminatum*	10	Plant pathogen	Domsch et al., 1980
432	*Hygrocybe sp.*	1	Biotrophic lifestyle with plants	Halbwachs et al., 2013
82	*Mortierella sp.*	9	Saprotrophs	Domsch *et al*., 1980

3.6. CONTINUED

OTU ID	Classification	Frequency	Ecological strategy	References
Sandscale Haws National Nature Reserve				
Barley straw				
256	*Phoma exigua*	2	Saprotrophs	Domsch *et al*., 1980
24	*Schizothecium carpinicola*	10	Saprotrophs and plant parasites	Webster and Weber, 2007
299	*Thelebolus sp.*	1	Coprophiles and psychrophilic	Domsch *et al*., 1980
51	*Fusarium acuminatum*	10	Plant pathogen	Domsch *et al*., 1980
43	*Exophiala sp.*	14	Saprotrophs	Domsch *et al*., 1980
6	*Podospora intestinacea*	13	Coprophiles	Webster and Weber, 2007
Hawthorn wood				
153	*Peziza ammophila*	3	Saprotroph	Weber and Webster, 2007
33	*Cadophora luteo-olivacea*	1	Plant pathogen	Gramaje *et al.*, 2011
36	*Cadophora malorum*	2	Plant pathogen	Sapdaro *et al.*, 2011
136	*Melanoleuca cinereifolia*	1	Saprotroph	Kirk *et al*., 2008
235	*Microdochium bolleyi*	1	Plant pathogen	Domsh *et al*., 1980
46	*Phaeoacremonium mortoniae*	1	Plant pathogen	Gramaje et al., 2007
17	*Cryptococcus sp.*	6	Saprotrophs	Domsch *et al*., 1980
37	*Cryptococcus sp.*	3	Saprotrophs	Domsch *et al*., 1980
91	*Cryptococcus sp.*	4	Saprotrophs	Domsch *et al*., 1980
341	*Dioszegia rishiriensis*	1	Saprotroph	Domsch *et al*., 1980
62	*Dioszegia sp.*	1	Saprotrophs	Domsch *et al*., 1980
236	*Glomus sp.*	1	Arbuscular mycorrhiza	Weber and Webster, 2007
197	*Rhodotorula aurantiaca*	1	Saprotroph	Weber and Webster, 2007
276	*Sporobolomyces roseus*	1	Saprotroph	Weber and Webster, 2007

3.6. CONTINUED

OTU ID	Classification	Frequency	Ecological strategy	References
Sandscale Haws National Nature Reserve Hawthorn wood				
315	*Fusarium oxysporum*	1	Plant pathogen	Domsch *et al*., 1980
307	*Dactylaria dimorphospora*	1	Saprotroph	Weber and Webster, 2007
87	*Penicillium spinulosum*	3	Saprotroph	Domsch *et al*., 1980
320	*Trichosporon porosum*	2	Saprotroph	Middelhoven *et al*., 2011
114	*Tubaria sp.*	1	Saprotroph	Weber and Webster, 2007
56	*Phanerochaete sp.*	2	Saprotrophs (white-rot fungi)	Weber and Webster, 2007
118	*Alatospora flagellata*	1	Aquatic saprotroph	Kirk et al., 2008
302	*Cercospora zeae-maydis*	1	Plant pathogen	Crous *et al*., 2006
227	*Discosia pseudoartocreas*	1	Plant pathogen	Crous and Damm, 2013
185	*Glomus sp.*	2	Arbuscular mycorrhizas	Weber and Webster, 2007
379	*Mariannaea elegans*	1	Saprotroph	Domsch *et al*., 1980
308	*Paraphaeosphaeria neglecta*	1	Plant symbiont	Verkley *et al*., 2014
252	*Penicillium canescens*	1	Saprotroph	Domsch *et al*., 1980
78	*Pezizella discreta*	1	Saprotroph	Ellis and Ellis, 1997
132	*Phaeosphaeriopsis sp.*	6	Saprotroph	Weber and Webster, 2007
245	*Phialocephala humicola*	2	Plant symbionts	Jong and Davis, 1972
111	*Pyrenochaeta sp.*	1	Plant pathogens, endophytics	http://www.doctorfungus.org/thefungi/
209	*Pyrenochaetopsis sp.*	2	Plant pathogens	Crous and Damm, 2013
128	*Fusarium culmorum*	2	Plant pathogen	Domsch *et al*., 1980
212	*Rhizofagus sp.*	3	Arbuscular mycorrhizas	Tisserant *et al*., 2014
151	*Trichoderma brevicompactum*	2	Saprotroph	Kraus *et al*., 2004

3.6. CONTINUED

OTU ID	Classification	Frequency	Ecological strategy	References
Sandscale Haws National Nature Reserve				
Hawthorn wood				
240	*Geoglossum fallax*	1	Saprotroph	http://fungus.org.uk
159	*Microdochium phragmitis*	2	Plant endophyte	Ernst *et al.*, 2011
335	*Psilocybe cf. subviscida/crobula*	1	Saprotroph	Weber and Webster, 2007
40	*Rhizophagus sp.*	5	Arbuscular mycorrhizas	Tisserant *et al.*, 2013
188	*Verticillium leptobactrum*	4	Saprotroph	Ragaieg *et al.*, 2011
Ainsdale sand dunes National Nature Reserve				
Barley straw				
210	*Mortierella sp.*	3	Saprotrophs	Domsch *et al.*, 1980
116	*Cladophialophora chaestopira*	11	Saprotroph	Badali *et al.*, 2008
244	*Candida sp.*	1	Saprotrophs, Plant endophytes, Animal associations	Weber and Webster, 2007
295	*Glomus sp.*	3	Arbuscular mycorrhizas	Weber and Webster, 2007
274	*Myrothecium sp.*	2	Saprotrophs	Domsch *et al.*, 1980
246	*Penicillium nothofagi*	2	Soil fungi	Houbraken *et al.*, 2011
105	*Phlebiella christiansenii*	1	Saprotroph	Kirk *et al.*, 2008
358	*Russula laccata*	1	Ectomycorrhiza	Kibby, 2004
109	*Thelephora sp.*	1	Ectomycorrhiza	Bâ *et al., 2002*
138	*Tolypocladium cylindrosporum*	7	Saprotroph	Weiser and Pillai, 1981
292	*Tomentella sp.*	2	Ectomycorrhiza	Kirk *et al.*, 2008
96	*Tomentella sp.*	2	Ectomycorrhiza	Kirk *et al.*, 2008
222	*Trichocladium sp.*	2	Saprotroph	Domsch *et al.*, 1980

3.6. CONTINUED

OTU ID	Classification	Frequency	Ecological strategy	References
Ainsdale sand dunes National Nature Reserve				
Barley straw				
89	*Cryptococcus terricola*	5	Saprotroph	Domsch *et al*., 1980
67	*Cryptococcus podzolicus*	8	Saprotroph	Domsch *et al*., 1980
48	*Mortierella elongata*	13	Saprotroph	Domsch *et al*., 1980
145	*Phialophora sp.*	3	Saprotrophs	Domsch *et al*., 1980
101	*Pilidium concavum*	2	Saprotroph (brown- rot fungus)	Lopes *et al*., 2010
131	*Pochonia suchlasporia*	7	Parasite of nematode eggs	Zare and Gams, 2001
77	*Epicoccum nigrum*	2	Plant pathogen	Weber and Webster, 2007
194	*Lecythophora sp.*	1	Animal pathogens and comensals on plants	Domsch *et al*., 1980
339	*Lewia infectoria*	1	Plant pathogen	Weber and Webster, 2007
195	*Verticillium sp.*	3	Saprotrophs and plant pathogens	Domsch *et al*., 1980
206	*Apodus deciduus*	3	Coprophilous	Kirk *et al*., 2008
178	*Exophiala sp.*	7	Saprotrophs, animal pathogens	Domsch *et al*., 1980
107	*Paecilomyces carneus*	6	Entomopathogenic fungus	Leles *et al.*, 2010
82	*Mortierella sp.*	9	Saprotrophs	Domsch *et al*., 1980
263	*Mycoarthris corallinus*	2	Plant pathogen	Hofstetter *et al.*, 2012
219	*Mortierella sp.*	4	Saprotrophs	Domsch *et al*., 1980

Table 3.7 Ecological strategies of fungi that were retrieved from bait materials which remained buried for 3, 6 or 12 months in sand-dune ecosystems ECM: ectomycorrhiza, AM: arbuscular mycorrhiza. Fungal species are listed in Table 3.6. *NR*: No bags retrieved.

Ecosystem/sampling time (months)	Three	Six	Twelve
Sandscale Haws National Nature Reserve			
Barley straw			
Mobile dunes	Saprotrophs, coprophiles, plant and animal pathogens	Saprotrophs and plant pathogens	Saprotrophs,animal and plant pathogens
Dune slacks	Saprotrophs, coprophiles and plant parasites	Root endophyte and animal and plant pathogens	Saprotrophs and corprophiles
Fixed dunes	Saprotrophs, coprophiles and plant parasites	*NR*	Saprotrophs, coprophilous and plant parasites
Hawthorn wood			
Mobile dunes	Saprotroph	Plant pathogens and saprotrophs	Saprotrophs, AM, plant parasites
Dune slacks	Saprotrophs, coprophiles, plant pathogens	Saprotrophs, plant pathogens, coprophilous, AM	*NR*
Fixed dunes	Saprotrophs, plant parasites and plant pathogens	Saprotrophs (a white-rot fungus), coprophilous and plant symbionts	Saprotrophs (white-rot and brown-rot fungi), arbuscular mycorrhiza, plant endophyte, plant pathogens, coprophiles
Ainsdale sand dunes National Nature Reserve			
Barley straw			
Dune slacks	Saprotrophs, coprophiles, ECM, AM and plant endophytes	*NR*	Saprotrophs (one brown-rot fungus), coprophiles plant pathogens and parasites, AM and animal pathogens
Fixed dunes	Saprotrophs, coprophiles, animal and plant pathogens, AM and ECM	Saprotrophs, coprophiles and plant pathogens	Saprotrophs, plant parasites, AM and animal pathogens

3.4 Discussion

Differences in sand-dune ecosystems related to soil pH and loss-on-ignition

The assessment of soil pH and loss-on-ignition (LOI) values of the sand-dune soils showed that these soils have different geochemical characteristics. The differences in soil pH and LOI values were expected as sand-dune soils present wide variations in their calcium carbonate ($CaCO_3$) content which is reflected in a variation in pH across the dune system (Ranwell and Boar, 1986). The differences in LOI values are a result of leaching of nutrients, the distance from the sea and/or the amount of organic matter accumulated in soils due to soil development with age (Willis, 1985).

The main differences were that the soil pH and LOI values of the mobile dunes were different from the other two ecosystems, *i.e*. the dune slacks and the fixed dunes, in both reserves (Table 3.2). The mobile-dune soils were alkaline and with the lowest LOI values. An explanation for this is that the mobile dunes are the ecosystems which are closer to the foreshore where the deposits of calcium carbonate from shells are higher (Willis, 1985). By contrast, all soils from dune slack/grasslands and from fixed dunes, in both study sites, were more acidic which could be attributed to the role of rain in nutrient leaching. Rain leaches soluble calcium carbonate and other nutrients. There is also a build-up of organic carbon from progressive plant colonization and growth associated with the loss of carbonate, therefore, leading to acidification (Willis 1985). In the current study, during one field sampling (January 2013) at both sites, the area in the dune slacks (Sandscale Haws and Ainsdale) and in the fixed dunes (Sandscale Haws) where the bait bags were placed, were flooded, thus indicating that rain could have indeed leached nutrients from the soils. Furthermore, the soil always tends to become more acidic with time (Ranwell and Boar, 1986) and with distance from the sea. In our study, the distance from the sea appeared to be an important factor which contributes to soil pH, and that is related to soil age, being that the soils that are closer to the sea were the youngest within the dune system. The mobile dunes are probably the youngest ecosystem in both reserves due to sand accretion (Skelcher, 2009; 2012).

The assessment of LOI of soils showed a pattern that has been commonly explained before: soils at inland parts of the dune system characteristically have higher losses on ignition than freshly blown sand near the shoreline sand (Brown, 1958; Willis, 1985). Our results are similar to previous studies as the mobile-dune ecosystems, in both

reserves, did contain the lowest amount of LOI if compared with the dune slacks and fixed dunes that are in the inland parts of the dune system. Moreover, soils from the dune slacks and fixed dunes at Sandscale Haws and from the dune slacks at Ainsdale had similar amount of organic matter (Table 3.2). This could be explained by the fact that the ecosystem that was named as 'fixed dunes' at Sandscale Haws was classified under the same NVC (*i.e*. SD16 *Salix repens – Holcus lanatus* dune-slack community) as the dunes slacks at both reserves.

Frequency of fugal taxa across different sand-dune soils

There were differences in the frequency (number of OTUs) of the fungal species retrieved from buried barley straw and hawthorn wood samples which were related to the ecosystem type: the mobile dunes had the lowest number of OTUS (*e.g.* 4-23 at Sandscale Haws; Table 3.3) compared with the number of OTUs retrieved from samples buried in the dune slacks and in the fixed dunes (*e.g*.11-28 in the dune slacks and 7-94 in the fixed dunes at Sandscale Haws; Table 3.3). Moreover, the fungal community in the mobile dunes grouped separately in the ordinations analysis (see later). A probable explanation for this is that the mobile dunes are a difficult habitat for species to inhabit because there are, for example, limited resources available for fungi to use, low organic matter content and severe environmental fluctuations, therefore only specialist fungi that have developed a number of adaptations (*e.g*. the development of a solid, subterranean aggregation of mycelium mixed with sand; Rotheroe, 1993) are capable of colonizing these harsh ecosystems. Our results are in line with those of Rotheroe (1993) who assessed the fungal diversity of Welsh sand dunes: from a total of 502 fungi, only six species of specialist fungi where found in the mobile 'yellow' dunes, *e.g. Peziza ammophila* (Ascomycota) and *Melanoleuca cinereifolia* (Basidiomycota). This was attributed to the lack of nutrients and environmental fluctuations in this area of the dune system. In our study, the mobile dunes had the lowest LOI when compared with the other two sand-dune ecosystems. This suggests that the low amount of available carbon in the mobile dunes limits the establishment of higher number of fungi as they need carbon for nutrition and energy.

Fungal community composition of bait materials and ecological function of fungi

Sand dune habitats are highly heterogeneous and offer a wide variety of microhabitats which fungal species utilize with different strategies to cope with stressful environments (Brown, 1958; Geml *et al*., 2014). Similar to other studies carried out in sand dunes (Mueller and Schmit, 2007; Dzal *et al*., 2011; Geml *et al*., 2014), our study showed a fungal community composition which comprised different fungal life strategies such as plant (*Fusarium oxysporum*, Hypocreales) or animal (*Pochonia* sp., Hypocreales) pathogens, coprophilous fungi (*Apodus deciduus,* Sordariales), mycorrhizas (see examples below) and saprotrophs (see examples below). However, this discussion is based on ectomycorrhizas (ECM) and saprotrophs because of their role in the global carbon cycle, as ECM and saprotrophs may be responsible for degrading different C and nutrient fractions in soil organic matter (Talbot *et al*., 2013).

Root-associated fungi

Our study showed the presence of two types of mycorrhizas at both reserves: arbuscular mycorrhizas (AM) and ectomycorrhizas (ECM). The arbuscular mycorrhizal fungal genus *Glomus* (Glomeromycota) was one of the two most commonly recovered genera from the bait bags (the other genus was *Cryptococcus* whose ecology will be explained later). Although it was surprising because AM need a living plant to survive, an explanation for this is that the bait bags were buried within the root zone (rhizosphere) of *Salix repens* which commonly forms AM in sand dunes (van der Heijden and Kuyper, 2001). Thus, the bait materials could become easily "contaminated" with AM spores or mycelia as a natural process in the field.

The study sites were characterised by different plant communities such as *S. repens, B. pendula* and *C. monogyna* which commonly form ECM in temperate regions (Carlile *et al*. 2001). The presence of ECM is a successful life strategy in these types of nutrient-poor soils (Webster and Weber, 2007); for fungi it may be the most important source of soluble carbon. ECM basidiomycetes were recovered such as *Thelephora* (Thelephorales), *Tomentella* (Thelephorales) and *Russula laccata* (Russulales) in the dune slacks in Ainsdale. This agrees with the fact that basidiomycetes commonly form ECM with *S. repens* in sand dunes (Geml *et al*., 2014).

