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Preliminary characterization of microbial functional diversity using sole-C-source utilization profiles in Tremoal do Pedrido mire (Galicia, NW Spain)

Caracterización preliminar de la diversidad microbiana funcional usando perfiles de utilización de fuente-única de carbono en el Tremoal do Pedrido (Galicia, NW España)
Caracterização preliminar da diversidade microbiana funcional usando perfis de utilização de fonte-única de carbono na turfeira Tremoal do Pedrido (Galicia, NO Espanha)

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ABSTRACT

Peatlands are a global carbon sink. It has been estimated that Northern peatlands store 257-342 Gt of carbon. Most carbon is in the form of peat organic matter whose transformation is largely dependent on microbial activity, and this in turn has a major influence on carbon cycling. The presence of two main physico-chemical environments in the peat deposits -the upper, aerobic, acrotelm and the lower, anaerobic catotelm- suggests that there could be differences in both the microbial activity and communities' composition with depth. In this preliminary study we analyzed the depth variation in microbial community functional diversity using sole–carbon–source utilization profiles, in a raised bog from Galicia (NW Spain). Substrate utilization was quantified by measuring the absorbance at 590 nm of the colour developed by the reduction of the tetrazolium dye contained in each carbon source. Substrates consumption was followed for 10 days and expressed as average well colour development (AWCD) and microbial diversity (Shannon-Wiener index, H). The highest activity (AWCD 1.6-2.0) and microbial diversity (H 3.3) were found at the surface (upper 2 cm) of the peatland. No substrate was used below a depth of 52 cm. Principal components analysis showed three main depth records of degradation: i) substrates (N-compounds, carbohydrates, carboxylic acids) only used at the surface of the peatland, ii) substrates (mostly carboxylic acids) used at the surface and at 46-48 cm, and iii) substrates (N-compounds and polymers) used from the surface to a depth of 48-52 cm. The overall kinetics of substrate utilization showed four patterns: i) asymptotic, ii) exponential (although of low activity), iii) linear, and iv) no reaction over the whole incubation period. Some differences were observed, both in intensity of substrate degradation and total time of reaction, when substrates were grouped according to the results of principal component analysis. These findings suggest a "stratification" of the microbial communities that may be controlled by the varying geochemical conditions (humidity, temperature, acidity, nutrient- and oxygen availability) with depth, and that the acrotelm/catotelm boundary is an effective barrier for oxidative degradation of the organic matter in peatlands.

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RESUMEN

Las turberas son un sumidero global de carbono. Se ha estimado que las de áreas boreales acumulan entre 257-342 Gt de carbono. La mayor parte del carbono se encuentra como materia orgánica de la turba, cuya transformación depende en gran medida de la actividad microbiana la cual, a su vez, tiene una importancia relevante en el ciclo del carbono. La existencia de dos ambientes físico-químicos principales -el acrotelm y el catotelm- en los depósitos turbosos, sugiere que puede haber diferencias tanto en la actividad como en la composición de las comunidades microbianas con la profundidad. En este estudio preliminar, analizamos la variación vertical de la diversidad microbiana funcional utilizando perfiles de utilización de fuente-única de carbono (sole-carbon-source profiles) en una turbera ombrotrófica de Galicia (NW España). La utilización de los substratos se cuantificó mediante la absorbancia a 590 nm del color desarrollado por la reducción del colorante de tetrazolio que contiene el substrato. El consumo de los sustratos se siguió durante 10 días y se expresó como la media del color desarrollado (AWCD) y la diversidad microbiana (índice de Shannon-Wiener, H). La mayor actividad (AWCD 1,6-2,0) y diversidad microbiana (H 3,3) se encontró en la superficie (< 2 cm) de la turbera. No hubo consumo de sustratos por debajo de 52 cm de profundidad. El análisis de los datos mediante componentes principales mostró tres patrones de degradación con la profundidad: i) substratos (compuestos de nitrógeno, carbohidratos, ácidos carboxílicos) solo degradados en la superficie de la turbera, ii) substratos (la mayoría ácidos carboxílicos) utilizados tanto en superficie como a 46-48 cm, y iii) substratos (compuestos de nitrógeno y polímeros) utilizados desde la superficie hasta una profundidad de 48-52 cm. La cinética general de degradación mostró cuatro patrones: i) asintótico, ii) exponencial (pero de bajo consumo), iii) lineal y iv) sin reacción en todo el periodo de incubación. Sin embargo, se encontraron diferencias en la intensidad de consumo y en el tiempo total de reacción cuando los substratos se agruparon en función de los resultados obtenidos en el análisis de componentes principales. Todo ello sugiere una "estratificación" de las comunidades microbianas, que podría estar controlada por la variación de las condiciones geoquímicas (contenido en humedad, temperatura, acidez y disponibilidad de nutrientes y oxígeno) en profundidad, y que el límite acrotelm/catotelm es una barrera efectiva para la degradación oxidativa de la materia orgánica en las turberas.

