Enhancing carbon sequestration in peatlands using exogenous phenolic inhibitors

A thesis submitted to Bangor University by

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in candidature for the degree of Philosophiae Doctor

September 2017

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Abstract

Northern hemisphere peatlands store approximately 455 Pg of organic carbon (C) and are an important component of the global C cycle, currently acting as sinks for atmospheric C, despite peatlands only covering 3% of the planet’s surface. The presence of phenolic compounds in the peatlands allows these stocks to accumulate, by slowing down the process of decomposition to below that of photosynthetic production. Phenolic inhibitors are abundant due to the unique properties in waterlogged peatlands, which have the ability to suppress phenol oxidase activity, which is among the few enzymes capable of degrading phenolic compound inhibitors. This permits accumulation of phenolic compounds, which, in turn, prevents hydrolase enzymes from breaking down organic matter and thus promoting sequestration of C in peatlands. This mechanism has been described as the “enzymic latch” and leads to carbon sequestration in peat. It is noteworthy that human carbon emissions, which exceed 8 Pg per year leading to global warming, can turn these wetlands from carbon sinks into important sources of greenhouse gases, namely carbon dioxide (CO₂) and methane (CH₄). To help prevent further climate change, this study examined whether manipulation of the enzymic latch might reduce the rate of decomposition by increasing the abundance of phenolic inhibitors in peat soil and thus reducing the rate of carbon release into the atmosphere. The study showed that inhibitory phenolic additions may have an effect on carbon sequestration in peatlands in the form of supplementary wood chips, which have proven promote carbon storage in peat matrices. The results also demonstrated the ability of waste materials such as crude oil to suppress CO₂ release that requires further study. Low molecular weight phenolic inhibitors may be more potent than high molecular weight in terms of effect on suppressing enzymic decomposition.
Acknowledgements

First of all, I would like to thank my supervisor Prof Chris Freeman for continued support and valuable advice and supported my work in this way and helped me get results of better quality during my study. I could not have imagined having a better advisor and mentor for my PhD study.

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Chapter 1: Introduction
1.1. Wetlands

About 5 to 8% of the Earth’s terrestrial surface or 7 to 10 million km$^2$ of land is classified as ‘wetland’ (Mitsch, 2011). Globally, wetlands vary in size from many tens or hundreds of km$^2$, such as the Pantanal in South America, to less than a metre, such as the riparian zone of a natural watercourse.

Ecologically, wetlands are among the most productive biological systems on the planet, similar to rain forests and coral reefs in this respect. In recent human and biological history, wetlands have been instrumental in transferring numerous genetic, biological, and chemical materials and providing rich sources and sinks for greenhouse gases (Mitsch, 2011). For example, Braekke (1981) observed that 62% of dissolved materials exported from a Norwegian watershed were chemically changed by passing through wetlands, even though the wetlands occupied just 24% of the catchment.

Often seen as “the kidneys of the landscape”, wetlands fall between two extremes in terms of where they receive their water. Some wetlands receive the majority of their water supply from precipitation. Alternatively, other wetlands may get a large portion of their water from groundwater releases which can also contain waste created by either human activity or natural processes. Wetlands, thus, help in times of either drought or floods and stabilize water supplies. In addition, wetlands are known to filter contaminated water sources, protect shorelines, and enrich underground water reserves (Mitsch, 2011). People on Earth are highly indebted to wetland services such as improved quality of water, flood control, shoreline and wildlife conservation (Mitra et al. 2005; Mitsch and Gosselink, 2007).

Wetlands play a unique and important role in global as well as local biogeochemistry. For instance, many species in peatland systems are significant reservoirs of important gases like CO$_2$, CH$_4$ and N$_2$O (Freeman et al. 1993).

There are many definitions of wetlands, comprehensive definition of wetlands according to the Ramsar Convention that states that “wetlands are areas of marsh fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low
tide does not exceed six metres” (Matthews 1993). Three key features often attached to the definition of wetlands:

1. Water table is either within the root zone or at the ground surface.
2. Wetlands have unique soil properties, with low oxygen content being the critical property.

Mitsch and Gosselink (2000a) describe eight distinct types of wetlands: (1) Coastal – consisting of mangrove, marsh, tidal freshwater, and saltmarsh, and (2) inland – riparian bionetworks, freshwater, freshwater marsh, peatland. (Mitsch and Gosselink, 2000a). Alternatively, the National Wetlands Working Group (1988) offers five broad wetland divisions (shallow water, swamp, marsh, fen, and bog), categorized according to their form. These can be further divided into seventy sub-types depending on surface conditions, vegetation patterns, types of water, and the conditions of mineral soil lying underneath (Table 1.1) (Brinson 1993).

**Table 1.1. Lists of the major terminology used in wetland-related literature.**

<table>
<thead>
<tr>
<th>Wetland</th>
<th>Land having the water table close to or above the surface, or which is saturated for a long period of time. Includes most peatlands.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peatland</td>
<td>Any ecosystem where in excess of 30-40 cm of peat has formed. Includes some wetlands but also organic soils where aquatic processes may not be operating (e.g. drained or afforested peatlands).</td>
</tr>
<tr>
<td>Mire</td>
<td>All ecosystems described as swamp, bog, fen, moor, muskeg or peatland, but often used synonymously with peatlands. Includes all peatlands, but some mires which have mineral substrate.</td>
</tr>
<tr>
<td>Fen</td>
<td>A mire which is influenced by water from outside its own limits, i.e. groundwater and surface flows.</td>
</tr>
<tr>
<td>Bog</td>
<td>A mire which receives water solely from rain and/or snow falling on its surface.</td>
</tr>
<tr>
<td>Marsh</td>
<td>Loose term usually referring to a fen with tall</td>
</tr>
</tbody>
</table>
Table

| Herbaceous vegetation | Loosely defined term with very wide range of usage. Usually referring to a fen and often implying forest cover. |

Adapted from Charman (2000).

1.2. Peatlands

Peatlands are the most extensive wetland type globally. Peatlands are set apart from mineral wetlands by their dominating living plant layer and thick layer of preserved plant detritus accumulated over time. Mineral wetlands, by contrast, do not have any considerable thickness or accumulated organic remains (Charman, 2002).

Northern hemisphere peatlands, historically, have sequestered atmospheric CO$_2$ quicker than it has been emitted by decomposition processes. This has taken place since the end of the last ice age, thus peatlands have accumulated vast stores of carbon (C). At present peatlands are estimated to contain 20 to 30% of world's global soil C stock, equating to more than 60% of the C pool in the atmosphere (Freeman, 2001, Yu, 2012).

A key control of atmospheric CO$_2$ and CH$_4$ concentrations is the exchange of these gases between the atmosphere and the soil. Peatland soils are basically a C sink, since C uptake through photosynthesis commonly surpasses C release as plant waste decomposes (Gorham, 1991). On the other hand, peatlands are usually a source of CH$_4$, due to methogenesis in the anaerobic peat profile by methanogens. Peatlands and other wetlands are a key component of the regulation of atmospheric carbon gases (Moore, 1997).

Anthropogenic alteration of wetlands is likely to be partly responsible for the observed increase in CO$_2$ and CH$_4$ in the atmosphere over the last few decades (Gorham, 1991).

The biogeochemistry of peatlands is unbalanced ecosystems where the production rate of organic matter exceeds biodegradation (Clymo, 1984, Gorham, 1991). This may be due to lack of oxygen, low nutrients, low temperatures and low pH (Gorham, 1991, McLatchey and Reddy, 1998, Yavitt et al., 2005, Laiho, 2006). However, Freeman et al. (2001) showed that the main factor of inhibit the decomposition is likely to be constraints on the activity of phenol
oxidases in peatlands. It is the anaerobic conditions in peat that inhibit the activity of phenol oxidase, thus preventing the breakdown of phenolic compounds. Phenolic compounds have been found to inhibit the activity of enzymes. This mechanism is known as the enzymic latch and has been utilised in peatland-geoengineering strategies designed to enhance C sequestration in peatlands (Freeman et al. 2001). *More details in section 3.*

1.2.1. Classification of peatlands

Since different kinds of biological and physical processes shape peatlands around the world, different types of peatlands exist. The extent of mire hydrologic isolation as a basis for classification is possibly the most commonly employed to this day and refers to the proportion of groundwater inflow the peatland receives in comparison to precipitation (Mitsch and Gosselink, 2000b). The system includes the following three categories of peatland:

- **Minerotrophic Peatlands:** it is swamps which receive water that has already been sifted through rich fens and mineral soils.
- **Mesotrophic Peatlands:** These peatlands fall between the minerotrophic and ombrotrophic extremes.
- **Ombrotrophic Peatlands:** Receive nutrients solely via rainfall.

1.3. Carbon cycling and storage

Fig.1.1. Illustrates the important elements of the C cycle within wetlands where many reactions involving C take place. The major processes are nitrate reduction in the anaerobic zone, sulphate reduction, iron reduction, fermentation, methanogenesis, and respiration in the aerobic zone (Kayranli et al., 2010). Between 45% and 50% of organic matter is C. One of the most important products of wetlands is dissolved organic matter (DOM). Microbial activity results in the mineralization of organic matter into inorganic forms. Carbohydrates turn into CO$_2$ by the process of respiration in plants and microbes. Similarly, carbohydrates are converted to other chemical compounds (e.g. CO$_2$, ethanol, lactic acid) by the process of fermentation. Organic C in a wetland is turned into compounds that include CH$_4$ and CO$_2$. 
There are 5 major C reservoirs found in wetlands. These are: gaseous end products (e.g. CO$_2$ and CH$_4$), microbial biomass C, dissolved organic carbon (DOC), particulate organic carbon, and plant biomass carbon.

Figure 1.1. The main elements of the carbon cycle in a wetland (Kayranli et al. 2010).

C is exported from wetlands via two major routes; as CO$_2$ emerging straight into the atmosphere, and as aquatic DOC draining the ecosystem in rivers and streams.

Wetlands are important sources of CH$_4$. There are three ways noted by Mitch and Gosselink (2000a) by which CH$_4$ loss from wetlands can take place. These are: i) ebullitive flux or diffusion, where bubbles of methane travel to the surface from the low levels of peat; ii) vascular system of plants can also transfer CH$_4$ it into the atmosphere; and iii) by a ‘scavenging’ process in which small methane bubbles in pore water form larger bubbles that are released into the atmosphere by surfacing.
Kayranli et al. (2010) report that approximately, $1.45 \times 10^{11} \text{ kg CH}_4 \text{-C yr}^{-1}$ is emitted to the atmosphere by natural wetlands. This is equal to around 25% of the entire release from natural and anthropogenic sources. Levels of CH$_4$ production are strongly influenced by factors like temperature and vegetation composition.

Gorham (1991) states that compared to the C under the surface, the amount available in live plant biomass is quite low, representing just 1.5% of the entire global peatland C pool.

Peatlands have been long understood as unbalanced ecosystems where the rate of organic material production surpasses its decay (Moore and Bellamy, 1974). This is because decomposition is inhibited and C is therefore able to accumulate. It is also assumed that many peatlands have been amassing soil C ever since the end of the last ice age (Harden et al. 1992).

Freeman et al. (2001) have carried out rigorous research that informs us that constraints on the enzyme phenol oxidase is the key cause of C being locked up in peatlands. This allows phenolic compounds to accumulate. These compounds inhibit the activity of the major biodegrading hydrolase enzymes in a process known as the “enzymic latch” (Freeman et al. 2001).

Molecular oxygen is required by the enzyme, phenol oxidase, to be able to break down phenolic compounds. Thus, the normal anoxic conditions present in many types of wetlands ensure phenol oxidase activity is restricted. However, when aerobic conditions are introduced e.g. as a result of a lowering of the water table, phenol oxidase activity is no longer suppressed, inhibitory phenolic compounds are reduced and decomposition is stimulated. The soil matrix will subsequently release C as DOC and CO$_2$ metabolic end-products (Jones and Mulholland 1998, Gorham, 1991).

1.4. Characteristics of wetland soils

Wetland soils are composed of varying proportions of mineral and organic constituents. Almost all wetland soils contain some organics, however, those soils with less than 20-35% organics are considered to be (on the basis of dry-weight) a mineral soil (Mitsch and Gosselink, 2000b).
Table 1.2. Physiochemical differences between mineral and organic soil taken from Mitsch and Gosselink 2000a and 2000b.

<table>
<thead>
<tr>
<th></th>
<th>Mineral soil</th>
<th>Organic soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic content (percent)</td>
<td>Less than 20 to 35</td>
<td>Greater than 20 to 35</td>
</tr>
<tr>
<td>Organic carbon (percent)</td>
<td>Less than 12 to 20</td>
<td>Greater than 12 to 20</td>
</tr>
<tr>
<td>pH</td>
<td>Usually circumneutral</td>
<td>Acidic</td>
</tr>
<tr>
<td>Bulk density</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Porosity</td>
<td>Low (45-55 %)</td>
<td>High (80 %)</td>
</tr>
<tr>
<td>Hydraulic conductivity</td>
<td>High (except for clays)</td>
<td>Low to high</td>
</tr>
<tr>
<td>Water holding capacity</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Nutrient availability</td>
<td>Generally high</td>
<td>Often low</td>
</tr>
<tr>
<td>Cation exchange capacity</td>
<td>Low, dominated by major cations</td>
<td>High, dominated by hydrogen ions</td>
</tr>
<tr>
<td>Typical wetland</td>
<td>Riparian forest, some marshes</td>
<td>Northern peatland</td>
</tr>
</tbody>
</table>

Due to the presence of water at or below the surface of wetland soils, anaerobic conditions usually persist. When soil pore spaces are filled with water, the rate at which oxygen diffuses through the soil is reduced. Oxygen diffusion has been reported to be 10,000 times slower in an aqueous solution compared to a dry soil (Gambrell and Patrick 1978).

However, an oxygen gradient does exist within wetland soils as the top layer of the soil, which is in contact with the atmosphere, is usually oxidised to a certain extent (Conrad 1996). That oxygen is always completely consumed from wetland soil waters is not necessarily true. Oxidized soils usually form a fine coating that is merely a few millimetres thick on the soil-water interface, at the surface. Ferric, manganous, nitrate, and sulphate ($\text{Fe}^{3+}$, $\text{Mn}^{4+}$, $\text{NO}_3^-$ and $\text{SO}_4^{2-}$), oxidized ions, are present in microlayers. However, reduced types like manganous salts, ferrous, sulphides, and ammonia dominate the lower anaerobic soil (Mitsch and Gosselink, 2000b).

A measure of the electron availability (or stress) in a solution, known as redox potential, or oxidation-reduction potential, is usually employed to quantify the degree of electrochemical reduction in wetland soils. It is not only in the presence of oxygen that oxidation occurs, but
may also happen when hydrogen is absent or more commonly, when an electron is emitted by a chemical. Reduction on the other hand refers to the loss of oxygen, or the gaining of hydrogen.

Since organic matter is one of the most concentrated compounds in wetland soils, its oxidation can happen when just a few terminal electron acceptors are present, including $\text{O}_2$, $\text{NO}_3^-$, $\text{Mn}^{2+}$, $\text{Fe}^{3+}$, or $\text{SO}_4^{2-}$. In the presence of oxygen, the rate of organic decomposition is quickest and slower for electron acceptors like sulphates and nitrates.

**Table 1.3.** Oxidised and reduced forms of elements in wetland soils, and the approximate redox potentials for transformations to, taken from (Mitsch and Gosselink 2000b).

<table>
<thead>
<tr>
<th>Element</th>
<th>Oxidised form</th>
<th>Reduced form</th>
<th>Approximate redox potential for transformation (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>$\text{NO}_3^-$ (nitrate)</td>
<td>$\text{N}_2\text{O}$ (nitrous oxide), $\text{N}_2$ (nitrogen), $\text{NH}_4^+$ (ammonium ion)</td>
<td>250</td>
</tr>
<tr>
<td>Manganese</td>
<td>$\text{Mn}^{2+}$ (manganic)</td>
<td>$\text{Mn}^{2+}$ (manganous)</td>
<td>225</td>
</tr>
<tr>
<td>Iron</td>
<td>$\text{Fe}^{3+}$ (ferric)</td>
<td>$\text{Fe}^{2+}$ (ferrous)</td>
<td>+100 - -100</td>
</tr>
<tr>
<td>Sulphur</td>
<td>$\text{SO}_4^{2-}$ (sulphate)</td>
<td>$\text{S}^-$ (sulfide)</td>
<td>-100 - -200</td>
</tr>
<tr>
<td>Carbon</td>
<td>$\text{CO}_2$ (Carbon dioxide)</td>
<td>$\text{CH}_4$ (methane)</td>
<td>Below -200</td>
</tr>
</tbody>
</table>

### 1.5. Decomposition

As discussed earlier, organic soils such as peatlands are net sinks of $\text{CO}_2$ due to suppressed decomposition of organic matter in an anoxic environment. The slow breakdown of organic material derived from plant senescence has allowed organic carbon to accumulate to the extent that it has exerted a net cooling effect on the planet.
Singh and Gupta (1977) list the major organisms that help decompose litter. These are: lumbricid worms, enchytraeid worms, macroarthropods, microarthropods, nematodes, protozoa, bacteria, actinomycetes, and fungi. As a result of their respiration, these decomposers are responsible for most of the nutrient replenishment in ecosystems and nutrient turn-over. These processes also include organic substances to be fully mineralised into inorganic ions (Killham 2001). Thus, Swift et al. (1979) reports that decomposers play two important roles: to mineralise important elements and to form the soil’s organic matter. Three main factors control litter decomposition. These are litter quality, climate, and the number and composition of the decomposer community (Coûteaux et al. 1995).