Saprotrophs

Saprotrophic fungi are the main organisms responsible for breaking down organic matter (Lynch and Thorn, 2006). They belong to both the phyla Ascomycota and Basidiomycota (Carlile *et al*., 2001; Deacon, 2006; Webster and Weber, 2007), however, saprotrophs that belong to the phylum Basidiomycota (informally basidiomycetes) are the main decomposers of lignin and they are difficult to isolate from soil and culture (Thorn *et al.*, 1996; Lynch and Thorn, 2006). In this current study, fungi that carried out the degradation of bait materials were determined, including wood rotting fungi, in several ecosystems. Firstly, fungi that grow on simple soluble nutrients were characterized, such as the yeasts *Cryptococcus* and *Rhodotorula*, and mitosporic fungi that might utilise hydrated cellulose and hemicelluloses such as *Cladosporium* species which grow on old and dead plant materials (Harvey, 1967). Some species of *Mortierella* were also retrieved which are amongst the commonest soil fungi and they are capable of decomposing chitin (Jackson, 1965). One of the species retrieved was *M. elongata* which is widely distributed especially in neutral soils (Ali *et al*., 1965; Ali *et al*., 1975) where it decomposes chitin (Jackson, 1965; Veldkamp, 1955).

Polymer-degrading species such as *Fusarium, Trichoderma, Penicillium* and *Chaetomium* (soft-rot fungus) are successful decomposers of cellulose in wheat straw (Harper and Lynch, 1985; Kabuyah, 2012) and of other cellulose-rich material buried in soils (Deacon, 2006). For example, *Fusarium* species such as *F. culmorum* have been reported from dunes (Moubasher and Mazen, 1971; Nicot, 1958; Pugh *et al*., 1963), salt marshes (Cooke, 1970; Park, 1972) and seawater (Roy, 1966). It is one of the first colonizers of wheat straw (Bruehl and Lai, 1966) and dead straw because of the relatively high carbon content of straws in cellulose and hemicelluloses (Butler, 1959; Garrett, 1963). *F. culmorum* is a soil inhabitant that is capable of decomposing cellulose and pectin and it has ahigh competitive saprotrophic ability (Butler, 1953; Lucas, 1955; Rao, 1959). It uses L-arabinose, a component of biopolymers such as hemicelluloses and pectin, as carbon sources (Ross, 1960). *Chaetomium* species are known as important agents in the decay of cotton and other cellulosic materials, and they caused soft rot in wood (Millner, 1975). They degrade herbaceous and lignified plant matter in soils (Millner, 1975), although some species can occur in dung (Lodha, 1964; Millner,

1975). They need substrates of a high C/N ratio where the carbon source is provided in an insoluble form such as starch or cellulose, for good sporulation (Millner, 1975). *Penicillium* species predominate in soils of temperate regions where they appeared to be more abundant at greater soil depths if compared with other genera. *P. canescens* was retrieved in this study which has been isolated from dunes (Nicot, 1958) and arable soils (Cassini *et al*., 1966). It has been observed on wood sticks exposed in soils (Gersonde and Kerner-Gang, 1968).

Another important saprotrophic fungus that was recovered was *Melanoleuca cinereifolia* (Agaricales). It is one of the saprotrohic species that is difficult to identify from macroscopic features alone, particularly because of its sand-dune habitat as its body becomes ingrained with sand, although it is commonly associated with marram grass (*Ammophila arenaria*; Rotheroe, 1993; Kirk *et al*., 2008). It was indeed retrieved from buried barley straw in the mobile dunes.

In dune slacks, saprotrophy is one of the nutritional modes present, such as one species that was retrieved in this study: *Psathyrella spadiceogrisea* (Agaricales) that is common in hardwood forests where it could be a wood degrader (Rotheroe, 1993; Watling and Rotheroe 1993). Wood is predominantly decomposed by basidiomycetes, whether brown-rot or white-rot fungi. Up until now, the only organisms known to decompose lignin completely are white-rot fungi. They are the most numerous among all the wood-decaying fungi (Crawford and Crawford, 1980; Berg and McClaugherty, 2003; Deacon, 2006; Webster and Weber, 2007). Barley straws and hawthorn wood were indeed colonized by two genera of white-rot fungi, *i.e. Phlebia* and *Phanerochaete* from the order Polyporales. Thus, it is suggested that the bait materials were being degraded by white-rot fungi, under field conditions. The diversity of degraders encountered in this study is in line with the classic assumption which suggests that there is a change in species composition of fungal communities as plant substrata undergo decay. So, there might be a substratum succession of fungi (Harper and Lynch, 1985; Dighton, 1997; Frankland, 1998) that are degrading buried barley straw and hawthorn wood in the field.

Fungal OTU richness

Accumulation curves failing to reach an asymptote (Fig. 3.4) suggest that sequencing depth was insufficient to characterise the full fungal community in both sites. However, all the diversity estimates indicated that most of the richness was indeed recovered and that there were not significant differences in fungal OTU richness between barley straw buried in the two different sites. At Sandscale Haws, estimates for OTU fungal richness for hawthorn wood were a little higher if compared with barley straw, particularly the Chao estimate. The difference in OTU richness between plant materials could be attributed to the ability of fungi to utilise the substrata as a source of carbon because hawthorn wood may need a more complex fungal community that includes polymer degrading fungi to attack the recalcitrant lignin (Deacon, 2006; Webster and Weber, 2007).

Association with ecosystem type/soil pH

There was a clear association of fungal communities with different ecosystem types at both sites which is likely to be attributed to the differences in plant community composition, soil pH and LOI of the ecosystems where the bait bags were buried. In Sandscale Haws, the mobile dunes ecosystem was classified by the National Vegetation Classification, UK, as an *Ammophila arenaria* mobile dune community, while the two other ecosystems, although separated by hundreds of metres, , belong to the same vegetation classification, i.e. *Salix repens – Holcus lanatus* or *Salix repens – Calliergon cuspidatum* dune-slack community. Thus, if fungal community composition is related to plant community composition because of direct interactions (Taylor *et al.*, 2014), it would be expected that similar ecosystems hold similar fungal communities. At Ainsdale, the vegetation composition also is likely to structure the fungal community composition because the bait bags were buried in very different ecosystems: the mobile dunes which is characterised by the presence of *Ammophila arenaria* community, in the dune grassland / slack with which has some *S. repens* patches and in a *Betula* (Birch*)* woodland (fixed dunes).

Differences in soil pH are related to differences in the distribution of specific fungal taxa in natural habitats (Brown, 1958; Rousk *et al*., 2009; Geml *et al*., 2014). Similar to

previous studies, our results suggested that alkaline *versus* more acidic sand-dune soils hold different fungal taxa. All the mobile dunes soils that were assessed had an alkaline pH and most of the fungal taxa retrieved from the bait samples buried at these sites were different from the fungal taxa retrieved from samples buried in the more acidic sites, *i.e*. from dune slacks or fixed dunes. Basidiomycete yeasts such as *Rhodotorula aurantiaca* and *Sporobolomyces roseus*, the basidiomycete *Melanoleuca cinereifolia*, and ascomycetes such as *Cadophora luteo-olivacea*, *Isaria farinosa* and *Microdochium sp* were only retrieved from the mobile 'alkaline' dunes.

Brown (1958) isolated soil fungi from different English sand-dune soils which had different soil pH values: species such as *Mortierella sp.*, *Penicillium sp.*, *Trichoderma sp.* and *Verticillium sp.* were generally isolated from acidic soils. Geml *et al*. (2014) showed the same trend in the 'preference' of some fungi in soils based on soil pH, *Penicillium sp.*, *Verticillium sp.* and *Trichosporon sp.* were identified from acidic sites. Our study is in line with these previous studies as the same species were also retrieved from neutral-slightly acidic soils. However, some fungal species that were previously reported from alkaline sites such as *Fusarium culmorum*, *F. oxysporum*, *Tomentella sp.*, *Phoma sp.*, *Exophiala sp.* and *Podospora sp.* (Brown, 1958; Geml *et al*., 2014), in our study, these species were retrieved form samples buried in slightly acidic soils. Even though some studies suggested that soil pH strongly correlates with the composition of fungal communities (Brown, 1958; Rousk *et al*., 2009; Geml *et al.*, 2014), in natural ecosystems it is challenging to determine the factors that structure soil communities as these could be structured directly by pH or indirectly *via* the interaction of pH with communities, *e.g.* bacterial, animal and fungal communities, and environmental factors such as nutrient availability, organic carbon availability and vegetation characteristics (Geml *et al.* 2014). For example, the amount of organic carbon which is higher in the final stages of the dune succession, because of an increase in the amount of vegetation (Webley *et al*., 1952), seems to be related to the frequency of some fungal species (Brown, 1958). In our study, both the dune slacks and fixed dune ecosystems are part of the final stages of a dune succession and the amount of organic carbon (measured as LOI) at these sites was higher if compared with the LOI in the mobile dunes. The fungal frequencies at these final stages were higher if compared with fungal frequencies in the mobile dunes at Sandscale Haws, by contrast, the fungal frequencies in the dune slacks

at Ainsdale were higher if compared with the fungal frequencies in the fixed dunes (Table 3.3).

There were significant differences in the fungal taxa retrieved depending on the type of bait material in Sandscale Haws This could be related to the use of the different available substrata for fungi because not all fungi are able to degrade/colonise the same resource, for example, fungi need to produce a particular set of enzymes to degrade the different types of woody plants (Lynch and Thorn, 2006; Webster and Weber, 2007) probably due to the different chemical composition, *e.g.* cellulose and lignin content, of plant groups (Pearl, 1967; Raven *et al*., 2005; Deacon, 2006). For example, hardwood plant species (angiosperms, among which hawthorn wood is one) have from 19% to 28% lignin content (Sarkanen and Ludwig,1971) and grasses (graminoids) appeared to have generally less lignin content, such as barley straw that has about 17% lignin content (Adapa *et al*., 2009). Moreover, the community composition growing on degrading materials is also dependent on the ecosystem where the material is located and the stage of the decomposition of the material.

Differences in fungal colonisation by bait material types, burial depth and sampling time

There were not significant differences (Table 3.4) in the fungal community composition based on the burial depth which was contrary to previous studies which showed that the fungal communities in the O (organic) horizon were taxonomically more diverse if compared with those in the A (mineral) horizon in forests (Kodowaki *et al*., 2014). An explanation for our results could be that sand-dune soils might have a simpler fungal community composition if compared with, for example, forest soils, probably due to the higher diversity of trees/plants in forests if compared with the diversity of trees/plants in sand dunes because plant diversity is considered one factor structuring soil fungal communities (McGuire *et al*., 2011).

Several studies reported that soil fungal community composition presents seasonal changes. A study (Bowen and Harper, 1989) showed that fungal species on wheat straw decomposing in the field fluctuated within a year. *Typhula sp.* (a lignin-decomposing basidiomycete) was isolated from samples at different sampling times and lignin-

decomposing basidiomycetes with slower extension rates than *Typhula* were isolated from samples recovered in spring (Bowen and Harper, 1989). Fungal isolates from buried wheat straw and dead wheat leaves differed depending on the burial time of the material in an arable soil in England (13, 22 or 32 weeks; Robinson *et al*., 1994). More recently, Taylor *et al*. (2010) found that the soil fungal community composition in Alaska had seasonal changes within a year. Similar to previous studies, the soil fungal community composition particularly in Ainsdale, appears to be structured within a year, as the statistical analyses carried out here (Table 3.4; Fig. 3.6C&D) suggested that the fungal communities differed among sampling periods.

3.5 Conclusions

In the United Kingdom, sand dunes are of considerable extent and variety, and some are nationally important, however, the mycological importance of the sand dune habitat in the UK has only been documented relatively sparsely with work being undertaken in Wales and Scotland (Rotheroe, 1993; Rotheroe *et. al*, 1987; Watling and Rotheroe, 1989; Rhind and Jones, 1999; Leake *et al*., 2004, but see Brown, 1958). The current study is one of the few carried out to characterise soil fungi in different sand-dune ecosystems in England, and is also one of the few studies using DNA sequencing for such characterisation, showing the relatively large diversity of soil fungi which is likely to be due to a variation in the vegetation and edaphic factors, *e.g*. soil pH, of the sand-dune ecosystems. The fungal diversity was shown both in terms of the number of OTUs (275) and the fungal life strategies (ecologies). Among the fungal ecologies, the mostly recovered OTUs were found either as forming fungal symbiosis with plants or as saprotrophs. Fungi have an important role in facilitating colonisation by higher plants and sustaining sand dune systems, therefore fungal symbiosis with plants is a key strategy for the maintenance of such ecosystems. Saprotrophy involving extremely limited resources in nutrient-poor soils should allow greater specialization and more subtle relationships making possible an increase of fungal species diversity.

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Supporting information

Table S 1. Number of bags containing two different types of bait materials (barley straw and hawthorn wood) that were buried in two National Nature Reserves, across different sand-dune ecosystems at different depth (5 or 11 cm) and retrieved three, six or twelve months later.

Plot ID[a]	Number of buried bags	Number of bags per sampling time (months): Three	Six	Twelve	Total number of bags retrieved
Sandscale Haws National Nature Reserve					
SBM	18 (9/9)[b]	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SBD	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SBF	18 (9/9)	6 (3/3)	NR	12 (6/6)	18 (9/9)
SHM	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SHD	18 (9/9)	6 (3/3)	6 (3/3)	NR	12 (6/6)
SHF	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
Ainsdale sand dunes National Nature Reserve					
ABM	18 (9/9)	4 (2/2)	NR	NR	4 (2/2)
ABD	18 (9/9)	6 (3/3)	NR	12 (6/6)	18 (9/9)
ABF	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)

[a]ID key: S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the type of buried bait material barley straw or hawthorn wood, M/D/F corresponds to the different sand-dune ecosystems where the bait materials were buried mobile dunes or dune slacks or fixed dunes. For example: SBM corresponds to a plot in Sandscale Haws (S), the buried bait material was barley straw (B) which was buried in the mobile dunes (M).

[b]The numbers between brackets (a/b) correspond to number of bags buried or retrieved at/from 5 or 11 cm depth below the soil surface (5cm / 11 cm). NR: no bags retrieved.

Table S 2: Overview of the 275 OTUs found colonising bait materials, *Hordeum vulgare* (barley) straw and *Crataegus monogyna* (hawthorn) wood, buried in two different sand-dune sites and throughout different ecosystems, including the sample ID where they were retrieved, their frequency (number of samples where each OTUs was present), number of reads and BLASTN top hits with accession numbers in GenBank.