KEY WORDS
Peatlands, Histosols, acrtotelm/ catotelm boundary, Ecoplates, metabolic functional diversity, AWCD-H-D

PALABRAS CLAVE
Turberas, Histosoles, límite acrotelm/ catotelm, Ecoplates, diversidad metabólica funcional, AWCD-H-D

RESUMO

As turfeiras são sumidouros globais de carbono, com as do Hemisfério Norte acumulando entre 257-342 Gt. A maior parte do carbono está na forma de matéria orgânica de turfa, cuja transformação depende em grande parte da atividade microbiana, e esta por sua vez tem uma grande influência sobre o ciclo do carbono. A existência de dois ambientes físicos-químicos principais -a acrotelm e a catotelm- nos depósitos turfosos, sugere que pode haver diferenças tanto na atividade como na composição das comunidades microbianas com a profundidade. Neste estudo preliminar, analisámos a variação vertical da diversidade microbiana funcional com o uso de perfis de utilização de fonte-única de carbono (sole-carbon-source profiles) numa turfeira ombrotrófica da Galiza (NO Espanha). A utilização dos substratos foi quantificada mediante a medição da absorvância a 590 nm da cor desenvolvida pela redução do corante de tetrazólio contido em cada fonte de carbono. O consumo dos substratos foi avaliado durante 10 dias e expresso como a média da cor desenvolvida (AWCD) e a diversidade microbiana (índice de Shannon-Wiener, H). A maior atividade (AWCD 1,6-2,0) e diversidade microbiana (H 3,3) observaram-se na superfície (<2 cm) da turfeira. Os substratos não foram consumidos abaixo de 52 cm de profundidade. A análise estatística por componentes principais mostrou três padrões principais de degradação com a profundidade: i) substratos (compostos de azoto, hidratos de carbono, ácidos carboxílicos) somente degradados na superfície da turfeira, ii) substratos (maioria ácidos carboxílicos) utilizados na superfície e em 46-48 cm, e iii) substratos (compostos azotados e polímeros) utilizados desde a superfície até a profundidade de 48-52 cm. A cinética geral de degradação mostrou quatro padrões: i) assintótica, ii) exponencial (apesar do baixo consumo), iii) linear e iv) sem reação durante todo o período de incubação. No entanto, algumas diferenças foram observadas na intensidade de degradação do substrato e no tempo total de reação, quando os substratos se agruparam em função dos resultados obtidos da análise por componentes principais. Estes resultados sugerem que pode haver uma "estratificação" das comunidades microbianas, que podem estar controladas pela variação das condições geoquímicas (teor de humidade, temperatura, acidez e disponibilidade de nutrientes e oxigénio) com a profundidade, e que o limite acrotelm/catotelm é uma barreira efetiva para a degradação oxidativa da matéria orgânica em turfeiras.