Decomposition in aerobic conditions employs biological carbon as its energy source and emits CO$_2$ and H$_2$O as end-products. In the absence of oxygen, decomposition happens through anaerobic channels that cause fermentative metabolism to occur by bacteria. Complex organic molecules, during fermentation, are divided into several low molecular weight organic acids, CO$_2$, and basic alcohols. These then function as substrates for other bacteria in anaerobic regime along with denitrifiers, reducers of SO$_4^{2-}$ and Fe, Mn, and methane-producing bacteria (Richardson and Vepraskas 2001).

Compared to anaerobic decomposition, aerobic decomposition is much more efficient and provides more energy for microorganisms. This allows decomposition of organic matter to occur much faster (Richardson and Vepraskas 2001).

1.6. Enzymes

Soil enzymes are the biological catalysts of organic matter decomposition (Dick and Tabatabai 1993). A soil’s enzyme activity relies not only on the abiotic factor (Skujiņš 1976) for extracellular enzymes, active enzymes within the dead cells, active enzymes and those related to the portions of the dead cell, but also the activity linked in living microbial cells (Ladd and Paul 1973, Nannipieri et al. 1978, 1979, 1983, Sparling et al. 1981a). Extracellular enzymes, or exoenzymes, which are found outside the cell, may be truly free of the cell they were secreted from, or they may remain associated with certain cell membranes, periplasmic spaces, cell walls, sheaths or capsules (Sinasabaugh et al, 1991). These enzymes are hugely important as they are able to cleave macromolecular organic matter into low
molecular weight molecules, allowing the activation of other enzymes – often those within cells (Rogers, 1961; Lock, 1990).

In soil science, there are two important organic polymers that are decomposed by particular soil enzymes suites – lignin and cellulose. Between 30 and 60% of plant material (dry weight) is accounted for by cellulose whose decay carries utmost significance for the global C cycle (Paul and Clark, 1989). Cellulose is a carbohydrate consisting of a chain of linked D-glucose units. To break down the polymer, cellulolytic enzymes are generated by several types of microorganisms (micro-fungi, bacteria, and actinomycetes). According to Chew and Obbard (2001), insoluble cellulose molecules are broken down into simple water-soluble mono- or disaccharide compounds which can then be utilised by microbes.

Lignin, a major constituent of wood, is composed of polymers and aromatic molecules and makes up 60% of peat soils (Paterson and Lundquist, 1985; Clymo, 1983; Kirk and Farrell, 1987).

Ander and Eriksson (1976) report that the most active degraders of lignin are the white-rot fungi that release water and CO₂, although only under aerobic conditions. Lignin decay in soil can be seen in a three-level process. Esterification of the exposed methoxyl groups is level one. The next level is the process of depolymerisation; the final level involves the splitting of the phenolic ring in the post-removal stage (Killham 2001). The resulting phenolic compounds are strong enzyme inhibitors owing to the power resonance which balances the aromatic rings bonds (Harwood and Parales 1996). One of the few enzymes that can decay phenolic materials is the extracellular enzyme phenol oxidase; however, its activity requires bimolecular oxygen (Freeman et al. 2001a, Freeman et al. 2004a).

1.7. Effect of climate change on wetlands

Due to the importance of peatlands as global C sinks, the effect of climate change on the stability of peatlands has received significant attention. All peatlands, often in spite of limited plant productivity, have served as long-term sinks of C for several thousand years (Holden 2005). These sinks store much more C per unit area than any other type of upland ecosystem (Weishampel et al. 2009). The C balance in peatlands involves several processes including autotrophic and heterotrophic respiration, primary productivity and export of the
DOC, that in turn relies on climatic forces. For instance, near the acrotelm (peat surface), losses due to respiration are influenced by temperature and usually rise as soil temperature increases. The catotelm (deeper peats) decompose slowly due to persistent anaerobic conditions. However, in drier and warmer climates, this peat may become more susceptible to carbon loss due to the lowering of the water level.

In general, ecosystems can be conserved through the restoration of peatland, and additionally reduce impact of climate change. In both cases, the water level is a key variable (Gitay et al. 2001). A lower water table and associated anaerobic conditions may lead to an increase in the rate of decomposition. During the summer period, C losses are reported to increase due to the lower water level in peatland environments (Schreader et al. 1998, Alm et al. 1999a, Moore et al. 2002).

On a global scale, the amount of C deposited in soil is inversely related to temperature (Knorr et al. 2005). The most crucial issue in these studies is the relationship between the air and soil temperature and the net primary productivity and decomposition of organic matter in soil. It is generally accepted that net basic generation (providing the input of soil organic C), and the organic matter decomposition of soil (determining soil organic C losses) increase as the temperature increases (Chivers et al., 2009; Updegraff et al., 2001; Kirschbaum 1995; and Shaver et al., 1992).

According to Kirschbaum (1995), a 1°C increase in temperature will cause a 10% loss of organic soil C in the areas of the globe where the current average temperature is 5°C, while the same rise in temperature at a site where the average temperature is 30°C would cause only a 3% loss of soil organic C. An increase in temperature can cause the net primary productivity to increase by stimulating photosynthesis and enhancing C inputs to the soil, whilst decomposition is enhanced through the stimulation of microbe and enzyme activities. In addition, any increase in concentrations of CO₂ emitted from peat soil decomposition could enhance net primary productivity, through increased photosynthesis, leading to increased C sequestration. Kirschbaum (1995) concluded that the increase in CO₂ emissions due to warming effects on decomposition will be greater in the cooler regions of the planet.
According to Freeman et al. (2001b), phenol oxidase activity is more efficient at higher temperatures, though this enzyme is recognized to be greatly limited in soils that are waterlogged. A 36% increase in activity was reported from a 10°C increase in temperature.

CO₂ production in dry soils can be restricted by the lack of water. On the other hand, in saturated soils, there is reduction in oxygen diffusion which can similarly restrict biotic functions (Jones and Mulholland, 1998).

1.8. Carbon sequestration and geo-engineering

Since rising atmospheric CO₂ concentrations are raising global temperatures (MacCracken and Luther 1985), a large number of initiatives that aim to store and capture carbon (sequestration) have been proposed.

Carbon capture and storage (CCS) denotes the gathering of released CO₂ from massive pollution sources (e.g. industrial sites and power stations for electricity generation from fossil fuels, and preventing it from entering the atmosphere). Such prevention is usually carried out by moving the captured greenhouse gas to long term areas like geological formations underground. CCS also includes the capture and storage of CO₂ already in the atmosphere. This is done with the help of geo-engineering methods.

Geoengineering is a term generally associated with large-scale projects designed to manipulate the Earth’s climate in order to counteract the current trend in rising atmospheric CO₂ concentrations and the increase in the Earth’s temperature that this is widely accepted to be causing (Wigley, 2006).

Geoengineering strategies can suitably be classified into 2 groups:

1) Carbon dioxide removal (CDR) strategies to address the basic issue of changing climate by reducing CO₂ emissions to the atmosphere or actively removing it from the atmosphere.

2) Solar Radiation Management (SRM) strategies based on the use of a mirror or screen in space to reflect a small percentage of the sun’s heat and light away from the Earth (Royal Society, 2009; Institute of Mechanical Engineers, 2009).
The strategies and techniques for CDR have been used to manage, preserve, and improve C sinks in terrestrial environments, to employ biomass for C sequestration, to utilize it as an energy source of C, to improve processes of natural weathering to eliminate the CO$_2$ from the atmosphere, and to directly manipulate the CO$_2$ from the air to improve oceanic CO$_2$ uptake. For example, oceanic CO$_2$ uptake can be increased by impregnation of the oceans with nutrients or by improving processes of upwelling of nutrient-rich waters, which will stimulate the growth of algae (Royal Society, 2009; Institute of Mechanical Engineers, 2009). The distinction between industrial carbon management (ICM) and geoengineering is both imprecise and interesting. In drawing the distinction, we may first consider climatic geoengineering as a category of response to the CO$_2$ climate problem. Figure 2 shows a simple schematic of the climate problem for which the response strategies are mitigation, geoengineering, or adaptation. In this scheme, geoengineering is any manipulation of the climate system that alters its response to anthropogenic forcing; the status of ICM is unclear because it resembles both conventional mitigation and geoengineering (Keith, 2000).

**Figure 2.2.** The climatic issue outlined above and its potential solutions (Keith, 2000). Three part schema of the climate problem. The horizontal arrows in the top row show the causal chain in this version of the anthropogenic climate problem. The vertical arrows and the bottom row define the modes of intervention.

Freeman et al. (2012) argued that geoengineering of peatlands offers a possible CDR strategy. It may be possible to engineer peatlands to sequester even more C than they already do by strengthening the natural C sequestering ability of the peat. Potential solutions must be thoroughly researched before being undertaken on a large scale, to
ensure there are no negative feedbacks to the functioning of the peatland and therefore its C balance.

1.9. Application of exogenous phenolic inhibitors

The ubiquity of phenolic compounds is a key feature of organic wetlands, especially peatlands. As strong inhibitors of hydrolytic enzymes and therefore overall soil decomposition (Freeman et al., 2001b), these compounds have received great attention in studies of soil organic matter decomposition.

Additions of exogenous phenolic inhibitors such as woody plant material that has a high content of phenolic concentration suggests that phenol oxidases can play an important role in the microbial conditioning and suppress enzymic decomposition by strengthen the enzymic latch (Freeman et al. 2012). The authors also offer insights into inhibition of hydrolase activity by material that is phenolic/humic; it suggests that there is some extent of inhibition and complexation (Freeman et al, 2001).

By increasing the total amount of phenolic inhibitors available C storage may be increased, which would result in increased efficiency of production of phenolic matter by the genetic modification of Sphagnum plants that assist phenolic synthesis to occur by increasing the expression of the initial enzyme (phenylalanine ammonia lyase, PAL). Accordingly, geoengineering can be of a variety of benefits to the global environment (Freeman et al. 2012).

Crude oil is another potential exogenous phenolic inhibitor which may supress decomposition and enhance carbon sequestration in peatlands. Crude oil contains a high concentration of phenolic compounds which, as outlined above, play an important role in regulating decomposition in wetlands. The overall microbial population diversity in oil-contaminated sites usually declines (Van Hamme et al., 2003), particularly, of those microbes participating in carbon and nitrogen cycling. The loss of microbial diversity and changes in community composition alter their functional processes. Although soil respiration can be stimulated by the enrichment of the biodegradable C source after new oil spills, an inhibitory effect on the hydrolase activities involved in nitrogen, phosphorus, or C cycles has been observed (Labud et al., 2007). It is also true that contaminants may well
serve as organic C sources, and an enrichment of oil-degrading microbial populations has been observed in most contaminated ecosystems (Margesin et al., 2000). A better understanding of the effect of crude oil on plant and soil microorganisms may be of help in enhancing C sequestration in wetlands. Freeman (2012) reported that improvements in C capture could potentially be achieved as peatland-geoengineering strategies through increasing abundance of exogenous phenolic inhibitors, or manipulating edaphic factors that slow down the microbial production in peatlands such as inorganic nutrient and pH that release their nutrients slowly, while maintaining low soil C turnover rates. As far as we are aware there have not been any studies published regarding the effects of crude oil on C cycling in soils.

1.10. Conclusion

In wetlands, the introduction of aerobic conditions stimulates phenol oxidase activity, thereby removing phenolic inhibition. This leads to an increase in hydrolase enzyme activities and microbial growth rates, and therefore decomposition of organic matter and CO₂ production. Consequently, one of the solutions that could inhibit the activity of enzymes is the use of exogenous phenolic inhibitors. Existing research suggests that use of these supplements may have a positive effect in reducing emissions of CO₂ into the atmosphere. The main aim of this project is to evaluate the potential for phenolic enrichment as a strategy for constraining decomposition and increasing the C sequestering ability of wetland soils even further. The experiments will determine the effects of these additions on all major fluxes of C (gaseous exchange of CO₂ and CH₄). A number of hypotheses have been proposed to investigate the potential of this strategy:

- Chapter 2: The addition of wood chips to peat soil will increase the concentration of phenolics, which will reduce enzyme activities, lower CO₂ emissions from decomposition and enhance C sequestration. Additionally, wood chips from different tree species will release different quantities of phenolic inhibitors and therefore have variable impacts on decomposition.

- Chapter 3: Soaking wood chips in lignin solution will lead to a greater input of phenolics to the peat and a greater suppression of hydrolase enzymes than the addition of wood chips only. Soaking wood chips in lignin solution will prevent the
increase in decomposition at the beginning of the experiment that was observed in chapter 2.

- Chapter 4: The addition of crude oil to peat soil will increase the concentration of phenolics, which will reduce enzyme activities, lower CO$_2$ emissions from decomposition and enhance C sequestration.

- Chapter 5: The higher the overall molecular weight of the phenolic compound, the greater the inhibition of peat decomposition processes which will provide information on differences in their inhibitory effect on extracellular hydrolase enzymes in peat slurries taken from UK peatlands.
1.11. References


Ross, D. J.1972. Effects of freezing and thawing of some grassland topsoils on oxygen uptakes and dehydrogenase activities. Soil Biology and Biochemistry. 4:115-117.


Chapter 2: Enhancing carbon sequestration in peatlands using contrasting low cost, phenolic-rich biomass supplements
Enhancing carbon sequestration in peatlands using contrasting low cost, phenolic-rich biomass supplements

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2.1. Abstract

The addition of phenolic compounds to peatland soils has been proposed as a means of enhancing the suppression of enzymes, reducing the rate of organic matter decomposition and increasing below-ground carbon sequestration. This study evaluated the potential of phenolic enrichment as a geoengineering strategy by adding wood chips to peat soil and determining the impacts on key components of organic matter decomposition and Sphagnum growth. All treatments increased the concentration of phenolics and the activities of B-D-glucosidase and Chitinase enzymes were significantly supressed by the increase in phenolics (p<0.01, p<0.05 respectively), especially in a treatment with Spruce chips mixed with peat. A reduced flux of carbon dioxide (CO₂) from the peat soil was also recorded. These results indicate that through the addition of phenolic compounds to wetland soils, it may be possible to inhibit extracellular enzyme activities in order to reduce the flux of CO₂ from soils to the atmosphere and increase carbon sequestration.

Keywords: Peatlands, Phenolics, Hydrolase enzymes, Carbon dioxide, Sphagnum farming, Geoengineering.
2.2. Introduction

Peatland ecosystems are freshwater wetlands which accumulate partially decomposed organic material derived from decaying plant matter. Globally, peatlands cover 3% of the land surface but the amount of carbon stored as peat is equivalent to 20% of the world’s soil carbon and 60% of the carbon currently in the atmosphere (Joosten et al. 2016). Peat forms where the water table is close to the ground surface, creating anaerobic conditions in the soil that are unfavourable for complete decomposition of plant matter. According to the enzymic latch theory (Freeman et al. 2001), the lack of oxygen prevents the enzyme phenol oxidase from breaking down phenolic compounds, which are released into the soil following plant senescence (Dickinson 1979, Sikora and Keeney 1977). These compounds are inhibitory to hydrolase enzymes (Appel, 1993; Wetzel, 1992), the main agents of decomposition in soils, therefore their suppressed activity causes organic matter to accumulate.

Large areas of peatlands have been disturbed by drainage, peat extraction and land use conversion for urbanisation, forestry and agriculture (Wichtmann et al. 2016). Degraded peatlands can represent a concern for the biogeochemical cycles, especially in the context of global warming, because some degraded peatlands no longer sequester carbon and, in the case of severe disturbances, can become net sources of carbon by oxidation of accumulated peat (Paul and Clark 1989, Laine and Minkkinen 1996, Waddington et al. 2002, Ramchunder et al. 2009). In densely populated areas of the world, like Europe, more than half of the present-day peatland expanse is degraded to a point where the ecosystem is no longer accumulating carbon, while in specific individual countries this proportion can be over 90% (Joosten 2016). In recent decades, awareness has grown concerning the importance of preserving peatland habitats (Joosten and Clarke 2002) to sustain their ecological services for future generations. In addition to conservation, solutions to reduce carbon emissions from degraded peatlands have also been investigated, including restoration (Waddington and Warner 2001, Strack and Zuback 2013, Quinty and Rochefort 2003, Bonn et al. 2014, Kotowski et al. 2016, Thom et al. 2016, Graf and Rochefort 2016, Anderson et al. 2017, Zhang et al. 2016, Dommain et al. 2016), rewetting (Tuittila et al. 1999, Soini et al. 2010, González et al. 2013, Wilson et al. 2016) and paludiculture on organic soils (Joosten et al. 2016b). In the last two decades, paludiculture of undecomposed Sphagnum
fibres, i.e. Sphagnum farming, earned interest because it reduces anthropic pressure on natural ecosystems and offers a sustainable after use for degraded peatlands (Joosten 1998, Campeau and Rochefort 2002, Gaudig and Hoosten 2002, Gaudig et al. 2014).