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
13	19	4456	80%	98%	*Lasiosphaeriaceae*	EU754958.1
43	14	513	100%	98%	*Exophiala sp.*	AB701673.1
21	14	2502	89%	96%	Annulatascaceae	JF519062.1
47	14	493	91%	100%	*Ilyonectria crassa*	KJ475469.1
7	13	13653	88%	100%	*Plectosphaerella cucumerina*	KF472138.1
6	13	14132	99%	98%	*Podospora intestinacea*	AY999121.1
48	13	436	100%	*99%*	*Mortierella elongata*	JF439485.1
12	13	3965	89%	99%	Chaetomiaceae	JX545185.1
25	11	1424	85%	97%	*Exophiala sp.*	GQ302685.1
116	11	67	100%	99%	*Cladophialophora chaestopira*	EU035406.1
51	10	427	99%	99%	*Fusarium acuminatum*	KF527822.1
24	10	1355	100%	97%	*Schizothecium carpinicola*	NR_103589.1
69	10	167	100%	95%	*Cladophialophora chaetospira*	HQ871875.1
9	10	9183	89%	88%	Sordariales	JF519062.1
15	9	3207	90%	100%	*Pyrenophora teres*	EF452469.1
1	9	25622	89%	99%	*Apodus deciduus*	AY681199.1
119	9	55	94%	87%	*Aquaticola sp.*	JF831462.1
45	9	548	89%	100%	*Cladosporium perangustum*	KJ620970.1
82	9	113	100%	99%	*Mortierella sp.*	KF225823.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
67	8	205	100%	99%	*Cryptococcus podzolicus*	FN428924.1
131	7	39	100%	99%	*Pochonia suchlasporia*	AB214658.1
53	7	215	88%	99%	*Phoma sp.*	KF646102.1
138	7	34	100%	100%	*Tolypocladium cylindrosporum*	AB208110.1
178	7	24	100%	99%	*Exophiala sp.*	GU055730.1
90	6	109	100%	98%	*Schizothecium aloides*	AY999120.1
2	6	23103	89%	96%	*Zopfiella tabulata*	AY999132.1
17	6	3064	100%	99%	*Cryptococcus sp.*	EF687931.1
104	6	63	97%	99%	*Mortierella sp.*	DQ093725.1
132	6	41	100%	99%	*Phaeosphaeriopsis sp.*	HQ630983.1
88	6	108	89%	99%	*Hebeloma sp.*	JQ724055.1
95	6	76	100%	79%	*Taifanglania inflata*	KC285890.1
107	6	61	99%	99%	*Paecilomyces carneus*	AB258369.1
49	5	463	100%	100%	*Microdochium sp.*	AJ246155.1
16	5	3087	99%	100%	*Sarocladium sp.*	KC753435.1
115	5	59	100%	99%	*Bipolaris eleusines*	DQ337382.1
130	5	40	100%	99%	*Tetracladium sp.*	JX545196.1
124	5	49	100%	96%	Ascomycota	HM239909.1
112	5	61	100%	89%	*Shiraia bambusicola*	AY536372.1
40	5	617	98%	98%	*Rhizophagus sp.*	FR772330.2
31	5	1065	91%	97%	*Hyaloscypha leuconica*	JN033451.1
61	5	160	97%	96%	*Tumularia sp.*	KF646104.1
89	5	93	100%	100%	*Cryptococcus terricola*	HE863717.1
59	5	269	100%	100%	*Lewia infectoria*	JF440601.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
26	4	711	88%	97%	Ascomycota	HM239685.1
200	4	15	100%	99%	*Heteroconium sp.*	KC989067.1
86	4	112	100%	87%	*Sebacina sp.*	EU909223.1
30	4	1134	89%	81%	*Cercophora sp.*	KF823604.1
91	4	98	100%	99%	*Cryptococcus sp.*	JX967365.1
183	4	19	100%	84%	Neonectria lucida	GU062255.1
71	4	184	90%	94%	*Exophiala sp.*	HQ631063.1
0	4	38264	83%	99%	Pezizomycotina	GU256218.1
8	4	10145	89%	93%	*Podospora appendiculata*	AY999126.1
123	4	48	98%	99%	*Rhizofagus sp.*	HG969317.1
147	4	30	100%	99%	Leotiomycetes	JF449683.1
181	4	18	100%	99%	Ascomycota	AY568066.1
188	4	18	100%	98%	*Verticillium leptobactrum*	AB214657.1
121	4	40	100%	100%	Basidiomycota	HM240154.1
93	4	60	95%	98%	*Chaetomium sp.*	KF313104.1
170	4	23	100%	99%	*Pochonia suchlasporia*	HG008759.1
64	4	217	100%	93%	*Monacrosporium ellipsosporum*	AY695065.1
76	4	160	100%	91%	*Apodus deciduus*	AY681199.1
219	4	14	100%	98%	*Mortierella sp.*	JX270421.1
140	3	32	100%	99%	*Cryptococcus sp.*	HG532069.1
10	3	6413	91%	80%	*Pseudallescheria boydii*	JX349252.1
58	3	271	100%	80%	*Pseudallescheria boydii*	GU566282.1
232	3	10	94%	97%	*Dactylaria appendiculata*	AY265339.1
50	3	437	99%	93%	*Podospora sp.*	KC753429.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
145	3	27	100%	98%	*Phialophora sp.*	JQ898559.1
216	3	12	80%	87%	*Trichosporon sp.*	JX898552.1
106	3	55	100%	85%	Glomeromycota	JF519527.1
195	3	14	100%	100%	*Verticillium sp.*	HE977537.1
11	3	4116	90%	99%	*Immersiella sp.*	JF519087.1
66	3	208	100%	92%	Ascomycetes	AY568066.1
206	3	12	100%	98%	*Apodus deciduus*	AY681199.1
186	3	19	100%	89%	Sordariales	JF519062.1
161	2	24	100%	98%	*Isaria farinosa*	DQ681344.1
272	2	8	100%	91%	*Sebacina vermifera*	DQ520096.1
264	2	5	100%	99%	*Trichoderma asperellum*	KF359587.1
162	2	17	100%	94%	*Exophiala sp.*	AB701673.1
27	2	641	89%	93%	*Chaetosphaeria sp.*	HQ630994.1
19	2	2788	89%	92%	*Schizothecium glutinans*	AY999116.1
127	2	43	90%	99%	*Trechisporales*	JF691365.1
122	2	48	100%	98%	*Schizothecium glutinans*	AY999116.1
256	2	6	100%	99%	*Phoma exigua*	GU062320.1
199	2	12	99%	95%	Helotiales	JX001625.1
187	2	14	100%	94%	*Cryptococcus dimennae*	HG008764.1
228	2	9	100%	94%	*Dactylaria sp.*	JF449852.1
383	2	5	99%	92%	*Cryptococcus sp.*	HQ890370.1
271	2	6	100%	83%	*Mingxiaea setariae*	AB118875.1
36	2	936	100%	100%	*Cadophora malorum*	JQ796752.1
99	2	83	89%	100%	*Cryptococcus stepposus*	JX188129.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
164	2	24	100%	96%	*Rhodotorula sp.*	FN812728.1
245	2	10	100%	99%	*Phialocephala humicola*	AB671502.2
320	2	6	100%	99%	*Trichosporon porosum*	KF285994.1
154	2	28	99%	98%	Helotiales	JX001625.1
56	2	276	100%	99%	*Phanerochaete sp.*	GU934592.1
300	2	6	100%	99%	Chaetosphaeriaceae	GU327452.1
128	2	44	99%	99%	*Fusarium culmorum*	KF576630.1
173	2	20	100%	99%	*Trichosporon sp.*	JX270381.1
209	2	10	100%	99%	*Pyrenochaetopsis sp.*	AB916515.1
246	2	10	100%	99%	*Penicillium nothafagi*	NR_121518.1
185	2	18	98%	98%	*Glomus sp.*	EF393596.1
352	2	6	100%	98%	Pleosporales	JF749176.1
151	2	26	100%	97%	*Trichoderma brevicompactum*	KJ000306.1
257	2	7	100%	94%	*Pyrenochaetopsis sp.*	AB916515.1
142	2	32	100%	85%	Helotiales	JQ318662.1
269	2	6	100%	84%	Ascomycota	HM240020.1
159	2	16	100%	99%	*Microdochium phragmitis*	AM502263.1
202	2	14	100%	90%	Sordariomycetidae	FJ708601.1
387	2	6	100%	88%	*Glomus sp.*	JQ218220.1
354	2	5	100%	83%	Coniochaetales	FJ439578.2
3	2	20342	84%	80%	*Aquaticola sp.*	JF831462.1
222	2	10	100%	100%	*Trichocladium sp.*	JF519272.1
96	2	46	100%	99%	*Tomentella sp.*	DQ990852.1
274	2	6	100%	98%	*Myrothecium sp.*	JN859396.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
292	2	6	100%	98%	*Tomentella sp.*	HM146874.1
182	2	17	100%	94%	Ascomycota	HM240020.1
213	2	8	100%	88%	*Sebacina sp.*	JX317492.1
54	2	270	100%	99%	Helotiales	HM059042.1
101	2	76	100%	99%	*Pilidium concavum*	KF255414.1
165	2	23	100%	93%	*Mycogone perniciosa*	KC964105.1
63	2	229	86%	89%	Basidiomycota	HM240215.1
84	2	111	100%	89%	*Phialophora sp.*	JQ247407.1
77	2	144	99%	99%	*Epicoccum nigrum*	EU272494.1
225	2	11	100%	93%	Chaetomiaceae	GU055625.1
79	2	132	100%	91%	Pleosporales	KF823605.1
4	2	19858	89%	82%	*Podospora sp.*	GU166458.1
263	2	8	100%	99%	*Mycoarthris corallinus*	AF128440.1
29	2	1175	89%	99%	*Halenospora varia*	KF156329.1
243	2	8	95%	99%	*Monacrosporium cionopagum*	AY944137.1
242	2	10	100%	88%	*Cyphellophora laciniata*	EU035416.1
74	2	176	100%	81%	*Chaetomium sp.*	HQ607819.1
168	1	21	100%	100%	*Massarina rubi*	AF383963.1
339	1	5	100%	100%	*Lewia infectoria*	JX421701.1
321	1	5	94%	99%	*Penicillium soppii*	KJ028790.1
294	1	7	100%	93%	*Microdochium nivale*	AM502266.1
177	1	19	100%	89%	*Torula caligans*	FJ478093.1
226	1	11	97%	94%	*Sarocladium sp.*	KC311484.1
35	1	989	87%	99%	*Sarocladium sp.*	KC311484.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
75	1	173	100%	80%	*Cephalotrichiella penicillata*	KJ869166.1
340	1	5	100%	80%	*Pseudallescheria boydii*	GU566282.1
169	1	21	100%	87%	Sordariomycetidae	FJ708601.1
174	1	19	100%	82%	*Pseudocatenomycopsis rothmanniae*	KF777185.1
203	1	13	94%	84%	*Rhodotorula ferulica*	AF444528.1
293	1	4	91%	93%	*Conlarium duplumascospora*	JN936996.1
297	1	3	100%	87%	Tremellales	FJ611954.1
94	1	50	100%	84%	Helotiales	KC694160.1
215	1	6	100%	84%	Ascomycota	DQ273344.1
279	1	7	100%	99%	*Doratomyces sp.*	JX270355.1
191	1	15	100%	96%	*Heteroconium sp.*	KC989067.1
316	1	5	100%	96%	*Schizothecium aloides*	AY999120.1
85	1	111	90%	94%	Sebacinales	GU256215.1
157	1	25	100%	86%	*Mycena capillaripes*	JF908489.1
60	1	251	100%	83%	*Pseudolachnella guaviyunis*	KJ834524.1
57	1	275	100%	86%	*Oliveonia pauxilla*	HQ441577.1
198	1	15	100%	84%	*Phialemonium dimorphosporum*	FJ441614.1
255	1	9	100%	82%	*Ramariopsis kunzei*	GU187552.1
299	1	3	100%	99%	*Thelebolus sp.*	AB916508.1
432	1	2	100%	99%	*Hygrocybe sp.*	FR750604.1
163	1	12	100%	96%	*Psathyrella spadiceogrisea*	DQ389682.1
208	1	7	100%	96%	*Devriesia sp.*	JF519086.1
301	1	3	100%	96%	*Myrmecridium schulzeri*	KC989072.1
234	1	5	100%	95%	*Apodus deciduus*	AY681199.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
305	1	3	100%	93%	*Mortierella sp.*	JX270478.1
286	1	4	100%	89%	*Erythrobasidium sp.*	EF060826.1
306	1	3	94%	86%	*Paraphoma dioscoreae*	KF251169.1
329	1	3	100%	86%	*Apodus deciduus*	AY681199.1
427	1	3	100%	82%	*Lasiosphaeria glabrata*	AY587915.1
270	1	4	100%	81%	*Rhodotorula sp.*	HM545717.1
214	1	6	100%	79%	Basidiomycota	HM240175.1
330	1	5	100%	93%	*Ceratobasidium sp.*	HQ687894.1
318	1	5	100%	89%	Ascomycota	HM239909.1
136	1	35	100%	100%	*Melanoleuca cinereifolia*	JF908356.1
33	1	997	100%	99%	*Cadophora luteo-olivacea*	KF156297.1
235	1	9	100%	99%	*Microdochium bolleyi*	AM502265.1
46	1	541	100%	97%	*Phaeoacremonium mortoniae*	JN693516.1
287	1	7	100%	89%	*Pleospora sp.*	JQ247387.1
276	1	7	100%	100%	*Sporobolomyces roseus*	HG008766.1
62	1	243	100%	99%	*Dioszegia sp.*	HF947085.1
171	1	19	100%	99%	*Dioszegia fristingensis*	EU517066.1
236	1	9	100%	99%	*Glomus sp.*	FJ769329.1
341	1	5	100%	99%	*Dioszegia rishiriensis*	AB545810.1
167	1	21	80%	97%	*Tremella phaeophysciae*	JN053479.1
197	1	15	100%	97%	*Rhodotorula aurantiaca*	AB026015.1
404	1	3	93%	92%	*Bensingtonia subrosea*	NR_073310.1
280	1	7	100%	91%	*Cryptococcus nemorosus*	FN428883.1
193	1	15	100%	99%	Ascomycetes	AM901919.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
275	1	7	100%	93%	*Schizothecium glutinans*	AY999116.1
315	1	5	100%	99%	*Fusarium oxysporum*	KJ653447.1
278	1	7	87%	99%	Ascomycetes	GU256218.1
307	1	5	99%	99%	*Dactylaria dimorphospora*	U51980.1
317	1	5	100%	91%	*Xenostigmina zilleri*	FJ839639.1
407	1	3	100%	80%	*Lasiodiplodia pseudotheobromae*	JN607093.1
114	1	29	94%	99%	*Tubaria sp*	KJ028790.1
42	1	579	100%	88%	*Dictyochaeta sp.*	JF449595.1
205	1	13	92%	99%	*Mortierella sp.*	AB638472.1
221	1	11	94%	99%	*Dictyosporium toruloides*	DQ018093.1
227	1	11	100%	99%	*Discosia pseudoartocreas*	KF777161.1
252	1	9	100%	99%	*Penicillium canescens*	KF156319.1
302	1	5	100%	99%	*Cercospora zeae-maydis*	JX143743.1
308	1	5	100%	99%	*Paraphaeosphaeria neglecta*	JX496107.1
78	1	143	100%	98%	*Pezizella discreta*	JF908571.1
111	1	61	100%	98%	*Pyrenochaeta sp.*	KC339228.1
333	1	5	83%	98%	*Tremella*	KC171330.1
379	1	5	100%	98%	*Mariannaea elegans*	AB855778.1
118	1	51	100%	97%	*Alatospora flagellata*	KC834041.1
331	1	5	100%	97%	*Leptodontidium orchidicola*	AF486133.1
139	1	33	100%	95%	*Glomus sp.*	AY236298.1
314	1	5	96%	94%	*Phialophora hyalina*	AB190398.1
400	1	5	100%	94%	Basidiomycota	GU328638.1
98	1	85	95%	92%	*Sebacina sp.*	EU910920.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
23	1	2391	89%	85%	*Phialophora sp.*	AF083199.1
351	1	3	98%	83%	*Glomus sp.*	GQ388518.1
258	1	7	99%	82%	*Rhinocladiella sp.*	KC965863.1
73	1	175	100%	95%	*Bisporella citrina*	JQ256414.1
249	1	9	100%	79%	*Nectria berolinensis*	HM534893.1
137	1	35	94%	80%	*Sebacina sp.*	HQ154321.1
100	1	83	82%	100%	*Cylindrotrichum sp.*	AM262411.1
327	1	5	85%	100%	*Leptosphaeria veronicae*	JF740255.1
240	1	9	100%	99%	*Geoglossum fallax*	EU784256.1
335	1	5	100%	98%	*Psilocybe cf. subviscida/crobula*	KC176337.1
336	1	5	95%	97%	*Chaetomium sp.*	KF313104.1
254	1	9	100%	95%	*Myrmecridium schulzeri*	KC989072.1
401	1	3	100%	90%	*Haptocillium balanoides*	EF546660.1
14	1	3695	99%	87%	Sordariomycetes	DQ273344.1
253	1	9	100%	80%	*Rhodoveronaea varioseptata*	EU041813.1
217	1	11	0%	79%	Sordariales	JF519062.1
244	1	5	100%	100%	*Candida sp.*	KC525814.1
267	1	4	100%	99%	Glomerales	KF206536.1
358	1	3	100%	99%	*Russula laccata*	JQ724007.1
55	1	147	89%	98%	*Hymenoscyphus sp.*	AB705232.1
105	1	33	100%	98%	*Phlebiella christiansenii*	EU118659.1
109	1	31	100%	97%	*Thelephora sp.*	GU184075.1
117	1	27	83%	96%	*Inocybe ochroalba*	EU326165.1
355	1	3	99%	96%	*Glomus sp.*	AM384979.1
149	1	14	84%	95%	*Ramariopsis sp.*	KF673106.1
288	1	4	87%	93%	*Harposporium bysmatosporum*	FJ380935.1

OTU ID	Frequency	Reads	Query coverage	Max identity	Classification	Accession
247	1	5	100%	85%	*Lophiostoma sp.*	JN904577.1
218	1	11	100%	99%	Nectriaceae	EF601613.1
398	1	5	86%	99%	Auriculariales	JF449733.1
231	1	9	100%	95%	*Tumularia sp.*	KF646104.1
189	1	15	83%	92%	*Preussia tetramera*	GQ203792.1
194	1	15	100%	100%	*Lecythophora sp.*	GU062289.1
283	1	7	100%	96%	Chaetomiaceae	JX545185.1
406	1	3	94%	94%	*Hydropisphaera erubescens*	FJ969800.1
233	1	9	100%	91%	*Dactylaria appendiculata*	AY681199.1
160	1	25	100%	94%	*Schizothecium glutinans*	AY999116.1
273	1	7	100%	89%	*Kernia pachypleura*	DQ318208.1
281	1	7	100%	77%	Sebacinales	KF359622.1
22	1	2423	88%	99%	*Coprinopsis urticicola*	HQ847015.1
334	1	5	95%	97%	Xylariales	GQ924056.1

Chapter 4

Paper 3. Fungal degradation of macromolecular materials in sand-dune soils.