PALAVRAS-CHAVE
Turfeiras, Histossolos, fronteira acrtotelm/ catotelm, Ecoplates, diversidade funcional metabólica, AWCD-H-D

1. Introduction

According to the definition of the Natura 2000 framework, peatlands are wetlands formed by the accumulation of peat and have current peat-forming vegetation. Peat accumulates because the net production of organic matter exceeds its decomposition. This fact makes peatlands a global carbon sink. They contain one third of the global soil carbon and 10% of the global freshwater (Bartalev et al. 2004a, b). Estimations of the total carbon reservoir in northern peatlands by different authors vary between 257-342 Gt (Tarnocai and Stolbovoy 2006). Although dominant in boreal areas, peatlands are also present in temperate and tropical latitudes, where they also play an important role in the water and carbon cycles. In Galicia, for example, it has been estimated that mountain peatlands have accumulated 10-16 Mt of carbon (Pontevedra Pombal et al. 2004), representing 4.7-7.5% of the carbon stored in forests biomass in Spain and 24-38% of the forests biomass in Galicia.

Carbon is fixed by photosynthesis and is stored initially in the acrotelm, i.e, the relatively young, well–aerated surface layer of the peat deposit (Ingram 1978). After that, carbon will be retained at depth in the anaerobic catotelm. However, not all the carbon that enters the acrotelm is later stored in the catotelm because of the loss by aerobic decomposition. About of 90-97% of the carbon fixed by living plants is typically lost by decomposition (Francez and Vasander 1995), a process that is carried out by microorganisms (Gilbert and Mitchell 2006). Despite their role as an atmospheric C sink, peatlands can also become a source of C to the atmosphere if the balance between decomposition and accumulation is modified, as it has been suggested to occur due to climate change (Tarnocai and Stolbovoy 2006). Predictions made using global circulation models indicate that the northern areas (where peatlands are extensive) will be the most affected by climate warming (Tarnocai and Stolbovoy 2006). Climate change could not only alter the carbon cycle in peatlands, but also enhance the export of stored contaminants in peat (i.e. Hg, Pb, organohalogens) to the aquatic systems or to the atmosphere (Martínez Cortizas et al. 2007).

Taking into account the huge amount of carbon accumulated in peat deposits and the risk of their change from sink to source, the importance of understanding all the aspects related to decomposition mechanisms is justified.

Peatlands are widely studied systems in different fields. As methods and techniques have evolved in recent years, there has been a large increase of knowledge regarding the diversity and composition of their microbiota (Andersen et al. 2013). Relatively few studies deal with peatland microbial communities as a whole, as most research was done on particular groups (e.g. methanotroph) (e.g. Dedysh 2009) and mostly for taxonomic purposes. Within this category there are studies related to the use of microbial indicators, such as testate amoebae, for the reconstruction of past environmental conditions in paleoecological studies (Charman et al. 1999; Charman 2001; Mitchell et al. 2001). In the functional approach, on the other hand, the focus is on the role of microorganisms in the cycling of nutrients (carbon and nitrogen) and organic matter decomposition, targeting isolated enzymes and is almost exclusively based on cultivation-based studies (Artz 2009). Despite the limitations of both approaches, the importance of peatlands as carbon reservoirs, sinks, and sources in the global carbon cycle (Andersen et al. 2013), and their relevance to the cycles of greenhouse gases (CO_2 and CH_4 in particular) justifies the development of a functional approach.

One possible approach to the characterization of microorganism activity in soils is to analyze the functional diversity of the microbial community using sole-carbon-source utilization profiles; and for this the Ecoplates from BIOLOG have been proposed as a simple and efficient method. The Biolog plates method was used, for example, to compare the metabolic activity of heterotrophic microbial communities from different habitats–water, soil and wheat rhizospheres (Garland and Mills 1991). Garland (1996) used BIOLOG plates to characterize the patterns produced by different microbiological samples from root exudates while Lupwayi et al. (1998) used them to investigate the effects of tillage and crop

rotation on the diversity and community structure of soil bacteria. Pietikäinen et al. (2000) studied the microbial substrate utilization pattern (using Biolog Ecoplates) to determine the effect of burning in the microbial community structure in humus layers. In the field of peat research, Fisk et al. (2003) used Ecoplates to compare patterns of soil microbial activity in northern peatlands which differed in vegetation communities.

