DOCTOR OF PHILOSOPHY

# Generating carbon credits through application of the enzymic latch mechanism in peatland carbon sequestration

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# Generating carbon credits through application of the enzymic latch mechanism in peatland carbon sequestration

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## Summary

Northern hemisphere peatlands are the most efficient carbon stores of any terrestrial ecosystem holding around 455 Pg of carbon – twice the amount found in the world's forest biomass. Such stocks accumulate because phenolic inhibitors in the peat-soil slow the rate of decomposition to below that of photosynthetic production. The disproportionate importance of phenolics in peatlands is related to the unique properties of waterlogged peat soils suppressing the activity of phenol oxidase; one of the few enzymes capable of breaking these inhibitors down. This permits accumulation of phenolic compounds that are potent inhibitors of hydrolase enzymes – major agents in the breakdown of organic matter. Phenol oxidases can therefore be considered an 'enzymic latch' holding in place climate-changing amounts of carbon. However, anthropogenic impacts and the effects of global warming are switching these organic wetlands from carbon sinks to significant sources of greenhouse gases (GHG); namely, carbon dioxide ($CO_2$) and methane ($CH_4$). This study investigates the possibility of manipulating the enzymic latch in order to strengthen the suppression of decomposition in peatlands and thereby maximising their carbon sequestering abilities.

It was found that adjusting the levels of photosynthetically active radiation (PAR) various peatland plants were subjected to did not alter the activity of phenol oxidases, as was hypothesised (due to the expected increase in root exudation brought about by maximised photosynthetic activity). Results also showed that managing floral communities of peatlands to strengthen the enzymic latch (by increasing phenolic concentrations and/or decreasing phenol oxidase activities) only produced a modest increase in carbon sequestration. Directly amplifying the levels of phenolics in the peat-matrix though proved to have a highly significant effect on suppressing enzymic decomposition and the resulting biogenic release of carbon-based trace gases. It was shown that although exudates from wood fragments were able to produce these inhibitory effects, adding a solution of high molecular weight industrially produced lignin (calcium lignosulphonate) was the most effective method of supplementing peat samples to achieve maximum suppression of organic matter breakdown. This effect was observed in peat taken from both temperate and boreal locations. Results from this study revealed that if supplementary phenolics were added to global peatlands as a method of geoengineering the additional amounts of carbon sequestered by peatlands could be transformed into 'carbon credits' to an estimated value of £42.25 billion.

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# Chapter 1

## Introduction: Peatlands, carbon markets and strengthening the enzymic latch

## 1.1 Carbon cycling in peatlands

### 1.1.1 Peatlands

Peatlands cover just three percent of the world's surface (Gorham 1991) yet they contain nearly a third of all the organic carbon found in the Earth's soils, which is equivalent to approximately two-thirds of the entire atmospheric carbon pool (IPCC 2007). Peatlands have sequestered this carbon over millennia due, primarily, to the accumulation of partially decomposed organic matter in anoxic soils (Clymo 1984) and it is estimated they continue to do so at an average rate of 0.07 to 0.096 Pg of carbon per year (1 Pg = $10^{15}$g) (Gorham 1991, Clymo et al. 1998). Decomposition of some soil organic matter (SOM) does take place in peatlands though, releasing carbon in aquatic (dissolved organic carbon, DOC), solid (particulate organic carbon, POC) and gaseous forms such as carbon dioxide ($CO_2$) and methane ($CH_4$). These two gases alongside nitrous oxide ($N_2O$) make up the main greenhouse gases (GHGs) emitted by peatlands. Most studies agree that although the natural processes in peatlands produce varying amounts of GHGs - depending on the ecosystem's condition, type and location - they are currently net carbon sinks at the global level (Kayranli et al. 2010). However, there are fears anthropogenic and environmental factors could be altering the balance, turning peatlands into carbon sources (Ise et al. 2008).

Peatland is generally considered to be a generic term for any freshwater wetland that accumulates partially decayed plant matter (usually to a depth of greater than 40cm), referred to as peat (Mitsch and Gosselink 2000). They are classified as organic wetlands and are estimated to be the most widespread group of wetlands, differing from mineral wetlands by having a living plant layer plus thick accumulations of preserved plant detritus. See Table 1 for a definition of the main types of peatland.

The unique biogeochemistry of peatlands make them unbalanced ecosystems: where the production rate of organic matter exceeds that of decomposition (Clymo 1984, Gorham 1991). Traditionally this impaired decay is attributed to anoxia (Gorham 1991, McLatchey and Reddy 1998, Yavitt et al. 2005), low nutrients, low temperatures and low pH (Gorham 1991, Laiho 2006). However, work by Freeman et al. (2001b) showed that constraints on the enzyme phenol oxidase could be the main inhibiting factor on decomposition. Phenol oxidases are among the few enzymes able to fully degrade phenolic compounds (McLatchey and Reddy 1998) and their activity can be suppressed by the conditions in peat; allowing the build up of phenolic compounds, which then prevent the major agents of nutrient cycling, hydrolase enzymes, from carrying out normal decay processes (Wetzel 1992, Appel 1993).

| | |
|---|---|
| **Wetland** | Land with the water table close to or above the surface, or which is saturated for a significant period of time. Includes most peatlands. |
| **Peatland** | 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 eg. drained or afforested peatlands. |
| **Mire** | All ecosystems described in English as swamp bog, fen, moor, muskeg and peatland, but often used synonymously with peatlands.<br>Includes all peatlands, but some mires may have mineral substrate.<br>Often used to describe an actively growing peatland. |
| **Fen** | A mire which is influenced by water from outside its own limits.<br>True fens or rich fens receive water which has passed through mineral soils and are known as **Minerotrophic Peatlands**.<br>Poor fens or transitional peatlands are known as **Mesotrophic Peatlands** and are between mineral-nourished (minerotrophic) and precipitation-dominated (ombrotrophic) peatlands. |
| **Bog** | A mire which receives water solely from precipitation. Some, such as raised bogs, are referred to as **Ombrotrophic Peatlands** and have developed peat layers higher than their surroundings. |
| **Marsh** | Loose term usually referring to a fen with tall herbaceous vegetation often with a mineral substrate. Can also refer to saline wetlands ie. saltmarshes |
| **Swamp** | Lose term with very wide range of usage. Usually referring to a fen and often implying forest cover. |

**Table 1. Definitions and descriptions of different types of wetlands.**

1.1.2 Peatland soils

Although nearly all soils have organic matter in them to some degree, mineral soils are generally considered to only have an organic content of 20 to 35 percent, on a dry-weight basis. On the other end of the scale, peat (composed of partially decomposed plant remains) consists of over 65 percent organic matter and less than 20 to 35 percent inorganic content (Charman 2002). The two types of soils can have very different physiochemical properties, which are expressed in Table 2.

| | **Mineral soil** | **Organic soil** |
|---|---|---|
| **Organic content** (%) | < 20 to 35 | > 20 to 35 |
| **Organic carbon** (%)<br>Estimated using<br>$\%C_{org} = \%OM / 2$<br>(where $\%C_{org}$ = percentage of organic carbon, and %OM = percentage of organic matter) | < 12 to 20 | > 12 to 20 |
| **pH** | Usually around neutral (pH 7) | Acidic (< pH7) |
| **Bulk density**<br>Defined as the dry weight of soil material per unit volume | High (1.0 – 2.0 g/cm$^3$) | Low (0.2 -0.3 g/cm$^3$ for decomposed organic soil. Low as 0.04 g/cm$^3$ for *Sphagnum*-based peat) |
| **Porosity**<br>Total volume of soil that consists of pore space | Low (45-55%) | High (80%) |
| **Hydraulic conductivity**<br>Rate of water movement through porous medium | High (except for clays) | Low to high |
| **Water holding capacity** | Low | High |
| **Nutrient availability** | Generally high | Often low<br>(Minerals tied up in organic forms, not bio-available to plants) |
| **Cation exchange capacity**<br>Sum of exchangeable cations (positive ions) that a soil can hold. | Low, dominated by major metal cations ($Ca^{2+}$, $Mg^{2+}$, $K^+$, and $Na^+$) | High, dominated by hydrogen ions ($H^+$) |
| **Typical wetland** | Riparian forest | Northern peatland |

**Table 2. Physiochemical differences between mineral and organic soils.**

Whether a soil is mineral or organic, long-term water saturation will usually result in anaerobic, or reduced, conditions, as microorganisms use up all the available molecular oxygen during respiration. Eventually all the available pore spaces in the soil structure are filled with water, slowing the diffusion of oxygen down by as much as 1,000 times - compared to a similar drained soil (Gambrell and Patrick 1978). However, an oxygen gradient does exist within peatland soils as the very top layer of the soil, which is often in contact with air or water, is usually oxidised to a certain extent (Conrad 1996). This microlayer contains oxidised products such as ferric, nitrate and sulphate ions ($Fe^{3+}$, , $NO_3^-$ and $SO_4^{2-}$ ). As the oxygen concentration reduces with depth, reduced forms dominate, like ferrous ($Fe^{2+}$) and manganous salts, ammonia and sulphides.

This gradient of oxidised-to-reduced chemical species can be measured by the redox potential, which quantifies the electron pressure, or availability, in a solution. The redox potential of any solution can be physically calculated as an electric potential in units of millivolts (mV). This is possible because oxidation not only occurs during the uptake of oxygen but also when hydrogen is removed, or a chemical gives up a negatively charged electron – while reduction is the opposite process of releasing oxygen, gaining hydrogen or gaining an electron.

Reduced substances can be oxidised by a number of terminal electron acceptors and not just oxygen. The rate of organic decomposition is slower though when chemical species such as nitrates and sulphates are the electron acceptor, compared to oxygen. Which terminal electron acceptor is used depends on availability but does follow a predictable sequence following aerobic respiration when oxygen itself is the terminal electron acceptor at a redox potential of 400 to 600 mV eg. $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$. Table 3 indicates the terminal electron acceptor sequence and the approximate

redox potentials for transformation. These different redox zones, characterised by the dominance of the electron acceptors ($O_2$, $NO_3^-$ , $Mn^{4+}$ , $Fe^{3+}$, $SO_4^{2-}$ and $CO_2$), theoretically create relatively well defined habitats for the different groups of trace gas-metabolising microorganisms (Conrad 1996).

| Element | Oxidised form | Reduced form | Approximate redox potential for transformation (mV) | Reaction | Type of respiration |
|---|---|---|---|---|---|
| Nitrogen | $NO_3^-$ (nitrate) | $N_2O$ (nitrous oxide), $N_2$ (nitrogen), $NH_4^+$ (ammonium ion) | 250 | $2NO_3 + 10e^- + 12H^+ \rightarrow N_2 + 6H_20$ | Nitrate reduction / Denitrification |
| Manganese | $Mn^{4+}$ (manganic) | $Mn^{2+}$ (manganous) | 225 | $MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + 2H_2O$ | Manganese reduction |
| Iron | $Fe^{3+}$ (ferric) | $Fe^{2+}$ (ferrous) | +100 until -100 | $Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$ | Iron reduction |
| Sulphur | $SO_4^=$ (sulphate) | $S^=$ (sulphide) | -100 until -200 | $SO_4^= + 8e^- + 9H^+ \rightarrow HS^- + 4H_2O$ | Sulphate reduction |
| Carbon | $CO_2$ (Carbon dioxide) | $CH_4$ (methane) | Below -200 | $CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2H_2O$ | Methanogenesis |

**Table 3. Oxidised and reduced forms of elements in wetland soils, and the approximate redox potentials for transformation.**

1.1.3 Carbon in peatlands

Globally, peatlands cover an area of around 400 million hectare (ha) but store between 390 and 528 Pg (Gorham 1991, Immirzi and Maltby 1992, Freeman et al. 2004b, Bragazza et al. 2006, Page et al. 2011). This is equivalent to around 60 to 78 percent of the entire atmospheric carbon pool and is twice the amount of carbon found in the entire world's forest biomass (Oechel et al. 1993, IPCC 2007, Parish et al. 2008). This quantity is equal to the levels of carbon that would be emitted to the atmosphere

from burning fossil fuels at the current annual global rate (approximately 7 Pg) for the next 75 years (Murdiyarso et al. 2010). Such figures mean that peatlands are the most efficient carbon stores of all terrestrial ecosystems and are second only to oceanic deposits as Earth's most important stores (Parish et al. 2008).

Peatlands have been called "unusual" ecosystems, interms of GHG scenarios, as although they sequester the GHG $CO_2$ from the atmosphere as peat they also emit $CO_2$, $CH_4$ and $N_2O$, in potentially large quantities (Gorham 1991). This is due to aerobic and anaerobic decomposition processes, which occur at varying rates, dependent on a number of environmental and biotic factors such as climate, chemical composition of the litter and nature and abundance of decomposing microorganisms (Couteaux et al. 1995, Limpens et al. 2008b, Kayranli et al. 2010). Although it is clear that more research is needed to gain a better understanding of the range of flux pathways (inputs and outputs) and the various forms of carbon (gases, solutes and particulates) involved in the carbon budget of peatlands (Billett et al. 2010, Kayranli et al. 2010) most research agrees peatlands have been a net sink of atmospheric carbon throughout the Holecene period to the present day (Moore 2002, Smith et al. 2004, Kayranli et al. 2010, Waddington et al. 2010).

It is feared the delicate balance between accumulation of peat and decay may cause peatlands to become net carbon sources following human interventions. Degradation of peatlands through drainage, fires, atmospheric deposition and exploitation (eg. for fuel and horticultural products) are resulting in growing GHG emissions. Carbon dioxide emissions from peatland drainage, fires and exploitation are currently estimated to be at least 3,000 million tonnes per annum, or equivalent to more than 10 percent of the global fossil fuel emissions (Parish et al. 2008). Many researches also warn that a warmer, drier climate as is being predicted by some climate

change models for many areas of the globe (especially over parts of Western Europe and North America), could lower the water table of some peatlands creating aerobic conditions in the peat matrix. This may affect the soil's biogeochemical process and lead to a rise in the rate of decomposition, increasing GHG emissions from the peatlands: switching more of them from being net carbon sinks to carbon sources (Lensi and Chalamet 1982, Wilson et al. 1989, Shaver et al. 1992, Freeman et al. 1993, Kirschbaum 1995, Freeman et al. 1997a, Updegraff et al. 2001, Holden 2006, Chivers et al. 2009, Koh et al. 2009, Backstrand et al. 2010). It is also feared that warmer temperatures will increase peatland permafrost thaw, diminishing the tundra's carbon storage capacity (Moore et al. 1998, Natali et al. 2011).

### 1.1.4 Peatlands in the UK

Estimates on the extent of UK peat vary from 1.5 million to 5 million ha (around 15 percent of the land area), depending on whether shallow peats, less than 1 metre in thickness, are included. This equates to an estimated minimum of between 2,302 and 3,121 Megatons (Mt) of carbon (Table 4; Billett et al. 2010, Lindsay 2010).

Work by Billett et al. (2010) suggests the carbon accumulation rate for the UK's peatland is comparable to the original figure suggested by Gorham's (1991) for Northern Hemisphere peatlands in general: around -23 g carbon $m^{-2}$ $yr^{-1}$ (negative fluxes indicate net uptake from the atmosphere). The authors concluded though, when they considered all flux pathways, the current accumulation rate of some of the UK's peatlands may be higher: -56 to -72 g carbon $m^{-2}$ $yr^{-1}$. However, large areas of the UK's peatlands have been subjected to land management practises that have negatively affected their capacity for carbon accumulation. Such activities include afforestation, drainage, prescribed burning, peat extraction, grazing, fertilisation and liming (Holden

et al. 2007). Indeed, an estimated 1.5 million hectares (ha) of the country's peat is believed to have been drained, with the practise of peatland drainage peaking in Britain in the 1970s (Milne and Brown 1997, Holden 2006). A report by Natural England went as far as to say that only one percent of England's peats (>40cm deep) can be considered "undamaged" (Natural England 2010) and a changing climate may even result in a decline in the distribution of some of country's actively growing peatlands (Clark et al. 2010a, Clark et al. 2010b). Fortunately, there are now many projects underway to restore areas of the UK's peatlands through restoration and rewetting projects, many of them thanks to Government polices and initiatives (DEFRA 2008, Lindsay 2010, Natural England 2010).

| Country | | Soil depth | | |
|---|---|---|---|---|
| | Area ($km^2$) | 0-100 cm (Mt Carbon) | >100 cm (Mt Carbon) | Total (Mt Carbon) |
| **Scotland** | 17,789 | 1104 | 516 | 1,620 |
| **England** | 4,246 | 296 | 123 | 419 |
| **Wales** | 732 | 67 | 52 | 119 |
| **Northern Ireland** | 1,873 | 90 | 54 | 144 |
| **UK** | 24,640 | 1,557 | 745 | 2,302 |

**Table 4. UK peatland area and carbon storage.** Taken from a review by Billett et al. (2010).

## 1.2 The UNFCCC and the Kyoto Protocol

For this thesis it is important to understand how peatlands feature in national GHG monitoring schemes and their potential role in carbon markets. It is therefore essential to consider the general mechanisms and main accounting systems of the UNFCCC and the Kyoto Protocol. Once this has been reviewed peatlands' current and potential future role will be considered.

### 1.2.1 The UNFCCC

During the United Nations Conference on Environmental Development (UNCED) at Rio de Janerio in June 1992 an international environmental treaty was produced, named the United Nations Framework on Convention on Climate Change (UNFCCC). Its main objective: "[The] stabilisation of greenhouse gas concentrations in the atmosphere at a level that would prevent dangerous anthropogenic interference with the climate system. Such a level should be achieved within a time-frame sufficient to allow ecosystems to adapt naturally to a climate change, to ensure that food production is not threatened and to enable economic development to proceed in a sustainable manner." (Article 2; UNFCCC 1992).

The GHGs considered by the UNFCCC to be the most important, due to their Global Warming Potentials (GWPS), are $CO_2$, $CH_4$, $N_2O$, sulphur, hexafluoride ($SF_6$), hydroflurocarbons (HFCs), and perfluorocarbons (PFCs).

### 1.2.2 The Kyoto Protocol

A total of 192 Parties signed-up to the UNFCCC treaty, which came into force in 1994. However, the UNFCCC itself has no enforcement mechanisms to ensure stabilisation of GHGs and sets no legally binding limits on GHG emissions for

individual countries, or Parties. Instead the UNFCCC makes provision for updates, or protocols, to set mandatory emission levels (Article 17; UNFCCC 1992). To-date the principal update is the Kyoto Protocol, which was negotiated amongst 84 countries and was adopted in Kyoto, Japan, on 11 December 1997 entering into force on 16 February 2005. The detailed rules for the implementation of the protocol were discussed by the Conference of the Parties (COP) serving as a meeting of the Parties to the Kyoto Protocol (CMP) and finally agreed at the seventh Conference of the Parties to the UNFCCC (commonly referred to as COP-7) in Marrakesh, Morocco, in 2001. These "Marrakesh Accords" were formally adopted by the CMP at its first session in Montreal, Canada in December 2005 (UNFCCC 2008).

*1.2.2.i The first commitment period*

The major feature of the original Kyoto Protocol is the setting of binding targets for 37 industrialized countries and the European Community, known as Annex I Parties, for reducing GHG emissions. These amounted to an average of five percent against 1990 levels over the five-year period of 2008 to 2012, known as the first commitment period, for the three most important gases: $CO_2$, $CH_4$, $N_2O$ (UNFCCC 1998).

Annex I Parties have to account for their total GHG emissions from a number of sources listed in Annex A to the Kyoto Protocol. These include GHG emissions from energy and industrial process, solvent and other product use, agriculture and waste sectors (UNFCCC 1998). Each Annex I Party has a specific emissions target detailed in Annex B of the Kyoto Protocol, set relative to its GHG emissions from the base year: 1990 for $CO_2$, $CH_4$ and $N_2O$ and either 1990 or 1995 for $SF_6$, HFCs and PFCs. A Party's reduction target is expressed as levels of allowed emissions, or

assigned amounts, over the 2008-2012 commitment period. These allowed emissions are divided into assigned amount units (AAUs), each of which represents an allowance to emit one metric tonne of $CO_2$ equivalent (t $CO_2$ eq or $CO_2$-e).

The Kyoto Protocol not only establishes the levels GHG reduction levels for those nations defined as Annex I Parties (generally the more developed countries) but it also outlines the basis of how these targets could be attained, stating in Article 3 paragraph 3: "The net changes in greenhouse gas emissions by sources and removals by sinks resulting from direct human-induced land-use change and forestry activities, limited to afforestation, reforestation and deforestation since 1990, measured as verifiable changes in carbon stocks in each commitment period, shall be used to meet the commitments under this Article of each Party included in Annex I" (UNFCCC 1998).

The main source referred to in this article relates to the emissions from fossil fuel consumption and Annex I Parties must meet their targets primarily through national reduction measures, ie. investing in less polluting technology etc. However, Parties must also take measures to protect and enhance emission removals from specific ecosystems which sequester carbon from the atmosphere, referred to as land use, land-use change and forestry (LULUCF). Article 3 paragraph 3 of the protocol refers to direct, human-induced, afforestation, reforestation and deforestation activities and accounting for these is mandatory for Annex I Parties. However, Article 3 paragraph 4 describes other activities which accounting for is optional, stating: "The Parties to this Protocol shall, at its first session or as soon as practicable thereafter, decide upon modalities, rules and guidelines as to how, and which, additional human-induced activities related to changes in greenhouse gas emissions by sources and

removals by sinks in the agricultural soils and the land-use change and forestry categories shall be added to, or subtracted from, the assigned amounts for Parties included in Annex I" (UNFCCC 1998).

Once an Annex I Party decides to account for one of these LULUCF activities, which include cropland management, grazing land management and/or revegetation, it must continue to do so for the full commitment period (Holden et al. 2007). In contrast to emissions from Annex A sources the protocol requires parties to account for emissions and removals from LULUCF activities by adding to or subtracting from their initial assigned amount. Net removals from LULUCF activities result in additional emission allowances called removal units (RMU), which a party may add to its assigned amount, and a party must account for any net emissions from LULUCF activities by cancelling Kyoto units. The quantity of emission allowances issued or cancelled is subject to specific rules which differ for each LULUCF activity (UNFCCC 1998, 2008).

Annex I Parties can also add to or subtract from their initial assigned amount - and therefore raise or lower the level of their allowed emissions over the commitment period - by trading Kyoto units with other parties in accordance with three market-based mechanisms.

These Kyoto Mechanisms are:

1) Emissions Trading (ET) – Article 17

2) The Clean Development Mechanism (CDM) – Article 12

3) Joint Implementation (JI) – Article 6

*1.2.2.ii The second commitment period*

In Doha, Qatar, on 8 December 2012, the "Doha Amendment to the Kyoto Protocol" was adopted (United Nations 2012). The amendment includes:

- New commitments for Annex I Parties to the Kyoto Protocol who agreed to take on commitments in a second commitment period from 1 January 2013 to 31 December 2020.
- A revised list of GHGs to be reported on by Parties in the second commitment period.
- Amendments to several articles of the Kyoto Protocol which specifically referenced issues pertaining to the first commitment period and which needed to be updated for the second commitment period.

Crucially, during the second commitment period, Parties committed to reduce GHG emissions by at least 18 percent below 1990 levels in the eight-year period from 2013 to 2020; however, the composition of Parties in the second commitment period is different from the first. The principle mechanisms for meeting these emission targets remains the same as for the first commitment phase, and are discussed below.

1.2.3 CDM and JI

Joint Implementation (JI), outlined in Article 6 of the Kyoto Protocol, is a project-based mechanism whereby one Annex I Party with higher than average emission reduction costs is able to invest in cheaper emission reduction measures in another Party and receive at least partial credit for in any emission reduction resulting from the investment (Bush and Harvey 1997). The unit associated with JI is called an emission reduction unit (ERU) and they are converted from existing AAU and RMUs

before being transferred. It is important to note that JI does not affect the total assigned amount of Annex I parties collectively, instead it redistributes the assigned amount among them.

Defined in Article 12 of the Kyoto Protocol, CDM is another project-based mechanism which allows Annex I Parties to implement an emission-reduction project in developing countries. Such projects must follow strict guidelines and accounting requirements to ensure any reductions or removals associated with projects are additional to what would otherwise occur in the absence of the projects. If successful, projects can earn saleable certified emission reduction credits (CERs), since unlike emission trading and JI, CERs increase both the total assigned amount available to the relevant Annex I Party and their allowable level of emissions. This mechanism is seen by many as pioneering as it is the first global, environmental investment and credit scheme of its kind. For any of these projects to be eligible the emission reduction or enhancements they produce must be "additional to any that would otherwise occur" (UNFCCC 2007).

### 1.2.4 Emissions trading

Emissions trading, as set out in Article 17 of the Kyoto Protocol, allows countries that have emission units to spare (emissions permitted to them but not "used") to sell this excess capacity to countries that are over their targets (UNFCCC 1998). This has therefore created a new commodity in the form of emission reductions or removals. Annex I Parties can create domestic or regional (between a group of Parties) schemes for emissions trading, though they are subject to Kyoto Protocol rules laid out in Article 17 of the Protocol and Decision 11 of the first session of the CMP (UNFCCC 1998).

Since $CO_2$ is the principal GHG, emission trading operating under Kyoto Protocol umbrella is generally referred to as trading in carbon and units of emission reductions are equal to one tonne of $CO_2$ – giving the popular term, "carbon credit". Carbon is now tracked and traded like any other commodity and this is known as the "carbon market". However, it is important to note that unlike other commodities "carbon" is politically generated and managed market with no physical commodity - only a dematerialised allowance certificate (Hill et al. 2008).

### 1.2.5 The EU Greenhouse Gas Emission Trading System

In 2000 the European Commission launched the European Climate Change Programme (ECCP) in order to identify and develop all the necessary elements and strategies needed to implement the Kyoto Protocol across the different countries in the European Union (EU). This led to the adoption of a wide range of new policies and measures, including the EU Greenhouse Gas Emission Trading System (EU ETS). Based on the European Emissions Trading Directive on October 13 2003, the EU ETS began operation on January 2005 and is the largest multi-national GHG emission trading system in the world (European Commission 2003).

The EU ETS only applies to combustion installations with a net heat supply in excess of 20MW (megawatts), such as industrial power stations, oil refineries, coke ovens, iron and steel plants, and factories making cement, glass, lime, brick, ceramics, pulp and paper (European Commission 2003). These industries – totalling about 11,500 installations - are believed to be responsible for around a third of the EU's GHG emissions and roughly 45 percent of the EU's $CO_2$ emissions (Braun 2009). Each participant is allocated a certain quantity of emission allowances in accordance with their historical emissions, minus a specified reduction commitment amount. Each

emission allowance entitles the party in question to emit one tonne of $CO_2$ (as in the Kyoto Protocol) within a specified period. At the end of this obligation period, the party must demonstrate that the extent of its emissions are covered by its emission allowances. Parties may acquire additional emission allowances, either by purchasing them from other participants in the EU ETS or by carrying over any remaining credit from one obligation period to the next. Those which emit more than their allowance have to make up the shortfall by acquiring the necessary allowances from other market participants. In this way, emissions trading gives market participants the flexibility to fulfil their reduction commitment either by their own efforts or through the purchase of additional reduction certificates (Braun 2009).

Allowances traded in the EU ETS are held in accounts in electronic registries set up by member states of the EU, although all of these registries are overseen by a Central Administrator at EU level who, through the Community Independent Transaction Log, monitors each transaction for any irregularities. In this way, the registries system keep track of the ownership of allowances in the same way as a banking system keeps track of the ownership of money.

This type of scheme is often referred to as “cap-and-trade” scheme because there is as an agreed aggregate cap on a particular sources' accepted level of pollution. These sources are then allowed to trade amongst themselves to determine which can produce the emissions and at what levels. For countries and companies in the EU, which is bound by the Kyoto Protocol, these types of schemes are mandatory with penalties for those not abiding to them. The EU ETS is not the only mandatory emission trading market though and countries such as America, South Korea, Japan, Australia and Canada are in varying stages of developing their own (Benwell 2009).

Many of the initiatives of EU ETS are developments and extensions of the emission permit trading system of the 1990 Clean Air Act Amendments (1990 CAAA) aimed at regulating sulphur dioxide ($SO_2$) emissions from United States thermal power plants. The system, of harnessing the forces of economic markets to achieve cost-effective environmental protection, proved incredibly effective in the US with 100 percent compliance in reducing $SO_2$ emissions (Benkovic and Kruger 2001, Kumar and Managi 2010).

There is also a rapidly growing “voluntary” emission trading market aimed at businesses and even individuals looking to offset the GHGs they produce. The units of trading in the voluntary sector are still equivalent to one tonne of carbon dioxide and are known as verified emissions reductions (VERs). Anyone can participate in the market and voluntary offset schemes can be defined as those aimed at generating GHG emission reductions not required by Kyoto Protocol’s derived regulation. Units are generated by projects that are assessed and verified by third party organisations, rather than through the official channels of the UNFCCC (DECC 2008). There have been recent improvements in the standardisation of the registries which monitor these VERs in order to increase trust in the system (Benwell 2009). In a review of the differences between the voluntary and mandatory markets Benwell (2009) states there are many reasons why parties would become involved in voluntary schemes, these include a genuine wish to reduce emissions; marketing opportunities from the “carbon neutral” or “green” image; shareholder and/or institutional pressure; threat of future climate litigation and a wish to potentially influence future regulations. However, it can be argued that the distinction between the two types of market are often blurred and the term "voluntary" is misleading, as although parties are not forced into entering into a

scheme, they are nevertheless enforced by law. The author concludes that both schemes are vital for the reduction of greenhouse gas emissions and calls for more hybridisation and the divide between the two to be abandoned.

### 1.2.6 The Kyoto Accounting system

At the end of the Kyoto Protocol's commitment period the total emissions by each Annex I Party is compared to its initial assigned amount to check if it has fulfilled its commitment. Each Party's available assigned amount is equal to its initial assigned amount, plus any additional Kyoto units acquired from other Parties through the Kyoto Mechanisms, or issued for net removals from a LULUCF activity, minus any units transferred to other Parties or cancelled for net emissions from a LULUCF activity (Decision 13/CMP.1) (UNFCCC 2006). Each Annex I Party is therefore required to have established and maintained a national GHG inventory and a registry to track its holdings of and transaction of Kyoto units. These are subject to numerous review and compliance procedures (Article 7, 5, 8 and 18) (UNFCCC 1998). The Intergovernmental Panel on Climate Change (IPCC) – an independent body made up of the world's leading scientists and experts who report directly to the UNFCCC – prepared guidelines for the format and accounting methodologies of national GHG inventories (GHGI) (IPCC 2006a).

### 1.2.7 Peatlands in the Kyoto Protocol

The IPCC's guidelines for an Annex I Party's GHG monitoring and accounting provides a broad range of emission factors (EF) that can be used to estimate emissions from different activities. The emission factors which they provide are called Tier I and, because they are guidelines for all countries, are generalisations which apply to broad

climate zones. However, the IPCC recommends that Parties should develop more detailed Tier II emission factors or Tier III modelling approaches that are relevant to their individual circumstances.

Chapter 7 of the IPCC National GHG Inventories states that emissions from peatlands that are managed for forestry, grazing, cultivation, extraction or development should be accounted for. A series of Tier I emission factors are provided for the main GHG emissions created through different activities in temperate peatlands. Some broad guidelines are also laid out for Tier II and III emission factors though the IPCC confess knowledge and literature on the subject is "sparse" (IPCC 2006b).

Under Article 3 of the Kyoto Protocol Annex I Parties have the option to include emission reductions delivered through a range of land management activities (eg. LULUCF) such as the improved management of existing forests, increasing soil carbon sequestration and restoration of degraded land. Peatland restoration is not explicitly included in the list of optional activities in Article 3.4 of the Protocol, therefore any emission savings delivered by peatland restoration would not count towards meeting a Party's Kyoto commitment. However, in 2009 at the UNFCCC's Conference of the Parties meeting in Copenhagen (COP-15) it was agreed in principle that Parties could voluntarily include "wetland re-wetting" as a LULUCF option in any post-2012 international protocol. In 2010 at COP-16 in Cancun, Mexico; 2011 at COP-17 in Durban, South Africa, and 2012 at COP-18 in Doha, Qatar, it was further agreed that in any future climate agreement it should be possible for Parties to reduce their emissions by rewetting drained peatlands - identified as sinks of GHG (Agenda item 3; United Nations 2010a). However, negotiators could not agree on the full details, and accounting for peatland drainage is currently still voluntary for participating Parties. Further discussions are therefore expected at future COP meetings to further clarify the

guidelines relating to LULUCF and peatlands in the second commitment period of the Kyoto Protocol (United Nations 2010c). Such decisions will affect GHG emission monitoring schemes under the national GHG inventories of signatories to the Kyoto Protocol, and will potentially become nationally appropriate mitigation actions (NAMAs) if managed correctly (Natural England 2010).

In the UK the Department of Energy and Climate Change (DECC) is responsible for the delivery of the Government's GHG inventory (GHGI) to the UNFCCC, in accordance with Article 5 of the Kyoto Protocol and European Commission Decision, in order to comply with the UK and EU's Kyoto Protocol commitments (DECC 2009). Currently, peatlands are classified under the grassland category of land use in the GHGI and numbers on emissions from peatlands only include extraction of peat for horticultural use, oxidation of drained lowland raised bogs and nitrous oxide emissions from the cultivation of histosols.

### 1.2.8 Reducing emissions from deforestation and forest degredation

Peatlands were also given support at the Cancun climate talks through an agreed text for the Reducing Emissions from Deforestation and Forest Degradation mechanism (REDD), which could offer opportunities for the protection and restoration of peatlands in developing countries such as Indonesia, Malaysia and Papua New Guinea (United Nations 2010b). REDD is a scheme proposed at COP-13 in Bali, 2007, which aims to offer financial incentives for developing countries to reduce GHG emissions by better management of forest resources (UN-REDD 2008). Such projects will create quantifiable units – similar to current CDM projects - which can be used in a form of carbon market in the second commitment period of the Kyoto Protocol.

REDD+ (or REDD Plus) moves REDD forward, following discussions and decisions by interested parties at recent COP meetings and includes the role of conservation, sustainable management of forests and enhancement of forest carbon stocks.

Indonesia, which has 21 million out of the 25 million ha of peatland in south-east Asia (IFCA. 2008), is developing demonstration activities involving peatlands for testing the potential of a global REDD carbon market. The Indonesian Ministry of Forestry, who are managing the activities are being given financial and technical support from a number if developed countries and the World Bank, (Couwenberg et al. 2010). More work though is needed to create adequate guidelines to assess the emissions from peatlands with the necessary accuracy, because to be tradable under REDD, or any other mechanism, GHG emission reductions have to be "results based, demonstrable, transparent and verifiable, and estimated consistently over time", as stated at COP-13 (United Nations 2008). This is a major challenge in terms of developing the necessary accounting and trading systems.

## 1.3 Peatlands' future role

Following decisions at recent COP meetings it is clear GHG emissions from peatlands are to feature more prominently in the Kyoto Protocol's second commitment period. For Annex I Parties, such as the UK, there are two main areas where these ecosystems could prove beneficial; carbon offsetting and in national GHG inventories.

### 1.3.1 National GHG Inventories

A report by International Union for Conservation of Nature (IUCN) Peatland Programme (IUCN 2009) suggested that the UK's peatlands could be delivering over 3 million tonnes of $CO_2$ sequestration per annum. However, the report estimates 10 million tonnes of $CO_2$ per year are being lost to the atmosphere from the country's damaged peatlands, equating to between three and 30 tonnes per ha per year depending on how badly the site is affected. The IUCN suggests there is sufficient evidence to show it is possible to halt significant amounts of this loss from peatlands through habitat restoration (eg. rewetting). Indeed, 1.5 million tonnes of $CO_2$ emission are already expected to be saved by 2015 thanks to the UK Biodiversity Action Plan's target to restore 845,000 ha of blanket bog. Although these figures may be useful as a benchmark, continued research in this area is essential.

Any increased $CH_4$ emissions associated with rewetting (Wilson et al. 2009) are likely to be small in relation to the overall GHG losses from a damaged peatland. Though again, more research is needed in this area as Baird et al (2009) concludes; restoration of a peatland does not necessary result in it becoming a carbon sink. The IUCN argue that such peatland restoration projects are a cost effective means of addressing climate change, compared with other carbon abatement methods such as afforestation and renewable energy. Summing up their findings the report says:

"Restoring peatlands can be considered a natural form of carbon capture and storage, preventing release of carbon from damaged bogs and preserving it for potentially millions of years."

Writing in 2009 the authors of the report highlighted that the methodologies for national GHG inventory accounting systems, under the Kyoto Protocol, did not fully address the GHG emission savings from peatland restoration. Thanks to decisions at the latest COP meetings, this is changing and the IPCC is actively looking into best practices for including GHG fluxes from peatlands.

It is necessary to clarify that although there may be some dispute in IUCN's figure of peatlands storing carbon for millions of years, due to the glacial-interglacial patterns, the reasoning of the authors is still valid: peatlands can potentially store and capture carbon for a period of time which may prove beneficial in stabilising the amounts of GHGs in the atmosphere.

Clearly for peatland restoration to feature in any national GHG inventory, monitoring of the activities must be measurable, reportable, and verifiable (MRV), ensuring that it is possible for the results of individual actions to be quantified and reported in a consistent and transparent way (Joosten and Couwenberg 2009). This will allow for appropriate verification, by a third party review, and ensure adequate information is available to assess progress against the objectives of the UNFCCC and the guidelines of the Kyoto Protocol. Unfortunately, for various reasons - such as the diversity of peatlands and climatic conditions where they occur, and the variability of parameters (weather, vegetation, etc) that control GHG emissions seasonally and between years – assessment of GHG fluxes from peatlands is often more complicated than monitoring industrial processes and even forest management practises. A report

by Joosten and Couwenberg (2009) concluded that despite the difficulties associated ensuring peatland restoration projects are MRV, affordable methodologies will be available for reliable baseline setting and monitoring in a post-2012 climate framework. This, they claim, will allow inclusion of peatland conservation and rewetting in climate policies. Such methodologies could involve applying various proxies (eg. waterlevel and vegetation) to GHG flux models. The models themselves are calculated with flux measurements from techniques including the chamber method, which usually enables measurements on a scale of up to one $m^2$; or eddy-covariance, which can assess GHG fluxes over larger areas (typically one $km^2$).

If Annex I Parties are able to include both the negative fluxes of GHG from existing peatlands and the emission reductions from restoring peatlands in LULUCF of their national GHG inventories, it could help reduce the amount of emission cuts – and therefore investment - they have to make from other sectors, such as industry. Figure 1 demonstrates how LULUCF activities are added to the initial assigned amount units, along with units obtained from the three Kyoto Mechanisms, to ensure a Party is in compliance of its Annex A emission targets - in accordance with Article 3 paragraph 1 of the Kyoto Protocol. With relatively large areas of peatland, the UK could benefit from this scheme, as well as other Annex I countries such as Finland.

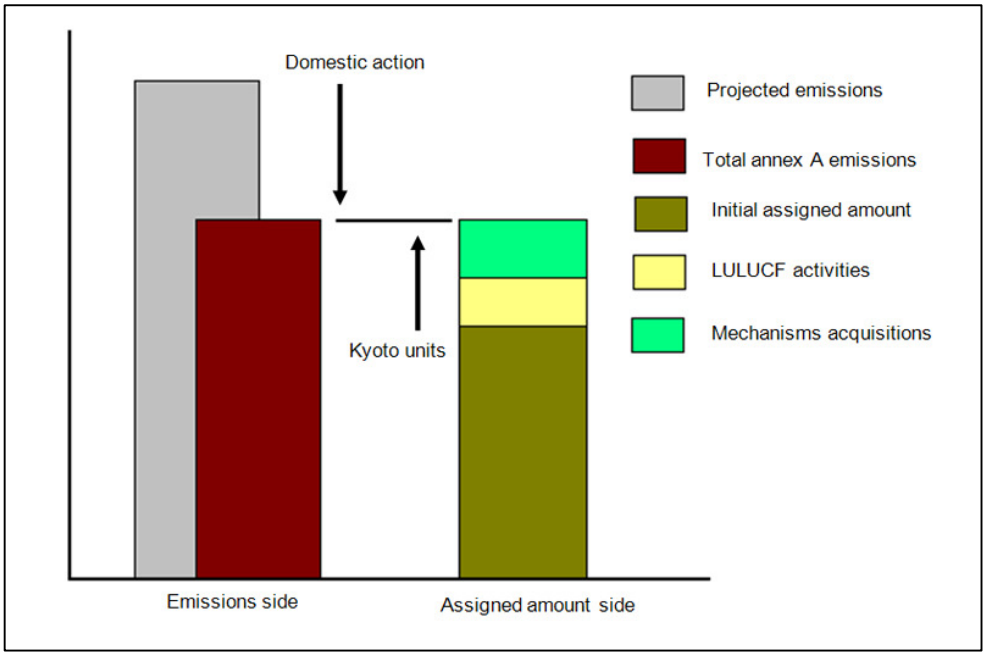



**Figure 1. Determination of an Annex I Party's compliance with Article 3, paragraph 1 of the Kyoto Protocol**. (UNFCCC 2008).

1.3.2 Carbon offsetting

Using ecological restoration projects to offset GHG emissions has been considered for over 20 years and can work alongside cap-and-trade schemes, such as the EU ETS. The first official biosequestration project involved reforestation in Guatemala in 1988, where one of the world's largest power companies, Applied Energy Services (AES), provided $2 million for the planting of 50 million trees in the Western Highlands region of the Central American country. AES won regulatory approval for the project which they planned would offset the $CO_2$ emissions of their coal-fired power station in Connecticut, USA, over its 40-year life span – somewhere around 14.1 million tonnes of carbon (Galatowitsch 2009, Wittman and Caron 2009).

Ecological restoration projects using peatlands for biosequestration could be a financially sound method for creating carbon credits to be used in carbon markets.

There are a growing number of both mandatory and voluntary markets that include biosequestration projects (see Table 5). The Kyoto Protocol's CDM and any similar mechanisms likely to feature in the protocol's second commitment period (eg. REDD) are the most important to the UK. As previously stated, discussions at COP-15 to 18, continually highlighted the importance of peatlands in such mechanisms and decisions at the last two COP meetings could see investment in peatland conservation and restoration in countries such as Indonesia, for the creation of Kyoto and REDD emission units.

There is some concern that allowing terrestrial sinks as an offset to emissions by developed countries removes some of the responsibility, at least temporarily, for dealing directly with the problem of fossil fuel GHG emissions. This may result in further delays in dealing with the main GHG sources, or as Grubb et al (1999) suggests, it could become a "distraction from the fundamental goals of sustainable development". However, Roulet (2000) argued that if the UNFCCC's objective is taken at face value and the stabilisation of GHG concentrations is the ultimate goal, then all sinks and sources, regardless of their origin and national implications should be accounted for and included in mitigation actions.

| Project | Project locations | Project developers |
|---|---|---|
| **International mandatory compliance schemes** | | |
| Clean Development Mechanism | Non-Annex I countries | Mostly large carbon service companies |
| **Mandatory cap-and-trade schemes with offsets** | | |
| Regional Greenhouse Gas Initiative (RGGI) | Nebraska, USA | Intergovernmental organisations for power station operators |
| Greenhouse Gas Abatement Scheme (GGAS) | New South Wales, Australia | Carbon service companies |
| **Voluntary GHG schemes** | | |
| Climate Action Reserve (CAR) | California, USA | Carbon service companies and government agencies |
| Climate, Community & Biodiversity (CCBS) | No restrictions | NGOs |
| Plan Vivo | Developing countries | Local NGOs |
| Voluntary Carbon Standard (VCS) | Restrictions where mandatory trading occurs | Large carbon service companies |
| VER+ | No restrictions | Carbon service companies |
| Voluntary Offset Standard (VOS) | Restrictions where mandatory trading occurs | Carbon service companies |

**Table 5. Carbon market schemes that include biosequestration projects**. Taken from sources including Galatowitsch (2009).

### 1.4 Future perspectives

For Annex I Parties such as the UK, management of peatland areas to specifically maintain and increase the carbon stocks found in their waterlogged organic soils, is set to become a progressively important aspect of GHG emission monitoring and accounting. Such carbon stewardship is likely to be a cost effective activity in helping meet national emission commitments under the second commitment period of the Kyoto Protocol. Work by Worrall et al. (2009) compared the market price of carbon to the cost, and effectiveness, of different peatland management practices – such as the cessation of grazing, revegetation and blocking of drainage ditches. It was

found that, depending on the price of carbon and the restoration technique used, restoring certain areas of the UK's peatland could prove profitable, creating carbon credits. The $CO_2$ equivalency units created though could be incorporated into a LULUCF scheme for a GHG inventory, instead of being used in an emissions market. It is important to note that Worrall et al. (2009) concluded that no single management technique is best for the carbon stewardship of all peatlands, To maximise their effectiveness as carbon sinks targeted actions are required: combining several management practises - an issue also raised by Ostle et al (2009). It is also important to note that Worrall et al. (2009) argued the profitability of using peatlands in carbon offsetting schemes heavily depends on the price of carbon.

LULUCF itself has already been improved to give more significance to peatlands, which could help such carbon stewardship projects. Volume 4 of the 2006 IPCC Guidelines refine LULUCF to include GHG emissions from agriculture, resulting in the Agriculture, Forestry and Other Land Use (AFOLU) sector. The IPCC say this change recognises that land-use changes can involve all types of land and the new guidelines are intended to improve "consistency and completeness in the estimation and reporting of greenhouse gas emissions and removals" (IPCC 2006b). One of the principal changes is the recognition of GHG emissions , specifically $CO_2$ and "non - $CO_2$" missions (eg. $N_2O$ from managed peatlands).

If CDM and REDD specifically take into account the role of peatlands, investment into projects in countries such as Indonesia could see areas of wetlands conserved and restored. The benefits of this may well go beyond curbing the world-wide increase in GHG emissions. Such ecosystems help clean polluted waters, stabilise water systems and support a host of unique flora and fauna – all of which may create

further economic, and environmental, benefits (Cornell 2010). If land owners can make more money from preserving an area of peatland, through a CDM or REDD project, they are less likely to develop the land for purposes such as palm oil plantations, which are major GHG emitters (Wicke et al. 2008). However, for this to become a reality it is essential that the revenue derived from peatland management and restoration competes with activities such as palm oil agriculture. If the created credits are then tradable and incorporated into the proposed compliance markets (rather than voluntary markets) it will help ensure a stable supply of funds at higher carbon prices, and allow projects to be buffered against uncertainties and fluctuations in related markets, such as the price of palm oil (Butler et al. 2009).

As discussed, there are issues that still need to be overcome to ensure any GHG emission reduction projects are measurable, reportable, and verifiable. However, our understanding and skills for measuring the peatland carbon cycle are continuously improving and are likely to increase dramatically if peatland restoration becomes attractive to large investors.

### 1.5 Peatlands and the voluntary carbon market

In the UK there are already proposals for an accredited voluntary carbon market involving peatlands. Known as the Peatland Carbon Code (PCC), the scheme aims to be run in a similar way to the hugely successful Forestry Commission's Woodland Carbon Code (WCC) which has been used as a standard for creating voluntary carbon credits with forests in the UK since 2011. The scheme works by verifying the amounts of carbon removed from the atmosphere through the planting of trees by landowners, and then finding willing partners to pay for this carbon (usually in

an effort to offset their own emissions). Within months of being established their projects had removed 1 million tons of $CO_2$, priced at around £25 a tonne. There are currently over 60 projects with clients including Marks and Spencer, Stagecoach and KwikFit.

The PCC, which hopes to offer a similar verification and brokering service, has already received support with the proposal being ranked as the UK's top opportunity for business to work with the natural environment in a 2012 report by the UK Government Research Councils' Valuing Nature Network. The report suggested that a PCC not only has strong market potential in the UK, but also could create the opportunity for the UK to export its expertise in this area to other ecosystem service markets globally.

The PCC currently plans to launch a Phase 1 code to the Corporate Social Responsibility market in the summer of 2013 and submit the code to be considered for inclusion in the government's GHG reporting guidelines – which provide advice to businesses on how to undertake corporate carbon reporting. This would enable businesses to formally record their peatland projects in their carbon reporting. Market research by the BRE Trust in 2009 suggested demand from UK companies wishing to voluntarily support land-based carbon reduction projects could exceed 10 million tonnes per year. However, the voluntary market in the UK is also set to undergo an unprecedented surge in interest because, as of April 2013, all companies listed on Main Markets of the London Stock Exchange have to account for their greenhouse gas (GHG) emissions. As part of this they will be actively encouraged to include voluntary carbon credits from UK based projects.

## 1.6 Increasing peatland carbon sequestration

Despite attempts over the years at enhancing carbon capture in the oceans and land-based afforestation schemes, no attempt has yet been made to optimise peatland carbon storage capacity. However, as recent studies suggest peatlands sequester carbon due to the inhibitory effects of phenolic compounds creating an 'enzymic latch' on decomposition, it may be possible to manipulate this biogeochemical process to maximise carbon sequestration. This would, potentially, produce novel peatland-geoengineering strategies which could be used to beneficially alter the planet's climate.

### 1.6.1 The enzymic latch

A key rate limiting step in the decomposition of organic matter (OM) in peat-soil is the activity of extracellular hydrolase enzymes (Burns 1982, Sinsabaugh 1994). These are produced by heterotrophic soil organisms in order to cleave large, complex, high molecular weight, OM macromolecules into their respective monomers, which can then cross cell membranes and be assimilated by the microbes (Rogers 1961, Swift et al. 1979). This is important as microbial uptake of OM is governed by molecular size and composition (Kaplan and Bott 1983), with microogranisms generally being unable to assimilate organic molecules with a molecular weight over 1,000 Da (Daltons; Confer and Logan 1998) and preferentially utilising low molecular weight molecules (Meyer et al. 1987). The activities of extracellular hydrolising enzymes can therefore prevent the excessive accumulation of detrital OM in the environment, whilst also supplying photosynthetic organisms with nutrients and give an indication of growth and development of microbial communities in the peat matrix (Gajewski and Chrost 1995). However, the production and activity of these essential extracellular hydrolase enzymes in soil can be inhibited by various biotic and abiotic factors (Burns

1978, Ladd 1978). In peatlands this inhibition has led to an unprecedented accumulation of high molecular weight, recalcitrant, aged organic carbon (Clymo 1983) and, specifically, a range of compounds known as polyphenols, or phenolics (Freeman et al. 2001a, Freeman et al. 2001b).

Phenolic compounds are a diverse group of organic compounds, defined by the presence of at least one aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents or their derivatives eg. esters and glycosides. Simple monomeric phenols, which include flavonoids and phenolic acids are produced universally within higher-order plants (Hattenschwiler and Vitousek 2000), while woody plants are the main producers of polyphenols, such as lignin and tannins (Haslam 1989, Faulon and Hatcher 1994).

The important role phenolics have on decomposition and nutrient cycling has long been realised (Horner et al. 1988) and in particular it has been documented that polyphenols are capable of inhibiting organic decomposition in a peatland environment (Freeman et al. 1990, Freeman et al. 2001b, Freeman et al. 2004b). This is brought about by the phenolic compounds forming complexes with protein molecules, serving as polydentate ligands, causing inactivation of hydrolase enzymes (the main agents of decomposition) through competitive and non-competitive inhibition (Wetzel 1992). The polyphenols also bind to the reactive sites of proteins and other organic and inorganic substrates, often rendering them inactive to further chemical activity and biological attack (Muscolo and Sidari 2006).

The extent to which such phenolic compounds accumulate within the peatland soil is in turn dependent on the activity of another set of enzymes capable of their elimination - through oxidation. Phenol oxidases are oxidative copper-containing enzymes that catalyse the release of reactive oxygen-radicals that can then promote a

variety of non-enzymic reactions to oxidise phenolic compounds (Thurston 1994, Claus 2004). Indeed phenol oxidases can instigate the oxidation of both complex polyphenols with outcomes ranging from partial oxidation and the release of oxidative intermediates, to the complete degradation of phenolic compounds to non-phenolic end products. In peatlands, phenol oxidases are produced by fungi, bacteria, actinobacteria and, to a lesser extent, plants (Gramss et al. 1999, Burke and Cairney 2002, Fenner et al. 2005a).

The enzymic latch mechanism therefore refers to how constraints on the activity of phenol oxidases in peatlands represent a fragile 'latch' holding back the decomposition and release of a vast 455 Pg store of carbon. These constraints prevent the following sequence of events, shown in Figure 2, whereby (Freeman et al. 2012): oxygen stimulates phenol oxidase [a] causing a decline in the abundance of inhibitory phenolic compounds [b] allowing stimulation of hydrolase enzymes [c] creating two responses; increased breakdown of low molecular weight labile dissolved organic carbon [d] and release of inorganic nutrients that were sequestered previously in the peat matrix [e]. Both these events provide substrates and nutrients for enhanced microbial activity [f], and hence increased production of hydrolase enzymes [g] and phenol oxidase [h], together with enhanced emissions of biogenic trace gases such as $CO_2$, $CH_4$ and $N_2O$ [i]. The key regulators of this pathway are therefore the phenolics and the factors influencing the emzymes capable of depleting these inhibitory compounds.

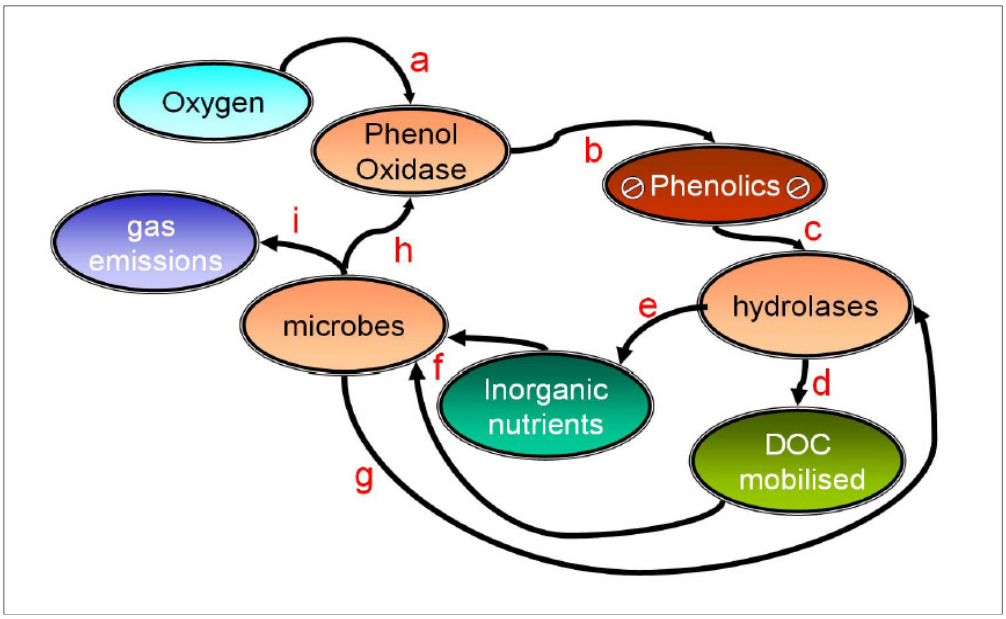



**Figure 2. The role of phenol oxidase in regulating peatland carbon storage through the enzymic latch mechanism.**

The enzymic latch mechanism has the potential to be used to increase carbon sequestration in two principle ways. The first is through strengthening the enzymic latch by increasing the abundance of phenolic inhibitors or manipulating edaphic factors that could slow down the microbial production of phenol oxidases and/or its activity (eg. inorganic nutrient availability and pH). Secondly, by increasing the amount of carbon influenced by the enzymic latch using methods to either increase peatland plant productivity or add forms of externally captured carbon.

### 1.6.2 Peatland-geoengineering

Geoengineering has been defined by the UK's Royal Society as "the deliberate large-scale intervention in the Earth's climate system, in order to moderate global warming" (Royal Society 2009). Geoengineering methods can be divided into two basic classes:

1. **Carbon dioxide removal** (CDR) techniques which remove $CO_2$ from the atmosphere.
2. **Solar radiation management** (SRM) techniques that reflect a small percentage of the sun's light and heat back into the space.

Suggested SRM techniques include increasing the reflectivity of the planet (known as albedo) by brightening human structures (eg. painting them white), planting crops with high reflectivity (Singarayer and Davies-Barnard 2012), covering desserts with reflective materials, enhancing marine cloud reflectivity (Latham et al. 2012), injecting sulphate aerosols into the lower stratosphere to mimic the effects of volcanic eruptions (Davidson et al. 2012) and even placing deflectors in space to reduce the amount of solar energy reaching the Earth (Goldblatt and Watson 2012). It has been suggested that such techniques would take only a few years to have an effect on the climate once deployed but as they do not treat the root cause of climate change (increased levels of GHG in the atmosphere) they would create an artificial balance between increased GHG concentrations and reduced solar radiation. This would therefore need constant maintenance - perhaps for centuries (Shepherd 2012).

Using a strengthening of the enzymic latch in peatlands though, would be classified as CDR geoengineering technique as it would involve the increased sequestration of carbon. Other land use management projects have been proposed including afforestaion and reforestation (Bonan 2008); however, unlike these methods peatlands are not vulnerable to rapid re-release of sequesterd carbon when trees die and decompose (Korner 2003).

The use of any geoengineering techniques is highly controversial due to the fact their effects, both intended and unintended, are not yet fully understood as no large scale projects have been undertaken (Bertram 2010). However, it has been argued that fighting the effects of climate change should involve a comprehensive three-point approach embracing mitigation, adaptation and geoengineering (referred to as the MAG approach). Indeed in their comprehensive report on the subject the Royal Society stated that while the overall reduction of GHG emissions is the safest and most predictable method of moderating climate change (and should therefore be policy-makers' priority) geoengineering is a potentially viable method for supporting these aims. They concluded that further research should be conducted, as a matter of some urgency, to investigate whether proposed techniques could be made available if it becomes necessary to reduce the rate of warming this century (Royal Society 2009).

## 1.7 Objectives of this thesis

The primary objective of this PhD is to determine if the enzymic latch in peatlands can be strengthened in order to increase the peat's ability to sequester carbon. This follows on from original work on the enzymic latch by Freeman et al. (2001b) and later proposals by the same author that increasing phenolic concentrations in the peat matrix can suppress extracellular hydrolase enzyme activities, thereby retarding microbial decomposition and the resulting release of biogenic carbon (Freeman 2011, Freeman et al. 2012). If it is found peatland carbon sequestration can be maximised, we will then quantify the potential $CO_2$-e which can be removed from the atmosphere and investigate the value of this on existing and developing carbon markets and accounting schemes. At the outset of this thesis we therefore put forward two key hypotheses.

1. Lowering phenol oxidase activities will increase the amounts of carbon stored in peat.
2. Supplementing peat with additional phenolic compounds will increase its carbon sequestering abilities.

To answer these two overarching hypothesis a number of specific objectives and hypothesis will be addressed and tested in the following chapters of the thesis.

- Chapter 2: The key protocols used for the biogeochemical analysis needed to answer the aims of this thesis will be synthesised, tested and developed.
- Chapter 3: It will investigated whether adjusting light levels peatland plants are grown under can affect phenol oxidase activity in the rhizosphere due to changes in photosynthesis levels altering rhizodeposition and thereby microbial activity.
- Chapter 4: The hypothesis that vegetation can be used to beneficially adjust

peatland carbon fluxes, will be answered by studying the biogeochemical cycles in peat taken from areas dominated by various plant species.

- Chapter 5 and Chapter 6: The inhibitory effect on organic matter decomposition, and the resulting biogenic trace gas production, of various phenolic compounds will be fully investigated and quantified. Including the required type, concentration and molecular weight of the phenolic matter for maximum suppression, and the effect of phenolic addition on different peatland-types.