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

Irma González González, Bart E. van Dongen and Clare H. Robinson. Fungal degradation of macromolecular materials in sand-dune soils.

Fungal degradation of macromolecular materials in sand-dune soils

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Abstract

The largest amounts of terrestrial carbon (approximately 1580 Gt carbon) can currently be found in soils which represent two to three times more carbon than is present in the biomass of terrestrial plants. Macromolecular material such as lignin and cellulose forms a substantial part of this soil carbon and its degradation is of importance in the global carbon cycling. However, little is known about the degradation of macromolecular materials in field conditions and, particularly the involvement of fungi in the degradation of cellulose-rich and lignin-rich materials, such as straw and wood, in soils which are relatively low in native organic matter, *e.g.* sand-dune soils. The extent to which lignin and cellulose are degraded in barley (*Hordeum vulgare*) straw and hawthorn (*Crataegus monogyna*) wood buried in sand-dune ecosystems for 12 months was assessed using gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. The assessment of the chemical composition of the materials showed clear differences in the presence of characteristic lignin moieties such as the p-hydroxyphenyls only found in grasses (barley straw). Visual analyses and weight losses of the retrieved bags showed a substantial degradation of the buried materials when compared with barley straw/hawthorn wood prior to burial. There were shifts in the $[Ac/Al]_S$, $[Ac/Al]_G$ and [S/G] lignin decomposition state proxies which showed that lignin oxidation occurred in the straw and the wood curls over a 12 month period, and the lignin degradation was probably induced by white-rot fungi. There was a general increase in the $[Ac/Al]_S$ and $[Ac/Al]_G$ ratios of both types of baits, and for some samples the increase was higher at each sampling time (e.g. sample from the mobile dunes at Sandscale Haws), There was also a decrease in the cellulose to lignin ratio over the 12-

month burial period which indicated that cellulose was also degraded. However, there were clear differences in the S/G ratio of both plant materials: for barley straw the ratio decreased over the 12-month period, by contrast in hawthorn wood it increased probably because of the contribution of tannins to the S6 moiety in hawthorn wood. Our data contribute tentatively to the theory which supports that white-rot basidiomycete fungi are involved in the degradation of lignin-rich materials in sand-dune ecosystems as the lignincellulosic material from bait samples was clearly degraded over a12-month period in the field in a similar manner to the typical fungal degradation that was previously described by other authors under laboratory conditions.

4.1 Introduction

The degradation of soil organic matter (SOM) plays an extremely important role in the global cycling of carbon. Part of the carbon that is fixed by plants in the form of CO_2, during photosynthesis, is transferred to the soil *via* dead plant matter (Jenkinson *et al.*, 1991). The rate of decomposition of plant matter and the annual input of plant debris determine the amount of carbon in soils (Post *et al*., 1982), soils being the largest reservoirs of terrestrial carbon (approximately 1580 Gt carbon; Schimel, 1994). The composition, properties and amount of plant litter are crucial factors that control the formation of SOM (Kögel-Knaber, 2002). Plant litter is composed of complex mixtures of organic components, mainly polysaccharides such as cellulose, aliphatic biopolymers, tannins and lignin (Scholes *et al*., 1997; Schimel, 1994). Of these macromolecules, cellulose is the most abundant plant polymer on earth and during its hydrolysis products, such as soluble sugars and organic acids that are essential sources of energy and carbon for microbial communities, are released (Leschine, 1995). Lignin is the second most abundant biopolymer on earth and it is highly resistant to degradation because of its complex structure which varies depending on the plant group, being classified as softwood, hardwood and grass lignins (Sarkanen and Ludwig, 1971. However, some fungi and bacteria can fragment these macromolecules by producing a variety of hydrolytic enzymes, e.g. fungi produce extracellular manganese and lignin peroxidase enzymes (Pérez *et al.,* 2002; Tomsovsky and Homolka, 2003; Martínez *et al*., 2005), and bacteria such as *Novosphingobium sp*. B-7 that also produces manganese peroxidase (MnP) and laccase (Lac; Chen *et al.,* 2012).

Fungi have the ability to break down major plant components, such as cellulose and lignin, which is the basis of their organic recycling role and makes fungal communities key-players in global ecosystem functioning (Krivtsov *et al*., 2006). The degradation of cellulose and lignin are coupled and carried out by three main wood-degrading groups of fungi: white-rot, brown-rot and soft-rot fungi (Berg and MacClaugherty, 2003; Schwarze, 2007). White-rot fungi (Basidiomycota) are able to degrade lignin by producing ligninolytic enzymes (Kirk and Farrell, 1987), such as laccases, manganese peroxidases and lignin peroxidases produced by the genus *Trametes* (Tomsovsky and Homolka, 2003).

Wood and cereal straws are mainly composed of cellulose, hemicellulose and lignin, which make them ideal "model" organic matter for assessing how macromolecular materials are degraded in natural conditions, as the degradation of materials that are cellulose-rich and lignin-rich is essential in the formation of soil carbon. At present, there is little information about the extent to which lignin and cellulose of plant materials, such as straw and wood, can be degraded in field conditions in soils which are carbon-poor, such as sand-dune soils (but see Kabuyah *et al*., 2012 and Bastow *et al*., 2008). Recent studies showed that in a temperate sand-dune grassland (Kabuyah *et al*., 2012) and in a tropical (Kabuyah, 2012) grassland soil, lignin and cellulose were degraded in bait materials over a burial period of 46 and 12 months, respectively. These studies suggested that lignin were degraded by white-rot fungi which was in line with previous laboratory research (Vane *et al.*, 2001; 2005; Vane, 2003; Martínez *et al*., 2005; Robertson *et al*., 2008).

Studies of degradation of macromolecular materials in the field appear scarce, therefore, the aim of the present study was to assess lignin and cellulose degradation of different types of plant materials that were buried in sand-dune soils, particularly with respect to typical lignin decay during white-rot fungal attack, as a function of both burial time and ecosystem types.

4.2 Methods

4.2.1 Study sites

The first study site was at Sandscale Haws National Nature Reserve (National Grid Reference SD200756; Fig. 4.1A & B, Appendix A), which comprises areas of different

sand-dune successional ecosystems, such as dune slacks, grassland and freshwater marsh and small areas of woodland and scrub with few disturbances. It is one of most botanically rich coastal sites in the UK where over 600 species of plant species have been recorded (Skelcher, 2012).

The second site was at Ainsdale Sand Dunes National Natural Reserve (Grid Reference: SD292110, Fig. 4.1A & C, Appendix A). This reserve protects important plant and animal biodiversity in England. The most typical sand dune ecosystems are well characterized: embryonic and mobile dunes, humid dune slacks, fixed dune grasslands and dune heath (Skelcher, 2009).

Figure 4.1. (A) Map of the United Kingdom pointing out the study sites. (B) Sandscale Haws and (C) Ainsdale Sand Dunes National Nature Reserves (NNR).

Three different ecosystems within each reserve were chosen for the experimental set up. The choice of the different ecosystems is based on the research about fungal succession in sand dunes made by Brown (1958), who described a pattern in ecological succession using classical techniques of fungal isolation on agar media.

Special notes about these ecosystems at Sandscale Haws NNR (Skelcher, 2012; Appendix A):

a) *Mobile dunes* in Sandscale Haws are found in the *Ammophila arenaria* mobile dune community. The soil profile is undifferentiated with accreting mobile dunes (personal observation). There is no active management to allow natural processes of accretion to occur. This area is classified as SD6 *Ammophila arenaria* mobile dune community under the National Vegetation Classification (NVC) for the United Kingdom.

b) *Dune grassland / slack* in Sandscale Haws is located within the *Salix repens – Calliergon cuspidatum* dune slack community. This area is grazed by cattle, sheep and rabbits. No scrub control has taken place in this area in the past five years. This area is classified as SD16 *Salix repens – Holcus lanatus* dune-slack community or SD15 *Salix repens – Calliergon cuspidatum* dune-slack community under the NVC.

c) T*he fixed dune* is located within the *Salix repens – Holcus lanatus* dune slack community. This area is grazed by cattle, rabbits and sheep. No control of the scrub has taken place in the past five years. This area is classified as SD16.

Special notes about these ecosystems at Ainsdale Sand Dunes NNR (Skelcher, 2009):

a) *Mobile dunes* have had scrub species cleared from them in the past and more than 100 years ago the dunes were built up artificially high by the use of sand trap fencing. The dune will have rolled inland over the top of a slack because of coastal erosion processes, perhaps as recently as the last 20 years, so a new dune has been made from an eroding old dune. This area is classified as SD6 *Ammophila arenaria* mobile dune community under NVC for the UK.

b) *Dune grassland / slack* has been mowed on several occasions and been grazed with sheep every winter since early 1990s. There were some *Salix repens* patches (personal observation). This area corresponds to the SD16 *Salix repens- Holcus lanatus* dune slack community under the NVC for the UK.

c) The fixed dune is located within a *Betula* (Birch) woodland that was used to store water up until 1965 and has had no management since then apart from grazing in winter by cows over the last 4 years.

In addition, two soil depths were chosen for the experimental set up. The selection of the depths 5 and 11 cm depends on the horizons' delimitations and characteristics which allow the presence of fungal communities. The 5 cm depth is the limit of the organic horizon. Bardgett (2005) pointed out that this horizon is biologically important since we can find decomposing litter with different biological communities associated which can

be more or less active (e.g. beneath a grassland soil, fungal communities can be driving litter decomposition rather than bacteria). The limit of rooting depth can be found around 11 cm and, for example, in sand alkaline dunes it is the limit of the "grey" sand horizon in semi-fixed "grey" dunes (Brown, 1958).

4.2.2 *Experimental set up and field sampling*

Hordeum vulgare L. (barley) straw internodes (0.08 to 0.10 g) or *Crataegus monogyna* (hawthorn) wood curls (0.10 g) were placed in 3 x 4 cm 50 μm nylon mesh bags (Figure 4.2). This size of bait bag was used to minimize the impact on the ecosystem and the mesh size was large enough to allow colonisation by fungi but small enough to avoid the colonization by fauna and plant roots. Each bag was attached to a nylon string, metal numbered tag and a metal tent peg to make bait bag collection as straightforward as possible. The *Hordeum vulgare* and *Crataegus monogyna* baits were unsterilized to maintain their natural chemical composition. Thus, the plant bait materials were naturally pre-colonised by fungi.

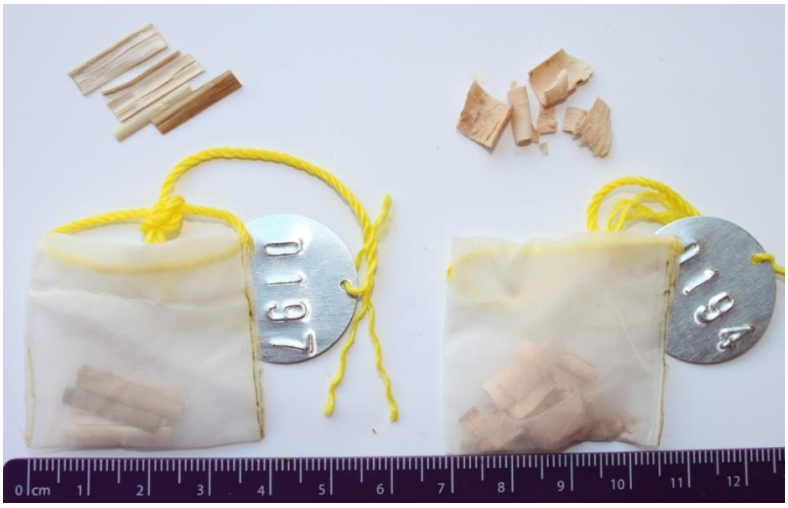

Figure 4.2. *Hordeum vulgare L.* (barley) straw internodes (top left) and *Crataegus monogyna* (hawthorn) wood curls (top right) prior to burial. Mesh bags (3 x 4 cm) filled with bait materials, *H. vulgare L.* (barley) straw (bottom left) and *C. monogyna* (hawthorn) wood (bottom right). Photo Irma González.

Nine 20 x 45 cm plots were set up across three different ecosystems, *i.e.* mobile dunes, dune grassland/slacks and fixed dunes, within both reserves. Six 20 x 45 cm plots were

set up at Sandscale Haws NNR, on the 16th and 22nd of May 2012, two plots in each of three different ecosystems. Each plot within an ecosystem corresponded to a different bait material burial, *i.e.* either *Hordeum vulgare L*. (barley) straw or *Crataegus monogyna* (hawthorn) wood. Three 20 x 45 cm plots were set up at Ainsdale Sand Dunes NNR, on the 7th of June 2012, for the burial of *Hordeum vulgare* straw, one plot in each of three different ecosystems. One hundred and sixty-two mesh bags were buried in total, 108 mesh bags at Sandscale Haws NNR and 54 mesh bags at Ainsdale Sand Dunes NNR, at depths of 5 and 11 cm below the soil surface evenly spread across the different ecosystems (Table S1).

The bait bags were retrieved 3, 6 or 12 months after burial in the field, to allow for different periods of burial to show any different decomposition patterns and fungal colonization over a year. However, not all bags could be retrieved as some areas where the plots where placed were either flooded or the sand accretion was too high which made it impossible to retrieve all bags. One hundred and forty-two bait bags were collected in total, from the majority of ecosystems and from both depths, but not from all the different sampling times (Table S1). Bags from the same ecosystem, sampling time and the two depths (5 and 11 cm) were essentially treated as replicate bags. For collection, each bait bag was placed separately in a pre-furnaced foil packet, then placed inside a Ziploc bag to prevent the material from drying out and to prevent contamination, stored in a coolbox containing frozen coolpacks in the field, transported to the laboratory and stored in the fridge at -20 ºC in the laboratory until needed for laboratory analyses.

4.2.3 GC-MS with TMAH thermochemolysis

Air-dried initial materials (barley straw and hawthorn wood) and recovered samples were ground with a ceramic mortar and pestle and analyzed in triplicate by GC-MS with TMAH thermochemolysis with the method described by Kabuyah *et al*. (2012). Thus, 7 µl of tetramethylammonium hydroxide (TMAH) and 2 µl of the hydrocarbon 5α-androstane internal standard (100 µl of a 0.256 mg ml^{-1} solution in dichloromethane) were added to 1 mg of sample. 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%

diphenyldimethylpolysiloxane; 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 700 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; Gauthier *et al.*, 2003; Vane *et al.*, 2005; Robertson *et al.*, 2008; Mason *et al.*, 2009; Kabuyah *et al.*, 2012) and naming of the TMH products followed the conventions established previously (Clifford *et al.*, 1995; Hatcher *et al.*, 1995).

The acid /aldehyde [Ac/Al]G parameter is a relative decomposition proxy for the guaiacyl lignin monomer which was measured using the peak areas of 3,4-dimethoxybenzoic acid, methyl ester (G6) and 3,4- dimethoxybenzaldehyde (G4). The acid/aldehyde [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), (Vane *et al.*, 2001; Kabuyah *et al.*, 2012). The syringyl/guaiacyl ratio (S/G) in TMH products from both buried materials describes a relative intensity ratio of TMAH thermochemolysis products.

To assess the relative contribution of cellulose present in bait materials, six common cellulose markers (guaiacol, 1,4-dimethoxybenzene, 2,6-dimethoxytoluene, 4-methoxybenzaldehyde, 3,5-dimethoxyphenol, 1,2,3-trimethoxy-5-methylbenzene) were identified and the relative peak areas were determined. The [cellulose:cellulose+lignin] ratios were determined using the sum of the six selected cellulose markers and six lignin markers (G4, G5, G6, S4, S5 and S6), using the method described by Kabuyah (2012).

4.2.4 Percentage of mass remaining after one year burial in the field

The contents of two bait bags per ecosystem were air-dried overnight and weighed on an analytical balance to determine the percentage of mass remaining after twelve months of burial in the field. Seven duplicates were analyzed, fourteen bait bags in total. An analysis of variance (two-way ANOVA) was performed in R v.3.0.3 (http://www.R.project.org/) using the *aov* function to assess the potential effects of material and ecosystem on the percentage of mass remaining.