In this paper we describe the preliminary results of the application of the Ecoplates method to a peat core, with the aim to study the microbial activity patterns with depth. As already mentioned, the upper part of the peat deposits contains two main geochemical environments that largely differ in water content, oxygen and nutrient availability, and thus also likely differ in their microbial activity. The peatland we sampled is located in NW Spain, a temperate area, and its functionality may serve as an analogue for northern peatlands under a scenario of climate warming.

With the previous considerations, the objectives of this work were: i) to characterize the microbial activity patterns at different depths in the upper part (100 cm) of the peat deposit where the most intense biogeochemical changes occur; ii) to characterize the kinetics of the decomposition of the different carbon substrates; and iii) to study the relationship between the diversity of microbial availability and the kinetics with other physico-chemical properties of the peat.

2. Materials and Methods

2.1. Location and sampling

The peat core (OBX) used for this study was sampled in June 2013 in Tremoal do Pedrido (29T 0619124 4812082 UTM), an ombrotrophic raised bog located in the Xistral Mountains (Northwestern Spain) at an elevation of 695 m a.s.l., and 29 km south of the northern coast of Galicia (**Figure 1**). The raised bog originated from an earlier minerogenic mire which started to developed on the fluvial terrace of the Pedrido River ~12,500 years ago (based on radiocarbon dating of the base of the core; data not shown). The mire shows the characteristic form of a raised dome surrounded by a lower elevation belt of minerotrophic peat (the fen lag). In Natura 2000 framework the mire habitat is classified as 7110 (Martínez Cortizas et al. 2009). In World Reference Base (IUSS-ISRIC-FAO 2006) it is classified as Hemic Ombric Histosol. The species most representative of the vegetation in the dome are *Sphagnum subnitens* Russ & Warnst., *Molinia caerulea* (L.), *Carex duriaeui* Steud. ex Kunze, *Agrostis stolonifera* L. and *A. curtisii* Kerguélen., followed by *Potentilla erecta* (L.), *Erica mackaiana* Bab., *Deschampsia flexuosa* (L.), *Carex panicea* L. and *Calluna vulgaris* (L.); while in the minerotrophic area of the fen lag the most representative species are *Sphagnum subsecundum* Nees., *S. denticulatum* Brid., *Juncus bulbosus* L. and *Carex echinata* Murray followed by *Molinia caerulea* (L.), *Eriophorum angustifolium* Honckeny., *Eleocharis multicaulis* (Sm.), *Deschampsia cespitosa* (L.) and *Agrostis hesperica* Romero, Blanca & Morales (Fraga Vila et al. 2001; Martínez Cortizas et al. 2009).

The mean annual temperature in the area is 9.5 ºC, and annual precipitation 1500 mm (695 m a.s.l.) (with very low seasonality) (Martínez Cortizas and Pérez Alberti 2000). The day of sampling (06/06/2013) air temperature was 30 ºC and relative humidity 35%.

Sampling was done with a Russian peat corer to a depth of 100 cm. To avoid disturbance of the peat core, two parallel hemi-cores were sampled at short distance (20-30 cm apart). The first core section included the upper 50 cm and the second one from 50 to 100 cm. Immediately

after sampling of each section the temperature was measured every 2 cm using a Mini Temp Ray tec MT4. Then the sections were wrapped in plastic film, protected in PVC hemi-tubes and taken to the laboratory. Once in the laboratory both sections were cut into 2 cm slices and placed in polyethylene bags and preserved at 4 ºC in a freezer until analysis.