# Chapter 2

## Methodologies for peatland enzyme assays and small-scale trace-gas analysis

## Chapter introduction

The activities of phenol oxidases and a suite of hydrolase enzymes (β-D-glucosidase, arylsulphatase, β-D-xylosidase, N-acetyl-β-D-glucosaminidase and phosphatase) were investigated throughout this thesis due to their importance in peatland decomposition. Whilst the fluxes of trace-gases ($CO_2$, $CH_4$ and $N_2O$) were measured from peat to quantify soil and microbial respiration.

Although all these procedures are relatively common in biogeochemistry and wetland science their complete methodologies have not been collated and described in detail. It was therefore decided to develop and test parts of the method to suit the requirements of this study and write two scientific papers outlining the methods, which would then make-up a single chapter. These would be the first publications whereby the methods had been thoroughly tested, evaluated, improved and synthesised in such a way.

Chapter 2.1 synthesises existing enzyme assays for wetland soils and is the result of method development to ensure the protocols are able to detect the low levels of enzyme activity observed in some of the peat-soils reported on in this chapter.

Chapter 2.2 presents a method for the collection of trace gases from small samples of peat-soil. Although, the principles of this protocol are frequently used in wetland science it has not been synthesised in such a way before; with the inclusion of gas chromatograph usage and a detailed explanation of the relevant gas flux equation. The chapter also presents an argument for the publication of method validation when reporting trace gas flux data - adding to the debate on the issue in the scientific community.

# 2.1 Optimal methodologies for extracellular enzyme assays from wetland soils

### 2.1.1 Abstract

Measurement of extracellular enzymic activity in wetland soils can give an indication of the ecosystems biogeochemical processes, and rates of nutrient and carbon cycling. Analysis of these have allowed researchers to gain an understanding of the ecosystems' microbial ecology and how it can be affected by environmental factors. Here, for the first time, we give a detailed description of all the assays necessary to determine the activity of a suite of key hydrolase enzymes and phenol oxidases. All the methods described have been thoroughly tested and, where necessary developed, to offer the accuracy required to determine potentially low enzyme activities in some wetland soils. These enzymes control the rates of decomposition and consequently the production of biogenic greenhouse gases. Knowing the processes responsible for the breakdown of organic matter is therefore essential if it becomes necessary to curb these emissions. Our protocols allow for cost effective analysis of a large number of samples and provide sufficient accuracy to determine differences between soil types. Demonstrated by our comparison of two typical upland soil types: peat and podzol. When coupled with contemporary microbial techniques these enzyme assays permit entire biochemical pathways to be determined, giving unparalleled knowledge on the processes involved in wetland ecosystems.

### 2.1.2 Introduction

#### 2.1.2.1 Background

For over 100 years scientists have studied soil enzymology, with the earliest scientific report on the subject being written by Albert Woods in 1899 entitled, "The destruction of chlorophyll by oxidizing enzymes" (Woods 1899). Over the decades scientists have broadened their understanding of how enzymes influence soil fertility and plant growth; while more recently their role in the carbon cycle and therefore climate change has become an increasingly popular area of research. The majority of these "free" or soluble soil enzymes originate from edaphic microorganisms where they are synthesised and then secreted. In turn, they can act as generators of signals to induce further enzyme production by other microorganisms and plants. Being extracellular has led to these types of enzymes being coined "abiotic" (derived from Greek and meaning "without-life") as they are independent of, and unregulated by, viable cells. Alongside free enzymes a host of other extracellular enzymes exist attached to the outer surfaces of viable and dead cells, adsorbed to clay minerals and associated with soil colloids (Burns 1982).

In relation to wetland biogeochemistry, which can involve the study of water alongside soil, the earliest enzyme studies were by aquatic ecologists who noted the presence of extracellular enzymes during studies of periphytic bacterial cultures in the early 1970s (Corpe and Winters 1972, Daatselaar and Harder 1974). Later, various extracellular enzymes were reported in marine sediments (Kobori and Taga 1979a, Meyer-Reil 1986) and sea water (Kobori and Taga 1979b, Somville and Billen 1983). The ecological significance of the enzymatic decomposition of dissolved organic matter (DOM) and particulate organic matter (POM) in marine and freshwater environments was demonstrated during the 1980s (Duddridge and Wainwright 1982,

Faubel and Meyerreil 1983, Graf et al. 1983, Hollibaugh and Azam 1983, Hoppe 1983, Lancelot and Billen 1984, Ammerman and Azam 1985, Hoppe et al. 1988) whilst Chróst and his colleagues demonstrated substantial enzymatic degradation of organic matter in a lake (Halemejko and Chrost 1984, Chrost and Overbeck 1987, Chrost 1989, Chrost et al. 1989). In rivers, extracellular enzymic actions on DOM and POM were measured in the early 1990s (Admiraal and Tubbing 1991, Munster et al. 1992) and, in particular, Sinsabaugh and his colleagues have conducted a pioneering series of studies to estimate litter decomposition rates based on extracellular enzyme activities (Sinsabaugh and Linkins 1990, Sinsabaugh et al. 1992, Sinsabaugh et al. 1994, Sinsabaugh and Findlay 1995, Sinsabaugh et al. 2009). Around this time attempts were made to relate enzyme activities to the biogeochemical properties of wetland ecosystems (Pind et al. 1994, Freeman et al. 1995, Freeman et al. 1996, Freeman et al. 1997a, Freeman et al. 1998) and since then wetland studies with diverse objectives have adopted enzymatic analysis. Some of these include looking at environmental controls on key decompositional processes. For example, water level drawdown by climate change or human intervention is one of the most widely studied topics concerning wetland enzymes (Freeman et al. 1996, Freeman et al. 1997a, Freeman et al. 1998, Williams et al. 2000, Burns and Ryder 2001, Corstanje and Reddy 2004, Mentzer et al. 2006, Song et al. 2007). In addition, effects of elevated $CO_2$ (Kang et al. 2001, Blank and Derner 2004, Freeman et al. 2004a, Fenner et al. 2007b, Kim and Kang 2008), $O_3$ (Williamson et al. 2010), UV (Thomas et al. 2009), temperature (Fenner et al. 2005b, Fenner et al. 2006, Fenner et al. 2007a), land use changes (Ye et al. 2009, Gao et al. 2010) and metals (Siciliano and Lean 2002, Duarte et al. 2008) have received attention, particularly in relation to potential climate change feedbacks. Enzyme activities have also been assessed to connect microbial ecology and

biogeochemical process such as GHG emissions (Freeman et al. 1997a, Freeman et al. 1998, Kang et al. 1998) or soil processes (Gutknecht et al. 2006, Drenovsky et al. 2008).

In this paper we describe in detail the enzymic assays required to measure two key extracellular enzyme groups involved in organic matter decomposition in wetlands - hyrdolases and phenol oxidases – to demonstrate it is possible to detect significant differences between two contrasting types of organic soil commonly found in the northern hemisphere: peat and podzol. To address this objective six peat and six podzol soil samples were taken from the same blanket bog site in the UK and measured for enzyme activities under controlled conditions which replicated field-soil temperatures.

2.1.2.2 Assays for the activity of hydrolase enzymes

*2.1.2.2.i Hydrolase enzymes*

Hydrolytic enzymes, or hydrolases, conduct the hydrolysis of proteins, starch, fats, nucleic acids, and other complex biomolecules. Hydrolysis (from the Greek for "breaking with water") involves the breaking of chemical bonds by the addition of a water molecule, with a hydrogen atom from the water attaching to one atom and the resulting hydroxyl attaching to its neighbour eg. $A$-$B$ + $H_2O$ →$A$-H + $B$-OH. In soil, extracellular hydrolysing enzymes control the rate substrates are degraded to become available for microbial or plant uptake, making them the main mediators of soil biological processes: organic matter decomposition, mineralisation and nutrient cycling (Marx et al. 2001).

In wetland science, the activities of five key hydrolase enzymes - produced by a variety of micro-organisms and even plant cells - are regularly investigated due to

their roles in carbon and nutrient cycling, these are; β-D-glucosidase, arylsulphatase, β-D-xylosidase, N-acetyl-β-D-glucosaminidase and phosphatase.

*β-D-glucosidase*

Glucosidases (or glycosyl hydrolases) catalyze the hydrolysis of the glycosidic linkage between a carbohydrate molecule and another group, releasing smaller sugars such as glucose ($C_6H_{12}O_6$). They are therefore important in providing labile carbon and energy sources to support microbial life in soil. As cellulases they are involved in the degradation of plant-derived cellulose ( $(C_6H_{10}O_5)n$). Specifically, β-D-glucosidase (β-D-glucoside-glucohydrolase; enzyme commission number, EC; 3.2.1.21), catalyses the hydrolysis of terminal, non-reducing 1,4-linked β-glucose residues (such as cellobiose and other water-soluble cellodextrins) to result in simple β-D-glucose: the stereoisomer of glucose most commonly found in nature (Deng and Popova 2011).

*Arylsulphatase*

Arylsulphatase (aryl-sulphate sulphohydrolase; EC 3.1.6.1) is a principle enzyme involved in the soil sulphur cycle. It catalyses the removal of the sulphate ion from a large number of arylsulphate esters (Press et al. 1985), in such forms as choline sulphate, phenolic sulphate and sulphated polysaccharides (Oshrain and Wiebe 1979). The enzyme is found in plants, fungi, bacteria and animals, but microorganisms are thought to be its main source in soil (Klose et al. 2011)

*β-D-xylosidase*

β-D-xylosidase (EC 3.2.1.37) is a hemicellulase catalysing the breakdown of hemicelluloses. Unlike cellulose, hemicellulose does not have a set structure; is

structurally weaker, and instead of containing only anhydrous glucose is constructed of many different sugar monomers – including xylan, made of β-D-xylose units. β-D-xylosidase's role is therefore in the final stage of the decomposition of hemicelluloses: catalyising the hydrolysis of xylobiose ($C_{10}H_{18}O_9$ ) to xylose ($C_5H_{10}O_5$) (Wittmann et al. 2004, Jordan and Wagschal 2010).

*N-acetyl-β-D-glucosaminidase*

N-acetyl-β-D-glucosaminidase (EC 3.2.1.96) is involved in the breakdown of chitin $(C_8H_{13}O_5N)_n$: a polymer of β-(1,4)-N-acetyl-D-glucosamine (NAG) units, which is the second most abundant polysaccharide in nature after cellulose. In wetland soils, chitin is a significant fraction of humus-bound nitrogen and therefore its decomposition, and that of other polymers containing NAG units, is an important processes of nitrogen cycling (Kang et al. 2005).

*Phosphatase*

A phosphatase is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid mono-esters into a phosphate ion and a molecule with a free hydroxyl group (dephosphorylation). The dominant phosphatase enzyme in most soils is phosphomonoesterase (acid phosphomonoesterase, EC 3.1.3.2), a relatively nonspecific phosphohydrolase that acts on a range of low molecular weight phosphorous compounds with a monoester bond (Turner et al. 2002).

*2.1.2.2.ii Principles of the assay*

The following assays utilise fluorogenic 4-methylumbelliferone (MUF) labeled substrates which fluoresce at 450 nm emission, 330nm excitation (Freeman et

al. 1995). While HPLC (high-performance liquid chromatography) has been proposed as a cleanup stage prior to analysis of difficult low activity samples (Freeman et al. 1998), or as an option for measuring several enzymes simultaneously (Freeman and Nevison 1999), the vast majority of assays undertaken continue to be based on the following fluorimetric principle. Upon enzymic hydrolysis of the bond between the MUF and any chemically attached compound the MUF is liberated (see Figure 1) and its fluorescence can be readily measured, using a fluorometer, and related to activity of the hydrolytic enzyme. Soil samples are therefore incubated for a set period of time after being made into a slurry with the relevantly labeled MUF substrate.

This methodology allows for easy correction of interferences caused by the highly coloured phenolic compounds, which are often present in high concentrations in wetland soils, and is far more sensitive than conventional colorimetric techniques making it particularly well-suited to low activity peatland samples (Freeman et al. 1995). However, the MUF-based method can be affected by quenching (decreases of the fluorescence intensity) in the detection of MUF in soil samples. The degree of quenching varies greatly, both temporally and spatially. For example, Freeman et al. (1995) found quenching in one soil type led to the reduction of fluorescence by 21 and 61 percent for May and October samples respectively; whilst quenching in another soil type was consistently less than 20 percent. Fluorescence is also known to be affected by pH and temperature (Guilbault 1990, Lakowicz 2006). Consequently, a calibration curve for each soil is required for quantification of MUF in any given sample; whereby all standards, blanks and samples are prepared and treated in exactly the same manner. Concentrations of MUF can then be calculated using this calibration curve and enzyme activities are expressed as millimoles (mmol) or micromoles (µmol) of MUF released per gram ($g^{-1}$) of soil, per hour ($h^{-1}$). Measuring the fluorescence of samples in

microplates, as in the described method, allows for the calibration curve and assays of all five enzymes to be conducted simultaneously for a large number of samples.

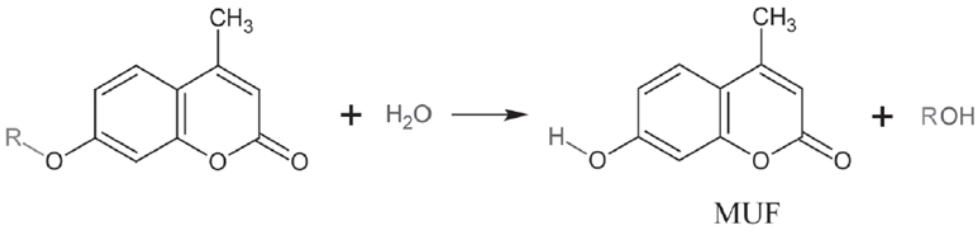



**Figure 1. Structure of 4-methylumbelliferone.** Measurement of hydrolase enzyme activities are based on the detection of 4-methylumbelliferone (MUF) released by enzymatic hydrolysis of specific substrates when incubated with soil samples. R indicates the substrate, specific to each enzyme.

2.1.2.3 Assay for the activity of phenol oxidases

*2.1.2.3.i Phenol oxidases*

Phenol oxidases are a group of enzymes capable of oxidising phenolic compounds from simple aromatics to complex polyphenols; with outcomes ranging from partial oxidation and the release of oxidative intermediates, to complete degradation and formation of non-phenolic end products, such as $CO_2$ (Duran et al. 2002). The suppression of phenol oxidases and resulting build-up of phenolic compounds in peatlands in particular has been demonstrated as the key reason why such ecosystems in the northern hemisphere have accumulated vast deposits of organic carbon since the end of the last ice age (Freeman et al. 2001a, Freeman et al. 2001b). Phenol oxidases have binding sites for aromatic compounds and, crucially as metalloprotein oxidoreductases, a distinctly different site containing copper for molecular oxygen (Duckworth and Coleman 1970). Litter and soil pools of phenol

oxidases consist of an array of different types of the enzyme which can originate from a number of sources; known microbial producers include fungi (Pelaez et al. 1995, Bending and Read 1997, Burke and Cairney 2002), bacteria (Hullo et al. 2001, Fenner et al. 2005a) including actinomycetes (Crawford 1978, Endo et al. 2003) while to a lesser extent certain plant roots have also been shown to directly exude them (Gramss et al. 1999). The grouping of phenol oxidases themselves are based upon subtle differences in their structure and oxidative potential, with the main classifications being the laccases, tyrosinases and catechol oxidases. All of them function by catalysing the reduction of $O_2$ to $H_2O$ using different phenolic compounds as the hydrogen donor (Burke and Cairney 2002, Duran et al. 2002). The resulting free radical from this oxidation process is converted to a quinone, either spontaneously or by further enzymic catalysis. The quinone and the free radical product can then undergo non-enzymatic coupling reactions leading to polymerisation (Duran et al. 2002, Prosser et al. 2011).

*2.1.2.3.ii Principles of assay*

In this method, based on that of Pind et al. (1994), the determination of phenol oxidase activity uses phenolic amino acid L-3,4-dihydroxy phenylalanine (L-DOPA) as the model substrate. The enzymic oxidation of this compound by all three major types of phenol oxidases produces the red pigment, dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone), which has a molar extinction coefficient of 3,700 $M^{-1}$ $cm^{-1}$ at 475nm (Mason 1948, Pomerantz and Murthy 1974). Its absorbance is determined on a spectrophotometer with greater activities of phenol oxidases producing more dopachrome and thereby higher absorbance readings (Figure 2).

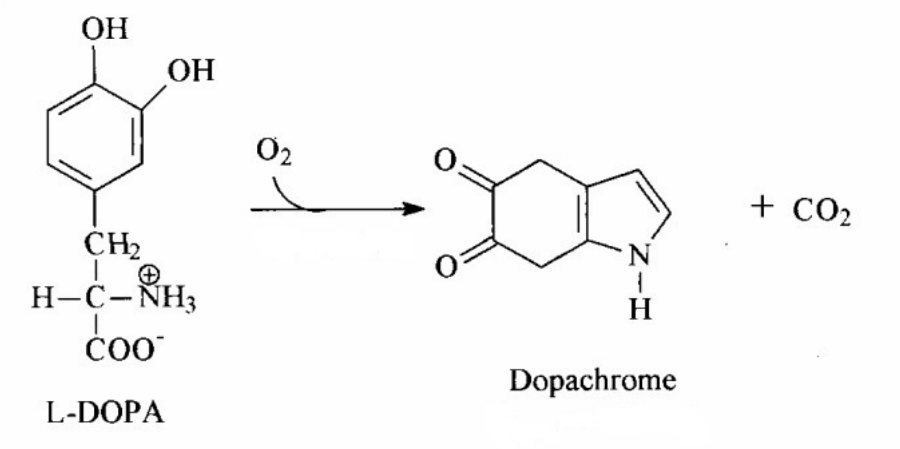



**Figure 2. Structure of L-3,4-dihydroxy phenylalanine.** The oxidation of L-3,4-dihydroxy phenylalanine (L-DOPA) to 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) by phenol oxidases during the enzymic assay.

### 2.1.3 Materials and methods

#### 2.1.3.1 Study site

Samples were extracted from the upland peat-dominated Peaknaze study site in the Peak District, northern England (1°54.5′ W, 53°28.3′ N). The site is located at around 440 m above sea level, receives a mean annual rainfall of 1m and has an average annual temperature of 8°C. Two soil samples were taken from plots on adjacent blanket peat and peaty podzol sites. The deep peat region is covered by a degraded vegetation of *Eriophorum vaginatum*, *Calluna vulgaris* and *Vaccinium myrtillus*; *Sphagnum* mosses are largely absent from the area, thought to be the result of historic sulphur pollution (Tallis, 1987). Mean peat depth across the site is 2.3m. The podzol site has a 5–8cm organic horizon, overlying a 20–40cm sandy E horizon and stony B horizon. Vegetation is mainly *Festuca Ovina* and *Vaccinium myrtillus*.

#### 2.1.3.2 Sample collection and preparation

Six soil samples (≥ 11g) were collected from a depth of 5 to 10cm, within a $1m^2$ area from each of the two experimental sites (podzol and peat soil areas) within a 24 hour period, placed in separate sealable plastic bags and transported to the laboratory in a cooled, insulated container. The field temperature of the ground at the location of sample collection was recorded. Such a sampling technique is considered acceptable for measuring enzyme activities of the peat profile with the highest microbial activities (Freeman et al. 1995). However, intact core samples can be taken and divided into ~10mm sections if more accurate zonation of the peat profile is to be considered.

In the laboratory the six soil samples from each soil type were thoroughly homogenised together with any adhering debris and macroinvertebrates being

removed. They were then kept in an incubator at the recorded field temperatures if the analysis was to be run immediately – otherwise they were stored in air-tight, dark conditions at 4$^{o}$C and returned to field temperatures for 12 hours before conducting any analyses. As soil samples were taken when the ground temperature was <15 $^{o}$C all laboratory work was performed in a large insulated container, using ice-bags to replicate field temperatures. Analyses were performed within 2 weeks of collection as previous tests have shown soil enzyme activities to be stable for this period of time.

2.1.3.3 Hydrolase enzyme assays

*2.1.3.3.i Reagents*

1. The MUF-labeled substrate solutions were prepared by dissolving the relevant amount of substrate (Table 1) to make 1L of 200µM 4-MUF phosphate solution and 400µM solutions for the other substrates in 20mL of Cellosolve solvent (ethylene glycol monomethyl ether) and then approximately 700 mL of sterile water. A magnetic stirrer was used if required. Volumes were then made up to 1,000 mL in a volumetric flask. These solutions are best made daily but can be stored for days or even weeks if kept in the dark at 4$^{o}$C. It is important that they are brought to the required field temperatures before they are used in any assays. Stability of the solutions can be checked by monitoring the fluorescence intensity prior to each time the substrates are used. The stated concentrations of the MUF-labelled substrates ensure optimal enzymic activity for wetland soils (Freeman et al. 1995).
2. For preparation of the calibration curve a 1,000µM MUF stock solution was made (referred to as MUF-free acid, due to lack of bonding with any substrate) by dissolving 0.1762g of 4-methylumbelliferone sodium salt (98%, M1508;

Sigma Aldrich Ltd, Dorset, UK) in 20mL of Cellosolve solvent and then approximately 700 mL of sterile water. A magnetic stirrer was used if required. Volumes were then made up to 1,000 mL in a volumetric flask. This standard solution is then diluted with sterile water to achieve the desired concentrations needed for the calibration curve. Dilutions were carried out in 2mL Eppendorf (Eppendorf UK Ltd, Stevenage, UK) tubes with final concentration taking into account the 1:6 dilution factor of the standard solution (50µL) in the microplate well (300µL). Concentrations of MUF derived during assays of wetland enzymes are typically <100µM when using the procedures described in this manuscript.

*3.* Sterile (deionised) water.

| **Substrate** | **Enzyme** | **Enzyme commission number (EC)** |
|---|---|---|
| **4-MUF β-D-glucopyranoside** | β-D-glucosidase | 3.2.1.21 |
| **4-MUF sulphate potassium salt** | Arylsulphatase | 3.1.6.1 |
| **4-MUF β-D-xylopyranoside** | β-D-xylosidase | 3.2.1.37 |
| **4-MUF N-acetyl-β-D-glucosaminide** | N-acetyl-β-D-glucosaminidase | 3.2.1.96 |
| **4-MUF phosphate** | Phosphatase | 3.1.3.2 |

**Table 1**. **The MUF-labelled substrates needed for measuring the specified enzyme's activity**.

*2.1.3.3.ii Procedure*

All soil samples were thoroughly homogenized by hand before six 1 g (±0.002) sub-samples were weighed-out; one for each of the hydrolase enzyme assays and another for the calibration curve. Each sub-sample was placed into a separate Stomacher bag (Seward, West Sussex, UK) and a final sample of soil (>1 g) was weighed and placed into a porcelain crucible for calculating dry weight and loss on ignition using standard procedures (Frogbrook et al. 2009). Seven millilitres of the relevant MUF substrate (maintained at field temperatures) was added to each Stomacher bag and placed into a laboratory paddle blender (Stomacher circulator, Seward) for 30 seconds. Seven millilitres of sterile water (maintained at field temperatures) was also added to the Stomacher bag containing the soil sub-sample for the calibration curve and treated in the same way. All the bags were incubated at field conditions for 45 minutes for phosphatase and 60 minutes for N-acetyl-β-D-glucosaminidase, β-D-xylosidase, Arylsulphatase, β-D-glucosidase and the calibration curve sample. If assaying all five enzymes at the same time it is advisable to add the substrates to those that require 60 minutes incubation time and wait 15 minutes before commencing the procedure for phosphatase. Two minutes before the incubation time came to an end the contents of each stomacher bag was thoroughly mixed by hand and the solution from each substrate bag used to fill one 1.5mL Safe-Lock Eppendorf tube. For the calibration curve sample two 2mL Safe-Lock Eppendorf tubes were filled with the solution. All tubes were immediately centrifuged at 14,000rpm for five minutes in a temperature controlled centrifuge, set to the field temperature. From the resulting supernatant of each substrate tube 250 µL was pipetted into a well of a black flat-bottomed 96 well Sterilin Microplate (Sterilin, Cambridge, UK); after ensuring each

well already contained 50 µL of sterile water - guaranteeing a comparable dilution to the standard solutions in the calibration curve.

The calibration curve itself was created by adding 50 µL of each of the standard solutions to a series of wells on the same microplate as the substrate samples. To each of these 250 µL of the supernatant, from the calibration curve Eppendorf tubes, was pipetted.

A fluorometer was used to determine the fluorescence of the MUF at 450 and 330nm excitation. Enzyme activity was then reported as per the equation in section 2.1.3.3.iii.

*2.1.3.3.iii Calculation*

As the activity of each hydrolase enzyme is expressed as µmol of MUF released $g^{-1}$ of soil $min^{-1}$ it was necessary, before completing the equation, to calculate the dry mass of the 1 g of wet soil used in the assay (calculated from the additional sub-sample of soil taken for this purpose in the sample preparation).

MUF concentrations in the reaction mixtures were calculated against the calibration curve constructed for each soil at the time of the assay. To produce this curve the average reading from zero MUF standards were subtracted from all other readings; then to change the calculated fluorescence value into enzyme activity the following calculation was applied:

Enzyme activity = $F / M_{dry} / t_{assay} / 8$

Where: F = fluorescence value

$M_{dry}$ = dry weight of 1 g of wet soil

$t_{assay}$ = time of assay eg 60 minutes or 45 for phosphatase.

8 = converts the original units from µM to µmol, due to the original

dilution (1:8) of the soil to substrate mixture in the Stomacher bags.

2.1.3.4 Phenol oxidase assays

*2.1.3.4.i Reagents*

1. A 10mM solution of phenolic amino acid L-3,4-dihydroxy phenylalanine (L-DOPA) analytical grade was prepared (no earlier than two hours before the analysis was be carried out) by dissolving 0.1972g in 100mL of sterile deionised water.
2. Sterile deionised water.

*2.1.3.4.ii Procedure*

All soil samples were thoroughly homogenized by hand before two separate 1 g (±0.002) sub-samples were weighed out and placed into separate Stomacher bags. The remainder of the soil (>1 g) was used for calculating the dry weight and loss on ignition of the soil according to using standard procedures (Frogbrook et al. 2009).

Sterile water (maintained at field temperatures), 9mL, was added to each Stomacher bag which were then placed separately into a laboratory paddle blender and homogenised for 30 seconds. 10mL of sterile water (maintained at field temperatures) was added to one of the bags and 10mL of L-DOPA (maintained at field temperatures) to the other. The former, which contained no model substrate were assigned as the blank samples. Blanks were used to determine the background absorbance of the sample at 475nm, necessary for the calculation of phenol oxidase activity in the samples with added substrate. Each bag was homogenized in the paddle blender for a further 30 seconds and then incubated at the relevant field temperature for 10 minutes.

It is important to note that the precise incubation time should be predetermined beforehand to ensure linear oxidation of L-DOPA, as the response may vary for different soil types.

After the allotted time both bags were removed from the incubator and homogenised gently by hand. From each bag three 1.5mL Safe-Lock Eppendorf tubes were filled and immediately centrifuged at 14,000rpm for five minutes, ideally in a temperature controlled centrifuge set to the required field temperature. 300 µL of the supernatant from each tube was pipetted into a well of a clear 96 well Sterilin Microplate and a spectrophotometer was used to measure the absorbance of the red pigment (dopachrome) at 475nm of all the wells.

*2.1.3.4.iii Calculation*

Phenol oxidase activity is expressed as µmol of product formed (dopachrome or 2-carboxy-2,3-dihydroindole-5,6-quinone, referred to in the equation as diqc) per minute ($min^{-1}$) per gram ($g^{-1}$) of soil (dry weight). Before undertaking this equation it was therefore necessary to calculate the dry mass of the 1g of wet soil used in this assay (calculated from the additional sub-sample of soil taken for this purpose in the sample preparation).

To calculate the enzyme activity; the average blank value was subtracted from the average substrate value, to give an average absorbance value (A), this was then used in the following formula which is based on the Beer-Lambert Law regarding the relationship between absorbance and transmission of ultraviolet and visible light:

$$c = A / \varepsilon b$$

where c = concentration of phenol oxidase (mols)

A = average absorbance

ε = molar absorption coefficient for phenol oxidases (i.e. 3,700 $M^{-1}$ $cm^{-1}$)

b = path length (when using 300µL in a 96 well Sterilin Microplate this is 0.865cm)

To express the activity of the phenol oxidases in the correct units the following equation was used:

$$\mu mol\ dicq\ g^{-1}\ min^{-1} = ((c \times (V_{total}/V_{peat}) \times 10 \times 1{,}000) / w) / t$$

where c = concentration of phenol oxidase (mols)

$V_{total}$ = total volume of solutions in Stomacher bag (ie. 20,000µL)

$V_{peat}$ = volume in Stomacher bag occupied by peat homogenate (ie. 10,000µL)

10 = dilution of peat used in original homogenate (i.e. 1 in 10)

1,000 = conversion of activity from µM to µmol

w = dry weight of 1 g of wet peat used in the assay (g)

t = length of reaction time (min) e.g. 10 minutes

This expresses phenol oxidase activity as µmol dicq $g^{-1}$ $min^{-1}$.

### 2.1.4 Results and discussion

Figure 3 and 4 show enzyme activities typical for these assays, soil types and locations; with acceptable standard deviations between replicates allowing for statistical comparisons between ecosystem types. For example, an independent t-test showed phenol oxidase activity was significantly lower in the peat site ($M$ = 0.147, $SE$ = 0.04) than the podzol site ($M$ = 0.765, $SE$ = 0.024), $t(10) = -13.28$, $p < 0.001$. β-D-glucosidase was higher in the peat ($M$ = 7.592, $SE$ = 0.787) than the podzol site ($M$ = 5.355, $SE$ = 0.485), $t(22) = 2.419$, $p = 0.024$; with a similar result for β-D-xylosidase (peat, $M$ = 13.805, $SE$ = 2.417; podzol, $M$ = 7.588, $SE$ = 0.533), $t(20) = 2.301$, $p$ = 0.032.

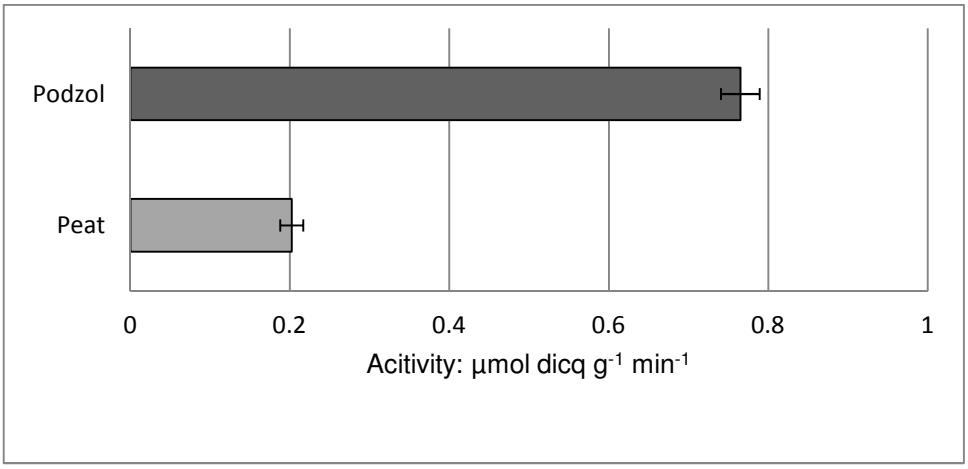



**Figure 3. Phenol oxidase activity in soil samples taken from two different locations.** Peat and podzol soil regions, from the Peaknaze study site in northern England, UK. Enzyme activities are reported as mean ($n$=6)along with ± standard error.

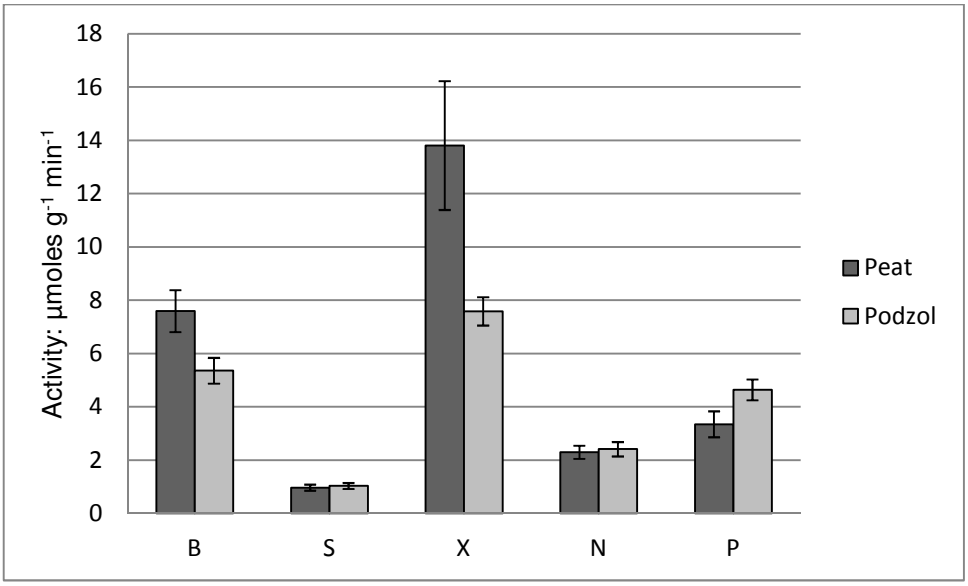



**Figure 4. The activity of hydrolase enzymes in soil samples taken from two different locations.** Peat and podzol soil regions, from the Peaknaze study site in northern England, UK. B = β-D-glucosidase; S= Arylsulphatase; X= β-D-xylosidase; N= N-acetyl-β-D-glucosaminidase; P= Phosphatase. Enzyme activities are reported as mean (*n*=6) along with ± standard error.

It is important to note that by avoiding buffering or altering the pH, and by using field temperatures for the incubation of the soil samples, these assays are best suited when the aim of the analysis is to mimic field conditions as closely as possible, not to provide optimal conditions for enzyme activities (Kourtev et al. 2002). Indeed, in one analysis it was found that phenol oxidase was actually higher in the assay conducted at the field temperature of 12$^{o}$C (0.151 µmol dicq g$^{-1}$ min$^{-1}$) compared to that at 20$^{o}$C (0.093 µmol dicq g$^{-1}$ min$^{-1}$).

When conducting the analysis for phenol oxidase activities it is necessary to test the linearity of L-DOPA oxidation over time for each soil type, before selecting the best time-period to conduct the bulk of the assays over. This time can vary from five

minutes (Pind et al. 1994) to an hour (Prosser et al. 2011). However, it has previously been shown that the assay should not be allowed to exceed one hour as the L-DOPA becomes unstable in the presence of oxygen and therefore reaction rates are no longer linear (Sinsabaugh et al. 1999). It was found that centrifuging samples at less than 10,000 rpm did not allow adequate separation of particulate organic matter (POM) and the supernatant of samples. There was little difference in absorbance measurements of the blank samples when they were centrifuged at 10,000 or 14,000rpm. However, using 14,000rpm ensures the supernatent of samples with high POM are free of any suspended sediment that would interfere with the absorbance measurement. Other equations to calculate phenol oxidase activity were tested. However, the equation given in this paper allows for more flexibility for subtle changes in the methodology such as use of different well plates (path length), dilutions of peat homogenate and length of reaction time.

The described methodology for analysis of hydrolase enzyme activities is based on Freeman et al. (1995) with adaptations to allow the assays to be conducted on a microplate. This saves costs in-terms of both labor and reagents, it also enables the activity of a whole suite of hydrolase enzymes to be tested from a large number of samples in a relatively short space of time: greatly increasing our knowledge and understanding of biogeochemical cycles in soil. The timings of the incubations used in the reported example are based on work by Freeman et al. (1995) but it is important to test that these are optimal for each soil-type beforehand, on a small number of sub-samples. If quenching of the MUF in samples prevents accurate fluorescence readings the soil samples can be diluted with sterile water as long as the MUF-free standards in the calibration curve are treated in the same way.

This study was conducted using organic wetland soils but may be applied to other wetland soil types. It may be necessary to perform linearity tests to determine optimal substrate concentrations prior to analysis. The substrate concentrations used in this study were based on linearity tests performed by Freeman et al. (1995) for peatland soils, with a lower concentration used for phosphatase compared to the other substrates due to saturation at higher concentrations. The main differences between the two wetland soil types used in this study were the soil moisture and organic matter contents (Table 1). The formulae presented for calculating the activities of phenol oxidase and hydrolase enzymes correct for the dry weight of soil and are recommended when assaying one particular soil type (i.e. with consistent soil properties). If comparing between soil types it is recommended enzyme activities are expressed per unit of soil organic matter (SOM) rather than soil dry weight, as presented in synthesis papers such as Sinsabaugh et al. (2008). Although there is scope for scaling our recommended protocol, for example to use different masses of peat and volumes of substrate, it is not recommended that less than 1 g of soil is used during the assay. Due to the fibrous nature of peat soil it can be difficult to prepare sub-samples of peat with equal masses which have the same water and soil organic matter contents; we found that 1g is the minimum sample mass for achieving consistent sample characteristics. Likewise, we do not recommend directly determining enzyme activities in a microplate, which has been reported by Marx et al. (2001) and others, because the particulate organic matter and suspended sediment content of wetland soils is high and it is essential that samples are filtered prior to analysis to prevent quenching of fluorescence and resulting underestimation of enzyme activities.

### 2.1.5 Conclusion

Our protocols for analysing the activity of a suite of hydrolase enzymes and phenol oxidases allow for in-depth research into wetland biogeochemistry. The activity of enzymes in wetland soils have been determined during a large number of studies encompassing a wide range of wetland soil types but there remains a lack of coherence in the methodological procedures employed. As a result it is currently difficult to reliably compare activities reported by different studies where dissimilar methods have been used. We are suggesting that there is a need for a common method of enzyme activity determination and propose a protocol that could be universally applied to wetland soils.

By measuring the activity of the enzymes determined in this study in soil, or pore-water, it is possible to get an indication of the rates of nutrient and carbon cycling in wetland soils. It would then be possible to derive the presence, health and metabolism of the different microbial groups which produce and secrete them (Dick and Burns 2011). However, in many cases the differences between samples being tested can be slight and it is therefore essential that robust and accurate assays are used.

Previous enzymic studies have enabled us to gain an understanding of the decompositional processes in wetlands and how these are affected by changing environmental factors; such as drought (Fenner and Freeman 2011), elevated $CO_2$, temperature and land-use changes. As our climate continues to change, understanding how these factors control biogeochemical cycles may allow us to prevent conditions that produce potentially damaging processes, ie. increased breakdown of organic matter leading to greater biogenic GHG emissions. There is also increasing emphasis on research programs aimed at investigating coupled cycling of macronutrients (C, N and P) (Whitehead and Crossman 2012); measurement of enzyme activities offers a

relatively rapid and straightforward means of investigating such cycles in soils and waters. When contemporary molecular techniques, such as metagenomic sequencing to determine microbial phylogenetics and functional genes (Mackelprang et al. 2011) are related to enzymic activity, entire biochemical pathways and associated processes can be determined. This combination of methods allows ecosystems to be understood at an unparalleled level and could even lead to the development of bio-geoengineering strategies to sequester carbon from the atmosphere (Freeman et al. 2012). The protocols outlined in this paper therefore give the level of accuracy needed to measure the activity of hydrolases and phenol oxidases for this research. Furthermore the procedures are cost effective, use standard laboratory equipment and allow for tens of samples to be analysed simultaneously.

# 2.2 Measurement of small-scale trace gas fluxes from peat samples

### 2.2.1 Abstract

The flux of key greenhouse gases between peatlands and the atmosphere has a globally significant effect on the Earth's climate. Understanding these fluxes is therefore crucial in our study of climate change. Here we present an inexpensive but versatile method to measure the trace gas fluxes from peat microcosm experiments. We believe that this is the most detailed and up-to-date description of the protocol on conducting both slurry and intact soil respiration analyses, including full descriptions of gas collection procedures, gas chromatograph instrument usage and the calculations involved in the final gas flux equation. We demonstrate that our methodologies produce robust data sets allowing for statistical analysis to be conducted for studying seasonal variations and differences between peat types. We also present an argument on the importance of including a method validation when wetland and environmental scientisits report on trace gas flux data.

## 2.2.2 Introduction

### 2.2.2.1. Background

Peatlands are the most important long-term depositories for carbon in the world and despite only covering three percent of the Earth's surface influence the global balance of the three main greenhouse gases (GHGs) – carbon dioxide, methane and nitrous oxide ($CO_2$, $CH_4$ and $N_2O$) (Gorham 1991, Augustin et al. 2011). Studying the relationship between peatlands and GHGs is therefore crucial if we are to understand how these organic wetlands influence our climate. Closed (or static) chambers and eddy covariance techniques are accepted methods for measuring such transfers of gases between the atmosphere and peatlands *insitu* (Minke et al. 2011); however, in many instances it is necessary to investigate fluxes from smaller volumes of peat and often after experimental manipulation of soil samples in the laboratory. To do this small intact peat soil samples or peat-based slurries can be used, whereby 1) a known amount of peat is measured and sealed in a chamber and, if a slurry is required, mixed with a liquid, 2) resulting gases are captured, 3) gases are analysed, and finally 4) the flux for each gas calculated. Despite this being a common procedure in biogeochemical studies there is no standardised methodology for any of the processes outlined. However, with increasing attention on the use of peatlands for carbon storage, national GHG accounting schemes and even geoengineering projects (Dunn and Freeman 2011) it is essential that interested parties start to report fully on their methodologies to ensure they are repeatable and comparable with others, and – wherever possible - standardised.

2.2.2.2 Trace gases

Biogeochemical cycles in peat produce numerous gaseous compounds (Table 1) which, with the exception of oxygen and nitrogen (the two most abundant components in the Earth's atmosphere), are referred to as trace gases. The majority of the trace gases formed in peatlands are low in concentration and are immediately taken in by plants and the soil, or involved in other chemical conversions. Concentrations of $CO_2$, $CH_4$ and $N_2O$, are the more significant however and, coupled with their importance as GHGs (increasing the radiative forcing of the atmosphere), make them the subject of most soil respiration studies.

| **Biogeochemical cycle** | **Gaseous compounds** |
|---|---|
| Carbon | • Carbon dioxide ($CO_2$)*<br>• Carbon monoxide (CO)<br>• Oxygen ($O_2$)<br>• Methane ($CH_4$)*<br>• Non-methane-hydrocarbons (NMHCs; e.g. isoprene, terpene, alcohols, ethers and esters) |
| Nitrogen | • Nitrous oxide ($N_2O$)*<br>• Nitrogen ($N_2$)<br>• Nitric and nitrogen oxide (NO and $NO_2$)<br>• Ammonia ($NH_3$) |
| Sulphur | • Hydrogen sulphide ($H_2S$), carbonyl sulphide (COS) and other inorganic sulphides<br>• Dimethyl sulphide ($(CH_3)_2S$), dimethyl sulphoxide ($(CH_3)_2SO$) and other organic sulphides<br>• Methanethol ($CH_3SH$)<br>• Carbon disulphide ($CS_2$) |
| Phosphorous | • Phosphine ($PH_3$) |

**Table 1. The main peatland biogeochemical cycles and the resulting gases produced.** Taken from Augustin et al. (2011). Gases marked with an asterisk are those most commonly analysed in peat-soil respiration experiments.

The gas generally produced by peat in the largest quantities is $CO_2$ (Billett et al. 2010), mainly due to the respiration of microorganisms in the oxic part of the soil continuously breaking down organic matter (Gammelgaard et al. 1992, Vasander and Kettunen 2006). In the deeper anoxic region $CH_4$ is formed by *Archaea* (methanogenic microorganisms) by the utilisation of a limited set of substrates – hydrogen and acetate being the most important (Segers 1998). Some of this $CH_4$ is oxidised to $CO_2$ in the uppermost layer of the peat by methanotrophic microorganisms (Freeman et al. 2002, Lai 2009). $N_2O$ production is derived from soil microbial processes of nitrification and denitrification (Freeman et al. 1993, Freeman et al. 1997b, Augustin et al. 2011). The former involving ammonia ($NH_3$) being oxidised to nitrate ($NO_3^-$) with $N_2O$ as a by-product, and the latter, occurring in anoxic conditions, transforming nitrate to $N_2O$, with most of this being further reduced to nitrogen ($N_2$). However, microorganisms involved in other processes such as aerobic denitrification, co-denitrification, methanotroph-dependent denitrification and in the course of dissimilatory nitrate reduction to ammonium, are all known to produce $N_2O$ as well as the chemical process of chemodenitrification (Regina et al. 1996, Robertson and Groffmann 2007, Kool et al. 2011).

In this study we outline methodologies for the collection, analysis and calculation of trace gas fluxes from peat microcosms: both slurries and intact soil samples. To demonstrate their use we investigate a) the differences in the gases produced from temperate fen and bog peat samples, and b) the effect of seasonality on soil respiration from peat taken from a blanket bog. These objectives were achieved by the collection of samples ($n \geq 4$) from the various fen and bog ecosystems and then analysed for trace gas emissions under controlled aerobic or anaerobic conditions at field-soil temperatures.

### 2.2.3 Materials and methods

#### 2.2.3.1 Site descriptions

The temperate bog site was the Migneint Valley in North Wales, UK. (3°48.8′ W, 52°59.6′ N). The area is a 200 $km^2$ Special Area of Conservation, incorporating one of the largest areas of blanket bog in Wales. The area is 460m above sea level and is predominantly *Sphagnum*-rich. *Calluna vulgaris* (common heather) and *Eriophorum vaginatum* (hare's-tail cotton grass) dominant blanket mire, with acid grasses on drier hillslopes, and *Juncus effusus* (soft rush) in riparian areas. The underlying geology of the ombrotrophic bog is a mixture of acid and basic volcanic rocks, Ordovician shales and mudstones. Annual rainfall is 2,400mm and in the peat the water table is usually within 100mm of (and often at) the ground surface. The study area has a pore water pH, at a depth of 100mm, in the range of 4.1 to 5.1. The mean peat depth across the site is 2,000mm (Evans et al. 2012).

The fen site was Llyn Padrig on the Isle of Anglesey, UK (4°26.19′W, 53°13.12′N) and is a moderately productive (mesotrophic) wetland, consisting of a small basin mire and remnant lake. The site suffered partial drainage in the latter years of the 19th century, resulting in a reduction of open water and a gradual increase in willow scrub as the floating mire terrestrialised. Surrounded by agricultural land the Site of Special Scientific Interest (SSSI) is around 50$km^2$ and 25m above sea level. The water table at the time of sample collection was at the surface of the peat.

#### 2.2.3.2 Sample preparation

The slurries were contained in 50mL centrifuge tubes (Fisher Scientific UK Ltd, Loughborough, UK). The centre of each tube's lid was drilled with a steel twist

5mm drill bit and a size nine Suba-Seal rubber septa (Sigma Aldrich Ltd, Dorset, UK) was fitted into the hole and glued, using commercially available superglue, securely into place. As the rubber closes the puncture, the septa allowed repeated piercing with a hypodermic needle.

To make the slurries, four peat-soil samples (>10g) were taken from within a $1m^2$ area at the field sites at a depth of between 50 and 100mm – the area of highest microbial activity (Freeman et al. 1995). The four peat samples from each study site were then thoroughly homogenised together, by hand, for 10 minutes. Any roots or woody pieces of plant, thicker than 1mm and over 10mm in length were removed. Four 10g sub-samples of this peat were put into the prepared centrifuge tubes along with 10mL of sterile deionised (ultra-pure) water. A clean glass rod was used to mix the slurry for two minutes, alternating between a clockwise and anti-clockwise motion. The tubes were then placed on a rotary shaker at 200 rpm for 30 minutes. After this time they were stored in dark conditions at stable field temperatures.

An intact peat-soil respiration analysis was also conducted in the adapted 50mL centrifuge tubes. However, instead of homogenising the peat samples and adding water, to make slurries, complete 10g pellets of peat were added, with as little disturbance as possible, to the centrifuge tubes. 16 bog peat samples (≥10g) were collected from a depth of between 50 and 100mm from a $4m^2$ area and placed into separate plastic bag, for transportation to the laboratory. If necessary, soil moisture content was kept constant throughout the experiment by adding ultra-pure water to ensure the original mass of the peat sample was kept constant, however; this is not usually necessary as soil respiration analysis is generally done only once, immediately after the peat is collected and added to the centrifuge tubes.

To perform either of the analyses in anaerobic conditions (eg. in order to replicate field conditions for samples taken from deeper, waterlogged peat depths) the slurries were placed in a zipper lock-style AtmosBag glove bag (Sigma Aldrich Ltd, Dorset, UK), the air was removed with a vacuum pump and oxygen-free-nitrogen was used to re-fill the bag. This process was repeated every 24 hours and the bag regularly checked for holes. The AtmosBag and the slurries, or soil respiration samples, were then kept in dark conditions at the required temperature.

2.2.3.3 Gas collection

A 10cm$^3$ gas syringe fitted with a two-way valve (Sigma Aldrich Ltd, Dorset, UK) and a short bevel hypodermic needle, was used to extract gas samples through the rubber septa in the lids of the centrifuge tubes. Ten mL of gas were collected from the tubes, with the two-way valve being closed before removing the needle from the septa. This was then transferred to labelled, pre-evacuated 5.9mL Exetainers (Labco Ltd, Lampeter, UK); the soda glass vials were fitted with screw caps with pierceable rubber septa. The resulting positive pressure increased accuracy during gas chromatography analysis and the constant pressure on the plunger of the syringe meant it was easier to check if the vials had remained gas-tight during filling.

Pre-evacuation of all atmospheric air was achieved by removing three full 20mL gas syringes of air from the Exetainers, after it was found this number of extractions was the most that could be realistically achieved (due to the negative pressure of the vacuum created in the vial) and repeatability of the results of a standard gas were good (Figure 1). A syringe-extraction method of evacuation was chosen over others, such as using a vacuum pump, due to its simplicity and ability to be used away

from the laboratory if required. Evacuated vials were left no longer than 48 hours before use and were stored in the dark at room temperature.

To calculate the movement of gases involving the slurries a number ($n$>4) of 10mL gas samples were taken from above the centrifuge tubes containing the samples at the start of the experiment; this background or starting gas concentration is hereafter known as Time 1 (T-1). Each centrifuge tube was capped securely with the septum-lids and the exact time noted. If anaerobic conditions were required gas samples were taken from inside the AtmosBag using the gloved access and ensuring all the necessary equipment (Exetainers®, syringe and caps) were placed in the bag the last time it was emptied and filled with nitrogen. The slurries were then kept in their normal conditions (dark and stable temperature) for a specific amount of time to allow evolved gases to accumulate, before 10mL of gas from the headspace above the slurry was collected, as described previously.

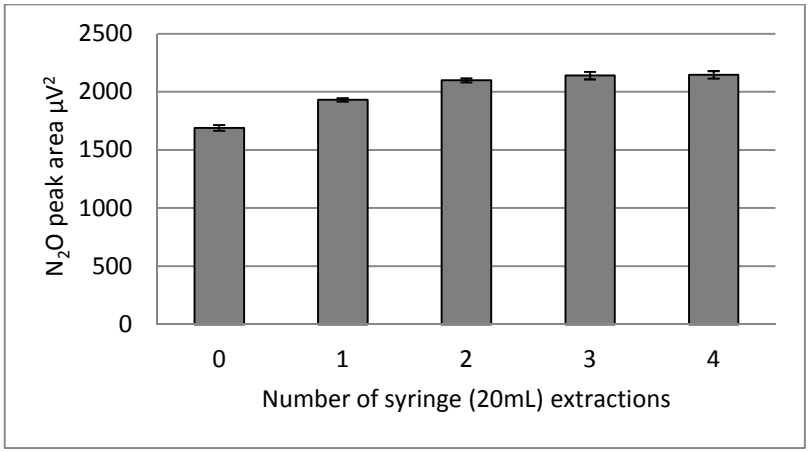



**Figure 1. Concentrations of $N_2O$ use to test the number of 20mL syringe extractions needed to evacuate the 5.9mL Exetainers prior to gas sample storage.** After a set amount of extractions (0 to 4) 10mL of a standard were injected into the vials and the samples analysed on the GC. Three evacuations were considered the optimal number as it was the most that could realistically be achieved, due to the pressure of the vacuum created and gave good repeatability of results. Further extractions showed no significant improvement in the latter, as indicated by the peak areas ($\mu V^2$) of these 1ppm $N_2O$ results. Error bars indicate ± standard error.

The amount of time allowed to elapse between the capping of the centrifuge tubes and gas sample extraction was determined by a test for the linearity of the production rate which was conducted with a sub-sample of the peat before the main experiment was carried out. Slurries were made and sealed, as described. Every 30 minutes for three hours gas samples were taken from a number of these slurries, analysed for trace gas concentration and displayed graphically as parts per million (ppm). The linearity between the T-1 gas samples and each of the other time periods was calculated using a standard line of best fit. The time period that gave the correlation coefficient ($R^2$) value closest to 0.9, for all of the trace gases analysed, was

chosen as the best time to extract a 10mL gas sample from the slurries for the rest of the experiment (Figure 2). Due to the small headspace of the centrifuge tubes, more than one extraction could not be taken to check the linearity of each sample's gas production.

The above gas sampling process was generally conducted two hours after slurry preparation, and then every 24 hours for up to 120 hours, although longer time periods could be used depending on the experiment. Care was taken not to disturb the slurries during and between gas collections.

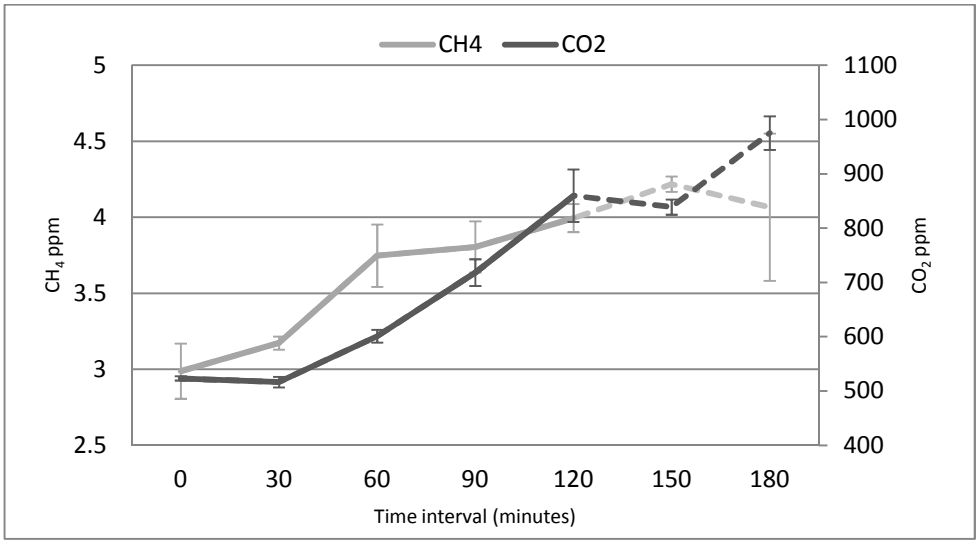



**Figure 2. Gas production from a typical 50:50 peat and ultra-pure water slurry in 12°C aerobic conditions.** This type of linearity test, lasting at least three hours (hatched line), would be conducted before any series of experiments to test for the linearity of gas production. A time which gave a correlation coefficient ($R^2$) value of 0.9 above, through all the time periods, for the relevant gases was chosen as the optimum time to take a gas sample for use in the flux calculation (solid line). In this example 120 minutes was chosen as the $R^2$ values were 0.92 and 0.9 for $CH_4$ and $CO_2$ respectively. $N_2O$ was not produced in significant amounts in this trial. Error bars indicate ± standard error.

2.2.3.4 Gas chromatography

Gas samples were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC) instrument, equipped with a flame ionisation detector (FID) with a $CO_2$ to $CH_4$ catalytic converter (methaniser), to measure concentrations of $CO_2$ and $CH_4$ and an electron capture detector (ECD) for $N_2O$. Two mL of gas from the Exetainers® containing the samples was injected via a 1041 on-column injector system, set at 40$^oC$ (the septum of which was changed after approximately 500 injections), onto a PoroPak QS (1.83m x 3.18mm) 80/100 column. The column oven temperature was set to 40$^oC$ and the carrier gas, oxygen free nitrogen, had a flow rate of 30mL $min^{-1}$. The flow was then split 50:50 (approximately) to the two detectors.

The temperature of the methaniser was 300$^oC$ and the FID was at 125$^oC$. The latter had a hydrogen flow of 25mL $min^{-1}$, 20mL $min^{-1}$ make-up gas (oxygen free nitrogen) and 300mL $min^{-1}$ of compressed air. The methaniser had a hydrogen flow of 5mL $min^{-1}$. The temperature of the ECD was 300$^oC$ with a constant flow of 20mL $min^{-1}$ of oxygen free nitrogen.

The column was heated to 150$^oC$ for two hours every 2,000 samples to remove any water condensate which may have developed in it, due to its relatively low operating temperature. After cooling back down to 40$^oC$ it is left for 24 hours before conducting more analyses.

$CH_4$, $CO_2$, and $N_2O$ (retention times 1.08, 1.87 and 2.25 minutes respectively) were quantified by comparison of peak area with that of the three standards of known concentration (Table 2), prepared by Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK), used in the preparation of a standard curve, which was only accepted if the correlation coefficient ($R^2$) value was greater than 0.98.

| | $CO_2$ | $CH_4$ | $N_2O$ |
|---|---|---|---|
| Standard A | 250 | 2 | 0.5 |
| Standard B | 500 | 25 | 1 |
| Standard C | 1,000 | 50 | 2 |

**Table 2. Concentrations (ppm) of the commonly used standard gases.** A certificate of analysis (CofA) was requested with each new batch of gases and the calibration curve altered accordingly to take into account the exact amounts.

Injection of the samples was achieved with a Combi PAL headspace auto-sampler (CTC Analytics, Zwingen, Switzerland) equipped with a 5mL syringe and specially constructed trays for holding 50 individual 5.9mL Exetainers. A minimum of three blank samples, containing atmospheric air at ambient pressure, were put on at the start of an each analysis run. These were followed by a set of the three standard gases, which were added in the same quantities as the sample gases to Exetainers evacuated in the same way as the samples. This series of standards was used to calibrate the GC. Every 10 samples a quality control standard was included and another set of the standard gases was added at the end of each batch of samples to check for instrument drift.

To validate the analytical procedures being carried out, the limits of detection (LoD) of the method GC instrument were determined using the accepted rule that any signal that can be accurately measured should be at least 2.1 times greater than the baseline noise (signal/noise=2.1) and limits of quantitation (LoQ) were calculated using a signal to noise ratio of 10:1 (ICH 2005). The reliability of the experimental results therefore depends on the analyte measurement being above the LoQ. Any

results below the LoD were discarded. The precision of analysis (coefficient of variation for replicate injections of standards) was calculated with a minimum of 10 standard gas samples ($n$=10) being analysed of a similar level to the experimental samples, if possible. Table 3 shows the validation results from the GC instrument used for development of the reported method.

| | **$CO_2$** | **$CH_4$** | **$N_2O$** |
|---|---|---|---|
| LoD | 2.1ppm | 23ppb | 50ngm |
| LoQ | 9.8ppm | 112ppb | 200ngm |
| Precision (C.V.%) | 1.5 | 2 | 4.7 |
| Linearity ($R^2$) | 0.98 | 0.99 | 0.94 |

**Table 3. An example of the validation requirements for trace gas analysis.** A limited validation of the method should be performed in the laboratory to establish Limit of Detetction (LoD), Limit of Quantitation (LoQ), precision of analysis (coefficient of variation, C.V.) and calibration curve linearity (correlation coefficient, $R^2$). Here we show accepted validation results from our method development.