4.3 Results

TMH products released in the initial bait materials during TMAH thermochemolysis

To determine the relative lignin and cellulose content of the *H. vulgare* straw and *C. monogyna*, the original samples were analysed by gas chromatography-mass spectrometry (GC-MS) with TMAH thermochemolysis. A suite of THM products was identified (Fig. 4.3,Table 4.1), similar to previous studies (Vane *et al*., 2001; 2005; Gauthier *et al*., 2003; Mason *et al*., 2009; Robertson *et al*., 2008; Kabuyah *et al*., 2012; Kabuyah, 2012). The partial chromatograms of the total ion current (TIC) for the TMAH thermochemolysis products showed differences between both types of plant materials. The lignin distribution in the pyrogram of barley straw was dominated by trans-3-(4-methoxyphenyl)-3-propenoate (P18), trans-3-(3,4-dimethoxyphenyl)-3-propenoate (G18), 3',4",5'-trimethoxyacetophenone (S5), the syringyl isomer unit cis-2-(3,4,5-trimethoxyphenyl)- 1-methoxyethylene (S7) and 4-vinylanisole moieties (P3; Fig. 4.3A, Table 4.1). By contrast, the lignin distribution in the pyrogram of hawthorn was dominated by the syringyl isomer units cis-2-(3,4,5-trimethoxyphenyl)- 1-methoxyethylene (S7) and (S8) trans- l-methoxy-2-(3,4,5-trimethoxyphenyl)ethylene and 3,4,5-trimethoxybenzaldehyde (S4) moieties, P3 and P18 could not be observed (Fig. 4.3A, Table 4.1). This difference in the lignin composition is clearly reflected in the [Ac/Al]S, [Ac/Al]G and S/G ratios which yield mean ($\underline{+}$ standard error) 0.9 ± 0.1, 0.2 ± 0.01, 2.3 ± 0.05 for barley straw, and 0.5 ± 0.01, 0.3± 0.01 and 2.1 ± 0.1 for hawthorn wood (Table S2).

The cellulose distribution in the pyrogram in barley straw was dominated by 3,5-dimethoxyphenol and 1,4-dimethoxybenzene. By contrast, the cellulose distribution in

the pyrogram of hawthorn was dominated by 1,2,3-trimethoxy-5-methylbenzene and 3,5-dimethoxyphenol (Fig. 4.3A, Table 4.1). The cellulose to lignin ratio in both original materials was comparable with ratios of 0.28 ± 0.02 (barley) and 0.27 ± 0.02 (hawthorn), respectively (Table S2).

Also, three trimethoxybenzene isomers (TMB1, TMB2 and TMB3) were observed in both pyrograms which are products that derived from A and B rings of condensed tannins (Nierop *et al*., 2005). However, TMB1 and TMB2 in the barley straw had a higher relative intensity if compared with TMB1 and TMB2 in the hawthorn wood, and one TMB3 was only detected in the barley straw (Fig. 4.3A; Table 4.1).

Lignin and cellulose ratios in the initial materials were calculated to be able to characterise the composition of the different bait materials and to determine any modification after burial in the field, as in previous studies (Kabuyah *et al*, 2012, Kabuyah, 2012). Initial samples of barley straw produced mean (± standard error) ratios of [Ac/Al] S, [Ac/Al] G, S/G and [cellulose:cellulose+lignin] of 0.9 ± 0.1, 0.23 ± 0.01, 2.34 ± 0.05 and 0.29 ± 0.02 respectively (Table S2) whereas initial samples of hawthorn wood had ranges of values for the same ratios of 0.49 ± 0.01, 0.27 ± 0.01, 2.14 ± 0.1 and 0.28 ± 0.02 respectively (Table S2).

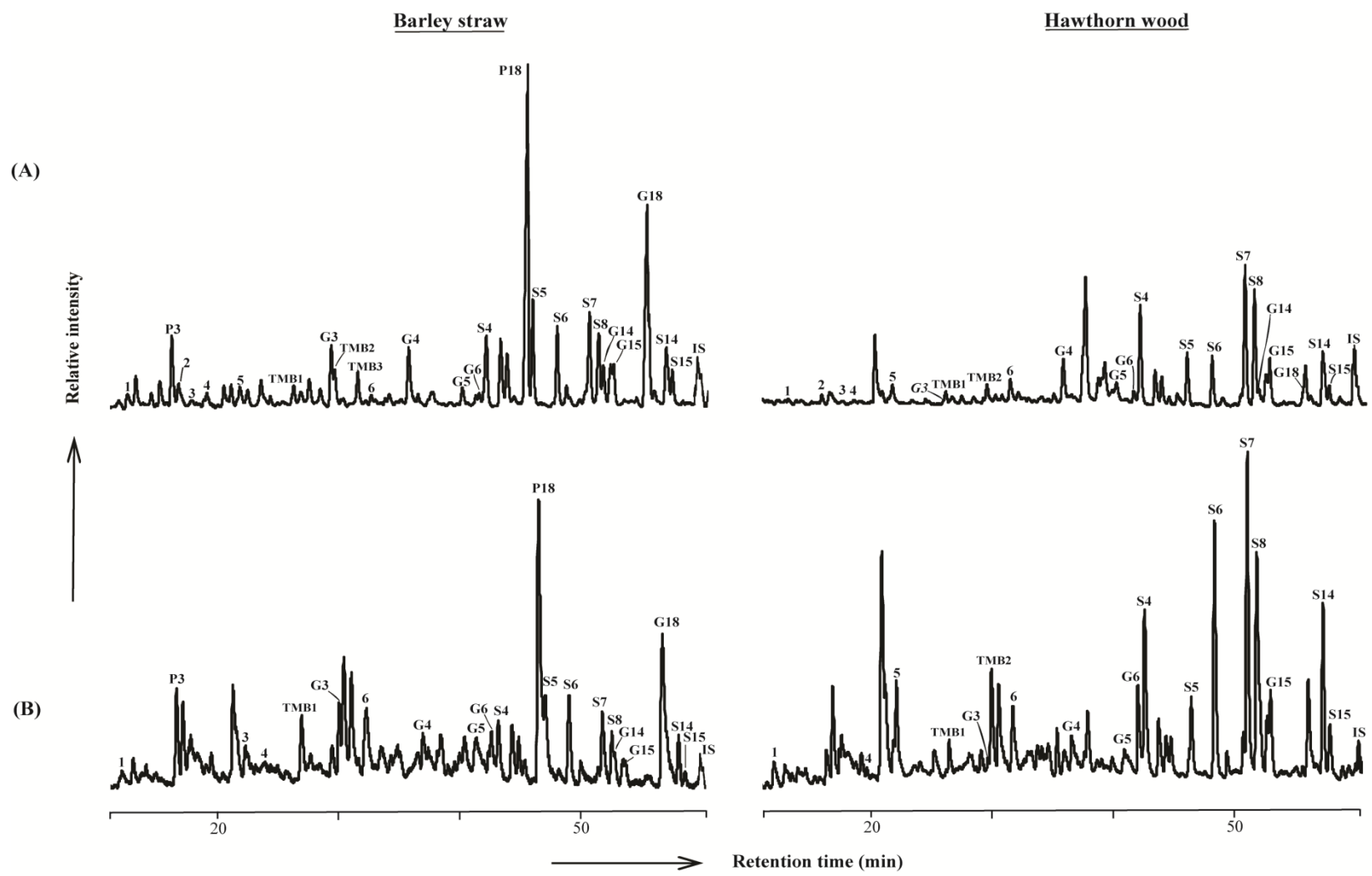
Barley straw
Hawthorn wood
(A)
(B)
Relative intensity
Retention time (min)
20
50
P18
G18
S7
S8
S6
S5
S4
S14
G14
G15
S15
IS
G6
G5
G4
G3
TMB1
TMB2
TMB3
P3
1
2
3
4
5
6

Figure 4.3. Partial chromatograms of the total ion current (TIC) for the TMAH thermochemolysis products from (A) original and (B) degraded materials, *Hordeum vulgare* L. (barley) straw and *Crataegus monogyna* (hawthorn) wood. Peak assignments of characteristic TMAH thermochemolysis products as in Clifford *et al*. (1995), Gauthier *et al*. (2003); Vane *et al*. (2001), del Río *et al*. (2007) and Kabuyah *et al*. (2012) and are listed in Table 4.1. IS denotes the internal standard 5α-androstane.

Table 4.1. List of TMAH thermochemolysis products (Clifford et al., 1995; Vane et al., 2001 & 2005; Gauthier et al. 2003; del Río et al., 1998 & 2007; Kabuyah et al., 2012).

Peak label	Compound	Source[a]	Characteristic ions
1	guaiacol	C	109,124,81
P3	4-vinylanisole	H	134,91,119
2	1,4-Dimethoxybenzene	C	123,138,95
3	2,6-Dimethoxytoluene	C	152,121,77
4	4-Methoxybenzaldehyde	C	135,136,77
5	3,5-Dimethoxyphenol	C	154,125,94
TMB1	1,2,3-Trimethoxybenzene	T	168,153,110
G3	3,4-dimethoxystyrene	G	164,149,91
TMB2	1,2,4-Trimethoxybenzene	T	153,168,125
TMB3	1,3,5-Trimethoxybenzene	T	168,139,125
6	1,2,3-Trimethoxy-5-methylbenzene	C	182,167,139
G4	3,4-dimethoxybenzaldehyde	G	151,165,166
G5	3,4-Dimethoxyacetophenone	G	137, 165, 180
G6	3,4-dimethoxybenzoic acid, methyl ester	G	165,181,196
S4	3,4,5-trimethoxybenzaldehyde	S	125,181,196
P18	*trans*-3-(4-Methoxyphenyl)-3-propenoate	H	161, 192, 133
S5	3',4'',5'-trimethoxyacetophenone	S	195,210,139
S6	3,4,5-trimethoxybenzoic acid, methyl ester	S	226,211,195
S7	*cis*-1-(3,4,5-Trimethoxyphenyl)- 2-methoxyethylene	S	209,224,181
S8	*trans*- 1-(3,4,5-Trimethoxyphenyl)-2-methoxyethylene	S	209,224,181
G14	erythro-1-(3,4-Demethoxyphenyl)-1,2,3-trimethoxypropane	G	166, 181, 270
G15	threo/erythro-1-(3,4-Demethoxyphenyl)-1,2,3-trimethoxypropane	G	166, 181, 270
G18	*trans*-3-(3,4-Dimethoxyphenyl)-3-propenoate	G	22,207,191
S14	*threo/erythro*-1 -(3,4,5-trimethoxyphenyl)- 1,2,3-trimethoxypropane	S	211,181,300
S15	*threo/erythro*-1 -(3,4,5-trimethoxyphenyl)- 1,2,3-trimethoxypropane	S	211,181,300

[a] C = cellulose, G = guaiacyl, S = syringyl, H = p-hydroxyphenyl and T = tannins.

Physical changes of plant materials upon burial in the field

The retrieved materials had different coloration patterns when compared with the original materials indicating that physical changes occurred over different periods of burial, thus suggesting (geo)chemical changes. The original barley straw had a beige/cream coloration, while barley straw retrieved at 6 months from the mobile dunes presented a distinctive pink-red coloration and black spots, the same bait material retrieved at 12 months from the fixed dunes had a darkened color (Fig. 4.4A). Original hawthorn wood samples also had a beige/cream color, by contrast to the coloration of the retrieved hawthorn from the mobile dunes at six-month sampling which had a brown coloration, and the same material retrieved from the fixed dunes at twelve-month sampling appeared to lose their colour as they were white (Fig. 4.4B).

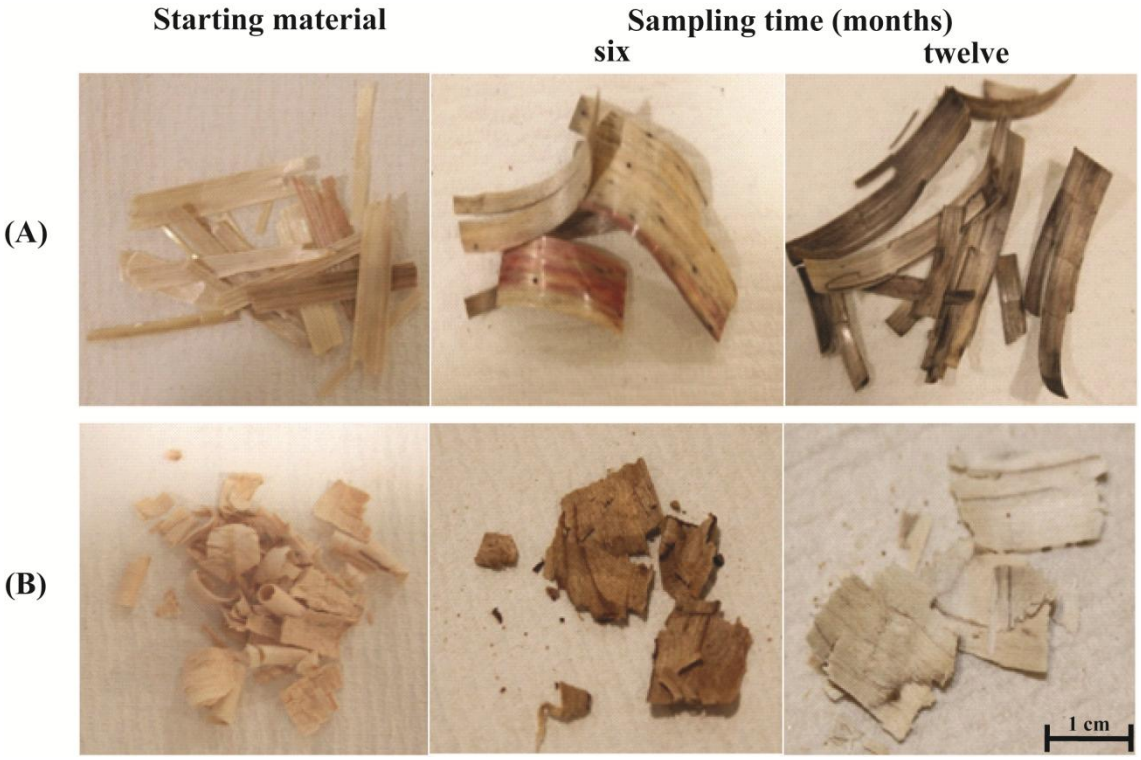

Figure 4.4. (A) *Hordeum vulgare* L (barley) straw and (B) *Crataegus monogyna* (hawthorn) wood starting bait materials (prior to burial) and after different sampling times in the mobile dunes and fixed–dunes at Sandscale Haws and Ainsdale Sand Dunes National Nature Reserves.

The percentage of mass remaining of both bait materials was assessed for those samples that remained buried for twelve months (Table 4.2). Based on the assessment of this remaining mass, between 11 and 51 % of the barley straw buried at Sandscale Haws and between 44 and 50% at Ainsdale Sand Dunes was still present after 12 months burial. In the case of hawthorn wood, between 28 and 47%, was still present after 12 months burial (Table 4.2). However, the two-way ANOVA showed that there were not statistically significant differences in the percentage of mass remaining based on the different type of bait material and/or ecosystem as the *p*-values for the different treatements: ecosystem ($F = 0.81$; $p = 0.056$), material ($F= 0.14$; $p = 0.717$) and ecosystem:material ($F= 0.36$; $p = 0.567$), were not significant.

Table 4.2. Percentage of mass remaining after one year barley straw and hawthorn wood burial in Sandscale Haws and Ainsdale sand dunes. Based on two measurements per ecosystem.

Sample ID[a]	Material	Ecosystem	Percentage of mass remaining
Sandscale Haws National Nature Reserve			
SBM12	Barley straw	Mobile dunes	51 ± 0.03
SBD12	Barley straw	Dune slacks	25 ± 0.01
SBF12	Barley straw	Fixed dunes	11 ± 0.01
SHM12	Hawthorn wood	Mobile dunes	47 ± 0.03
SHF12	Hawthorn wood	Fixed dunes	28 ± 0.01
Ainsdale sand dunes National Nature Reserve			
ABD12	Barley straw	Dune slacks	50 ± 0.01
ABF12	Barley straw	Fixed dunes	44 ± 0.01

[a]ID key: S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the bait material barley straw or hawthorn wood, M/D/F corresponds to the different sand-dune ecosystems where the bait materials were buried mobile dunes or dune slacks or fixed dunes, and 12 is the sampling time in months. For example: SBM12 corresponds to a sample buried in Sandscale Haws (S), the buried bait material was barley straw (B) which was buried in the mobile dunes (M) and retrieved 12 months later.

