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The role of molecular weight in the enzyme-inhibiting effect of phenolics: the significance in peatland carbon sequestration

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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 water-logged peat soils suppressing the activity of phenol oxidase; one of the few enzymes capable of breaking these inhibitors down (a role often referred to as the "enzymic latch"). 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 the importance of the molecular weight of phenolics on levels of inhibition of microbial decomposition in peat. We found the higher the molecular weight, of a phenolic compound, the greater its inhibitory effect on the breakdown of organic matter.

1. 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., 2008). 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 (Fenner and Freeman, 2011; Freeman et al., 2001a; Freeman et al., 2001b).

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 of 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 decaying processes of soil organic matter (SOM) (Freeman et al., 2001b; Wetzel, 1992). Extracellular enzymes, such as hydrolase enzymes which are produced by a range of bacteria (Fenner et al., 2005) 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

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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).

We suggest that supplementing peat soil with phenolics using a range of different molecular weight phenolic compounds will provide information on differences in their inhibitory effect on extracellular hydrolase enzymes in peat slurries taken from an ombrotrophic blanket bog in the UK. This, we hypothesise, will modify rates of OM breakdown changing gaseous effluxes of carbon-based trace gases, namely the CO_2 and CH_4 released from microbial respiration.

2. Methods

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 460 m 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 2400 mm and in the peat the water table is usually within 100 mm of (and often at) the ground surface. The study area has a pore water pH, at a depth of 100 mm, in the range of 4.1–5.1. The mean peat depth across the site is 2000 mm (Evans et al., 2012). Peat for the experiments were collected between June and September (summer). Soil samples (>200 g), from a depth of 50–100 mm, 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 cool, 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 h of collection.

A series of 1-1 peat-solution slurries were created in 50 mL centrifuge and consisted of 10 g samples of peat (which had previously been homogenised by hand for 10 min) and 10 mL 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 (e.g. 50 g in 1000 mL): cinnamic acid (molecular weight, M_W; 148.16 daltans, Da), gallic acid (M_W 170.12); flavone (M_W 222.24), tannic acid (M_W 1,701.20), sodium (Na) lignosulphonic acid (M_W 8000), Na lignosulphonic acid, (M_W 12,000) or calcium (Ca) lignosulphonic 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.

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 h 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.

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 h. Fluxes were calculated with an initial background gas sample (T-1) being collected from inside the centrifuge tubes containing the slurries, before lids-with a size #9 Suba-Seal rubber septa (Sigma Aldrich Ltd, Dorset, UK) through their centre-were secured onto the tubes, forming an air-tight seal. Sixty minutes later a 10 mL gas sample (T-2) was taken from the headspace of the centrifuge tubes through the septum–after a series of preliminary tests showed that CO_2 and CH_4 concentrations increased linearly for up to 60 min.

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 (1.83 m × 3.18 mm) 80/100 column. The column oven temperature was set to 40 °C and the carrier gas, oxygen free nitrogen, had a flow rate of 30 mL min^{-1}. The temperature of the methaniser was 300 °C and the FID was at 125 °C. The latter had a hydrogen flow of 25 mL min^{-1}, 20 mL min^{-1} make-up gas (oxygen-free nitrogen) and 300 mL min^{-1} of compressed air. The methaniser had a hydrogen flow of 5 mL min^{-1}.

The activity of a range of hydrolase enzymes (β-D-glucosidase, sulphatase, β-D-xylosidase, N-acetyl-β-D-glucosaminidase and phosphatase) were calculated from a series of peat slurries made with varying concentrations of Ca lignosulphonic acid using the protocol as outlined by Dunn et al. (2014). These slurries were made and stored in the same way as described before, and were left for 24 h before the assays were conducted. Ca lignosulphonic acid was selected due to its high molecular weight and the results from the flux experiments.

Relationships between variables were measured using Pearson's correlation coefficient (r), while significant differences between results were determined by independent t-tests. All statistical tests were performed using PASW Statistics 18 (currently IBM SPSS; IBM Corporation, New York, USA).

3. Results

From Fig. 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 h of incubation. This negative corre-

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