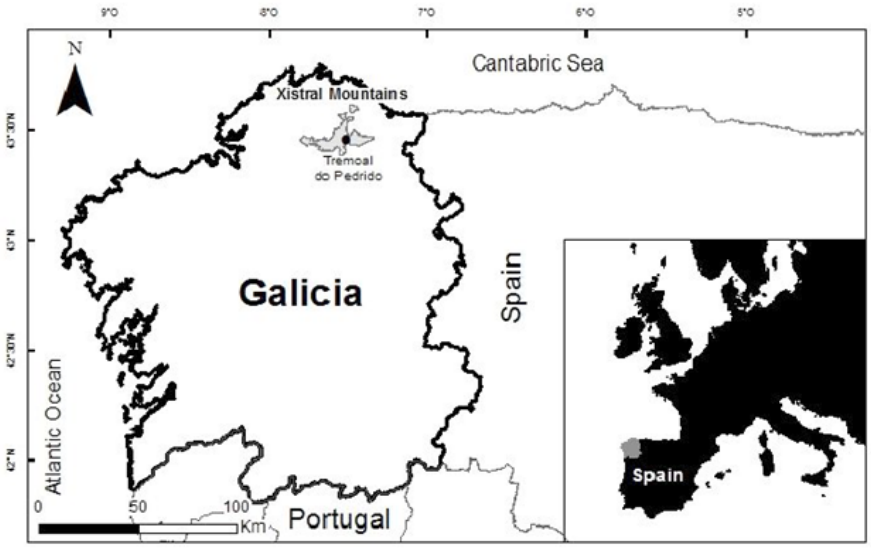

Figure 1. Location map of the Xistral Mountains and the Tremoal do Pedrido mire.

2.2. Characterization of the peat core

To do a basic physico-chemical characterization of the OBX core, the fresh samples were analyzed for water content, peat density, ash content and pH in water and KCl. We used the protocols described in (Pontevedra Pombal 2002). These results were compared with those obtained in a previous study of another core (TPD, sampled and analyzed in 2000) from the same bog and 20 m apart, in order to provide a general characterization of the vertical variations in the peat deposit. For the physico-chemical characterization of TPD, the above-mentioned protocols were also used. Carbon and N were determined on dry milled and homogenized samples using an elemental analyzer LECO TruSpec CHN.

2.3. Microbial metabolic fingerprinting

To study the microbial activity in the core we used Ecoplates from Biolog. Ecoplates are designed to obtain a community metabolic fingerprint which indicates differences in community composition (Campbell et al. 1997). Of the wide range of Biolog plates, we selected Ecoplates because they contain more ecologically relevant (i.e. root exudates) substrates. Each microplate contains 96 wells that are triplicates of 31 organic substrates, which can be grouped into six chemical guilds (Dobranic and Zak 1999) (**Table 1**) and a blank. The organic compounds were selected because they are highly discriminating between soil microbial communities (Hitzl et al. 1997), while the blanks were represented by 3 wells with water to serve as a control (Lagomarsino et al. 2007). When a

substrate is used, a color is developed because microbial respiration reduces a tetrazolium dye that is included with the carbon source. Preston-Mafham et al. (2002), in their review of the application of sole-carbon-source utilisation profiles to analyse microbial functional diversity, gave five main recommendations: 1) to reduce the time between sampling and inoculation; 2) use equivalent sample sizes; 3) inoculate the plates with a soil suspension; 4) take multiple point readings to allow full kinetic analysis; and 5) take into account inoculum density. Of these we followed the first four recommendations, as described below. Although it may pose some limitations to the interpretation of our results, for this preliminary study of the application of Ecoplates to peat research we did not make an assessment of the inoculum density. We believe that this approach better reflects the conditions of the natural environment of the peat deposit.