2.2.3.5 Flux calculation

The exchange of gases between the peat-slurries, or intact samples, and the headspace in the centrifuge tubes can, in principle, occur in both directions. It therefore depends on what is chosen as the reference as to whether a particular flux (rate of change in a gas concentration over time) is expressed as positive or negative. By convention climate science takes the atmosphere as the reference so any gas movement

from the peat-slurry to the headspace will be expressed as a positive flux and movement of gas into the peat, will be negative.

Calculating the gaseous fluxes from the initial background concentrations was achieved by the following equation (Levy et al. 2011):

$$\text{Flux } (\mu g\ g^{-1}\ s^{-1}) = \frac{\delta C}{\delta t} \times \left(\frac{V \times M}{m \times V_{mol}}\right)$$

Where:

- **δC** is rate of change in the gas concentration: T-1 minus T-2.
- **δt** is the change in time from the background reading to the final measurement (in seconds).
- **V** is volume of the headspace of the chamber ($m^3$). When using the centrifuge tubes method this is commonly $0.00004926 m^3$.
- **M** is the molecular weight of the gas (16.04, 44.01 and 44.01 g $mol^{-1}$ for $CH_4$, $CO_2$ and $N_2O$ respectively).
- **m** is the mass of the peat sample used in grams (or total mass of peat and liquid used for the slurries).
- $\mathbf{V_{mol}}$ is the volume of a mole of gas (air) at a given temperature ($m^3\ mol^{-1}$) calculated by:

$$p \times (R \times K)$$

Where **p** is air pressure (kPa); **R** is equal to 8.314 (the ideal gas constant) and **K** is temperature (Kelvin).

The equation first calculates the difference between T-2 and T-1 per second as µmol/mol $s^{-1}$ (accepting that one ppm is equal to one µmole of gas/mole of air). Using

the gas constant from the Ideal Gas Law the density of the air in the headspace of the chamber is calculated in mol $m^{-3}$. This in-turn is used to calculate the moles of air per unit, in this case per gram of peat-slurry (mol $g^{-1}$), by dividing the density by the volume of the headspace of the chamber and multiplying by the number of grams of peat sample (20g in total for the slurries, which include the peat and liquid). The molar flux (µmol $g^{-1}$ $s^{-1}$) of the gas is then given by multiplying the result of the µmol/mol $s^{-1}$ by the mol $g^{-1}$. Molar fluxes are then changed to mass fluxes by multiplying the former by the molecular weight of the relevant gas. The units can then be adjusted and increased, if necessary, to suit the analysis such as, mg $CO_2$ $g^{-1}$ $hour^{-1}$.

2.2.3.6 Statistical considerations

Significant difference between the various results were determined by independent *t*-tests using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA). To ensure the reliability of the *t*-statistic it was checked that the usual assumptions of parametric tests were not violated. The *t*-statistic was reported along with the degrees of freedom and the significance value (*p*) with a value of less than 0.05 taken to be a statistically significant result, as this represents a 95% level of confidence.

### 2.2.4 Results

The $CO_2$ fluxes of the peat-slurries (Figure 3A) showed that those made using bog peat rose slightly, but not significantly, over the 120 hour period: from a mean average (*M)* of 4.35 (standard error, *SE,* 0.57) to 5.39 (*SE* 0.39) µg of $CO_2$ $g^{-1}$ $h^{-1}$. Whilst those made with peat taken from the fen site increased markedly between 24 hours and 48 hours (*M* 4.2 µg $g^{-1}$ $h^{-1}$, *SE* 0.52, to 8.52, *SE* 0.92) and then steadily declined during the remaining three days, with the final mean flux being 3.19 µg of $CO_2$ $g^{-1}$ $h^{-1}$ (*SE* 0.01). It was noteworthy that there was no statistically significant difference between the two fluxes at the beginning of the time period, according to an independent *t*-test ($t$ (6) = 0.197, $p$ = 0.85), but there was at the end ($t$ (3.01) = 5.714, $p$ = 0.011): the flux from fen slurries being 40% of that from the bog samples.

In contrast $CH_4$ fluxes (Figure 3B) were dramatically different at the start of the experiment ($t$ (6) = -10.457, $p < 0.001$), with the mean average flux from fen slurries being 9.86-fold higher than that of the bog slurries. However, there was no statistical difference after 120 hours ($t$ (3.30) = 1.184, $p$ = 0.315; fen *M* 4.92 ng of $CH_4$ $g^{-1}$ $h^{-1}$, *SE* 2.97; bog *M* 8.53, *SE* 0.66), following the decline of fen fluxes and rise in the bogs'.

Both fen and bog $N_2O$ fluxes increased over time (Figure 3C), from a similar initial flux at 24 hours (fen *M*. 0.63 ng of $N_2O$ $g^{-1}$ $h^{-1}$, *SE* 0.13; bog *M* 1.32, *SE* 0.38; $t$ (3.71) = -1.718, $p$ = 0.167). While after 120 hours the bog slurries had a flux over three times greater than the fen ($t$ (6) = 6.90, $p > 0.001$; bog *M* 8.14 ng $g^{-1}$ $h^{-1}$, *SE* 0.79; fen *M* 2.38, *SE* 0.29).

The soil respiration results (Figure 4) showed that the $CO_2$ flux from the peat samples in summer were 97% higher than those in the winter: a mean average flux of 9.91 µg of $CO_2$ $g^{-1}$ $h^{-1}$ (*SE* 1.33) compared to 5.03 µg $g^{-1}$ $h^{-1}$ (*SE* 0.39). An independent

*t*-test showed that these two fluxes were statistically different; $t$ (17.55) = 3.513, $p$ = 0.003.

Both seasons' $CH_4$ fluxes were negative, with the summer flux having a greater negative value than the winter: -0.41 μg $g^{-1}$ $h^{-1}$ (*SE* 0.04) compared to -0.13 (*SE* 0.05) ($t$ (30) = - 4.332, $p > 0.001$).

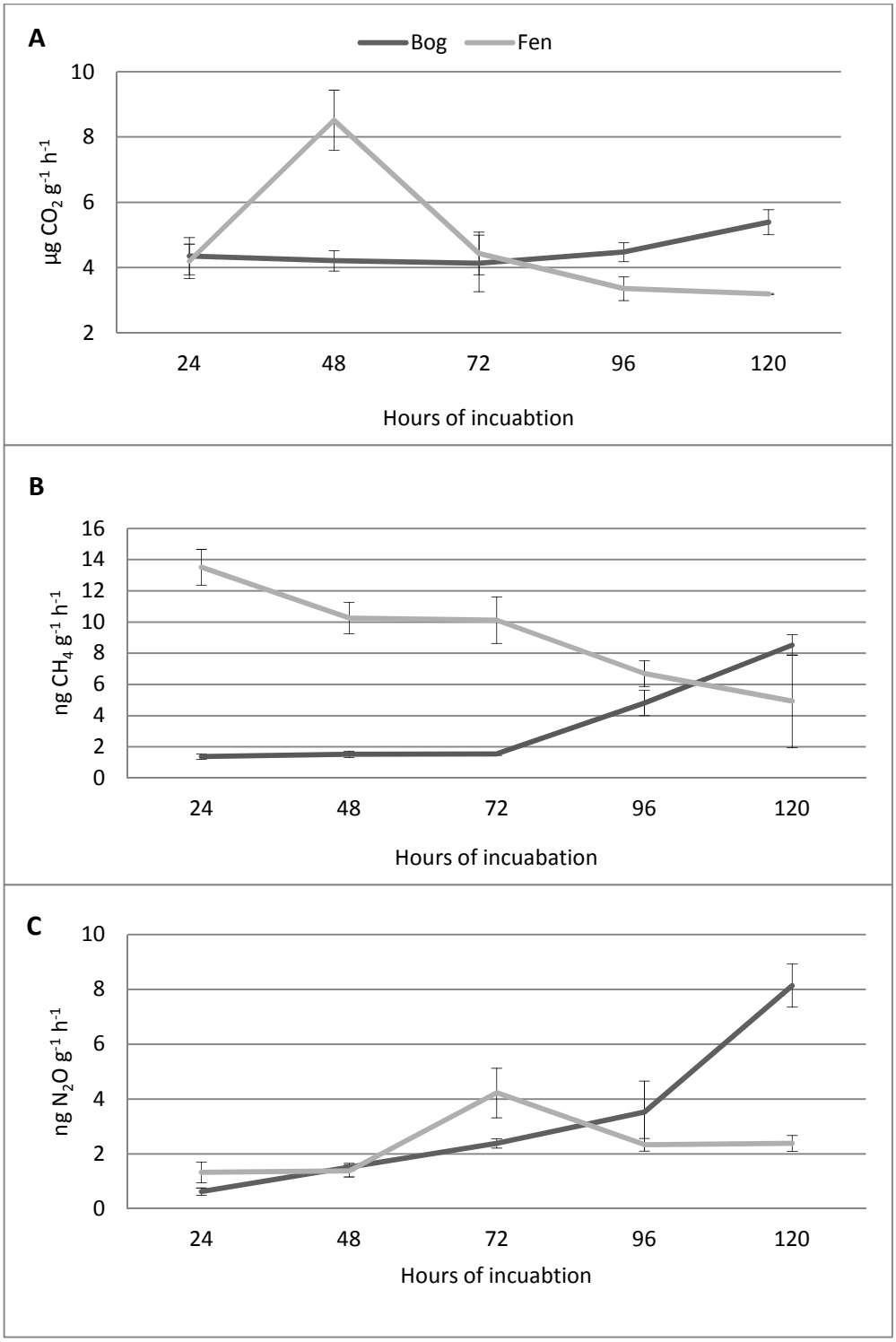



**Figure 3. Trace gas fluxes from fen and bog peat-slurries.** 50:50 peat-water slurries were made and fluxes measured every 24 hours for five days. **A** - $CO_2$; **B** – $CH_4$; **C** – $N_2O$. Slurries were incubated in dark, anaerobic conditions at field-soil temperature (12$^{o}$C). Mean averages are shown ($n$ = 4) and error bars indicate ± standard error.

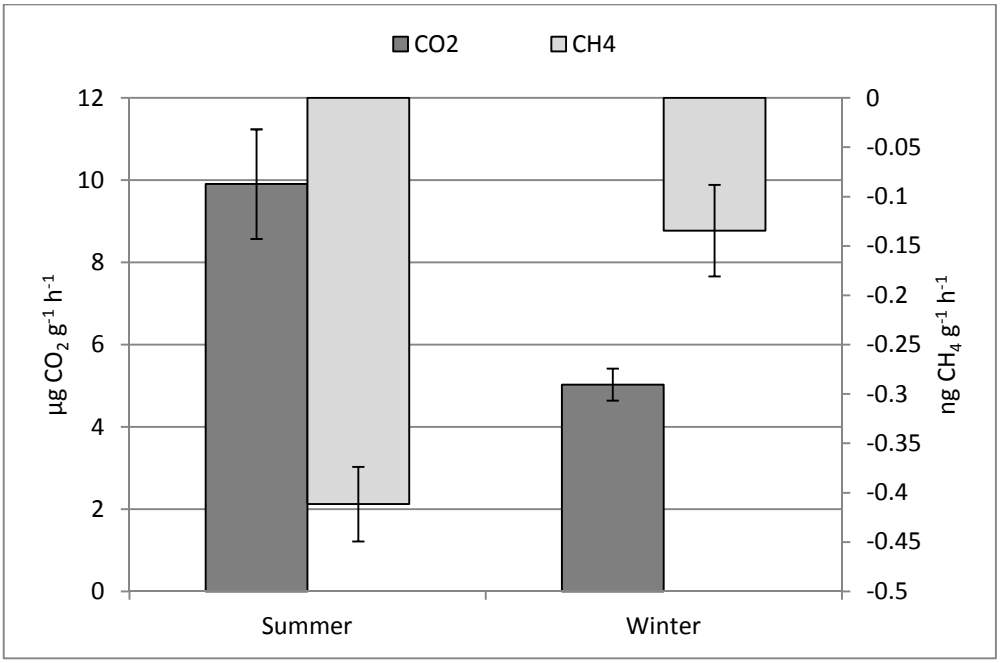



**Figure 4. Carbon-based trace gas fluxes from intact peat-soil samples.** Soil respiration measurements were taken from peat collected in the summer (July, 2011) and the winter (December, 2011). Peat samples were incubated under aerobic conditions, in the dark, at field-soil temperatures. Mean averages are shown ($n$ = 16) and error bars indicate ± standard error.

### 2.2.5 Discussion

Significant differences were detected in the measured trace gas fluxes from both the peat-slurry experiments (investigating the differences between organic wetland soil types) and intact peat-soil respiration measurements (looking at the effect of seasonality in peat samples from a blanket bog).

The flux results from the slurries show that over the 120 hour time period of the experiment, peat from fens produce higher amounts of $CO_2$ than bogs (Figure 3A) - similar to previous studies of wetland types (Moore and Knowles 1989). The large flux from the fen at 48 hours suggests that a pulse of labile carbon was mineralised, with the following decline in fluxes indicating a subsequent lack of appropriate microbial substrate. Our results (Figure 3B) also support previous studies which show peat from bogs have a low capacity for $CH_4$ production, due to the microbiological and chemical properties of the acidic and fibric soil, (Moore and Knowles 1990, Kang and Freeman 2002), while emissions from fen-peat are relatively high due to the more favourable pH, greater nutrient abundance and higher net primary productivity (NPP) (Roulet et al. 1992, Kang et al. 1998). Nitrous oxide fluxes (Figure 3C) were characteristically low from both peat types (Moore 1994). However, as the organic matter in the slurries decomposed over the experimental time period the $N_2O$ fluxes increased in both the slurries (Freeman et al. 1993).

Due to the waterlogged and anaerobic conditions our peat-slurry samples were incubated under, no $CH_4$ consumption by methanotrophs was observed. However, the intact peat-soil respiration measurements showed that our methodology can detect negative fluxes accurately; with significant differences in $CH_4$ consumption being identified between the two seasons. Due to increased methanotroph activity in warmer temperatures (Lai 2009) methane oxidation was likely higher in the samples taken in

July than those in December. Similarly, higher microbial activity can explain the greater $CO_2$ emissions in the summer peat samples. Levels of $N_2O$ were below the limit of detection.

The results from our study show the success of our methodologies – despite the relatively low concentrations of the gases involved (eg. 0.2ppm of $N_2O$). Furthermore the inexpensive equipment used requires minimum training for effective use and allows aerobic and anaerobic conditions, across a wide range of temperatures, to be simulated.

Making slurries from peat samples has the advantage over traditional soil respiration analysis of allowing substances to be added and thoroughly mixed into the peat to investigate their effects on microbial activity, or adding $^{13}C$ labelled compounds to investigate carbon pathways and decomposition rates, and when water-extractable samples need to be taken from the soil following trace gas flux analysis.

Our methods allow a wide-range of soil respiration monitoring and manipulation experiments to be conducted quickly and cost effectively, potentially increasing our understanding of the feedback loops peatland gas fluxes have on our climate. However, to ensure the reliability of such experiments, method validation (LoD, LoQ and precision of analysis) should be referred to when presenting any trace gas fluxes obtained with a GC instrument and any measurements below the LoD discarded and any below the LoQ reported as such. It is hoped the methodologies described here will be used to establish a standardised methodology for collecting, analysing and reporting on gas fluxes from microcosm experiments in a laboratory setting.

**Chapter conclusion**

The methods described in this chapter for measuring and analysing trace gases were used throughout this thesis, with slight alterations to the flux equation depending on the masses of peat used. It was also used when measuring fluxes from closed chambers placed over peat cores, substituting mass of peat for area covered by the chamber.

All hydrolase enzyme assays in this thesis were conducted as outlined in the relevant part of this chapter. Some of the phenol oxidase analyses used a slightly different method, which gave robust and accurate results when enzyme activities were relatively high but were not as precise when activities were low. However, they used less peat-soil sample, which was often an issue for concern. When the alternative method was used in the thesis the methodology is described in full for the relevant chapter.

Crucially the method developments, findings and method validations presented in this chapter prove the accuracy and robustness of the key protocols used in this thesis. Thus ensuring the reliability of any findings - a vital issue when presenting any data on GHG emissions, due to the potential political and financial ramifications increasingly associated with this area of science.

# Chapter 3

## Can light intensity regulate phenol oxidase activity in the rhizosphere of a blanket bog

## 3.1 Abstract

Plants exude an array of chemicals from their roots into the surrounding soil. These root exudates, which include labile carbon compounds and oxygen, are known to affect microbial communities in the rhizosphere - with increased rhizodeposition leading to greater microbial activities. As rates of photosynthesis are often correlated to root exudation we investigated whether adjusting photosynthetic levels, by controlling the amount of light peatland plants were grown under, would affect phenol oxidase activity in the rhizosphere of peatland plants (due to the enzyme being produced by a range of microorganisms and requiring oxygen to function). Any increase in phenol oxidase activity would open the 'enzymic latch' in pealtands leading to the release of carbon, and therefore greenhouse gases, through organic matter decomposition. We therefore wanted to answer the question 'could adjusting light levels reaching blanket bog plants result in the suppression of decomposition, brought about lower levels of photosynthesis?'. To answer this a range of typical blanket bog plants were grown in conditions of varying photosynthetically active radiation (PAR) for six weeks. Peat-soil samples were taken every week from the rhizosphere of each plant and analysed for phenol oxidase activity. Unfortunately, we did not find a strong relationship between varying light levels and phenol oxidase activity for any of the plant species – suggesting light does not regulate the enzyme's activity in the rhizosphere of a blanket bog. Interestingly though, we did observe significant differences in phenol oxidase activities between the species of plants tested - suggesting we could encourage the growth of certain species over others in order to maximise the carbon storage capabilities of peatlands.

## 3.2 Introduction

Peatlands cover just three percent of the world's surface (Gorham 1991) yet they contain nearly a third of all the organic carbon found in the Earth's soils, equivalent to approximately two-thirds of the entire atmospheric carbon pool (IPCC 2007). Peatlands have sequestered this carbon over millennia due, primarily, to the accumulation of partially decomposed organic matter in anoxic soils (Clymo 1984) and it is estimated they continue to do so at an average rate of 0.07 to 0.096 Pg (1 Pg = $10^{15}$g) of carbon per year (Gorham 1991, Clymo et al. 1998). Decomposition of some soil organic matter (SOM) does take place in peatlands though - to varying degrees - releasing carbon in aquatic (dissolved organic carbon, DOC), solid (particulate organic carbon, POC) and gaseous forms such as carbon dioxide ($CO_2$) and methane ($CH_4$) (Gorham 1991, Freeman et al. 2001a, Freeman et al. 2001b, Worrall et al. 2003). The flux of these latter two key greenhouse gases (GHGs) between peatlands and the atmosphere has a globally significant effect on the Earth's climate. Globally these wetlands currently act as a net sink for atmospheric $CO_2$ (Kayranli et al. 2010) and crucially, unlike other ecosystems (such as forests) they are potent long term repositories for this carbon (Holden 2005, Dunn and Freeman 2011, Freeman et al. 2012). The actual mechanism allowing this carbon accretion, the 'enzymic latch' (Freeman et al. 2001b, Fenner and Freeman 2011), is so called because the sequestration results from a suppression of the normal enzymic decomposition pathways. This unusual stalling of SOM breakdown is the result of a disproportionate concentration of enzyme-inhibitory phenolic compounds in the peat-matrix, which accumulate due to constraints on phenol oxidase enzymes (Freeman et al. 2001b), impeding activities of the major agents of decomposition; namely, hydrolase enzymes (Freeman et al. 2004b). Removal of these limitations on phenol oxidases, such as

oxygen availability, temperature and pH, can initiate a biogeochemical cascade of degradation (Freeman et al. 1997a, Freeman et al. 2001a, Freeman et al. 2004b, Fenner and Freeman 2011). Conversely, manipulation to strengthen this enzymic latch has the potential to promote enhanced peatland carbon sequestration (Freeman et al. 2012). Such amplified suppression of decomposition in peat-soils would increase SOM accumulation, maximise the carbon storage intensity per unit area of peatlands and offer a new approach to geoengineering – acting as a cost efficient $CO_2$ removal (CDR) scheme (Royal Society 2009). We propose one method for doing this involves altering the amount of light available for photosynthesis to peatland plants. Preventing optimal rates of photosynthesis would decrease root exudation and thus further lower phenol oxidase activity in the upper layer of the acrotelm, or rhizosphere.

Root exudation is part of the rhizodeposition process of plants and involves the release of ions (eg. $H^+$), inorganic acids, oxygen, water and a wide-variety of carbon-based compounds; depending on the species of plant and its age, along with external factors such as biotic and abiotic stresses (Bais et al. 2006). These organic compounds can be separated into 1) low-molecular weight compounds, which include amino acids, organic acids, sugars, phenolics and an array of secondary metabolites; and 2) high-molecular weight compounds like mucilage and proteins (Badri and Vivanco 2009). Known as the 'rhizosphere priming effect' (RPE) (Kuzyakov 2002) these rhizodeposits are known to affect microbial communities and their activities in the soil surrounding the roots (Shackle et al. 2000) - an area known as the rhizosphere, which is split into three zones: endorhizosphere (root tissue, including the epdiermis and cortical layers), rhizoplane (the root surface with the epidermis and mucilage) and ectorhizosphere (the soil near the root) (Badri and Vivanco 2009). Amplified rhizodeposition has been

shown to increase microbial activity in the rhizopshere (Oger et al. 2004) and as most phenol oxidases in peat-soil are produced by bacteria (Burke and Cairney 2002) and fungi (Fenner et al. 2005a), we hypothesise that higher rhizodeposition will lead to higher extracellular phenol oxidase activity. Previous studies have shown that increased rates of photosynthesis increase rhizodeposition (Rovira 1969, Graham et al. 1982); therefore by repressing photosynthetic activity by altering the amount of light reaching peatland plants, it may be possible to curtail microbial activity and the production of phenol oxidase. The enzyme's activity may also be suppressed under sub-optimal photosynthesising conditions due to increased soil hypoxia, as oxygen (essential for phenol oxidase activity) is also a common root exudate (Bertin et al. 2003). The release of oxygen from the roots of some wetland plants, known as radial oxygen loss (ROL) has been shown to be correlated to photosynthetic activity (Lai et al. 2012). The intensity of the light, or photon flux density (PFD), which promotes optimal rates of photosynthesis is different depending on the plant species. Increasing the PFD beyond this point results in photosynthetic saturation, as the rate of photosynthesis does not increase, and eventually photoinhibitory damage, due to the over excitation of the photosynthetic reaction centres (Björkman and Demmig-Adams 1994). It was not known what the optimal PFD for photosynthesis was for the two vascular plant species used in our study but it was hypothesised it would be around 750 µmoles $m^{-2}$ $sec^{-1}$, the equivalent of a sunny midsummer's day at the chosen blanket bog site in North Wales, UK (personal communication, CEH 2013).

Being a dominant peatland plant, the effect of light on *Sphagnum* mosses was also investigated. As bryophytes they do not have roots in the same way as vascular plants, however; they do exude organic carbon which can increase microbial activity in the peat-soil (Shackle et al. 2000, Fenner et al. 2004). And with bryophytes being

generally shade-loving plants (Davey and Rothery 1997) it was hypothesised phenol oxidase activities would be highest in plants grown under lower light conditions, as they would reach photosynthetic saturation, or even be affected by photoinhibitory damage, at higher PFDs.

To investigate how varying the light intensity would affect phenol oxidase activities in the rhizosphere of peatland plants through rhizodeposition, intact peat mesocosms were collected from a blanket bog in the UK. Each had one of the three experimental plant species growing in it- *Calluna vulgaris*, *Eriophorum vaginatum* or mixed *Sphagnum* moss species. These typical peatland plants were chosen due to thier dominance of the study site. The mesocosms were kept for six weeks under experimental conditions in a specially constructed growth room which controlled the levels of light the plants received. Peat-soil samples were taken every week from the rhizosphere of each mesocosm and analysed for phenol oxidase activity.

If it was discovered that altering the PFD reaching peatland plants could increase the levels of carbon sequestered by peatlands it would require methods to shade or intensify vast areas of land. However, as a geoengineering project this may not be inconceivable, in terms of physical practicability and/or expense, when compared to other Solar Radiation Management (SRM) methods currently being considered. These include increasing the albedo of the desert, stratospheric aerosols and even the manufacture and release of space-based reflectors (Royal Society 2009). Indeed one of the unintended consequences of some SRM projects may be a reduction in the Photosynthetically Active Radiation (PAR) reaching peatlands.

## 3.3 Materials and methods

### 3.3.1 Study site and sample collection

Mesocosms of peat-soil (22,800mm$^2$ x 220mm) and vegetation, were collected in the first week of June, 2011, from the Migneint Valley in North Wales, UK. (3°48.8′ W, 52°59.6′ N). The area is a 200 km$^2$ Special Area of Conservation, incorporating one of the largest areas of blanket bog in Wales. The area is 460m above sea level and is predominantly a *Sphagnum*-rich, *Calluna vulgaris* (common heather) and *Eriophorum vaginatum* (hare's-tail cotton grass) dominant blanket mire, with acid grasses on drier hillslopes, and *Juncus effusus* (soft rush) in riparian areas. The underlying geology of the ombrotrophic bog is a mixture of acid and basic volcanic rocks, Ordovician shales and mudstones. Annual rainfall is 2,400mm and in the peat the water table is usually within 100mm of (and often at) the ground surface. The study area has a pore water pH, at a depth of 100mm, in the range of 4.1 to 5.1. The mean peat depth across the site is 2,000mm (Evans et al. 2012).

The mesocosms (held within black plastic containers) were collected so that they were dominated by one of the three experimental vegetative species - according to a score of 5 based on the Braun-Blanquet cover-abundance scale (Wikum and Shanholtzer 1978). The species studied were mixed *Sphagnum* moss species, *Calluna vulgaris* and *Eriophorum vaginatum*: referred to as *Sphagnum*, *Calluna* and *Eriophorum* (respectively) throughout this study. All groups of mesocosms containing one of the species were taken from an area of approximately 25m$^2$ and the entire study area was approximately 1,000m$^2$ of relatively level ground with similar peat composition, structure and hydrology. 15 mesocosms of each plant species were collected and care was taken to ensure they were obtained from regions of similar water-table level and microtopography, in the relevant 25m$^2$ area.

Using one of the plastic containers with its end removed as a guide each monolith was carefully extracted, to ensure the surface vegetation was kept intact and compaction of the soil was avoided to prevent any changes to its physical structure.

### 3.3.2 Experimental set-up and sampling regime

Once the mesocosms had been collected they were returned to the laboratory, approximately 30 miles from the study site, and were allowed to settle in a secure outdoor location for two weeks. They were then placed in a climate controlled room (set, due to constraints on the room's temperature control, to 18$^{o}$C) with commercially available growth lights, set to give 12 hour conditions of 1,000 (± 20) µmoles $m^{-2}$ $sec^{-1}$ PAR and 12 hours of darkness, for one week. Soil samples were taken (4g) every week from a depth of 50mm within the mesocosms, using a 10mm soil-borer and taking care to minimise any disturbance to the vegetation or soil structure.

The mesocosms were then randomly assigned a light regime for the next six weeks which meant they were either kept under the full 1,000 (± 20) µmoles $m^{-2}$ $sec^{-1}$ PAR conditions, or they were shaded – using horticultural mesh – so they received 750 (± 20) µmoles $m^{-2}$ $sec^{-1}$ PAR or 500 µmoles $m^{-2}$ $sec^{-1}$ PAR. The lower and higher light levels were chosen as they were 250 µmoles $m^{-2}$ $sec^{-1}$ either side of the equivalent PAR recorded during a sunny midsummer's day at the study site (750 µmoles $m^{-2}$ $sec^{-1}$). All other conditions were kept the same. Water levels in the mesocosms were checked daily and kept consistent with the level of the water table in field at time of collection, using laboratory prepared distilled water.

3.3.3 Phenol oxidase activity

The activity of extracellular phenol oxidases in the peat-soil from the different mesocosms was determined every week using 10 mM L-DOPA (dihydroxy phenylalanine) (Sigma-Aldrich Co., Ltd, Dorset) solution as a substrate, based on the procedure of Pind et al. (1994) and Williams et al. (2000).

Homogenates, made from 1g of peat (from every mesocosm) and 9mL of ultra-pure water, were prepared in separate Stomacher bags (Seward, West Sussex, UK) and a laboratory paddle blender (Stomacher circulator, Seward) was set to mix them for 30 seconds at normal speed setting, in order to minimise cell disruption. Aliquots, 750µL, of the homogenate were transferred into two 1.5mL Safe-Lock Eppendorf tubes (Eppendorf, Stevenage, UK) along with 750µl of 10 mM L-DOPA solution. The reaction tubes were incubated at the temperature of the soil in the microcosms (measured at the time of sample collection date) for nine minutes (after preliminary tests showed that phenol oxidase activity was linear up to this time). After incubation the tubes were centrifuged at 10,000rpm for five minutes and 300µL of each supernatant was pipetted into three wells of a clear 96 well Sterilin Microplate (Sterilin, Cambridge, UK) and absorbance measured at 475nm. Mean absorbance of the three control wells was then subtracted from that of the three L-DOPA wells for each sample and phenol oxidase activity calculated using Beers Law and the molar absorbency coefficient (3,700) for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate (diqc) (Mason 1948) and expressed as µmol of diqc produced $minute^{-1}$ $g^{-1}$ sample (dry weight). Dry weight used in each assay was determined by taking the mean dry weight of 1g of the original peat sample dried at 105°C to constant weight. A pH buffer has commonly been used in the protocols of other enzyme studies. However

in order to maintain conditions closely resembling those in the field, no buffer was added to control the pH of the phenol oxidase assay in this study.

### 3.3.4 Phenolic compound concentrations

The concentration of water extractable phenolics from the peat samples were assayed every week using a modified version of the spectrophotometric method developed by Box (1983). To conduct the assays, 1g samples of the peat were added to 9mL of ultra-pure water, homogenised using a laboratory paddle blender for 30 seconds and then incubated for a further 60 minutes. The solution was thoroughly mixed again, centrifuged at 10,000rpm and the resulting supernatant used for the analysis of phenolic concentration.

Within the 300µL wells of a flat-bottomed 96 well microplate, replicates of 250µL of the supernatant sample, 37.5µL of $Na_2CO_3$ solution (200 mg $L^{-1}$) and 12.5µL of Folin-Ciocalteau phenol reagent were dispensed and mixed thoroughly. A standard curve was prepared by adding the same chemicals to 0 to10 mg $L^{-1}$ phenol solutions. After two hours of incubation at room temperature, the colour change of the reactants was measured at 750nm absorption and the concentration of dissolved phenolics of each sample derived from the absorbance curve of the phenol standards (mg $L^{-1}$). Samples out of range of the standard curve were re-measured using a standard curve with a higher upper concentration or by diluting the supernatant sample (eg 1:10).

It has been suggested that there is no single optimal protocol for quantification of total phenolic concentrations (Yu and Dahlgren 2000). However, we selected the Folin-Ciocalteau method because it has been shown to be superior to most other methods (Yu and Dahlgren 2000) and, crucially, it has been used in previous used for

similar peat-soil samples (Freeman et al. 2004b, Fenner et al. 2005a). Although the method may have limitations (Yu and Dahlgren 2000), because we ensured the same extraction and quantification methods were applied to all peat samples, comparisons should prove reliable.

### 3.3.5 Statistical considerations

Relationships between variables were measured using Pearson's correlation coefficient ($r$), while significant differences between results were determined by independent one-way analysis of variance (ANOVA) tests and repeated measure ANOVAs. All statistical tests were performed using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA).

To ensure the reliability of the correlation coefficient and the $F$-ratio of the independent ANOVA, it was checked that the usual assumptions of parametric tests were not violated. The experimental design ensured that data was in a continuous, interval ratio format and measurements from the different treatment groups were independent. The normality of data distribution was checked by plotting the data graphically, looking at the values of skewness and kurtosis, and with the Sharpio-Wilk test (with a significant value less than 0.05 indicating a deviation from normality). For the independent ANOVA the assumption of homogeneity of variance was checked using the Levene's test, with a significant value greater than 0.05 indicating the variances were significantly equal, and therefore the assumption was tenable. If either the assumption of normality or homogeneity of variance was violated the data was transformed (eg. $\log(X_i + y)$, where $y$ was a given number added to all results positive). However, there is evidence to suggest that when group sizes are equal ANOVA is robust enough to cope with non-normally distributed data (Donaldson 1968, Field

2009) and therefore the *F*-ratio was taken from the Welch test, when the assumption of homogeneity of variances was violated, which is considered more robust in such circumstances (Field 2009).

For the repeated measures ANOVA the assumption that the phenol oxidase activities and phenolic concentration from the same mesocosms over the duration of the experiment are independent was obviously violated. The assumption of sphericity was therefore tested for, to confirm the level of dependence between experimental conditions is relatively equal, using the Mauchly's test – which tests the hypothesis that the variances of the differences between conditions are equal. A probability value of less than 0.05 of the Mauchly's test statistic therefore indicated there were significant differences between the variances of differences and, therefore, the condition of sphericity was not met. When this occurred the Greenhouse-Geisser correction was applied to the *F*-ratio.

For both the ANOVA tests the *F*-ratio was reported along with the degrees of freedom used to assess it, namely the degrees of freedom for the effect of the model and the degrees of freedom for the residuals of the model. To compare the various experimental light treatments against each other *post hoc* tests were performed. A significance value of less than 0.05 was again considered to be a statistically significant result.

The Pearson's correlation coefficient (*r*) was reported along with the significance value.

For displaying on the graphs standard error (*SE*), or standard deviation of the mean, was calculated by:

$$SE = \frac{\sigma}{\sqrt{n}}$$

where σ is the standard deviation of the parent population (derived by the square root of the group's variance from the mean) and *n* is the number of observations, or samples.

### 3.4. Results

The results from Figure 1 and analysis using repeated measures ANOVAs suggest that for all three plant species investigated there were no statistically significant differences on the effect of light intensity on phenol oxidase activity over the six week experimental time period (Figure 1): *Sphagnum, F* (8.16, 48) = 1.448, $p$ = 0.207; *Eriophorum*, *F* (9.22, 36) = 1.797, $p$ = 0.105; *Calluna*, *F* (10, 48) = 1.545, $p$ = 0.153. There was also no significant differences, for any of the species (*Sphagnum* (*F* (2, 14) = 2.601, $p$ = 0.115); *Eriophorum F* (2, 5.9) = 3.056, $p$ = 0.123; *Calluna* (F (2, 14) = 0.453, p = 0.646), when only the final week's enzyme activities were taken into account for each of the three light intensities. However, when all the weekly results were combined for the respective plant species and light intensity groups (Figure 4), a series of one-way ANOVAs indicated that there was statistically less phenol oxidase activity in the rhizospheres from the *Calluna* mesocosms at 50% full light (*M* = 0.03 µmol dicq $g^{-1}$ $min^{-1}$, *SE* = 0.004), compared to those under full light conditions (*M* = 0.05, *SE* = 0.007): *F* (2, 64.35) = 5.157, $p$ = 0.008, with a relevant planned contrast of *t* (64.49) = -1.170, $p$ = 0.004.

The light treatments only changed the final phenol oxidase activity in the rhizosphere of the *Calluna* mesocosms, with all the samples showing a decline (*F* (3, 12.5) = 13.488, $p < 0.001$), compared to before the light treatments were administered. In both the *Sphagnum* and *Eriophorum* mesocosms, activities remained statistically unaltered after six weeks.

Water extractable phenolic concentrations from the rhizospheres of the different plant groups were also not affected by light intensity, with all three intensities showing no significant difference from each other over the six weeks (Figure 2):

*Sphagnum, F* (9.77, 46) = 0.837, $p = 0.594$; *Eriophorum*, *F* (8.93, 48) = 1.887, $p = 0.081$; *Calluna*, *F* (9.55, 48) = 0.612, $p = 0.789$. Similarly to phenol oxidase activities, there were no significant differences, for any of the plant species, when only the final week's enzyme activities were taken into account for each of the three light intensities (Table 1). Unlike, the enzyme results though all the samples taken from the plant groups, irrespective of the intensity of the light they received, showed a significant change in phenolic concentration at the end of the experiment, compared to the start. Concentrations in the *Sphagnum* (*F* (3, 26) = 6.134, $p = 0.003$) and *Eriophorum* (*F* (3, 24) = 8.226, $p = 0.001$) rhizospheres all declined after six weeks, while they increased in the *Calluna* (*F* (3, 26) = 23.62, $p < 0.001$) samples.

When the phenol oxidase activity of all the plant species from the final week of the experiment (week six) were grouped together and plotted against their relevant phenolic concentrations (Figure 3), there was a statistically significant negative correlation for those mesocsoms kept in 100% light and those kept in 75% light ($r = -0.69$, $p$ (one-tailed) = 0.005, and $r = -0.608$, $p$ (one-tailed) = 0.018, respectively), albeit of a moderate size. There was no statistically significant correlation between phenol oxidase activity and phenolic concentrations at 50% full light: $r = -0.395$, $p$ (one-tailed) = 0.073.

By amalgamating all the weekly results for peat-soil phenol oxidase activity for each plant species under the separate light intensity treatments, differences between species were identified (Figure 4). At 100% full light there were statistically significant differences between the species (*F* (2, 55.9) = 9.161, $p < 0.001$), and post hoc comparisons, using the Games-Howell test, revealed that the enzyme activity in the

rhizosphere of the *Calluna* mesocosms were significantly less than the *Sphagnum* ($p$ = 0.16) and *Eriophorum* ($p$ = 0.003) samples. Both the other two light treatments, 75 and 50 % full light, showed the same effects ($F$ (2, 50.73) = 30.099, $p < 0.001$ and $F$ (2, 48.78) = 19.391, $p < 0.001$, respectively) with the former also indicating that *Sphagnum* samples had significantly less phenol oxidase activity than those from the *Eriophorum* mesocosms ($p < 0.001$). When phenolic concentrations (Table 1) were analysed in a similar way, no significant differences were observed between species.

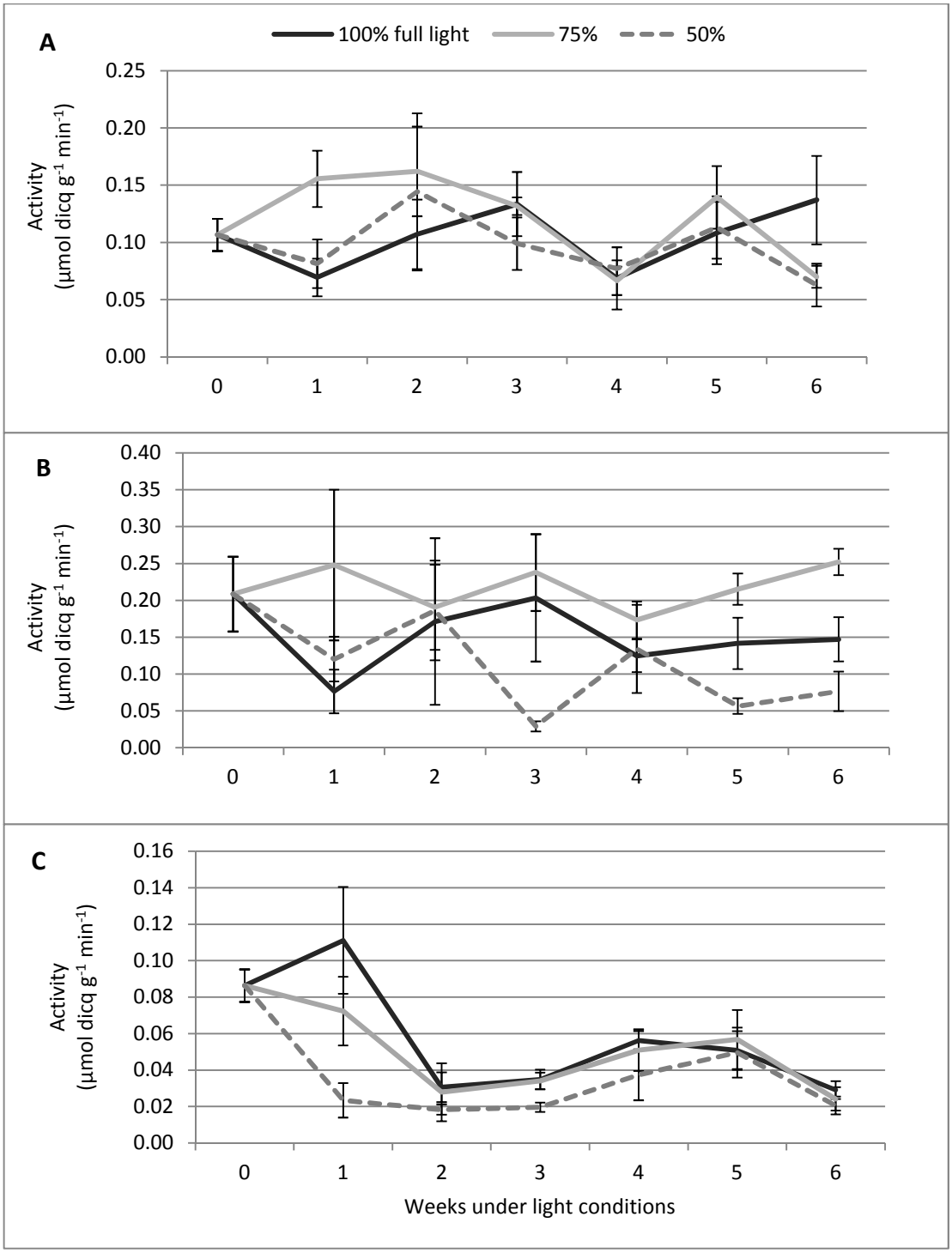



**Figure 1. Phenol oxidase activity in the rhizosphere of different peatland plant species over a six week period under varying light conditions.** Peat-soil samples taken from a depth of 5cm from mesocosms containing **A** = *Sphagnum* species; **B** = *Eriophorum*; **C** = *Calluna*. Mean averages are shown ($n$ = 5) and error bars indicate ± standard error.

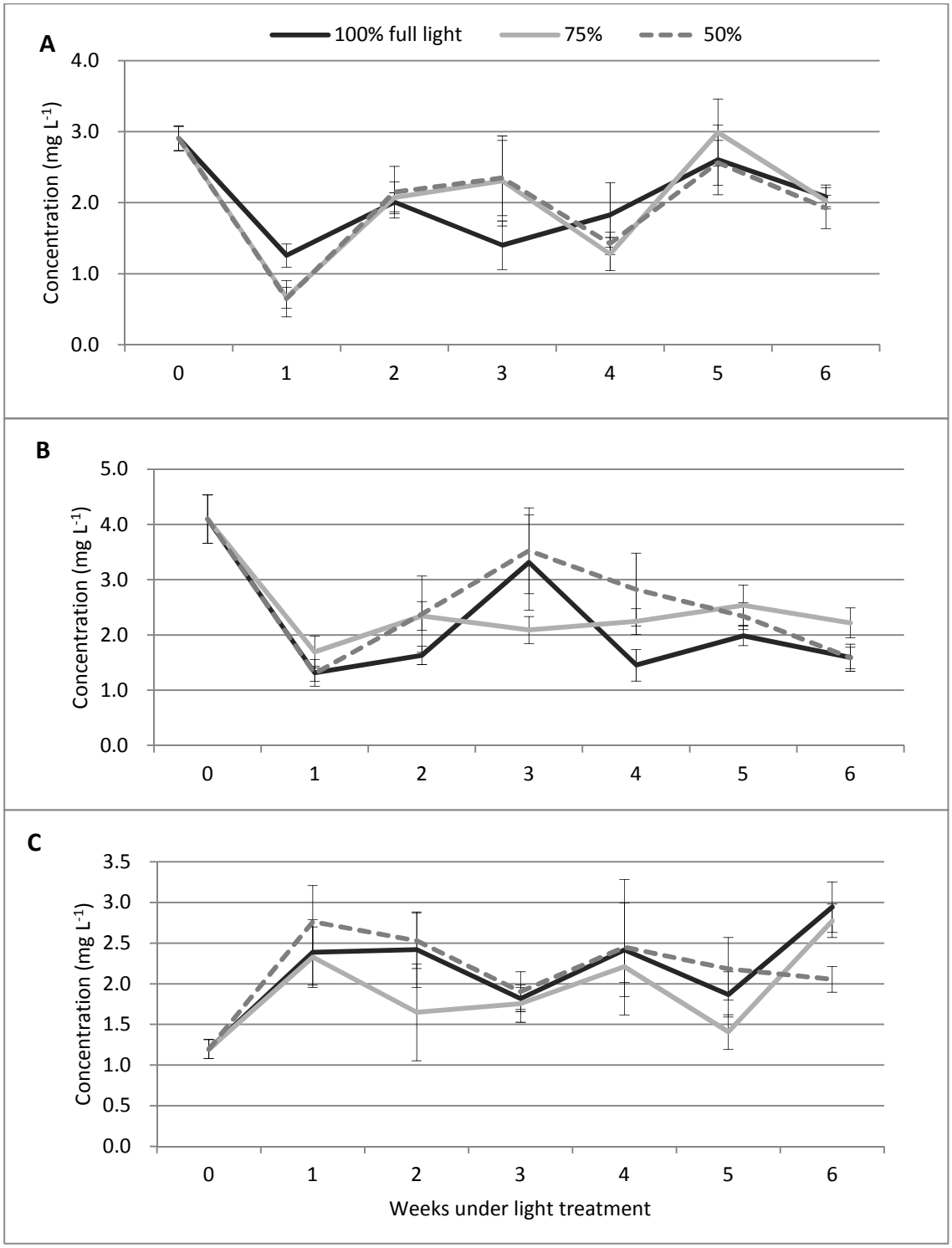



**Figure 2. Water extractable phenolic compound concentrations (mg $L^{-1}$) from the rhizosphere of different peatland plant species over a six week period under varying light conditions.** Peat-soil samples taken from a depth of 5cm from mesocosms containing **A** = *Sphagnum* species; **B** = *Eriophorum*; **C** = *Calluna*. Mean averages are shown ($n$ = 5) and error bars indicate ± standard error.

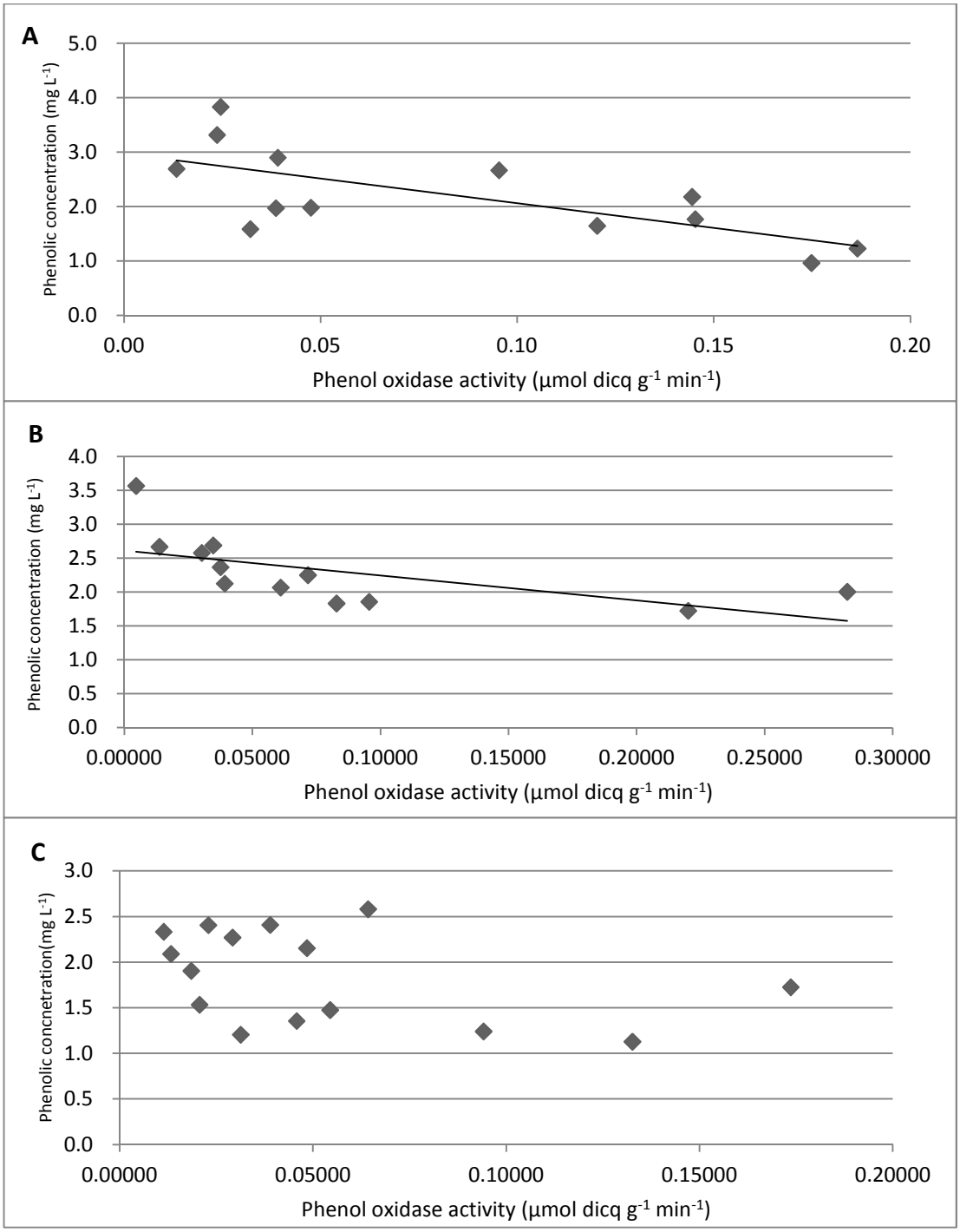



**Figure 3. Investigating the relationship between phenol oxidase activity and water extractable phenolic compound concentrations from the rhizosphere of a blanket bog under varying light conditions.** Peat-soil samples taken from a depth of 5cm from mesocosms containing *Sphagnum*, *Eriophorum*, and *Calluna* species. Data for all species, from week six, are shown in each graph. **A** = 100% full light; **B** = 75% full light; **C** = 50% full light.

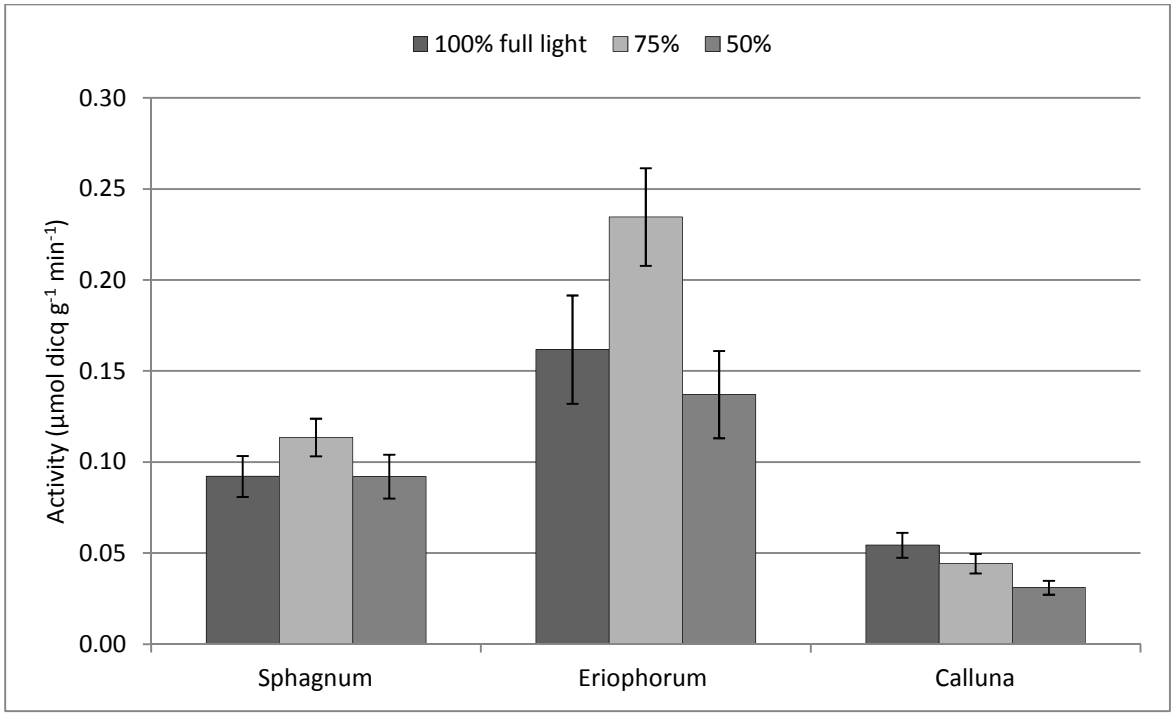



**Figure 4. Phenol oxidase activity in the rhizosphere of peatland plant species.** Mean averages from the entire six week experiment are shown ($n$ = 35). Error bars indicate ± standard error.

| | *Sphagnum* | *Eriophorum* | *Calluna* |
|---|---|---|---|
| 100% full light | **1.990** (0.138) | **2.163** (0.215) | **2.119** (0.160) |
| 75% | **2.002** (0.176) | **2.606** (0.265) | **1.917** (0.140) |
| 50% | **2.042** (0.176) | **2.605** (0.255) | **2.171** (0.165) |

**Table 1. Water extractable phenolic concentrations in the rhizosphere of peatland plant species.** Mean averages from the entire six week experiment are shown ($n$ = 35) in bold. Error bars (in parentheses) indicate ± standard error.

### 3.5. Discussion

It has long been accepted that light intensity affects vascular plant root exudates into the rhizosphere; with higher levels of light, and the associated higher rates of photosynthesis, generally resulting in increased rhizodeposition (Rovira 1969, Graham et al. 1982); including the release of $CO_2$ (Kuzyakov and Cheng 2001) and oxygen (Christensen et al. 1994, Sorrell 1999). As root exudates can substantially increase microbial activity in the rhizosphere (Shackle et al. 2000, Oger et al. 2004) it was hypothesised that when light levels were optimal for photosynthesis by the species, production and release of phenol oxidases would increase from the communities of bacteria (Fenner et al. 2005a) and fungi (Burke and Cairney 2002), and even from the plants themselves (Gramss et al. 1999). When coupled with root oxygen release, raising the redox potential of the soil, these conditions would increase the enzymes' measurable activity in the peat-soil by 'opening' the enzymic latch (Freeman et al. 2001b). The same was predicted in the mesocosms containing *Sphagnum* species, as although being bryophytes and not having roots like higher plants *Sphagnum* species are also known to exude organic carbon which can increase microbial activity in the peat (Fenner et al. 2004). However, our results showed that the only significant effect photon density had on phenol oxidase activities were found when the mean average of the whole six weeks was considered, with *Calluna* samples at 50% full-light showing a 43% drop in activity compared to those at 100% full-light (Figure 4). Otherwise there were no significant differences in the enzyme's activity between the photon density treatments for any of the plants over the experimental time period. This could indicate that none of the range of light levels we used were optimal for photosynthesis in any of the species - though it should still have been possible to identify differences between those samples at full light (250 µmoles $m^{-2}$ $sec^{-1}$ above the equivalent of a bright sunny

day) compared to those shaded and only receiving 50% full light (or 250 µmoles $m^{-2}$ $sec^{-1}$ less than a sunny day). However, as there were no visual differences in growth rates or senescence observed during the experiment this may, when coupled with the similar phenol oxidase activities, provide evidence that wetland plants from blanket bogs in the UK are adapted to maximise their photosynthetic abilities across a wide range of light intensities. This supports long-standing evidence that plants, including *Sphagnum*, have evolved mechanisms for acclimatising to different environmental (specifically levels of PAR) conditions, and can optimise their growth to suit these by reallocating resources and/or changing their morphology (Arp 1991, Bonnett et al. 2010). As a result, root exudation may have remained comparable across our three light regimes, whereas carbon allocation to other parts of the plant (eg. leaves) may have been affected by the treatments. Moreover, our study also agrees with previous research showing that despite bryophytes generally being considered shade-adapted plants, reaching photosynthetic light saturation at low irradiances of between 30 and 300 µmoles $m^{-2}$ $sec^{-1}$ (Davey and Rothery 1997), some species of open, sun-exposed habitats, such as *Sphagnum* species, may not reach saturation until 1,000 µmoles $m^{-2}$ $sec^{-1}$ (Proctor 2002). It may also have been possible that light treatments were actually having an effect on rates of photosynthesis and root exudation levels, but any effect on phenol oxidase activity was offset by a correlated release of phenolic compounds, exuded from the roots of the *Calluna* or *Eriophorum* (Dakora and Phillips 2002) and *Sphagnum* tissues (Rasmussen et al. 1995). These inhibitory chemicals would have suppressed the activity of hydrolase enzymes and the further release of inorganic nutrients needed by phenol oxidase-producing microorganisms (Fenner and Freeman 2011). However, this explanation seems unlikely as we did not observe any significant

differences in leachable phenolic compound concentrations for the light treatments in any of the plant species (Figure 2 and Table 1).

*Calluna* was the only species to show an overall decline in phenol oxidase activity over the six week experimental time period (Figure 1), correlating with an overall decline in leachable phenolic compound concentrations (Figure 2). This coincides with work by Yan et al. (2008) who recorded lower concentration of DOC from *Calluna* dominated peat, and could be due to the observed structure and morphology of *Calluna* roots, the roots hairs of which were at a greater depth than *Eriophorum* or *Sphagnum*. Therefore any increase in phenol oxidase activity may 1) not have been measurable with our sampling regime and 2) been suppressed by increased anaerobic conditions found at greater depths in the peat-soil profile. Indeed average phenol oxidase activities were found to be lower in all the *Calluna* samples compared to the other two plant species investigated (Figure 4).

To answer the main question of our study, our results suggest that light intensity does not regulate phenol oxidase activity in the rhizosphere of a blanket bog. Interestingly though we did observe significant differences in the enzyme activity between the plant species themselves. For all light regimes, *Eriophorum* had the higher phenol oxidase activities, followed by *Sphagnum* and then *Calluna* (*Eriophorum* > *Sphagnum* > *Calluna*). If phenol oxidase activity is used as a proxy for the decomposition of organic matter, and thereby release of both gaseous ($CO_2$) and aqueous (dissolved organic carbon, DOC) carbon from peatlands (Freeman et al. 2004b), our results suggest that heather is the most desirable plant species to maximise the ecosystem's carbon sequestration capabilities. With lower phenol oxidase activities

allowing the build-up of hydrolase enzyme-inhibitory phenolic concentrations (Fenner and Freeman 2011).

Before conclusions are drawn about the potential of altering light-densities across areas of peatland for geoengineering projects, further interdisciplinary research should be undertaken to continue the work of this pilot study. For instance rates of photosynthesis must be measured and a wider range of photon densities should be used, including going lower than 500 µmoles $m^{-2}$ $sec^{-1}$ and higher than 1,000 µmoles $m^{-2}$ $sec^{-1}$, in order to determine where each wetland plant species has its maximum and minimum photosynthetic rate, and where they reach photosynthetic light saturation. Alongside phenol oxidase and phenolic compound concentration measurements, carbon pathways must also be analysed from the samples, with regular $CO_2$ fluxes and DOC measurements being taken. However, perhaps the most obvious factor which needs to be investigated further is the experimental time-period. Our investigation was conducted over a relatively short period: six weeks. It could be that this is not long enough for any changes in light levels to have an effect on rhizodeposition and any resulting phenol oxidase activity. Clearly a longer experiment is needed, taking in an entire growing season or, preferably, a whole year.

Our results do provide support for the use of the enzymic latch mechanism to maximise carbon sequestration in peatlands, with the significant negative correlations between phenol oxidase activities and leachable phenolic compound concentrations observed for 100% and 75% full-light treatments, when the results of all the plant species were amalgamated (Figure 3). This is consistent with Freeman's previous work looking at the relationship between phenolic compounds and phenol oxidase (Freeman et al. 2001a, Freeman et al. 2001b, Freeman et al. 2004b, Fenner and Freeman 2011)

and confirms that when phenol oxidase activity is low phenolics accumulate. Therefore, our study suggests the most promising use of peatland plants as a bio-geoengineering project would be to encourage the growth of certain species over others in order to minimise the activity of phenol oxidases in the peat soil. We would suggest the order of peatland plants to achieve maximised carbon sequestration is therefore; *Calluna* > *Sphagnum* > *Eriophorum*.