Lignin and cellulose degradation after burial of samples

To determine lignin and cellulose degradation of buried bait materials, *H. vulgare* (barley) straw and *C. monogyna* (hawthorn) wood, the bait samples collected at different sampling times and from different sand-dunes ecosystems were analysed by TMAH thermochemolysis. Similar to previous studies (Vane *et al.*, 2001; 2005; Gauthier *et al.*, 2003; Mason *et al.*, 2009; Robertson *et al.*, 2008; Kabuyah *et al.*, 2012; Kabuyah, 2012), a suite of THM products was identified (Fig. 4.3, Table 4.1).The pyrograms of the decayed samples showed differences in the relative abundance of products. Generally, characteristic lignin moieties such as 3,4-dimethoxybenzaldehyde (G4) and 3,4,5-trimethoxybenzaldehyde (S4) increased after one year of decay in both plant materials, whereas other lignin moieties decreased such as methyl 3,4-dimethoxybenzoate (G6) and 3,4,5-trimethoxybenzoic acid, methyl ester (S6). Typical cellulose moieties showed modification as some of these moieties were below detection limit which suggests that they were degraded, therefore not possible to quantify, *e.g.* 1,4-dimethoxybenzene in barley straw or 2,6-dimethoxytoluene in hawthorn wood. Other TMH products such as the TMB isomers showed also some modifications: TMB1 had a higher relative abundance in all decayed samples if compared with the initial materials, TMB2 was below detection limit after the decay of barley straw samples, however, the relative intensity of the TMB2 in the decayed hawthorn wood samples was higher if compared with the initial material. TMB3 was also below detection limits in decayed barley straw samples, thus also suggesting that this product underwent degradation after burial of barley straw samples in the field (Fig. 4.3B; Table 4.1).

Crataegus monogyna (hawthorn wood) and *Prunus armeniaca* (apricot wood) belong to the family Rosaceae, this means that both species potentially share some chemical and biological characteristics. Undecayed apricot wood contains tannins as the form of three trimethoxybenzene isomers (TMB1, TMB2 and TMB3) which are products of gallic acid and 2,4,6-trihydroxybenzoic acid (precursors of hydrolysable and condensed tannins; Joll *et al.*, 2003), as it has been shown using pyrolysis GC-MS with TMAH thermochemolysis and solid–state ^{13}C nuclear magnetic resonance (NMR). Although these three TMB isomers could also originate from tannin, lignin and polysaccharides, in apricot wood their origin is most likely tannins (Vane *et al.*, 2005). Original and

degraded hawthorn woods contained two TMB isomers, similar to apricot wood (Fig. 4.3, Table 4.1) which were higher in the degraded hawthorn.

Values of the [Ac/Al]S, [Ac/Al]G, S/G and [cellulose:cellulose+lignin] ratios were analysed in decayed barley straw and hawthorn wood bait samples to estimate any modification from the initial values (Vane *et al*., 2001; 2005; Kabuyah *et al*., 2012; Kabuyah, 2012). [Ac/Al]S ratios of both bait materials were higher than the values of initial materials (Fig. 4.5A, Table S2). In barley straw samples, it increased from 0.9 ± 0.1 to 1.3 ± 0 (samples SBD6 and SBD12) at Sandscale Haws and from 0.9 ± 0.1 to 1.4± 0.2 (sample ABD12**)** at Ainsdale**.** In hawthorn samples, it increased from 0.5 ± 0.01 to 0.9 ± 0.2 (sample SHM12**;** Fig. 4.5A, Table S2). Moreover, some clear patterns were observed in the increase of the [Ac/Al]S values at some ecosystems: in hawthorn wood samples retrieved from the mobile dunes at Sandscale Haws, this [Ac/Al]S ratio was increasing at each sampling time, it increased from 0.7 ± 0.03 at 3 months to 0.8 ± 0.1at six months and 0.9 + 0.2 at twelve months (Fig.4.5A, Table S2), and in the same material but retrieved from the dune slacks the increase was from 0.5 ± 0.01 at three months to 0.6 ± 0.01 at six month (it was not possible to retrieve samples at twelve months). A similar pattern was observed for barley straw samples recovered from the dune slacks and the fixed dunes at Ainsdale: the [Ac/Al]S ratio increased from 1.3 ± 0.3 at 3 months to 1.4 + 0.2 at 12 months in the dune slacks, and from 0.8 ± 0.1 at three months, to 1.1 ± 0.1 at six months and 1.3 ± 0.2 at twelve months in the fixed dunes (Fig. 4.5A, Table S2). The [Ac/Al]S ratio of barley straw recovered from Sandscale Haws did not show any evident pattern as the values increased or decreased regardless the sampling time (Fig.4.5A, Table S2). The [Ac/Al]G values of the decayed materials were also higher than the original ratio. In barley straw samples the [Ac/Al]G value increased from 0.2 ± 0.01 to 0.9 ± 0.2 (sample SBD6**)** at Sandscale and from 0.2 ± 0.01 to 0.7 ± 0.1 (ABF12**)** at Ainsdale. The [Ac/Al]G values of hawthorn samples increased from 0.3± 0.01to 1.3 ± 0.4 (sample SHM12**;** Fig. 4.5B, Table S2). The increase in the [Ac/Al]G values of hawthorn wood and barley straw samples retrieved from Sandscale Haws showed a similar pattern to the [Ac/Al]S ratio. By contrast, at Ainsdale, only the [Ac/Al]G values of barley straw samples retrieved from the fixed dunes showed a pattern related to sampling time: it increased from 0.3 ± 0.03 at three months to 0.5 ± 0.1 at six months and 0.7 ± 0.1 at twelve months (Fig. 4.5; Table S2).

S/G values of retrieved barley straw showed a general decreased: from 2.3 ± 0.05 to 1.7 ± 0.1 (samples SBM3 and SBF3**)** at Sandscale and from 2.3 ± 0.05 to 1.4 ± 0.1 (sample ABD12**)** at Ainsdale (Fig. 4.5C; Table S2). Barley straw samples retrieved from Ainsdale showed a clear pattern in the S/G shifts: the S/G ratios of the samples retrieved from the fixed dunes decreased from 1.8 ± 0.05 at three months, to 1.7 ± 0.2 at six months and 1.6 ± 0.04 at twelve months, while the samples retrieved from the dunes slacks decreased from 1.5 ± 0.1 at three months to 1.4 ± 0.1 at twelve months (Fig. 4.5C; Table S2). In Sandscale Haws, there were not clear patterns in the S/G shifts of barley related to sampling time, for example, the S/G ratio of samples retrieved from the dune slacks was 1.7 ± 0.2 at three months, 2.1 ± 0.2 at six months and 1.9 ± 0.4 at twelve months (Fig. 4.5C, Table S2). By contrast, the S/G ratios of hawthorn wood samples were generally higher than the original material: it increased from 2.1 ± 0.1 to 4.1 ± 0.4 (sample SHF3), however, there was not an evident pattern in the changes of the values of this ratio that was related to the sampling time (Fig. 4.5C). For example, S/G values of hawthorn retrieved from the fixed dunes changed from 4.1 ± 0.4 at three months to 2.6 ± 0.4 at six months and 3.5 ± 0.3 at twelve months (Fig. 4.5C, Table S2).

The [cellulose:cellulose+lignin] ratio for all retrieved bait materials showed a general decrease from the original ratios: barley straw ratio decreased from 0.29 ± 0.02 to 0.16 ± 0.01(sample SBM6**)** at Sandscale, and from 0.29 ± 0.02 to 0.21 ± 0.01 (ABF12**)** at Ainsdale, while in hawthorn this ratio decreased from 0.28 ± 0.02 to 0.15 ± 0.01 (sample SHM3, Fig. 4.5D; Table S2). Even though most of the ratios of the retrieved barley straw samples were in the boundaries of the original ratio, some patterns could be observed. For example, in the fixed dunes at Ainsdale, the ratio values of the retrieved barley decreased at each sampling time from 0.28 ± 0.02 at three months, to 0.24 ± 0.03 at six months and 0.21 ± 0.01at twelve months (Fig. 4.5 D; Table S2). In the case of barley samples retrieved from the dune slacks at Sandscale Haws, the ratio increased from 0.22 ± 0.01 at three months, to 0.23 ± 0.01at six months and 0.26 ± 0.03 at twelve months (Fig. 4.5D, Table S2). The [cellulose:cellulose+lignin] ratio of hawthorn wood did not show a general pattern that could be related to sampling time, for example, the ratio values of hawthorn retrieved from the mobile dunes decreased from the original ratio from 0.28 ± 0.02 to 0.15 ± 0.01 at three months, then it increased to 0.17± 0.01 at six months and to 0.23 ± 0.03 at twelve months. By contrast, the [cellulose:cellulose+lignin] ratio of hawthorn samples retrieved from the fixed dunes

decreased from 0.18 ± 0.01at three months, to 0.16 ± 0.03 at six months and to 0.17 ± 0.02 at twelve months (Fig. 4.5 D; Table S2).

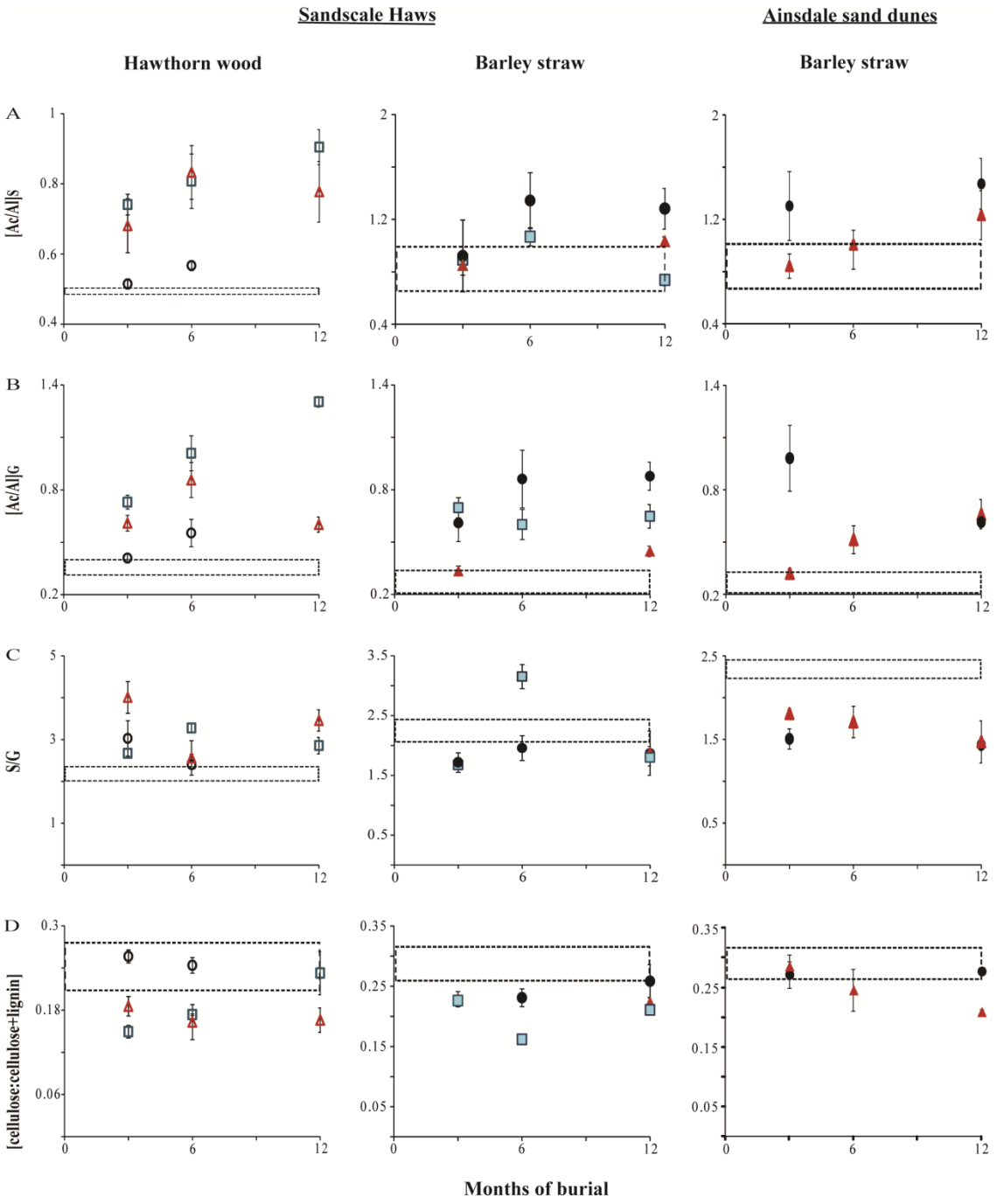

Figure 4.5. (A) [Ac/Al]S), (B) [Ac/Al]G, (C) S/G and (D) [cellulose:cellulose+lignin] ratios in TMH products from barley straw and hawthorn wood buried in Sandscale Haws and Ainsdale sand dunes collected after 3, 6 or 12 months, see methods for an explanation of the determination of all the ratios. The different symbols represent the different ecosystems: mobile dunes (squares), dune slacks (circles) and fixed dunes (triangles). The rectangular dashed boxes indicate the original ratios of the undecayed barley straw and hawthorn wood based on 3 measurements. Some samples were unable to be retrieved (Table S1).

4.4. Discussion

Chemical comparisons of barley straw and hawthorn wood

A suite of TMH products was identified in barley straw and hawthorn wood (Fig. 4.3, Table 4.1), particularly TMH products that are characteristic of cellulose and lignin degradation which have been previously identified in a variety of plant groups such as wheat straw, hemp, flax and apricot wood (Vane *et al*., 2001; 2005; del Río et al., 2007; Kabuyah *et al*., 2012; Kabuyah, 2012; Table 4.1). The bait materials used in this study belong to two different vascular plant groups: barley straw (*H. vulgare* L.) is a grass that belongs to the plant group monocots while hawthorn wood (*C. monogyna*) is a small shrub from the eudicot group (Raven *et al*., 2007). Monocots generally contain all three types of lignin moieties *i.e.* p-hydroxyphenyls, syringyl and guaicyl moieties (Clifford *et al*., 1995) and these three moieties were indeed identified in barley straw (*H. vulgare*; Fig. 4.3, Table 4.1). Moreover, similar to other monocots (*e.g.* juncus, *Juncus effusus* L.; Clifford *et al*., 1995) the most abundant moieties in barley straw were *trans*-3-(4-methoxyphenyl)-3-propenoate (P18) and *trans*-3-(3,4-dimethoxyphenyl)-3-propenoate (G18; Fig. 4.3, Table 4.1). By contrast, in members of the plant group eudicots, the p-hydroxyphenyls are generally not (abundantly) present and the most abundant moieties are the syringyl units (Clifford *et al*., 1995; del Río *et al.* 1998). In line with previous analyses of alder (*Alnus sp*., Clifford *et al*., 1995), birch (*Betula* sp.*;* del Río *et al.* 1998) and apricot wood (*Prunus armeniaca*; Vane *et al*., 2005) the p-hydroxyphenyl moieties could indeed not be detected in the hawthorn wood pyrolysates and the most abundant moieties were the syringyl units (*e.g.* S7, S8 and S4, Fig. 4.3, Table 4.1). The differences in the chemical composition of the bait materials is likely to result in differences in the changes of the syringyl and guaiacyl acid-aldehyde ratios (as could be seen later), and particularly in differences in the shift of the S/G ratio because of the contribution of tannins to the S6 moiety since tannins are abundantly present in hardwood (Vane *et al.,* 2005), such as hawthorn wood, in the form of three trimethoxybenzene (TMB) isomers (Fig. 4.3).

Similar to previous studies (Clifford *et al.*, 1995; Vane *et al.*, 2005) TMB isomers which originate from tannins were found in both bait materials. The presence of these TMB isomers is meaningful to highlight because of their potential contribution to some of the acid/aldehyde ratios that are used to assess lignin degradation such as the

[Ac/Al]$_S$ ratio and S/G because a potential contribution of 3,4,5-trimethoxybenzoic acid from hydrolysable tannins to S6 may be possible (Vane *et al*., 2001; Filley *et al*., 2006). Particularly, in hardwood trees, such as hawthorn wood, the presence of tannins could have affected the S/G ratio observed (Vane *et al*., 2005). In contrast, grasses contain very low amounts of condensed hydrolysable tannins (Harper and Lynch, 1981; Robertson *et al*., 2008) meaning that, in the case of the barley straw, any interference with the lignin ratio by tannins is not expected.