Therefore, the presence of Sphagnum in the treatments used to reduce the rate of decomposition in peat is a major factor in this study. In a Sphagnum farm, the goal is to produce undecomposed Sphagnum fibres as fast as possible, on a cyclic and renewable basis. To achieve this objective, productivity of Sphagnum must be optimized while decomposition minimized. Sphagnum species possess different intrinsic characteristics affecting their growth and decomposition rates and reduce decomposition of dead plant matter. Poikilohydric Sphagnum plants can maintain waterlogging in surface peat and their tissues also contain an oxopolysaccharide (Painter 1983) and phenolic compounds (Rasmussen et al. 1995) that suppress soil heterotroph and extracellular enzyme activity (Freeman et al. 2001a, Verhoeven and Toth 1995), thereby impeding decomposition. Similarly, microbes can be manipulated in peatlands by adding inhibitory phenolics such as woody plants (rich in phenolics) which have the potential to reduce decomposition rates (Freeman et al. 2012) and will have the ability to reduce CO₂ rates and decomposition process because of the species production of lignin rich woody plant tissue (Van and Boon 1994, Huang et al. 1998) and thus strengthening the enzymic latch mechanism (Freeman et al. 2001b). Woody Plants are the principle producers of polyphenols (Haslam 1989). While in lignins, which is the most stable of polyphenols, where the polyphenols can have the biggest effect on litter recalcitrance in long-term and therefore control over fine scale decomposition dynamics (Horner et al. 1988), suggesting greater opportunities with the aim of suppressing organic decomposition and thereby strengthening the enzymic latch (Freeman et al. 2001, Freeman et al. 2004). Wood fragments of Larch, Spruce and Cedar ((Larix laricina, Picea mariana, Thuja occidentalis) which are common in Canadian peatlands have been selected as phenolic inhibitors to promote CO₂ sequestration.

In this study, we investigate a new approach to maximize the carbon sequestration in peat with the presence of Sphagnum mosses: the use of phenolic enrichment (i.e., wood chips), as a strategy for strengthening the “enzymic latch mechanism” that controls the rate of decomposition in peatland soils. In order to strengthen the enzymic latch, we must enhance
increase carbon sequestration, and this can be achieved by through two main ways. First by strengthening the enzymic latch by increasing abundance of phenolic inhibitors, or manipulating edaphic factors that slow decomposition in peatlands (eg. inorganic nutrient availability, labile C inputs and pH). The second way is by increasing the amount of C influenced by the enzymic latch, using methods to either increase peatland plant productivity, or create forms of externally captured carbon (Freeman et al. 2012).

2.3. Hypotheses
- The addition of wood chips to peat soil will increase the concentration of phenolic compounds.
- The increase in phenolic compounds will lead to lower hydrolase enzyme activities and reduced CO₂ fluxes.

2.4. Methods
2.4.1. Experimental design and implementation of treatments
To test the effect of exogenous phenolic inhibitors on Sphagnum papillosum productivity, a completely randomized design experiment with seven treatments was implemented in the greenhouses at Laval University, Canada. The treatments tested were: adding wood chips from 3 tree species (Larix laricina, Picea mariana, Thuja occidentalis, all common in Northern American bogs) at the surface of the peat (treatments 2 to 4), mixing woodchips from the three same tree species within the top ten centimetres of peat (treatments 5 to 7), and no amendment of the peat (treatment 1). For all of the treatments, Sphagnum papillosum fragments were added on top of the amended (or not) peat substrate. All treatments were repeated five times for a total of 35 experimental units (EU). Each EU is a mesocosm (plastic bin) measuring 60 x 40 x 36 cm equipped with a perforated drain and pipe to allow the water table to be maintained at the desired level. Each EU was filled with approximately 20 cm of bulk fibric peat (von post : H3-4) collected from a vacuum extracted peatland located approximately 30 km from the greenhouses (46°42’N 71°03’W). Mature trees from the three targeted species were harvested in another natural peatland (46°46’N 71°00’W) and the trunks (without needles) were processed through a wood chipper to produce woodchips of 1 to 2 cm diameter. Approximately 500 g of (dry) woodchips were either spread on top of the peat (“surface” treatments) or mixed
with the top 10 cm of peat (“mixed” treatments). *Sphagnum papillosum* was hand collected in fragments of about 10 cm in a natural peatland in the same site as the trees were harvested. *Sphagnum* fragments were spread on top of the amended peat in a ratio of 1:10 – i.e. 1 m² of *Sphagnum* collected in a natural peatland spread over 10 m² of mesocosms. Mesocosms were watered twice per week with rainwater to maintain the water table at 10 cm below the peat surface. Greenhouses conditions were set at 22°C during the day and 18°C during the night with a constant 75% relative humidity and a photoperiod of 15 hours. The experiment took place over a 10 month period (July 2013 to May 2014).

2.4.2. Sampling
Gas flux sampling was undertaken at approximately monthly intervals using a static headspace technique. The opaque chambers (19 cm height x 13 cm width x 21 cm length) included a suba-seal for sampling. Each chamber was placed on the peat/*Sphagnum* surface, pressed down gently to create a seal and sampled after 1 hour had elapsed (after a series of preliminary tests showed that CO₂ and CH₄ concentrations increased linearly up to this time period). A 20 ml sample of gas was extracted and injected into a 12 ml exetainer (Labco, Buckinghamshire, UK). Several samples of air from the greenhouse were also collected periodically during the flux analysis. Soil samples were collected from each EU from September 2013 to May 2014. This was achieved by extracting approximately 200 g by hand from a depth of 5-10 cm and placing in a sandwich bag which was then sealed and stored at 4°C. All gas and soil samples were shipped to the Bangor University, UK, for analysis. *Sphagnum papillosum* cover was visually estimated at the end of the experiment. All *Sphagnum* fragments were then collected in each mesocosm, cleaned from any woodchip or peat residues and weighed for biomass measurements.

2.4.3. Laboratory analyses
The gas samples collected in the exetainers were analysed using a Varian model 450 gas chromatograph (GC) instrument. The GC system is designed for the analysis of the three main greenhouse gases (CH₄, CO₂, N₂O), being equipped with a methaniser (temperature 380°C) and flame ionisation detector (FID, 125°C) for CO₂ and CH₄ and an electron capture detector (ECD, 300°C) for N₂O. Two ml of gas was extracted from the exetainers using a CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland) equipped with five ml
syringe and added to the column injector system (100°C). Gases were separated on a 1.83 m x 3.18 mm PoroPak QS 80/100 column (40°C). Oxygen-free Nitrogen, at a flow rate of 30 ml min\(^{-1}\), was used as the carrier gas. Gas fluxes were calculated by subtracting the mean values of the greenhouse atmosphere samples from each of the one hour chamber values and expressing the flux values as mg m\(^{-2}\) h\(^{-1}\).

Soil samples were analysed for the concentration of phenolic compounds and the activity of five key hydrolytic enzymes (Table 2.1). Samples were prepared for the analysis of phenolics using a water extraction method similar to that described by Chantigny (2003). The soil samples were homogenised by hand and five grams placed in a 50 ml centrifuge tube (Fisher Scientific, Loughborough, UK) and 40 ml of deionised water added. The tubes were placed on a KS501 orbital shaker (Ika, Staufen, Germany) at a speed of 300rpm for 24 hours, after which they were centrifuged at 5000rpm for 30 minutes on a Sorvall ST16R centrifuge (Thermo Fisher, Altricham, UK). The supernatant was filtered through 0.45 µM syringe filters (Phenomenex, Macclesfield, UK) and analysed for phenolics using a method adapted from Box (1983). To separate 1.5 ml centrifuge tubes, 1 ml of sample was added, followed by 50 µl of Folin-Ciocalteau phenol reagent (Sigma, Gillingham, UK) and 0.15 ml of Na\(_2\)CO\(_3\) (200 g L\(^{-1}\)) (Sigma) to buffer the reaction. The process was repeated for calibration standards made from phenol compound (Sigma) in the range 0.5 to 30 mg L\(^{-1}\). After 1.5 hours 300 µL of each sample and standard were transferred to wells of a clear 96 well microplate (Triple Red, Long Crendon, UK) and absorbance measured at 750 nm on a Spectramax M2e spectrophotometer (Molecular Devices, Wokingham, UK).

Hydrolase enzyme activities were determined by following Dunn et al (2014). All substrates were obtained from Glycosynth (Warrington, UK), prepared by dissolving the relevant amount of substrate (Table 2.1) to make 1L of 200μM 4-MUF phosphate solution and 400μM solutions for the other substrates in 20mL of ethylene glycol monomethyl ether (Sigma) and deionised water and stored at 4°C until required. A standard solution of 1000 µM MUF-free acid was prepared using 4-methylumbelliferone sodium salt (Sigma) and a dilution series made in 2 ml microcentrifuge tubes in the range 0-100 µM. Soil samples and substrates were placed in an incubator set to the mean temperature of the soil in the greenhouses (~20°C) the day before the assays were undertaken. The soil samples were homogenised by hand and one gram placed in six separate stomacher bags (Seward, Worthing, UK), one for each of the five hydrolase enzyme substrates and the standard
solution. To each stomacher bag seven mL of the appropriate substrate or deionised water (standard solution) was added and the bags homogenised in a Stomacher 80 (Seward) for 30 seconds. The bags were incubated at field temperature for 60 minutes (45 minutes for phosphatase due to saturation at higher concentrations) and 1.5 ml of solution centrifuged at 14,000rpm. For the substrate solutions 50 µL of deionised water was pipetted into wells of a 96-well black microplate (Scientific Laboratory Supplies, Yorkshire, UK), followed by 250 µL of supernatant from the substrate bags. For the standards, 50 µL of each MUF-free standard solution was pipetted into the microplate followed by 250 µL of supernatant from the peat/deionised water bag. The microplate was then measured on the Spectramax M2e plate reader, analysing the fluorescence at 330 nm excitation and 450 nm emission. The instrument creates a calibration curve from the standards to calculate the enzyme concentration of the samples. From these values the enzyme activities are calculated and expressed as µmol g⁻¹ min⁻¹. Soil samples were also analysed for dry weight and organic content by weighing samples in crucibles and following the standard methods detailed in Frogbrook et al. (2009).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme commission number (EC)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-glucosidase</td>
<td>3.2.1.21</td>
<td>4-MUF β-D-glucopyranoside</td>
</tr>
<tr>
<td>Arylsulphatase</td>
<td>3.1.6.1</td>
<td>4-MUF sulfate potassium salt</td>
</tr>
<tr>
<td>β-D-xylosidase</td>
<td>3.2.1.37</td>
<td>4-MUF β-D-xylopyranoside</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase (Chitinase)</td>
<td>3.2.1.96</td>
<td>4-MUF N-acetyl-β-D-glucosaminide</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>3.1.3.2</td>
<td>4-MUF phosphate</td>
</tr>
</tbody>
</table>

Table 2.1. Enzyme names, commission numbers and substrates used during assays.

2.4.4. Statistical analyses

The effect of the exogenous phenolic inhibitors on the measured soil parameters (phenolics concentration, hydrolase enzyme activities and greenhouse gas fluxes) were determined using one-way ANOVA and Tukey HSD post-hoc tests in R v3.3.1. Pearson correlation was used to test for significant relationships between the key variables. Most data met the
homogeneity and normality assumptions, which were tested using the Bartlett and Shapiro Wilk tests, but those that did not were log-transformed.

The effect of the exogenous phenolic inhibitors on *Sphagnum papillosum* cover and biomass was analysed by one-way ANOVA for a randomized complete block design using the MIXED procedure of SAS (SAS Statistical System Software, v. 9.2, SAS Institute Inc., Cary, NC, USA.) Following the ANOVAs, protected Fisher’s LSDs were run when a significant difference between treatments was found. Data met the homogeneity and normality assumptions. All treatments were included in the analyses except for the bare peat treatments where no fragments were introduced.

2.5. Results

Data were analysed by calculating the change in the measured parameters between September 2013 and May 2014, a period of 8 months. Figures 2.1-2.5 present the mean change in Phenolics, β-glucosidase and CO₂ emissions over this period.

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Conductivity</th>
<th>β-glucosidase</th>
<th>Sulphatase</th>
<th>Xylosidase</th>
<th>Chitinase</th>
<th>Phosphatase</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>F value</td>
<td>9.37</td>
<td>0.40</td>
<td>5.59</td>
<td>2.25</td>
<td>1.26</td>
<td>1.29</td>
<td>0.39</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 2.2. Main effect results of ANOVA analyses (P values are only displayed for significant effects).

<table>
<thead>
<tr>
<th>Species</th>
<th>Phenolics</th>
<th>β-glucosidase</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larch</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Spruce</td>
<td>&lt;0.001</td>
<td>0.024</td>
<td>0.005</td>
</tr>
<tr>
<td>Cedar</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Post-hoc results for parameters where a significant main effect was recorded for the Species factor

2.5.1. Phenolics

For all but 1 treatment (Larch, surface) greater increases were observed in the concentration of phenolic compounds compared to the Control (Figure 2.1). There was a significant main effect of treatment on Phenolics (Table 2.2) and post-hoc analysis revealed
that both the “Spruce, mixed” (5x greater) and “Cedar, mixed” (3.6x greater) treatments led to a significantly greater increase in Phenolics than that which occurred in the Control (Table 2.3).

Figure 2.1. Mean change in concentration of Phenolics (mg of phenol per gram dry weight of peat) over the period September 2013 to July 2014 (n=5). Significance levels: *=p<0.05, **=p<0.01, ***=p<0.001

2.5.2. Extracellular hydrolases

A significant mean treatment effect was measured only for the enzyme β-glucosidase, with no significant main effects for Sulphatase, Xylosidase, Chitinase or Phosphatase (Table 2.2). For β-glucosidase, the activity decreased more in all of the treatments compared to the Control (Figure 2.2). Similar to the results for Phenolics, post-hoc analysis revealed that the “Spruce, mixed” treatment resulted in the greatest change in β-glucosidase activity (almost 20x greater decrease than the Control), however there was not a concurrent significant impact of the “Cedar, mixed” treatment on β-glucosidase activity.
Figure 2.2. Mean change in activity of β-glucosidase over the period of approximately eight months (September 2013 to May 2014) (n=5). Significance levels: *=p<0.05, **=p<0.01, ***=p<0.001

2.5.3. CO₂ fluxes

The flux of CO₂, which was measured every month, showed a spike at the beginning of the experiment and a high degree of variability between treatments. This may have been a disturbance effect or may have resulted from an initial flush of labile carbon from the wood chips. Therefore, the CO₂ flux data was analysed in the same way as the soil data, by calculating the difference from September 2013 to May 2014, to allow time for the soil to stabilise. The flux increased slightly in the Control but decreased in all of the treatments with wood chips added (Figure 2.3) and there was a significant main treatment effect (Table 2). Post-hoc analysis demonstrated that both the “Larch, surface” and “Spruce, surface” (but not “Cedar, surface” or any of the “mixed” treatments) led to a significant reduction in CO₂ flux compared to the Control. CH₄ and N₂O fluxes were also measured, but the variability between and within each treatment was consistently high throughout the experiment, so these data have not been analysed further.
A significant negative relationship was observed between the mean change in both Phenolics concentration and β-glucosidase activity (Figure 2.5; $r = -0.51$, $p=0.002$) but significant relationships were not observed between Phenolics and any of the other hydrolase enzymes or the CO$_2$ flux ($p>0.05$). Figure 4 demonstrates that the strongest treatment effect was observed for the Mixed treatments.
2.5.4. Vegetation

No significant effect of phenolic enrichments treatment was recorded on Sphagnum cover nor biomass. Sphagnum productivity was thus similar between the control treatment and the treatments with added woodchips. The presence of woodchips (or any tree species, either at the surface or mixed with the peat) did not impede on Sphagnum growth in conditions where the water table was maintained relatively high (10 cm below the surface).

2.6. Discussion

This experiment has demonstrated that it is possible to increase the concentration of phenolic compounds and reduce enzyme activities and CO\(_2\) emissions from peat through the addition of exogenous phenolic compounds in the form of wood chips. Previous research suggests that phenolic supplements can be applied to suppress microbial metabolism and therefore reduce the CO\(_2\) fluxes through either by naturally produced anaerobic compounds in peat or the addition of analogue polyphenolic waste materials such as wood chips (Freeman et al. 2012).
Mixing the wood chips into the peat was found to be a more effective method of increasing phenolics concentration than surface application. This is possibly attributed to the form of phenolics when reactions with peat which the structure of compound can influence their fate in soils by dissolved phenolics, allowing them to be processed quickly into simple, assimilable forms (Schmidt et al. 2011, Gleixner 2013). Of the three wood materials, Spruce had the greatest impact in terms of elevating phenolics and suppressing enzymes, and for the “mixed” treatments only, but Larch had a slightly greater impact in reducing the CO₂ emissions, and only for the “Surface” treatment. There seems to be a general tendency for the activities of most enzymes to decrease with increasing depth in peat profiles (Freeman et al. 1995). Previous experimental work demonstrated that, on its own, Cedar released approximately 3.5 times as much phenolics into water as the other two wood types (data not shown), however it did not significantly decrease β-glucosidase activity or CO₂ emissions and did not increase the phenolics concentration of the peat soil as much as the Spruce for either the “surface” or “mixed” treatment.

The high CH₄ and variable CO₂ fluxes for some treatments suggests that in addition to the release of phenolic compounds the wood chips may also leach low molecular weight carbon that may stimulate microbial growth. This would counteract the impact of the phenolics and may impair the ability of wood chips to ultimately lead to reduced soil decomposition.

Freeman et al. (2012) have proposed that enhanced carbon storage in ecosystems, particularly in peatlands, is feasible by modifying phenolic contents in order to inhibit decomposition of organic matter by a mechanism known as the “enzymic latch” (Freeman et al. 2001). They proposed that increases in phenolic content in peat ecosystems can be achieved either by increased expression of phenolic inhibitors from peatland plants or by enhancement of enzyme latch by physicochemical modification (Min et al. 2015). Yoo and Kang (2012) reported that the addition of biochar with high phenolics content represents a further approach to stabilize soil organic matter (SOM) in terrestrial ecosystems by inhibiting enzyme activities. Phenol oxidases activity and phenol metabolism have been shown to respond positively to seasonal inputs of organic matter (Peters and Colwell 1989, Sinsabaugh and Linkens 1990, Sinsabaugh et al. 1991). For example, the strong positive correlation between the soluble phenolic content and Phenol oxidases activity in the Carex-
derived peat suggests a linkage between substrate availability and enzyme activity. This suggests that the manipulating of phenolic abundance in the peat could be used to strengthen the enzymic latch and offer a new approach to geoengineering (Freeman et al. 2012) acting as a cost efficient biological CO$_2$ removal (CDR) geoengineering techniques (Royal Society 2009).