# Chapter 4

## Can plant diversity manipulation be used to increase carbon sequestration in a peatland ecosystem?

## 4.1 Abstract

Plants are known to influence carbon fluxes from peatlands both i) directly through respiration and ii) by the production of litter and root exudates, which are then broken down by microbes within the peat matrix. In this study we investigated whether three different plant species typical of a UK blanket bog - *Calluna vulgaris, Juncus effusus* and mixed *Sphagnum* species - would affect the carbon sequestering abilities of peat samples taken from directly beneath them. To quantify this we measured fluxes of soil derived $CO_2$ and $CH_4$, and extractable levels of dissolved organic carbon from peat samples taken from areas dominated by one of the three plant communities. It was found that there were significant differences between the carbon fluxes from the different sites, which we attributed to changes brought about by the vegetation on the pH, phenolic concentrations and extracellular enzyme activities found in the peat matrix. Peat taken from *Sphagnum*-dominated areas emitted less $CO_2$ than the other two sample groups, as was its overall DOC concentrations and phenol oxidase activities. Conversely, *Juncus*-peat had the highest $CO_2$ and $CH_4$ fluxes, along with the greatest phenol oxidase activities. Taking all the results into consideration the plants were ranked in order of their ability to sequester carbon to the peat soil within which they were growing: *Sphagnum* > *Calluna* > *Juncus*. These results suggest that plant community structures could be altered in order to maximise a peatland's ability to be used as a carbon store should they be managed as part of a carbon stewardship scheme or a geoengineering project.

## 4.2 Introduction

Northern peatlands play a key role in the global carbon cycle. Fluxes of carbon dioxide ($CO_2$) and methane ($CH_4$) between these organic wetlands and the atmosphere affect our climate (Frolking et al. 2006); while fluvial exports of dissolved organic carbon (DOC) influence nutrient cycling, and associated $CO_2$ release, in freshwater and marine systems (Hansell et al. 2004, Fenner et al. 2007b). Preventing the release of these greenhouse gases (GHGs) and carbon has been proposed as a method of "carbon stewardship" to create carbon offsetting credits (Worrall et al. 2009, Dunn and Freeman 2011), and even as climate-cooling geoengineering projects (Freeman et al. 2012).

The biogeochemical cycles responsible for these carbon flows, and the abiotic factors controlling them, have been intensely researched and debated over the past two decades (reviews by Limpens et al. 2008a, Kayranli et al. 2010), along with the effect vegetation has on the direct fluxes of GHG and DOC (eg. Roura-Carol and Freeman 1999, Moore et al. 2002, Neff and Hooper 2002, Strom et al. 2003, Ward et al. 2009, Henneberg et al. 2012). A growing number of studies have also looked at the effect plants have on peatland biogeochemistry (eg. Ward et al. 2007, Sutton-Grier and Megonigal 2011). Although Ward et al. (2007) concluding that peat decomposer communities were not particularly sensitive to vegetation community composition, our previous work (Dunn et al. in preparation; Chapter 3) and indeed later studies by Ward (Ward et al. 2010) suggested this area warranted further study.

In this study, we investigate how plants affect the three main pathways of carbon efflux from peat-soil: $CO_2$, $CH_4$ and DOC. These are all produced from the decomposition of organic matter in the peat matrix, and plants can influence this rate of breakdown in two principle ways. The first is the quantity and quality of the soil litter -

formed from dead plant tissues - in terms of the nutrient content, carbon to nitrogen ratio (C:N) and degradability (Bragazza et al. 2006, Limpens et al. 2008a). The second is the quality and quantity of root exudation - part of the rhizodeposition process in plants and involves the release of ions, inorganic acids, oxygen, water and a wide-variety of carbon-based compounds (Bais et al. 2006). These organic compounds can be separated into 1) low-molecular weight compounds, which include amino acids, organic acids, sugars, phenolics and an array of secondary metabolites; and 2) high-molecular weight compounds like mucilage and proteins (Badri and Vivanco 2009). Known as the 'rhizosphere priming effect' (RPE; Kuzyakov 2002) the released labile carbon is known to stimulate microbial communities, increasing their activities in the surrounding soil resulting in the amplification of organic matter breakdown (Shackle et al. 2000). To increase carbon storage in peatlands it is therefore desirable to encourage the dominance of plant species which may facilitate the suppression of decomposition of this litter or soil organic matter (SOM). We hypothesise that the plant communities, or species, most suited for this are those that strengthen the 'enzymic latch' (Freeman et al. 2001b). This can be achieved by either 1) inhibiting extracellular phenolic compound oxidation through production of recalcitrant, phenolic-rich litter and/or polyphenol secondary metabolites in root exudation, or 2) limiting the amount of oxygen available in the peat matrix, necessary for the functioning of phenol oxidases (Freeman et al. 2004b), which can be transported into organic soils by plants via aerenchyma tissues and out through their roots (often referred to as radial oxygen loss; ROL). Such strengthening of the enzymic latch would impede the activity of hydrolase enzymes, the main suite of agents responsible for the breakdown of organic matter (Freeman et al. 2004b).

In this study we wanted to determine whether three typical UK blanket bog plant species (*Calluna vulgaris, Juncus effusus* and mixed *Sphagnum* species) could be ranked in order of their ability to increase carbon sequestration in the peat matrix in which they grow. To calculate this we measured $CO_2$, $CH_4$ and DOC production from peat samples taken from two different depths, 10cm and 30cm, to ensure we were observing interactions in the peat which were directly affected by the roots (rhizosphere). The work was carried out at two contrasting times of the year, summer and winter, to ensure we considered the seasonal differences in plant activity in the form of growth and photosynthesis. In addition, extractable phenolic concentrations and enzyme activities were analysed to evaluate if the enzymic latch was being strengthened as hypothesised. A number of other specific hypotheses were also tested:

1. Peat taken from areas dominated by *Juncus* plants will have the lowest concentrations of phenolic compounds, which will result in the highest phenol oxidase and hydrolase enzyme activities, and result in the higher production of $CO_2$ compared to peat taken from *Calluna* or *Sphagnum* dominated areas. These features will be observed in the *Juncus* samples due to the availability of labile litter and root exudates, plus high ROL from these aerenchymatous, productive rushes (Sorrell 1999, Ervin and Wetzel 2000, Zhai et al. 2013).

2. Peat taken from areas dominated by *Juncus* plants will have the lowest $CH_4$ fluxes due to the more oxygenated environment (from high ROL) inhibiting the activity of methanogens and increasing $CH_4$ oxidation (Hanson and Hanson 1996, Fridovich 1998).

3. Peat taken from areas dominated by *Juncus* plants will have the highest levels of water-extractable DOC due to the higher microbial breakdown of SOM, brought about by the presence of labile-carbon and the oxygenated environment.

4. Peat taken from areas dominated by *Calluna* plants will have the lowest rates of $CO_2$ and DOC production due to the species' production of lignin-rich woody plant tissue (van der Heijden and Boon 1994, Huang et al. 1998): strengthening the enzymic latch mechanism (Freeman et al. 2001b)

If it is discovered that there are significant differences between the carbon sequestering capabilities of the different peat samples, it is hoped our findings could assist with land management practices should areas of peatland be designated for carbon storage projects – often referred to as $CO_2$ removal (CDR; Royal Society 2009).

## 4.3 Materials and methods

### 4.3.1 Study site

Peat samples for the experiments were taken from the Migneint Valley in North Wales, UK. (3°48.8′ W, 52°59.6′ N). The area is a 200 $km^2$ Special Area of Conservation, incorporating one of the largest areas of blanket bog in Wales. The area is 460m above sea level and is predominantly a *Sphagnum*-rich. *Calluna vulgaris* (common heather) and *Eriophorum vaginatum* (hare's-tail cotton grass) dominant blanket mire, with acid grasses on drier hillslopes, and *Juncus effusus* (soft rush) in riparian areas. The underlying geology of the ombrotrophic bog is a mixture of acid and basic volcanic rocks, Ordovician shales and mudstones. Annual rainfall is 2,400mm and in the peat the water table is usually within 100mm of (and often at) the ground surface. The study site has a pore water pH, at a depth of 100mm, in the range of 4.1 to 5.1. The mean peat depth across the site is 2,000mm (Evans et al. 2012).

### 4.3.2 Sample collection

Botanical surveys were conducted using the Braun-Blanquet cover-abundance scale (Wikum and Shanholtzer 1978), with areas approximately 25$m^2$ scoring 5 (75 to 100% cover) by the required vegetation being selected for sample collection. The dominant species selected for this study were mixed *Sphagnum* moss species, *Calluna vulgaris* and *Juncus effusus*: referred to as *Sphagnum*, *Calluna* and *Juncus* (respectively) throughout this study. The entire study area was approximately 1,000$m^2$ of relatively level ground with similar peat structure and hydrology.

Four peat-soil samples (>50g) were taken from within each of the selected areas at depths of 10cm and 30cm, by the use of a hand trowel to excavate small pits, during the summer (August) and winter (February). Care was taken to ensure the

samples from each of the 25m$^2$ areas were obtained from regions of similar peat-structure, water-table level and microtopography,. Samples were placed in separate sealable plastic bags and transported to the laboratory in a cooled, insulated container. The temperature of the soil at the location of sample collection was recorded. Back in the laboratory the soil samples had any adhering debris and macroinvertebrates removed from them before being stored at 4$^o$C until they were needed for analysis.

4.3.3 Soil respiration

To assess the rates of soil respiration of the different sites and depths during the two seasons 10g samples of peat were placed into 50mL centrifuge tubes (Fisher Scientific UK Ltd, Loughborough, UK) and lids, with a centrally-located size nine Suba-Seal rubber septum (Sigma Aldrich Ltd, Dorset, UK), were secured onto them forming an air-tight seal. The tubes were incubated, in the dark and at field soil temperatures, for 90 minutes (after preliminary tests demonstrated that $CO_2$ and $CH_4$ concentrations increased linearly up to this time). A 10cm$^3$ gas syringe, fitted with a two-way valve (Sigma Aldrich Ltd, Dorset, UK) and a short bevel hypodermic needle, was used to collect a 10mL gas sample through the septa of each tube (considered time-two; T-2, for flux calculations) which were transferred to labelled pre-evacuated 5.9mL Exetainers® (Labco Ltd, Lampeter, UK). In order to calculate carbon-based trace gas fluxes initial background gas measurements (considered as time-one; T-1) were taken from the headspace above the peat sample in the centrifuge tubes before the lids were placed onto them.

Gas samples were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC) instrument, equipped with a flame ionisation detector (FID)

with a $CO_2$ to $CH_4$ catalytic converter (methaniser), to measure concentrations of $CO_2$ and $CH_4$. Two millilitres of gas from the exetainers containing the samples was injected via a 1041 on-column injector system, set at 40°C onto a PoraPak QS (1.83m x 3.18mm) 80/100 column. The column oven temperature was set to 40°C and the carrier gas, oxygen free nitrogen, had a flow rate of 30mL $min^{-1}$.

The temperature of the methaniser was 300°C and the FID was at 125°C. The latter had a hydrogen flow of 25mL $min^{-1}$, 20mL $min^{-1}$ make-up gas (oxygen free nitrogen) and 300mL $min^{-1}$ of compressed air. The methaniser had a hydrogen flow of 5mL $min^{-1}$.

$CH_4$ and $CO_2$, (retention times 1.08 and 1.87 minutes respectively) were quantified by comparison of peak area with that of the three standards of known concentration (Table 1), prepared by Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK), used in the preparation of a standard curve, which was only accepted if the correlation coefficient ($R^2$) value was greater than 0.98.

| | **$CO_2$** | **$CH_4$** |
|---|---|---|
| **Standard A** | 250 | 2 |
| **Standard B** | 500 | 25 |
| **Standard C** | 1,000 | 50 |

**Table 1. Concentrations (ppm) of the standard gases.** A certificate of analysis (CofA) was requested with each new batch of gases and the calibration curve altered accordingly to take into account the exact amounts.

Calculating the gaseous fluxes from the initial background (T-1) concentrations was achieved by the following equation (Levy et al. 2011):

$$\text{Flux } (\mu g\ g^{-1}\ s^{-1}) = \frac{\delta C}{\delta t} \times \left(\frac{V \times M}{m \times V_{mol}}\right)$$

Where $\delta C$ is rate of change in the gas concentration (T-2 minus T-1); $\delta t$ is the change in time from the background reading to the final measurement (in seconds); $V$ is volume of the headspace of the chamber ($m^3$); $M$ is the molecular weight of the gas (16.04 and 44.01 g $mol^{-1}$ for $CH_4$ and $CO_2$ respectively); $m$ is the mass of the peat; $V_{mol}$ is the volume of a mole of gas (air) at a given temperature ($m^3\ mol^{-1}$) calculated by:

$$p \times (R \times K)$$

Where $p$ is pressure (kPa); $R$ is equal to 8.314 (the ideal gas constant) and $K$ is temperature (Kelvin).

4.3.4 Phenol oxidase activity

The activity of extracellular phenol oxidases in peat from the different sites and depths during the two seasons was determined using 10 mM L-DOPA (dihydroxy phenylalanine) (Sigma-Aldrich Co., Ltd, Dorset) solution as a substrate, based on the procedure of Pind et al. (1994) and Williams et al. (2000).

Homogenates, made from 1g of peat (from every sample) and 9mL of ultra-pure water, were prepared in separate Stomacher bags (Seward, West Sussex, UK) and a laboratory paddle blender (Stomacher circulator, Seward) set to mix them for 30 seconds at normal speed setting, in order to minimise cell disruption. Aliquots, 750µL, of the homogenate were transferred into two 1.5mL Safe-Lock Eppendorf® tubes (Eppendorf, Stevenage, UK) along with 750µl of 10 mM L-DOPA solution. The reaction tubes were incubated at field soil temperature (measured at the time of sample

collection date) for nine minutes (after preliminary tests showed that phenol oxidase activity was linear up to this time). After incubation the tubes were centrifuged at 10,000rpm for five minutes and 300μL of each supernatant was pipetted into three wells of a clear 96 well Sterilin Microplate (Sterilin, Cambridge, UK) and absorbance measured at 475nm. Mean absorbance of the three control wells was then subtracted from that of the three L-DOPA wells for each sample and phenol oxidase activity calculated using Beers Law and the molar absorbency coefficient (3,700) for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate (diqc) (Mason 1948) and expressed as μmol of diqc produced $minute^{-1}$ $g^{-1}$ sample (dry weight). Dry weight used in each assay was determined by taking the mean dry weight of three 1g of original peat sample dried at 105$^{o}$C to constant weight. A pH buffer has commonly been used in the protocols of other enzyme studies. However in order to maintain conditions closely resembling those in the field, no buffer was added to control the pH of the phenol oxidase assay in this study.

4.3.5 Hydrolase enzyme activities

The activity of five extracellular hydrolase enzymes in peat from the different sites and depths during the two seasons was determined by utilising fluorogenic 4-methylumbelliferyl (MUF) labelled substrates which fluoresce at 450 nm emission, 330nm excitation, and using methods based on work by Freeman et al. (1995). Homogenates of 1g of the peat sample and 7mL of the relevant MUF substrate (see Table 2) were made using a laboratory paddle blender.

All the samples were incubated at field soil temperature, for 45 minutes for phosphatase and 60 minutes for N-acetyl-β-D-glucosaminidase, β-D-xylosidase,

Arylsulphatase, β-D-glucosidase. Two minutes before the incubation time came to an end the contents of each stomacher bag was thoroughly mixed by hand and the solution from each substrate bag used to fill one 1.5mL Safe-Lock Eppendorf tube, which were then centrifuged at 14,000rpm for five minutes in a temperature controlled centrifuge, set to the required temperature. From the resulting supernatant of each substrate tube 250µL was pipetted into a well of a black flat-bottomed 96 well microplate; after ensuring each well already contained 50 µL of ultra-pure water - guaranteeing a comparable dilution to the standard solutions in the calibration curve. A fluorometer was used to determine the fluorescence of the MUF at 450 and 330nm excitation.

| **Substrate** | **Enzyme** |
|---|---|
| 4-MUF β-D-glucopyranoside | β-D-glucosidase |
| 4-MUF sulphate potassium salt | Arylsulphatase |
| 4-MUF β-D-xylopyranoside | β-D-xylosidase |
| 4-MUF N-acetyl-β-D-glucosaminide | N-acetyl-β-D-glucosaminidase |
| 4-Methylumbelliferyl phosphate | Phosphatase |

**Table 2. The MUF-labeled substrates needed for measuring the specified enzyme's activity.**

Standards for the calibration curve were prepared in a similar way to the substrate samples; with 1g of the peat sample being added to 7mL of ultra-pure water and homogenised in the laboratory paddle blender for 30 seconds. From this 1.5 mL was centrifuged at 14,000rpm for five minutes before 250µL of the resulting supernatant was transferred from each vial to six separate wells on a black flat-bottomed 96 well microplate. Fifty microlitres of varying concentrations of a MUF-

free acid stock solution were added to the supernatant to create a concentration curve of 0-100µM, from which the enzyme activity was determined.

Enzyme activities were expressed as MUF produced (µmol $g^{-1}$ $min^{-1}$), correcting for the dry weight of the peat.

4.3.6 Phenolic compound concentrations

The concentration of water extractable phenolics from the peat samples were assayed using a modified version of the spectrophotometric method developed by Box (1983). To conduct the assays, 1g samples of the peat were added to 9mL of ultra-pure water, homogenised using a laboratory paddle blender for 30 seconds and then incubated for a further 60 minutes. The solution was thoroughly mixed again, centrifuged at 10,000rpm and the resulting supernatant used for the analysis of phenolic concentration.

Within the 300µL wells of a flat-bottomed 96 well microplate, replicates of 250µL of the supernatant sample, 37.5µL of $Na_2CO_3$ solution (200 mg $L^{-1}$) and 12.5µL of Folin-Ciocalteau phenol reagent were dispensed and mixed thoroughly. A standard curve was prepared by adding the same chemicals to 0 to10 mg $L^{-1}$ phenol solutions. After two hours of incubation at room temperature, the colour change of the reactants was measured at 750nm absorption and the concentration of dissolved phenolics of each sample derived from the absorbance curve of the phenol standards (mg $L^{-1}$). Samples out of range of the standard curve were re-measured using a standard curve with a higher upper concentration or by diluting the supernatant sample (eg 1:10).

4.3.7 Dissolved Organic Carbon

To measure the water extractable dissolved organic carbon (DOC) concentrations of the peat samples from the different sites and depths 4g of each sample were placed in a stomacher bag and 36mL of ultra-pure water was added to them. Samples were then homogenised in the stomacher machine for 30 seconds. Each sample was filtered to remove particulate materials, firstly using glass microbe filters and then using sterile membrane filters (0.45μm pore size).

Filtered water samples were then analysed with an Analytical Sciences Ltd Thermalox TOC-TN analyser, using a carrier gas of high purity oxygen at a flow rate of 180mL $min^{-1}$ and an injection volume of 15μL. A six-point calibration was performed, using 1 to 100 $mgL^{-1}$ of potassium hydrogen phthalate (total organic carbon, TOC). Samples were acidified with 1M HCl and sparged for 90 seconds with the carrier gas to remove all inorganic carbon prior to analysis.

4.3.8 pH

To measure the pH, 1g of each sample was added to 9mL of ultra-pure water and homogenised thoroughly using a laboratory paddle blender for 30 seconds. A Mettler Toledo FE20 Desktop pH meter, which was calibrated with two buffers of known pH (4 and 7), was used to measure the pH of each water-soil homogenate. For data analysis pH values were transformed to H+ concentrations (μmol $L^{-1}$) assuming pH is the negative logarithm of the hydrogen ion concentration.

4.3.9 Statistical considerations.

Relationships between variables were measured using Pearson's correlation coefficient (r), while significant differences between results were determined by independent analysis of variance (ANOVA) tests. All statistical tests were performed using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA).

To ensure the reliability of the correlation coefficient and the *F*-ratio it was checked that the usual assumptions of parametric tests were not violated. The experimental design ensured that data was in a continuous, interval ratio format and measurements from the different treatment groups were independent. The normality of data distribution was checked by plotting the data graphically, looking at the values of skewness and kurtosis, and with the Sharpio-Wilk test (with a significant value less than 0.05 indicating a deviation from normality). For the *F*-ratio the assumption of homogeneity of variance was checked using the Levene's test, with a significant value greater than 0.05 indicating the variances were significantly equal, and therefore the assumption was tenable. If either the assumption of normality or homogeneity of variance was violated data was transformed (eg. $\log(X_i + y)$, where $y$ was a given number added to all results to make them positive). However, there is evidence to suggest that when group sizes are equal ANOVA is robust enough to cope with non-normally distributed data (Donaldson 1968, Field 2009) and therefore the *F*-ratio was taken from the Welch test, when the assumption of homogeneity of variances was violated, which is considered more robust in such circumstances (Field 2009).

When reporting the results of the ANOVA the *F*-ratio was used along with the degrees of freedom used to assess it, namely the degrees of freedom for the effect of the model and the degrees of freedom for the residuals of the model. To compare the various experimental treatments against each other Tukey *post hoc* tests were

performed, when group variances were similar, otherwise the Games-Howell procedure was used. A significance value ($p$) of less than 0.05 was considered to be a statistically significant result, as this represents a 95% level of confidence.

The Pearson's correlation coefficient ($r$) was reported along with the significance value.

For displaying on the graphs standard error ($SE$), or standard deviation of the mean, was calculated by:

$$SE = \frac{\sigma}{\sqrt{n}}$$

where σ is the standard deviation of the parent population (derived by the square root of the group's variance from the mean) and $n$ is the number of observations, or samples.

## 4.4 Results

Figures 1 and 2 show that peat taken from directly beneath *Juncus* dominated areas of bog had the highest $CO_2$ and $CH_4$ fluxes, when the sum of winter and summer results were taken into account. All summer $CO_2$ fluxes were higher than winter fluxes: by 1.5 to 4.3 times at 10cm. Differences between $CO_2$ fluxes at the two depths (10cm and 30cm) were consistently more pronounced in the winter samples although during both seasons *Sphagnum* showed the greatest difference between depths, with the 10cm flux being 11.6 times greater than the 30cm flux in the winter, compared to double the amount in the summer. When a one-way analysis of variance (ANOVA) was used to explore comparisons between the effect of different plant communities on peat gas fluxes at each depth and season it was found that only the results for winter at 30cm did not show any statistical differences (Table 3A).

All $CH_4$ fluxes from peat directly beneath *Juncus* communities were positive, indicating $CH_4$ was being produced instead of rather than consumed to give the negative fluxes seen in all the other results except from those samples taken from 30cm during the winter (Figure 2). A series of ANOVA tests indicated that at each depth and season there were significant differences between the fluxes (Table 3A), although not between *Sphagnum* and *Calluna*, according to the relevant post-hoc tests.

It is important to note that the differences in some of the $CH_4$ concentration results used to calculate the $CH_4$ fluxes (ie. T-1 and T-2) were close to the limit of quantification for the gas chromatograph instrument used (112ppb).

Figure 3 showed that when both winter and summer concentrations were included together the peat from *Sphagnum* dominated areas had the lowest concentrations of water-extractable DOC (Figure 3). However, ANOVA tests showed

there was only a statistically significant difference with the samples from 10cm taken in the summer (Table 3B) and in this situation *Juncus* had the highest concentration of leachable DOC: 1.7 times higher than both *Calluna* and *Sphagnum*.

Water-extractable phenolic concentrations were highest overall in peat samples where the dominant plant species was *Calluna* (Figure 4). Total summer concentrations were also higher than those in winter for all species. However, there were no statistically significant differences between any of the peat samples when seasonal, depth groups were explored using ANOVA (Table 3B).

Total phenol oxidase activity was highest in *Juncus* dominated peat (Figure 5). In summer at 10cm (which showed the only statistically significant difference: $F$ (2, 8) = 5.682, $p$ = 0.029) the enzyme was 2.5 times more active under *Juncus* than *Calluna* (Table 3C). *Juncus* was also the only species which had lower phenol oxidase activity in the winter than the summer and it was noticeable that there was only a small difference between *Juncus*' two depths at winter (0.00045 µmol dicq $g^{-1}$ $min^{-1}$) compared to the other two species.

There were no statistical differences between the $H^+$ concentrations (Figure 6) for any of the species' respective depths in the samples collected during the winter (Table 3B). In the summer samples taken from a depth of 10cm there were significant differences ($F$ (2, 9) = 18.493, $p < 0.001$) with *post hoc* tests showing *Calluna* had significantly higher concentrations (and therefore lower pH values) than *Juncus* (p = 0.001) and *Sphagnum*. At 30cm an ANOVA indicated there was again significant

differences ($F$ (2,9) = 4.740, $p$ = 0.039) but this time *Calluna* was only significantly higher than *Sphagnum*.

*Juncus* had the lowest activity levels for all the hydrolase enzymes compared to the other two species (Figures 6A, B and C), with the largest, statistically significant (Table 3C) differences being seen in the winter at 10cm. There was little total difference between *Calluna* and *Sphagnum*, except for β-D-glucosidase where, due largely to high activity at 30cm in winter, *Calluna* was the most active.

The results from both depths and both seasons were amalgamated before relationships between various factors was investigated using Pearson's correlation. There were no significant correlations for *Calluna* (including hydrolase enzyme activities, which for the sake of clarity are not shown in Table 4) and only a weak negative correlation between phenolic concentrations and phenol oxidase activity ($r$ = -0.034, $p$ = 0.906) (Table 4A). *Juncus* had a surprising positive correlation between these latter two factors (r = 0.737, p = 0.002), which coincided with positive correlations between phenolic concentrations and $CO_2$ and $CH_4$ production (Table 4B). However, H+ concentrations were significantly, though not strongly, negatively correlated to $CO_2$ fluxes ($r$ = -0.605, $p$ = 0.013) and phenol oxidase activities ($r$ = 0.529, $p$ = 0.043), meaning these two factors increased as pH dropped. Phenolic concentrations from the *Sphagnum* samples, showed a non-significant weak negative correlation with phenol oxidase activity ($r$ = -0.239, $p$ = 0.372) and a significant but moderate negative correlation with $CH_4$ fluxes, again there was a positive correlation with $CO_2$ fluxes. Similar to *Juncus*, concentrations of H+ in *Calluna* (Figure 4B) were negatively correlated to phenol oxidase activities ($r$ = -0.433, $p$ = 0.094) and $CO_2$ ($r$ = -0.560, $p$ = 0.024), though positively to $CH_4$.

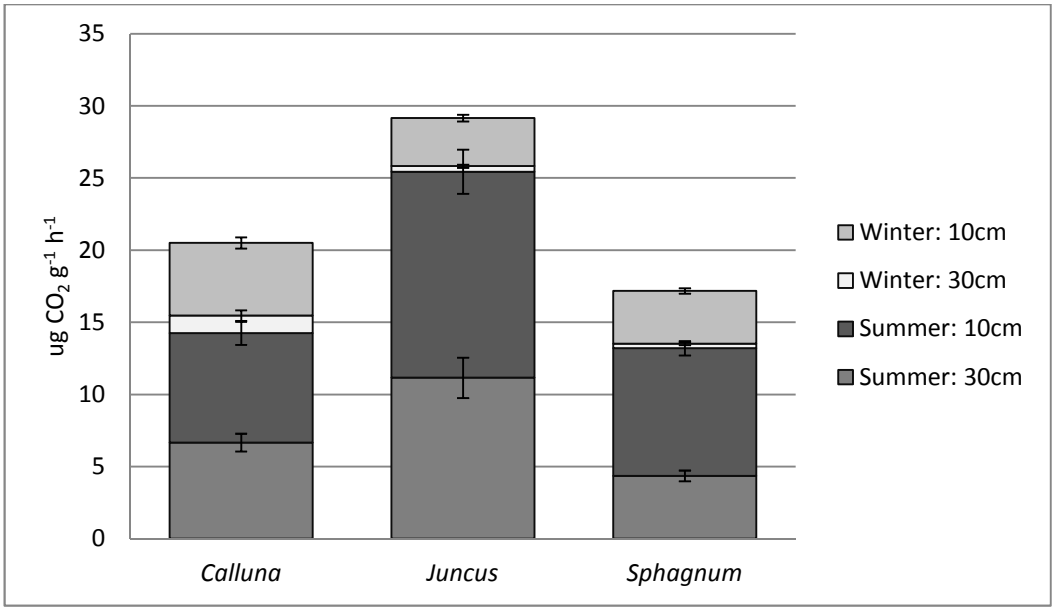



**Figure 1. The mean microbial $CO_2$ fluxes from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. Fluxes were measured using 10g of homogenised peat sealed in a 50mL chamber. ± standard error are indicated.

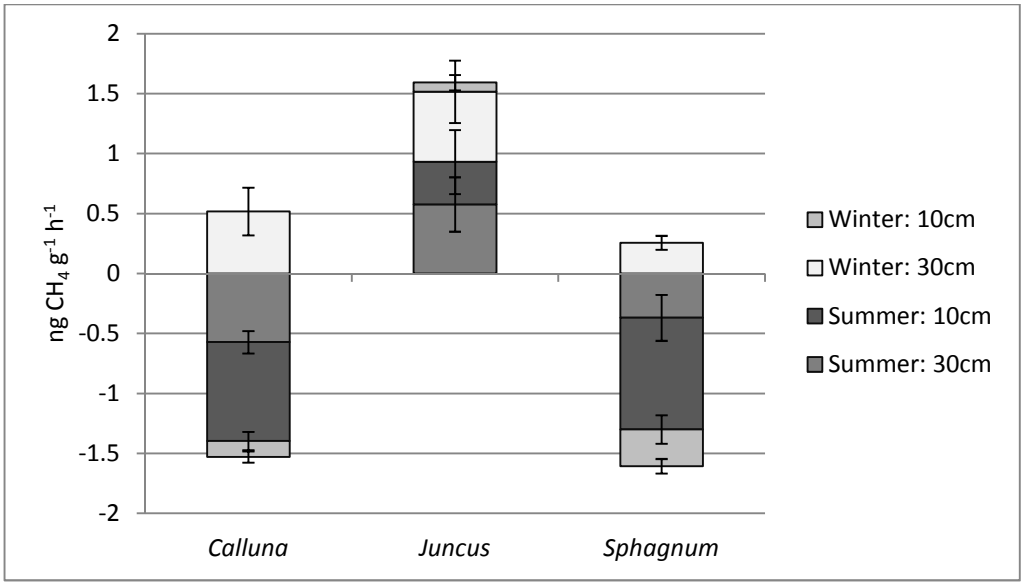



**Figure 2. The mean microbial $CH_4$ fluxes from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. Fluxes were measured using 10g of homogenised peat sealed in a 50mL chamber. ± standard error are indicated. It is important to note that the differences in some of the concentration results used to calculate these fluxes were close to the limit of quantification for the instrument used.

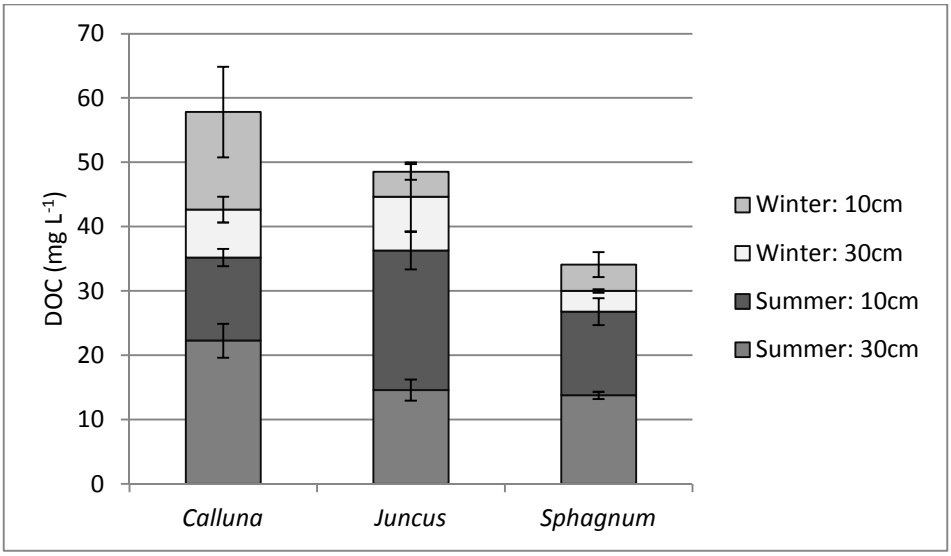



**Figure 3. The mean water-extractable DOC concentrations from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. Leachable DOC was extracted from 1:9 peat to Milli-Q pure water homogenisations. ± standard error are indicated.

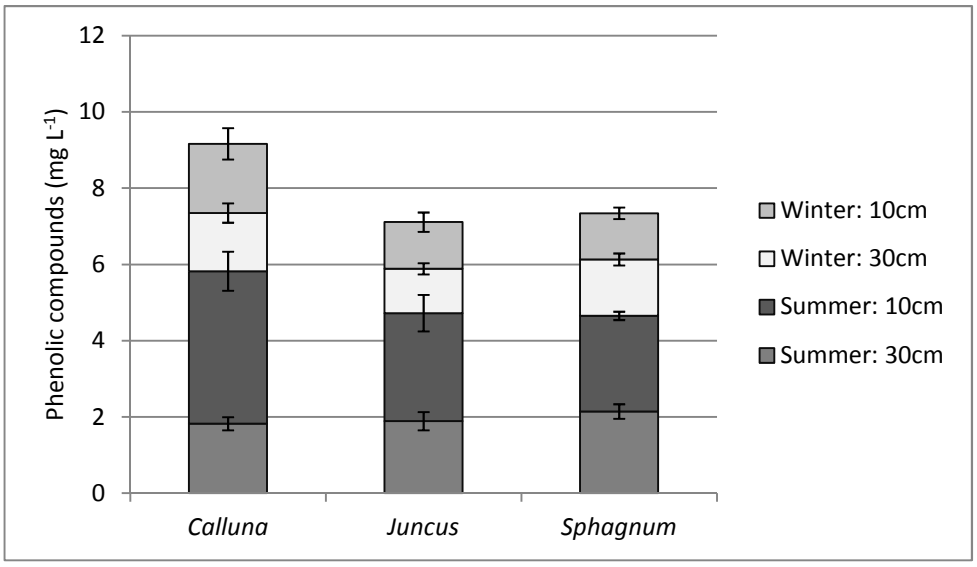



**Figure 4. The mean water-extractable phenolic concentrations from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. Leachable phenolic compounds were extracted from 1:9 peat to Milli-Q pure water homogenisations. ± standard error are indicated.

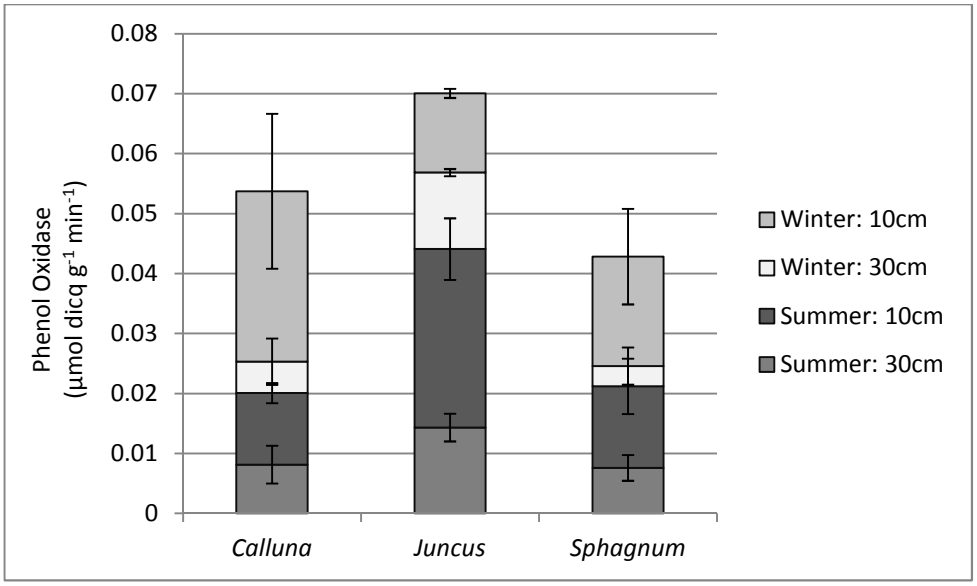



**Figure 5. The mean phenol oxidase activity from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. ± standard error are indicated.

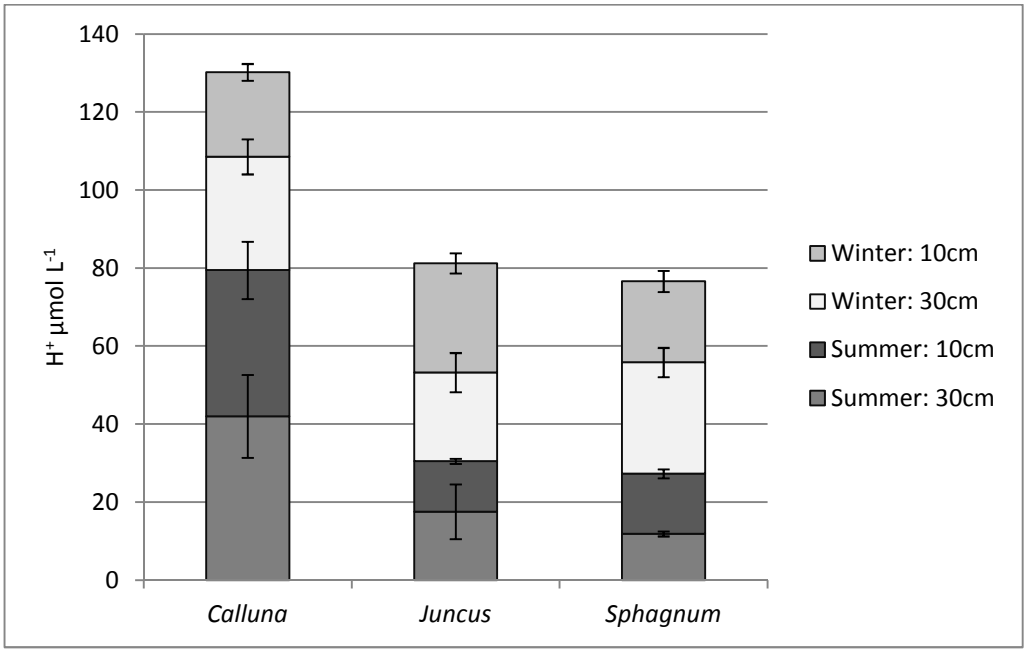



**Figure 6. The mean $H^+$ ion concentrations (µmol $L^{-1}$) from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. ± standard error are indicated.

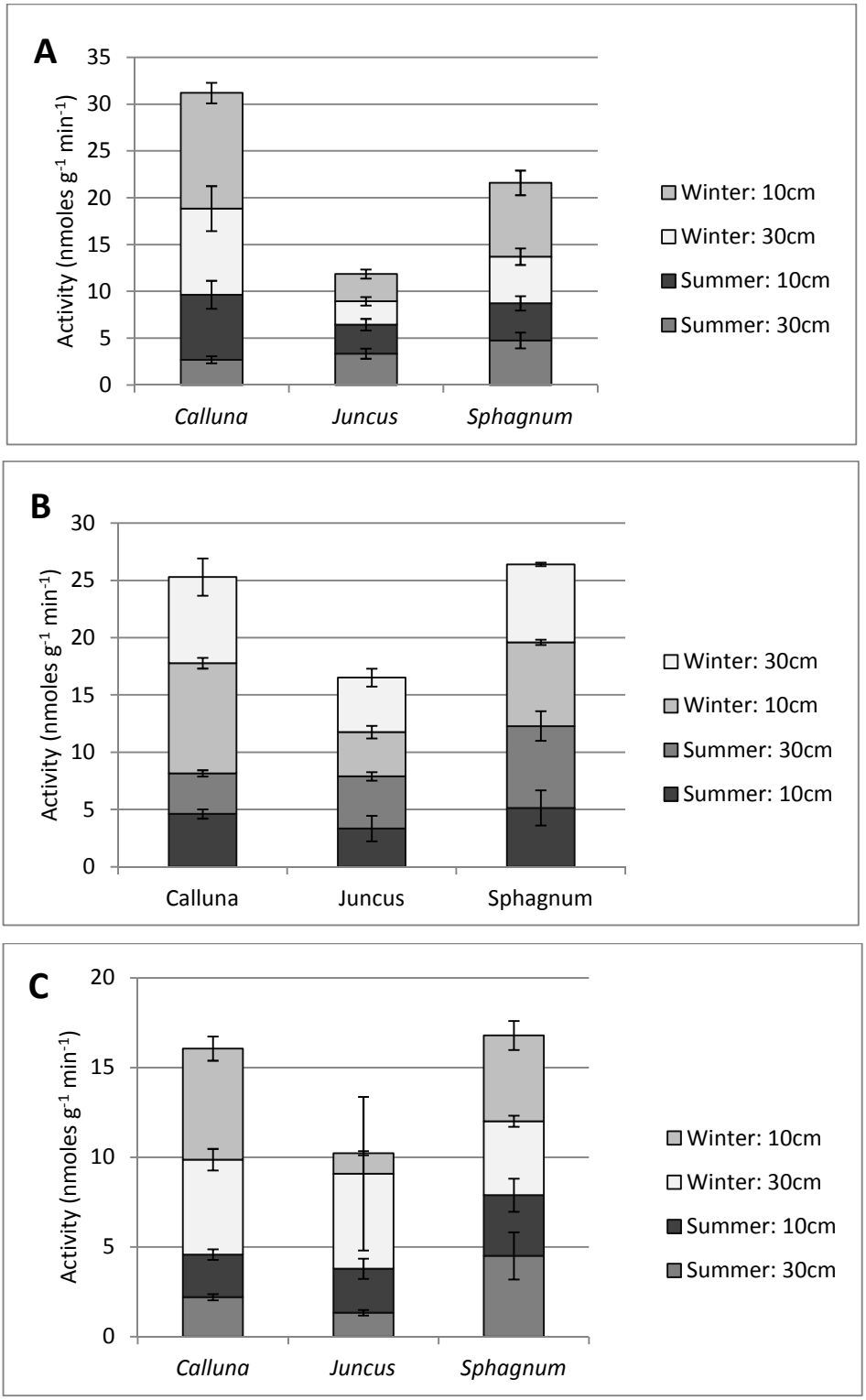



**Figure 7. Mean hydrolase enzyme activities from peat directly beneath different peatland plant communities.** Peat samples were taken during July (summer) and January (winter) and from a depth of 10cm and 30cm. ± standard error are indicated. **A**: β-D-glucosidase; **B**: β-D-xylosidase and **C**: N-acetyl-β-D-glucosaminidase.

**A**

| Analysis | ANOVA result | Post-hoc tests | | Significant |
|---|---|---|---|---|
| | | Comparison | *p* value | |
| | **$CO_2$ flux** | | | |
| Summer 10cm | *F* (2, 16.99) = 7.25 (Welch) *p* = 0.005 | | | * |
| | | *Calluna* → *Juncus* | 0.005 | * |
| | | *Juncus* → *Sphagnum* | 0.016 | * |
| | | *Sphagnum* → *Calluna* | 0.392 | - |
| Summer 30cm | *F* (2, 18.4) = 15.37 (Welch) *p* < 0.001 | | | * |
| | | *Calluna* → *Juncus* | 0.02 | * |
| | | *Juncus* → *Sphagnum* | 0.001 | * |
| | | *Sphagnum* → *Calluna* | 0.01 | * |
| Winter 10cm | *F* (2, 28.27) = 6.95 (Welch) *p* = 0.004 | | | * |
| | | *Calluna* → *Juncus* | 0.003 | * |
| | | *Juncus* → *Sphagnum* | 0.513 | - |
| | | *Sphagnum* → *Calluna* | 0.012 | * |
| Winter 30cm | *F* (2, 27.64) = 2.815 (Welch) *p* = 0.77 | | | - |
| **$CH_4$ flux** | | | | |
| Summer 10cm | *F* (2, 23.86) = 9.64 (Welch) *p* = 0.001 | | | * |
| | | *Calluna* → *Juncus* | 0.002 | * |
| | | *Juncus* → *Sphagnum* | 0.001 | * |
| | | *Sphagnum* → *Calluna* | 0.729 | - |
| Summer 30cm | *F* (2, 20.56) = 10.657 (Welch) *p* = 0.001 | | | * |
| | | *Calluna* → *Juncus* | 0.001 | * |
| | | *Juncus* → *Sphagnum* | 0.011 | * |
| | | *Sphagnum* → *Calluna* | 0.613 | - |

| | | | | |
|---|---|---|---|---|
| Winter 10cm | $F$ (2, 45) = 10.923 $p$ < 0.001 | | | * |
| | | *Calluna* → *Juncus* | 0.034 | * |
| | | *Juncus* → *Sphagnum* | <0.001 | * |
| | | *Sphagnum* → *Calluna* | 0.105 | - |
| Winter 30cm | $F$ (2, 21.22) = 3.58 (Welch) $p$ = 0.038 | | | * |
| | | *Calluna* → *Juncus* | 0.245 | - |
| | | *Juncus* → *Sphagnum* | 0.447 | - |
| | | *Sphagnum* → *Calluna* | 0.053 | - |

**B**

| Analysis | ANOVA result | Post-hoc tests | | Significant |
|---|---|---|---|---|
| | | Comparison | *p* value | |
| **DOC** | | | | |
| Summer 10cm | $F$ (2, 8) = 5.288 $p$ = 0.0.34 | | | * |
| | | *Calluna* → *Juncus* | 0.047 | * |
| | | *Juncus* → *Sphagnum* | 0.049 | * |
| | | *Sphagnum* → *Calluna* | 1 | - |
| Summer 30cm | $F$ (2, 4.55) = 4.376 (Welch) $p$ = 0.087 | | | - |
| Winter 10cm | $F$ (2, 3.82) = 1.07 (Welch) $p$ = 0.429 | | | - |
| Winter 30cm | $F$ (2, 2.72) = 2.152 (Welch) $p$ = 0.275 | | | - |
| **Phenolics** | | | | |
| Summer 10cm | $F$ (2, 3.49) = 3.556 (Welch) $p$ = 0.144 | | | - |
| Summer 30cm | $F$ (2, 9) = 0.707 $p$ = 0.144 | | | - |
| Winter 10cm | $F$ (2, 9) = 1.401 $p$ = 0.295 | | | - |
| Winter 30cm | $F$ (2, 9) = 1.042 $p$ = 0.392 | | | - |
| **H+** | | | | |
| Summer 10cm | $F$ (2, 9) = 18.493 $p$ < 0.001 | *Calluna* → *Juncus* | 0.001 | * |
| | | *Juncus* → *Sphagnum* | 0.842 | - |
| | | *Sphagnum* → *Calluna* | 0.002 | * |
| Summer 30cm | $F$ (2, 9) = 4.740 $p$ = 0.039 | *Calluna* → *Juncus* | 0.099 | - |
| | | *Juncus* → *Sphagnum* | 0.850 | - |
| | | *Sphagnum* → *Calluna* | 0.042 | * |
| Winter 10cm | $F$ (2, 9) = 2.481, $p$ = 0.139 | | | - |
| Winter 30cm | $F$ (2, 9) = 0.620, $p$ = 0.559 | | | - |

**C**

| Analysis | ANOVA result | Post-hoc tests | | Significant |
|---|---|---|---|---|
| | | Comparison | *p* value | |
| **Phenol Oxidase** | | | | |
| Summer 10cm | $F$ (2, 8) = 5.682 $p$ = 0.029 | | | * |
| | | *Calluna* → *Juncus* | 0.034 | * |
| | | *Juncus* → *Sphagnum* | 0.052 | - |
| | | *Sphagnum* → *Calluna* | 0.947 | - |
| Summer 30cm | $F$ (2, 9) = 2.120 $p$ = 0.176 | | | - |
| Winter 10cm | $F$ (2, 9) = 0.778 $p$ = 0.488 | | | - |
| Winter 30cm | $F$ (2, 9) = 2.978 $p$ = 0.102 | | | - |
| **β-D-glucosidase** | | | | |
| Summer 10cm | $F$ (2, 9) = 3.820 $p$ = 0.063 | | | - |
| Summer 30cm | $F$ (2, 9) = 2.887 $p$ = 0.108 | | | - |
| Winter 10cm | $F$ (2, 9) = 20.975 $p$ < 0.001 | | | * |
| | | *Calluna* → *Juncus* | < 0.001 | * |
| | | *Juncus* → *Sphagnum* | 0.019 | * |
| | | *Sphagnum* → *Calluna* | 0.033 | * |
| Winter 30cm | $F$ (2, 9) = 5.087 $p$ = 0.033 | | | * |
| | | *Calluna* → *Juncus* | 0.028 | * |
| | | *Juncus* → *Sphagnum* | 0.499 | - |
| | | *Sphagnum* → *Calluna* | 0.171 | - |
| **β-D-xylosidase** | | | | |
| Summer 10cm | $F$ (2, 9) = 0.680 $p$ = 0.531 | | | - |
| Summer 30cm | $F$ (2, 5.3) = 4.87 (Welch) $p$ = 0.063 | | | - |
| Winter 10cm | $F$ (2, 9) = 43.851 $p$ < 0.001 | | | * |
| | | *Calluna* → *Juncus* | < 0.001 | * |
| | | *Juncus* → *Sphagnum* | 0.001 | * |
| | | *Sphagnum* → *Calluna* | 0.012 | * |
| Winter 30cm | $F$ (2, 4.18) = 2.954 (Welch) $p$ = 0.159 | | | - |

| **N-acetyl-β-D-glucosaminidase** | | | | |
|---|---|---|---|---|
| Summer 10cm | $F$ (2, 5.08) = 0.484<br>(Welch) $p$ = 0.642 | | | - |
| Summer 30cm | $F$ (2, 5.35) = 8.219<br>(Welch) $p$ = 0.023 | | | * |
| | | *Calluna* → *Juncus* | 0.022 | * |
| | | *Juncus* → *Sphagnum* | 0.181 | - |
| | | *Sphagnum* → *Calluna* | 0.326 | - |
| Winter 10cm | $F$ (2, 4.19) = 31.382<br>(Welch) $p$ = 0.003 | | | * |
| | | *Calluna* → *Juncus* | 0.009 | * |
| | | *Juncus* → *Sphagnum* | 0.039 | * |
| | | *Sphagnum* → *Calluna* | 0.422 | - |
| Winter 30cm | $F$ (2, 4.82) = 1.318<br>(Welch) $p$ = 0.349 | | | - |

**Table 3 A-C. Results from a series of one-way ANOVAs exploring the effect plant communities have on microbial activity in peat-soil.** Before running any analysis it was checked that the usual assumptions of parametric tests were not violated. The assumption of homogeneity of variance was checked using the Levene's test, with the Welch result (indicated) used when equal variances could not be assumed. To compare the various experimental treatments against each other Tukey *post hoc* tests were performed, when group variances were similar, otherwise the Games-Howell procedure was used. *F*-ratios are presented along with the degrees of freedom for the effect of the model and the degrees of freedom for the residuals of the model. Statistical significance ($p < 0.05$) is indicated with *.

# A

| | | Phenolics | $CO_2$ | $CH_4$ | DOC | P.Ox |
|---|---|---|---|---|---|---|
| $H^+$ | Correlation | 0.088 | 0.472 | 0.072 | 0.302 | -0.241 |
| | Significance | 0.756 | 0.065 | 0.792 | 0.294 | 0.368 |
| | Sample size | 15 | 16 | 16 | 14 | 16 |
| Phenolics | Correlation | | 0.392 | -0.267 | 0.278 | -0.034 |
| | Significance | | 0.148 | 0.336 | 0.358 | 0.906 |
| | Sample size | | 15 | 15 | 13 | 15 |
| $CO_2$ | Correlation | | | -0.423 | 0.347 | 0.166 |
| | Significance | | | 0.102 | 0.224 | 0.540 |
| | Sample size | | | 16 | 14 | 16 |
| $CH_4$ | Correlation | | | | -0.244 | -0.150 |
| | Significance | | | | 0.401 | 0.580 |
| | Sample size | | | | 14 | 16 |
| DOC | Correlation | | | | | 0.197 |
| | Significance | | | | | 0.500 |
| | Sample size | | | | | 14 |

# B

| | | Phenolics | $CO_2$ | $CH_4$ | DOC | P.Ox |
|---|---|---|---|---|---|---|
| $H^+$ | Correlation | -0.384 | $-0.605^{*}$ | -0.482 | 0.154 | $-0.529^{*}$ |
| | Significance | 0.142 | 0.013 | 0.069 | 0.615 | 0.043 |
| | Sample size | 16 | 16 | 15 | 13 | 15 |
| Phenolics | Correlation | | $0.601^{*}$ | $0.665^{**}$ | 0.395 | $0.737^{**}$ |
| | Significance | | 0.014 | 0.007 | 0.181 | 0.002 |
| | Sample size | | 16 | 15 | 13 | 15 |
| $CO_2$ | Correlation | | | $0.648^{**}$ | 0.126 | 0.412 |
| | Significance | | | 0.009 | 0.682 | 0.127 |
| | Sample size | | | 15 | 13 | 15 |
| $CH_4$ | Correlation | | | | 0.090 | 0.456 |
| | Significance | | | | 0.781 | 0.101 |
| | Sample size | | | | 12 | 14 |
| DOC | Correlation | | | | | 0.084 |
| | Significance | | | | | 0.795 |
| | Sample size | | | | | 12 |

**C**

| | | Phenolics | $CO_2$ | $CH_4$ | DOC | P.Ox |
|---|---|---|---|---|---|---|
| **$H^+$** | Correlation | -0.457 | -0.560* | 0.653** | -0.701** | -0.433 |
| | Significance | 0.075 | 0.024 | 0.006 | 0.002 | 0.094 |
| | Sample size | 16 | 16 | 16 | 16 | 16 |
| **Phenolics** | Correlation | | 0.588* | -0.446 | 0.662** | -0.239 |
| | Significance | | 0.017 | 0.083 | 0.005 | 0.372 |
| | Sample size | | 16 | 16 | 16 | 16 |
| **$CO_2$** | Correlation | | | -0.777** | 0.730** | 0.217 |
| | Significance | | | .000 | 0.001 | 0.418 |
| | Sample size | | | 16 | 16 | 16 |
| **$CH_4$** | Correlation | | | | -0.633** | -0.401 |
| | Significance | | | | 0.008 | 0.124 |
| | Sample size | | | | 16 | 16 |
| **DOC** | Correlation | | | | | 0.154 |
| | Significance | | | | | 0.569 |
| | Sample size | | | | | 16 |

**Table 4 A-C. Results from a Pearson correlation exploring the effect plant communities have on microbial activity in peat-soil**. The two seasons (summer and winter) and both depths (10cm and 30cm) were amalgamated for each of the plant species: **A** = Calluna, **B** = Juncus and **C** = Sphagnum. Relationships between the different factors were conducting using Pearson's correlation. $H^+$ = $H^+$ mol $L^{-1}$; $CO_2$ = µg of $CO_2$ $g^{-1}$ $h^{-1}$; $CH_4$ = ng of $CH_4$ $g^{-1}$ $h^{-1}$; DOC = dissolved organic carbon (mg $L^{-1}$). Correlation significant at the 0.05 level (2-tailed) indicated with *, and at the 0.01 level (2-tailed) with **.

## 4.5 Discussion

In this study the preparation of the peat-soil samples (ie. removal of live roots) eliminated potential rhizopshere respiration, or root derived $CO_2$, from being measured. This allowed us to attribute all $CO_2$ effluxes to microbial decomposition of SOM (soil derived $CO_2$) which is strongly influenced by litter type and rhizosphere interactions between root exudation and microbial communities (Cheng et al. 2003), and have either a stimulatory (priming effect) or a suppressive influence on the breakdown of SOM (Van Veen et al. 1991, Cheng 1999). The higher fluxes of $CO_2$ seen in peat taken from areas of blanket bog dominated by *Juncus* (Figure 1) were expected, due to the species being a relatively productive, fast growing plant (Ervin and Wetzel 2000), producing labile litter, which has often already been acted upon by degraders before reaching the peat surface (Kuehn et al. 2000), and low-molecular weight root exudates (Zhai et al. 2013). Crucially, ROL is high from the roots of *Juncus* plants (Sorrell 1999) providing more aerobic conditions and increasing the redox potential in the rhizosphere, compared to *Calluna* or *Sphagnum* dominated areas (Chabbi 1999, Fenner et al. 2007a). This was reflected in the higher (accumulated) phenol oxidase activities (Figure 5), which require oxygen to function (Freeman et al. 2001b). However, this was not significantly correlated with significantly higher hydrolase enzyme activities (Figure 6, Table 3A and 4B) as was hypothesised according to the emzymic latch mechanism (Freeman et al. 2001b). We suggest this may be due to the extremely labile nature of the carbon compounds produced by the *Juncus* plants, which would require little production of extracellular enzymes from the peat-soil microbes. To test this hypothesis in future studies it would have been necessary to quantify microbial population density in addition to measuring extracellular enzyme activities. Phenolic compound concentration results for *Juncus*

also did not support our initial hypotheses as they were not significantly lower in the *Juncus* samples, despite the higher phenol oxidase activities, although this may simply indicate that the litter from these rushes contains less phenolics than lignin-rich woody heather (van der Heijden and Boon 1994, Huang et al. 1998) or *Sphagnum* moss which produces sphagnum-acid (trans-sphagnum acid ethyl ester) (Rasmussen et al. 1995). This may also explain why despite *Sphagnum* samples having the lowest accumulated $CO_2$ flux, of the three plant types (Figure 1), they did not have the lowest phenolic compound concentrations (Figure 4). Indeed, it is noteworthy that *Juncus* samples were the only ones that showed a positive correlation between phenolic concentrations and phenol oxidase activities, in contrast with the albeit non statistically significant negative correlations displayed by *Calluna* and *Sphagnum* samples (Table 4A-C). Phenol oxidase activities in *Juncus* samples did show a significant, though moderate, negative correlation with $H^+$ concentrations ( $r$ = -0.529, $p$ (two-tailed) = 0.043), indicating that enzyme's activity in the rhizosphere of *Juncus* plants may be retarded by low pH conditions.

From our $CO_2$ flux results it could be summarised that the plants, in order of their ability to create conditions that limit microbial SOM decomposition, is: *Sphagnum* > *Calluna* > *Juncus*. However, the differences between $CO_2$ fluxes at different depths, which can be explained by microbial activities generally being higher in the acrotelm than the lower depths of the catotelm (Freeman et al. 1995), and the observed effects of seasonality, highlight the importance of analysing samples from different depths and seasons to ensure accuracy when reporting soil derived $CO_2$ fluxes from an ecosystem.

We hypothesised that the increased aerobic conditions created in the rhizosphere by oxygen being transported through the *Junucs* plants and out of the roots by aerenchymous tissues (Sorrell 1999), would produce the lowest accumulated $CH_4$ fluxes of any of the species tested. The addition of oxygen in the rhizopshere can influence anaerobic respiration and thereby suppress the production of the final end product, $CH_4$, in three main ways: 1) oxygen can supply electron acceptor substrates to microbial functional groups with more energetically favourable pathways (denitrification, iron reduction and sulphate reduction) (Laanbroek 2010, Sutton-Grier and Megonigal 2011); 2) oxygen can directly inhibit $CH_4$ production by oxygen toxicity (Fridovich 1998) and 3) it enables $CH_4$ oxygenation by methanotrophs (Hanson and Hanson 1996). However, our results showed that areas dominated by *Juncus* had higher accumulated $CH_4$ emissions (Figure 1) than either *Calluna* or *Sphagnum* samples. There was also no significant difference between the corresponding depths of the *Calluna* and *Sphagnum* areas (Table 3A). Indeed, unlike the other species, all the *Juncus* samples recorded a positive flux, irrespective of depth or season, indicating $CH_4$ was being produced by methanogens rather than consumed by methanotrophs. Moreover, the relatively high levels of phenol oxidase activity in the *Juncus*-dominated areas (Figure 5) suggest there was certainly oxygen present in the these samples and any possible pockets of anoxic zones containing methanogens, or even the presence of a limited number of methanogens able to operate in aerobic conditions (review by Bridgham et al. 2013), are unlikely to account for our observed $CH_4$ fluxes from *Juncus*.