Besides the presence of lignin and tannin moieties in the original materials, six abundant cellulose moieties could also be identified, such as guaiacol, 1,4-dimethoxybenzene, 2,6-dimethoxytoluene, 4-methoxybenzaldehyde, 3,5-dimethoxyphenol and 1,2,3-trimethoxy-5-methylbenzene (Fig. 4.3, Table 4.1), which was in line with previous studies that identified the same moieties as breakdown products of cellulose in wheat straw after GC-MS with TMAH thermochemolysis (Gauthier *et al*., 2003; Kabuyah, 2012). Plant materials are composed of both lignin and cellulose (Raven *et al*., 2007),cellulose being found in greater amounts than lignin, although the cellulose to lignin proportion is distinct depending on the type of plant (Harper and Lynch, 1981; Raven *et al*., 2007; Adapa *et al*., 2009) which could be evaluated using the [cellulose:cellulose+lignin] ratio. This ratio was slightly different in the original materials: 0.29 $\pm$ 0.02 in barley straw and 0.28 $\pm$ 0.02 in hawthorn wood (Fig. 4.5, Table S2).

Physical changes of plant materials upon burial in sand-dune soils

The changes in the original colour of materials (Fig. 4.4) suggest that they are colonised by microbial communities because the mesh size of the bags that contained the bait materials was small enough to avoid colonisation by small animals and large enough to allow mycelia and bacterial colonisation. Some of the retrieved hawthorn wood bait materials that remained buried in the field for one year showed a bleached appearance, which suggests the presence of white-rot fungi, since white-rot fungi cause a white or bleached appearance on the materials they are attacking (Finley, 1998). Besides a discoloration, a significant drop in the mass remaining for both materials up to 90% of the original material present (Table 4.2), indicated that the OM present was indeed degrading (Table 4.2), although the drop in the mass remaining could not be related

either to the type of bait material or the ecosystem were the bait was buried (ANOVA p-values were not statistically significant). This is in line with previous studies which showed that mass changes were a result of microbial degradation (Kabuyah, 2012; Tascioglu *et al.*, 2013). Tascioglu *et al.* (2013) for instance, measured the mass of wood specimens treated with various extracts, their results showed mass losses of wood because of 16-week exposure to either the white-rot fungus *Trametes versicolor* or the brown-rot fungus *Fomitopsis palustris.* Additionally in our study, and in line with Tascioglu *et al*. (2013), DNA-based analysis for characterizing the fungal mycelia colosing the bait materials showed that fungal mycelia were indeed present on the retrieved materials and it was possible to characterise saprotrophs (fungal degraders) among which were wood-rotting fungi (*i.e. Pilidium concavum* and *Phanerochaete sp*., González González *et al.,* in preparation).

Comparison between the degradation of barley straw and hawthorn wood

To determine if the loss of OM is caused by the degradation of lignin and/or cellulose, the bait materials retrieved after three, six and months were analysed using THM with TMAH and compared to the original compositions. Similar to other studies using THM with TMAH to assess lignin and cellulose degradation by fungi (Vane *et al*., 2001; 2005; del Río et al., 1998; 2007; Kabuyah *et al*., 2012; Kabuyah, 2012), the distribution and relative intensity of the different moieties of barley straw and hawthorn wood changed upon the degradation of the bait materials buried in the soil and all ratios that are used to indicate lignin side chain oxidation such as the [Ac/Al]$_S$, Ac/Al]$_G$ and S/G ratios and to monitor cellulose degradation showed modifications.
The analyses showed both similarities and differences in the degradation pattern of the different types of bait materials buried across the same ecosystems at the same reserve (Sandscale Haws). The similarities were related to the relative increase and decrease of some moieties: in both types of baits there was a relative increase in the relative intensity of the S6 and G6 moieties and a relative decrease of G4 and cellulose moieties upon degradation (Fig. 4.3B). Particularly, most of the cellulose products were below detection limit in the degraded materials (*e.g.* 2,6-dimethoxytoluene) suggesting that cellulose decayed faster than lignin moieties in both types of baits. This is supported by shifts in the [cellulose:cellulose+lignin] ratios which showed general decreases for both materials if compared with the original ratios (Fig.4.5D, Table S2). This is in line with previous studies which showed that cellulose is decaying faster than lignin (Berg *et al*.,

1982; Kabuyah, 2012): after a year of Scots pine needle litter decomposing in a boreal Scots pine forest, Berg *et al*. (1982) for instance, showed that only 20% of cellulose remained in pine needle litter whereas above 40% of lignin remained in the same plant litter. Lignin removal or modification during litter degradation provides access to other carbohydrates, especially to cellulose and hemicelluloses (Steffen *et al*., 2007), indicating that it is likely that once lignin was modified/ or slightly removed from the bait materials the microbial communities had full access to use less complex polymers such as cellulose. Recently, we showed that putative fungal cellulose modifiers were identified colonizing both bait materials (Chapter 3) including the yeast *Cryptococcus* that might utilise hydrated cellulose (Harvey, 1967). Therefore, from these results it seems likely that cellulose was a primary organic matter source for the fungal communities colonising both types of bait materials.

Besides the shift in the relative abundance of lignin and cellulose moieties, there was also a general increase in both [Ac/Al]S and [Ac/Al]G ratios of the decayed barley straw and hawthorn wood if compared with the ratios of the original materials (Fig.4.5A&B, Table S2). Comparable with previous analyses in laboratory-based (Kirk and Farrell, 1987; Vane *et al.*, 2001; Robertson *et al.*, 2008) and field experiments (Kabuyah *et al.*, 2012; Kabuyah, 2012), the general increase in guaiacyl and syringyl acid-to-aldehyde ratios indicates that the fungi present induced the oxidative cleavage of the Cα-Cβ bond at the Cα position to yield aldehydes which were then oxidized to carboxylic acid in a manner similar to the attack by white-rot fungi (Kirk and Farrell, 1987; Vane *et al.*, 2001; 2003; Kabuyah *et al*., 2012). Although the G6 moiety may be generated as a TMAH reaction product from native lignin, the increase in [Ac/Al]G values, of both materials, upon decay were most likely to have resulted from formation of aromatic aldehydes from alcohol groups by oxidative cleavage at the Cα bonds and subsequent oxidation of the aldehyde acid (Vane *et al.*, 2003).
It is important to highlight that the increase in the [Ac/Al]G ratio was two times larger than the increase of the [Ac/Al]S for all the decaying materials (Table S2), this result was similar to Vane *et al.* (2001) who also noticed a larger increase in the value of [Ac/Al]G ratio if compared with the [Ac/Al]S ratio of decayed wheat straw. A probable explanation for our results is that syringyl-lignin is located in the secondary cell wall of plants which make it more easily degraded than guaiacyl-lignin that is located in the middle lamella (Sarkanen and Ludwig, 1971; Fergus and Goring, 1979; Syafii and

Yoshimoto, 1991). Moreover, guaiacyl units are more resistant to cleavage because they have a free C5 position in the aromatic ring available for very strong carbon-carbon bonds which makes them more resistant to depolymerisation than syringyl units which are less condensed and have a lower redox potential if compared with the guaiacyl units (Kirk and Farrell, 1987; Vane *et al*., 2001; del Río *et al.*, 2007; Lourenço *et al.*, 2012).

Although in both bait materials the same general trends were observed, the analyses also indicated differences, particularly in the way in which the shifts in the [Ac/Al]S and Ac/Al]G ratios occurred: the [Ac/Al]S ratio of the degraded hawthorn showed clear patterns in the way it changed over the burial time. For most of the retrieved samples, there was a general increase in the [Ac/Al]S ratios at each sampling time, for example, the ratio increased from 0.5 ± 0.01 of the original material to 0.7 $\pm$ 0.03 at three months, 0.8 $\pm$ 0.1 at six months and 0.9 $\pm$ 0.2 at twelve months in the mobile dunes (Fig. 4.5). By contrast, the [Ac/Al]S ratio of most of the retrieved barley straw samples remained comparable to the ratio of the original material (about 0.9 ± 0.1). This suggests that type of material attracted different fungal communities which were able to degrade the bait materials differently. For example, only white-rot fungi colonised hawthorn wood samples (Chapter 3).

Interestingly, the shift of the S/G ratio of both bait materials was completely different. The S/G ratio describes the ratio of methylated syringyl derivatives to guaiacyl derivatives and is generally used to indicate lignin breakdown in plants (Vane *et al.*, 2001). Barley samples showed a general decrease in the S/G ratio if compared with the ratio for original barley straw (Fig. 4.5C, Table S2). This is probably caused by a preferential decay of syringyl units upon fungal decay in agreement with previous studies (Vane *et al.*, 2001, 2003, 2005 & 2006; Steffen *et al.*, 2007; Kabuyah *et al.*, 2012). In contrast, the S/G ratios of decomposed hawthorn wood generally increased over the same period (Fig. 4.5C, Table S2) which could potentially be due to a tannin contribution to S6 moiety (Mason *et al*., 2009). Mason *et al.* (2009) examined how depth-related profiles of the lignin and tannin-derived phenols changed when moorland was afforested. They also measured an increase in the S/G ratio which was related to a decrease in guaiacyl and increasing in syringyl contributions, furthermore, they pointed out that the S6 moiety could have a contribution of gallic acid (3,4,5-trihydroxybenzooic acid). Gallic acid is an important substructure of hydrolysable and

condensed tannins, which was broken down with increasing soil depth and that could yield S6 upon thermochemolysis (Hernes *et al*., 2001). Filley *et al.* (2006) advised that caution needs to be taken when acid/aldehyde ratios are used to infer fungal alterations of lignin because of the potential contribution of 3,4,5-trimethoxybenzoic acid from hydrolysable tannins to S6. Barley straw does contain tannins in low amounts which do not interfere with the [Ac/Al]S ratio (Brandon *et al.*, 1982). Hawthorn wood, however, may contain a great proportion of both hydrolysable and condensed tannins, as discussed earlier, meaning that S6 might have a contribution of tannins or other degraded phenolics. Thus the S6 moiety could have indeed a contribution of tannins which produced an increase of S/G ratio of decaying hawthorn wood.

To summarise, the comparison of the degradation of two types of bait plant materials showed some similarities and clear differences in the shifts of the ratios that are used to monitor lignin and cellulose degradation. Particularly the differences could be attributed to the type of bait because they belong to different plant groups meaning that their chemical composition is different, therefore their degradation might not be similar. Moreover, fungi degrade differently the resources because of their ability to produce different set of enzymes to attack different types of materials.

Effects of environmental conditions in the degradation of barley straw

To monitor the potential effect of different environmental conditions on the rates of lignin and cellulose degradation, barley straw was buried at different reserves, across three different ecosystems, and analysed using GC-MS with TMAH thermochemolysis, similar to previous studies (Vane *et al*., 2001; 2005; del Río et al., 1998; 2007; Kabuyah *et al*., 2012; Kabuyah, 2012). Similar to what was explained earlier for barley straw buried at Sandscale, in the case of barley buried at the different reserves/ecosystems the [Ac/Al]S and Ac/Al]G ratios were generally higher and the S/G ratios and [cellulose:cellulose+lignin] ratio were lower if compared with the ratios of the original material (Fig. 4.5).

However, some patterns were observed that could potentially be related to the ecosystem type where barley was buried: all the ratios of the samples buried in the fixed dunes (at both reserves) and all the ratios of barley straw buried in the dune slacks at Ainsdale appeared to change linearly over the 12-month burial time, for example, the

[Ac/Al]S ratio of barley buried in the dune slacks at Ainsdale changed from an initial value of 0.9 ± 0.1 to 1.3 ± 0.3 at three months and 1.4± 0.2 at twelve months (Fig. 4.5; Table S2). By contrast, all the ratios of barley buried in the mobile dunes and dune slacks at Sandscale fluctuated, for example, the [Ac/Al]S of barley buried in the dune slacks at Sandscale changed from 0.9 ± 0.3 at three months, 1.4 ± 0.2 at six months and 1.3 ± 0.2 at twelve months (Fig. 4.5, S2). Moreover, the highest changes of the ratios that could be observed were in the [Ac/Al]S and [Ac/Al]G ratios of those barley samples retrieved from the dunes slacks: for the [Ac/Al]S ratio around 1.4± 0.2 at both reserves, and for the [Ac/Al]G ratio between 0.9 ± 0.2 at Sandscale and 1.1 ± 0.1 at Ainsdale (Fig. 4.5, S2).

The dune slacks site at Ainsdale in our study was a similar site to the grassland site of a previous study (Kabuyah et al., 2012) where it took 46 months to see similar rates of wheat straw degradation if compared to the degradation of wheat straw in a tropical grassland where it took only 12 months to see the same rates of degradation. In line with this previous study, the soils where barley straw and hawthorn wood were buried had different geochemical characteristics such as different ion concentrations (*e.g.* the "richest" ecosystem regarding ion concentration was the dune slacks in Sandscale Haws and the fixed dunes in Ainsdale), similar pH and relatively different loss-on-ignition values (Chapter 2). Moreover, it was observed that barley samples were colonised by different fungal communities depending on the ecosystem type (at both reserves) and sampling time (at Aisndale; Chapter 3).

Therefore, the differences observed in the degradation of barley buried at different ecosystems/reserves could potentially indicate that different environmental conditions and differences in the fungal communities colonising the bait materials (in this case barley straw) affected the degradation process of macromolecular materials such as lignin and cellulose.

4.5 Conclusions

Hordeum vulgare L. (barley) straw and *Crataegus monogyna* (hawthorn wood) curls were used as "model" resources for assessing the fungal degradation of lignin and cellulose under field conditions. Our results showed shifts in all the relative state proxies that are used to monitor lignin degradation, [Ac/Al]S, [Ac/Al]G and S/G, after

different burial times over a 12-month burial period, and cleavage of Cα-Cβ bonds which is an indication of 'white-rot' fungal attack. Cellulose was also degraded and its degradation was faster than that of lignin as shown by the absence of some cellulose moieties in the degraded samples.

The plant materials were degraded differently, the main difference was the shifts in the S/G ratio that was lower than the original material for barley straw, by contrast the S/G ratio of the degraded hawthorn samples were higher if compared with the ratio of the original material.

Despite the fact that *C.monogyna* wood curls are indeed degrading and being colonised by fungal communities, it is challenging to attribute the degradation pattern to a particular group of fungi using the acid/aldehyde ratios because of the potential contribution of 3,4,5-trimethoxybenzoic acid from hydrolysable tannins, or other degraded phenolics, to the S6 moiety. This highlights the need for the use of an alternative technique that allows distinguishing among degraded lignin, tannins and other phenolics, *e.g.* with the use of ^{13}C-labelled TMAH thermochemolysis.

Environmental conditions were shown to affect the rates of lignin and cellulose degradation of barley straw buried at different ecosystems/reserves as the samples buried in the dune slacks (Ainsdale) and in fixed dunes (Ainsdale and Sandscale) changed linearly over the burial time, in the other ecosystems the ratios fluctuated without any evident pattern.

The degradation of macromolecular structures such as lignin and cellulose is of particular importance in the global carbon cycle. Therefore, the study of this process in carbon-poor soils could help in our understanding of the carbon input from these types of soils. Decomposer (saprotrophic) fungi are key players in carbon and nutrient cycling in the ecosystems, although, lignin and cellulose degradation *in situ* should be a cooperative process among different microorganisms: for example, ascomycetes, bacteria and 'white-rot' fungi which could have different roles (Šnajdr, 2010).

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Supporting information

Table S 1. Number of bags containing two different types of bait materials (barley straw and hawthorn wood) that were buried in two National Nature Reserves, across different sand-dune ecosystems at different depth (5 or 11 cm) and retrieved three, six or twelve months later.

Plot ID[a]	Number of buried bags[b]	Number of bags per sampling time (months)[b]: Three	Six	Twelve	Total number of bags retrieved[b]
Sandscale Haws National Nature Reserve					
SBM	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SBD	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SBF	18 (9/9)	6 (3/3)	NR	12 (6/6)	18 (9/9)
SHM	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
SHD	18 (9/9)	6 (3/3)	6 (3/3)	NR	12 (6/6)
SHF	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)
Ainsdale sand dunes National Nature Reserve					
ABM	18 (9/9)	4 (2/2)	NR	NR	4 (2/2)
ABD	18 (9/9)	6 (3/3)	NR	12 (6/6)	18 (9/9)
ABF	18 (9/9)	6 (3/3)	6 (3/3)	6 (3/3)	18 (9/9)

a) ID key: S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the buried bait material barley straw or hawthorn wood, M/D/F corresponds to the different sand-dune ecosystems where the bait materials were buried mobile dunes or dune slacks or fixed dunes. For example: SBM corresponds to a plot in Sandscale Haws (S), the buried bait material was barley straw (B) which was buried in the mobile dunes (M).

b) The numbers between brackets (a/b) correspond to number of bags buried or retrieved at/from 5 or 11 cm depth below the soil surface (5cm / 11 cm). NR: No bags retrieved.