2.7. Conclusion
Our results show that the addition of supplementary phenolic compounds, especially when mixed into the peat soil, is an effective method of strengthening the enzymic latch and enhancing carbon sequestration. Further research is required including testing the effect of phenolic addition on a larger scale and whether phenolic compounds result in more inhibition when added to the peat surface or when mixed into the substrate. Longer-term monitoring would also be beneficial, and alternative tree species investigated as a source of wood chips.

2.8. Acknowledgement

Financial support was provided by the Natural Sciences and Engineering Research Council of Canada, the Canadian Sphagnum Peat Moss Association and its members. We thank Noemie D’Amour and Christiane Dupont for greenhouse gas and vegetation sampling.
2.9. References


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Chapter 3: Optimising carbon sequestration in peatlands using lignin in combination with other low cost, phenolic-rich biomass supplements
Optimising carbon sequestration in peatlands using lignin in combination with other low cost, phenolic-rich biomass supplements

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3.1 Abstract

Peatlands store approximately one-third of all soil organic carbon (390–455 Pg), and play an important role in reducing climate change. Peatlands are therefore a powerful carbon capture and storage agent, through sequestration of anthropogenic CO₂ emissions. However, drying can lead to large decreases in storage, leading to net carbon loss during droughts, causing increased CO₂ emission rates, due to increased microbial activity and decomposition. In order to capture carbon in peat soil more further, recent studies suggest that peatland carbon sequestration is due to the inhibitory effects of phenolic compounds that create an ‘enzymic latch’ on decomposition, enhancing the suppression of enzymes and therefore increasing below-ground carbon sequestration. The use of lignin solution and wood chips from different tree species as phenolic inhibitors was investigated as a means of further reducing the rate of decomposition in peat, suppression of both enzyme activities and CO₂ emissions. In this study we found that there was a high concentration of phenolic inhibitors in the first four months, but no significant effect of phenolic additions on enzyme activity.
3.2. Introduction

More than one third of the world’s soil carbon is stored in peatlands, despite only occupying approximately 3% of the world’s land surface area (Page et al. 2011, Yu, 2012. Gorham et al. 2012, Charman et al. 2013). Within the last few thousand years, peatlands have effectively sequestered carbon at a rate of approximately 24-32 g C/m²/yr, due to the incomplete decomposition of plant material within the waterlogged and anaerobic soil matrix (Loisel et al. 2014). If peatlands experience water table drawdown due to drainage and/or drought, oxygen ingress can stimulate the decomposition of organic matter by opening the “Enzymic Latch” (Freeman et al. 2001). The shortage of oxygen under conditions of water saturation prevents the enzyme phenol oxidase (POX) from breaking down phenolic compounds (Freeman et al. 2001), which are released into the soil following plant senescence (Dickinson 1979, Sikora and Keeney 1977). These compounds are inhibitory to hydrolase enzymes (Wetzel 1992, Appel 1993), the principle agents of decomposition in soils; therefore their suppressed activity causes organic matter to accumulate. POX therefore plays an important role in regulating soil carbon sequestration and mobilization (Freeman et al. 1996, 2001a, 2004). Moreover, some phenolic compound-degrading microorganisms (for example, fungi) are only capable of achieving this in the presence of a readily degradable substrate functioning as their primary energy source (Paul and Clark 1989). Any increase in its decomposition can also reduce carbon storage directly due to the fact that phenolic compounds can form 50–60% of the whole dry mass of peat (Clymo 1983), with decomposition rates highly influenced by litter quality (Rydin and Jelgum 2013, Malterer et al. 1992, Strakova et al. 2011).

A number of studies of past environmental and climate changes in peatlands have been studied using various biological, physical and chemical proxies (study of past climates) (Chambers et al. 2012). Anthropogenic carbon emissions to the atmosphere currently exceed 8 Pg y⁻¹ and to prevent further climate change, substantial cuts are necessary (IPCC 2007). IPCC emission scenarios suggest that if there are no policies to reduce climate change, CO₂ concentrations may reach as high as 535 to 983 ppm by 2100, potentially resulting in a 1.1 to 6.4°C rise in global temperatures (IPCC 2013). In this context, the importance of restoring degraded peatlands, and their carbon sequestration capacity, is a sensible natural solution aimed at reducing atmospheric CO₂ concentrations (Freeman et al.

Since phenolic compounds are key to the suppression of decomposition in peatlands, the addition of phenolics as a means of enhancing carbon sequestration is receiving growing attention (Chapter 1, Schmidt et al. 2011, Freeman et al. 2012). Lignin is the most stable of the polyphenols and can have the biggest long-term impact on decomposition rate (Horner 1988). Woody plants are the primary producers of polyphenols (Haslam 1989) and Chapter 2 of this thesis has shown that Spruce wood chips are particularly effective in releasing phenolic compounds into the surrounding peat matrix. During this preliminary experiment we observed a significant reduction in hydrolase enzyme activities and CO$_2$ emissions following supplementation of soil with phenolics. However, there was an initial disturbance effect resulting from the addition of wood chips which led to a short-term increase in decomposition rates (as indicated by elevated CO$_2$ flux). The present study builds on the results of the previous chapter and investigates the efficacy of soaking the woodchips in lignin solution prior to addition as a means of preventing the initial disturbance effect. It is hypothesized that the lignin solution will act as a readily available source of phenolics that will supress decomposition in the initial stages, with the wood chips acting as a longer-term source of phenolics for a sustained effect. This experiment also investigated the effect of lignin addition on the survival of seeded *Sphagnum*. A living and photosynthesising moss layer is critical to ensuring a peatland is able to effectively sequester carbon long-term (Fenner et al. 2004). In this regard, *Sphagnum spp.* are especially important and are the most extensive of all peatland bryophyte taxa (Andrus 1986, Gajewski et al. 2001). *Sphagnum* tissues are the single most important carbon sink in bogs (Heijmans et al. 2002) due to their low decomposition rates (Kuhry and Vitt 1996). The *Sphagnum* moss representing 10–15% of the terrestrial carbon stock, and the most efficient species group at reducing belowground carbon turnover, stating the importance of Sphagnum species for carbon to accumulated in peatlands (Clymo and Hayward 1982).
3.3. Methods

3.3.1. Experimental design

The experiment was performed in a controlled greenhouse set at 22°C during the day and 18°C during the night with a constant 75% relative humidity and a photoperiod of 15 hours, at Laval University, Canada. A fully randomised design experiment with 10 treatments was used to analyse how exogenous phenolic inhibitors would impact on carbon sequestration. The treatments are described in (Table 3.1).

<table>
<thead>
<tr>
<th>Treatment name</th>
<th>Sphagnum species</th>
<th>Wood chips added?</th>
<th>Lignin solution used?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Peat</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>RUB-Control</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>RUB-Wood</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>RUB-5</td>
<td>Rubellum</td>
<td></td>
<td>Yes (5%)</td>
</tr>
<tr>
<td>RUB-10</td>
<td></td>
<td></td>
<td>Yes (10%)</td>
</tr>
<tr>
<td>RUB-15</td>
<td></td>
<td></td>
<td>Yes (15%)</td>
</tr>
<tr>
<td>MAG-Control</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MAG-Wood</td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>MAG-5</td>
<td>Magellanicum</td>
<td></td>
<td>Yes (5%)</td>
</tr>
<tr>
<td>MAG-10</td>
<td></td>
<td></td>
<td>Yes (10%)</td>
</tr>
<tr>
<td>MAG-15</td>
<td></td>
<td></td>
<td>Yes (15%)</td>
</tr>
</tbody>
</table>

Table 3.1. Design of experiment that includes 10 treatments.

A total of 40 experimental units (EU) were prepared. Each EU was a large plastic box measuring 60 x 40 x 36 cm equipped with a perforated drain and pipe to allow the water table to be maintained at the desired level. On 8th June 2015, each EU was filled with
approximately 20 cm of bulk fibric peat (von post: H3-4) from a vacuum extracted peatland (46°42'N 71°03'W) located approximately 30 km from the greenhouses. Spruce trees were harvested in another natural peatland (46°46'N 71°00'W) and the trunks (without needles) were processed through a wood chipper to produce woodchips of 1 to 2 cm of diameter. *Sphagnum rubellum* and *Sphagnum magellanicum* were hand collected in fragments of about 10 cm in a natural peatland in the same site as the trees were harvested.

Lignin solution was prepared at 5, 10 and 15% w/v concentrations using calcium lignosulfonate, a high molecular weight by-product of the paper milling industry. The compound for this study was sourced from Borregaard (Sarpsborg, Norway). Wood chips were soaked in lignin solution for approximately four days. On 9th June 2015, approximately 600 g (dry weight) of either soaked or non-soaked woodchips were mixed into the top 10 cm of peat within each EU. On 10th June 2015, *Sphagnum* fragments were spread on top of the amended peat in a ratio of 1:10 – i.e. 1 m² of *Sphagnum* collected in the natural peatland spread over 10 m² of mesocosm. Mesocosms were watered twice per week with rain water to maintain the water table at 10 cm below the peat surface. The experiment took place over a 9 month period (July 2014 to April 2015).

3.3.2. Sampling

Gas flux sampling was undertaken at approximately monthly intervals using a static headspace technique. Measurements of net ecosystem exchange (NEE) were undertaken using transparent chambers, ecosystem respiration (ER) using black bin bags. NEE represents the carbon balance of an ecosystem, with a mean positive value indicating net carbon emissions from the soil to the atmosphere and a mean negative value the opposite (i.e. net carbon sequestration). The chambers (19 cm height x 13 cm width x 21 cm length) included a suba-seal for sampling. Each chamber was placed on the peat/*Sphagnum* surface, pressed down gently to create a seal and sampled after 1 hour had elapsed. A 20 mL sample of gas was extracted and injected into a 12 mL Exetainer (Labco, Buckinghamshire, UK). Several samples of air from the greenhouse were also collected periodically during the flux analysis. Soil samples were collected from each EU in July 2015, October 2015 and April 2016 (the latter being a composite of all replicates for each treatment). This was achieved by extracting approximately 200 g by hand from a depth of 5-10 cm and placing in a
sandwich bag which was then sealed and stored at 4°C. All gas and soil samples were shipped to Bangor University, UK, for analysis. *Sphagnum rubellum* and *magellanicum* cover were visually estimated at the end of the experiment. All *Sphagnum* fragments were then collected in each mesocosm, cleaned from any woodchip or peat residues and weighed for biomass measurements.

### 3.3.4. Laboratory analyses

The gas samples collected in the Exetainers were analysed using a Varian model 450 gas chromatograph (GC). The GC system is designed for the analysis of the three main greenhouse gases (CH$_4$, CO$_2$, N$_2$O), being equipped with a methaniser (temperature 380°C), flame ionisation detector (FID, 125°C) for CO$_2$ and CH$_4$ and an electron capture detector (ECD, 300°C) for N$_2$O. Two mL of gas was extracted from the exetainers using a CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland) equipped with a five mL syringe and added to the column injector system (100°C). Gases were separated on a 1.83 m x 3.18 mm PoroPak QS 80/100 column (40°C). Oxygen-free Nitrogen, at a flow rate of 30 mL min$^{-1}$, was used as the carrier gas. Gas fluxes were calculated by subtracting the mean values of the greenhouse atmosphere samples from each of the one hour chamber values and expressing the flux values as mg m$^{-2}$ h$^{-1}$.

Soil samples were analysed for the concentration of phenolic compounds and the activity of several key hydrolytic enzymes. Samples were prepared for the analysis of phenolics using a water extraction method similar to that described by Chantigny (2003). The soil samples were homogenised by hand and five grams placed in a 50 ml centrifuge tube (Fisher Scientific, Loughborough, UK) and 40 mL of deionised water added. The tubes were placed on a KS501 orbital shaker (Ika, Staufen, Germany) at a speed of 300rpm for 24 hours, after which they were centrifuged at 5000rpm for 30 minutes on a Sorvall ST16R centrifuge (Thermo Fisher, Altricham, UK). The supernatant was filtered through 0.45 µm syringe filters (Phenomenex, Macclesfield, UK) and analysed for phenolics using a method adapted from Box (1983). To separate 1.5 mL centrifuge tubes, 1 mL of sample was added, followed by 50 µL of Folin-Ciocalteau phenol reagent (Sigma, Gillingham, UK) and 0.15 mL of Na$_2$CO$_3$ (200 g L$^{-1}$) (Sigma) to buffer the reaction. The process was repeated for calibration standards made from phenol compound (Sigma) in the range 0.5 to 30 mg L$^{-1}$. After 1.5 hours 300 µL of each...
sample and standard were transferred to wells of a clear 96 well microplate (Triple Red, Long Crendon, UK) and absorbance measured at 750 nm on a Spectramax M2e spectrophotometer (Molecular Devices, Wokingham, UK).

Hydrolase enzyme activities were determined by following Dunn et al. (2014) (see Chapter 2). All substrates were obtained from Glycosynth (Warrington, UK), prepared by dissolving in ethylene glycol monomethyl ether (Sigma) and deionised water and stored at 4°C until required. A standard solution of 1000 µM MUF-free acid was prepared using 4-methylumbelliferone sodium salt (Sigma) and a dilution series made in 2 mL microcentrifuge tubes in the range 0-100 µM. Soil samples and substrates were placed in an incubator set to the mean temperature of the soil in the greenhouses (~20°C) the day before the assays were undertaken. The soil samples were homogenised by hand and one gram placed in six separate stomacher bags (Seward, Worthing, UK), one for each of the five hydrolase enzyme substrates and the standard solution. To each stomacher bag seven mL of the appropriate substrate or deionised water (standard solution) was added and the bags homogenised in a Stomacher 80 (Seward) for 30 seconds. The bags were incubated at field temperature for 60 minutes (45 minutes for phosphatase) and 1.5 mL of solution centrifuged at 14,000 rpm. For the substrate solutions 50 µL of deionised water was pipetted into wells of a 96-well black microplate (Scientific Laboratory Supplies, Yorkshire, UK), followed by 250 µL of supernatant from the substrate bags. For the standards, 50 µL of each MUF-free standard solution was pipetted into the microplate followed by 250 µL of supernatant from the peat/deionised water bag. The microplate was then measured on the Spectramax M2e plate reader, analysing the fluorescence at 330 nm excitation and 450 nm emissions. The instrument creates a calibration curve from the standards to calculate the enzyme concentration of the samples. From these values the enzyme activities are calculated and expressed as µmol g⁻¹ min⁻¹. Soil samples were also analysed for dry weight and organic content by weighing samples in crucibles and following the standard methods detailed in Frogbrook et al. (2009).

### 3.3.5. Statistical analyses

The data was tested for significance of two factors on several measured parameters; Sphagnum (3 levels – none, Rubellum, Magellanicum) and Treatment (5 levels – control, wood chips only, wood chips & 5% lignin, wood chips & 10% lignin, wood chips & 15%...
lignin). The analysis was two-way ANOVA and Tukey HSD post-hoc tests, run in R v3.3.1. If a significant Treatment effect was found, the focus on post-hoc analysis was in comparing the Control treatment to those with additional phenolics. Pearson correlation was used to test for significant relationships between the key variables. Most data met the homogeneity and normality assumptions, which were tested using the Bartlett and Shapiro Wilk tests, but those that did not were log-transformed.

### 3.4. Results

Statistical analyses revealed no significant effect of Sphagnum or Treatment on any of the data collected in July 2015. However, significant effects were observed for the October 2015 samples, which are summarised below (Table 3.2).

<table>
<thead>
<tr>
<th>Measured parameter</th>
<th>Factor</th>
<th>F Value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content</td>
<td>Sphagnum species</td>
<td>0.125</td>
<td>0.883</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2.840</td>
<td>0.040</td>
</tr>
<tr>
<td>Soil Organic Matter</td>
<td>Sphagnum species</td>
<td>0.087</td>
<td>0.917</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
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<td>0.299</td>
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<tr>
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<td>Sphagnum species</td>
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<td>Treatment</td>
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<td>0.000</td>
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<td>Sphagnum species</td>
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<td></td>
<td>Treatment</td>
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</tr>
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<td></td>
<td>Treatment</td>
<td>7.207</td>
<td>0.000</td>
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<tr>
<td>β activity</td>
<td>Sphagnum species</td>
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</tr>
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<td></td>
<td>Treatment</td>
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<td>Sphagnum species</td>
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<td>0.438</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>3.717</td>
<td>0.013</td>
</tr>
<tr>
<td>Variable</td>
<td>Factor 1</td>
<td>Factor 2</td>
<td>p-value</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>N activity</td>
<td>Sphagnum species</td>
<td></td>
<td>1.055</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td></td>
<td>1.206</td>
</tr>
<tr>
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<td>Sphagnum species</td>
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<td>0.878</td>
</tr>
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<td>Sphagnum species</td>
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<td>5.491</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td></td>
<td>0.787</td>
</tr>
<tr>
<td>GPP</td>
<td>Sphagnum species</td>
<td></td>
<td>11.421</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td></td>
<td>0.275</td>
</tr>
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Table 3.2. Results of two-way ANOVA analysis testing the effects of two factors on several dependent variables.