Table 1. Carbon sources used as substrate in Ecoplates.
Substrates are classified in chemical guilds according to Dobranic and Zack (1999)

A1 water	**A2 Cbh** β-Methyl-D-Glucoside	**A3 CbA** D-Galactonic Acid γ-Lactone	**A4 AmA** L-Arginine
B1 Misc Pyruvic Acid Methyl Ester	**B2 Cbh** D-Xylose	**B3 CbA** D-Galacturonic Acid	**B4 AmA** L-Asparagine
C1 Polym Tween 40	**C2 Cbh** i-Erythritol	**C3 CbA** 2-Hydroxy Benzoic Acid	**C4 AmA** L-Phenylalanine
D1 Polym Tween 80	**D2 Cbh** D-Mannitol	**D3 CbA** 4-Hydroxy Benzoic Acid	**D4 AmA** L-Serine
E1 Polym α-Cyclodextrin	**E2 Cbh** N-Acetyl-D-Glucosamine	**E3 CbA** γ-Hydroxybutyric Acid	**E4 AmA** L-Threonine
F1 Polym Glycogen	**F2 CbA** D-Glucosaminic Acid	**F3 CbA** Itaconic Acid	**F4 AmA** Glycyl-L-Glutamic Acid
G1 Cbh D-Cellobiose	**G2 Misc** Glucose-1-Phosphate	**G3 CbA** α-Ketobutyric Acid	**G4 Amin** Phenylethyl-amine
H1 Cbh α-D-Lactose	**H2 Misc** D,L-α-Glycerol Phosphate	**H3 CbA** D-Malic Acid	**H4 Amin** Putrescine

Amino acids (AmA); Amines (Amin); Carboxylic acids (CbA); Carbohydrates (Cbh); Miscellaneous (Misc); Polymers (Polym).

With the aid of the temperature record we selected 11 peat sections (0-2 cm, 10-12 cm, 20-22 cm, 26-28 cm, 32-34 cm, 38-40 cm, 46-48 cm, 52-54 cm, 62-64 cm, 78-80 cm and 96-98 cm) as representative of the vertical variations. Three of the sections were done in duplicate (0-2 cm, 20-22 cm and 96-98 cm). Immediately after sampling -the same day-, fixed volumes of peat were taken from the center of each selected peat slice using a syringe (1.6 cm of diameter and 2 cm in thickness = 4.02 cm^3 of peat). Each plug was transferred to Falcon tubes and 30 ml of sterile MiliQ water were added. Samples were allowed to stir for 13 h. Then they were filtered through sterile quantitative analysis filters ALBET 140 (pore diameter 15-20 µm) and 100 µL of the water extracts were inoculated into each well of the Ecoplates. All used materials were sterilized using an autoclave at 121 ºC during 1 hour.

Monitorization of the changes in the consumption of the substrates was done measuring the absorbance at 590 nm with the aid of a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA). The first measurement (time zero, T0) was taken immediately after inoculation. Then, the microplates were incubated at constant temperature (26 ºC) during 10 days. A preliminary test performed on peat that had been stored in a refrigerator showed no evidence of substrate consumption (i.e. development of color) in the first three days, thus the second measurement was set at 65 h (time 1, T1), and then readings were done twice every day.

Considering that microplates are only able to compare microbial functional diversity in different soils or states and not to obtain direct information on microbial community function (Preston-Mafham et al. 2002), we used the first peat section as a reference to compare those taken at the other depths because it showed the highest response (i.e. absorbance) and the largest number of substrates reacted.

2.4. Statistical methods and calculations

A principal components analysis (PCA) was performed on the average absorbance of each carbon substrate (after subtraction of the blank) for the readings obtained at 185 h of incubation, using a varimax rotation to maximize the loadings of the variables and provide the best separation among the components (Tabachnick and Fidell 1989). In some cases (5% of the whole data set), one of the three replicates of each microplate was found to be a gross outlier (Baxter 1999) and was not included to calculate the average. Only in a few occasions (1% of the data set) two of the replicates did not react at all but one showed color development. In these cases we used the absorbance of the reacting well, assuming that the presence of reaction indicates that for the given peat layer there must be microorganisms capable of substrate consumption. The PCA was done using SPSS 20.0 software.

To express the overall absorbance of the Ecoplates we calculated the AWCD (Preston-Mafham et al. 2002; Fisk et al. 2003; Stefanowicz 2006; Weber and Legge 2010) for each of the 12 reading times to get an approximation to the kinetics of the consumption of the substrates. Taking the advantage of the PCA results, we also calculated AWCD values for the most representative substrates (those with loadings greater than 0.7) of each component, as it will be described later.