However, organic carbon (as an electron donor) is also a primary controlling factor for methanogenesis in peatlands, and this in-turn is controlled by litter type (Bridgham and Richardson 1992), quality and quantity of root exudates (Dorodnikov

et al. 2011) and plant productivity (Updegraff et al. 2001, Laanbroek 2010, Sutton-Grier and Megonigal 2011). Our results therefore suggest that when only microbial respiration in peat is taken into account the quantity and quality of the organic carbon, produced by the plants, are the key controlling factor for methanogenesis - and areas of peatland dominated by *Juncus* provide the best environment for methanogenic microbes. This is despite the likelihood of this peat being the most oxygenated out of the three sample types tested, due to the high ROL from *Juncus* roots (Sorrell 1999). Further work should now be carried out to analyse the peat litter for organic acid content (eg. amounts of acetate) and the presence of elements and compounds that can directly or indirectly affect $CH_4$ production, such as nitrogen and sulphates (Freeman et al. 1994, Dowrick et al. 2006, Gauci and Chapman 2006), to explain this further.

The pH of the samples may also have had an effect on the activities of the methanotrophs and methanogens. *Sphagnum* samples were significantly correlated to H+ concentration and $CH_4$ flux, albeit moderately ($r = 0.653$, $p$ (two-tailed) = 0.006), suggesting lower pH increases $CH_4$ production in the rhizosphere of *Juncus*; contrary to previous studies (Dunfield et al. 1993, Bergman et al. 1998). Such a correlation was not seen in the *Juncus* samples or, to the same degree, in the *Calluna* peat (Table 3A, B).

Obviously, the plants themselves are major factors in moderating the flux of $CH_4$ between peat and the atmosphere (Henneberg et al. 2012 and references therin, Gray et al. 2013), with previous studies showing that aerenchyma can act as conduits for the gas - allowing $CH_4$ to bypass zones of aerobic methanotrophy (Shannon et al. 1996, Laanbroek 2010). There is also some debate as to whether arenchymateous plants, such as *Juncus* result, in higher (Schimel 1995) or lower (Roura-Carol and Freeman 1999) $CH_4$ fluxes from peatlands compared to other wetland plants. These

factors should be taken into account when advising land managers on the most effective plant communities for carbon sequestration in peatlands, but from our results the plants, in order of their ability to suppress methanogenesis in the peat they grow in, is: *Sphagnum* = *Calluna* > *Juncus*.

Our results show *Calluna* rather than *Juncus* had the highest accumulated concentrations of water-extracteble DOC. However, Table 3B shows that the only statistically significant difference was between the samples taken at 10cm during the summer ($F$ (2,8) = 5.288, $p$ = 0.034), where *Juncus* was 67% higher than both *Calluna* and *Sphagnum*. It was expected that *Juncus* would have high levels of DOC due to the previously described quantity and quality of root exudates and labile nature of the litter, especially during summer when growth rates of the rush are highest (Wetzel and Howe 1999); but the concentrations from *Calluna* were surprising and contrary to previous studies (Yan et al. 2008). This may be due to the levels of leachable phenolic concentrations from *Calluna* which, although were the highest of the species when accumulated (Figure 4), were not significantly different to the respective depths and seasons of *Juncus* and *Sphagnum* samples (Table 3B), and therefore suppression of hydrolase enzymes was limited (Figure 6A-C). One explanation for these relatively high levels of hydrolase enzymes observed in samples taken from *Calluna* dominated sites is drainage. Despite our best efforts to find an area of pristine blanket bog, large areas of the Mignient site have had ditches cut into the peat in an effort to drain the land during the last century. Although, many of these have since been filled in it is still unclear as to the full and long term effects of peat drainage. In some studies a lowering of the water table in peatlands have been shown

to increase DOC production, which has been attributed to amplified biological activity and rates of decomposition (Tipping et al. 1999, Pastor et al. 2003, Strack et al. 2008).

The H+ concentration, or pH, is also known to suppress the production and release of DOC from peatlands (Evans et al. 2005, Evans et al. 2012), though our results only showed DOC had a significantly negative relationship with the $H^+$ concentration in *Sphagnum* samples ($r$ = -0.701, $p$ = (two-tailed) 0.002).

If our results represent a true picture of what is happening in peatlands, then the plants in order of their ability to create a conditions that limit water-extractable DOC release from peatlands is: *Sphagnum* > *Juncus* ≥ *Calluna.* As with $CO_2$ production, DOC release is clearly affected by temperature (season samples collected) and depth of the peat samples. This again highlights the importance of taking these factors into consideration when discussing carbon turnover in peatlands.

To answer the main question of our study, our results do indeed suggest that plant types affect carbon cycling in a blanket bog, which has implications for carbon sequestration. Taking into account the main carbon fluxes, we conclude that the order of plant species in terms of their ability to sequester carbon is: *Sphagnum* > *Calluna* > *Juncus*. The latter should be discouraged from growing by land owners looking to manage areas of peatland for carbon stewardship due to its tendency to produce labile litter and root exudates which promote release of $CO_2$, $CH_4$ and DOC. The lack of statistical differences of the corresponding depth and season, for any of the three carbon pathways, between *Calluna* and *Sphagnum* permits the argument that there is little to choose between the two regarding their sequestering abilities. However, when the accumulated DOC values are considered, and the higher activities of the hydrolase enzyme, β-D-glucosidase (winter 10cm samples were significantly higher in *Calluna*

compared to *Sphagnum*, see Table 3C), we judged *Sphagnum* species to be the best wetland plant for preventing the loss of carbon in blanket bogs. Therefore carbon stewardship programmes looking to maximise carbon sequestration of peatlands should encourage the growth of *Sphagnum* mosses on their bog - with the caveat that further research in the field, taking into account living vegetation, supports the results of the belowground carbon fluxes.

Although subtly adjusting the vegetation of peatlands may eventually prove a popular $CO_2$ removal scheme with landowners it may not offer the levels of carbon sequestration necessary to halt, or even reverse, climate change. To use peatlands for a geoengeneering project on this scale it is likely we will need to develop a robust method of directly manipulating the enzymic latch in order to further suppress phenol oxidase activity and thereby decomposition of peat litter. We propose adjusting the pH or the levels of phenolics in the peat matrix (Freeman et al. 2012) may prove to be the best ways to go about this.

# Chapter 5

## Phenolic enrichment of peat to strengthen the enzymic latch

## 5.1 Abstract

Northern peatlands store 455 Pg of carbon – a third of the entire global carbon store. Carbon accumulates because phenolic inhibitors slow the rate of decomposition to below that of photosynthetic production. The disproportionate importance of phenolics in peatlands is related to the unique properties of waterlogged peat soils suppressing the activity of phenol oxidase; one of the few enzymes capable of breaking these inhibitors down. This permits accumulation of phenolic compounds that are potent inhibitors of hydrolase enzymes – major agents in the breakdown of organic matter. In our study we investigate whether the application of natural phenolic inhibitors can further suppress microbial decomposition in peat. Calcium lignosulphonate, a high molecular weight by-product of the paper milling industry, was found to suppress the activities of hydrolase enzymes and phenol oxidases, along with $CO_2$ production, in peat slurries. While fragments of birch wood had similar effects on cores of peat taken from a blanket bog in the UK. Our findings suggest that phenolic supplementation could offer a new bio-geoengineering technique: harnessing the unique biogeochemistry of northern peatlands to increase their rates of carbon sequestration by up to 76%.

## 5.2 Introduction

Peatlands are unbalanced ecosystems where rates of production exceed levels of decomposition, leading to accretion of around 455 Pg of carbon (1 Pg = $10^{15}$g) in the northern hemisphere (Gorham 1991, Limpens et al. 2008a). Traditionally, this impaired decay was attributed to the general anoxia (Gorham 1991, McLatchey and Reddy 1998), low nutrients, low temperatures and low pH associated with peat soils (Gorham 1991, Laiho 2006). But it is now believed that constraints, such as oxygen, on phenol oxidase enzymes is the latch which keeps this vast carbon stock 'locked-up' and prevents it from being released from these wetlands in gaseous or fluvial forms (Freeman et al. 2001a, Freeman et al. 2001b, Fenner and Freeman 2011).

Phenol oxidases are among the few enzymes able to fully degrade phenolic compounds (McLatchey and Reddy 1998) so any suppression of their activity allows the build-up phenolics. These compounds in the peat (dead organic matter) have the ability to inhibit the main agents of carbon and nutrient cycling, hydrolase enzymes, from carrying out normal decay processes of soil organic matter (SOM) (Wetzel 1992, Freeman et al. 2001b). Extracellular enzymes, such as hydrolase enzymes which are produced by a range of bacteria (Fenner et al. 2005a) and fungi (Burke and Cairney 2002), are the primary means by which soil microbes degrade complex organic compounds into smaller molecules they can assimilate. In addition to allowing microbes to access energy and nutrients present in complex substrates, extracellular enzymes catalyse the initial, rate-limiting step of decomposition and nutrient mineralisation (Burns 1982, Sinsabaugh 1994). This dual role of enzymes in soils means that changes in enzyme activities and production can affect ecosystem processes directly.

Phenolics are a class of chemical compounds consisting of a hydroxyl functional group (—OH) bonded directly to an aromatic hydrocarbon group, consisting of six carbon atoms attached to the same number of hydrogen atoms ($C_6H_6$), known as a benzene ring. The simplest of the class is carbolic acid, $C_6H_5OH$, which is often referred to as phenol. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule.

A wide variety of phenolic compounds are produced as secondary metabolites by plants; they include phenolic acids, flavonoids, tannins and the less common stilbenes and lignans. There are currently more than 8,000 known phenolic structures found in plants, ranging from the simple molecules of phenolic acids to the highly polymerised substances such as tannins (Dai and Mumper 2010). Phenolic acids can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Tannins are usually also subdivided into two groups; 1) hydrolysable tannins and 2) condensed tannins. Hydrolysable tannins are compounds containing a central core of glucose or another polyol esterified with gallic acid, also called gallotannins, or with hexahydroxydiphenic acid, also called ellagitannins (Dai and Mumper 2010). Lignin is the most common member of the flavonoids and is an essential component of wood. It has a highly complex macromolecule that occurs in the plant cell wall and is associated with cellulose and hemicellulose (Faulon and Hatcher 1994).

Amongst many other functions, plants need phenolics for pigmentation, growth, reproduction and resistance to pathogens (Lattanzio et al. 2006). However, it is the role phenolics play as regulators of soil processes, in terms of their inhibitory effects on extracellular hydrolase enzymes controlling decomposition and nutrient

cycling (Freeman et al. 2001b), that is crucial to peat formation and stability, and the topic of this study.

Although peatlands currently act as a net sink for atmospheric carbon (Kayranli et al. 2010) and crucially unlike other ecosystems (such as forests), they are potent long term repositories for carbon (Holden 2005), the breakdown of organic matter (OM) to carbon dioxide ($CO_2$) and methane ($CH_4$) still occurs, to varying degrees, especially in disturbed and damaged peats (Kayranli et al. 2010). Therefore, following initial proposals by Freeman et al. (2012) we hypothesise that a manipulation or strengthening of the enzymic latch using phenolics can be used to promote enhanced peatland carbon sequestration. Amplified suppression of decomposition in peatsoils has the potential to increase peat accumulation, maximise the carbon storage intensity per unit area of peatlands and offer a new approach to geoengineering – acting as a cost efficient biological $CO_2$ removal (CDR) scheme (Royal Society 2009).

We hypothesise that supplementing peat soil with phenolics will suppress the activity of extracellular hydrolase enzymes. This will reduce rates of OM breakdown resulting in lower effluxes, from microbial respiration, of carbon-based trace gases, namely $CO_2$ and $CH_4$. In order to investigate this we will add various phenolic compounds, including whole fragments of wood, to both peat slurries and peat cores taken from an ombotrophic blanket bog in the UK.

## 5.3 Materials and methods

### 5.3.1 Study site and sample collection

#### *5.3.1.1 Migneint Valley*

Peat samples for the experiments were taken from the Migneint Valley in North Wales, UK. (3°48.8′ W, 52°59.6′ N). The area is a 200 $km^2$ Special Area of Conservation, incorporating one of the largest areas of blanket bog in Wales. The area is 460m above sea level and is predominantly a *Sphagnum*-rich, *Calluna vulgaris* and *Eriophorum vaginatum* dominant blanket mire, with acid grasses on drier hillslopes, and *Juncus effusus* in riparian areas. The underlying geology of the ombrotrophic bog is a mixture of acid and basic volcanic rocks, Ordovician shales and mudstones. Annual rainfall is 2,400mm and in the peat the water table is usually within 100mm of (and often at) the ground surface. The study area has a pore water pH, at a depth of 100mm, in the range of 4.1 to 5.1. The mean peat depth across the site is 2,000mm (Evans et al. 2012).

#### *5.3.1.2 Peat-soil samples*

Peat for the experiments requiring peat-slurries were collected between June and September (summer). Soil samples (>200g), from a depth of 50 to 100mm, were taken (by the use of a hand trowel to excavate small pits) from five different locations within 25$m^2$ of each other with similar above-ground plant species composition, peat-composition, water-table level and microtopography. Samples were placed in sealable plastic bags and transported to the laboratory in a cooled, insulated container. The field temperature of the ground at the location of sample collection was recorded. Back in the laboratory the soil samples were amalgamated, homogenised thoroughly

by hand, and had any adhering debris and macroinvertebrates removed from them. They were kept in an incubator at the recorded field temperatures before beginning the experiments within 24 hours of collection.

*5.3.1.3 Peat cores*

Sixteen microcosms, consisting of intact cores – or monoliths - of peat soil (110mm diameter and 400mm depth) and vegetation, were collected at the start of March (2011) from an area approximately 100m$^2$. Areas selected to extract the cores from were carefully chosen to ensure they had similar characteristics, principally the vegetation type (consisting of mixed *Sphagnum* species), peat structure and compoistion, and water table height, which was at the surface of the litter layer (at the base of the living *Sphagnum* stems) at time of collection. The cores were extracted using a serrated knife and the plastic core-liner acting as templates. Each cylinder was slowly lowered to its full depth into the peat profile and then carefully extracted with the peat inside it. This ensured the surface vegetation was kept intact and avoided compaction of the soil, thus preventing any changes to its physical structure. The cores were then placed inside larger plastic holding cylinders (150mm diameter and 400mm depth) which had a water-tight seal on the bottom. The peat cores were secured tightly inside the holding cylinders to ensure no accidental lateral or horizontal movement. All the cores were transported to the outdoor experimental facility (approximately 30 miles from the field site) and allowed to acclimatise for four weeks in order to minimise disturbance effects. Water from each site was collected on a fortnightly basis and used to keep the water level of the cores at, or just below, the soil surface – this was facilitated by the use of holes drilled into the side of

the holding cylinders. A randomised block design was used when placing the cores randomly in a grid system and for assigning treatments.

## 5.3.2 Peat-slurry experiments

### *5.3.2.1 Peat-slurry creation*

The 50:50 peat-solution slurries were created in 50mL centrifuge tubes (Fisher Scientific UK Ltd, Loughborough, UK) and consisted of 10g samples of peat (which had previously been homogenised by hand for 10 minutes) and 10mL of the relevant treatment solution. For the control slurries this solution consisted of ultra-pure water and the phenolic treatment slurries consisted of a 5% weight/volume (w/v) solution of ultra-pure water with one of the following dissolved into it (eg. 50g in 1,000mL): cinnamic acid (molecular weight, $M_W$, 148.16), gallic acid ($M_W$ 170.12); flavone ($M_W$ 222.24), tannic acid ($M_W$ = 1,701.20), sodium lignosulphonate acid ($M_W$ 8,000), sodium lignosulphonate acid, ($M_W$ 12,000) or calcium lignosulphonate acid ($M_W$ 49,100). These compounds were chosen as although they are all natural-based products, which can be found in organic matter, the refined experimental compounds are all inexpensive and commercially available. A 5% w/v solution of the phenolic compounds was used following the success of initial concentration experiments and the fact this was considered a maximum useable concentration, taking into account the ability of the compounds to dissolve in water.

A clean glass rod was used to mix the slurry for two minutes, alternating between a clockwise and anti-clockwise motion, before they were placed on a rotary shaker at 200rpm for 30 minutes. To maintain anaerobic conditions the slurries were then immediately placed in a zipper lock-style AtmosBag glove bag (Sigma Aldrich Ltd, Dorset, UK), the air was evacuated with a vacuum pump and oxygen-free-

nitrogen was used to re-fill the bag. This process was repeated every 24 hours and the bag regularly checked for holes. The AtmosBag and the slurries were kept in dark conditions at a stable temperature consistent with the recorded temperature of the soil during sample collection.

*5.3.2.2 Microbial respiration*

To measure the microbial respiration from the peat slurries carbon-based gas fluxes ($CO_2$ and $CH_4$) were measured after the samples had been incubated under the experimental conditions for 24 hours.

A 100mm$^3$ gas syringe fitted with a two-way valve (Sigma Aldrich Ltd, Dorset, UK) and a short bevel hypodermic needle, was used to collect the 10mL gas samples which were transferred to labelled pre-evacuated 5.9mL Exetainers (Labco Ltd, Lampeter, UK). These were stored at room temperature in the dark for no longer than one week before analysis.

To calculate fluxes (the rate of flow) an initial background gas sample (T-1) was collected from inside the centrifuge tubes containing the slurries, before lids - with a size nine Suba-Seal rubber septa (Sigma Aldrich Ltd, Dorset, UK) through their centre – were secured onto the tubes, forming an air-tight seal. 60 minutes later a 10mL gas sample (T-2) was taken from the headspace of the centrifuge tubes through the septa – after a series of preliminary tests showed that $CO_2$ and $CH_4$ concentrations increased linearly for up to 60 minutes.

*5.3.2.3 Trace gas analysis*

Gas samples were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC) instrument, equipped with a flame ionisation detector (FID)

with a $CO_2$ to $CH_4$ catalytic converter (methaniser), to measure concentrations of $CO_2$ and $CH_4$. Two millilitres of gas from the Exetainers containing the samples was injected via a 1041 on-column injector system, set at 40°C onto a PoroPak QS (1.83m x 3.18mm) 80/100 column. The column oven temperature was set to 40°C and the carrier gas, oxygen free nitrogen, had a flow rate of 30mL $min^{-1}$.

The temperature of the methaniser was 300°C and the FID was at 125°C. The latter had a hydrogen flow of 25mL $min^{-1}$, 20mL $min^{-1}$ make-up gas (oxygen free nitrogen) and 300mL $min^{-1}$ of compressed air. The methaniser had a hydrogen flow of 5mL $min^{-1}$.

$CH_4$ and $CO_2$, (retention times 1.08 and 1.87 minutes respectively) were quantified by comparison of peak area with that of the three standards of known concentration (Table 1), prepared by Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK), used in the preparation of a standard curve, which was only accepted if the correlation coefficient ($R^2$) value was greater than 0.98.

| | **$CO_2$** | **$CH_4$** |
|---|---|---|
| **Standard A** | 250 | 2 |
| **Standard B** | 500 | 25 |
| **Standard C** | 1,000 | 50 |

**Table 1. Concentrations (ppm) of the standard gases.** A certificate of analysis (CofA) was requested with each new batch of gases and the calibration curve altered accordingly to take into account the exact amounts.

Calculating the gaseous fluxes from the initial background (T-1) concentrations was achieved by the following equation (Levy et al. 2011):

$$\text{Flux } (\mu g\ g^{-1}\ s^{-1}) = \frac{\delta C}{\delta t} \times \left(\frac{V \times M}{m \times V_{mol}}\right)$$

Where $\delta C$ is rate of change in the gas concentration (T-2 minus T-1); $\delta t$ is the change in time from the background reading to the final measurement (in seconds); $V$ is volume of the headspace of the chamber ($m^3$); $M$ is the molecular weight of the gas (16.04 and 44.01 g $mol^{-1}$ for $CH_4$ and $CO_2$ respectively); $m$ is the mass of the peat-slurry used in grams (total mass of peat and liquid used); $V_{mol}$ is the volume of a mole of gas (air) at a given temperature ($m^3\ mol^{-1}$) calculated by:

$$p \times (R \times K)$$

Where $p$ is pressure (kPa); $R$ is equal to 8.314 (the ideal gas constant) and $K$ is temperature (Kelvin).

*5.3.2.4 Phenol oxidase*

The activity of extracellular phenol oxidases was determined using 10 mM L-DOPA (dihydroxy phenylalanine) (Sigma-Aldrich Co., Ltd, Dorset) solution as a substrate, based on the procedure of Pind et al. (1994) and Williams et al. (2000).

Homogenates made from the 1g peat-slurry samples, were added to 9mL of ultra-pure water, were prepared in separate Stomacher bags (Seward, West Sussex, UK) and a laboratory paddle blender (Stomacher circulator, Seward) was set to mix them for 30 seconds at normal speed setting, in order to minimise cell disruption. Aliquots, 750µL, of the homogenate were transferred into two 1.5mL Safe-Lock Eppendorf tubes (Eppendorf, Stevenage, UK) along with 750µl of 10 mM L-DOPA solution. The reaction tubes were incubated at field temperature (measured at the time

of sample collection date) for nine minutes (after preliminary tests showed that phenol oxidase activity was linear up to this time). After incubation the tubes were centrifuged at 10,000rpm for five minutes and 300μL of each supernatant was pipetted into three wells of a clear 96 well Sterilin Microplate (Sterilin, Cambridge, UK) and absorbance measured at 475nm. Mean absorbance of the three control wells was then subtracted from that of the three L-DOPA wells for each sample and phenol oxidase activity calculated using Beers Law and the molar absorbency coefficient (3,700) for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate (diqc) (Mason 1948) and expressed as nmol of diqc produced $minute^{-1}$ $g^{-1}$ sample (dry weight). Dry weight used in each assay was determined by taking the mean dry weight of three 1g of original peat-slurry sample dried at 105°C to constant weight. A pH buffer has commonly been used in the protocols of other enzyme studies. However in order to maintain conditions closely resembling those in the field, no buffer was added to control the pH of the phenol oxidase assay in this study.

*5.3.2.5 Hydrolase enzymes*

The activities of five hydrolase enzymes was determined by utilising fluorogenic 4-methylumbelliferyl (MUF) labelled substrates which fluoresce at 450 nm emission, 330nm excitation, and using methods based on work by Freeman et al. (1995) and Kang (1999). Homogenates of 1g of the peat-slurry sample (prepared as before) and 7mL of the relevant MUF substrate (see Table 2) were made using a laboratory paddle blender.

| Substrate | Enzyme |
|---|---|
| 4-MUF β-D-glucopyranoside | β-D-glucosidase |
| 4-MUF sulphate potassium salt | Arylsulphatase |
| 4-MUF β-D-xylopyranoside | β-D-xylosidase |
| 4-MUF N-acetyl-β-D-glucosaminide | N-acetyl-β-D-glucosaminidase |
| 4-Methylumbelliferyl phosphate | Phosphatase |

**Table 2. The MUF-labelled substrates needed for measuring the specified enzyme's activity.**

All the samples were incubated at field temperature (measured at the time of sample collection date), for 45 minutes for phosphatase and 60 minutes for N-acetyl-β-D-glucosaminidase, β-D-xylosidase, Arylsulphatase, β-D-glucosidase. Two minutes before the incubation time came to an end the contents of each stomacher bag was thoroughly mixed by hand and the solution from each substrate bag used to fill one 1.5mL Safe-Lock Eppendorf tube, which were then centrifuged at 14,000rpm for five minutes in a temperature controlled centrifuge, set to the required temperature. From the resulting supernatant of each substrate tube 250µL was pipetted into a well of a black flat-bottomed 96 well black microplate; after ensuring each well already contained 50 µL of ultra-pure water - guaranteeing a comparable dilution to the standard solutions in the calibration curve. A fluorometer was used to determine the fluorescence of the MUF at 450 and 330nm excitation.

Standards for the calibration curve were prepared in a similar way to the substrate samples; with 1g of the peat-slurry sample being added to 7mL of ultra-pure water and homogenised in the laboratory paddle blender for 30 seconds. From this 1.5 mL was centrifuged at 14,000rpm for five minutes before 250µL of the resulting

supernatant was transferred from each vial to six separate wells on a black flat-bottomed 96 well microplate. Fifty microlitres of varying concentrations of a MUF-free acid stock solution were added to the supernatant to create a concentration curve of 0-100µM, from which the enzyme activity was determined.

Enzyme activities were expressed as MUF produced (µmol $g^{-1}$ $min^{-1}$), correcting for the dry weight of the peat.

*5.3.2.6 Phenolic compound concentrations*

The concentration of water extractable phenolics from the peat-slurries samples were assayed using a modified version of the spectrophotometric method developed by Box (1983). To conduct the assays, 1g samples of the peat-slurries (prepared as before) were added to 9mL of ultra-pure water, homogenised using a laboratory paddle blender for 30 seconds and then incubated for a further 60 minutes. The solution was thoroughly mixed again, centrifuged at 10,000rpm and the resulting supernatant used for the analysis of phenolic concentration.

Within the 300µL wells of a flat-bottomed 96 well microplate, replicates of 250µL of the supernatant sample, 37.5µL of $Na_2CO_3$ solution (200 mg $L^{-1}$) and 12.5µL of Folin-Ciocalteau phenol reagent were dispensed and mixed thoroughly. A standard curve was prepared by adding the same chemicals to 0 to10 mg $L^{-1}$ phenol solutions. After two hours of incubation at room temperature, the colour change of the reactants was measured at 750nm absorption and the concentration of dissolved phenolics of each sample derived from the absorbance curve of the phenol standards (mg $L^{-1}$). Samples out of range of the standard curve were re-measured using a

standard curve with a higher upper concentration or by diluting the supernatant sample (eg 1:10).

### 5.3.3 Peat-core experiments

#### *5.3.3.1 Sampling regime*

Once the cores had been allowed to acclimatise for four weeks trace gas fluxes were measured. This procedure was repeated after another week and at the same time pore water was collected from all of the cores for analysis of dissolved forms of carbon (see section 2.3.4.). Experimental treatments were then added to groups of four cores.

The experimental treatments consisted of 'injecting' dried pieces of birchwood spatulas (150mm long, 17mm wide, 2mm thick and with a mass of 2.5g) into the top of the peat core. The birchwood spatulas were inserted fully, lengthways, into the peat soil until the top of each one was level with the litter layer at the base of the *Sphagnum* stems. Three different amounts of birchwood spatulas were added to different groups of bog and fen cores: three spatulas (7.5g), six spatulas (15g) and 12 spatulas (30g). A group of control cores were left with no treatments injected into them. The birchwood spatulas were obtained from a medical suppliers in order to ensure they were clean (though not sterile) and free of any artificial preservatives.

After the treatments had been in the cores for four weeks trace gas fluxes were measured in the same way as before and this continued weekly, for a total of 24 weeks, which incorporated the ecosystems' main growing season over the summer. Once the last gas flux measurements had been taken, pore water was extracted from the peat cores and analysed in the same way as before. The cores were then removed

from their core-liners and samples of soil extracted to be analysed for enzymic activity.

*5.3.3.2 Trace gas fluxes*

Whole core trace gas fluxes ($CO_2$ and $CH_4$) were measured by sealing the cores with an opaque headspace chamber (1,600 $cm^3$). A 20mL gas syringe fitted with a short bevel hypodermic needle was used to extract gas samples through a septum at the top of the chamber. 20mL of gas was taken as soon as the chamber was placed onto the cores, to give an initial background concentration (T-1). Gas taken from the chambers were injected into pre-vacuumed 12mL glass Exetainers. Evolved gases were then allowed to accumulate over 60 minutes (after a series of preliminary tests showed that $CO_2$ and $CH_4$ concentrations increased linearly up to this time period) before another 20mL of gas was taken from the chambers and transferred to labelled, evacuated Exetainers (T-2).

Gas samples were analyzed by gas chromatography using the previously explained methodology and instrumentation.

*5.3.3.3 Pore water collection*

Pore water was extracted from the peat soil by the use of suction samplers. These were inserted to a depth of 75mm below the litter layer at the base of the *Sphagnum* stems as soon as the cores were brought from the field to the experimental facility and were left in place throughout the experiment. To construct the samplers Plastipak syringes (2mL) (Fisher Scientific UK Ltd, Loughborough, UK) were cut off 15mm from the luer tip. The tip was retained, packed with glass wool and connected to 200mm of Tygon autoanalyser tubing (Sigma Aldrich Ltd, Dorset, UK).

To collect pore water samples a 20mL Plastipak syringe was fitted to the tubing and suction applied for up to four hours to allow the uptake of water. Following collection all samples were filter sterilised using 0.2µm diameter membranes (Whatman, Kent, UK) and refrigerated (4$^{o}$C) immediately to minimise bacterial degradation and remove particulate organic carbon (POC; Fenner 2002).

*5.3.3.4 pH and conductivity*

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 $L^{-1}$) assuming pH is the negative logarithm of the hydrogen ion concentration.

Conductivity of the pore water samples were taken using a Primo 5 conductivity meter, which was calibrated with a solution of known conductivity (1,413µS $cm^{-1}$). Both analyses were conducted before the pore water was filtered.

*5.3.3.5 Phenolic compound concentrations*

The concentration of water soluble phenolics in the pore water samples was calculated in the same way as previously described minus the steps of extracting the water soluble phenolics from the peat-slurry solution.

*5.3.3.6 Soil sampling and enzyme assays*

Samples of peat were collected from directly around the hole created during the removal of the peat-cores, from a depth of 50 to 75mm. These were stored and

transported as described previously. Within 24 hours the activities of phenol oxidase and hydrolase enzymes were conducted as before.

At the end of the 24-week experimental time period the same enzyme assays were conducted on peat-soil samples taken from the peat-cores (from the same depth) when they were removed from their core-liners.

### 5.3.4 Statistical considerations

Outliers within the raw data sets were removed using the method of Davies and Goldsmith (1972) and checked graphically. Relationships between variables were measured using Pearson's correlation coefficient ($r$), while significant differences between results were determined by independent $t$-tests and independent analysis of variance (ANOVA) tests. All statistical tests were performed using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA).

To ensure the reliability of the correlation coefficient, $t$-statistic and $F$-ratio it was checked that the usual assumptions of parametric tests were not violated. The experimental design ensured that data was in a continuous, interval ratio format and measurements from the different treatment groups were independent. The normality of data distribution was checked by plotting the data graphically, looking at the values of skewness and kurtosis, and with the Sharpio-Wilk test (with a significant value less than 0.05 indicating a deviation from normality). For the $t$-statistic and $F$-ratio the assumption of homogeneity of variance was checked using the Levene's test, with a significant value greater than 0.05 indicating the variances were significantly equal, and therefore the assumption was tenable. If either the assumption of normality or homogeneity of variance was violated data was transformed (eg. $\log(X_i + y)$, where $y$

was a given number added to all results positive). However, there is evidence to suggest that when group sizes are equal ANOVA is robust enough to cope with non-normally distributed data (Donaldson 1968, Field 2009) and therefore the *F*-ratio was taken from the Welch test, when the assumption of homogeneity of variances was violated, which is considered more robust in such circumstances (Field 2009).

The *t*-statistic was reported along with the degrees of freedom and the significance value (*p*) with a value of less than 0.05 taken to be a statistically significant result, as this represents a 95% level of confidence. When the hypothesis being tested was directional, a one-tailed *t*-test was used to give the significance value, rather than the normal two-tailed test. Effect sizes were calculated by converting the *t*-statistic into an *r*-value by:

$$r = \sqrt{\frac{t^2}{t^2 + df}}$$

where *t* is the given *t*-value and *df* is the degrees of freedom used in the *t*-test.

For the independent ANOVA the *F*-ratio was reported along with the degrees of freedom used to assess it, namely the degrees of freedom for the effect of the model and the degrees of freedom for the residuals of the model. To compare the various experimental treatments against each other planned comparisons were performed. A significance value of less than 0.05 was again considered to be a statistically significant result.

The Pearson's correlation coefficient (*r*) was reported along with the significance value.

For displaying on the graphs standard error (*SE*), or standard deviation of the mean, was calculated by:

$$SE = \frac{\sigma}{\sqrt{n}}$$

where σ is the standard deviation of the parent population (derived by the square root of the group's variance from the mean) and *n* is the number of observations, or samples.

**5.4 Results**

From Figure 1A it can be seen that the average $CO_2$ fluxes from peat slurries were inversely related to the molecular weight of the added phenolic material, following 24 hours of incubation. This negative correlation is modest (Taylor 1990) but highly significant ($r$ = -0.554, $p$ (one-tailed) = 0.001). A one-tailed independent t-test also showed there was a significant difference ($t$ (6) = 9.715, $p < 0.001$) with a large effect size $r$ = 0.94 between the $CO_2$ flux of the control slurries and those containing the Ca Lignosulphonate acid. Although $CH_4$ fluxes from the same slurries, showed a similar negative correlation with molecular weight (Figure 1B), the Pearson's correlation coefficient showed that this was not statistically significant: $r$ = -0.268, $p$ (one-tailed) = 0.084.

When varying concentrations of a Ca Lignosulphonate acid solution were added to peat to form slurries, and incubated for 24 hours, all the hydrolase enzyme activities (except for β-D-xylosidase: $r$ = -0.081, $p$ (one-tailed) = 0.388) showed a statistically significant negative correlation with the solutions' concentration (Figure 2): β-D-glucosidase, $r$ = -0.854, $p$ (one-tailed) < 0.001; sulphatase, $r$ = -0.666, $p$ (one-tailed) = 0.003; N-acetyl-B-D-glucosaminidase, $r$ = -0.447, $p$ (one-tailed) = 0.047; phosphatase, $r$ = -0.557, $p$ (one-tailed) = 0.015. An independent one-tailed $t$-test of the control slurries (0% w/v) and those containing a 5% w/v Ca Lignosulphonate solution indicated there was statistically significant difference between the enzyme activities of β -D-glucosidase ($t$ (4) = 10.072, $p < 0.001$), sulphatase ($t$ (4) = 15.989, $p < 0.001$) and phosphatase ($t$ (4) = 4.006, $p$ = 0.008). All three showed a reduction in activity when the phenolic compound was present at this concentration; β-D-glucosidase by 78%, sulphatase by 96% and phosphatase by 52%. N-acetyl-β-D-

glucosaminidase showed a gradual increase in enzyme activity as the concentration of the Ca Lignosulphonate supplement rose to 3% w/v, however concentrations above this retarded activity; with a 5% w/v concentration reducing activity by 11% compared to the control, although this was not statistically significant ($t$ (4) = 0.514, $p$ = 0.317). Similarly, β -D-xylosidase activities increased with concentration until 3% w/v before declining, but this time a 5% w/v concentration did not lower activities below that of the control, though the 35% increase was not statistically significant ($t$ (4) = -2.401, $p$ = 0.074).

The activity of phenol oxidases (Figure 3) showed a statistically significant negative correlation with concentration ($r$ = -0.624, $p$ (one-tailed) = 0.007) with a 5% w/v Ca Lignosulphonate solution suppressing activity by 73%, compared to the control slurries ($t$ (4) = 10.964, $p$ < 0.001). Figure 3 also indicates that a 5% w/v Ca Lignosulphonate acid solution increased phenolic compounds present in the slurries by 8,717% (Control, $M$ = 4.767, $SE$ = 0.784; 5% solution, $M$ = 420.3, $SE$ = 14.199).

The pH of the control slurries, after 24 hours was 4.54, while the 5% w/v slurries were 4.15; a difference of 0.39 of a pH unit. However, this was not statistically significant ($t$ (4) = 2.272, $p$ = 0.063).

Figure 4 and analysis using ANOVA shows that the amount of wooden spatulas in the mesocosms had a significant effect on the amount the $CO_2$ flux over the 24 weeks of the experiment ($F$ (3,46) = 4.286, $p$ = 0.010). A planned contrast revealed that those mesocosms with six spatulas had a significantly lower average $CO_2$ flux over the experimental time period, compared to those with no wooden additions (control mesocosms): $t$ (24) = -2.674, $p$ = 0.007 (one-tailed). The addition of

wooden spatulas did not however have a statistically significant effect ($F$ (3,78) = 1.014, $p$ = 0.391) on the $CH_4$ fluxes of the mesocosms (Table 3).

Although there was no significant difference in the phenolic concentrations between the peat mesocosms before the wooden spatulas were added at Week 0 (Table 4; (3,12) = 1.469, $p$ = 0.272), ANOVA planned contrasts indicated there was a statistically significant difference between the phenol oxidase activity (Figure 5) and the phenolic compound concentrations (Figure 6) in the mesocosms with no wooden spatulas added to them and those with six added ($t$ (10) = -2.038, $p$ = 0.035 and $t$ (24) = 1.188, $p$ = 0.004 (one-tailed) respectively) after the 24 weeks of the experiment .

Hydrolase enzyme activities at Week 0 showed no significant differences between the groups of mesocosms. At the end of the experimental time period mesocosms with six wooden spatulas had generally lower activities than the controls (Table 5). However, only the activity of B-D-glucosidase was significantly lower, as although the overall ANOVA was not statistically significant ($F$ (3,12) = 1.613, $p$ = 0.238) planned contrasts revealed mesocosms with six wooden spatulas in them had significantly lower levels of enzyme activity than those without: $t$ (12) = -1.989, $p$ = 0.035 (one-tailed). Moreover Figure 5 shows a near perfect negative correlation of B-D-glucosidase activity with phenol oxidase activity in the mesocosms.

Finally, Table 6 shows there were no significant differences between the pH and conductivity between the mesocosm groups before the wooden spatulas were added, and afterwards at the end of Week 24. All mesocosms showed an increase in pH and a drop in conductivity.

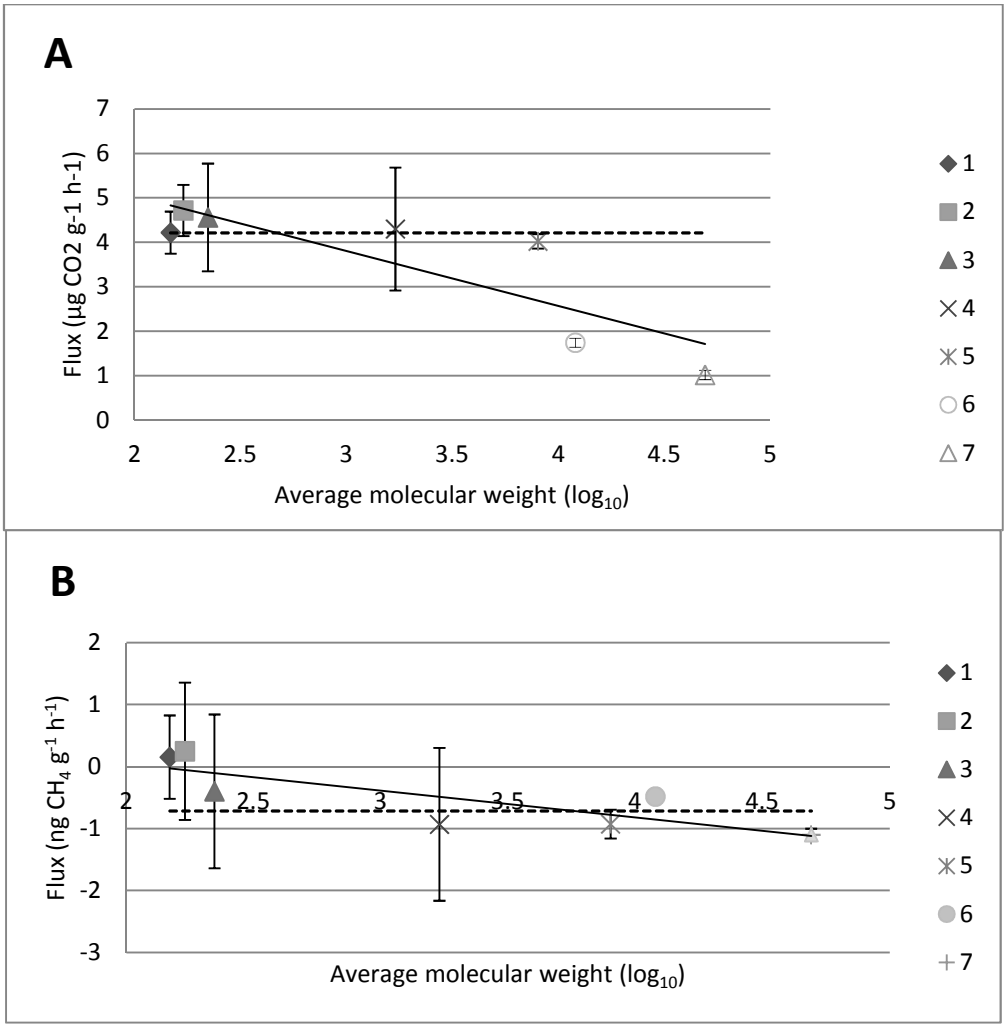



**Figure 1. $CO_2$ and $CH_4$ fluxes from peat slurries after 24 hours of having supplementary phenolic compounds added to them.** A 5% w/v solution, consisting of the phenolic compound and sterile water, was added to bog peat and made into slurries. These were then incubated in dark, anaerobic conditions for 24 hours before gas fluxes were calculated. Mean average of fluxes (*n*=4) are shown and error bars indicate ± standard error. The hatched line represents the mean average $CO_2$ (**A**) or $CH_4$ (**B**) flux from control slurries containing no additional phenolic material. 1 = Cinnamic acid ($M_W$ 148.16); 2 = Gallic acid ($M_W$ 170.12); 3 = Flavone ($M_W$ 222.24); 4 = Tannic Acid ($M_W$ = 1,701.20); 5 = sodium lignosulphonate acid ($M_W$ 8,000); 6 = sodium lignosulphonate acid, ($M_W$ 12,000); 7 = Calcium Lignosulphonate acid ($M_W$ 49,100).

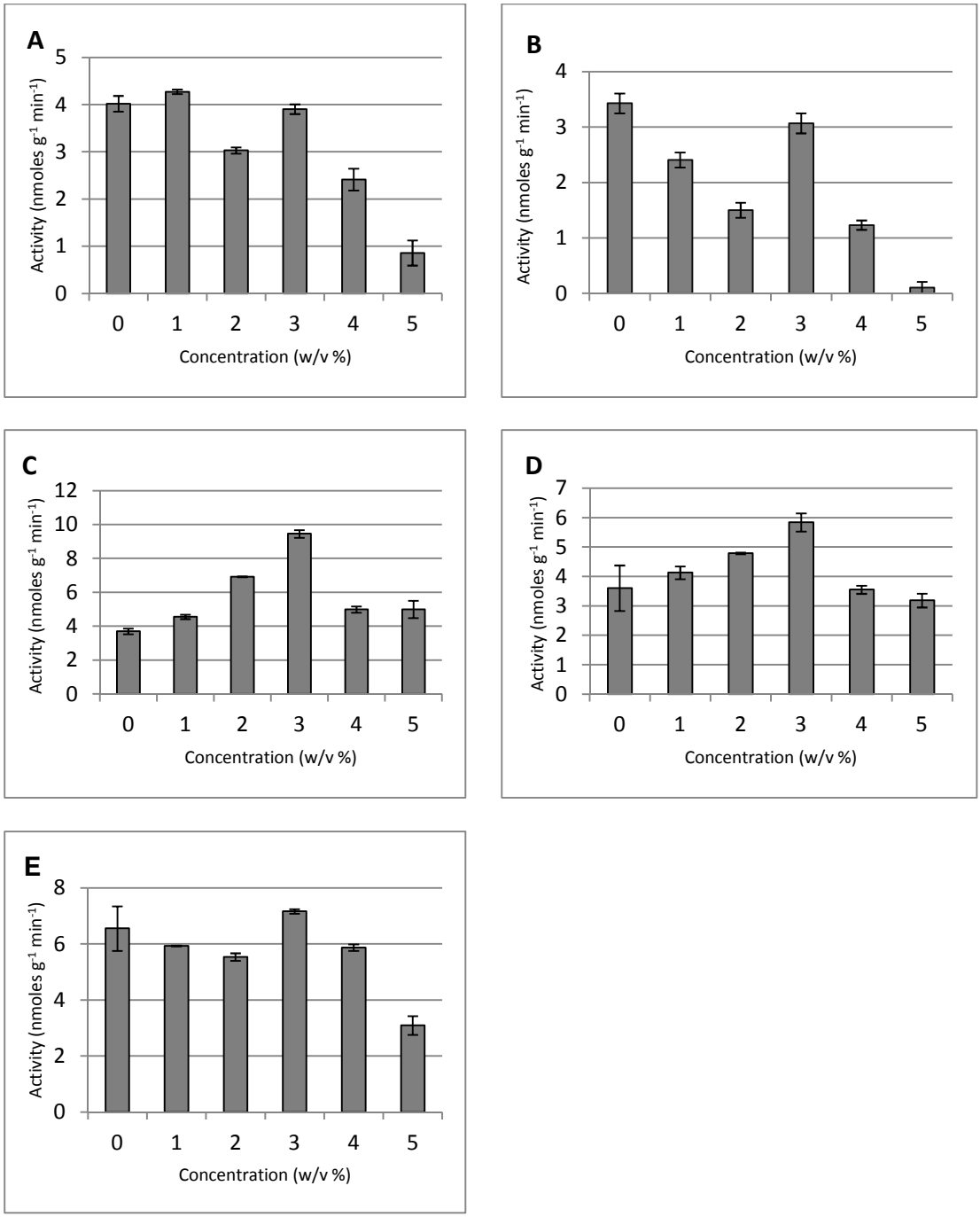



**Figure 2. Hydrolase enzyme activities in peat slurries made with different concentrations (w/v %) of a Ca Lignosulphonate acid solution.** Slurries were made with bog peat slurries. They were then incubated in dark, anaerobic conditions for 24 hours before gas fluxes were measured. Mean average of activities (*n*=4) are shown and error bars indicate ± standard error. **A** = β-D-glucosidase; **B** = sulphatase; **C** = β-D-xylosidase; **D** = N-acetyl-B-D-glucosaminidase; **E** = phosphatase.

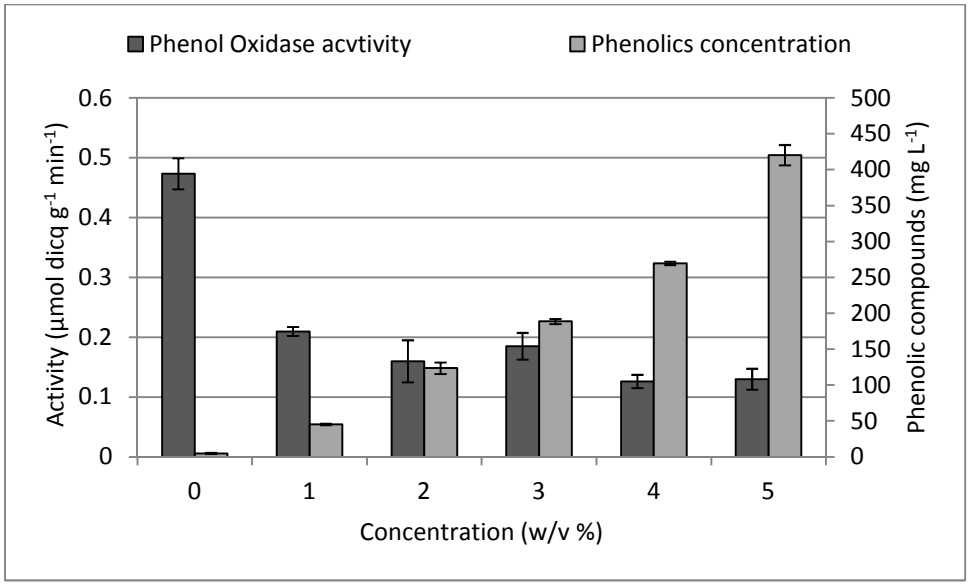



**Figure 3. Phenol oxidase activities and phenolic compound concentrations in peat slurries following addition of different concentrations (w/v %) of Ca Lignosulphonate acid.** Slurries were made with bog peat slurries. They were then incubated in dark, anaerobic conditions for 24 hours before gas fluxes were calculated. Mean averages of activities and concentrations (*n*=4) are shown and error bars indicate ± standard error.

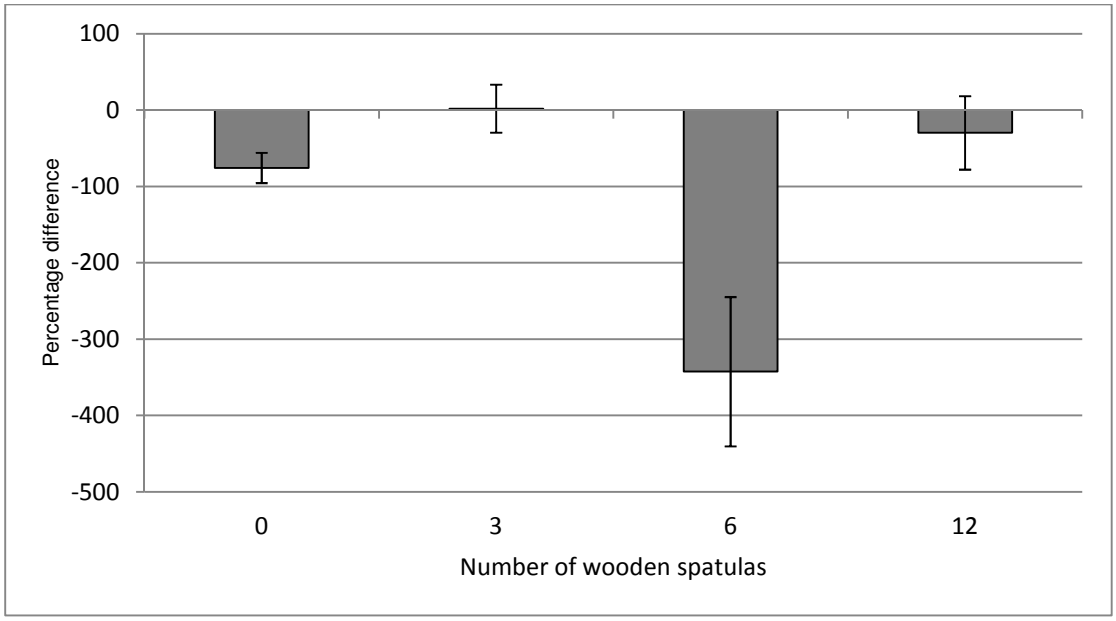



**Figure 4. Percentage difference in average $CO_2$ flux from peat mesocosms during 24 weeks of treatment, compared to pre-treatment fluxes.** Mean averages (*n*=4) are shown and error bars indicate ± standard error.

| Number of wooden spatulas | Percentage difference in $CH_4$ fluxes |
|---|---|
| 0 | **331.2** (122.173) |
| 3 | **208.9** (85.5) |
| 6 | **508.5** (261.6) |
| 12 | **431** (153.2) |
| ANOVA | $F$ (3,78) = 1.014, $p$ = 0.391 |

**Table 3. Percentage difference in average $CH_4$ flux from peat mesocosms during 24 weeks of treatment compared to pre-treatment fluxes.** Mean averages (*n*=4) are shown in bold and numbers in parentheses indicate ± standard error.

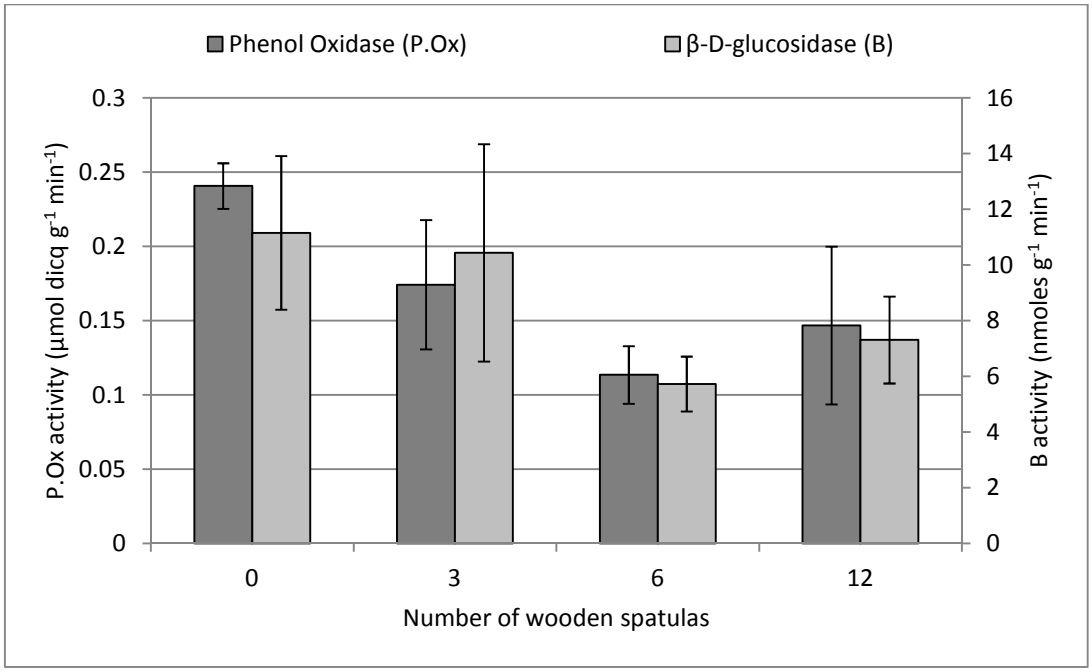



**Figure 5. Phenol oxidase and β-D-glucosidase activity of peat mesocosms following inclusion of wooden spatulas for 24-weeks.** Measurements were taken from peat-soil samples, taken from a depth of between 5 and 10 cm from the surface. Results show mean average (*n*= 4) and error bars indicate ± standard error. B on the secondary *x*-axis represents β-D-glucosidase activity.

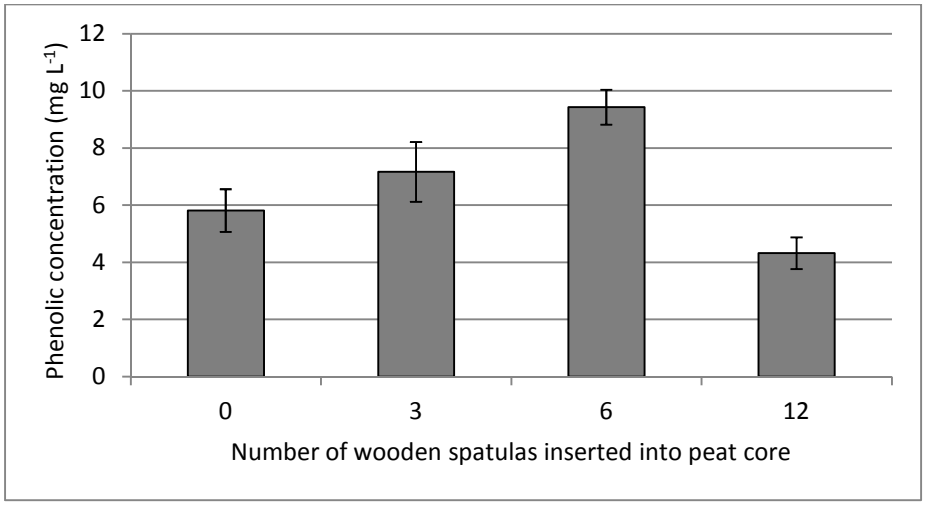



**Figure 6. Phenolic concentration of peat mesocosms following inclusion of wooden spatulas for 24-weeks.** Results show mean average (*n*= 4) and error bars indicate ± standard error.

| Number of wooden spatulas | Phenolic compound concentration (mg $L^{-1}$) |
|---|---|
| 0 | **7.003** (1.558) |
| 3 | **7.114** (1.564) |
| 6 | **8.173** (1.330) |
| 12 | **4.313** (0.8514) |
| ANOVA | $F$ (3,12) = 1.469, $p$ = 0.272 |

**Table 4. Concentration of phenolic compounds before wooden spatulas were added (Week 0).** Phenolic compound concentrations were taken from pore water. Mean averages (*n*=4) are shown in bold and numbers in parentheses indicate ± standard error.

| Wooden spatulas | Week 0 | | | | | Week 24 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | B | S | X | N | P | B | S | X | N | P |
| 0 | **6.612** (0.765) | **11.787** (1.672) | **3.774** (0.450) | **1.676** (0.350) | **14.3178** (2.014) | **<u>11.156</u>** (2.757) | **2.668** (0.371) | **4.194** (0.766) | **3.263** (0.365) | **33.274** (18.325) |
| 3 | **7.581** (0.804) | **10.801** (1.564) | **4.070** (0.383) | **1.904** (0.211) | **14.780** (0.576) | **10.437** (3.904) | **3.896** (1.622) | **4.971** (0.621) | **4.085** (0.855) | **41.739** (20.395) |
| 6 | **6.548** (0.464) | **9.617** (1.037) | **3.071** (0.409) | **1.646** (0.160) | **11.228** (1.818) | **<u>5.724</u>** (0.990) | **1.801** (0.139) | **4.169** (0.712) | **2.638** (0.464) | **17.444** (9.671) |
| 12 | **5.820** (0.880) | **10.504** (0.667) | **3.794** (0.796) | **1.643** (0.077) | **11.903** (0.747) | **7.309** (1.563) | **3.136** (0.589) | **3.511** (0.641) | **3.603** (0.503) | **56.461** (32.123) |
| ANOVA | $F$ (3,12) 0.940 $p$ = 0.452 | $F$ (3,12) 0.474 $p$ = 0.706 | $F$ (3,12) 0.631 $p$ = 0.609 | $F$ (3,12) 0.318 $p$ = 0.812 | $F$ (3,12) 1.772 $p$ = 0.206 | $F$ (3,12) 1.613 $p$ = 0.238 | $F$ (3,12) 1.103 $p$ = 0.386 | $F$ (3,12) 0.755 $p$ = 0.540 | $F$ (3,12) 1.112 $p$ = 0.382 | $F$ (3,12) 0.627 $p$ = 0.612 |

**Table 5. Hydrolase enzyme activities in peat-soil taken from mesocosms before treatment addition and 24 weeks later.** Peat-soil samples were taken from a depth of between 5 and 10 cm from the surface of the mesocosms. Mean averages ($n$=4) are shown in bold and numbers in parentheses indicate ± standard error. Underlined results indicate a statistically significant ($p < 0.05$) planned contrast in ANOVA. Enzymes: **B** = β-D-glucosidase; **S** = Arylsulphatase; **X** = β-D-xylosidase; **N** = N-acetyl-β-D-glucosaminidase; **P** = Phosphatase.

| Number of wooden spatulas | pH | | | Conductivity | | |
|---|---|---|---|---|---|---|
| | Week 0 | Week 24 | Change (%) | Week 0 | Week 24 | Change (%) |
| 0 | **4.56** (0.17) | **5.31** (0.21) | **+16.4** | **129.75** (8.33) | **65.25** (9.17) | **-37.7** |
| 3 | **4.62** (0.20) | **5.8** (0.34) | **+25.5** | **107.25** (18.56) | **64.75** (10.9) | **-39.6** |
| 6 | **4.59** (0.12) | **5.46** (0.18) | **+19.0** | **96.75** (15.37) | **74** (5.58) | **-23.5** |
| 12 | **4.78** (0.05) | **5.28** (0.29) | **+10.46** | **82.5** (10.62) | **64.5** (3.71) | **-21.8** |
| ANOVA | $F$ (3,12) = 0.436, $p$ = 0.731 | $F$ (3,12) = 0.505, $p$ = 0.686 | | $F$ (3,12) = 0.730, $p$ = 0.554 | $F$ (3,12) = 0.341, $p$ = 0.796 | |

**Table 6. pH and conductivity of mesocosm pore water with different numbers of wooden spatulas added to them.** Samples were taken from pore water, collected from a depth of 10cm from below the surface. Pre-treatment (Week 0) measurements were taken immediately before the wooden spatulas were added. Mean averages ($n$=4) are shown in bold and numbers in parentheses indicate ± standard error.