The only parameters for which a significant impact of Sphagnum species was found were Net Ecosystem Exchange (NEE) and Gross Primary Productivity (GPP). Post-hoc analysis revealed that the significant effects were only found when comparing the bare peat treatment with both Sphagnum species (i.e. the presence of Sphagnum significantly affected the NEE and GPP fluxes compared to no Sphagnum), whereas there was no difference between the two Sphagnum species. Therefore, for the purposes of graphical presentation, the data from the two separate species has been averaged for all measured parameters.

Figures 3.1-3.5 show measured parameters for which significant differences were found between the Control treatment and treatments with supplementary wood chips/lignin solution, with black circles indicating specifically which treatments were significantly different.
3.4.1. Water chemistry

For phenolics, the general trend was for higher concentrations across all six treatments in October 2015 compared to July 2015, and much lower concentrations by April 2016. Mean concentrations ranged from 0.19-1.13 mg L\(^{-1}\). In October 2015 the concentration of phenolics in all three treatments containing lignin solution were higher than the control, but post-hoc comparisons indicated that only the ‘wood + 10% lignin’ treatment had significantly higher phenolics than the control \((p = 0.043)\). By April 2016, the differences between treatments had diminished, with only the ‘wood + 15% lignin’ being slightly higher than the Control (Figure 3.1).

![Figure 3.1. Water extractable phenolics concentrations across all six treatments and all three sampling dates. The presence of a black circle indicates which treatments were significantly different to the Control.](image)

\(pH\) was higher in the wood only treatment compared to the Control, especially in October 2015, but not significantly so (Figure 3.2). The three treatments with soaked lignin chips added were all approximately 0.2 of a unit lower than the Control in October 2015, but only the 5% treatment was significantly lower \((p = 0.043)\). These differences had disappeared by April 2016 and, in fact, the lignin treatments had a pH slightly higher than the Control.
Figure 3.2. Water extractable pH across all six treatments and all three sampling dates. The presence of a black circle indicates which treatments were significantly different to the Control.

For conductivity, in October 2015, the wood only treatment was significantly lower than the Control ($p = 0.011$), but both ‘wood + 5% lignin’ and ‘wood + 10% lignin’ treatments were significantly higher (by about 100%) than the Control in October 2015 ($p = 0.035$, $p = 0.002$ respectively). The conductivity was also higher for the ‘wood + 15% lignin’ treatment but not significantly so. By April 2016 the conductivity values across the treatments had decreased and were relatively similar (Figure 3.3).
Figure 3.3. Water extractable conductivity across all six treatments and all three sampling dates. The presence of a black circle indicates which treatments were significantly different to the Control.

3.4.2. Extracellular hydrolases

Of the five analysed hydrolase enzymes, there were no significant treatment effects for four of them (β-D-glucosidase, Arylsulphatase, β-D-xylosidase and N-acetyl-β-D-glucosaminidase. However, there was significantly greater Phosphatase activity (Figure 3.4) for all three treatments with lignin-soaked wood chips added ($p=0.000$, $p=0.037$, $p=0.001$ respectively) in October 2015. By April 2016, the activities had reduced significantly for all treatments but the treatments with added wood chips and/or lignin had reduced by twice as much.
Figure 3.4. Phosphatase enzyme activities across all six treatments and all three sampling dates. The presence of a black circle indicates which treatments were significantly different to the Control.

3.4.3. Gas fluxes

There were no significant treatment effects for either NEE, ER or GPP for CO₂, or CH₄ and N₂O emissions. Figure 3.5 shows the ecosystem respiration flux. Fluxes were slightly higher for the four wood/lignin addition treatments compared to the Control in July 2015 and relatively similar in October 2015. By April, some treatments were comparable and some were higher than the Control.

Figure 3.5. Ecosystem Respiration flux across all six treatments and all three sampling dates. The presence of a black circle indicates which treatments were significantly different to the Control.
3.5. Discussion:

In this study, we aimed to build on the results of Chapter 2, where significantly reduced enzyme activities and emissions of carbon dioxide were recorded following addition of wood chips to peat soil – albeit after an initial period of high CO₂ release. Wood chips were soaked in different concentrations of lignin solution in order to prevent that flush by supplying the peat with both a readily available source of phenolics (from the lignin solution) to complement the slow-release of phenolics from the wood chips. However, for the wood chips only treatment in this experiment, our data showed that the phenolics concentration was slightly lower (but not significantly) than the control at all 3 time points, therefore this experiment has not been as successful as that presented in Chapter 2. The data showed that in the first 4 months (July to October) the wood chips soaked in lignin solution raised the phenolics concentration compared to the Control, but the effect was only significant for the 10% treatment. Despite increased phenolics concentrations, there was no suppression of enzyme activities or greenhouse gas emissions. This is unexpected given the theory behind phenolic suppression and the results of Chapter 2.

Because lignin, as a phenolic compound, would be considered a substrate for phenol oxidase, so it is likely that peatland fungi possess the ability to degrade a wide variety of recalcitrant substrates, particularly phenolic compounds (Williams and Crawford 1983, Golovchenko et al. 2013). Unexpectedly, the Phosphatase enzyme activity actually increased after adding lignin soaked wood chips. This may be due to the harsh conditions (e.g. low pH and low nutrient availability) lowering microbial and plant PO₄³⁻ uptake, thus allowing phosphate to accumulate (Kang and Freeman 1998). Another reason could be phosphate would be promptly utilised by microorganisms and plants, and phosphatase production is induced due to the low phosphate availability. There are many reports of an inverse relationship between phosphatase activity and inorganic phosphate availability (Siuda and Chróst 1987, Cotner and Wetzel 1991) and of a correlation between organic phosphorus mineralization and phosphatase activity. On this basis, Sinsabaugh and Moorhead (1994) suggested increased activity is a response to low environmental availability of phosphorus, and Chróst (1991) suggested that derepression of alkaline phosphatase when inorganic phosphate is limiting.
The lignin solution treatment lowered the pH and raised the conductivity in October 2015. This might have had other effects on the enzyme activity because pH can change the interactions between enzymes and stabilising matrices in the soil (eg. clay or humus) and therefore modify their rates of activity, which explains why free enzymes have a different optimal pH than clay-bound enzymes (McLaren and Estermann 1957).

However, this study indicates that the concentration of ‘wood + 10% lignin’ treatment in the first 4 months of enrichment may have an impact on carbon sequestration. Though there were no statistically significant differences in enzymes activity and gas emission between treatments, activity measurements for the carbon fluxes in the ‘wood + 10% lignin’ treatments were lower than both other treatments. This result tends to suggest that it is important to determine the quantity of lignin added to the peat as well as the persistence of reaction of these phenolic supplements in ecosystems to be able to suppress the rate of decomposition. In experiments that rely on exogenous additions to peat soil that may alter chemical and physical properties, there is a clear need for a more robust test to determine how widely applicable this approach may be.

3.6. Conclusion

This study has shown that the addition of wood chips and the lignin treatment resulted in relatively high phenolic concentrations compared to the control, especially the wood chips mixing lignin in the first 4 months. By measuring the activity of the enzymes determined in this study in peat soil, no significant differences for any enzyme was found, except for Phosphatase enzyme which showed increase marked in the wood chips mixing lignin treatment. Similarly, there were no statistically significant differences in CO₂ fluxes although slightly decreased CO₂ was observed in the wood + 10% lignin’ treatments. From our results, we can conclude that peat with additional phenolics whether in the form of industrial lignin or chips from wood fragments could have effect on carbon sequestration, assuming a suitable quantity of phenolic treatment as was the case with the wood + 10% lignin treatment.
3.7. References


Chapter 4: Crude oil application as an ecoengineering strategy to increase carbon sequestration in peatlands
Crude oil application as an ecoengineering strategy to increase carbon sequestration in peatlands

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4.1. Abstract

Peatlands store vast quantities of carbon due to low rates of decomposition, allowing organic matter to accumulate. However, many peatlands have been degraded by human activity which has threatened their status as a carbon sink. Crude oil is typically regarded as a soil contaminant, but its high phenolic content means that it could act to suppress enzyme activity and optimise carbon sequestration in peatlands. In the present study, the effects of crude oil additions on peat enzyme activities and carbon cycling are investigated using 36 peat mesocosms to which different volumes of crude oil (2 ml, 10 ml, 20 ml, 40 ml and 80 ml) are added and the effects monitored over a 10 month period. Phenol oxidase and hydrolase enzyme activity, CO2 flux and phenolic compound concentration measurements were made to assess any changes in microbial processing resulting from differences in the peat soil after crude oil addition. As expected, phenolic compound concentrations tended to be higher in the crude oil treatments than the control, although differences were not statistically significant. The crude oil had no effect on extracellular hydrolase enzymes at 10 cm and 20 cm depths. By the end of the experiment, one of the crude oil treatments showed significantly higher (p < 0.001) CO2 flux (net ecosystem exchange) than the control.

Keywords: Peatlands, Phenolics, Hydrolase enzymes, Carbon dioxide, Crude oil, Ecoengineering.
4.2. Introduction

Crude oil is considered a significant soil pollutant due to its widespread use as fuel and in other products and the rising number of spillages associated with its utilization (Samanta et al. 2002). Plants, microorganisms and all other living creatures including humans can be seriously harmed by exposure to such hydrocarbons (Mendoza 1998, Andreoni et al. 2004). This hazard increases as degradation proceeds of hydrocarbons in soil contaminated (Thiele-Bruhn and Brümm 2005, Eibes et al. 2006).

Several studies have been conducted examining the effects of crude oil on wetland vegetation. How vegetation reacts to the effects of crude oil and the extent to which it can regain its original form depends on several factors. One of the most significant factors is the toxicity of the oil, which depends on the quantity and type of oil, whether it can move to the intercellular spaces as well as in the vascular system resulting in a lower rate of photosynthesis (Lin and Mendelssohn 1996, Hester and Mendelssohn 2000, Pezeshki et al. 2000, Baker 1970). Short-term depressions in photosynthesis and mortality are the potential impacts of oil contamination on peatland plants (Baker 1970, Pezeshki and DeLaune 1993). A number of plant functions are affected by oil including water circulation, metabolism and nutrient uptake (McCown and Denek 1972). Oil contamination can also cause cell damage (Prendeville and Warren 1977), which may result in a reduction in gas exchange between the atmosphere and roots (Stebbing 1970). Burns and Teal (1979) found that oil can remain in salt marsh sediments for long time periods following oil spills. In addition to this, crude oil also has an impact on the soil microorganisms (Alexander and Webb 1985a). These communities of microorganisms help in the circulation of energy within food webs and also help to produce nutrients in the peat soils that assist in the growth of plants. Therefore, even after the growth of plants, crude oil may have long-term effects on peat soil function. Microbial activity is very significant in maintaining nutrient cycling in soils and any exposure to crude oil will directly affect the growth of plants and ecosystem functions (Bauer et al. 1991, Ladd et al. 1996, Schloter et al. 2003). Assessment of microbial biomass, enzyme activities or soil respiration can provide an indication of the presence and activity of microorganisms. Monitoring these parameters can also help to assess the strength and duration of the impact of crude oil on soil health (Brohon et al. 2001, Eibes et al. 2006).
Despite these negative impacts, it is possible that crude oil could be used to restore the carbon sequestering capacity of degraded peatlands. This is because crude oil contains a high concentration of phenolic compounds which play an important role in regulating enzyme activities in wetland soils. More than 20 phenolic compounds have been identified in crude oil (Ioppolo et al. 1992, Taylor et al. 1997). These compounds can regulate carbon cycling by inhibiting the rate of microbial decomposition processes. This happens through suppressing the activity of hydrolase enzymes in peatland soils (Freeman et al. 2004). Microbial population diversity in oil-contaminated sites usually declines (Van Hamme et al., 2003), with microbes participating in carbon and nitrogen cycling particularly affected. Although soil respiration can be stimulated by the enrichment of the biodegradable carbon source after oil spills, an inhibitory effect on the activities of hydrolase enzymes involved in nitrogen, phosphorus, or carbon cycles has been observed (Labud et al. 2007). It is also true that crude oil may be a source of organic carbon, and a growth in the oil-degrading microbial community has been observed in a number of contaminated ecosystems (Margesin et al. 2000). A better understanding of the effects of crude oil on plant and soil microorganisms is necessary to establish the potential for enhanced carbon sequestration in wetlands. Freeman et al (2012) reported that improvements in carbon capture could potentially be achieved through peatland ecoengineering strategies involving increasing the abundance of exogenous phenolic inhibitors, or manipulating edaphic factors that slow decomposition in peatlands. This, it is suggested, can be achieved by the addition of solid waste (e.g. coir, apple pulp from cider production and green compost) and liquids (e.g. olive waste, paper waste, green compost leachate) that release their nutrients slowly, while maintaining low soil carbon turnover rates.

The objectives of the present study were i) to assess the impact of crude oil as a source of exogenous phenolic inhibitors on carbon sequestration in peat soil and (ii) to demonstrate that waste materials can offer a useful source of low cost phenolic inhibitors.

4.3. Materials and Methods

4.3.1. Sample collection

Peat mesocosms were collected from the Migneint blanket bog in Snowdonia, North Wales, UK (UK Grid ref. SH7715143781); a 200 km² Special Area of Conservation. The site is
approximately 460 m above sea level and receives an average of 2400 mm of precipitation per year. Peat depth averages 2.0 m and vegetation is dominated by *Sphagnum papillosum*, *Calluna vulgaris* and *Eriophorum vaginatum* (Evans et al. 2012).

In total, forty mesocosms were collected in 11 cm x 40 cm drainpipes on 4th September 2014 from a relatively flat and uniform area of blanket bog. Each mesocosm was collected so that the vegetation was at the surface of the drainpipe and pre-drilled holes were positioned at 5 cm below this level to allow the water level in the mesocosms to mimic the mean natural water table height of this site. A cap was placed on the bottom of each mesocosm to seal the contents. Tens of litres of water from the nearest river (Afon Ddu) were also collected, to maintain a constant water table throughout the experiment. The mesocosms and water were carefully transported back to the laboratory.

4.3.2. Experimental design

The mesocosms were each placed inside a second drainpipe of greater width and similar height, which was sealed at the bottom to prevent water loss. The mesocosms were then placed on the roof of the Brambell Building, Bangor, UK (UK Grid ref. SH5771271892) and river water poured into the outer drainpipe until the level reached the holes of the inner drainpipe. Throughout the experiment watering was carried out on a weekly basis, or more often during the summer, to maintain a constant water table within the peat soil. The mesocosms were left until April 2015 to allow time for stabilisation and for any disturbance effect resulting from their collection and transport to have dissipated. Between April and August 2015 greenhouse gas fluxes and water chemistry (see below for methodological details) were monitored on several occasions in order to generate sufficient baseline pre-treatment data. Based on these data, four of the mesocosms were discarded and not used for the experiment and, using a random number generator, the other 36 mesocosms were divided into 6 treatments of 6 replicates each.

A sample of crude oil was obtained from a refinery in Saudi Arabia and kept in a cool, dark environment before the experiment began. Crude oil was added to all but the control treatment with varying frequency and volume, as detailed in Table 4.1, by injecting the oil to just below the soil surface (5 cm to 10 cm) using a plastic syringe. The oil additions were always performed at the end of the day once sampling had finished. The first (and only
additions for the Oil-2, Oil-10 and Oil-20) additions of oil took place on 21\textsuperscript{st} August 2015 and finished (for the Oil-40 and Oil-80 treatments) on 11\textsuperscript{th} December 2015.

**Table 4.1. Details of crude oil application for different treatments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of oil added each time (ml)</th>
<th>Frequency of addition</th>
<th>Total volume of oil added by end of experiment (ml)</th>
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<tr>
<td>Control</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Oil-2</td>
<td>2</td>
<td>Once</td>
<td>2</td>
</tr>
<tr>
<td>Oil-10</td>
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<td>Once</td>
<td>10</td>
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<tr>
<td>Oil-20</td>
<td>20</td>
<td>Once</td>
<td>20</td>
</tr>
<tr>
<td>Oil-40</td>
<td>10</td>
<td>Every 4 weeks</td>
<td>40</td>
</tr>
<tr>
<td>Oil-80</td>
<td>10</td>
<td>Every 2 weeks</td>
<td>80</td>
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</table>

**4.3.3. Sampling regime**

Monitoring of the mesocosms took place more frequently once the crude oil additions had begun, with weekly greenhouse gas flux measurements for the first month, followed by fortnightly and then monthly measurements until April 2016 and approximately fortnightly porewater measurements until January 2016. One final set of greenhouse gas and water chemistry analyses was run in July 2016.

At the end of the experiment the mesocosms were removed from the roof and taken apart in order to undertake various analyses of soil biogeochemistry. For each mesocosm, a 3 cm deep segment of peat was extracted at both 10 cm and 20 cm, placed in separate plastic bags and stored at 4°C for 24 hours until analysis.

**4.4.4. Analytical procedures**

4.4.4.1. Mesocosm monitoring

To evaluate gas fluxes from the peat soil, transparent plastic chambers measuring 19 cm height x 13-21 cm width (Really Useful Boxes\textsuperscript{®}, West Yorkshire, UK) were used. The top of each chamber had a suba-seal valve fitted with a teflon septa to enable sampling with a
plastic syringe and needle. The chambers were placed onto the cores and locked into place, left for a period of one hour and a 20 ml sample of the headspace was extracted and immediately injected into a pre-evacuated 12 ml Exetainer (Labco, Buckinghamshire, UK). Several background samples of air were taken from outside the chambers during the procedure so that a flux for each greenhouse gas could be calculated. Gas fluxes were measured as both net ecosystem exchange (NNE), where the plastic chamber was left uncovered to allow light penetration, and as ecosystem respiration (ER), whereby black bin bags were placed on top of each chamber immediately after it had been attached to block any light and therefore exclude photosynthetic processes. By calculating NEE and ER it would then be possible to calculate gross photosynthetic production (GPP).