As a measure of the number of substrates utilized (substrate richness) and diversity of the extent of utilization of particular substrates (substrate evenness) we applied the Shannon-Wiener index (H) (Zak et al. 1994; Stefanowicz 2006) and the transformation proposed by Jost (2006), also known as “effective number of species” (MacArthur 1965), to obtain true diversity measures.

3. Results

3.1. Physico-chemical characterization

Peat temperature showed a continuous decline with the depth (**Figure 2**) from a maximum of 14.8 ºC at the surface to a minimum of 6.8 ºC at 94-98 cm of depth, with a small increase at 40-52 cm (up to ~11 ºC). From 70 to 100 cm, the temperature was almost constant.

The depth variation of the volumetric water content (WC) (**Figure 2**) shows two distinct sections. In the upper 55 cm of the core WC values were almost constant with an average value of 54.8±6.7%. Below 55 cm average WC increased to 72.2±11.4% but showed a larger variability. The minimum value was found at the surface (44%) and the maximum at 98-100 cm (94%).

Figure 2 shows the pH in water (pHw) and in KCl (pHk) values with depth. As expected, pHw is higher than pHk, but in both cases they showed little variation with depth. pHw varies between

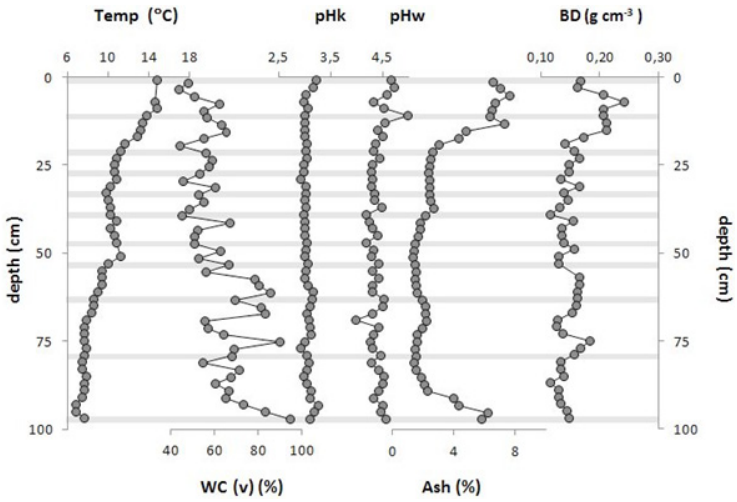

Figure 2. OBX core depth records of: temperature (Temp); volumetric water content (WC (v)); pH in water (pHw) and KCl (pHk); bulk density (BD); ash content (Ash). The shadows indicate the depths at which the samples were taken for Ecoplates.

3.98 and 4.98, while pHk is between 1.5 (in the upper 10 cm) and 1.2 units lower.

Peat bulk density (BD) ranged between 0.08 and 0.23 g cm^{-3}, the higher values were found in the top 25 cm of the core (Figure 2). Ash content is typically higher in the upper 18 cm (6-12%) and below it is fairly stable (around 2%).

The depth records of the physico-chemical properties measured in both cores (OBX and TPD) can be found in the supporting material. The quite similar BD and ash content records (r 0.87 and 0.79 respectively) indicate that there is a good stratigraphical correlation between the OBX and the TPD cores, and thus other properties analyzed in the later can also be of help to characterize the changes with depth in the OBX core; for example organic carbon (C), and nitrogen (N) contents and C/N ratios (supporting material). Organic carbon content ranges between 45% and 52.9%, and N between 1.32% and 2.64%. Both are inversely correlated (r = 0.92). Organic carbon content increases and N content decreases rapidly from the surface to 40 cm of depth. Below 40 cm contents show little variation. The C/N ratios record is almost identical to that of C (r 0.93), with values varying between 17.5 at the surface and around 30-40 below 40 cm (supporting material).