## 5.5 Discussion

In this study we aimed to discover if the addition of supplementary phenolic compounds to the peat matrix could strengthen the enzymic latch (Freeman et al. 2001b): decreasing SOM decomposition by suppressing extracellular enzyme activities, resulting in lower rates of carbon-based trace gas production ($CO_2$ and $CH_4$) and increased net sequestration of carbon as SOM. In the initial slurry experiments a range of phenolic compounds were made into a 5% w/v solution with pure-water and added to peat, taken from the most microbially-active depth of a blanket bog (Freeman et al. 1995). The phenolic types chosen were 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).

Our initial experiment indicated that the presence of certain phenolics did indeed affect the release of trace gases from the peat slurries (Figure 1). Samples containing Ca lignosulphonate showed the greatest suppression of both $CO_2$ and $CH_4$ compared to a set of control slurries (with no additional phenolics), though only the former was statistically significant. Figure 1 also showed that there was a moderate negative correlation between molecular weight ($M_w$) of the phenolic compounds and $CO_2$ efflux, which corroborates previous studies which have shown organic matter (OM) with a high molecular weight (>1,000 apparent $M_w$ distribution) can inhibit the metabolism of low $M_w$ OM (Freeman et al. 1990, Freeman and Lock 1992). The lignin, Ca lignosulphonate, had the highest $M_w$ 49,100 Daltons (Da) and also had the lowest effluxes of both $CO_2$ and $CH_4$ (though only the former was statistically significant compared to the control samples). It was surprising that lignin appeared to have the greatest effect on SOM decomposition (if $CO_2$ production is taken as a

proxy), as although lignin is the most stable of the polyphenols it is tannin which has previously been suggested is the principle controller over decompositional dynamics (Horner et al. 1988). Though in this study the tannin used did not significantly lower $CO_2$ production.

Ca lignosulphonate is a form of industrial lignin and is a by-product of chemical pulp mills in the paper manufacturing industry (Ouyang et al. 2006). It is a soluble brown powder and produced in very large quantities, with some figures suggesting that there is as much as 50 million tons of industrial lignin (lignosulphonate and alkali lignin) produced every year in the world (Xiao et al. 2001), but only 10% of this is used by other industries (Ouyang et al. 2006). Preparation of any 'unchanged' lignin from the cell walls of vascular plants is not easy; due to the complex and irregular structure of the polymer, which is intimately mixed with carbohydrate components (Brunow 2001). Therefore, whilst taking this into consideration, the availability and affordability of Ca lignosulphonate make it an excellent experimental lignin for use in our study. The low trace gas fluxes from slurries containing Ca lignosulphonate, which we observed, can be attributed to the inhibitory effect the lignin had on extracellular hydrolase enzyme activities in the peat slurries (Figure 2), a characteristic that has been well documented in peatlands (eg. Freeman et al. 2001a, Freeman et al. 2001b). 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). The polyphenols can also 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). Suppressing degradation in this way therefore prevents microbial respiration and the resulting

release of trace gases. It is important though to note that no work was done to consider any potential effect the calcium or sodium ions, as part of the various lignosulphonate compounds, were having on the biogeochemistry of the peat samples. It is likely they will affect the ion concentrations of the peat slurries so this needs to be investigated more fully.

Our results showed that the activity of several hydrolase enzymes were severely curtailed by the presence of a 5% w/v Ca lignosulphonate solution, including β-D-glucosidase which are involved in the generation of labile glucose from cellulose (Deng and Popova 2011), and so key in determining microbial activity and therefore $CO_2$ efflux from peat soils. The results of the concentration test (Figure 2) suggest that a 5% w/v Ca lignosulphonate solution is required to significantly inhibit OM degradation and $CO_2$ release. Higher concentrations of Ca lignosulphonate solution were not tested due to the difficulty of getting the lignin into solution and the wish to keep the concentration to practical and inexpensive amounts, should we wish to apply it on a larger field scale.

Our results also showed phenol oxidase activities were dramatically affected by the presence and concentration of Ca lignosulphonate (Figure 3) with a significant negative relationship being observed between the two. Phenol oxidases are oxidative copper-containing enzymes that catalyse the release of reactive oxygen radicals, which in turn, promote a variety of non-enzymatic reactions to oxidise phenolic compounds (Thurston 1994, Claus 2004). They instigate the oxidation of both complex polyphenols, such as lignin, and simple phenolic compounds, with outcomes ranging from partial oxidation and the release of oxidative intermediates to the complete degradation of phenolic compounds to non-phenolic end products (Freeman et al. 2012). By increasing the levels of phenolics in the peat matrix a rise in phenol

oxidase activity may therefore have been expected in our slurries, but the opposite was observed. However, this does concur with findings elsewhere showing that certain levels of phenolics released by lignified organic matter, commonly known as 'extractives', can inactivate phenol oxidase (Scalbert 1991). These extractives are believed to inhibit phenol oxidase-catlysed degradation of phenolics by binding essential metals, radical scavenging and antioxidant properties (Scalbert 1991, Heinonen 2007).

Interestingly, both β-D-xylosidase and N-acetyl-β-D-glucosaminidase activities increased with Ca lignosulphonate concentration up to 3% w/v, and the former's activities remained higher at 5% w/v than those of the control slurries. The supplementary lignin was not therefore able to suppress all enzymic activity, which could be due to the selective and specialist nature of hydrolase enzymes enabling them to use different substrates across a wide range of environments (Nybroe et al. 1992, Lee et al. 1999, Allison and Vitousek 2004).

Previous work has shown that pH plays an important role in carbon cycling (Dunfield et al. 1993, Bergman et al. 1998) and maybe even the enzymic latch in peatlands (Williams et al. 2000, Dunn et al. in preparation). Although the slurries made with the Ca lignosulphonate solution did have a lower pH, statistical tests suggested this was not significant so it could be predicted that the additional phenolics were the key suppressor of the enzyme activities and resulting GHG effluxes.

The reason for choosing to investigate whether adding wood (instead of Ca lignosulphonate) to a set of peat cores had an effect on GHG release, was to see if extractives from the wooden spatulas would include a wider range of inhibitory phenolic compounds (Kai 1991) and would therefore be able to suppress a larger

number of hydrolase enzymes. Interestingly though the results for cores containing six wooden spatulas were comparable and displayed similar relationships to the 5% w/v Ca lignosulphonate slurries: $CO_2$ fluxes were reduced compared to control cores (Figure 4) and phenol oxidase activities (Figure 5) were suppressed along with those of β-D-glucosidase (Table 5). However, GHG fluxes and enzyme activities of the other experimental cores, those with three or 12 wooden spatulas added to them, were not significantly affected suggesting correct dosage of phenolic enrichment is essential to achieve a significant suppression of organic matter breakdown. Three wooden spatulas was clearly too few to allow enough extractives to affect the biogeochemical cycles in the peat - demonstrated by the slight and non-significant increase in phenolic concentration after 24-weeks (Figure 6). While observation of the cores with 12 wooden spatulas inserted into them suggested that the upper layer of the peat had become severely damaged with gaps and holes being created in the surface. This made it difficult for the vegetation (mixed *Sphagnum* species) to recover over the experimental time period and could have increased oxygenation of the peat along with enabling extractives to be leached out of the upper layers during watering and precipitation events.

The peat cores which had wooden spatulas inserted into them all showed an average percentage increase in phenolic concentrations over the experimental time period (Figure 6 and Table 4). This was slight in the case of three and 12 spatulas (0.73 and 0.29 percent respectively) but as high as 15.27% in the cores with six spatulas in them. Control cores showed a 16.96% decrease though, suggesting that extractives from the wood do indeed increase levels of phenolics in the peat matrix. The final phenolic concentrations of the different groups (Figure 6) also appear to be negatively correlated with the activity of the hydrolase enzyme β-D-glucosidase

(Figure 5) corroborating previous work on the enzyme latch which indicate that phenolics inhibit the activity of hydrolases (Freeman et al. 2001b, Freeman et al. 2004b) and, similar to our slurry experiments, phenol oxidase activity (Figure 5).

All pore water pH values increased over the experimental time period (Table 6) though there were no significant differences between the different treatments, with the control cores actually having a lower pH (5.31) than those with six spatulas (5.46). It would usually be expected that the samples with the lowest (or suboptimal) pH would have the lowest levels enzyme activity (Pind et al. 1994) and therefore OM decomposition rates. As this was not the case it suggests that concentrations of phenolic compounds has the strongest inhibitory effect on enzymic decomposition in peatlands.

Levels of conductivity (EC) from all groups of cores dropped by between 21.8 and 39.6% over the experimental time period, with no statistical differences between the groups. This suggests that the addition of wood was not affecting concentrations of available nutrients, which may be important should we move our experiment up to field trials and wish to maintain floral communities.

To answer the main question from our study: have we developed a method for maximising carbon sequestration in peat? Clearly from our results we can conclude that supplementing peat with additional phenolics - whether in the form of industrial lignin or extractives from wood fragments - can suppress enzyme activity and resulting release of GHG fluxes (namely $CO_2$) from SOM decomposition by as much as 76% in the Ca lignosulphonate slurries and 47% in peat cores containing six wooden spatulas (compared to a set of controls). Methane remained largely unaffected by the presence of additional phenolics, possibly due to the compounds' main inhibitory effects being on the breakdown of the more labile carbon in the peat litter.

Further research is needed to answer some of the questions that this study poses, especially in terms of larger plot or field scale trials: which compound is more effective and practical to use, industrial lignin or intact wood; does the wood type make a difference due to differing levels of lignin and other phenolic compounds present; does increasing the surface area of the wood in contact with the peat soil increase its inhibitory affect; what are the effects on DOC entering water courses and what are the effects on plant communities and biodiversity? Other biogechemical questions also need to be answered such as are the phenolics directly inhibiting microbial metabolism alongside extracellular enzyme activity (Fenner and Freeman 2011).

Our findings have demonstrated that phenolic inhibition in peatland soils currently falls substantially below its full potential, and that there is potential to significantly increase carbon storage capacity through phenolic supplementation. Application of our findings at a global scale could be considered potentially 'climate-changing' if used as a unique form of geoengineering.

Northern peatlands currently capture $1.7x10^{15}$ g carbon $yr^{-1}$ through photosynthetic litter production (Reader and Stewart 1972). However, at present they only sequester $1x10^{14}$ g carbon $yr^{-1}$, or 9%, of that in the long-term. This inefficiency arises because substantially less carbon enters long-term storage, 34 to 52 g carbon $m^{-2}$ $yr^{-1}$, than is initially captured from net primary production (NPP), which can reach 489g carbon $m^{-2}$ $yr^{-1}$ (Reader and Stewart 1972, Wieder 2001). A powerful strengthening of the enzymic latch mechanism by manipulating the phenolic abundance in peatlands through our methods could significantly increase this storage amount by up to 76% equating to an increase in their long-term carbon storage to 92 g

carbon $m^{-2}$ $yr^{-1}$, or $1.76x10^{14}$ g carbon $yr^{-1}$. However, it is important to point out that our estimates do not include any reference to the carbon in the wood or industrial lignin which is used in the phenolic supplementation process. This is likely to remain unaffected by decomposition for a long period of time in the peat due to the effects of the enzymic latch, and may therefore increase the total amount of sequestered carbon substantially (Freeman et al. 2012).

It is essential to point out that our research is at the preliminary stage and much further interdisciplinary research work now needs to be conducted before firm conclusions are reached. Longer experimental time-scales, plot and field experiments need to be conducted along with investigations into microbial community shifts and adaptations which are likely to occur when additional phenolics are added to the peat. Other key issues, such as how the existing vegetation on peatlands will be affected and, in turn, effect decomposition rates with large-scale phenolic-enrichment, also need to be studied. However, deliberate strengthening of the enzymic latch in peatlands does present a promising prophylactic bio-geoengineering approach to the prevention of further rises in atmospheric $CO_2$ levels, offering a safe and cost-effective land-based $CO_2$ removal technique (CDR) which now requires further research should we require a 'Plan B' to reduce the rate of global warming this century (Royal Society 2009).

# Chapter 6

## A respirometric pulse-chase experiment using $^{13}C$-labelled litter to calculate decomposition rates in temperate and boreal peats

## 6.1 Abstract

Peatlands in the northern hemisphere are under threat from a range of anthropogenic and climatic factors. It is feared that degradation may destabilise the estimated 455 petagrams (Pg; 1 Pg = 1 billion metric tons) of carbon stored as organic soil within these ecosystems. Such vast amounts of peatland carbon accrue principally because of constraints on the enzyme phenol oxidase. This permits accumulation of plant-produced phenolic compounds, which are potent inhibitors of hydrolase enzymes – major agents in the breakdown of organic matter. However, it is unknown whether current litter phenolic concentrations fall short of those required to achieve the full potential capacity of peatlands for carbon sequestration. Here we show, through a respirometric pulse-chase experiment using $^{13}C$ labelled litter, that a further 99.6% suppression of decomposition can be achieved by supplementing peat with additional phenolics. The widespread assumption that the long term capacity of peatlands to sequester carbon will remain secure provided we maintain conditions that are waterlogged, acidic and anoxic alone can therefore no longer be relied upon. Additionally, we must also consider the need to maintain an adequate litter phenolic content. Moreover, supplementary phenolics were also able to suppress microbial respiration in boreal peat samples and have the potential to reduce carbon losses from Arctic peatlands from 100 megatonnes (Mt) each year to just 6 Mt. Our findings suggest that phenolic supplementation of northern peatlands could offer a new bio-geogengineering technique to beneficially alter our planet's climate.

## 6.2 Introduction

Northern peatlands store 455 Pg of yet occupy just 3% of the planet's surface (Gorham 1991, Limpens et al. 2008b). These vast global repositories of organic matter (OM) are able to accumulate principally because of phenolic inhibitors slowing the rate of decomposition to below that of photosynthetic production (Freeman et al. 2001b). In lower latitude (temperate) peatlands this build-up of phenolic compounds is related to the unique properties of waterlogged peat soils suppressing the activity of phenol oxidase; one of the few enzymes capable of breaking these inhibitors down (Freeman et al. 2004b) - thus creating an 'enzymic latch' mechanism, locking in place climate-altering amounts of carbon (Freeman et al. 2001b). However, concerns have been expressed that this carbon stock may destabilise, releasing carbon in either a gaseous (Gorham 1991, Freeman et al. 2001b) or aqueous form (Freeman et al. 2001a, Worrall et al. 2003) with some studies suggesting such a destabilisation may already be underway (Freeman et al. 2001a). It is therefore unclear whether current litter phenolic contents are sufficient to ensure optimal carbon sequestration rates.

In sub-Arctic, or boreal, peatlands low temperatures represent an obvious constraint on the breakdown of organic matter (Bridgham et al. 1998, Updegraff et al. 2001, Chivers et al. 2009), some of which has been cryopreserved for up to 16,500 years (MacDonald et al. 2006a). Here too though, there are fears that up to 277 Pg of boreal carbon (Schuur et al. 2008) is vulnerable to release, as high latitudes are expected to experience the strongest effects of global warming over the coming century (Meehl et al. 2007). We hypothesise that it may be possible to replace the existing 'cryo-enzymic latch' currently present in circumarctic peatlands with a comparable 'chemo-enzymic latch', similar to that found in temperate peatlands, consisting of phenolic inhibitors.

Phenolics, which are a class of chemical compounds consisting of a hydroxyl functional group (-OH) bonded to an aromatic hydrocarbon group, are produced as secondary metabolites by plants (Dai and Mumper 2010) where they have a range of functions including roles in pigmentation, growth, reproduction and resistance to pathogens (Lattanzio et al. 2006). However, once in the peat-soil via dead plant matter, they are known to act as powerful inhibitors of the main agents of decomposition; namely extracellular hydrolase enzymes (Freeman et al. 2001b, Freeman et al. 2004b). In the decomposition of OM (consisting of litter made from dead plant matter) polymeric materials such as lipids, proteins, and carbohydrates are primarily hydrolyzed by these extracellular hydrolases, excreted by heterotrophic microbes in the peat-soil. The hydrolytic enzymes hydrolyse their respective polymers into smaller molecules, primarily monomeric units, which are then consumed by microbes. The production and activities of these extracellular enzymes are therefore often the rate-limiting step in decomposition processes. It has been hypothesised that manipulation of the phenolic abundance in the peat could be used to strengthen the enzymic latch and even offer a new approach to geoengineering (Freeman et al. 2012; Dunn et al. in preparation) - acting as a cost efficient biological $CO_2$ removal (CDR) scheme (Royal Society 2009).

In our earlier work (Dunn et al. in preparation) we showed that lignin (a member of the flavonoid group of phenolics, which is an integral part of the secondary cell wall of plants and a major component of wood) can suppress the release of $CO_2$ from peat by as much as 76%. However, these results were the total $CO_2$ efflux from the peat, which could have included any chemical and/biological breakdown of the additional lignin solution. To quantify the actual breakdown of OM in the peat a respirometric pulse-chase experiment using $^{13}C$ labelled litter was

devised. This allowed us to accurately determine the inhibitory effect of our industrial lignin supplement on decomposition. The rate at which the labelled litter decomposed by was measured from analysing the release of $^{13}C$-enriched carbon dioxide ($^{13}C$-$CO_2$) and methane ($^{13}C$-$CH_4$) from the peat samples - the result of respiration from metabolising microbes assimilating the $^{13}C$ labelled litter.

The lignin used in this study was Ca lignosulphonate a by-product of chemical pulp mills in the paper manufacturing industry (Ouyang et al. 2006). It is a soluble brown powder and produced in very large quantities, with some figures suggesting that there is as much as 50 million tons of industrial lignin (lignosulphonate and alkali lignin) produced every year in the world (Xiao et al. 2001), but only 10% of this is used productively by other industries (Ouyang et al. 2006). Previous work by the authors suggest the compound successfully inhibited enzymic activity and $CO_2$ production from peat taken from a UK blanket bog (Dunn et al. in preparation). Ca lignosulphonate also makes an ideal experimental compound for our study because if it proves successful there is an inexpensive, plentiful supply of it.

We hypothesise that current litter phenolic concentrations are not sufficient to allow temperate peat to reach its maximum carbon sequestration rate. This will be determined by supplementing peat samples taken from a UK blanket bog with additional phenolics (lignin), which contains $^{13}C$ labelled litter (OM matter). The rate of this OM decomposition will be established through analysis of the efflux of $^{13}C$ enriched trace gases - respired by heterotrophic microbes. For our hypothesis to be accepted peat samples containing the additional lignin will have lower effluxes of these trace gases. We also hypothesise that the additional lignin will inhibit the activities of a range of hydrolase enzymes. For the boreal peat samples we

hypothesise that the supplementary phenolics will allow the vulnerable, highly thermally sensitive peats of this region to become as resilient to decomposition as their temperate peat counterparts.

If these hypotheses are accepted we may be on the first step to developing a method for geoengineering northern peatlands in an effort to alter our planet's climate.

## 6.3 Materials and methods

### 6.3.1 Study sites and sample collection

#### *6.3.1.1 Temperate peat*

Temperate peat samples were taken from the Migneint, an extensive blanket bog in relatively good condition located in North Wales, UK. (3°48.8′ W, 52°59.6′ N). Average annual rainfall is 2.4m and at 460m above sea level the mire is dominated by *Calluna vulgaris*, with *Eriophorum vaginatum* above a deep layer of mixed *Sphagnum* species. The mean peat depth across the site is 2m (Evans et al. 2012).

Peat samples were collected during November (winter) and July (summer) from a depth of 50 to 100mm (by the use of a hand trowel to excavate small pits) from five separate locations dominated by *Sphagnum* species within a $25m^2$ area. Care was taken to ensure samples were taken from areas with similar peat-composition, water-table levels and microtopography. samples were placed in sealable plastic bags and transported to the laboratory in a cooled, insulated container. The soil temperature at the location of sample collection was recorded. Back in the laboratory the samples were amalgamated, homogenised thoroughly by hand, and had any adhering debris and macroinvertebrates removed from them. They were kept in an incubator at the recorded field temperatures before beginning the experiments within a week of collection.

#### *6.3.1.2 Boreal peat*

Peat cores (50mm x 300mm) were taken from three sites in Alaska, USA. Site A was a nutrient rich fen located outside the boundaries of the Bonanza Creek Experimental Forest, approximately 35km south-east of Fairbanks, Alaska (64.82$^{o}$N,

147.87°W). The site has a mean annual temperature of -2.9°C and a mean annual precipitation of 269mm (30% as snow), it lacks trees and is dominated by a diverse community of brown moss, *Sphagnum* and emergent (eg. *Equisetum* and *Carex*) species (Turetsky et al. 2008). Site B a nutrient poor thermokarst bog, where the permafrost started thawing 20-years ago, 32km south-west of Fairbanks (64.41°N, 148.19°W). There are standing dead spruce and tarmarack trees present (Wickland et al. 2006). Finally Site C a low productivity black spruce site, with poor drainage and deep organic layer (0.9m), near to the Bonanza Creek Experimental Forest (Wickland et al. 2010). Samples were all collected in the winter when the peat was frozen and they were maintained at -18°C until required.

6.3.2 Peat slurry creation

The slurries were created in 50mL centrifuge tubes (Fisher Scientific UK Ltd, Loughborough, UK). The centre of the lids of each tube was fitted with a size nine Suba-Seal® rubber septa (Sigma Aldrich Ltd, Dorset, UK). As the rubber closes the puncture, the septa allowed repeated piercing with a hypodermic needle.

To make the 50:50 peat-solution slurries, 10g samples of peat (which had previously been amalgamated and homogenised by hand for 10 minutes) were put into the centrifuge tubes along with 10mL of the relevant solution. For the control slurries this solution consisted of ultra-pure water and for the phenolic treatment slurries consisted of a 5% weight/volume (w/v) solution - made with Ca lignosulphonate dissolved into ultra-pure water eg. 50g in 1,000mL.

A clean glass rod was used to mix the slurry for two minutes, alternating between a clockwise and anti-clockwise motion, before they were placed on a rotary shaker at 200rpm for 30 minutes. To perform the experiment under anaerobic

conditions the slurries were then immediately placed in a zipper lock-style AtmosBag glove bag (Sigma Aldrich Ltd, Dorset, UK), the air removed with a vacuum pump and oxygen-free-nitrogen was used to re-fill the bag. This process was repeated every 24 hours and the bag regularly checked for holes. The AtmosBag and the slurries were kept in dark conditions at a stable temperature consistent with the demands of the experiment and/or the recorded temperature of the soil during sample collection.

To extract gas samples from the headspace of the centrifuge tubes a 20cm$^3$ gas syringe fitted with a two-way valve (Sigma Aldrich Ltd, Dorset, UK) and a short bevel hypodermic needle, was used to extract gas through the rubber septa in the lids of the centrifuge tubes. For the boreal peat warming experiment 10mL of gas was collected from the headspace of the centrifuge tubes, with the two-way valve being closed before removing the needle from the septa, and transferred to labelled pre-evacuated 5.9mL Exetainers (Labco Ltd, Lampeter, UK), whilst the $^{13}$C labelled litter experiments required the extraction of 20mL (or as close as possible to this amount) of gas from the centrifuge tubes, which were transferred to labelled pre-evacuated 12mL Exetainers.

### 6.3.3 Respirometric pulse-chase experiment

#### *6.3.3.1 $^{13}$C-labelled litter production*

The labelled litter was produced by pulse labelling with $^{13}CO_2$ (99 atom % $^{13}$C) typical peatland plants (*Sphagnum*, *Juncus* and *Calluna* species) in a closed chamber system (Livingston and Hutchinson 1995). The plants were subjected to a five hour pulse at a concentration of 350 to 400ppm on a day when levels of photosynthetically active radiation (PAR) were relatively high (1,000 μmol m$^{-2}$ s$^{-1}$), ensuring ideal conditions for photosynthetic assimilation of $^{13}C$-$CO_2$ (Ostle et al.

2000). The aboveground plant biomass was then removed, dried, milled and homogenised to create a $^{13}C$ labelled litter.

*6.3.3.2 Respirometric $^{13}C$ flux analysis*

The $^{13}C$ labelled litter was added to the 50:50 peat slurries either a) 24 hours after the slurries had been made with the relevant solution (for the experiments involving boreal peat and the temperate peat collected in the winter) or b) during the mixing process of the slurry making (as in the case of the temperate peat collected in the summer). For the latter, all the slurries were originally made with ultra-pure water and after 24 hours 0.5g of the phenolic compound (Ca lignosulphonate) was added to them (the equivalent of the original 5% w/v Ca lignosulphonate solution). Due to the observed effect Ca lignosulphonate had on the pH of the slurries, the pH of some of the summer-peat slurries was also adjusted using small amounts of concentrated hydrochloric acid (HCl) to lower it and saturated calcium hydroxide ($Ca(OH)_2$) to increase the pH of slurries containing the Ca lignosulphonate solution.

Gas samples were taken regularly over the 120 hour course of the experiment and each time the slurries were sealed and incubated for 60 minutes (after a series of preliminary tests showed that $CO_2$ and $CH_4$ concentrations increased linearly for up to 90 minutes) before a gas sample was taken from the headspace of the centrifuge tube, as previously described. Gas samples were analysed using a trace gas isotope ratio mass spectrometer (IRMS) for $CO_2$ and $CH_4$ concentrations (ppm) and $^{13}C/^{12}C$-$CO_2$ ratios expressed in parts per million (‰) from the defined international PDB standard, calculated by:

$$\partial^{13}\mathrm{C} = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1{,}000$$

where R is the abundance ratio of $^{13}C$ to $^{12}C$ and the standard is V-PDB (Craig 1957). $\partial^{13}C$-$CO_2$ and $\partial^{13}C$-$CH_4$ effluxes were calculated (using $\partial^{13}C$-$CO_2$ as the example) with the mixing equation (Subke et al. 2004, Kammer and Hagedorn 2011):

$$\partial^{13}\text{C-CO}_2 \text{ efflux } (‰) = \frac{\left(^{13}\text{C}_{flask} \times \text{CO2}_{flask}\right) - \left(^{13}\text{C}_{ambient} \times \text{CO2}_{ambient}\right)}{\text{CO2}_{flask} - \text{CO2}_{ambient}}$$

6.3.4 Warming boreal-peat gas fluxes

For the warming experiments the boreal peat cores were sectioned into smaller frozen intact micro-cores (approximately 30mm x 30mm x 80mm), consisting of peat from between 50 and 100mm below the original surface level. These were placed into individual gastight 50mL cylinders under dark, anaerobic conditions. A set of the micro-cores were subjected to gradual warming (-18$^{o}$C to +15$^{o}$C) over a one month period and gas samples regularly analysed for flux rates. The original water content of the micro-cores was kept constant, by the addition of ultra-pure water (based on the mass of micro-cores). This approach contrasted with all known published studies of this type, which a) began measuring gas fluxes at temperatures already above 0$^{o}$C, and b) used peat slurries or homogenates rather than samples with an intact soil structure (Table 1).

To calculate fluxes (the rate of flow) at least two gas measurements were required; an initial one (T-1) and another after a known amount of time has passed (T-2). Due to the limited headspace of the centrifuge tubes T-1 was taken from inside the tubes with the lid off, after this had been taken the lid was secured so an air-tight seal was formed. A series of preliminary tests showed that $CO_2$ and $CH_4$ concentrations

increased linearly for over 60 minutes. An incubation time of 60 minutes was therefore chosen for the experiments.

Gas samples were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC) instrument, equipped with a flame ionisation detector (FID) with a $CO_2$ to $CH_4$ catalytic converter (methaniser), to measure concentrations of $CO_2$ and $CH_4$. Two millilitres of gas from the Exetainers containing the samples was injected via a 1041 on-column injector system, set at 40$^{o}$C onto a PoroPak QS (1.83m x 3.18mm) 80/100 column. The column oven temperature was set to 40$^{o}$C and the carrier gas, oxygen free nitrogen, had a flow rate of 30mL min$^{-1}$.

The temperature of the methaniser was 300$^{o}$C and the FID was at 125$^{o}$C. The latter had a hydrogen flow of 25mL min$^{-1}$, 20mL min$^{-1}$ make-up gas (oxygen free nitrogen) and 300mL min$^{-1}$ of compressed air. The methaniser had a hydrogen flow of 5mL min$^{-1}$.

| Peat type | Experiment conditions | Experiment temperature range (°C). | Author |
|---|---|---|---|
| TB, SB, F, S | LE, slurries, AC ($N_2$) | 0 - 35 | (Dunfield et al. 1993) |
| TS | LE, AC ($N_2$) | 5 - 35 | (Westermann 1993) |
| BP, TP | LE, slurries, AC ($N_2$) | 2 - 20 | (Yavitt et al. 1997) |
| BP, F | LE, slurries, AC ($N_2$) | 0 - 20 | (Bergman et al. 1998) |
| TP | LE, slurries, AC ($N_2$) | 4 - 30 | (van Hulzen et al. 1999) |
| BP | LE, slurries | 4 - 60 | (Metje and Frenzel 2005) |
| PP, ThW, ThE | FE | 0 - 14 | (Wickland et al. 2006) |
| BF | FE, top open chambers | 0 - 20 | (Turetsky et al. 2008) |
| BB, BF | FE, infrared radiation lamps | 4 - 20 | (White et al. 2008) |

**Table 1. Review of previous experiments investigating the effects of increasing temperatures on methane production in peat, or organic soils.** TB, TS, TP temperate: bog, swamp, peat; SB subarctic bog; F fen, S swamp; BP, BB, BF boreal: peat, bog, fen; PP permafrost plateau; ThE, ThW thermokarst: edges, wetland. LE laboratory experiment, FE field experiment, AC ($N_2$) anoxic conditions in pure $N_2$ atmosphere.

$CH_4$ and $CO_2$, (retention times 1.08 and 1.87 minutes respectively) were quantified by comparison of peak area with that of the three standards of known concentration (Table 2), prepared by Scientific and Technical Gases Ltd (Newcastle under Lyme, Staffordshire, UK), used in the preparation of a standard curve, which was only accepted if the correlation coefficient ($R^2$) value was greater than 0.98.

| | $CO_2$ | $CH_4$ |
|---|---|---|
| **Standard A** | 250 | 2 |
| **Standard B** | 500 | 25 |
| **Standard C** | 1,000 | 50 |

**Table 2. Concentrations (ppm) of the standard gases.** A certificate of analysis (CofA) was requested with each new batch of gases and the calibration curve altered accordingly to take into account the exact amounts.

Calculating the gaseous fluxes from the initial background (T-1) concentrations was achieved by the following equation (Levy et al. 2011):

$$\text{Flux } (\mu g\ g^{-1}\ s^{-1}) = \frac{\delta C}{\delta t} \times \left(\frac{V \times M}{m \times V_{mol}}\right)$$

Where $\delta C$ is rate of change in the gas concentration (T-2 minus T-1); $\delta t$ is the change in time from the background reading to the final measurement (in seconds); $V$ is volume of the headspace of the chamber ($m^3$); $M$ is the molecular weight of the gas (16.04 and 44.01 g $mol^{-1}$ for $CH_4$ and $CO_2$ respectively); $m$ is the mass of the peat-slurry used in grams (total mass of peat and liquid used); $V_{mol}$ is the volume of a mole of gas (air) at a given temperature ($m^3\ mol^{-1}$) calculated by:

$$p \times (R \times K)$$

Where $p$ is pressure (kPa); $R$ is equal to 8.314 (the ideal gas constant) and $K$ is temperature (Kelvin).

6.3.5 Peat-soil analysis

For measuring the effects of the Ca lignosulphonate solution on the various types of peat, 50:50 peat-solution slurries were made and stored in the same way as before (using dark and anaerobic conditions at field or required warming temperature). After 24 hours of incubation a sample of the slurry was removed and placed on absorbent tissue paper for 30 seconds, to remove excess liquid. One gram samples of this were then used for the enzymic or phenolic assays; with a mean sample value for each test, from the respective slurry, being derived from three sub-samples.

Dry weights of the peat slurries were determined by taking the mean dry weight of three 1g sub-samples, prepared in the same way, and dried at 105$^{o}$C to constant weight.

Warmed boreal peat samples were made using frozen peat, which thawed during the process of making the slurry, and were then incubated in dark, anaerobic conditions at +5$^{o}$C for 24 hours before the phenolic and enzyme assays were conducted.

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).

*6.3.5.1 Phenol oxidase*

The activity of extracellular phenol oxidases was determined using 10 mM L-DOPA (dihydroxy phenylalanine) (Sigma-Aldrich Co., Ltd, Dorset) solution as a substrate, based on the procedure of Pind et al. (1994) and Williams et al. (2000).

Homogenates made from the 1g peat-slurry samples added to 9mL of ultra-pure water were prepared in separate Stomacher bags (Seward, West Sussex, UK) and

a laboratory paddle blender (Stomacher circulator, Seward) was set to mix them for 30 seconds at normal speed setting, in order to minimise cell disruption. For each peat-slurry sample two bags were prepared in the same way. Into one of these stomacher bags 10mL of sterile water was added to one of the bags and 10mL of L-DOPA (both of which had been maintained at field temperatures or required warming temperature) to the other. The former, which contained no model substrate were assigned as the blank samples. Blanks were used to determine the background absorbance of the sample at 475nm, necessary for the calculation of phenol oxidase activity in the samples with added substrate. Each bag was homogenized in the paddle blender for a further 30 seconds and then incubated at the relevant temperature for nine minutes (after preliminary tests showed that oxidation of L-DOPA was linear up to this time).

After the allotted time both bags were removed from the incubator and homogenised gently by hand. From each bag three 1.5mL Safe-Lock Eppendorf tubes were filled and immediately centrifuged at 14,000rpm for five minutes, in a temperature controlled centrifuge set to the required temperature. 300μL of each supernatant was pipetted into three wells of a clear 96 well Sterilin Microplate (Sterilin, Cambridge, UK) and a spectrophotometer was used to measure the absorbance of the red pigment (dopachrome) at 475nm. Mean absorbance of the three control wells was then subtracted from that of the three L-DOPA wells for each sample and phenol oxidase activity calculated using Beers Law and the molar absorbency coefficient (3,700) for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate (diqc) (Mason 1948) and expressed as μmol of diqc produced minute$^{-1}$ g$^{-1}$ sample (dry weight).

*6.3.5.2 Hydrolase enzymes*

The activities of five hydrolase enzymes was determined by utilising fluorogenic 4-methylumbelliferyl (MUF) labelled substrates which fluoresce at 450 nm emission, 330nm excitation, and using methods based on work by Freeman et al. (1995) and Kang (1999). Homogenates of 1g of the peat-slurry sample and 7mL of the relevant MUF substrate (see Table 3) were prepared using a laboratory paddle blender.

| **Substrate** | **Enzyme** |
|---|---|
| 4-MUF β-D-glucopyranoside | β-D-glucosidase |
| 4-MUF sulphate potassium salt | Arylsulphatase |
| 4-MUF β-D-xylopyranoside | β-D-xylosidase |
| 4-MUF N-acetyl-β-D-glucosaminide | N-acetyl-β-D-glucosaminidase |
| 4-Methylumbelliferyl phosphate | Phosphatase |

**Table 3. The MUF-labelled substrates needed for measuring the specified enzyme's activity.**

All the samples were incubated at field temperature (measured at the time of sample collection date) or the experimental-warming temperature, for 45 minutes for phosphatase and 60 minutes for N-acetyl-β-D-glucosaminidase, β-D-xylosidase, Arylsulphatase, β-D-glucosidase. Two minutes before the incubation time came to an end the contents of each stomacher bag was thoroughly mixed by hand and the solution from each substrate bag used to fill one 1.5mL Safe-Lock Eppendorf tube, which were then centrifuged at 14,000rpm for five minutes in a temperature controlled centrifuge, set to the required temperature. From the resulting supernatant

of each substrate tube 250µL was pipetted into a well of a black flat-bottomed 96 well microplate; after ensuring each well already contained 50 µL of ultra-pure water - guaranteeing a comparable dilution to the standard solutions in the calibration curve. A fluorometer was used to determine the fluorescence of the MUF at 450 and 330nm excitation.

Standards for the calibration curve were prepared in a similar way to the substrate samples; with 1g of the peat-slurry sample being added to 7mL of ultra-pure water and homogenised in the laboratory paddle blender for 30 seconds. From this 1.5 mL was centrifuged at 14,000rpm for five minutes before 250µL of the resulting supernatant was transferred from each vial to six separate wells on a black flat-bottomed 96 well microplate. Fifty microlitres of varying concentrations of a MUF-free acid stock solution were added to the supernatant to create a concentration curve of 0-100µM, from which the enzyme activity was determined.

Enzyme activities were expressed as MUF produced (µmol $g^{-1}$ $min^{-1}$), correcting for the dry weight of the peat.

*6.3.5.3 Phenolic concentrations*

The concentration of water extractable phenolics from the peat-slurries samples were assayed using a modified version of the spectrophotometric method developed by Box (1983). To conduct the assays, 1g samples of the peat-slurries were added to 9mL of ultra-pure water, homogenised using a laboratory paddle blender for 30 seconds and then incubated for a further 60 minutes. The solution was thoroughly mixed again, centrifuged at 10,000rpm and the resulting supernatant used for the analysis of phenolic concentration.

Within the 300µL wells of a flat-bottomed 96 well microplate, replicates of 250µL of the supernatant sample, 37.5µL of $Na_2CO_3$ solution (200 mg $L^{-1}$) and 12.5µL of Folin-Ciocalteau phenol reagent were dispensed and mixed thoroughly. A standard curve was prepared by adding the same chemicals to 0 to10 mg $L^{-1}$ phenol solutions. After two hours of incubation at room temperature, the colour change of the reactants was measured at 750nm absorption and the concentration of dissolved phenolics of each sample derived from the absorbance curve of the phenol standards (mg $L^{-1}$). Samples out of range of the standard curve were re-measured using a standard curve with a higher upper concentration or by diluting the supernatant sample (eg. 1:10).

6.3.6 Statistical considerations

Significant difference between the various results were determined by independent *t*-tests and repeated measures analysis of variance (ANOVA) test, using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA). To ensure the reliability of the *t*-statistic and *F*-ratio it was checked that the usual assumptions of parametric tests were not violated. The experimental design ensured that data was in a continuous, interval ratio format and measurements from the different treatment groups – for the experiments requiring analysis with *t*-tests - were independent. The normality of data distribution was checked by plotting the data graphically, looking at the values of skewness and kurtosis, and with the Sharpio-Wilk test, with a significant value less than 0.05 indicating a deviation from normality. The assumption of homogeneity of variance was checked using the Levene's test, with a significant value greater than 0.05 indicating the variances were significantly equal, and therefore the assumption was tenable. If either the assumption

of normality or homogeneity of variance was violated data was transformed (eg. log10).

For the repeated measures ANOVA the assumption that the $\partial^{13}C\text{-}CO_2/CH_4$ effluxes from the same slurries over the duration of the experiment are independent was obviously violated. The assumption of sphericity was therefore tested for, to confirm the level of dependence between experimental conditions is relatively equal, using the Mauchly's test – which tests the hypothesis that the variances of the differences between conditions are equal. A probability value of less than 0.05 of the Mauchly's test statistic therefore indicated there were significant differences between the variances of differences and, therefore, the condition of sphericity was not met. When this occurred the Greenhouse-Geisser correction was applied to the *F*-ratio.

The *t*-statistic was reported along with the degrees of freedom and the significance value (*p*) with a value of less than 0.05 taken to be a statistically significant result, as this represented a 95% level of confidence. When the hypothesis being tested was directional, a one-tailed *t*-test was used to give the significance value, rather than the normal two-tailed test. Effect sizes were calculated by converting the *t*-statistic into an *r*-value by:

$$r = \sqrt{\frac{t^2}{t^2 + df}}$$

where *t* is the given *t*-value and *df* is the degrees of freedom used in the *t*-test.

The repeated measures ANOVA tests used the different measurements taken at each time point as the within-subject variables, and the treatment groups of the slurries as the between subject factor. The *F*-ratio was reported along with the degrees of freedom used to assess it, namely the degrees of freedom for the effect of the model and the degrees of freedom for the residuals of the model. To compare the various experimental treatments against each other *post hoc* tests were conducted with

the Bonferroni correction for the *t*-statistic being used due to the sphericity-related complications of repeated measure ANOVAs. A significance value of less than 0.05 was again considered to be a statistically significant result.

For displaying on the graphs standard error (*SE*), or standard deviation of the mean, was calculated by:

$$SE = \frac{\sigma}{\sqrt{n}}$$

where σ is the standard deviation of the parent population (derived by the square root of the group's variance from the mean) and *n* is the number of observations, or samples.

Percentage differences between the $^{13}C$ of peat-soil $CO_2$ and $CH_4$ effluxes from the slurries were calculated from the total areas under the curve produced by the relevant graphs in GraphPad Prism 6 (GraphPad Software Inc).

## 6.4 Results

The addition of a phenolic compound (5% weight/volume Ca lignosulphonate solution) to temperate peat-slurries, when the peat used was collected in the winter (Figure 1A), significantly reduced the $\partial^{13}C$ of $CO_2$ efflux compared to those slurries with no phenolic supplements ($F$ (2.13, 21.33) = 24.067, $p < 0.001$). However, the supplementary phenolics had no effect on the $\partial^{13}C$ of $CH_4$ efflux: $F$ (1.89, 60) = 1.026, $p < 0.417$), with both control and supplemented samples showing a steady increase after 72 hours. Before the $^{13}C$ labelled litter was added to the slurries there was no significant difference ($t$ (6) = 2. 384, p = 0.54) between control and phenolic supplemented slurries' $\partial^{13}C$ of $CO_2$ efflux; with the mean average of the former being -10.75 $\partial^{13}C$-$CO_2$ ($SE$ = 2.26). There was also no significant difference ($t$ (5.24) = -1.437, $p$ = 0.208) for the $\partial^{13}C$ of $CH_4$ efflux ($M$ = -43.16, $SE$ = 10.70).

The 5% w/v Ca lignosulphonate solution retarded all observed enzymic activity in the peat (Table 4), with the exception of β-D-xylosidase, which showed a non-statistically significant ($t$ (4) = -2.401, $p$ = 0.074) 35% increase (at a 95% confidence level). Sulphatase was affected the most by the presence of the addition phenolics with treated slurries having 97% less activity than controls ($t$ (4) = 15.989 $p$ <0.001). β-D-glucosidase activity was similarly curtailed by 79% ($t$ (4) = 10.072, $p <$ 0.001); phosphatase by 53% ($t$ (4) = 4.006, $p$ = 0.008) and N-acetyl- β -D-glucosaminidase by 12%, although this was not considered significant according to a independent one-tailed t-test: $t$ (4) = 0.514, $p$ = 0.317. The activity of phenol oxidases was suppressed by 73%, compared to the control slurries ($t$ (4) = 10.964, $p < 0.001$) and the 5% w/v Ca lignosulphonate solution increased phenolic compounds present in

the slurries by 8,717% (Control, $M$ = 4.767, $SE$ = 0.784; 5% solution, $M$ = 420.3, $SE$ = 14.199).

When peat from the temperate location was collected in the summer and made into 50:50 (soil:solution) slurries (Figure 2A) the Ca lignosulphonate had the same effect as before in suppressing the $\partial^{13}C\text{-}CO_2$ efflux. However, this time the $^{13}C$ labelled litter was added at the start of the experiment and all the slurries were made with sterile water, then after 24 hours the treatments were added. These results also indicate that lowering the pH of slurries (with HCl), so they were the same as those slurries with the supplementary phenolics (Table 5; pH $M$ = 3. 0, $SE$ = 0.014), did not reduce the $\partial^{13}C\text{-}CO_2$ efflux to the same degree. Also increasing the pH (with CaOH) of a set of slurries with Ca lignosulphonate already added to them (so they were similar to control slurries; pH $M$ = 3.3, $SE$ = 0.026), had the same effect as those slurries with just the supplementary phenolics. A repeated measures ANOVA showed that there was statistically significant differences between the treatments shown in Figure 2; $F$ (7.61, 80) = 15.987, $p < 0.001$. Furthermore, *post hoc* tests indicated that all the treatments were significantly different from each other ($p < 0.01$) except the phenolic supplementary group and those with the additional phenolics and the CaOH ($p = 0.993$).

Unlike the temperate peat collected in the winter, the additional phenolics did reduce the $\partial^{13}C\text{-}CH_4$ efflux from the summer peat-slurries, along with the phenolic slurries with adjusted pH (Figure 2B). Again, simply lowering the pH with HCl did not have the same inhibitory effects as the increasing the phenolic concentration of the slurries. A repeated measures ANOVA showed there were significant differences between the treatments ($F$ (6.95, 80) = 15.452, $p < 0.001$) though *post hoc* tests indicated these differences were not between the slurries made with Ca

lignosulphonate and Ca lignosulphonate plus CaOH ($p$ = 0.513), or the control slurries and those with HCl in them ($p$ = 0.991): both pairs giving similar $\partial^{13}$C-$CH_4$ efflux measurements to each other.

The pH of the slurries taken at the start of the experiment were all lower than the measurements taken at the end of the experimental time period (120 hours). All the pHs increased by between 22.3 (Ca lignosulphonate solution slurries) and 29.4% (control slurries).

When the peat microcosms were warmed from -18°C to +10°C the $CO_2$ fluxes increased with every temperature increase (Figure 3). The fluxes of those microcosms kept frozen (-18°C) remained similar throughout the time period of the experiment – with site A's final flux being 0.4 µg $CO_2$ $g^{-1}$ $h^{-1}$ (*SE,*0.3); B's 0.22 (*SE*, 0.13), and C's 0.46 (*SE*, 0.16). These final -18°C fluxes were also significantly lower than those of the relevant warmed microcosms, at +10°C, with the flux of site A being 4.44 µg $CO_2$ $g^{-1}$ $h^{-1}$ (*SE*, 0.53; $t$ (33) = 17.074, $p < 0.001$); B, 2.07 (*SE*, 0.21; $t$ (26.39) = 8.338l, $p < 0.001$) and C being 2.10 (*SE,* 0.14; $t$ (26.39) = 8.338, $p < 0.001$).

From Figure 4 it can be seen that the $\partial^{13}$C-$CO_2$ efflux (‰) from thawed boreal peat slurries, when $^{13}$C labelled litter was added, is curtailed by the presence of additional phenolics. For all three sites there was no significant difference, before the litter was added, in the $\partial^{13}$C-$CO_2$ efflux between the control slurries and those made with a 5% w/v Ca lignosulphonate solution: The controls from the nutrient rich fen (mean average) were -19.88 ‰ (*SE*, 2.51) while the phenolic supplemented ones were -22.62 (*SE*, 0.2); the thermokarst bog's controls were -23.22 (*SE*, 0.28) and the phenolic slurries, -23.34 (*SE*, 0.47), and the control slurries from the black spruce site

were -22.82 (*SE*, 0.30) and their phenolics slurries were -22.10 (*SE*, 0.53). Figure 4 and statistical analysis, using repeated measures ANOVA, indicated there were large differences between the phenolic supplemented slurries and controls (Site A, $F$ (1.45, 24) = 9.335, $p$ = 0.01; Site B, $F$ (1.01, 24) = 24.230, $p$ = 0.003, and Site C, $F$ (1.91, 24) = 7.175, $p$ = 0.01), with the latter having much lower $\partial^{13}C$-$CO_2$ effluxes – indeed, the final effluxes (at 120 hours) for these treated slurries showed no significant difference from the initial (0 hour) effluxes. By comparison, the control slurries' final efflux, for all sites, showed a large increase: Site A, 196.69 (*SE*, 15.06); Site B, 577.08 (*SE*, 114.11) and Site C, 235.28 (*SE*, 12.02).

The $\partial^{13}C$ of peat slurry $CH_4$ efflux however, did not show any significant difference between the control and phenolic supplemented slurries, irrespective of site (A, $F$ (2.06, 30) = 1.274, $p$ = 0.315; B, $F$ (1.53, 30) = 0.962, $p$ = 0.393; C, $F$ (1.25, 30) = 4.446, $p$ = 0.065). It was also noteworthy that all the final $\partial^{13}C$-$CH_4$ effluxes remained similar to the initial measurements, before the $^{13}C$ labelled litter was added, with independent t-tests showing only the control slurries from Site C had statistically different effluxes over the time period ($t$ (6) = -3.431, $p$ = 0.014). For all the other samples there were no significant differences between the measurements taken at 0 hours and those at 120 hours: Site A control slurries, $t$ (3) = 1.257 $p$ = 0.298, lignosulphonate slurries, $t$ (3) = -1.244, $p$ = 0,302; Site B control, $t$ (6) = -1.386, $p$ = 0.215, lignosulphonate $t$ (6) = -1.922, $p$ = 0.103; Site C, lignosulphonate $t$ (6) = -1.408, $p$ = 0.209.

As can be seen from Table 6, the activity of all the hydrolase and phenol oxidase enzymes measured were dramatically retarded by the presence of the lignosulphonate solution. With some of them, including β -D-xylosidase, being so low

they were below detectable limits on the instrument. All the observed reduction in activity were statistically significant ($p \leq 0.001$) with large effect sizes ($r > 0.85$). As expected, the addition of 5% w/v Ca lignosulphonate solution increased the phenolic compound concentration in the slurries by several thousand percent (as much as 6,600% for Site B), compared to control slurries with no supplementary lignosulphonate.

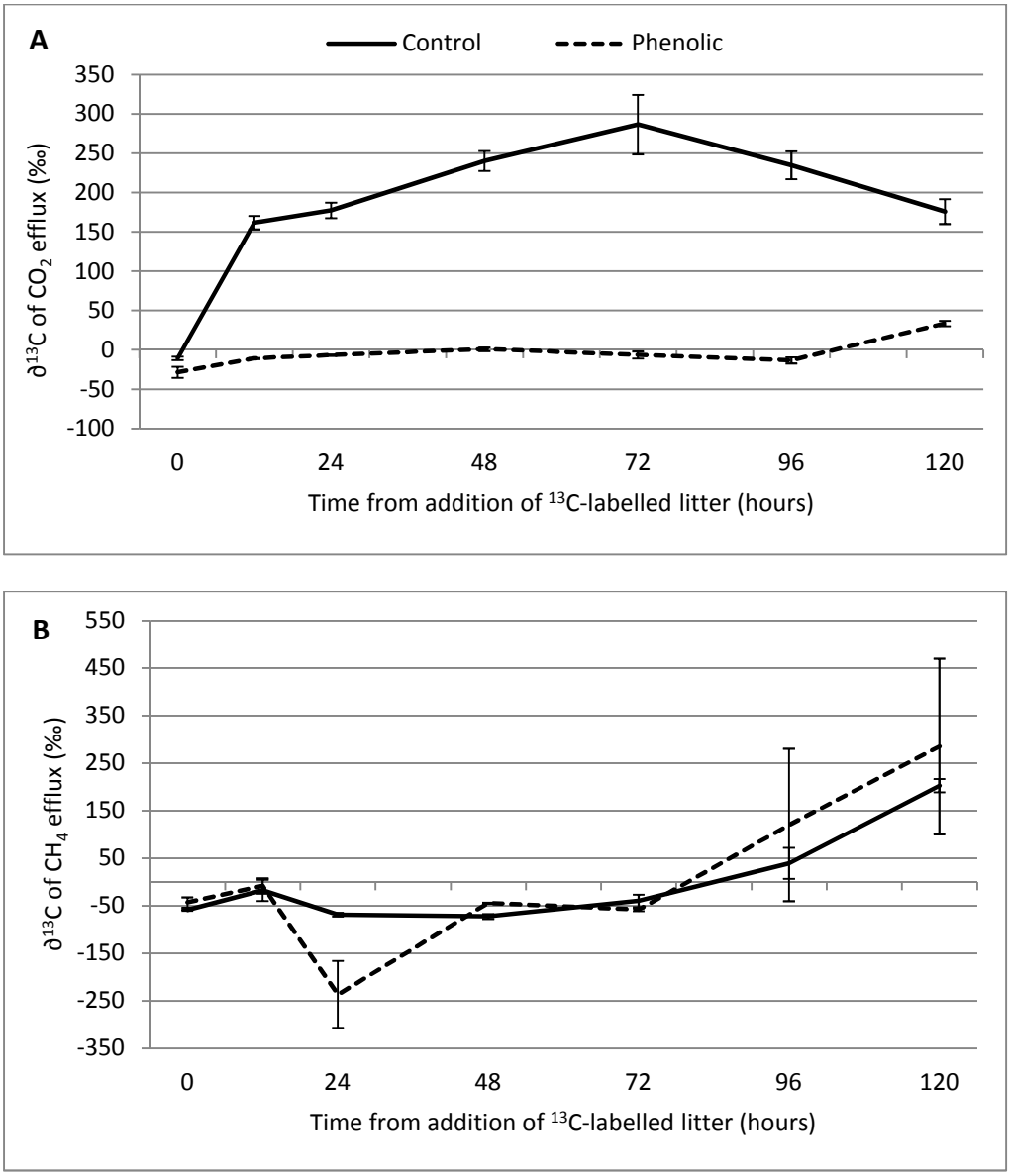



**Figure 1. Effect of phenolic supplements on litter decomposition in winter-collected bog-peat slurries; $^{13}$C labelled litter after 24 hours.** The $\partial^{13}$C of peat-soil $CO_2$ efflux (‰) (**A**) and $\partial^{13}$C of peat-soil $CH_4$ efflux (‰) (**B**) from 50:50 peat slurries made with $^{13}$C-labelled organic matter, to give relative decomposition rates. Phenolic samples were made with a 5% w/v lignin solution; controls were made with sterile pure water. Peat used in the slurries was collected in the winter (November). Slurries were incubated for the duration of the experiment under dark anaerobic conditions at 10$^{o}$C. Mean averages are shown ($n$ =6) and error bars indicate ± standard error.

| | Control | Phenolic | Difference |
|---|---|---|---|
| β-D-glucosidase | **4.018** (0.168) | **0.858** (0.265) | ↓ **78.65%** $t$ (4) = 10.072, $p$ < 0.001 $r$ = 0.98 |
| Sulphatase | **3.429** (0.180) | **0.106** (0.106) | ↓ **96.91%** $t$ (4) = 15.989, $p$ < 0.001 $r$ = 0.99 |
| β -D-xylosidase | **3.701** (0.169) | **4.995** (0.511) | ↑ **34.96%** $t$ (4) = -2.401, $p$ = 0.074 $r$ = 0.77 |
| N-acetyl- β -D-glucosaminidase | **3.604** (0.775) | **3.188** (0.234) | ↓ **11.54%** $t$ (4) = 0.514, $p$ = 0.317 $r$ = 0.25 |
| Phosphatase | **6.557** (0.796) | **3.100** (0.334) | ↓ **52.72%** $t$ (4) = 4.006, $p$ = 0.008 $r$ = 0.89 |
| Phenol Oxidase | **0.473** (0.026) | **0.130** (0.018) | ↓ **72.52%** $t$ (4) = 10.964, $p$ < 0.001 $r$ = 0.98 |
| Phenolics | **4.767** (0.784) | **420.3** (14.200) | ↑ **8,716.87%** $t$ (4) = -29.22, p < 0.001 $r$ = 0.99 |

**Table 4. Effect of a phenolic supplement on the enzyme activity of bog-peat slurries.** Phenol oxidase activity (µmol 2-carboxy-2, 3-dihydroindole-5,6-quinone formation $min^{-1}$ $g^{-1}$ peat), hydrolase activities (µmol methylumbelliferone formation $min^{-1}$ $g^{-1}$ peat) and phenolic compound concentrations (mg $L^{-1}$ ) are reported as mean averages (numbers in bold text) along with ± standard error (numbers in parentheses). Slurries were incubated for 24 hours under dark anaerobic conditions at $10^{o}$C.

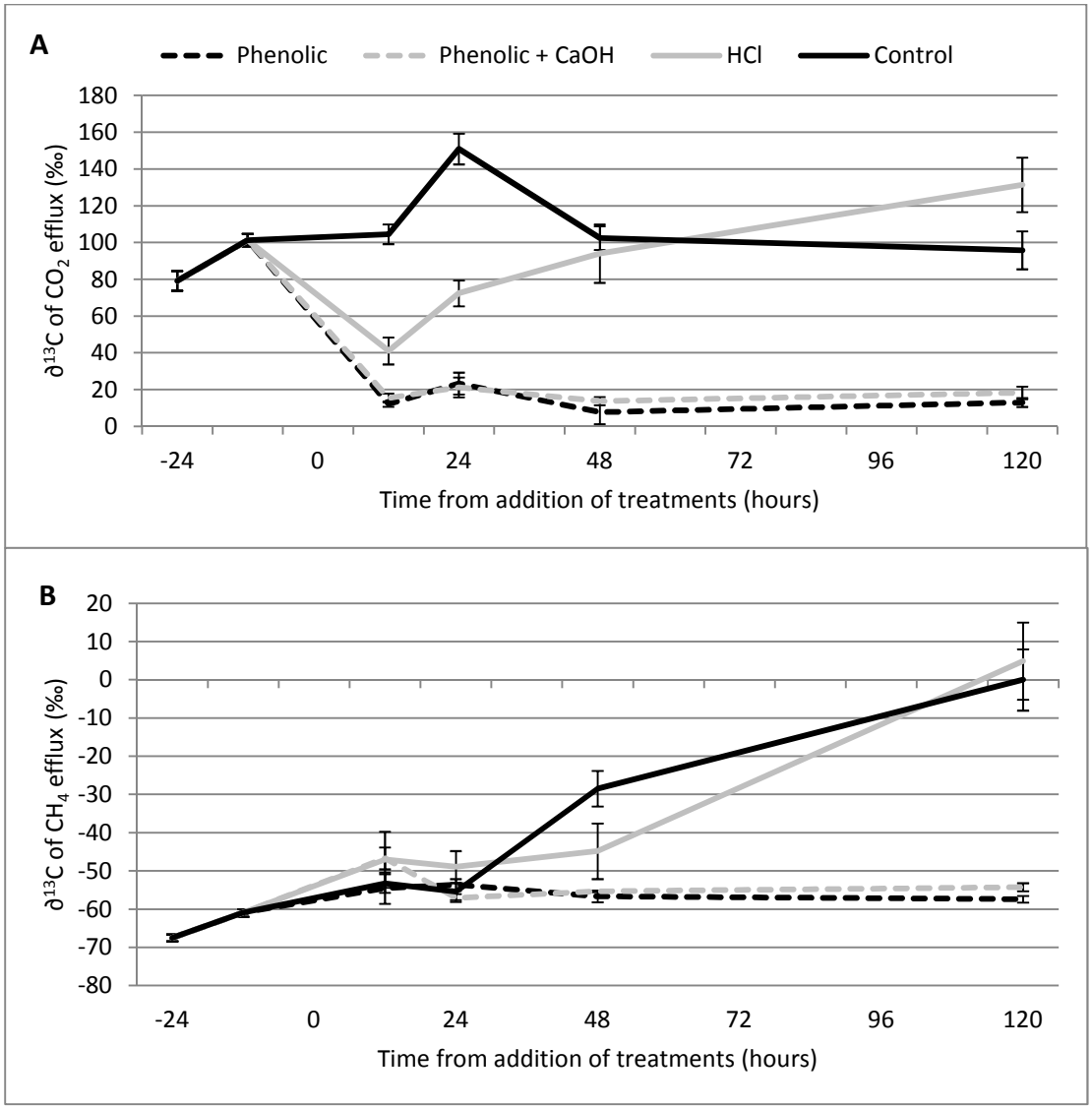



**Figure 2. Effect of phenolic supplements on litter decomposition in summer-collected bog-peat slurries; treatment added after 24 hours.** The $\partial^{13}$C of peat-soil $CO_2$ efflux (‰) (**A**) and $\partial^{13}$C of peat-soil $CH_4$ efflux (‰) (**B**) from 50:50 peat slurries made with $^{13}$C-labelled organic matter, to give relative decomposition rates. Phenolic samples were made with a 5% w/v lignin solution (Ca lignosulphonate); controls were made with sterile pure water. Peat used in the slurries was collected in the summer (July). The $^{13}$C-labelled litter was added after the slurries had been made for 24 hours. Slurries were incubated for the duration of the experiment under dark anaerobic conditions at 10$^{o}$C. Mean averages are shown ($n$ =6) and error bars indicate ± standard error.

| | Initial pH | Final pH | Percentage change |
|---|---|---|---|
| Control | **3.285** (0.026) | **4.250** (0.111) | ↑ 29.4% |
| HCl added | **2.968** (0.016) | **3.75** (0.0214) | ↑ 26.4% |
| Phenolic supplement | **2.998** (0.014) | **3.667** (0.105) | ↑ 22.3% |
| Phenolic supplement with CaOH | **3.435** (0.03) | **4.333** (0.105) | ↑ 26.1% |

**Table 5. pH of experimental peat-slurries.** Concentrated hydrochloric acid was used to adjust relevant slurries to a similar pH of those with a phenolic supplement (Ca lignosulphonate). Calcium hydroxide was added along with the phenolic supplement to increase the pH of slurries to the level of the controls. Mean average pH values are given ($n$ = 6) in bold. Percentage changes were calculated by converting pH values to $H^+$ concentrations. Numbers in parentheses indicate ± standard error.