All gas samples were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC). The GC system was set up for greenhouse gas (methane (CH₄), carbon dioxide (CO₂) and nitrous oxide (N₂O)) analysis using only one column and two different detectors; a flame ionisation detector (FID) for a CO₂ and CH₄ and an electron capture detector (ECD) for N₂O. A 2 ml sample of gas was removed from the Exetainer using a syringe and injected into the column injector system coupled with a 1.83 m x 3.18 mm (inside diameter) stainless-steel column packed with PoroPak QS 80/100. The operational temperature of the detector and column was 40°C. Nitrogen was used as a carrier gas at a flow rate of 30 ml min⁻¹. The temperature of the methaniser was 300°C and the FID was at 125°C. The latter had a hydrogen flow of 25 ml min⁻¹ make-up gas (oxygen free nitrogen) and 300 ml min⁻¹ of compressed air. The methaniser had a hydrogen flow of 5 ml min⁻¹. The temperature of the ECD was 300°C with a constant flow of 20 ml min⁻¹ of oxygen-free nitrogen. The retention times for CH₄, CO₂, and N₂O were 1.08, 1.87 and 2.25 min, respectively. The total time for one sample was set to 3.3 min to give sufficient time for sample collection, injection, a 2.5 min chromatogram and data collection. Peak height was used for quantification of CH₄ and N₂O, and peak area for CO₂. These data gave the best linear range for the analysis. In addition, three standards of known concentration (CO₂, CH₄ and N₂O), prepared by Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK) were used to confirm accuracy.

Soil porewater samples were collected from a depth of approximately 10 cm using rhizone suction samplers (Rhizopshere, Wageningen, The Netherlands). Approximately 20 ml of
sample was collected, immediately analysed for pH and conductivity using SevenEasy and FiveGo probes, respectively (both Mettler Toledo, Leicester, UK) and filtered through a 0.45 µM syringe filter (Triple Red, Buckinghamshire, UK) into a (HDPE) plastic vial. The samples were stored at 4°C until analysis. Porewater samples were analysed for phenolic concentrations using the method of Box (1983), adapted for 300 µl microplate wells.

4.4.4.2. Soil analysis

The soil samples collected at 10 cm and 20 cm depths were analysed for water and organic content, enzyme activities and water extractable phenolics concentrations. Prior to the first analysis, each soil sample was homogenised by hand for 3 minutes and large roots and stones removed.

To calculate water and organic (loss on ignition) content of soil samples, standard methods similar to those outlined in Frogbrook et al. (2009) were followed. Porcelain crucibles were half filled (~10 g) with soil samples and heated at 105°C for 24 hours and 550°C for 6 hours, with the crucibles weighed at each stage and the mass loss calculated.

Hydrolase enzyme activities were determined by following Dunn et al. (2014). All substrates were obtained from Glycosynth (Warrington, UK), prepared by dissolving in ethylene glycol monomethyl ether (Sigma) and deionised water and stored at 4°C until required.

Soil samples were prepared for the analysis of water extractable phenolics using a similar extraction method to that described by Chantigny (2003). Five grams of peat were placed in a 50 ml centrifuge tube (Fisher Scientific, Loughborough, UK) and 40 ml of deionised water added. The tubes were placed on a KS501 orbital shaker (Ika, Staufen, Germany) at a speed of 300rpm for 24 hours, after which they were centrifuged at 5000rpm for 30 minutes on a Sorvall ST16R centrifuge (Thermo Fisher, Altricham, UK). The supernatant was filtered through 0.45 µM syringe filters (Phenomenex, Macclesfield, UK) and stored at 4°C until analysis. Phenolic compounds were determined according to the method described above.

4.4.5. Statistical analysis

Due to baseline (pre-treatment) variability between each mesocosm, for each measured parameter the treatment data was standardised similar to that described by Jones et al.
(2009) i.e. the mean value for the baseline period (which comprised at least four time points) for each mesocosm was subtracted from the value at each time point once the treatments began. The post-treatment data is therefore expressed as a change in the value of the measured parameter from the baseline period.

Most of these calculated data met the homogeneity of variance and normality assumptions, which were tested using the Bartlett and Shapiro Wilk tests, but those that did not were log-transformed. Data points were removed if they were outliers using the method of Davies and Goldsmith (1972). An overall two-way ANOVA was used to test for the factors Treatment (6 levels; Control, Oil-2, Oil-10, Oil-20, Oil-40, Oil-80) and Date for each measured parameter. Following this, one-way ANOVA was run to test the effect of Treatment on each date. If a significant main effect was observed, a pairwise t test was run to determine which of the treatments where oil was added were significantly different from the Control. All tests were run in R v 3.3.1. A $p$ value of 0.05 was used to determine significance.

4.5. Results

4.5.1. Mesocosm monitoring

Crude oil was added to the mesocosms on 21st August 2015 and a further three times (until 13th November 2015) for the Oil-40 treatment and a further seven times (until 27th November 2015) for the Oil-80 treatment. Figures 4.1-4.2 present porewater chemistry (phenolics and pH) and figures 4.3-4.5 greenhouse gas fluxes for the monitoring period, which involved sampling every 2-4 weeks until January 2015 (porewaters) and April 2015 (greenhouse gases), with a final set of samples collected in the summer (July 2016).

4.5.2. Phenolics and pH

In the first few weeks after the initial addition of crude oil it was observed that concentrations of phenolic compounds were higher in all treatments compared to the control apart from the 80 ml (10 ml every 2 weeks) treatment (Figure 4.1). Differences between treatments were largely consistent until January 2016 but then the phenolics concentration increased sharply to July 2016 for the Oil-40 and Oil-80 treatments. All treatments were above the Control by the end of the experiment, with the Oil-40 treatment being significantly so on the last sampling date ($p < 0.01$).
All six treatments generally displayed similar trends for pH for the first few weeks (Figure 4.2). As with phenolics, the Oil-40 and Oil-80 diverged from the rest, including the Control, by July 2016, with the Oil-40 treatment being significantly higher on this date ($p < 0.05$).

![Figure 4.1](image.png)

**Figure 4.1.** Change in porewater phenolics concentration from baseline period during treatment phase for peat mesocosms supplied with varying quantities of crude oil. Significant differences at specific dates between the Control and treatments with crude oil added are indicated by different coloured stars (* = $<0.05$, ** = $<0.01$, *** = $<0.001$).
Figure 4.2. Change in porewater pH from baseline period during treatment phase for peat mesocosms supplied with varying quantities of crude oil. Significant differences at specific dates between the Control and treatments with crude oil added are indicated by different coloured stars (* = <0.05, ** = <0.01, *** = <0.001).

4.5.3. Greenhouse Gases

Measurements of net ecosystem exchange (NEE) were undertaken using transparent chambers, ecosystem respiration (ER) using opaque chambers, and gross photosynthetic potential (GPP) derived from both of these fluxes. NEE represents the carbon balance of an ecosystem, with a mean positive value indicating net carbon emissions from the soil to the atmosphere and a mean negative value the opposite (i.e. net carbon sequestration). Figure 4.3 shows the mean NEE fluxes of all six treatments over the course of sampling; the NEE was mostly negative, indicating the mesocosms were usually sequestering carbon, but there was a big increase on the final sampling date (4th July 2016) for all treatments, when all fluxes were positive. For the first three months there were no significant differences between the six treatments but by 17th November 2015 the Oil-80 treatment had a significantly higher flux than the Control (p < 0.01) and on 8th December 2015 both the Oil-40 (p < 0.05) and Oil-80 (p < 0.01) were significantly higher than the Control. Neither of these treatments were significantly different to the Control on the next and penultimate
sampling occasion (3rd March 2016) but on 4th July 2016 the NEE flux was almost five times greater than the Control and this difference was highly significant ($p < 0.001$). Despite the NEE flux of the Oil-40 treatment being more than twice as high as the Control treatment this was not statistically significant.

Figure 4.4 shows the ecosystem respiration (ER) fluxes over time for the six treatments. The general trend was higher fluxes in the warmer, summer months (August 2015 and July 2016) and lower fluxes in the colder months. The only date on which significant differences between the oil addition treatments and Control were detected was on the final sampling date, 4th July 2016. Both the Oil-40 and Oil-80 treatments had an ER flux approximately half that of the Control ($p < 0.05$).

A gross photosynthetic potential GPP flux was calculated by subtracting the ER value from the NEE and is shown in Figure 4.5. The trend over time was similar to the ER flux data, with the greatest negative flux in the summer months. At all time points none of the oil addition treatments were significantly different to the Control, even on 4th July 2016 when the Oil-40 and Oil-80 treatments strongly diverged. The lack of significance is probably due to the high standard error of the Control treatment on this date.

![Figure 4.3](image)

**Figure 4.3.** Net Ecosystem Exchange (NEE) of CO$_2$ from the first time point after adding crude oil. Significant differences at specific dates between the Control and treatments with crude oil added are indicated by different coloured stars ($* = <0.05$, **$ = <0.01$, ***$ = <0.001$).
**Figure 4.4.** Ecosystem Respiration (ER) of CO₂ from the first time point after adding crude oil. Significant differences at specific dates between the Control and treatments with crude oil added are indicated by different coloured stars (* = <0.05, ** = <0.01, *** = <0.001).

**Figure 4.5.** Gross Photosynthetic Potential (GPP) of CO₂ from the first time point after adding crude oil. Significant differences at specific dates between the Control and treatments with crude oil added are indicated by different coloured stars (* = <0.05, ** = <0.01, *** = <0.001).
4.5.4. Soils

At the end of the experiment, samples of peat from within each mesocosm were extracted at two depths (10 cm and 20 cm) and several analyses conducted to further investigate the impacts of the crude oil additions on decomposition processes within the soils. Two-way ANOVA tested for the effects of Treatment (each of the six aforementioned treatments) and Depth (10 v 20 cm) and if a significant treatment effect was observed a further one-way ANOVA and post-hoc analysis was run for each depth separately. The results of the two-way ANOVA analysis are presented in Table 4.2. Here, the effect of the Treatment factor is discussed. Of the nine measured soil parameters a significant Treatment effect was only observed for pH. One Way-ANOVA revealed a significant treatment effect for the 20 cm data ($F=4.24, p=0.0049$) but not the 10 cm data (although the latter was significant at the 10% level). For 20 cm, post hoc analysis showed the Control and Oil-80 treatments to be significantly different ($p < 0.05$), with the Oil-80 pH value being approximately 0.3 greater than the Control pH value. The Oil-80 treatment also had the highest pH of all six treatments at 10 cm, and the overall trend between treatments was similar to that for 20 cm, but the difference compared to the Control was not quite significant. There were no significant effects of treatment on soil water or organic content, phenolics (despite the significant differences observed by the end of the experiment for the porewaters) or enzyme activities.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>Factor</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content</td>
<td>Treatment</td>
<td>0.90</td>
<td>0.489</td>
</tr>
<tr>
<td></td>
<td>Depth</td>
<td>10.62</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Treatment:Depth</td>
<td>0.44</td>
<td>0.822</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>1.44</td>
<td>0.244</td>
</tr>
<tr>
<td>Soil organic matter content</td>
<td>Depth</td>
<td>30.41</td>
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</tr>
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<td></td>
<td>Treatment:Depth</td>
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<td>0.408</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>5.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH</td>
<td>Depth</td>
<td>2.13</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>Treatment:Depth</td>
<td>1.32</td>
<td>0.265</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Treatment</td>
<td>Depth</td>
<td>Treatment:Depth</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------</td>
<td>-------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>Phenolics</strong></td>
<td>0.72</td>
<td>29.05</td>
<td>1.36</td>
</tr>
<tr>
<td><strong>β-glucosidase</strong></td>
<td>0.50</td>
<td>0.50</td>
<td>1.21</td>
</tr>
<tr>
<td><strong>Sulphatase</strong></td>
<td>0.96</td>
<td>16.24</td>
<td>1.62</td>
</tr>
<tr>
<td><strong>Xylosidase</strong></td>
<td>0.96</td>
<td>0.96</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Chitinase</strong></td>
<td>7.58</td>
<td>7.58</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Phosphatase</strong></td>
<td>38.76</td>
<td>38.76</td>
<td>1.33</td>
</tr>
<tr>
<td><strong>Phenol Oxidase</strong></td>
<td>1.73</td>
<td>1.73</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 4.2. Results of two-way ANOVA, testing the effects of the Treatment and Depth factors and their interaction on various measured parameters. Values presented in bold are statistically significant.
4.6. Discussion

The results of this study have shown a clear effect of adding exogenous phenolic compounds, via crude oil, on peatland carbon cycling and emphasise the important role that phenolics compounds play in regulating the emission of CO$_2$ from soil. The current study has demonstrated that adding crude oil to peat will suppress decomposition and reduce the flux of CO$_2$ to the atmosphere. The treatments which were supplied with the greatest quantity of crude oil (Oil-40 and Oil-80) demonstrated higher phenolic compounds and reduced ER CO$_2$ fluxes by the end of the experiment. Freeman et al (2012) have proposed that enhanced carbon storage in ecosystems, particularly in peatlands, is feasible by modifying soil phenolic content in order to inhibit decomposition of organic matter by a mechanism known as the “enzymic latch” (Freeman et al. 2001). They proposed that increases in phenolic content in peat ecosystems can be achieved either by increased expression of phenolic inhibitors from peatland plants or by enhancement of enzyme latch by physicochemical modification (Min et al. 2015). This study suggests that it may prove possible to achieve reduced soil decomposition rates, which would be expected to lead to enhanced below-ground carbon storage, through exogenous phenolic addition with low cost supplements. Some studies have showed that if the oil is toxic enough, or added in sufficient quantity, to cause mortality of the decomposers (invertebrate or microbial), then decomposition can be inhibited with the absence of photosynthesis (Siddiqui and Adams 2002). Despite the statistically significant decrease in ER, the NEE flux of both the Oil-40 and Oil-80 actually increased sharply at the end of the experiment, much more so than the control treatment. This suggests the peat became less efficient at sequestering carbon, which, given the decrease in ER, must be because the vegetation became less able to take up carbon through photosynthesis. The increase in NEE compared to the Control of the Oil-40 and particularly the Oil-80 treatments must mean that the larger quantity of crude oil damaged the vegetation and impaired photosynthesis (i.e. the carbon input pathway) to a greater extent than the phenolics in the crude oil reduced the CO$_2$ soil decomposition flux (i.e. the carbon output pathway). This is highlighted in the data for the GPP flux, which showed a dramatically impaired rate of GPP for both the Oil-40 and Oil-80 treatments by the end of the experiment. We observed during the experiment that the vegetation became affected
by the crude oil, with reduced growth and much less of the typically vibrant green colour of healthy mosses.

Crude oil can cause environmental damage through several mechanisms, including affecting gas exchange and net photosynthesis. Oil can inhibit the rate of photosynthesis as described by Riedhart (1961) who found that oil-treated plants showed increased CO$_2$ fluxes. By monitoring the vegetation (Sphagnum) of mesocosms, it has been observed that the degree of photosynthesis inhibition may be affected by the quantity of crude oil addition (Riehl and Wedding 1959). The result of the present study also indicates that high concentrations of crude oil (Oil-40 and Oil-80) can result in death of most plant species. The tissue that is situated on the top layer of ground of the wetland vegetation can be badly affected by crude oil as it is the first one to absorb the oil. Crude oil may also affect tissues below the ground as a result of reduced gas exchange between peat soil and atmosphere which can disrupt root membranes (DeLaune et al. 2003). It can also impact on microbial processes which in turn modifies biogeochemical processes (Leahy and Colwell 1990, Shin et al. 2000). A complicated group of microorganisms can make use of a variety of electron acceptors (e.g., oxygen, nitrate, sulphate and iron oxyhydroxides) in order to stimulate the degradation process of saturated and aromatic hydrocarbons (Widdel and Rabus 2001). Generally, the presence of crude oil in wetlands reduces the diversity of microorganisms and increases the abundance of species that have the ability to utilize carbon sources.

Despite the increase in phenolics we did not measure any impacts on hydrolase enzymes. pH was observed to increase in both the porewater samples and soil water extracts in the Oil-40 and Oil-80 treatments. Guggenberger et al. (1994) and Njoku et al. (2009) also found that petroleum contaminated soil has higher pH compared with the natural background. It is possible that this may enhance decomposition as conditions become more favourable for soil microbes.

Many carbon capture and storage strategies involve significant financial investment. Relatively little attention has been paid to the potential of ecoengineering strategies to enhance carbon sequestration in peatlands, which, in their undisturbed state are natural carbon sinks. Our study suggests there is potential for crude oil addition to enhance carbon sequestration in peatland environments, but that care must be taken to avoid toxicity in
surface vegetation. Further research is necessary to establish the complex biogeochemical response of peatlands to crude oil application.

4.7. Acknowledgements

This research was conducted as part of Adel Alshehri’s PhD project, which was funded by the government of Saudi Arabia.
4.8. References


Chapter 5: The effects of phenolic compound molecular weight on carbon sequestration in peatlands
The effects of phenolic compound molecular weight on carbon sequestration in peatlands

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5.1. Abstract

Northern hemisphere peatlands represent about one-third of the global soil carbon pool. Phenolic inhibitors allow carbon to accumulate in peat soil due to their ability to slow the rate of decomposition. Phenol oxidases are one of the few enzymes that are capable of decomposing inhibitory phenolic compounds. Phenol oxidase suppression in peat soils is known to cause the accumulation of phenolic compounds, which, in turn, can prevent hydrolase enzymes from breaking down organic matter. Here we investigate using phenolic inhibitors of different molecular weights, the effect of molecular weight on the activity of β-glucosidase enzyme and the consequent reduction in carbon dioxide release. We investigate the importance of the molecular weight of phenolic and examine whether higher molecular weight phenolic compounds show a greater inhibitory effect on levels of inhibition of microbial decomposition in peat. In this study, we found that the molecular weight of phenolic inhibitors does not determine how much the compound supresses decomposition, which appears to be random. The inhibitory effect of Sodium salicylate (Mw 160.1 g/mol) was greatest on β-glucosidase enzyme activity and carbon dioxide fluxes.