Table S 2. Mean ratios (n=18) of *Hordeum vulgare* L. (barley) straw and *Crataegus monogyna* (hawthorn) wood samples buried in Sandscale Haws and Ainsdale sand dunes NNR. Starting material ratios were based on three measurements.

Sample ID[a]	Location	Burial time (months)	[Ac/Al]S[b]	[Ac/Al]G[b]	S/G[b]	[Cellulose:cellulose+lignin][b]
Sandscale Haws National Nature Reserve						
Barley straw: initial values			*0.9 ± 0.1*	*0.2 ± 0.01*	*2.3 ± 0.05*	*0.29 ± 0.02*
SBM3	Mobile dunes	3	0.9 ± 0.03	0.7 ± 0.1	1.7± 0.1	0.26 + 0.01
SBM6	Mobile dunes	6	1.1 ± 0.1	0.6 ± 0.1	3.2± 0.2	0.16 ± 0.01
SBM12	Mobile dunes	12	0.7 ± 0.05	0.7 ± 0.1	1.8 ± 0.1	0.21 ± 0.01
SBD3	Dune slack	3	0.9 ± 0.3	0.6 ± 0.1	1.7 ± 0.2	0.22 ± 0.01
SBD6	Dune slack	6	1.4 ± 0.2	0.9 ± 0.2	2.1 ± 0.2	0.23 ± 0.01
SBD12	Dune slack	12	1.3 ± 0.2	0.1± 0.1	1.9 ± 0.4	0.26 ± 0.03
SBF3	Fixed dunes	3	0.9 ± 0.1	0.3 ± 0.03	1.7 ± 0.1	0.23 ± 0.01
SBF12	Fixed dunes	12	1.1 ± 0.03	0.5 ± 0.03	1.9 ± 0.1	0.22 ± 0.01
Hawthorn wood: initial values			*0.5 ± 0.01*	*0.3± 0.01*	*2.1 ± 0.1*	*0.28 ± 0.02*
SHM3	Mobile dunes	3	0.7 ± 0.03	0.7 ± 0.04	2.7 ± 0.2	0.15 ± 0.01
SHM6	Mobile dunes	6	0.8 ± 0.1	1.1 ± 0.2	3.3 ± 0.1	0.17± 0.01
SHM12	Mobile dunes	12	0.9 ± 0.2	1.3 ± 0.4	2.9 ± 0.2	0.23 ± 0.03
SHD3	Dune slack	3	0.5 ± 0.01	0.4 ± 0.03	3.1 ± 0.4	0.26 ± 0.01
SHD6	Dune slack	6	0.6 ± 0.01	0.6 ± 0.08	2.4 ± 0.3	0.24 ± 0.01
SHF3	Fixed dunes	3	0.7 ± 0.1	0.6 ± 0.04	4.1 ± 0.4	0.18 ± 0.01
SHF6	Fixed dunes	6	0.8 ± 0.1	0.9 ± 0.1	2.6 ± 0.4	0.16 ± 0.03
SHF12	Fixed dunes	12	0.7 ± 0.1	0.6 ± 0.1	3.5 ± 0.3	0.17 ± 0.02

Table S 2. CONTINUED

Sample ID[a]	Location	Burial time (months)	[Ac/Al]S[b]	[Ac/Al]G[b]	S/G[b]	[Cellulose:cellulose+lignin][b]
Ainsdale Sand Dunes National Nature Reserve						
Barley straw: initial values			*0.9 ± 0.1*	*0.2 ± 0.01*	*2.3 ± 0.05*	*0.29 ± 0.02*
ABD3	Dune slack	3	1.3 ± 0.3	1.1 ± 0.2	1.5 ± 0.1	0.27 ± 0.02
ABD12	Dune slack	12	1.4± 0.2	0.6 ± 0.03	1.4 ± 0.1	0.28 ± 0.01
ABF3	Fixed dunes	3	0.8 ± 0.1	0.3 ± 0.03	1.8 ± 0.05	0.28 ± 0.02
ABF6	Fixed dunes	6	1.1 ± 0.1	0.5 ± 0.1	1.7 ± 0.2	0.24 ± 0.03
ABF12	Fixed dunes	12	1.3 ± 0.2	0.7 ± 0.1	1.4 ± 0.04	0.21 ± 0.01

a) ID key: S/A corresponds to the name of the reserve: Sandscale Haws or Ainsdale sand dunes, B/H corresponds to the bait material barley straw or hawthorn wood, M/D/F corresponds to the different sand-dune ecosystems where the bait materials were buried mobile dunes or dune slacks or fixed dunes and 3/6/12 is the sampling time in months. For example: SBM3 corresponds to a sample buried in Sandscale Haws (S), the buried bait material was barley straw (B) which was buried in the mobile dunes (M and was retrieved 3 months later.

b) [Ac/Al]S, [Ac/Al]G, S/G and [Cellulose:cellulose+lignin] ratios as determined in Vane *et al*. (2001), Kabuyah *et al*.(2012) and Kabuyah (2012). See methods section.

Chapter 5

Conclusions and further work

5.1. Conclusions

The main objectives of this work were to: (i) to assess the geochemical characteristics of soils from different sand-dune ecosystems, (ii) characterise the diversity of fungal mycelia colonising different types of bait materials buried across different sand-dune soils and collected at different periods of time, (iii) investigate how lignin and cellulose are degraded by these mycelia and compare the rates of lignin and cellulose degradation of the bait materials by reserve, ecosystem and sampling time.

In summary, this work showed that:

- Soils from sand-dune ecosystems within two English reserves (Sandscale and Ainsdale) showed clear differences in their geochemical composition, such as different ion concentration, contrasting pH (alkaline *vs.* more acidic sites) and different loss-on-ignition values.
- It was possible to characterise the fungal mycelia colonising bait materials buried in sand-dune soils (Sandscale and Ainsdale, UK), using 454 pyrosequencing. The fungal communities present on the bait materials were different depending on the type of bait material (at Sandscale), ecosystem/soil characteristics where the material was buried, *e.g. Melanoleuca cinereifolia* was only retrieved from samples buried in the mobile dunes (at Sandscale and Ainsdale) and the time that the material remained buried in the field (at Ainsdale).
- The identified fungi belong to groups with different ecologies such as coprophiles ectomycorrhizas, arbuscular mycorrhizas, plant and animal pathogens, and saprotrophs. Among the saprotrophs, wood-rotting fungi (basidiomycetes) were identified which are difficult to isolate from soils and are considered to be the main agents responsible for lignin modification/degradation.
- Field-based experiments in different sand-dune ecosystems/soils indicated that lignin can be degraded in barley straw and hawthorn wood, most likely by white-rot fungi (earlier supported by the identification of white-rot fungi using

454 pyrosequencing), as shown by the shifts in the [Ac/Al] $_S$, [Ac/Al] $_G$ and [S/G] relative lignin decomposition state proxies as determined by GC-MS with tetramethylammonium hydroxide (TMAH) thermochemolysis. . Cellulose could also be degraded as assessed by the shifts in the [cellulose:cellulose+lignin] ratio. The results confirm that even in relatively low carbon environments such as sand-dune soils, fungi are involved in the degradation of lignin and cellulose over time.

- Lignin and cellulose degraded relatively differently depending on the type of plant material, ecosystem/soil characteristics where the material was buried and fungal communities present on the bait materials, as shown by the differences in the shifts of the ratios that are used to monitor lignin and cellulose degradation (*i.e.* [Ac/Al]$_S$, [Ac/Al]$_G$, [S/G] and [cellulose:cellulose+lignin] ratios).
- Cellulose was degraded faster than lignin based on the comparison of relative amounts of cellulose and lignin moieties in degraded field samples over time.

5.2. Further work

Based on our results, the following suggestions for future research can be made:

For the DNA-based analysis (454 pyrosequencing), to analyse a greater number of samples per ecosystem type and sampling time. .

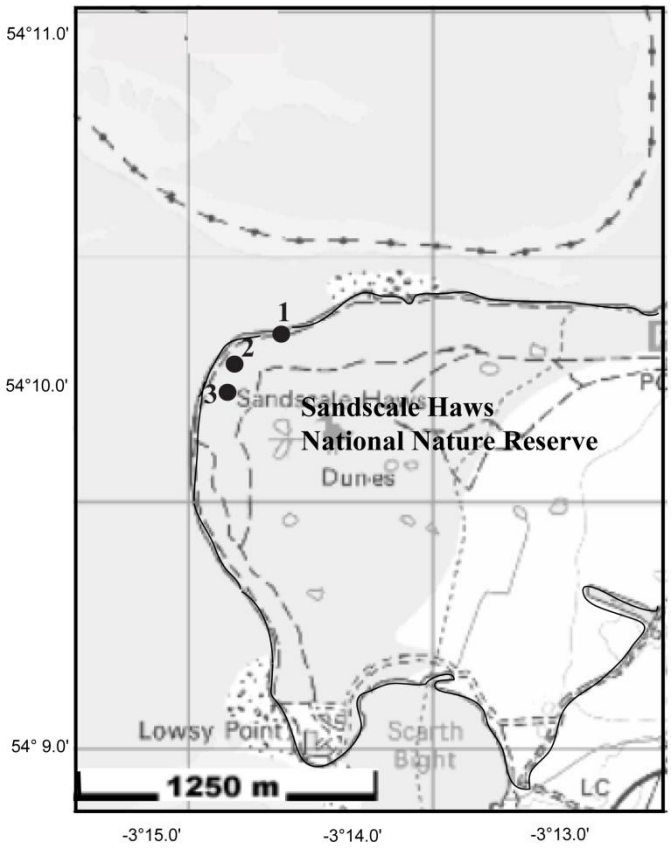
To analyse the complete ITS (ITS1 and ITS2, separately) region to avoid any exclusion in the amplification of fungal groups that could be related to potential bias introduced by the primers that were used.

Setting up the same field experiments for assessing fungal communities present in other sand-dune ecosystems and at other reserves to allow comparisons based on the geographic location of the sites.

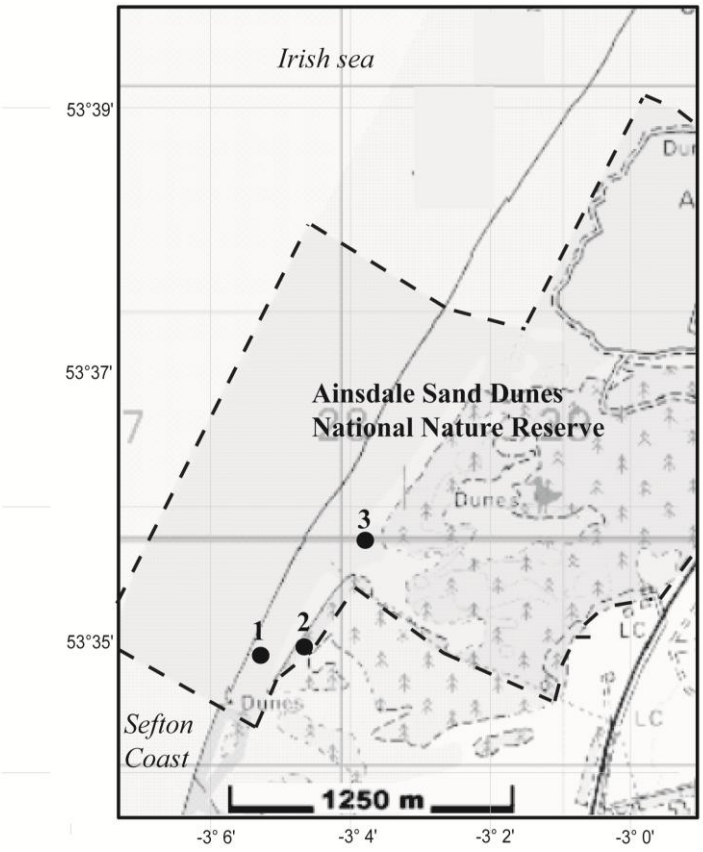
Setting up additional field experiments with the same number of bait bag samples containing the same starting material of barley straw and hawthorn wood, but sampling over a longer period of time (at several intervals over 24 months) which would help to determine further decomposition rates at the different ecosystems and allow us to conduct a clearer comparative study of fungal decomposition.

Sampling for actinomycetes to evaluate their potential involvement in lignin modification/degradation.

Appendix (A): Maps of the study sites pointing out the location of the different ecosystems across the reserves, 1. Mobile dunes, 2. Dune slacks and 3. Fixed dunes, and landscape photos corresponding to each ecosystem.




1. Mobile dunes © C. Robinson
2. Dune slacks © I. González
3. Fixed dunes © C. Robinson


Irish sea
53°39'
53°37'
53°35'
Ainsdale Sand Dunes
National Nature Reserve
Dunes
Dunes
LC
LC
1
2
3
Sefton
Coast
1250 m
-3° 6'
-3° 4'
-3° 2'
-3° 0'

Appendix (B): DNA isolation protocol, DNeasy Plant mini kit, Qiagen.

To grind the content of a mesh bag (bait material) using a sterilised ceramic mortar and pestle. Transfer the ground sample into a 2 ml- Eppendorf tube. Note: Wash the mortar and pestle with ethanol (70%), leave it to evaporate before using it to grind a different sample. All tweezers used for handling the samples have to be sterilised and washed with ethanol (70%), and left to evaporate before using it to handle a different sample.

Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) disrupted material tissue and vortex vigorously. No tissue clumps should be visible. Vortex or pipette further to remove any clumps.
Note: Do not mix Buffer AP1 and RNase A before use.

Incubate the mixture for 10-12 min at 65°C. Mix 2 or 3 times during incubation by inverting tube. This step lyses the cells.

Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 min on ice. This step precipitates detergent, proteins, and polysaccharides.

Centrifuge the lysate for 5 min at 14,000 rpm. Some plant materials can generate very viscous lysates and large amounts of precipitates during this step. After centrifugation, apply supernatant to QIAshredder Mini spin column and continue with step 5.

Pipette the lysate into the QIAshredder Mini spin column placed in a 2 ml collection tube, and centrifuge for 2 min at 14,000 rpm. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 7.

Transfer the flow-through fraction from step 11 into a new 2 ml Eppendorf tube without disturbing the cell-debris pellet.

Add 1.5 volumes of Buffer AP3/E to the cleared lysate, and mix by pipetting. For example, to 450 µl lysate, add 675 µl Buffer AP3/E. Reduce the amount of Buffer AP3/E accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AP3/E, but this will not affect the DNeasy procedure. Note: It is important to pipet Buffer AP3/E directly onto the cleared lysate and to mix immediately.

Pipette 650 µl of the mixture from step 8, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 8000 rpm, and discard the flow-through.* Reuse the collection tube in step 10.

Repeat step 9 with remaining sample. Discard flow-through* and collection tube. Place the DNeasy Mini spin column into a new 2 ml collection tube, add

500 µl Buffer AW, and centrifuge for 1 min at 8000 rpm. Discard the flow-through and reuse the collection tube in step 11.

Add 500 µl Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 min at 14,000 rpm to dry the membrane. It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube. Note: Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flowthrough, as this will result in carryover of ethanol.

Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube and pipet 50 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at 8000 rpm to elute.

Repeat step 12 once.

The microcentrifuge tube is reused for the second elution step to combine the eluates.

Appendix (B): Conference contributions

- **González González, I**., B.E. van Dongen and C.H. Robinson. 2014. Diversity of fungi in sand-dune soils. The Third Metabarcoding Spring School and Workshop. Poster presentation. Tromsø, Norway.

- **González González, I**., B.E. van Dongen and C.H. Robinson. 2014. Fungal degradation of macromolecular materials in sand-dune soils. The Third Metabarcoding Spring School and Workshop. Oral presentation. Tromsø, Norway.

- **González González, I.,** B.E. van Dongen and C.H. Robinson. 2013. Ecological function of fungi in sand-dune soils. Conference organised by the School of Earth, Atmospheric and Environmental Sciences. Oral presentation. Manchester, UK.

- **González González**, I., B.E. van Dongen and C.H. Robinson. 2013. Ecological function of fungi: Degradation of macromolecular materials in sand-dune soils. The 26th International Meeting on Organic Geochemistry (IMOG 2013). Poster presentation. Tenerife, Spain.

- **González González, I.,** B.E. van Dongen, A. Brewsher and C.H. Robinson. 2012. Molecular fungal diversity and its ecological function in sand-dune ecosystems. Conference organised by the School of Earth, Atmospheric and Environmental Sciences. Poster Presentation. Manchester, UK.