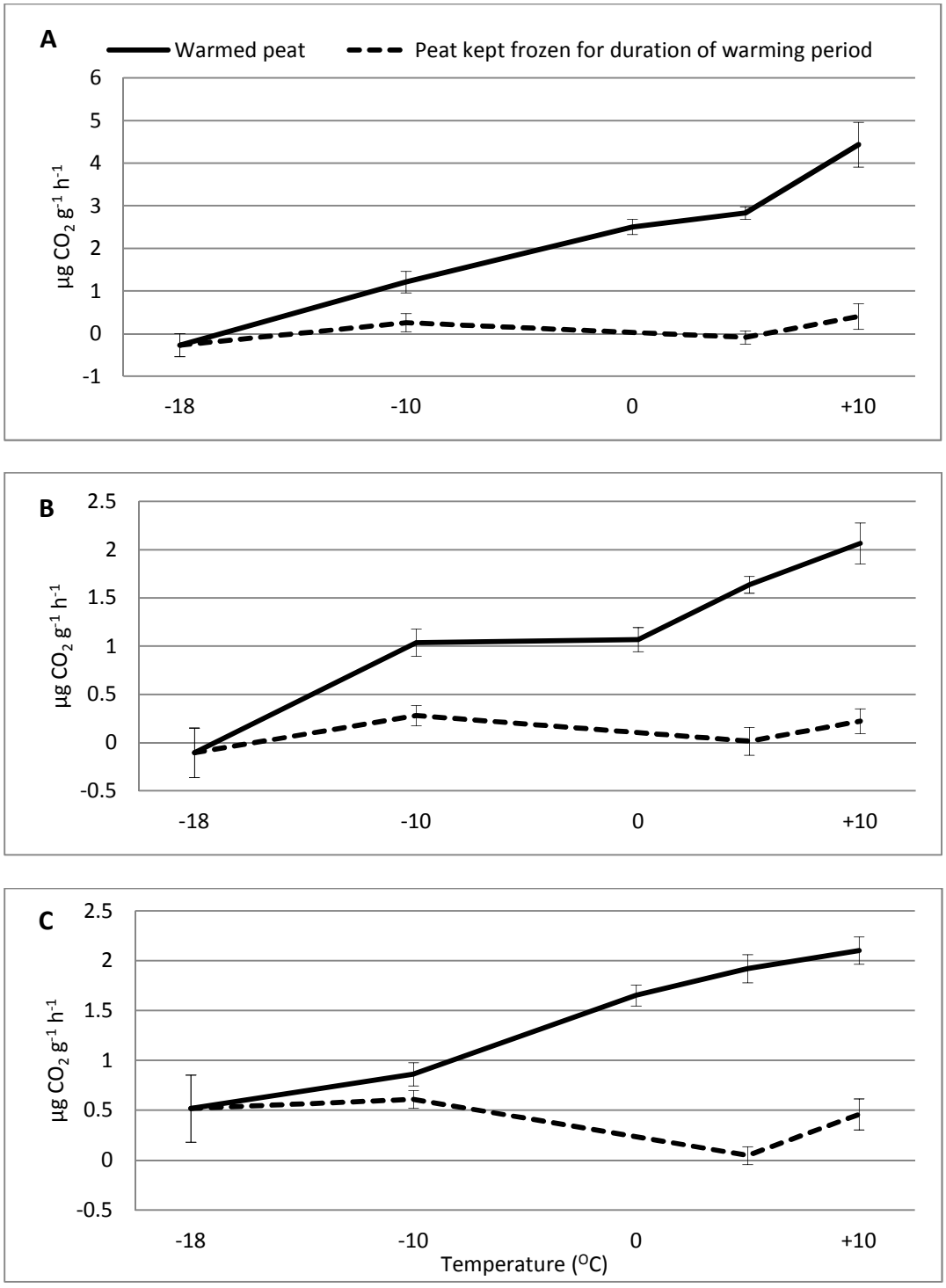



**Figure 3. $CO_2$ flux from thawed Arctic peat.** Samples were gradually warmed from -18 to +10°C. Control samples were kept at -18°C for the duration of the experiment. Peat used: **A**, nutrient rich fen; **B**, nutrient poor thermokarst bog where the permafrost thawed 20-years ago, or **C** a low productivity black spruce site. Mean averages are shown ($n$ =18) and error bars indicate ± standard error.

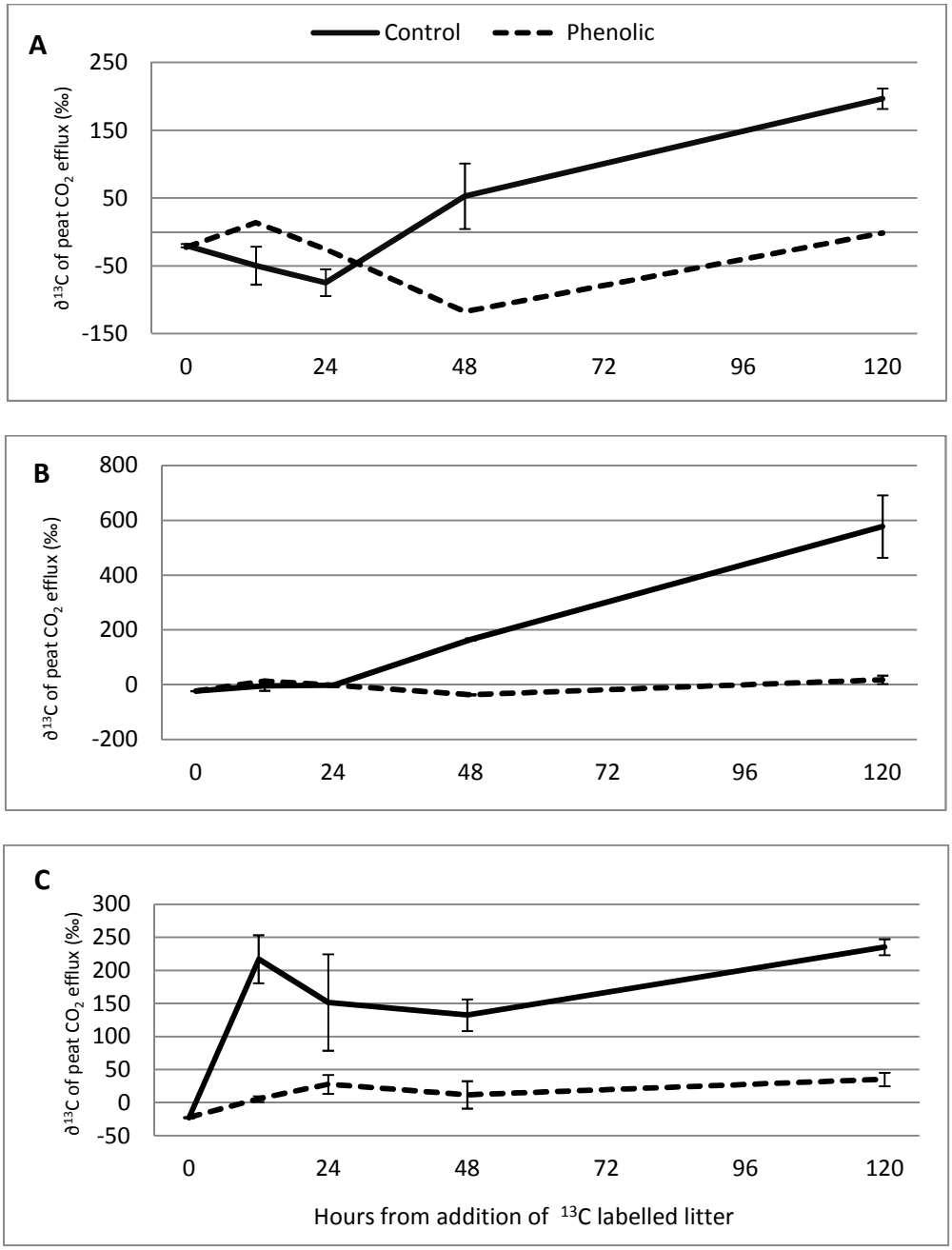



**Figure 4. Effect of a phenolic supplement on litter decomposition in Arctic peat slurries at +5°C; $CO_2$ efflux.** The $\partial^{13}C$ of peat-soil $CO_2$ efflux (‰) from 50:50 peat slurries made with $^{13}C$-labelled organic matter, to give relative decomposition rates. Phenolic samples were made with a 5% w/v lignin solution; controls were made with sterile pure water. Peat used: **A**, nutrient rich fen; **B**, nutrient poor thermokarst bog where the permafrost thawed 20-years ago, or **C** a low productivity black spruce site. Mean averages are shown ($n$ =4) and error bars indicate ± standard error.

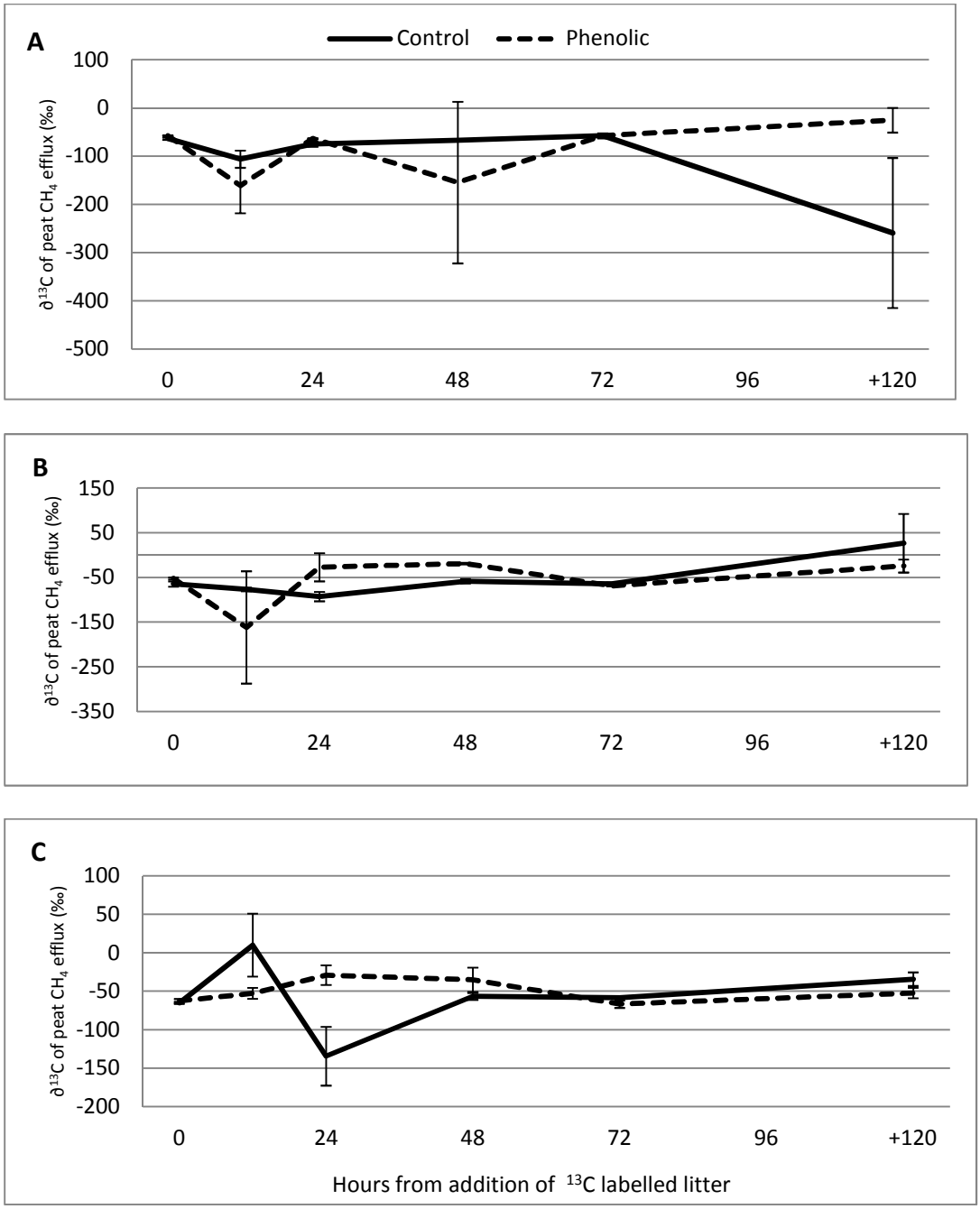



**Figure 5. Effect of a phenolic supplement on litter decomposition in Arctic peat slurries at +5°C; $CH_4$ efflux.** The $\partial^{13}C$ of peat-soil $CH_4$ efflux (‰) from 50:50 peat slurries made with $^{13}C$-labelled organic matter, to give relative decomposition rates. Phenolic samples were made with a 5% w/v lignin solution; controls were made with sterile pure water. Peat used: **A**, nutrient rich fen; **B**, nutrient poor thermokarst bog where the permafrost thawed 20-years ago, or **C** a low productivity black spruce site. Mean averages are shown ($n$ =4) and error bars indicate ± standard error.

| | Site | Control | Phenolic | Difference |
|---|---|---|---|---|
| β-D-glucosidase | A | **11.750** (0.585) | **0** | ↓ **100%** |
| | B | **0.651** (0.017) | **0** | ↓**100%** |
| | C | **0.718** (0.002) | **0.071** (0.02) | ↓ **90.11%** $t$ (8) = 16.48, $p$ < 0.001 $r$ = 0.99 |
| β -D-xylosidase | A | **2.676** (0.265) | **0** | ↓ **100%** |
| | B | **0.427** (0.026) | **0** | ↓ **100%** |
| | C | **0.570** (0.016) | **0** | ↓ **100%** |
| N-acetyl- β -D-glucosaminidase | A | **5.281** (0.044) | **0.532** (0.471) | ↓ **89.9%** $t$ (8) = 12.52, $p$ < 0.001 $r$ = 0.98 |
| | B | **0.624** ±0.005 | **0** | ↓ **100%** |
| | C | **0.543** ±0.002 | **0.257** ±0.024 | ↓ **52.67%** $t$ (8) = 5.56, $p$ = 0.001 $r$ = 0.89 |
| Phenol Oxidase | A | **0.065** (0.005) | **0.025** (0.004) | ↓ **61.26%** $t$ (6) = 6.131, $p$ < 0.001; $r$ = 0.93 |
| | B | **0.047** (0.009) | **0.013** (0.003) | ↓ **72.34%** $t$ (4) = 3.32, $p$ = 0.029; $r$ = 0.86 |
| | C | **0.038** (0.002) | **0.016** (0.006) | ↓ **57.9%** $t$ (4) = 9.49, $p$ = 0.001; $r$ = 0.96 |
| Phenolics | A | **51.563** (10.688) | **1547.13** (21.785) | ↑ **2,900.47%** $t$ (14)= -61.633, $p$ < 0.001 $r$ > 0.99 |
| | B | **25.06** (6.927) | **1,680.69** (18.76) | ↑ **6,606.66%** $t$ (14)= -82.786, $p$ < 0.001 $r$ > 0.99 |
| | C | **30.81** (11.05) | **1,709.31** (18.91) | ↑ **5,447.9%** $t$ (14)= -76.64, $p$ < 0.001 $r$ > 0.99 |

**Table 6. Effect of a phenolic supplement on the enzyme activity of Arctic peat slurries at +5°C.** Phenol oxidase activity (µmol 2-carboxy-2, 3-dihydroindole-5,6-quinone formation $min^{-1}$ $g^{-1}$ peat), hydrolase activities (µmol methylumbelliferone formation $min^{-1}$ $g^{-1}$ peat) and phenolic compound concentrations (mg $L^{-1}$ ) are reported as mean averages along with ± standard error (in parentheses). Peat taken from sites: **A**, nutrient rich fen; **B**, nutrient poor thermokarst bog where the permafrost thawed 20-years ago, or **C** a low productivity black spruce site.

## 6.5 Discussion

Heterotrophic decomposer microbes in peat metabolise organic matter (OM) release, as by-products, $CO_2$ and $CH_4$. Measuring the rate by which these gases are released from a sample of peat soil can therefore give an indication of microbial activity in terms of their OM breakdown rates: decomposition. The addition of our $^{13}$C-labelled litter to the experimental peat slurries allowed mineralisation of the carbon compounds within it to be measured separately from the decomposition of the existing peat litter and even any breakdown of the Ca lignosulphonate, due to the analysis of $^{13}C\text{-}CO_2$ and $^{13}C\text{-}CH_4$ effluxes. The use of labelled litter is therefore a targeted respirometric technique for measuring microbial respiration as an indicator of peat litter decomposition.

Our initial results showed that the addition of lignin dramatically reduced the breakdown of the labelled litter to $CO_2$ by 99.6% during the 120 hour time period (Figure 1A). This can be seen by the rapid increase in the $\partial^{13}C\text{-}CO_2$ from the control slurries as the $^{13}$C-labelled litter is broken down and eventually released by the microbes as $CO_2$. Conversely the slurries containing the supplementary lignin did not show a significant rise in $\partial^{13}C\text{-}CO_2$ suggesting the labelled litter is not being degraded. This can be attributed, as we hypothesised, to the suppression of hydrolase enzymes in the peat soil by the additional phenolics in the form of the lignin (Table 4). This inhibitory characteristic of phenolic compounds in peatlands has been well documented (eg. Freeman et al. 2001a, Freeman et al. 2001b). 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). The polyphenols can also 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). Suppressing degradation in this way therefore impedes microbial respiration and the resulting release of trace gases. Our results showed that the addition of lignin to peat slurries retarded the activity of all but one of the hydrolase enzymes we analysed, especially sulphatase and β-D-glucosidase, the latter of which is involved in the turnover of labile carbon (Deng and Popova 2011) and so is critical in regulating $CO_2$ efflux from peat soils. Only the activities of β-D-xylosidase were not suppressed, compared to a set of control slurries, although the increase seen was not statistically significant. Activities were also only assayed after 24 hours. Longer time periods (ie. 120 hours) should be measured to determine whether there is any lag in the inhibitory effect of the phenolics for extracellular enzymes involved in the breakdown of more recalcitrant forms of carbon.

Our results also showed phenol oxidase activities were severely curtailed by the presence of lignin. Phenol oxidases are oxidative copper-containing enzymes that catalyse the release of reactive oxygen radicals, which in turn, promote a variety of non-enzymatic reactions to oxidise phenolic compounds (Thurston 1994, Claus 2004). They instigate the oxidation of both complex polyphenols, such as lignin, and simple phenolic compounds, with outcomes ranging from partial oxidation and the release of oxidative intermediates to the complete degradation of phenolic compounds to non-phenolic end products (Freeman et al. 2012). By increasing the levels of phenolics in the peat-soil a rise in phenol oxidase activity may therefore have been expected in our slurries, but the opposite was observed. However, this does concur with findings elsewhere showing that certain levels of phenolics released by lignified organic matter, commonly known as 'extractives', can inactivate phenol oxidase (Scalbert 1991). These extractives inhibit phenol oxidase-catalysed degradation of phenolics by

binding essential metals, radical scavenging and antioxidant properties (Scalbert 1991, Heinonen 2007).

The second set of experiments showed that mineralisation of the labelled OM to $CO_2$ was occurring in all slurries before the addition of the lignin after 24 hours (Figure 2A). However, the supplementary phenolics effectively 'switched off' this decomposition within 12 hours. Crucially our results demonstrate that pH is not the principle controller of the enzymic latch, as although the addition of Ca lignosulphonate does lower the pH of the peat slurries (Table 5) when a set of slurries not containing lignin were adjusted with HCl to match the pH of the lignin slurries the production of $CO_2$ was reduced over the initial 24 hours, but then returned to the same level as the control. Furthermore, raising the pH of slurries containing the lignin, with CaOH, had the same effect on $CO_2$ production as those slurries made just with lignin. This strongly suggests that although pH is considered a key regulator of decomposition and the carbon cycle (eg. Dunfield et al. 1993, Bergman et al. 1998) it is not as important for the breakdown of OM as levels of phenolics in peatlands. Lowering the pH of peat may have a short term effect on rates of decomposition but it appears to be the presence of phenolic compounds which ultimately controls long term decomposition in peatlands.

The ability of supplementary phenolics to suppress OM breakdown so dramatically suggests phenolic inhibition in peatland soils currently falls substantially below its full potential, and that substantial increases in storage capacity could be achieved through phenolic supplementation. Moreover, it is not just peat from a temperate peatland which is affected by a strengthening of the enzymic latch. We showed that $CO_2$ production from samples taken from boreal peatlands were also severely curtailed by the addition of lignin (Figure 4A-C) which again can be

attributed to the inhibitory effects of phenolics on the activities of hydrolases and enzyme activities (Table 6). This finding has huge global importance due to the increased amounts of greenhouse gases expected to be released from high latitude peatlands as the region's temperature continues to rise (Meehl et al. 2007). Indeed our own results on the issue of thawing boreal peat (Figure 3A-C) clearly demonstrate the steady rise of $CO_2$ from boreal peat samples undergoing warming from -18$^{o}$C to + 10$^{o}$C.

Interestingly the supplementary phenolics did not appear to negatively affect the efflux of $CH_4$ from the boreal peat samples (Figure 5A-C). However, it is possible that $CH_4$ fluxes from thawing sub-Arctic soils (eg. Dunfield et al. 1993, Bergman et al. 1998, Metje and Frenzel 2005) have been overestimated (Dunn et al. in preparation - see Appendix 1). The finding that there was also no effect on the $CH_4$ fluxes from the temperate peat samples which were collected in the winter (Figure 1B) but there were in the summer samples (Figure 2B) is likely to be as a result of the methanogenesis being correlated, to varying degrees, with temperature (Williams and Crawford 1984, Moore and Dalva 1993, van Winden et al. 2012).

Unlike the production of $CO_2$ from the breakdown of the labelled litter, which was initially high and remained so for 120 hours in the control slurries (Figure 2A), levels of $\partial^{13}$C-$CH_4$ began modestly and then continued to rise. Therefore, there was no immediate decline in the $\partial^{13}$C-$CH_4$ results, as seen in the $\partial^{13}$C-$CO_2$, when the lignin was added. Instead a clear prevention of any increase in the efflux was observed (Figure 2B).

It has been estimated that northern peatlands currently capture 1.7x10$^{15}$g carbon yr$^{-1}$ through photosynthetic litter production (Reader and Stewart 1972).

However, at present they only sequester $1x10^{14}$g carbon $yr^{-1}$ or 9%, of that in the long-term. This inefficiency arises because substantially less carbon enters long-term storage, 34 to 52g carbon $m^{-2}$ $yr^{-1}$, than is initially captured from net primary production (NPP), which can reach 489g carbon $m^{-2}$ $yr^{-1}$ (Reader and Stewart 1972, Wieder 2001). If $CO_2$ production is taken as a proxy for OM decomposition rates the addition of our lignin treatment has the potential to suppress the breakdown of OM (litter) entering a temperate peatland system by as much as 99.6% (Figure 1A). This equates to 487g carbon $m^{-2}$ $yr^{-1}$, or $1.69x10^{15}$g carbon $yr^{-1}$, equal to all the carbon from global transport emissions (Barker et al. 2007).

To quantify the effect a strengthening of the enzymic latch could have on boreal peatlands; previous studies have shown a thermally-induced reanimation of microbial decomposition in circumarctic-peatlands could destabilise organic carbon (Schuur et al. 2008) which has been cryopreserved for up to 16,500 thousand years (MacDonald et al. 2006b). This could return as much as $1x10^{14}$g of carbon per year to the atmosphere as heterotrophically respired $CO_2$ (Dorrepaal et al. 2009). Again, we have demonstrated that our method to strengthen the enzymic latch has the potential to prevent this release by as much as 94%, depending on the type of boreal peatland (Figure 4); thereby reducing the estimated release of $1x10^{14}$g of carbon per year to just $6x10^{12}$g.

Our results present us with two significant conclusions. The first, as we hypothesised, is that both boreal and temperate peatland carbon sequestration is severely undermined by lower-than-optimal litter phenolic content. The widespread assumption that the long term capacity of peatlands to sequester carbon will remain secure provided we maintain conditions that are waterlogged, acidic and anoxic alone

(Gorham 1991) can no longer be relied upon. Additionally, we must also consider the need to maintain an adequate litter phenolic content. Leading on from this our second finding shows that, again as we hypothesised, the addition of supplementary phenolics, in the form of industrial lignin, can increase carbon sequestration by preventing the breakdown of fresh labile OM in the $^{13}$C-labelled litter - due to the inhibition of both hydrolase and phenol oxidase extracellular enzymes. Deliberate strengthening of the enzymic latch in peatlands in this way presents a promising prophylactic bio-geoengineering (Royal Society 2009) approach for reducing the radiative forcing on the atmosphere from all northern hemisphere peatlands - including, perhaps most significantly, areas of circumarctic peat which are expected to undergo the effects of long-term warming (Meehl et al. 2007). Application of our findings at a global scale could therefore be considered potentially 'climate-changing' if used as a unique form of geoengineering.

# Chapter 7

## Final discussion

## 7.1 Overview

Peatlands have always had an effect on our planet's climate, indeed it has been estimated that peat-accumulating wetlands sequester such significant amounts of carbon that they have lowered global temperatures by between 1.5 and 2.8°C over the past 10,000 years (Holden 2005). It is therefore surprising that as the dangers of anthropogenic global warming became apparent over the last few decades more has not been done, practically or legislatively (Chapter 1), to protect and manage areas of peatland for carbon capture and storage. Fortunately, this now seems to be being redressed (Chapter 1 and Appendix 2) and, taking the UK, as an example, efforts are being made to restore damaged and drained areas of peatland (Natural England 2010) with a major driving force being the desire to improve their carbon sequestering abilities. The ultimate aim of these projects is generally to return the wetland to one resembling an original 'pristine' peatland. However, even in undamaged peatlands only about 9% of the carbon fixed in photosynthesis survives decay before being buried in the anoxic waterlogged peat by the plants that continue to grow above, (Reader and Stewart 1972). The work in this thesis has shown that it may be possible to increase the amount of carbon undergoing long-term storage by strengthening the enzymic latch with supplementary phenolic compounds.

## 7.2 Method development

### 7.2.1 Enzyme assays

The measurement of extracellular enzyme activities has been a key component of this study and over the years several protocols have been developed for the assays of hydrolases and phenol oxidases. However, these had not been comprehensively described in a peer reviewed journal since the 1990s (Pind et al. 1994, Freeman et al. 1995, Freeman 1997). It was also found the most commonly used phenol oxidase assay (described in Chapters 3 to 5) was unable to detect the lower levels of activities observed in Chapter 6. The described protocol in Chapter 2.1 was therefore developed and is now the standard method used in the Wolfson Carbon Capture Laboratory. Its only disadvantage is that it requires double the amount of peat (2g) compared to the original method. This can be an issue if conducting an experiment which requires multiple peat samples to be taken over a period of time from a limited and finite host sample, as in Chapter 2. Using less than 1g of peat in each stomacher bag would not be recommended due to the difficulties in accurately weighing wet fibrous peat.

In all of the methods applied, we have avoided modifying assay conditions towards values that are optimal for the enzymes. For example, the procedures do not alter the pH as there is evidence that the activities of both phenol oxidases (Pind et al. 1994, Williams et al. 2000) and hydrolases (Kang and Freeman 1999) increase when the pH is higher than that usually found in an acidic peat bog. As highlighted by Kang and Freeman (1999) the alternative approach, namely modifying conditions towards enzymic optima, gives information on potential enzyme activity or quantity of active enzyme in the sample. However, the *actual* activity may be markedly different. Optimising for factors such as pH and temperature could therefore lead to

overestimates of the enzyme activities occurring in natural conditions (Freeman et al. 1995).

7.2.2 Gas fluxes

Many experiments throughout this thesis required the determination of rates of soil respiration from small samples of peat. In Chapter 4 this was simply to quantify differences between peat types, while in Chapters 5 and 6, the effects of supplementing peat-soils with various treatments was investigated. A standardised method using readily available laboratory equipment in a time efficient manner was required. And also, one that did not require large amounts of peat-soil as availability of samples was often limited.

The advantage of measuring soil respiration from slurries to this research project, rather than intact peat samples, is that experimental compounds can be added to the samples, such as Ca lignosulphonate, either directly as a solid (powder) or a solution - and evenly distributed due to the thorough homogenisation of the samples. Disadvantages to this method are that the water content of the peat is altered and the homogenisation can disrupt the peat matrix and structure. This destroys specific redox zones of the peat and could alter the activities of microbial communities, for example decreasing rates of $CH_4$ oxidation by methanotrophs and increasing anaerobic methanogenesis (Megonigal et al. 2005). However, as similar slurry-type analyses have been used in many previous peatland studies it was considered the best method for testing the effect of the experimental treatments in this thesis.

Calculating the gas flux using the equation from Chapter 2.2 takes into consideration various factors, which can be changed depending on the experimental conditions; for instance, temperature and mass of peat or peat slurry. Mass can also be

interchanged with surface area of the peat available for gaseous exchange. However, in most situations mass is easier to ascertain accurately. As flux equations quantify a movement of gas over a set period of time from a known amount of substrate it would have been preferable to take a number of gas samples from the centrifuge tubes containing the samples over a period of time (eg. every half an hour) to ensure linearity of the gas concentrations but due to the limited size of the headspace above the peat samples in the tubes, this was not practicable.

Following research for this chapter it became clear that there the majority of studies presenting trace gas results from peatlands, which use gas chromatography (GC) analysis, do not present data on the limits of detection (LoD), limits of quantitation (LoD) and the precision of analysis (coefficient of variation, C.V) of their GC instrument (Table 3 in Chapter 2.2). If these have not been checked and recorded it may mean that some presented results may not be justified. Due to the growing importance of reporting GHG emissions from ecosystems for climate change legislation, it is imperative that such issues are resolved to ensure the science is fully accountable and transparent.

### 7.3 Using vegetation to strengthen the enzymic latch

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). Senesced plant tissue forms peat-soil litter (Limpens et al. 2008a), while root exudates provide soil microbes and enzymes with a cocktail of chemicals, gases, labile carbon compounds and enzymes that influence rates of carbon and nutrient cyling (Ladd 1978, Bais et al. 2006). Manipulating this release of root exudates with the aim of

suppressing decomposition, and thereby strengthening the enzymic latch, was therefore chosen as a starting point for the thesis. Particularly after a recent study by Fontaine et al. (2007) suggested that preventing fresh labile plant-derived carbon from entering the soil profile could inhibit the microbial mineralisation of older carbon in the soil profile. As photosynthetic activity is a key regulator of the rate of root exudation (Rovira 1969) it was hypothesised that adjusting the level of photosynthetically active radiation (PAR) that peatland plants were subjected to would alter the enzymic activities of the peat in the rhizosphere (Chapter 3). However, this was not found to be the case suggesting peatland plants have adapted to maximise their rates of metabolism for the average light intensity they usually grow in. Instead it was found there were significant differences between plant species at each of the different light levels. This led to the initial hypothesis in Chapter 4 that plant diversity manipulation could be used to increase carbon sequestration in peatlands.

Plant community structures can be, and currently are, purposefully adjusted across areas of peatland in the UK, through processes such as raising or lowering of the water table, controlled grazing and targeted burning (Natural England 2010). If these types of methods could be applied for selecting plants which are able to maximise carbon sequestration of the peat they are growing in then this may present a new form of land management, whereby peatlands are managed for carbon storage - so called carbon stewardship (Worrall et al. 2009). The study in Chapter 4 concluded that *Sphagnum* moss was the most efficient species group at reducing belowground carbon turnover, which supports previous work stating the importance of *Sphagnum* species for carbon accretion in peatlands (Clymo and Hayward 1982, Heijmans et al. 2002, Fenner et al. 2004). This has been attributed to the polyphenols produced by

*Sphagnum* mosses as secondary metabolites, as although they lack lignin, a derivative of cinnamic acid called *trans*-sphagnum acid (Verhoeven and Liefveld 1997) is produced. All species of *Sphagnum* are thought to contain this polyphenol - although the actual amount varies depending on species, season and location in the plant itself - and all can secrete it into the external medium (Rudolph and Samland 1985).

Interestingly, although it was concluded that *Juncus* was the least desirable plant to have on an area of peatland for belowground carbon sequestration (due to high fluxes of $CO_2$, $CH_4$ and DOC), phenolic concentrations were not significantly lower, and hydrolase enzymes were not higher than the *Sphagnum* or *Calluna* peat samples - as would be expected with the enzymic latch mechanism. It was proposed this was because of the labile nature of the root exudates and litter produced by the fast-growing, productive *Juncus* plants encouraging high microbial activity (Fontaine et al. 2007).

Results from chapters 3 and 4 suggest that plants have a direct effect on carbon cycling in the peat directly beneath them (rhizosphere). However, any increase in sequestered carbon achieved through encouraging the growth of *Sphagnum* mosses over *Juncus* plants is likely to be modest. 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* growing. New strains of the mosses could be

selected for 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. To observe how increasing the absolute amount of phenolic inhibitors could affect carbon cycling the experiments described in Chapters 5 and 6 were conducted.

### 7.4 Increasing phenolic concentrations in peat

It is widely accepted that phenolics are potent inhibitors of enzymic activity in peatlands, with previous work highlighting their importance in suppressing the activities of hydrolase enzymes (Freeman et al. 2001a, Freeman et al. 2001b). However, as explained in Chapter 5 and 6 there are many forms of phenolics, from simple phenol acids to complex lignin compounds (Dai and Mumper 2010). It was correctly hypothesised that the higher the molecular weight of the experimental phenolic compound, the greater the negative effect on biogenic trace gas production and enzyme activity it would have (Chapter 5). This corresponded with previous work showing the presence of high molecular weight compounds in organic matter act as a barrier to the breakdown of lower molecular weight organic matter (Freeman et al. 1990, Freeman and Lock 1992).

The Calcium lignosulphonate, which was found to be the most-inhibitory of the phenolic compounds tested (Chapter 5), is an amorphous light-yellow-brown powder obtained from the sulphite pulping of softwood. Its structure consists of a

sulphonated random polymer of three aromatic alcohols: conifer alcohol, *p*-coumaryl alcohol, and sinapyl alcohol, of which coniferyl alcohol is the principle unit. It is non-toxic (being used in various industries including food manufacturing) and as it is produced in very large amounts as a by-product of the paper milling industry it is in plentiful supply and cheap to purchase (Xiao et al. 2001). As a form of lignin (a natural polymer) it is also not an alien compound with which to enrich a peatland. Lignin (from the Latin word 'lignum' meaning wood) is a major component of vascular plants and forms an essential part of all wood stems, which include *Calluna* and other heathland shrubs and trees.

Interestingly the experiments in Chapter 5 showed that the addition of Ca lignosulphonate suppressed $CO_2$ release from the peat samples by 76%. However, as the activity of some of the hydrolase enzymes were severely curtailed (Chapter 5, Figure 2) it was suspected that decomposition rates of the peat-soil litter was being under-estimated. There was no way of identifying if the measured $CO_2$ originated from the microbial breakdown of the peat litter or Ca lignosulphonate itself - especially as we were not conducting analyses for any of the extracellular enzymes specifically involved in the initial stages of the breakdown of lignin, such as lignin peroxidase. A method for quantifying the actual decomposition of peat litter was therefore required and the $^{13}C$-labelled litter experiment, described in Chapter 6, was devised. This experiment showed that the lignin was indeed dramatically suppressing organic matter breakdown to carbon-based trace gases by as much as 99.6%.

Both Chapters 5 and 6 revealed that not only was the Ca lignosulphonate solution suppressing hydrolase enzyme activities, as hypothesised, but it was also retarding the activity of extracellular phenol oxidases. This was unexpected because lignin, as a phenolic compound, would be considered a substrate for phenol oxidase.

However, this could be an example of typical substrate suppression and previous work has suggested that certain levels of phenolics can inactivate phenol oxidases (Scalbert 1991). Interestingly, this inhibition of phenol oxidases was also observed in the peat cores with six wooden spatulas added to them (Chapter 5, Figure 5).

**7.5 Effect of pH**

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. Enzymes are amphoteric globular proteins, being composed of both acidic and basic groups of amino acid side-chains. Like all proteins enzymes are subject to different states of ionisation depending on the pH and ionisation constants of these various reactive groups. In order for enzymes to function at their optimal levels the binding and catalytic sites must be in their proper state of ionisation - in some instances this is in the unionised state and in others, the ionised states (Frankenberger and Johanson 1982). Altering the hydrogen ion concentration of a soil can therefore affect their activities by the reaction of ionising or deionising reactive groups in the active centre of the enzyme. This can be both reversible or irreversible. In some situations, when changes to pH are extreme, the overall structure of the enzyme itself can be irreversibly altered, rendering it defunct, as high H+ or OH- concentrations can disrupt the protein's ionic and hydrogen structural bonds (Frankenberger and Johanson 1982). In addition, pH can also change the interactions between enzymes and stabilising matrices in the soil (eg. clay or humus) and therefore modify their rates of activity, which suggests why free enzymes have a different optimal pH than clay-bound enzymes (McLaren and Estermann 1957).

As described in section 7.2.1 there have been numerous studies investigating the effect of pH on peatland enzyme activities, including phenol oxidase, and similarly to Williams et al. (2000) in Chapter 4 some evidence (ie. in *Juncus* samples) was found to suggest that phenol oxidases were constrained by low pH and that the plants had an effect on peat litter pH. This finding could be investigated further; however, the results in Chapter 6 (Figure 2) show that although lowering the pH with HCl did initially suppress organic matter breakdown, after just 24 hours it began to recover (Figure 2). Crucially, the pH of these samples remained comparable with the phenolic treatment slurries after 120 hours (Table 5) - suggesting the low pH was not suppressing litter breakdown. Conversely enriching the peat samples with phenolics maintained decomposition suppression for the full experimental time period. 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.

**7.6 Methane fluxes**

Production of $CH_4$ (methanogenesis) in peat occurs when either of the two following reactions are facilitated by certain microbes (methanogens):

1. $CO_2$ is used as an electron acceptor to produce $CH_4$. Referred to as $H_2$-$CO_2$-dependent or hydrogenotrophic methanogenesis.

   $CO_2 + 8e^- + 8H^+ \rightarrow CH_4 + 5H_2O$

2. Acetate is used as an electron acceptor. Referred to as acetoclastic methanogenesis or acetotrophy.

   $CH_3COOH + 4H_2 \rightarrow 2CH_4 + 2H_2O$

These reactions generally require anaerobic, or extremely reduced, conditions after other terminal electron acceptors have been reduced - such as oxygen ($O_2$), nitrate ($NO_3^-$) and sulphate ($SO_4^{2-}$) (Mitsch and Gosselink 2007). For ombrotrophic peatlands $H_2$-$CO_2$-dependent methanogenesis is thought to be the main pathway for $CH_4$ production (Galand et al. 2005). However, there is evidence that there is a seasonal shift from $H_2$-$CO_2$-dependent methanogenesis dominance in the winter to acetoclastic methanogenesis during warmer seasons due to the warmer temperature and increased concentrations of acetate (Avery et al. 1999).

As $CH_4$ is one of the terminal products of decomposition in a peatland it was hypothesised that enriching peat with phenolics would also affect its production. However, results from Chapters 5 and 6 showed there was little effect on the gas's flux. This is likely due to the reservoirs of $CO_2$ and acetate already stored in the peat (Hines et al. 2001) available for methanogens to utilise. It is noteworthy that the peat taken during the summer period in Chapter 6 did show organic matter breakdown to $CH_4$ was suppressed though (Chapter 6 - Figure 2B). This may be because the percentage of $CH_4$ produced by acetoclastic methanogenesis is higher during this sampling time (Avery et al. 1999) due to the input of fresh, labile organic matter which favours this type of methanogenesis (Megonigal et al. 2005). Any perturbation in the biodegradation chain of this organic matter (through the addition of phenolics) is likely to be reflected by more reduced acetotrophy than hydrogenotrophic methanogenesis, as there is evidence to suggest the latter assimilates carbon from older sources (Megonigal et al. 2005). Thus, by phenolics suppressing organic matter breakdown in peat they *are* affecting methanogenesis, especially acetoclastic methanogenesis but this is only reflected when the latter process is dominant.

## 7.7 Putting a value on enhanced peatland carbon sequestration

By suppressing decomposition in peatlands using the described method of enriching peat with Ca lignosulphonate it was calculated in Chapter 6 that $1.69x10^{15}$ g carbon $yr^{-1}$ (1.69 Pg, or 1.69 billion tonnes, $yr^{-1}$) of additional carbon could be sequestered by the world's northern peatlands.

Figures from the UK's Department of Energy and Climate Change (DECC) document, 'Carbon valuation in UK policy appraisal: a revised approach' (2009), suggest that the official short-term estimates for the trading price of carbon is £25, with a range of £14 to £31, per tonne (t). This would mean the additional carbon sequestered by peatlands could be worth, in the region of: £42,250,000,000 (£42.25 billion).

## 7.8 Using peatlands for geoengineering projects

Geoengineering is the deliberate large-scale intervention in the Earth's climate system in order to moderate global warming. In 2009 the Royal Society produced an in-depth report on the subject and concluded that current GHG emission reduction policies and practices are insufficient; geoengineering projects may therefore be essential if we are to avoid the dangers of a changing climate. All currently suggested schemes though struggle to strike the right balance between the key requirements of affordability, effectiveness, safety and timeliness. Conversely peatland-geoengineering could score highly in all the criteria laid out by the Royal Society - see Table 1 for a comparison of peatland-geoengineering and other suggested $CO_2$ removal (CDR) geoengineering techniques.

Initial laboratory tests show the scientific robustness of the methods for suppressing enzymic decomposition using manipulation of a natural biogeochemical process. The materials used to achieve this (Ca lignosulphonate or wood - Chapters 5 and 6) are cheap and easily transportable and the peatlands themselves have no other major land-use pressures on them (such as agriculture). Crucially, unlike other proposed bio-geoengineering projects, such as afforestation, the carbon sequestered using our methods will be prevented from entering the atmosphere indefinitely.

If these treatments were to be used on a global scale then some disturbance to undamaged or pristine peatlands is inevitable. However, as argued in Appendix 3 this may be an unfortunate but essential consequence if we are wish to reduce significant amounts of anthropogenic GHGs.

| Technique | Advantages | Disadvantages |
|---|---|---|
| **Peatland enzymic latch manipulation**<br><br>Suppressing microbial decomposition of organic matter in peatlands by strengthening the enzymic latch | • Low cost<br>• Preliminary experiments successful<br>• Low risk of unanticipated environmental effects<br>• Reversible - $CO_2$ can be re-released (eg. burning)<br>• Several different methods of strengthening the enzymic latch which can be adapted depending on the location and type of peatland<br>• Uses 'low value' land, so little competition with other land uses<br>• Long-term storage of large amounts of carbon<br>• 'Carbon stewardship' may pay for peatland conservation and management | • Restrained by area of global peatlands<br>• May be necessary to 'damage' pristine peatlands<br>• Droughts my negate the effect of the enzymic latch |
| **Afforestation**<br><br>Establishment of a forest or stand of trees | • Low cost<br>• Proven record of success<br>• Begins $CO_2$ reductions immediately<br>• Low risk of unanticipated environmental effects<br>• Reversible- $CO_2$ can be re-released (eg. burning) | • Restrained by availability of sequestration sites<br>• Competition with other land uses, especially agriculture<br>• Biodiversity implications<br>• Limited potential for carbon removal<br>• Sequestered carbon not secure for long periods of time |
| **Bioenergy with carbon capture and sequestration (BECS)**<br><br>Biomass is harvested and used as fuel with the resulting $CO_2$ being captured and sequestered eg. in geological formations | • Low to medium costs<br>• Low to medium risk of unanticipated environmental effects<br>• Uses existing knowledge and technology<br>• Produces a useable source of energy | • Costs reliant on various factors such as fertiliser and transportaion costs<br>• More expensive than fossil fuel carbon capture and storage (CCS) as biofuels can be more expensive<br>• Slow to reduce global temperatures |

| | | |
|---|---|---|
| | | • Potential land-use conflicts<br>• Limited by plant productivity |
| **$CO_2$ removal from ambient air**<br><br>Using an industrial process to capture $CO_2$ from ambient air to produce a pure $CO_2$ stream for use or disposal | • Very low risk of unanticipated environmental effects<br>• No inherent limit on the size of the effect achievable<br>• Reversible - process can be stopped and captured $CO_2$ re-released<br>• Very little competition with other land uses | • Work still needed to find cost most efficient and effective method<br>• Requires substantial infrastructure construction<br>• Additional carbon storage locations required<br>• Transportation costs of captured $CO_2$ need to be considered |
| **Ocean fertilisation**<br><br>Introduction of nutrients to the upper ocean to increase marine primary productivty | • No competition with other land uses | • Not likely to be very effective<br>• May reduce biological carbon uptake elsewhere in the oceans<br>• Low long-term carbon storage potential<br>• Not expected to be very cost-effective<br>• Slow to reduce global temperatures<br>• Substantial prior research required to investigate environmental impacts, efficacy and verifiability<br>• High potential for unintended and undesirable ecological consequences |

**Table 1. A comparison of different carbon dioxide removal (CDR) techniques for geoengineering the climate.**

## 7.9 Final conclusions

This thesis has shown that strengthening the enzymic latch in peatlands is achievable and can increase their ability to sequester carbon. Attempts to achieve this through manipulating light levels and peatland plant communities were largely unsuccessful. However, phenolic enrichment of peat - in the form of high molecular weight lignin - can retard carbon cycling in peat samples by as much as 99%. Thus, peatlands have the potential to sequester an additional 1.69 Pg of carbon a year, equivalent to around one-and-a-half times the current emissions of transport (Barker et al. 2007) and worth £42.5 billion on current and projected carbon markets.

The key findings, based on the results and conclusions from the thesis are represented in Figure 1, and described as follows. The addition of lignin, namely Ca Lignosulphoante [A] and the use of wood fragments in the form of wooden spatulas, or fragments, can all increase the phenolic abundance in peat (Chapter 5 - Figure 3 and Chapter 6 - Table 4, 5 and 6; and Chapter 5 - Figure 6 respectively). This increase in phenolics leads to suppression in the activities of hydrolases [F] (Chapter 5 - Figures 2 and 5, and Table 5; Chapter 6 - Table 4 and 6) resulting in a decline in the breakdown of organic matter [K] into smaller compounds which can be used for microbial metabolism. This lack of available carbon and other essential elements to microbes curtail their activity [N] leading to a suppression of biogenic trace gas emissions from peatlands [O]. Additionally a reduction in microbial activity will reduce the numbers of extracellular enzymes they produce [L] and [M], further inhibiting decomposition. The deliberate increasing in phenolics also suppresses the activities of phenol oxidases [H] (Chapter 5 - Figure 3 and 5; Chapter 6 - Table 4 and 6) and as these are one of the few groups of enzymes capable of breaking down phenolic compounds [I] (Freeman et al. 2001b) this can lead to a continued build-up

of inhibitory phenolics which research for Chapter 1 showed can suppress the metabolism of microbes themselves (Fenner and Freeman 2011) [G] (Chapter 1 - Figure 2).

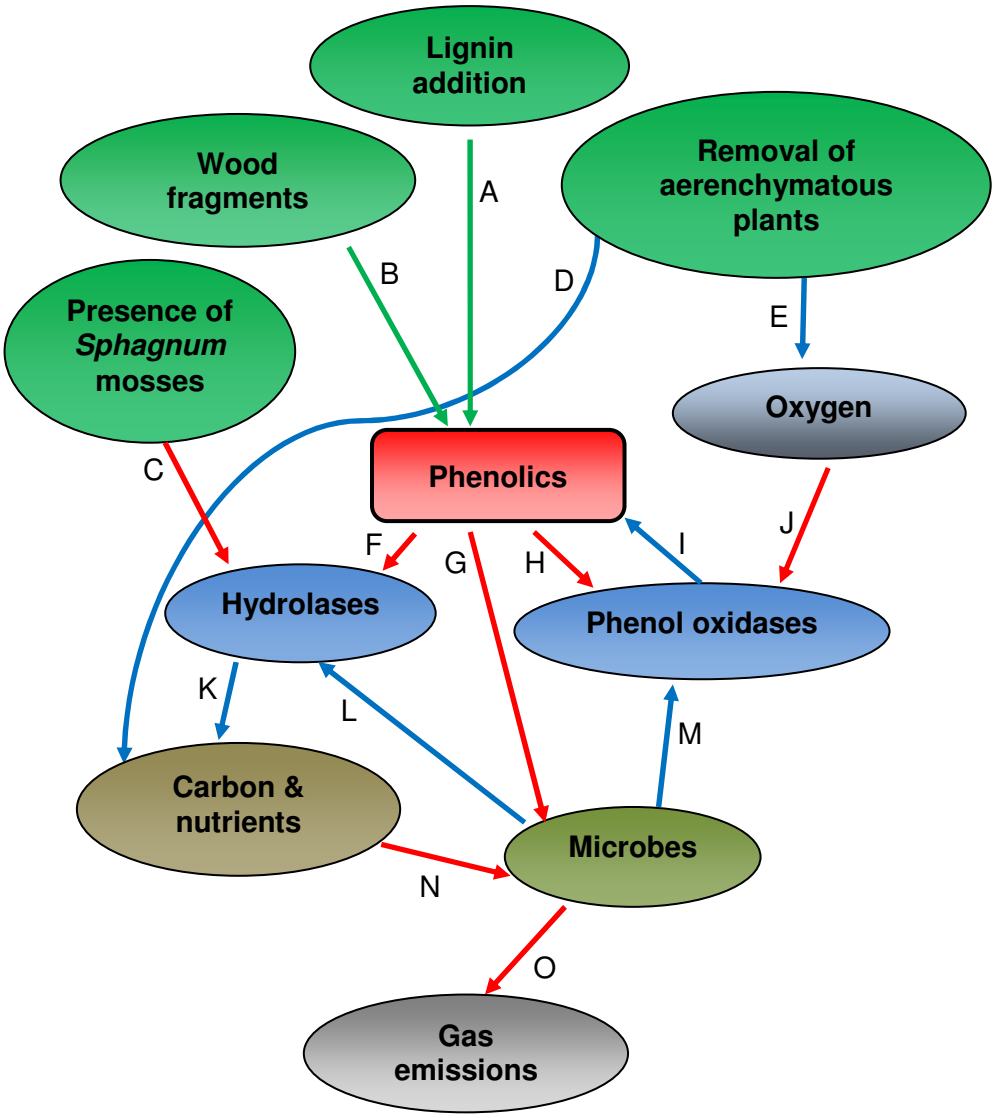



**Figure 1. Key findings from the thesis, based on the results and conclusions from the various studies.** A schematic diagram showing how the enzymic latch can be strengthened to suppress carbon-based trace gas emissions. Green arrows indicate deliberate increases. Red arrows show a suppression and blue arrows indicate a limiting effect or release.

Although previous studies suggest *Sphagnum* mosses can also increase concentrations of polyphenols in peat (Clymo and Hayward 1982, Rudolph and Samland 1985, Verhoeven and Liefveld 1997, Naumova et al. 2013) there was little evidence of this from results in Chapters 3 and 4. However, it did appear that carbon loss was constrained in *Sphagnum* dominated peat (Chapter 4) which could be attributed to lower pH suppressing enzyme activities [C] (Clymo 1984). Finally, by removing fast growing aerenchymatous plants from peatlands it may be possible to reduce the release of labile carbon compounds [D] and oxygen [E] (Ladd 1978, Bais et al. 2006), necessary for the activity of phenol oxidase, into the soil matrix (conclusions from Chapter 3 and 4).

Crucially this study also showed the inhibitory effects of phenolic enrichment were not restricted to one type of peatland. Similar suppression of decomposition was recorded from peat taken from various boreal peatland sites (Chapter 6) with the proposed mechanism having the potential to reduce carbon losses from Arctic peatlands from 100 megatonnes each year to just six. Thus a bio-geoengineering technique that enhances the security of one of Earth's most vulnerable carbon stores has been developed. This work has therefore opened up a new field of research - "applied planetary medicine" - whereby strategies are developed to restore the healthy functioning in our planet's biogeochemical pathways through techniques analogous to those widely adopted during human medical interventions.

Looking back to the two key hypotheses at the start of this thesis it can be concluded that there is limited evidence to suggest plants can be used to strengthen the enzymic latch, either by using light intensities to alter root exudation or by

manipulating floral communities, in order to reduce phenol oxidase activities. However, our second hypothesis is tenable: phenolic enrichment of peat can increase the ecosystem's ability to sequester carbon.

### 7.10 Future work

Although this study has shown the potential benefits enriching peat with additional phenolics has on increasing carbon sequestration more work now needs to be carried out to investigate the effects of this treatment on a plot or field scale. Factors such as vegetation and fluctuations in the water table may influence the effectiveness of the treatment. Indeed, which treatment would be best suited to field conditions - lignin compound or wood addition - needs to be investigated. If lignin is chosen would it be better to add the treatment as a solution or in pellet-form? And, crucially, unlike the addition of timber which is likely to remain as a solid item in the peat for hundreds of years - as evident from the discovery of preserved wooden artefacts in peatlands (Lageard et al. 1995) - it needs to be determined how long the inhibitory effects of supplementary lignin compounds would last for. It also needs to be determined whether the compounds will be quickly leached from the peat matrix and enter nearby watercourses with detrimental effects.

The concentration that either wood or lignin compounds would have to be added to areas of peatland to achieve worthwhile suppression of decomposition needs to be studied. For instance it was calculated that to achieve the levels of $CO_2$ suppression observed in the peat cores in Chapter 5, 1.6 tons of wood fragments would need to be added to a $1km^2$ area of peatland (or 105,000 wooden spatulas). This may well be unrealistic so more research is needed; including looking at the

effects of different types of wood, what size wood fragments should be used and to what depth they should be injected into the peat. If a Ca lignosulphonate solution is to be added to peat the ideal concentration has to be calculated, which may be different depending on the method of application. For instance preliminary tests conducted on peat cores showed that the pouring of a concentrated lignin solution over *Sphagnum* mosses caused senescence, which took the community several months to recover from.

Although the results involving the effect of vegetation on the enzymic latch were inconclusive, this area of research warrants further work. Not least because this may be the method most likely to achieve support from the public, and therefore policy makers (Royal Society 2009), should a peatland geoengineering project be required within the near future to help sequester the ever increasing concentrations of GHGs in the atmosphere. More types of peatlands, and different plant species should be investigated along with longer term monitoring.

An interesting area of future study could be the combining of different phenolic compounds to 'knock-out' entire suites of enzymes and even microbial communities. As the Ca lignosulphonate did not suppress all hydrolase enzymes analysed it is hypothesised that a mixture of different phenolics could work together to target specific enzymes with a bespoke lignin solution for peatland carbon sequestration.

# Appendices

# Appendix 1

## Methane emissions from thawing boreal peatlands

**High-latitude soils are likely to undergo the effects of strong warming over the coming century, leading to an expected doubling in methane emissions from thawing permafrost. However, we have found methanogens in the active layer of boreal peat permafrost are highly adapted to low temperatures, and that as a result warming induces a decline in methane emissions, rather than the expected increase. Our findings suggest that concerns over the initial feedback effects of thawing frozen peat may have been substantially overestimated.**

It is widely acknowledged that the soils at the highest latitudes will undergo the strongest warming over the next century leading to an expected doubling in methane ($CH_4$) emissions from thawing permafrost (Johansson et al. 2006, Zhuang et al. 2006, Meehl et al. 2007). Permafrost (perennially frozen ground) covers nearly a quarter of the exposed land surface of the northern hemisphere. Extensive areas of this region are comprised of peat: an organic soil of partially-decomposed plant material. Such peatlands contain up to 277 petagrams (Pg; 1 Pg = 1 billion metric tons) of carbon: around a quarter of the world's soil carbon pool (Schuur et al. 2008).

Despite freezing temperatures these sub-Arctic or boreal peatlands currently produce significant amounts of greenhouse gases (GHGs) including $CH_4$ (Smith et al. 2004) - formed by methanogenic archaea. Previous studies suggest thawing of frozen peat causes an increase in the release of carbon, primarily through accelerated microbial decomposition of organic matter (Schuur et al. 2008). Of the gaseous

products anticipated to increase with warming $CH_4$ raises greatest concern as the gas has 25 times more warming potential than $CO_2$ over a 100 year period.

Intact frozen cores of peat were taken from three boreal peatlands in Alaska, USA, for this study. Alpha (α) site was a nutrient rich fen; Beta (β) was a nutrient poor thermokarst bog, where the permafrost thawed from the environment 20 years-ago, and Gamma (γ) site was a low productivity black spruce site. Analysis of the peat soil from the different sites show typical ranges of pH (4.82 to 5.596), conductivity (15.5 to 43 μS $cm^{-1}$) percentage of water content (82.35 to 91.45) and organic matter (82.06 to 95.64) expected of high-latitude peatlands.

The intact cores were maintained under frozen conditions and transported to Bangor University in the UK, where they were stored at -18$^{o}$C for seven days. Each core was then split into smaller sections forming complete micro-cores (50mm x 200mm) from two different layers – below the frozen *Sphagnum* growth (considered 0cm) to a depth of 50mm and from 5mm to 100mm deep. The cores were cut in a room maintained at -10$^{o}$C to prevent premature thawing. A small amount of peat from each micro-core was retained for pH and conductivity measurements and the final mass of each micro-core determined for use in flux calculations and moisture loss.

Each micro-core was placed in 50mL centrifuge tubes, flushed with oxygen free nitrogen for 20 seconds at a regular pressure, sealed and placed at -18$^{o}$C for seven days. This created anaerobic conditions inside the centrifuge tubes, which then were maintained by regular flushing before and after each sampling occasion. Gas samples were collected at the start and end of a 45 minute incubation period using a syringe and 5$cm^3$ stored in Labco Exetainers (after preliminary tests showed gas concentrations were linear until this time period).