Keywords: Peatlands, Phenolics, Hydrolase enzymes, Carbon dioxide, Molecular weight, Salicylate, Geoengineering.
5.2. Introduction

Phenolic compounds are utilized and involved in various plant processes. Even though they can be produced naturally as a consequence of the breakdown of humic substances, tannins and lignins, various industrial procedures, involving manufacture of drugs, textiles, dyes, pesticides and paper, are the principal source of these compounds in the environment (Neilson et al. 1991, Moore and Ramamoorthy 1984). Natural sources of these compounds include plants (Hattenschwiler and Vitousek 2000) which produce secondary metabolites including phenolic acids, flavonoids, tannins and lignins. Because of the broad definition of phenolics (occurrence of at least one aromatic ring and hydroxyl group), to date over 8000 phenolic compounds have been identified (Dai and Mumper 2010), including simple, low molecular compounds and complex, highly polymerized compounds. The number of aromatic rings and chemical structure are used to categorize phenolic compounds. There are two divisions of the phenolic acid: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Tannins are normally also sub-grouped into two classes; 1) hydrolysable tannins and 2) condensed tannins. Hydrolysable tannins are substances comprising a main core of glucose or some other polyol esterified with gallic acid, termed gallotannins, or with hexahydroxydiphenic acid, termed ellagitannins (Dai and Mumper 2010). Lignin is the most common compound associated with the flavonoids and is an important constituent of wood. It has a large intricate macromolecule that exists in the plant cell wall and is linked with cellulose and hemicellulose (Faulon and Hatcher 1994). The structure of phenolics comprises one or more aromatic rings, to which one or more hydroxyl functional groups are attached. After accumulating in the soil, phenolics can regulate below ground biogeochemical processes, including soil organic matter (SOM) decomposition (Freeman et al. 2001 and Toberman et al. 2010) and nutrient cycling (Schimel et al. 1998 and Kraus et al. 2004).

According to Freeman et al (2012), phenolic compounds could be used as a geoengineering tool to enhance carbon sequestration in terrestrial ecosystems. Peatlands sequester organic carbon below ground because of the inhibitory effects of phenolic compounds on enzymic decomposition processes; termed the ‘enzymic latch’. Constraints on a single class of enzymes, phenol oxidases, has been proposed as the principal factor responsible for the
accumulation of vast carbon deposits held in peatlands (Freeman et al. 2001a, Freeman et al. 2001b, Fenner and Freeman 2011).

Phenol oxidases are one of the few enzymes that are capable of decomposing inhibitory phenolic compounds. These enzymes require oxygen to function effectively (McLatchey and Reddy 1998). Peat tends to be anoxic, therefore phenol oxidase is suppressed, which permits an accumulation of phenolic compounds, which, in turn, prevent hydrolase enzymes from breaking down organic matter (Wetzel 1992, Appel 1993), thereby facilitating sequestration of large amount of carbon (UNEP / GRID Arendal 2009). Phenolic compounds are present in all vegetation, making up to 60% of plant dry mass on average (Cates and Rhoades 1977).

The simplest phenolic, phenol for instance, consists of one aromatic ring with no additional carbon and is part of a group of basic phenols, the simplest form of phenolic compounds. Phenolic acids consist of a simple structure based around a C6-C1 skeleton, and include gallic acid, vanillic acid and syringic acid. Lignin as one of the most common compounds in plants exhibiting various arrangements of C6-C3 structure. Phenolics can be present in the soil either as soluble form, which can move easily in the soil solution, a sorbed form, which reversibly binds to the soil particles and proteins, or in polymerized form, consisting of humic substances connected with other soil organic matter. As several phenolic compounds including phenolic acids and tannins are soluble, they tend to remain in solution in soil pore water (Hebatpuria et al. 1999).

Reversible sorption of phenolics to soil particles occurs by way of hydrophobic, hydrogen and ionic bonding (Appel 1993). Humic constituents are produced by the polymerization of phenolics with other phenolics or soil organic matter (Kraus et al. 2003). Recent studies suggest that the state of phenolic compounds (dissolved or particulate), not their chemical structure, can impact their fate in soil (Schmidt et al. 2011, Gleixner 2013), challenging the conventional classification of phenolics as a slow, recalcitrant carbon source in carbon dynamics climate modelling (Parton et al. 1987). For instance, dissolved phenolics in the soil solution may be more likely to come into contact with microorganisms than sorbed or polymerized phenolics. By contrast, physically and chemically protected phenolics can persist longer than dissolved forms, influencing soil pH, nutrient accessibility, and enzyme
actions. This complex picture highlights the importance of further research into the role of phenolics in SOM decomposition.

This study aims to investigate whether higher molecular weight phenolic compounds, show a greater inhibitory effect on the peat decomposition processes which release the CO$_2$ and CH$_4$ from microbial respiration and will provide information on differences in their inhibitory effect on extracellular hydrolase enzymes in peat slurries taken from a blanket bog in the UK.

5.3. Materials and Methods

In total of eleven phenolic-based compounds of varying molecular weight were tested for their impacts on soil enzyme activities (Table 5.1).

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>94.11 g/mol</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td>154.12 g/mol</td>
</tr>
<tr>
<td>Sodium salicylate</td>
<td>160.1 g/mol</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>170.12 g/mol</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>1701.1 g/mol</td>
</tr>
<tr>
<td>Lignosulfonic acid sodium salt</td>
<td>8000 g/mol</td>
</tr>
<tr>
<td>Lignin alkali</td>
<td>10,000 g/mol</td>
</tr>
<tr>
<td>Sodium lignosulphonate acid</td>
<td>12,000 g/mol</td>
</tr>
<tr>
<td>Lignosulfonic acid calcium salt</td>
<td>18,000 g/mol</td>
</tr>
<tr>
<td>Calcium Lignosulphonate acid</td>
<td>49,000 g/mol</td>
</tr>
<tr>
<td>Lignosulfonic acid sodium salt</td>
<td>52,000 g/mol</td>
</tr>
</tbody>
</table>

Table 5.1. List of phenolic compounds inhibitors
These compounds are all commercially available, synthesised versions of naturally occurring phenolic compounds associated with vascular plant metabolism or degradation (Hattenschwiler and Vitousek 2000, Lattanzio et al. 2006) and were chosen to represent a range of molecular weights, from low to high, which are easily soluble in water. For each compound, a 5% weight/volume (w/v) solution was created by adding 0.25 g compound and 50 mL ultrapure water to a centrifuge tube (Fisher Scientific UK Ltd, Loughborough, UK), to create a final concentration of 5 g/L, and mixed on an orbital shaker at 200rpm for 24 hours to ensure the compounds were completely dissolved. For each compound, 10 mL of solution was poured into four separate centrifuge tubes, each containing 10g samples of peat which had been homogenized by hand for 10 minutes. There was also a control treatment, for which no additional compound was added. The peat solutions were mixed using an orbital shaker at 200rpm for 24 hours. The mixtures were kept in the dark under anaerobic environments (in a nitrogen environment within a zip lock type AtmosBag glove bag (Sigma Aldrich Ltd, Dorset, UK)) and at constant field temperature.

Following the incubation period, the flux of greenhouse gases was determined using a laboratory closed chamber technique. This involved replacing the centrifuge tube lids with ones containing a rubber septum and taking a 10 mL sample of gas after the lids had been in place for 1 hour. The gas samples were removed using a syringe and hypodermic needle and injected into pre-evacuated 6 mL Exetainers (Labco Ltd, Buckinghamshire, UK). The same procedure was repeated for 4 empty centrifuge tubes, which would provide the background concentrations necessary to calculate fluxes (the rate of flow).

The gas samples were analysed for CO$_2$ using a Varian 450 gas chromatograph (GC). The GC consists of a methaniser (temperature 380°C) and flame ionisation detector (FID) at 125°C. Two mL of gas was extracted from the Exetainers using a CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland) and injected onto a 1.83 m x 3.18 mm PoroPak QS 80/100 column (40°C). Oxygen-free nitrogen, at a flow rate of 30 mL/min, was used as the carrier gas. Gas fluxes were calculated after subtracting the mean background concentration from the concentration after 1 hour of incubation.

Using a technique described by Freeman et al (1995) and Dunn et al (2014), the activity of the hydrolytic enzyme β-glucosidase was determined. For each replicate, one gram of peat
was measured into stomacher bags and 7 mL of 400 μM 4-MUF β-D-glucopyranoside substrate added. This was homogenized for 30 seconds using a Seward Stomacher Model 80. Alongside this, a stomacher bag containing 1 gram of peat and 7 mL of ultrapure water was homogenized by the same procedure. The stomacher bags were then kept at field temperature for 1 hour before pipetting 1.5 mL of solution from every stomacher bag into 1.5 mL centrifuge tubes and centrifuging at 10,000 rpm for 5 minutes. 0.3 mL of the supernatant from each vial was transferred to a micro-plate and the fluorescence determined at 330 nm excitation and 450 nm emission, with a slit setting of 2.5 nm, using a SpectraMax M2e spectrophotometer (Molecular Devices, Wokingham, UK). For calibration standards, the supernatant from the stomacher bag that contained ultrapure water was transferred to wells of the microplate containing 50 μL of known concentrations of MUF-free acid (5 to 100 μM), which allowed for the calculation of the concentration of enzyme product in the samples and subsequently the activity of β-glucosidase, in μmol MUF g⁻¹ min⁻¹.

Samples were analysed for phenolics using a method adapted from Box (1983). To separate 1.5 mL centrifuge tubes, 1 mL of sample of the peat-slates was added, followed by 50 μL of Folin-Ciocalteau phenol reagent (Sigma, Gillingham, UK) and 0.15 mL of Na2CO3 (200 g/L) (Sigma) to buffer the reaction. The process was repeated for calibration standards made from phenol compound (Sigma) in the range 0.5 to 30 mg/L. After 1.5 hours 300 μL of each sample and standard were transferred to wells of a clear 96 well microplate (Triple Red, Long Crendon, UK) and absorbance measured at 750 nm on a SpectraMax M2e spectrophotometer (Molecular Devices, Wokingham, UK).

The pH of the pore water samples were taken using a Mettler Toledo FE20 Desktop pH meter, which was calibrated with two buffers of known pH (4 and 7). For data analysis pH values were transformed to H⁺ concentrations (μmol/L) assuming pH is the negative logarithm of the hydrogen ion concentration.
5.3.1. Study site and sampling

Peat samples for this study were collected from the Migneint blanket bog, in Snowdonia, Wales, UK. The area is dominated by *Sphagnum papillosum, Calluna vulgaris* and *Eriophorum vaginatum*. Annual rainfall is 2.4 m and the site is 460 m a.s.l. The mean peat depth across the site is 2.0 m and mean water table depth approximately -8 cm (Evans et al. 2012).

Several peat samples were collected in April 2016 across a representative 20 m$^2$ area of the bog, at a depth of 5-30 cm. After the removal of large root samples were placed inside sealable plastic bags and transported to the laboratory where they were stored at 4°C until analysis. They were kept in an incubator at the recorded field soil temperature 24 hours prior to the start of the experiments.

5.3.2. Statistical analyses

The effect of supplementing peat soil with phenolics using a range of different molecular weight phenolic compounds on levels of inhibition of microbial decomposition in peat (phenolics concentration, hydrolase enzyme activities and greenhouse gas fluxes) were determined using one-way ANOVA and Tukey HSD post-hoc tests in R v3.3.1. Relationships between variables were measured using Pearson’s correlation coefficient (r). Most data met the homogeneity and normality assumptions, which were tested using the Bartlett and Shapiro Wilk tests, but those that did not were log-transformed. All statistical tests were performed using the MIXED procedure of SAS (SAS Statistical System Software, v. 9.2, SAS Institute Inc., Cary, NC, USA.). Following the ANOVAs, protected Fisher’s LSDs were run when a significant difference between treatments was found. Data met the homogeneity and normality assumptions. All treatments were included in the analyses, the experimental design ensured that data were in a continuous, interval ratio format and measurements from the different treatment groups were independent.
5.4. Results

There was a significant main effect of molecular weight on all six dependent variables (Table 5.2), but few significant correlations (Table 5.3). The pH of the water extracts ranged between 3.460 – 5.122 (Table 5.2), with sodium lignosulphonate acid (Mw 12000 g/mol) having the highest pH and gallic acid (Mw 170 g/mol) the lowest. Post-hoc analysis revealed all eleven treatments were significantly different compared to the Control, but there was no significant correlation between molecular weight and pH. pH correlated significantly with β-glucosidase activity and N\textsubscript{2}O emissions, but only weakly.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>F value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>755.150</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Phenolics</td>
<td>509.395</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>18.176</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td>8.852</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CH\textsubscript{4}</td>
<td>2.436</td>
<td>0.017</td>
</tr>
<tr>
<td>N\textsubscript{2}O</td>
<td>26.582</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 5.2. Results of one-way ANOVA analysis to test the effect of Molecular Weight on several dependent variables.

<table>
<thead>
<tr>
<th>Molecular Weight</th>
<th>pH</th>
<th>Phenolics</th>
<th>β-glucosidase</th>
<th>CO\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson Correlation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>Pearson Correlation</td>
<td></td>
<td>.278\textsuperscript{*}</td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td></td>
<td>.031</td>
<td></td>
</tr>
<tr>
<td>Phenolics</td>
<td>Pearson Correlation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>Pearson Correlation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3. Results of Pearson correlation analysis, showing significant correlations only.
Figure 5.1. pH of water extracts after mixing peat samples with phenolic-based treatments of varying molecular weight. Control (---).

The concentration of phenolics in solution varied dramatically depending on the molecular weight of the compound, reflecting the extent to which phenolic components comprised the structure of the molecule. Post-hoc analysis revealed all but 3 of the compounds had significantly different phenolic concentrations compared to the Control; Tannic acid (Mw 1701 g/mol), Lignosulfonic acid sodium salt (Mw 8000 g/mol) and Lignin, alkali (Mw 10000 g/mol). All other treatments had significantly higher phenolic concentrations compared to the Control, especially those of low molecular weight. Phenolic concentration did not correlate with any other variable, including molecular weight.
The activity of $\beta$-glucosidase was lower in all eleven treatments compared to the control, although post-hoc analysis showed that the effect was significant for only 6 of those; Phenol (Mw 94 g/mol), 3,4-Dihydroxybenzoic acid (Mw 154 g/mol), Sodium salicylate (Mw 160 g/mol), Gallic Acid (Mw 170 g/mol), Lignosulfonic acid calcium salt (Mw 18,000) and Calcium Lignosulphonate acid (Mw 49000). The greatest suppression was for Sodium salicylate, for which the activity of $\beta$-glucosidase was actually undetectable.
Despite all of the treatments lowering β-glucosidase activity compared to the Control, suggesting impaired decomposition processes, there was no comparable effect on CO₂. All but 2 treatments actually had greater CO₂ emissions compared to the Control, but only one was significant; Gallic Acid (Mw 170 g/mol). The emission of CO₂ from the Sodium salicylate treatment was below detectable levels. CO₂ emissions did not correlate with any other measured variable.

**Figure 5.4.** CO₂ emissions from peat slurries after mixing peat samples with phenolic-based treatments of varying molecular weight. Control (-----).

5.5. **Discussion**

The main objective of this study was to test whether molecular weight influences the extent to which phenolic-based compounds are able to suppress decomposition processes in peat. As it has already been proven that the addition of phenolic compounds to peat can suppress decomposition (Chapter 1 and 3), examining the influence of molecular weight is a relevant avenue of research that could lead to refinement of the phenolic addition to peat approach as a valid geoengineering tool (Freeman et al. 2012). There is a general trend for the inhibitory effects of polymeric phenolics to increase with molecular weight, however, the effects of monomeric phenolics can either inhibit or enhance activities. Our data shows no consistent impact of molecular weight, with no significant correlations observed between
molecular weight and the two parameters that we used to determine the rate of decomposition; β-glucosidase activity and CO₂ emission.

The addition of Sodium salicylate to peat resulted in both β-glucosidase activity and CO₂ emissions falling to undetectable levels, suggesting that this compound is a potent inhibitor of microbial activity which warrants further research.

Sodium salicylate is a sodium salt of salicylic acid which is manufactured by a direct solid gas (Kolbe-Schmitt) reaction between Sodium phenolate and Carbon dioxide (Lehman 2009). Previous studies have revealed that Salicylate inhibits the activities of a number of cellular enzymes and in some instances the mechanisms of inhibition have been established (Smith 1968). Exogenously added Salicylic acid changed the biogeochemical process in peat soil, which further influenced the soil bacterial metabolic activity, and changed the functions of bacterial communities, which might be related to decomposition inhibition by phenolic compounds added (Freeman et al. 2001).

Our data showed that there was no direct correlation between the molecular weight of phenolic compounds and enzymes activity or CO₂ fluxes, which can be attributed to the complex and irregular structure of the polymer, which is intimately mixed with carbohydrate components (Brunow 2005). Moreover, the phenolic compounds form complexes with protein molecules, serving as polydentate ligands, causing inactivation of the hydrolase enzymes through competitive and non-competitive inhibition (Wetzel 1992). Therefore, the structure of Sodium salicylate makes it an excellent experimental compound in our study since the polyphenols can bind to the reactive sites of proteins and other organic and inorganic substrates rendering them inactive to further chemical activity and biological attack (Muscolo and Sidari 2006).
5.6. References


Chapter 6: Final discussion
6.1. Introduction

Peatlands play an important role in reducing climate change, due to their ability to sequester carbon. It is estimated that over the past 10,000 years the atmospheric carbon stored in peats has served to reduce global temperatures by about 1.5–2.8°C (Holden 2005). Despite the seriousness of global warming and a number of attempts in recent decades to mitigate against climate change (Chapter 1), no serious attempts have been made to improve and manage carbon sequestration in peatlands. From this point of view, the aim of this project was to restore degraded peatlands using phenolic supplements to enhance carbon sequestration using Canadian peat (Chapter 2 and Chapter 3) and Welsh peat (Chapter 4 and Chapter 5). The use of phenolic inhibitors was not limited to natural materials such as wood, but also included waste materials such as crude oil (Chapter 4). Data in this thesis have shown that it is possible to promote carbon sequestration in peatlands by adding inhibitory phenolic compounds.

6.2. The effect of exogenous phenolic compounds on extracellular enzymes activity

Enzymes are indicators of microbial metabolism and biochemical processes, and have great importance in peatland systems. Phenolic compounds are thought to be recalcitrant to decomposition, and inhibit the activity of hydrolase enzymes (Sun 2010). The measurement of extracellular enzyme activities has been a key component of this study, since they allow for the examination of “enzymic latch” mechanisms which suggest that exogenous phenolic inhibitors can be added to the peat to suppress enzymic decomposition (Freeman et al. 2001). In Chapter 2, a significant negative relationship was observed between the mean change in both phenolics concentration and β-glucosidase activity (Figure 2.5). The concentration of phenolics was manipulated by adding Spruce wood chips to Canadian peat that have already suppressed microbes and thus inhibited the most important hydrolase enzyme, β-glucosidase activity. Freeman et al. (2012) states that it is possible to strengthen the enzymic latch by increasing the abundance of phenolic inhibitors, or manipulating edaphic factors that slow decomposition in peatlands. However, significant relationships were not observed between phenolics and any of the other hydrolase enzymes or the CO₂ flux. We suggest that there was an initial disturbance effect resulting from the addition of
wood chips which led to a short-term increase in decomposition rates (as indicated by elevated CO$_2$ flux), so we have suggested soaking the wood chips in lignin solution prior to addition as a means of preventing the initial disturbance effect. It is hypothesized that the lignin solution will act as a readily available source of phenolics that will supress decomposition in the initial stages, with the wood chips acting as a longer-term source of phenolics for a sustained effect (Horner 1988). It is therefore surprising that as phenolic concentration increased, there was no suppression of enzymes activities (Chapter 3) given the theory behind phenolic suppression and the results of Chapter 2. This may be due to the sensitivity of the hydrolases enzymes to other conditions such as temperature, pH, and the nature and strength of ions which may have varied between samples (Michaelis and Menten 1913). As highlighted by Kang and Freeman (1999) the alternative approach, namely modifying conditions towards the enzymic optima, gives information on potential enzyme activity in the sample. However, the actual activity may be markedly different. Optimising factors such as pH and temperature could therefore lead to overestimataion of the enzyme activities occurring in natural conditions (Freeman et al. 1995). Moreover, the exposure of lignin to aerobic conditions over a prolonged period may have led to structural changes since lignin is known to be very sensitive to oxidation (Chang and Allan 1971). The present study also investigated the relationship between increases in phenolic concentrations and hydrolase enzymes activity. In general, there was no clear correlation between increased concentration of phenolic compounds and inhibition of enzyme activity despite the diversity of phenolic compounds used, including crude oil (Chapter 4) and commercially available phenolic compounds (Chapter 5), which proved that the sodium salicylate compound had a clear effect in terms of suppressing β-glucosidase activity compared to the control, but there is no significant correlation between this compound and the activity of β-glucosidase. However, as mentioned above, other factors may directly or indirectly influence enzyme activity when the concentration of phenolics is increased in peat. It is possible that the enzymes protocol could be improved by taking into account the experimental conditions; for instance, temperature, pH and also the chemical structure of samples (Bisswanger 2014). Additionally, our results suggest that when phenolic compounds are added to the peat, the quantity and quality of the compounds should be considered, because this will affect enzyme suppression and, in turn, decomposition (Freeman et al. 2001).
6.3. Effect of exogenous phenolic compounds supplements on carbon dioxide fluxes

All the samples in this study were analyzed for carbon dioxide (CO$_2$) flux as a significant indicator of their impact on climate change. For example, peatland carbon fluxes are affected by warming (Silvola et al. 1996, Fenner et al. 2006, Kim et al. 2012), elevated carbon dioxide concentrations (Fenner et al. 2007, Ellis et al. 2009b) and water table (Silvola et al. 1996, Fenner and Freeman 2011). It may be possible to enhance this effect by maintaining the lack of oxygen whilst adding supplementary phenolic compounds to the soil to increase hydrolase enzyme inhibition and lower soil CO$_2$ emissions even further. The key to restricting the rise in the carbon flux is held by the “enzymic latch” (Freeman et al. 2001a). Lovett et al. (2006) defined net ecosystem exchange (NEE) as gross primary production (GPP) – ecosystem respiration (ER). Defined in this way, NEE is conceptually simple and analogous to NPP (photosynthesis minus the respiration of primary producers) which has been measured in Chapters 3 and 4. In Chapter 3, there was no significant effect with the wood chips soaked in lignin solution treatment for NEE, ER or GPP over the period of experiment, while the crude oil treatments which were supplied with the greatest quantity of crude oil (Oil-40 and Oil-80) demonstrated higher phenolic compounds and reduced ER CO$_2$ fluxes by the end of the experiment (Chapter 4). Our study found that, for NEE, the addition of supplementary phenolic compounds of both wood chips mixed into the peat soil and sodium salicylate compound are effective in strengthening the enzymic latch and enhancing carbon sequestration (Chapters 2 and 5), possibly due to the structure, quantity and quality of the phenolic compounds added. However, the method used to measure CO$_2$ varied from one experiment to another, as well as the type of peat (Canadian peat in Chapters 2 and 3) and the materials used such as the mesocosoms (Chapter 4) or centrifuge tubes (Chapter 5), which may have influenced the result. The advantage of measuring soil respiration from slurries (Chapter 5) in this research project, rather than intact peat samples, is that experimental compounds can be added to the samples either directly as a solid (powder) or a solution, and evenly distributed during the thorough homogenisation of the samples. The disadvantages to this method are that the water content of the peat is altered and the homogenisation can disrupt the peat matrix and structure. Moreover, temperature and mass of peat or peat slurry with surface area of the peat available for gaseous exchange may be important factors in the accuracy of the results.
6.4. Effect of vegetation on carbon sequestration

The vegetation growing on an area of peat plays a critical role in controlling the soil’s biogeochemistry (Ward et al. 2009, Sutton-Grier and Megonigal 2011). In certain types of peat (e.g. those supporting *Sphagnum* vegetation) factors such as the presence of plant toxins may be important in governing rates of decomposition. It is thought that the preservation of 'bog bodies' is due to the properties of certain degradation products of *Sphagnum*, namely oxopolysaccharide and phenolic compounds which allow tissue preservation and suppress microbial activities in soil (Borsheim et al. 2001, Painter 1983, 1991, Freeman et al. 2001a). Additionally, *Sphagnum* mosses produce a number of phenolic compounds to inhibit microbial and fungal growth and the decomposition of organic matter (Bragazza et al. 2006, Turetsky et al. 2008). In this thesis, it was necessary to have vegetation in peat samples because of its importance in suppressing microbial enzymic decomposition and thus promoting sequestration of huge stores of carbon (UNEP / GRID 2009). Our results showed (Chapters 2, 3 and 4) that *Sphagnum* had no apparent effect on carbon suppression in peat samples. Firstly, in Chapter 4, the vegetation was affected by the crude oil, with reduced growth and much less of the typically vibrant green colour of healthy mosses during the experiment. Secondly, in Chapter 3 there were two types of *Sphagnum* (*Sphagnum rubellum* and *Sphagnum magellanicum*) used in order to ascertain the best *Sphagnum* to reduce decomposition rates in peat, but no significant effect was observed in terms of lowering carbon dioxide flux. The reason may be due to the biomass of the *Sphagnum* which may have been affected by clipping it and putting it in boxes to conduct the necessary analyses. As noted by Clymo (1970) there is no clear division of live and dead plant material in *Sphagnum*, so this is an arbitrary but consistent approach. However, if peatlands are to be used in a geoengineering project, aimed at mitigating anthropogenic carbon emissions, then a more radical method of strengthening the enzymic latch and suppressing decomposition must be found. One way of achieving this could be encouraging *Sphagnum* moss to produce and secrete more inhibitory phenolic compounds. Agriculture has perfected methods of growing a huge range of plants for human use and plant breeders have developed varieties of crops which are vastly more productive than their wild counterparts. There is therefore the possibility of transferring these skills to *Sphagnum* cultivation. New strains of the mosses could be selected to produce higher concentrations
of trans-sphagnum acid and optimal Sphagnum growing conditions could be created in peatlands for the plants' growth (ie. controlling water table levels, micro-topography etc). Freeman et al (2012) has even suggested a genetic modification of Sphagnum to promote the accumulation of trans-sphagnum acid by increasing the expression of the initial enzyme (phenylalanine ammonia lyase, PAL) involved in its production.

6.5. Effect of increasing phenolic concentrations in peat soil

The ubiquity of phenolic compounds is a key feature of organic wetlands, especially peatlands. As strong inhibitors of hydrolytic enzymes and therefore overall soil decomposition (Freeman et al. 2001), these compounds have received great attention in studies of soil organic matter decomposition. Since phenolics are potent inhibitors of enzymes, they have received tremendous attention (Wetzel 1992 and Appel 1993) and their inhibitory activities are a critical reason behind the low level of peat decay. A serious biochemical challenge is posed by phenolic substances because of the resonance energy that stabilizes the carbon–carbon bonds of aromatic rings within phenols (Harwood and Parales 1996). However, our data found that most exogenous phenolic compounds supplements in this study (Spruce and Cedar mixed, lignin solution, Oil-40 and Oil-80, most of phenolic compounds in chapter 5) result in a great increases in the concentration of phenolic compounds. A negative significant relationship in Chapter 2 is reported between mean change in concentration of phenolics and mean change in β-glucosidase activity (Figure 2.5) and also a decrease in CO₂ fluxes compared to the control (Figure 2.3). Our results in Chapter 5 also show that sodium salicylate, which was found to be the most inhibitory of the phenolic compounds tested in terms of suppression of β-glucosidase activity and CO₂ fluxes. This supports the findings of a study by Freeman et al (2012) which concludes that manipulation of the phenolic abundance in the peat could be used to strengthen the enzymic latch. This was further demonstrated in Chapter 4 where crude oil (Oil-40 and Oil-80) application resulted in higher phenolics levels and reduced ER CO₂ fluxes by the end of the experiment. Freeman et al (2012) suggest that with suitable incentives, though piecemeal, small-scale interventions, landscape scale changes can be achieved by engaging landowners in the carbon sequestration process. Adding supplements whether in the form of acids, phenolics, nutrients or even using modified Sphagnum plants will also require research and investment in a suitable method of deployment.
6.6. Effect of pH

In peatlands, at a low pH, carboxyl groups occur mainly in the undissociated form. Uncharged molecules are inhibitory to microorganisms because they may pass the cell membrane and act as an uncoupler (Ghose and Wikénet 1955). Thus, the low pH of peatlands has long been acknowledged to be linked to the suppressed rates of decomposition in peatlands, due in part to the effect of hydrogen ion (H^+) concentrations on enzymes. Indeed, enzyme activities are known to increase exponentially with rising pH (McLatchey and Reddy 1998, Laiho, 2006, Pind et al. 1994). In this present study, the effect of treatments of different phenolic compounds on pH values strongly suggests that differences in pH is unlikely to lead to degradation of phenolic compounds as we found in Chapter 5 with pH change, the concentration of phenolic compounds was significant increase compared to the control except (Tannic acid), this can be excepted due to some studies beyond this simple negative relationship between phenolics and decomposition. Fierer et al (2001) found out that low molecular phenolic compounds and some tannins could serve as a labile substrate, promoting microbial biomass. The H^+ concentration is known to influence soil, plant and microorganism systems (Husson 2013). For example, the dynamics of organic matter (Blossfeld et al. 2010, Grybos et al. 2009) and phosphatase enzyme release can be affected (Castaldi et al. 2010, Christophoridis and Fytianos 2006, Kjaergaard et al. 2012, Maine et al. 1992) as occurred in Chapter 3. Very few studies are available however on the effect of such factors in constructed wetlands (Braskerud et al. 2005). However, (Sinsabaugh 2010) found that there is a positive relationship between phenolics degrading enzyme activities and soil pH across ecosystems. Likewise, Pind et al. (1994) have reported that phenol oxidase activity increases as the pH of peat soils increases. Our data showed that the acidic pH of many peatlands were likely to be relatively unfavourable for the degradation of phenolic materials. It is worth mentioning that pH in most laboratory experiments remained homogeneous with inhibitory phenolic compounds samples in the peat during the experiment period as it did not have a direct effect on the suppression of microbial decomposition even with low value of pH, on the contrary, the enrichment of phenolic compounds to the peat had a role effective in maintaining suppression of decomposition. It was therefore concluded that the presence of inhibitory
phenolic compounds in peat were more important than low pH for curbing decomposition rates in these organic wetlands.

6.7. Implementing geoengineering techniques in peatlands

Since rising atmospheric CO\textsubscript{2} concentrations has been associated with rising global temperatures, a large number of initiatives that aim to store and capture carbon (sequestration) have been put in place. IPCC emission scenarios suggest that without policies to reduce climate change, atmospheric CO\textsubscript{2} concentrations could reach 421 ppm to 936 ppm by the year 2100 with potentially 1.1 to 6.4°C of additional global warming (IPCC 2013). Geoengineering could help reduce the future extent of climate change in terms of CO\textsubscript{2} emissions. Geoengineering is a term generally associated with large-scale projects designed to manipulate the Earth’s climate in order to counteract the current trend in rising atmospheric CO\textsubscript{2} concentrations and the increase in the Earth’s temperature that this is widely accepted to be causing (Wigley 2006). Freeman et al. (2012) suggest that increased C storage could be achieved by increasing the absolute amount of phenolic inhibitors present in peat substrates through the promotion of exogenous phenolic supplements (such as exogenous phenolic compounds in this thesis) or through the genetic modification of Sphagnum plants responsible for phenolic synthesis (Sphagnum rubellum and Sphagnum magellanicum - Chapter 3). The advantages of using phenolic supplements as a peatland geoengineering strategy (peatland enzymic latch manipulation) are their low cost, the fact that they are easily transportable and several different methods of strengthening the enzymic latch could be employed simultaneously (Royal Society, 2009). In this study, the materials used to achieve this strategy are phenolic inhibitors (Woodchips, Lignin solutions or Sodium salicylate – Chapters 2, 3 and 5). We suggest more research needs to be dedicated to this field in terms of investigating mechanism of peatland-geoengineering strategy which have out initial investigations suggest the ability to increase carbon capture and long-term sequestration in peat-forming terrestrial ecosystems.
6.8. Conclusion

The main aim of this thesis was to explore potential mechanisms to enhance carbon sequestration in peatlands using exogenous phenolic compounds. Experiment methods involved the addition of phenolic compounds as a means of harnessing the enzymic latch mechanism.

Main conclusions that can be drawn from this thesis are as follows:

- Our data show that the wood chips from 3 tree species (Larch, Spruce, Cedar) planted in Northern American bogs have a significant effect on carbon sequestration of peat. We attribute this to their high concentrations of phenolic compounds. A significant negative relationship was observed between the mean change in phenolics concentration and β-glucosidase activity. Moreover, “Larch, surface” and “Spruce, surface treatments” led to significant reductions in CO₂ flux.

- The addition of wood chips soaked in lignin solution (Spruce wood chips mixed with lignin solution) increased phenolics concentrations compared to the control in the first 4 months. The wood + 10% lignin treatment had significantly higher phenolics than the control three months after adding this treatment (in October 2015). Despite the high content of phenolic compounds, there was no suppression in the enzymes activity or CO₂ flux. We also observed that Phosphatase enzyme activity increased after adding lignin soaked wood chips, especially in the wood + 10% lignin treatment.

- Phenolic concentrations of peat mesocosms after adding crude oil gradually increased until the end of the experiment for all six treatments, especially Oil-40 and Oil-80 treatments. Our data showed that both Oil-40 and Oil-80 treatments were significantly higher than the Control in NEE fluxes at the end of experiment. While on the contrary in ER fluxes was significant decreased also at the end of experiment.

- There were no significant correlations between molecular weight of phenolic compounds and both β-glucosidase activity and CO₂ emission. The addition of Sodium salicylate, a low molecular weight compound, suppressed the activity of β-glucosidase enzyme and also CO₂ production in peat slurries, suggesting that this compound is a potent inhibitor of microbial activity which warrants further research.
6.9. References


Appendices
Figure 1. The location of the peat soil samples from the Canadian peatland used in the Chapter 2 and 3.

Figure 2. Wood chips soaked lignin was prepared at 5, 10 and 15% w/v concentrations used in Chapter 3.
Figure 3. 44 boxes of peat-soil and two kinds of *Sphagnums* (Magellanicum and Rubellum) in greenhouse at Laval University, Canada.

Figure 4. Peat samples for the crude oil experiment (Chapter 4) were taken from the Migneint Valley in North Wales, UK.