The process of collecting gas samples was repeated for three days at -18°C before the micro-cores were transferred to a Sanyo incubator, model MIR 152, at -10°C. Gas samples were collected in the same way every day for three days. After this the incubator temperature was increased by five degrees Celsius and the process repeated until +15°C was reached. Gas samples were analyzed by gas chromatography using a Varain model 450 gas chromatograph (GC) equipped with a flame ionisation detector (FID) with a $CO_2$ to $CH_4$ catalytic converter. A Poropak QS column was used at 40°C with nitrogen as a carrier gas at 30mL $min^{-1}$. The micro-cores were kept in the dark throughout the experiment. The mass of each micro-core was checked regularly and kept to the original weight by the addition of Milli-Q filtered water. A set of micro-cores from each peat type was maintained at -18°C conditions and gas samples were collected from these as above throughout the experimental time period. This approach contrasts with all prior laboratory studies of this type, which a) begin measuring gas fluxes at temperatures already above 0°C, and b) use peat slurries or homogenates rather than samples with an intact soil structure. Our approach also contrasts with prior field experiments that maintain areas of peat a few degrees Celsius above normal field temperatures (see Table 1).

Statistical analysis were made by a series of analysis of variance (ANOVA) test followed by pairwise tests using the least significant difference (LSD) method.

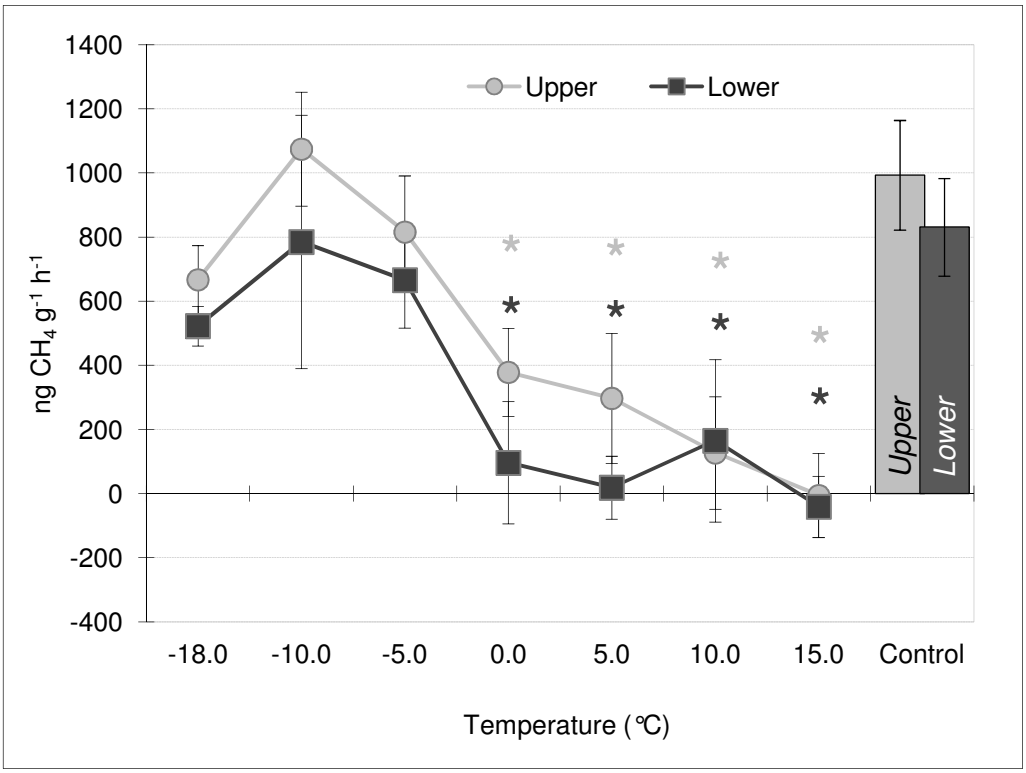
Contrary to expectations, $CH_4$ fluxes declined as temperatures increased - irrespective of depth or peat site (Figures 1A-C). At 15°C all $CH_4$ fluxes were significantly lower than those of the control micro-cores which were maintained at -18°C ($p<0.05$). The upper layer of site γ showed the greatest reduction in flux, dropping 2.1 mg $CH_4$ $g^{-1}$ $hr^{-1}$ between -18°C and 15°C; whilst the permafrost sample which showed the greatest reduction was the lower layer of site β, dropping 1.2 mg

$CH_4$ $g^{-1}$ $hr^{-1}$. There was no consistently significant difference of $CH_4$ fluxes between the two peat depths at each site ($p$<0.05), and all sites followed similar rates of reduction in their $CH_4$ fluxes. Temperate peat soil subjected to freezing at -18$^{o}$C showed a 50 percent drop in $CH_4$ production when measured in the same way.

Our findings indicate that methanogenic archaea communities in the active layer of permafrost and thermokarst soils are psychrophiles, specialised to metabolise under the harsh conditions of frozen Arctic soils which are not suited to warmer temperatures (Morozova et al. 2007).

A reduction in this magnitude of such a potent GHG is likely to prove critical for modelling the future of Earth's climate and suggest that we may need to reevaluate current assumptions about the nature of positive feedback to global warming from peatland permafrost thawing.

**1. A**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Control
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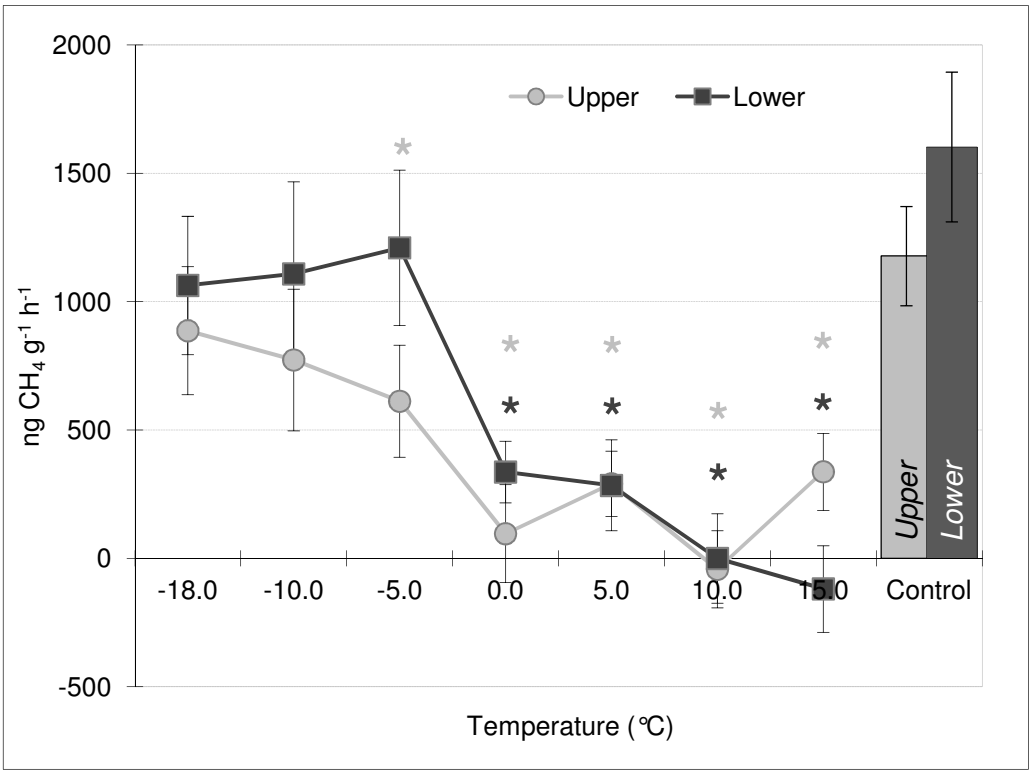
Temperature (℃)

**1. B**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**1.C**

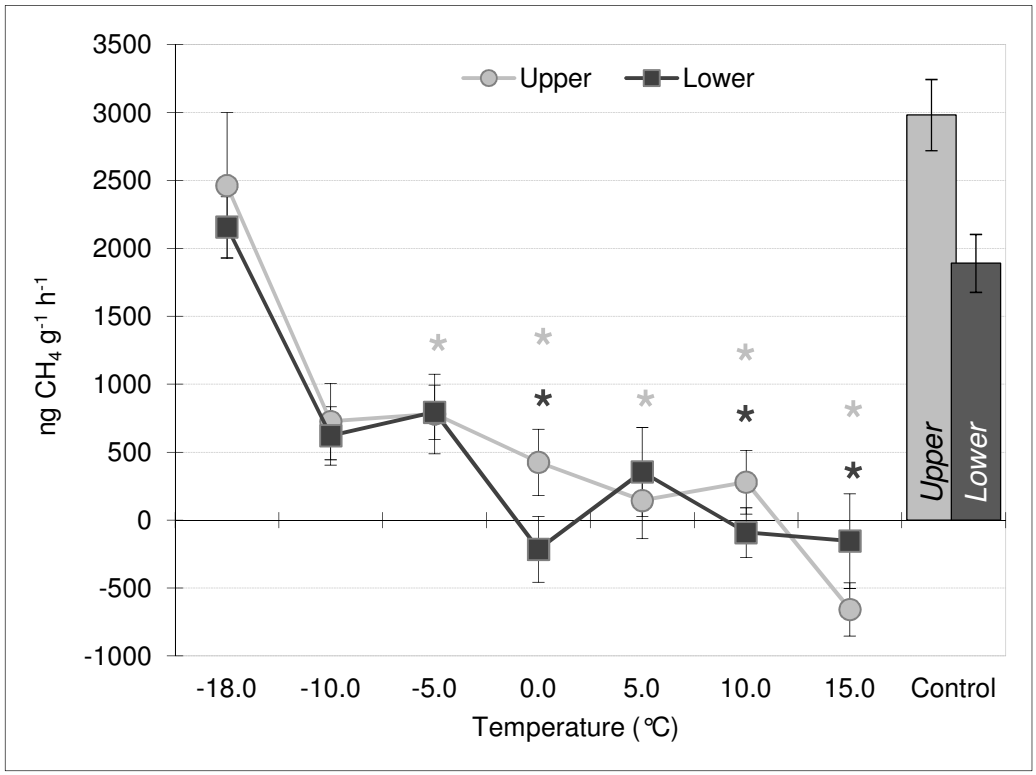


**Figure 1. Methane fluxes from warming boreal peat cores.** The $CH_4$ fluxes from peat micro-cores, taken from three sites in Alaska - a nutrient rich fen (α), a nutrient poor thermokarst bog (β) and a low productivity black spruce site (γ) (panels A – C respectively). Fluxes from two layers of the peat are shown: an upper layer of 0-5cm and lower layer 5-10cm. Control results represent the average fluxes taken at regular intervals over the experimental time period. Asterixis indicate a significant difference ($p$<0.05) between the $CH_4$ flux of sample and the site's control. Error bars indicate ± standard error.

| Peat type | Experiment conditions | Experiment temperature range ( °C). | Methane flux relation to experimental temperature | Author |
|---|---|---|---|---|
| TB, SB, F, S | LE, slurries, AC ($N_2$) | 0 - 35 | ↑ | Dunfield *et al*. (1993) |
| TS | LE, AC ($N_2$) | 5 - 35 | ↑ | Westermann (1993) |
| BP, TP | LE, slurries, AC ($N_2$) | 2 - 20 | ↑ | Yavitt *et al*. (1997) |
| BP, F | LE, slurries, AC ($N_2$) | 0 - 20 | ↑ | Bergman *et al*.(1998) |
| TP | LE, slurries, AC ($N_2$) | 4 - 30 | ↑ | Bergman *et al*. **(1999)** |
| BP | LE, slurries | 4 - 60 | ↑ | Metje & Frenzel (2005) |
| PP, ThW, ThE | FE | 0 - 14 | No relation | Wickland *et al*. (2006) |
| BF | FE, top open chambers | 0 - 20 | No relation | Turetsky *et al*. (2008) |
| BB, BF | FE, infrared radiation lamps | 4 - 20 | No relation | White *et al*.(2008) |

**Table 1. Review of previous experiments investigating the effects of increasing temperatures on the methane production in peat, or organic soils.** TB, TS, TP temperate: bog, swamp, peat; SB subarctic bog; F fen, S swamp; BP, BB, BF boreal: peat, bog, fen; PP permafrost plateau; ThE, ThW thermokarst: edges, wetland. LE laboratory experiment, FE field experiment, AC ($N_2$) anoxic conditions in pure $N_2$ atmosphere.

# Appendix 2

## Peatlands: our greatest source of carbon credits?

REVIEW

# Peatlands: our greatest source of carbon credits?

*Carbon Management* (2011) 2(3), 289–301

**Christian Dunn[†1] & Chris Freeman[1]**

Peatlands are the most efficient carbon stores of all terrestrial ecosystems, containing approximately 455 Pg of carbon, which is twice the amount found in the world's forest biomass. The majority of this carbon is stored in the saturated peat soil. Pristine peatlands are still sequestering carbon at a rate of 0.096 Pg carbon per year; however, anthropogenic degradation of peatlands through draining, fires and exploitation can increase the production of GHGs, switching peatlands from net sinks to net sources of carbon. Conservation of peatlands throughout the UK and the rest of the world is clearly essential for limiting GHG emissions and it is therefore surprising that accounting for emissions from peatlands does not feature prominently in the UNFCCC's Kyoto Protocol. Discussions at Conference of the Parties (COP) COP-15 and COP-16 look set to make amends for this oversight in any post-2012 climate change legislation, with peatlands becoming important factors in national GHG inventories, the agriculture, forestry and other land use (AFOLU) sector, and in the creation of internationally accredited carbon credits. Using figures from The Economics of Ecosystems and Biodiversity (TEEB), the world's peatlands can be valued at up to US$18 billion. However, this sum does not take into account pending UNFCCC decisions. The detailed mandatory inclusion of peatlands in national GHG inventory schemes and in accredited carbon markets could see their value rise even further. This review looks at the current GHG emission-monitoring legislation regarding peatlands, with special focus given to those in the UK. It discusses the importance of peatlands in carbon sequestration, reviews how peatlands feature in current GHG emission-monitoring schemes, concentrating on those associated with the Kyoto Protocol, and considers how peatlands may feature in national GHG emission-monitoring schemes and carbon markets in the future.

Peatlands cover just 3% of the world's surface [1], yet they contain nearly a third of all the organic carbon found in the earth's soils, which is equivalent to approximately two-thirds of the entire atmospheric carbon pool [2]. Peatlands have sequestered this carbon over millennia due, primarily, to the accumulation of partially decomposed organic matter in anoxic soils [3] and it is estimated they continue to do so at an average rate of 0.07–0.096 Pg of carbon per year (1 Pg = $10^{15}$g) [1,4]. However, decomposition of some soil organic matter (SOM) does take place in peatlands, releasing carbon in solid and gaseous forms such as $CO_2$ and methane. These two gases alongside nitrous oxide make up the main GHGs emitted by peatlands. Most studies agree that although the natural processes in peatlands produce varying amounts of GHGs, depending on the ecosystem's condition, type and location, they are currently net carbon sinks on the global level [5]. However, there are fears that anthropogenic and environmental factors could be altering the balance, turning peatlands into carbon sources [6]. Despite peatlands having the highest carbon density of any terrestrial ecosystem [7], they do not feature prominently in GHG emission-monitoring schemes under the Kyoto Protocol. Therefore, any emission savings created through peatland management or restoration projects do not necessarily count towards a Party's Kyoto commitment. Recent decisions have moved to readdress this issue in any post-2012 climate change legislation.

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This review focuses on the current GHG emission-monitoring legislation regarding peatlands, with particular focus given to those in the UK. It has been written for scientists and economists involved with the conservation of peatland habitats. The aims of the key sections are;

- To briefly discuss the importance of peatlands in carbon sequestration;
- To review how peatlands feature in current GHG emission monitoring schemes concentrating on those associated with the Kyoto Protocol;
- To consider how peatlands may feature in national GHG emission monitoring schemes and carbon markets in the future.

## Carbon cycling in peatlands

### Definition & types of peatlands

Peatland is generally considered to be a generic term for any freshwater wetland that accumulates partially decayed plant-matter peat (usually to a depth of greater than 40 cm), referred to as peat [8]. They are classified as organic wetlands and are estimated to be the most widespread group of wetlands differing from mineral wetlands by having a living plant layer plus thick accumulations of preserved plant detritus. See Table 1 for a definition of the main types of peatland.

The unique biogeochemistry of peatlands make them unbalanced ecosystems (i.e., where the production rate of organic matter exceeds that of decomposition) [1,3]. Traditionally, this impaired decay is attributed to anoxia [1,9,10], low nutrients, low temperatures and low pH [1,11]. Work by Freeman *et al.* also showed that constraints on the enzyme phenol oxidase could be the main inhibiting factor on decomposition [12]. Phenol oxidases are among the few enzymes able to fully degrade phenolic compounds [9] and their activity can be suppressed by the conditions in peat; allowing the build up of phenolic compounds, which then prevent the major agents of nutrient cycling, hydrolase enzymes, from carrying out normal decay processes [13,14].

**Key terms**

Peatland: A type of wetland where in excess of 30–40 cm of peat (partial decomposed organic matter) has formed.

Kyoto Protocol: The principal protocol to the UNFCCC, which sets out binding targets for industrialized countries to cut their GHG emissions over the 5-year period of 2005–2008, compared with a specified base year.

Wetland: Land with the water table close to or above the surface, or which is saturated for a significant period of time.

UNFCCC: The United Nations Framework on Convention on Climate Change produced by the United Nations Conference on Environmental Development (UNCED) in 1992, which aims to stabilize GHG concentrations in the atmosphere.

### Carbon in peatlands

Globally, peatlands cover an area of approximately 400 million hectare (ha) but store approximately a third of all the organic carbon found in the Earth's soils, estimated to be between 390 and 528 Pg (Pg = $1 \times 10^{15}$ g) [1,15–18]. This is equivalent to approximately 60–78% of the entire atmospheric carbon pool and is twice the amount of carbon found in the entire world's forest biomass [2,7,19]. This quantity is equal to the levels of carbon that would be emitted to the atmosphere from burning fossil fuels at the current annual global rate (~7 Pg in 2007) for the next 75 years [20]. Such figures mean that peatlands are the most efficient carbon stores of all terrestrial ecosystems and are second only to oceanic deposits as the Earth's most important stores [7].

Peatlands have been called 'unusual' ecosystems, in-terms of GHG scenarios, as although they sequester the GHG $CO_2$ from the atmosphere as peat, they also emit, in potentially large quantities, $CO_2$, methane and nitrous oxide [1]. This is due to complicated aerobic and anaerobic decomposition processes, which occur at varying rates, dependent on a number of environmental and biotic factors such as climate, chemical composition of the litter and nature and abundance of decomposing microorganisms [21,22]. A full analysis of these processes is beyond the scope of this article; however, Kayranli *et al.* have produced a comprehensive review of the relevant carbon cycles and pathways [5]. Although it is clear that more research is needed to gain a better understanding of the range of flux pathways (inputs and outputs) and the various forms of carbon (gases, solutes and particulates) involved in the carbon budget of peatlands [5,23], most research agrees peatlands have been a net sink of atmospheric carbon throughout the Holecene period to the present day [5,24–26].

It is feared the delicate balance between accumulation of peat and decay may cause peatlands to become net carbon sources following human interventions. Degradation of peatlands through drainage, fires, atmospheric deposition and exploitation (e.g., for fuel and horticultural products) are resulting in a growing source of anthropogenic GHG emissions. $CO_2$ emissions from peatland drainage, fires and exploitation are currently estimated to be at least 3,000 million tons per annum, or equivalent to more than 10% of the global fossil-fuel emissions [7].

Many researches also warn that a warmer, drier climate as is being predicted by some climate change models for many areas of the globe (especially over parts of Western Europe and North America), could lower the water table of some peatlands creating aerobic conditions in the peat matrix. This may affect the soil's biogeochemical process and lead to a rise in the rate of decomposition, increasing GHG emissions from the peatlands: switching more of them from being net-carbon sinks to carbon sources [27–37]. It is also feared that warmer temperatures will increase peatland permafrost thaw, diminishing the tundra's carbon-storage capacity [38,39].

### Peatlands in the UK

Estimates on the extent of UK peat vary from 1.5 to 5 million ha (~15% of the land area), depending on whether shallow peats, less than 1 m in thickness, are included. This equates to an estimated minimum of between 2,302 and 3,121 Mt of carbon (Table 2) [23,40].

Work by Billett *et al.* [23] suggests the carbon-accumulation rate for the UK's peatland is comparable to the original figure suggested by Gorham's [1] for Northern Hemisphere peatlands in general: approximately -23 g carbon $m^{-2}$ $yr^{-1}$ (negative fluxes indicate net uptake from the atmosphere), although the authors concluded when they considered all flux pathways, that the current accumulation rate of some of the UK's peatlands may be as high as -56 to -72 g carbon $m^{-2}$ $yr^{-1}$. However, large areas of the UK's peatlands have been subjected to land management practices that have negatively affected their capacity for carbon accumulation. Such activities include afforestation, drainage, prescribed burning, peat extraction, grazing, fertilization and liming [41]. Indeed, an estimated 1.5 million ha of the country's peat is believed to have been drained, with the practice of peatland drainage peaking in Britain in the 1970s [35,42]. A report by Natural England went as far as to say that only 1% of England's peats (>40 cm deep) can be considered 'undamaged' [43] and a changing climate may even result in a decline in the distribution of some of country's actively growing peatlands [44,45]. Fortunately, there are now many projects underway to restore areas of the UK's peatlands through restoration and rewetting projects, many of them thanks to Government polices and initiatives [40,43,46].

## The UNFCCC & the Kyoto Protocol

To understand how peatlands feature in national GHG monitoring schemes and their potential role in carbon markets, it is essential to understand the general mechanisms and main accounting systems of the United Nations Framework on Convention on Climate Change (UNFCCC) and the Kyoto Protocol. Once this has been reviewed, peatlands current and potential role will be considered.

### The UNFCCC

During the United Nations Conference on Environmental Development (UNCED) at Rio de Janerio in June 1992, an international environmental treaty was produced, named the UNFCCC. Its main objective: *"[The] stabilisation of greenhouse gas concentrations in the atmosphere at a level that would prevent dangerous anthropogenic interference with the climate system. Such a level should be achieved within a time-frame sufficient to allow ecosystems to adapt naturally to a climate change, to ensure that food production is not threatened and to enable economic development to proceed in a sustainable manner."* (Article 2 [47]).

**Table 1. Wetland definitions (taken from various sources).**

| Term | Definition |
|---|---|
| Wetland | ▪ Land with the water table close to or above the surface, or which is saturated for a significant period of time. Includes most peatlands but also ecosystems or mineral substrates, flowing and shallow waters |
| Peatland | ▪ 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). |
| Mire | ▪ All ecosystems described in English as swamp bog, fen, moor, muskeg and peatland, but often used synonymously with peatlands<br>▪ Includes all peatlands, but some mires may have mineral substrate<br>▪ Often used to describe an actively growing peatland |
| Fen | ▪ A mire, which is influenced by water from outside its own limits<br>▪ True fens or rich fens receive water that has passed through mineral soils and are known as minerotrophic peatlands<br>▪ Poor fens or transitional peatlands are known as mesotrophic peatlands and are between mineral-nourished (minerotrophic) and precipitation-dominated (ombrotrophic) peatlands |
| Bog | ▪ A mire, which receives water solely from precipitation. Some, such as raised bogs, are referred to as ombrotrophic peatlands and have developed peat layers higher than their surroundings |
| Marsh | ▪ Loose term usually referring to a fen with tall herbaceous vegetation often with a mineral substrate. Can also refer to saline wetlands (i.e., saltmarshes) |
| Swamp | ▪ Lose term with very wide range of usage. Usually referring to a fen and often implying forest cover |

The GHG considered by the UNFCCC to be the most important, due to their global warming potentials (GWPs), are $CO_2$, methane, nitrous oxide, sulphur, hexafluoride ($SF_6$), hydroflurocarbons (HFCs), and perfluorocarbons (PFCs).

### The Kyoto Protocol

A total of 192 Parties have currently signed up to the UNFCCC treaty, which came into force in 1994. However, the UNFCCC itself has no enforcement mechanisms to ensure stabilization of GHG and sets no legally binding limits on GHG emissions for individual countries, or Parties. Instead, the UNFCCC makes provision for updates, or protocols, to set mandatory emission levels (Article 17 [47]). To date, the principal update is the Kyoto Protocol, which was negotiated amongst 84 countries and was adopted in Kyoto, Japan, on 11 December 1997 entering into force on 16 February 2005. The detailed rules for the implementation of the protocol were discussed by the Conference of the Parties (COP) serving as a meeting of the Parties to the Kyoto Protocol (CMP) and finally agreed at the seventh conference of the Parties to the UNFCCC (commonly referred to as COP-7) in Marrakesh, Morocco, in 2001.

Table 2. UK peatland area and carbon storage.

| Country | Soil depth | | | |
|---|---|---|---|---|
| | Area (km²) | 0–100 cm (Mt carbon) | >100 cm (Mt carbon) | Total (Mt carbon) |
| Scotland | 17,789 | 1104 | 516 | 1620 |
| England | 4246 | 296 | 123 | 419 |
| Wales | 732 | 67 | 52 | 119 |
| Northern Ireland | 1873 | 90 | 54 | 144 |
| UK | 24,640 | 1557 | 745 | 2302 |

Data from [23].

These 'Marrakesh Accords' were formally adopted by the CMP at its first session in Montreal, Canada in December 2005 [48].

The major feature of the Kyoto Protocol is that it sets binding targets for 37 industrialized countries and the European Community, known as Annex I Parties, for reducing GHG emissions. These amount to an average of 5% against 1990 levels over the 5-year period 2008–2012 (known as the first commitment period) for the three most important gases – $CO_2$, methane and nitrous oxide [49].

Annex I Parties have to account for their total GHG emissions from a number of sources listed in Annex A to the Kyoto Protocol. These include GHG emissions from energy and industrial process, solvent and other product use, agriculture and waste sectors [49]. Each Annex I Party has a specific emissions target detailed in Annex B of the Kyoto Protocol, which is set relative its GHG emissions from the base year: 1990 for $CO_2$, methane and nitrous oxide and either 1990 or 1995 for $SF_6$, HFCs and PFCs. A Party's reduction target is expressed as levels of allowed emissions, or assigned amounts, over the 2008–2012 commitment period. These allowed emissions are divided into assigned amount units, each of which represents an allowance to emit 1 metric ton of $CO_2$ equivalent (t $CO_2$ eq or $CO_2$ equivalency).

The Kyoto Protocol not only establishes the GHG-reduction levels for those nations defined as Annex I Parties (generally the more developed countries), but it also outlines the basis of how these targets could be attained, stating in Article 3 paragraph 3: "*The net changes in greenhouse gas emissions by sources and removals by sinks resulting from direct human-induced land-use change and forestry activities, limited to afforestation, reforestation and deforestation since 1990. measured as verifiable changes in carbon stocks in each commitment period, shall be used to meet the commitments under this Article of each Party included in Annex I.*" [49]

Key terms

**$CO_2$ equivalency:** the amount of $CO_2$ that would have the same global warming potential over a specific time period (usually 100 years) for a given amount and type of another GHG (e.g., methane). The term is often abbreviated to $CO_2$-e

The main source referred to in this article relates to the emissions from fossil fuel consumption and Annex I Parties must meet their targets primarily through national reduction measures; for example, investing in less polluting technology. However, Parties must also take measures to protect and enhance emission removals from specific ecosystems that sequester carbon from the atmosphere, referred to as land use, land-use change and forestry (LULUCF). Article 3 paragraph 3 of the protocol refers to direct, human-induced, afforestation, reforestation and deforestation activities and accounting for these is mandatory for Annex I Parties. However, Article 3, paragraph 4, describes other activities, for which accounting is optional, stating: "*The Parties to this Protocol shall, at its first session or as soon as practicable thereafter, decide upon modalities, rules and guidelines as to how, and which, additional human-induced activities related to changes in GHG emissions by sources and removals by sinks in the agricultural soils and the land-use change and forestry categories shall be added to, or subtracted from, the assigned amounts for Parties included in Annex I.*" [49]

Once an Annex I Party decides to account for one of these LULUCF activities, which include cropland management, grazing land management and/or revegetation, it must continue to do so for the full commitment period [41]. In contrast to emissions from Annex A sources, the protocol requires Parties to account for emissions and removals from LULUCF activities by adding to or subtracting from their initial assigned amount. Net removals from LULUCF activities result in additional emission allowances called removal units, which a Party may add to its assigned amount, and a Party must account for any net emissions from LULUCF activities by canceling Kyoto units. The quantity of emission allowances issued or cancelled is subject to specific rules, which differ for each LULUCF activity [48,49].

Annex I Parties can also add to or subtract from their initial assigned amount – and therefore raise or lower the level of their allowed emissions over the commitment period – by trading Kyoto units with other Parties in accordance three market-based mechanisms.

These Kyoto mechanisms are:

- Emissions Trading (ET): Article 17;
- The Clean Development Mechanism (CDM): Article 12;
- Joint Implementation (JI): Article 6.

### CDM & Joint Implementation

Joint Implementation (JI), outlined in Article 6 of the Kyoto Protocol, is a project-based mechanism whereby one Annex I Party with a higher-than-average emission reduction costs is able to invest in cheaper emission reduction measures in another Party and receive at least partial

credit for in any emission reduction resulting from the investment [50]. The unit associated with JI is called an emission-reduction unit and they are converted from existing amount units and removal units before being transferred. It is important to note that JI does not affect the total assigned amount of Annex I Parties collectively; instead, it redistributes the assigned amount among them.

Defined in Article 12 of the Kyoto Protocol, CDM is another project-based mechanism that allows Annex I Parties to implement an emission-reduction project in developing countries. Such projects must follow strict guidelines and accounting requirements to ensure any reductions or removals associated with projects are additional to what would otherwise occur in the absence of the projects. If successful, projects can earn saleable certified emission-reduction credits, since. unlike emission trading and JI, emission-reduction credits increase both the total assigned amount available to the relevant Annex I Party and their allowable level of emissions. This mechanism is seen by many as pioneering since it is the first global, environmental investment and credit scheme of its kind. For any of these projects to be eligible the emission reduction or enhancements they produce must be *"additional to any that would otherwise occur"* [51].

### ▪ Emissions trading

Emissions trading, as set out in Article 17 of the Kyoto Protocol, allows countries that have emission units to spare (emissions permitted to them but not 'used') to sell this excess capacity to countries that are over their targets [49]. This has therefore created a new commodity in the form of emission reductions or removals. Annex I Parties can create domestic or regional (between a group of Parties) schemes for emissions trading. These are subject to Kyoto Protocol rules laid out in Article 17 of the Protocol and Decision 11 of the first session of the CMP [49].

Since $CO_2$is the principal GHG, emission trading operating under the Kyoto Protocol umbrella is generally referred to as trading in carbon and units of emission reductions are equal to 1 ton of $CO_2$ – giving the popular term, 'carbon credit.' Carbon is now tracked and traded like any other commodity and this is known as the 'carbon market'. However, it is important to note that unlike other commodities 'carbon' is a politically generated and managed market with no physical commodity, only a dematerialized allowance certificate [52].

### ▪ The EU GHG Emission Trading System

Under the Kyoto Protocol, the EU agreed to an 8% reduction in their GHG emissions, compared with the levels in 1990. The EU aims to achieve its target by distributing different rates among its member states. The EU has also offered to increase its emissions reduction to 30% by 2020, on the condition that other major emitting countries in the developed and developing worlds commit to do their 'fair share' under a future global-climate agreement [101].

In 2000, the European Commission launched the European Climate Change Program (ECCP) in order to identify and develop all the necessary elements and strategies needed to implement the Kyoto Protocol. This has led to the adoption of a wide range of new policies and measures, including the EU Greenhouse Gas Emission Trading System (EU ETS). Based on the European Emissions Trading Directive on 13 October 2003, the EU ETS began operation on January 2005 and is the largest multi-national GHG emission trading system in the world [53].

The EU ETS only applies to combustion installations with a net heat supply in excess of 20 MW, such as industrial power stations, oil refineries, coke ovens, iron and steel plants, and factories making cement, glass, lime, brick, ceramics, pulp and paper [53]. These industries (totaling approximately 11,500 installations) are believed to be responsible for approximately a third of the EU's GHG emissions and approximately 45% of the EU's $CO_2$ emissions [54]. Each participant is allocated a certain quantity of emission allowances in accordance with their historical emissions, minus a specified reduction commitment amount. Each emission allowance entitles the Party in question to emit 1 ton of $CO_2$ (as in the Kyoto Protocol) within a specified period. At the end of this obligation period, the Party must demonstrate that the extent of its emissions are covered by its emission allowances. Parties may acquire additional emission allowances, either by purchasing them from other participants in the EU ETS or by carrying over any remaining credit from one obligation period to the next. Those who emit more than their allowance have to make up the shortfall by acquiring the necessary allowances from other market participants. In this way, emissions trading gives market participants the flexibility to fulfill their reduction commitment either by their own efforts or through the purchase of additional reduction certificates [54].

Allowances traded in the EU ETS are held in accounts in electronic registries set up by member states of the EU, although all of these registries are overseen by a Central Administrator at EU level who, through the Community Independent Transaction Log, monitors each transaction for any irregularities. In this way, the registries system keep track of the ownership of allowances in the same way as a banking system keeps track of the ownership of money.

This type of scheme is often referred to as 'cap-and trade' scheme because there is as an agreed aggregate cap on a particular source's accepted level of pollution. These sources are then allowed to trade amongst themselves to determine which can produce the emissions

and at what levels. For countries and companies in the EU, which is bound by the Kyoto Protocol, these types of schemes are mandatory with penalties for those not abiding to them. However, the EU ETS is not the only mandatory emission trading market, and countries such as the USA, South Korea, Japan, Australia and Canada are in varying stages of developing their own [55].

Many of the initiatives of EU ETS are developments and extensions of the emission permit trading system of the 1990 Clean Air Act Amendments (1990 CAAA) aimed at regulating sulfur dioxide ($SO_2$) emissions from US thermal power plants. The system, of harnessing the forces of economic markets to achieve cost-effective environmental protection, proved incredibly effective in the US with 100% compliance in reducing $SO_2$ emissions [56,57].

There is also a rapidly growing 'voluntary' emission trading market aimed at businesses and even individuals looking to offset the GHG they produce. The units of trading in the voluntary sector are still equivalent to 1 ton of $CO_2$ and are known as verified emissions reductions. Anyone can participate in the market and voluntary offset schemes can be defined as those aimed at generating GHG emission reductions not required by Kyoto Protocol's derived regulation. Units are generated by projects that are assessed and verified by third party organizations, rather than through the official channels of the UNFCCC [58]. There have been recent improvements in the standardization of the registries that monitor these verified emissions reductions in order to increase trust in the system [55]. In a review of the differences between the voluntary and mandatory markets, Benwell states there are many reasons why Parties would become involved in voluntary schemes and these include a genuine wish to reduce emissions; marketing opportunities from the 'carbon neutral' or 'green' image; shareholder and/or institutional pressure; and threat of future climate litigation [55] and a wish to potentially influence future regulations. However, it can be argued that the distinction between the two types of market are often blurred and the term 'voluntary' is misleading, since although Parties are not forced into entering into a scheme, they are nevertheless enforced by law. The author concludes that both schemes are vital for the reduction of GHG emissions and calls for more hybridization and the divide between the two to be abandoned.

### ▪ The Kyoto accounting system

At the end of the Kyoto Protocol's commitment period, the total emissions by each Annex I Party will be compared with its initial assigned amount to check if it has fulfilled its commitment. Each Party's available assigned amount is equal to its initial assigned amount, plus any additional Kyoto units acquired from other Parties through the Kyoto mechanisms, or issued for net removals from a LULUCF activity, minus any units transferred to other Parties or cancelled for net emissions from a LULUCF activity (Decision 13/CMP.1, Annex, Paragraphs 11, 12 and 14 [102]). Each Annex I Party is therefore required to have established and maintained a national GHG inventory and a registry to track its holdings of and transaction of Kyoto units. These are subject to numerous review and compliance procedures (Article 7, 5, 8 and 18 [49]). The Intergovernmental Panel on Climate Change (IPCC) – an independent body made up of the world's leading scientists and experts who report directly to the UNFCCC – prepared guidelines for the format and accounting methodologies of national GHG inventories (GHGI) [59].

### ▪ Peatlands in the Kyoto Protocol

The IPCC's guidelines for an Annex I Party's GHG monitoring and accounting provides a broad range of emission factors that can be used to estimate emissions from different activities. The emission factors that they provide are called Tier I and, because they are guidelines for all countries, are generalizations that apply to broad climate zones. However, the IPCC recommends that Parties should develop more detailed Tier II emission factors or Tier III modeling approaches that are relevant to their individual circumstances.

Chapter 7 of the IPCC National GHGI states that emissions from peatlands that are managed for forestry, grazing, cultivation, extraction or development should be accounted for. A series of Tier I emission factors are provided for the main GHG emissions created through different activities in temperate peatlands. Some broad guidelines are also laid out for Tier II and III emission factors although the IPCC confess knowledge and literature on the subject is 'sparse' [60].

Under Article 3 of the Kyoto Protocol, Annex I Parties have the option to include emission reductions delivered through a range of land management activities (e.g., LULUCF), such as the improved management of existing forests, increasing soil-carbon sequestration and restoration of degraded land. Peatland restoration is not explicitly included in the list of optional activities in Article 3.4 of the protocol; therefore, any emission savings delivered by peatland restoration would not count towards meeting a Party's Kyoto commitment.

However, in 2009, at the UNFCCC's Conference of the Parties meeting in Copenhagen (COP-15) it was agreed in principle that Parties could voluntarily include 'wetland re-wetting' as a LULUCF option in any post-2012 international protocol. In 2010, at COP-16 in Cancun, Mexico, it was further agreed that in any future climate agreement, it should be possible for

Parties to reduce their emissions by rewetting drained peatlands, identified as sinks of GHG (Agenda item 3 [103]). However, negotiators could not agree on the full details and it was not determined whether accounting for peatland drainage would be mandatory or voluntary. Further discussions are therefore expected at COP-17 in Durban, South Africa, in 2011, to clarify the guidelines relating to LULUCF and peatlands in the second commitment period of the Kyoto Protocol (e.g., after 2012) [104]. Such decisions will affect GHG emission-monitoring schemes under the national GHGI of signatories to the Kyoto Protocol, and will become nationally appropriate mitigation actions (NAMAs) if managed correctly [43].

In the UK, the Department of Energy and Climate Change (DECC) is responsible for the delivery of the Government's GHGI to the UNFCCC, in accordance with Article 5 of the Kyoto Protocol and European commission decision, in order to comply with the UK and EU's Kyoto Protocol commitments [61]. Currently, peatlands are classified under the grassland category of land use in the GHGI and numbers on emissions from peatlands only include extraction of peat for horticultural use, oxidation of drained lowland raised bogs and nitrous oxide emissions from the cultivation of histosols.

### ▪ Reducing emissions from deforestation and forest degradation

Peatlands were also given support at the Cancun climate talks through an agreed text for the reducing emissions from deforestation and forest degradation (REDD) mechanism, which could offer opportunities for the protection and restoration of peatlands in developing countries such as Indonesia, Malaysia and Papua New Guinea [105].

Reducing emissions from deforestation and forest degradation is a scheme proposed at COP-13 in Bali, Indonesia, in 2007, which aims to offer financial incentives for developing countries to reduce GHG emissions by better management of forest resources [62]. Such projects will create quantifiable units, similar to current CDM projects, which can be used in a form of carbon market in the second commitment period of the Kyoto Protocol. REDD Plus (REDD+) moves REDD forward, following discussions and decisions by interested Parties at recent COP meetings and includes the role of conservation, sustainable management of forests and enhancement of forest carbon stocks.

Indonesia, which has 21 out of the 25 million hectares of peatland in Southeast Asia [63], is developing demonstration activities involving peatlands for testing the potential of a global REDD-carbon market. The Indonesian Ministry of Forestry, which is managing the activities, are being given financial and technical support from a number of developed countries as well as the World Bank, [64]. However, more work is needed to create adequate guidelines to assess the emissions from peatlands with the necessary accuracy, because to be tradable under REDD or any other mechanism, GHG emission reductions have to be *"results-based, demonstrable, transparent and verifiable, and estimated consistently over time"*, as stated at COP-13 [106]. This is a major challenge in terms of developing the necessary accounting and trading systems (Table 3).

## Peatlands' future role

Following the decisions at COP-15 and COP-16, it is clear that GHG emissions from peatlands are to feature more prominently in the Kyoto Protocol and any successor for the second commitment phase. For Annex I Parties, there are two main areas where these ecosystems could prove beneficial: carbon offsetting and in national GHGI.

### ▪ National GHGI

A report by International Union for Conservation of Nature (IUCN) Peatland Programme [107] suggested that the UK's peatlands could be delivering over 3 million tons of $CO_2$ sequestration per annum. However, the report estimates that 10 million tons of $CO_2$ per year are being lost to the atmosphere from the country's damaged peatlands, equating to 3–30 tons per ha per year, depending on how badly the site is affected. The IUCN suggests that there is sufficient evidence to show it is possible to halt significant amounts of this loss from peatlands through habitat restoration (e.g., rewetting). Indeed, 1.5 million tons of $CO_2$ emissions are already expected to be saved by 2015 as a result of the UK Biodiversity Action Plan's target to restore 845,000 ha of blanket bog.

Although these figures may be useful as a benchmark, continued research in this area is essential. Any increased methane emissions associated with rewetting are likely to be small in relation to the overall GHG losses from a damaged peatland [65]. Although again, more research is needed in this area, as Baird *et al.* conclude; restoration of a peatland does not necessary result in it becoming a carbon sink [66]. The IUCN argue that such peatland restoration projects are a cost-effective means of addressing climate change, compared with other carbon-abatement methods, such as afforestation and renewable energy. Summarizing their findings the report states, *"Restoring peatlands can be considered a natural form of carbon capture and storage, preventing release of carbon from damaged bogs and preserving it for potentially millions of years."*

Writing in 2009, the authors of the report highlighted that the methodologies for national GHG inventory accounting systems, under the Kyoto Protocol, did

not fully address the GHG emission savings from peatland restoration. As a result of decisions at COP-15 and COP-16, this is likely to change and it is hoped that a further ruling on this will be made at COP-17.

It is necessary to clarify that although there may be some dispute in IUCN's figure of peatlands storing carbon for millions of years, owing to the glacial-interglacial patterns, the reasoning of the authors is still valid: peatlands can potentially store and capture carbon for a period of time, which may prove beneficial in stabilizing the amounts of GHG in the atmosphere.

**Table 3. Key dates relating to peatlands in the Kyoto Protocol and climate change legislation**

| Key Dates | Event |
|---|---|
| 1992 | **Rio de Janerio, Brazil** The United Nations Conference on Environmental Development (UNCED) produce the international environment treaty, the United Nations Framework on Convention on Climate Change (UNFCCC) |
| 1994 | UNFCCC treaty comes into force |
| 1997 | **Kyoto, Japan** Signatories of the UNFCCC devise the Kyoto Protocol, which sets out mandatory emission levels for Parties |
| 2001 | **Marrakesh, Morocco** Detailed rules for implementation of Kyoto Protocol discussed at the seventh UNFCCC's COP or COP-7. Rules become known as Marrakesh Accords |
| 2000 | European Commission launches European Climate Change Programme (ECCP) to identify and develop necessary strategies to implement Kyoto Protocol |
| 2005, January | European Union GHG Emissions Trading Scheme (EU ETS) begins operation |
| 2005, February | Kyoto Protocol comes into force |
| 2007 | **Bali, Indonesia** At UNFCCC's COP-13, Reducing Emissions from Deforestation and Forest Degradation (REDD) is proposed |
| 2008 | First commitment period of the Kyoto Protocol starts Parties to show that during this period they have reduced their GHG emissions by an agreed amount |
| 2009 | **Copenhagen, Denmark** At UNFCCC's COP-15, agreement in principle that Parties can voluntarily include wetland re-wetting as a land use, land-use change and forestry (LULUCF) option in any post-2012 international climate-change protocol |
| 2010 | **Cancun, Mexico** At UNFCCC's COP-16 further agreement for Parties to include any emission reductions created through peatland re-wetting (now classified as GHG sinks) in future climate legislation |
| 2011 | **Durban, South Africa** UNFCCC's COP-17 to further discuss the role of peatlands in future post-2012 climate legislation |
| 2012 | First commitment period of the Kyoto Protocol ends |

COP: Conference of the Parties.

Clearly, for peatland restoration to feature in any national GHG inventory, monitoring of the activities must be measurable, reportable and verifiable (MRV), ensuring that it is possible for the results of individual actions to be quantified and reported in a consistent and transparent way [108]. This will allow for appropriate verification, by a third party review, and ensure adequate information is available to assess progress against the objectives of the UNFCCC and the guidelines of the Kyoto Protocol. Unfortunately, for various reasons, such as the diversity of peatlands and climatic conditions where they occur, as well as the variability of parameters (e.g., weather and vegetation) that control GHG emissions seasonally and between years, assessment of GHG fluxes from peatlands is often more complicated than monitoring industrial processes and even forest management practices. A report by Joosten and Couwenberg concluded that despite the difficulties associated ensuring peatland restoration projects are MRV, affordable methodologies will be available for reliable baseline setting and monitoring in a post-2012 climate framework [108]. This, they claim, will allow inclusion of peatland conservation and rewetting in climate policies. Such methodologies could involve applying various proxies (e.g., waterlevel and vegetation) to GHG flux models. The models themselves are calculated with flux measurements from techniques including the chamber method, which usually enables measurements on a scale of up to 1 $m^2$; or eddy-covariance, which can assess GHG fluxes over larger areas (typically 1 $km^2$).

If Annex I Party are able to include both the negative fluxes of GHG from existing peatlands and the emission reductions from restoring peatlands in LULUCF of their national GHGI, it could help reduce the amount of emission cuts, and therefore investment, they have to make from other sectors, such as industry. Figure 1 demonstrates how LULUCF activities are added to the initial assigned amount units, along with units obtained from the three Kyoto mechanisms, to ensure a Party is in compliance of its Annex A emission targets, in accordance with Article 3 paragraph 1 of the Kyoto Protocol. With relatively large areas of peatland, the UK could benefit from this scheme, as well as other Annex I countries such as Canada and Finland.

### ▪ Carbon offsetting

The use of ecological restoration projects to offset GHG emissions has been considered for over 20 years and this can work alongside cap-and-trade schemes, such as the EU ETS. The first official biosequestration project involved reforestation in Guatemala in 1988, where one of the world's largest power companies, Applied

not fully address the GHG emission savings from peatland restoration. As a result of decisions at COP-15 and COP-16, this is likely to change and it is hoped that a further ruling on this will be made at COP-17.

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Energy Services, provided US$2 million for the planting of 50 million trees in the Western Highlands region of the Central American country. Applied Energy Services, won regulatory approval for the project which they planned would offset the $CO_2$ emissions of their coal-fired power station in Connecticut, USA, over its 40-year life span, somewhere around 14.1 million tons of carbon [67,68].

Ecological restoration projects using peatlands for biosequestration could be a financially sound method for creating carbon credits to be used in carbon markets. There are a growing number of both mandatory and voluntary markets that include biosequestration projects (Table 4). The Kyoto Protocol's CDM and any similar mechanisms likely to feature in the protocol's successor (e.g., REDD, are the most important to the UK. As previously stated, discussions at COP-15 and COP-16 highlighted the importance of peatlands in such mechanisms. Decisions at COP-17 could see investment in peatland conservation and restoration in countries such as Indonesia, for the creation of Kyoto and REDD emission units.

There is some concern that allowing terrestrial sinks as an offset to emissions by developed countries removes some of the responsibility, at least temporarily, for dealing directly with the problem of fossil fuel GHG emissions. This may result in further delays in dealing with the main GHG sources, or as Grubb *et al.* said, it could become a *"distraction from the fundamental goals of sustainable development"* [69]. However, Roulet argued that if the UNFCCC's objective is taken at face value and the stabilization of GHG concentrations is the ultimate goal, then all sinks and sources, regardless of their origin and national implications, should be accounted for and included in mitigation actions [70].

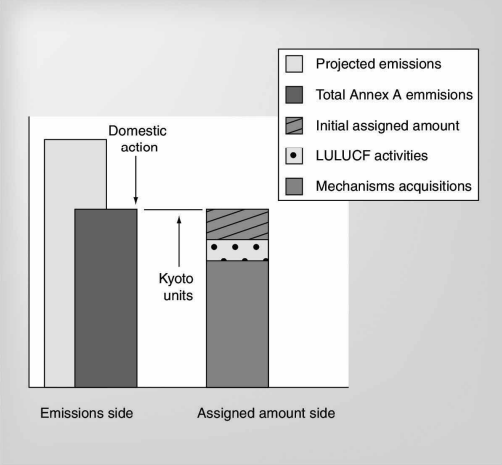


**Figure 1. Determination of an Annex I Party's compliance with Article 3, paragraph 1, of the Kyoto Protocol.**
LULUCF: Land use, land-use change and forestry.
Reproduced with permision from the UNFCCC [48].

**Table 4. Carbon market schemes that include biosequestration projects.**

| Project | Project locations | Project developers |
|---|---|---|
| **International mandatory compliance schemes** | | |
| Clean Development Mechanism | Non-Annex I countries | Mostly large carbon service companies |
| **Mandatory cap-and-trade schemes with offsets** | | |
| Regional GHG Initiative (RGGI) | Nebraska, USA | Not yet established |
| GHG Abatement Scheme (GGAS) | New South Wales, Australia | Carbon service companies |
| **Voluntary cap-and-trade schemes with offsets** | | |
| Chicago Climate Exchange (CCX) | Restrictions where mandatory trading occurs | Carbon service companies and NGOs |
| **Voluntary GHG schemes** | | |
| Climate Action Reserve (CAR) | California, USA | Carbon service companies and government agencies |
| Climate, Community and Biodiversity (CCBS) | No restrictions | NGOs |
| Plan Vivo | Developing countries | Local NGOs |
| Voluntary Carbon Standard (VCS) | Restrictions where mandatory trading occurs | Large carbon service companies |
| Verified emissions reductions+ | No restrictions | Carbon service companies |
| Voluntary Offset Standard (VOS) | Restrictions where mandatory trading occurs | Carbon service companies |

NGO: Non-governmental organization.
Data from [67].

Energy Services, provided US$2 million for the planting of 50 million trees in the Western Highlands region of the Central American country. Applied Energy Services, won regulatory approval for the project which they planned would offset the $CO_2$ emissions of their coal-fired power station in Connecticut, USA, over its 40-year life span, somewhere around 14.1 million tons of carbon [67,68].

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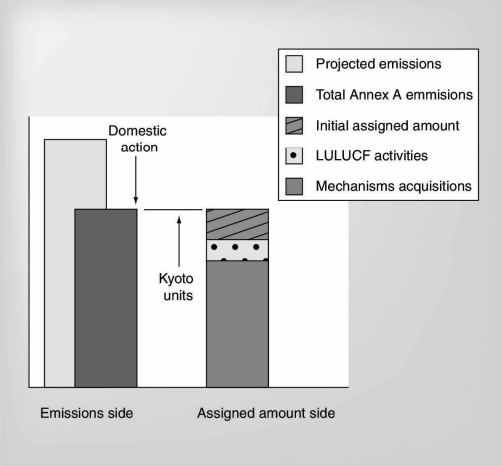


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Key term

Carbon stewardship: Management of an ecosystem to maximize carbon sequestration.

## Future perspectives

For Annex I Parties such as the UK, management of peatland areas to specifically maintain and increase the carbon stocks found in their waterlogged organic soil, is set to become a progressively important aspect of GHG emission monitoring and accounting. Such carbon stewardship is likely to be a cost-effective activity in helping to meet national emission commitments under any post-2012 successor to the Kyoto Protocol. Work by Worrall *et al.* compared the market price of carbon to the cost and effectiveness of different peatland management practices, such as the cessation of grazing, revegetation and blocking of drainage ditches [71]. It was found that, depending on the price of carbon and the restoration technique used, restoring certain areas of the UK's peatland could prove profitable, creating carbon credits. The $CO_2$ equivalency units created though could be incorporated into a LULUCF scheme for a GHG inventory, instead of being used in an emissions market. It is important to note that Worrall *et al.* concluded that no single management technique is best for the carbon stewardship of all peatlands. To maximize their effectiveness as carbon sinks, targeted actions are required: combining several management practices, an issue also raised by Ostle *et al.* [72]. It is important to note that Worrall *et al.* argued the profitability of using peatlands in carbon offsetting schemes heavily depends on the price of carbon.

Land use, land-use change and forestry itself has already been improved to give more significance to peatlands, which could help such carbon stewardship projects. Volume 4 of the 2006 IPCC Guidelines refine LULUCF to include GHG emissions from agriculture, resulting in the agriculture, forestry and other land use sector. The IPCC say this change recognizes that land-use changes can involve all types of land and the new guidelines are intended to improve *"consistency and completeness in the estimation and reporting of greenhouse gas emissions and removals"* [60]. One of the principal changes is the recognition of GHG emissions, specifically $CO_2$ and 'non-$CO_2$' emissions (e.g., $N_2O$, from managed peatlands).

If CDM and REDD specifically take into account the role of peatlands, investment into projects in countries such as Indonesia could see areas of wetlands conserved and restored. The benefits of this may well go beyond curbing the worldwide increase in GHG emissions. Such ecosystems help clean polluted waters, stabilize water systems and support a host of unique flora and fauna, all of which may create further economic and environmental benefits [73]. If land owners can make more money from preserving an area of peatland, through a CDM or REDD project, they are less likely to develop the land for purposes such as palm oil plantations, which are major GHG emitters [74]. However, for this to become a reality, it is essential that the revenue derived from peatland management and restoration competes with activities such as palm oil agriculture. If the created credits are then tradable and incorporated into the proposed compliance markets (rather than voluntary markets) it will help ensure a stable supply of funds at higher carbon prices, and allow projects to be buffered against uncertainties and fluctuations in related markets, such as the price of palm oil [75].

As discussed, there are issues that still need to be overcome to ensure any GHG emission-reduction projects are measurable, reportable and verifiable. However, our understanding and skills for measuring the peatland carbon cycle are continuously improving, and are likely to increase dramatically if peatland restoration becomes attractive to large investors.

So how much money could the world's peatlands be worth? A recent study by The Economics of Ecosystems and Biodiversity (TEEB) speculated the value of inland wetlands (e.g., peatlands such as bogs, fens and swamps) at between US$1,000–45,000 per ha per year [109]. If it is assumed peatlands cover an area of approximately 400 million ha [18] this equates to a total value of the world's peatlands of between US$400,000 million–18 billion. However, TEEB did not fully take into account recent and pending UNFCCC decisions. It is highly likely that the introduction of a carbon market and accounting system involving peatlands will see this value rise even further.

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*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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## Executive summary

**Peatlands**

- Peatlands are a type of organic wetland and are the largest terrestrial store of carbon owing to the build-up of semi-decomposed plant material.
- Peatlands are estimated to contain 455 Pg of carbon – two-thirds of the amount present in the atmosphere.
- It is feared that anthropogenic degradation of peatlands may switch the ecosystems from net carbon sinks to net carbon sources.
- Land management practises, such as re-wetting, can reduce GHG emissions from damaged peatlands.

**Current legislation**

- The United Nations Framework on Convention on Climate Change (UNFCCC) Kyoto Protocol aims to reduce Annex I Parties' main GHG emissions over the first commitment period (2005-2012) by agreed amounts from base year (1990) levels.
- The UNFCCC have created market-based mechanisms to help Annex I Parties achieve these targets.
- Despite the amounts of labile carbon stored in peatlands, the ecosystems and their management do not feature prominently in UNFCCC accounting legislation.
- Decisions at COP-15 and COP-16 raised the profile of peatlands, highlighting their importance in global GHG emissions.

**Future perspective**

- Peatlands look set to become important factors in UNFCCC GHG inventories, reducing emissions from deforestation and forest degradation plus and agriculture, forestry and other land use sectors.
- Targeted management of peatlands in both Annex I and Annex II Parties could lead to the creation of accredited carbon credits.
- If profitable, carbon stewardship could help prevent the loss and destruction of peatlands.
- The mandatory inclusion of peatlands in national GHG inventory schemes and accredited carbon markets could see their ecosystem value rise from the previously estimated US$18 billion.

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- ▪▪ of considerable interest


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## Appendix 3

# The need to consider geoengineering techniques using peatlands

WSP
June 2012
SECTION 2

SWS FYI

## The need to consider geoengineering techniques using peatlands

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The co-founder of the Nobel Prize-winning IPCC, Sir John Houghton, claims global warming represents the "single greatest threat mankind has ever faced" (Houghton, 2010). Such assertions suggest it is the obligation of world leaders and scientists to help safeguard the lives of the planet's seven billion people. Any "war on climate change" may mean making decisions and implementing actions which would be unnecessary and even un-palatable in "peace-time".

As it is widely accepted that anthropogenic increases in the production of greenhouse gas (GHG) emissions are the major contributing factor to current global warming, the obvious and, arguably, most important action should be a drastic rethink in our consumption and use of the world's resources (IPCC, 2007). However, it is feared that emissions will not be reduced at the rate or magnitude required to prevent some of the more apocalyptic climate predictions from becoming reality (Royal Society, 2009). Something must therefore be done now to reduce the amounts of GHGs in our atmosphere.

In a lecture last year Professor Chris Freeman suggested several geoengineering techniques to harness and improve the carbon sequestering characteristics of peatlands as a way of removing significant amounts of these excess GHGs (Freeman, 2011). Indeed, by increasing the concentrations of phenolics in peat soils Freeman predicts an extra 1.7x1015 g yr-1 of carbon can be sequestered - equivalent to around one-and-a-half times the current emissions produced by transport. Methods to do this include storing phenolic material in the peatlands themselves and using genetically modified Sphagnum species to amplify phenol production. Like all geoengineering techniques, because of the expense and any potential unforeseen consequences, the hope is they will never need to be used on a large scale due to a global census to reduce GHG emissions. However, a detailed report by the Royal Society (2009) concluded that further research into "low risk" geoengineering methods should be undertaken in case their

implementation is needed within this century. This should include "carefully planned and executed experiments". Using the criteria laid out by the Royal Society the techniques suggested by Freeman are likely to fall into the "low risk" geoengineering category; they are also likely to be cheaper and more cost effective than techniques such as space reflectors and mechanical carbon dioxide removers.

Responding to the ideas in Freeman's lecture, Runkle (2012) gave a well-argued call for caution in the use of peatland geoengineering techniques. I wholeheartedly agree with many of the issues raised in Runkle's discussion, in this publication. However, subtle modifications of peatland ecosystems and use of only restored peatlands for carbon sequestration projects (as Runkle suggests) may not remove the significant amounts of carbon from the atmosphere that could otherwise be achieved. Clearly this is only an issue if we accept some of the more dire of global warming predictions, but can we afford not to take these threats seriously and at least start to investigate all preventative avenues? As Runkle points out, the history of ecological interventions is littered with unintended consequences and any research into geoengineering techniques must do all it can to prevent falling into this trap too. It has even been mooted geoengineers should sign a form of Hippocratic oath before undertaking research because of the potential for widespread harm (Lovelock, 2008).

As an admirer of our planet's unique peatland ecosystems, do I want to do anything that could damage them? No; but the debate on whether we should use peatlands in geoengineering presents a serious moral dilemma which the wetland and biogeochemistry community must answer: *should we leave our pristine peatlands untouched, while average global temperatures continue to rise, if they have the potential to significantly reduce GHGs levels*? I acknowledge that we must ensure our most important peatlands continue to be protected and conserved to some level, but it is essential to realise we may have the ability to prevent the catastrophic effects of climate change that have been predicted. So should we stand by and wait for unprecedented political change or another area of science to come to the rescue? I would argue not. We as wetland scientist have a duty to care for our valuable peatland environments, but we also have a duty to formulate a "Plan B" for the planet, that could be put in place quickly and effectively should the need ever arise.

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