3.2. Microbial functional diversity: Ecoplates

The results of absorbance at the end of the incubation period for each substrate (average of the three replicates) and depths analyzed are shown in the supporting material. Substrates codes are according to Table 1. In the upper peat sample all compounds reacted except for γ-hydroxybutyric acid (E3); while low absorbance was found for D,L-a-Glycerol phosphate (H2). Also, almost all substrates had the highest reactivity (i.e. absorbance) in the upper peat sample. The other depths analyzed showed a large variability in the degradation of the substrates. For example, the peat section at 10-12 cm showed low activity (i.e. average absorbance) compared to the upper ones. Samples corresponding to depths 20-22 cm and 26-28 cm presented high to medium absorbance values for most polymers and almost half of the carbohydrates (see supporting material). Samples of depths 32-34 cm and 38-40 cm showed almost no substrate utilization. Moreover at 46-48 cm and 52-54 cm there was a relatively high activity in half of the substrates (see supporting material).

Eleven substrates (mostly amino acids, polymers and carbohydrates) reacted at greater depth (until 48 cm) (see supporting material). Of these, only

Table 2. Diversity index at the end of the incubation period for each sample

Samples	AWCD	H	D
00-02	1.97	3.33	27.9
00-02r	1.64	3.31	27.3
10-12	0.57	1.21	3.3
16-18	0.11	0.31	1.4
20-22	0.56	1.24	3.5
20-22r	0.34	0.78	2.2
26-28	0.60	1.20	3.3
32-34	0.13	0.37	1.4
38-40	0.08	0.26	1.3
46-48	0.89	1.77	5.8
52-54	0.14	0.39	1.5
62-64	0.08	0.26	1.3
78-80	0.09	0.29	1.3
96-98	0.13	0.42	1.5
96-98r	0.07	0.24	1.3

Average well colour development (AWCD); Shannon-Wiener index (H); Effective number of species (D); Duplicates (r).

Tween 40 (C1) and N-acetyl-D-glucosamine (E2) showed a significant reaction until 54 cm. Peat samples below 54 cm showed no activity in the whole incubation period, except for glycogen (F1).

AWCD results obtained for the last reading time showed three patterns (**Table 2**). The superficial sample (0-2 cm) showed the highest values (2.0-1.6). Samples from intermediate depths (10-12 cm, 20-22 cm, 26-28 and 46-48 cm) showed AWCD values between 0.6 and 0.9. The remaining samples had values between 0.1 and 0.3.

The Shannon-Wiener index (H) of entropy at the end of the incubation period showed a similar pattern to the AWCD (**Table 2**). The upper sample (0-2 cm) had a value of 3.3; intermediate depths (10-12 cm, 20-22 cm, 26-28 and 46-48 cm) had values between 1.2 and 1.8. The remaining samples had values lower than 0.8. The AWCD and H are highly correlated (r = 0.98). In both cases, duplicate samples showed the same variation among them that was observed for the absorbance values.

The difference between the maximum and minimum D values is greater than those of H and AWCD, resulting in a larger differentiation between samples. The upper sample (0-2 cm) had an average value of 27.6; intermediate depths (10-12 cm, 20-22 cm, 26-28 and 46-48 cm) had values between 2.2 and 5.9. The remaining samples had values lower than 1.5 (**Table 2**).

Considering the number of substrates that have reacted, AWCD, H and D values, the duplicates of surface (0-2 cm) and deeper samples (96-98 cm) presented quite similar results (**Table 2**). On the other hand, the duplicate of the sample 20-22 cm showed quite different values (almost a 2-fold difference).

3.3. PCA analysis of functional diversity

A certain pattern of substrate degradation is intuited from **Table 2**, but to reduce the dimensionality of the data set and to investigate into the causes of depth variability, we performed a principal components analysis (PCA) on the average absorbance of the substrates for all peat depths at the end of the incubation period. Substrate E3 (γ-hydroxybutyric acid) was not considered because of its lack of reaction. Four components explained 91.2% of the total

Table 3. Substrates grouped according to PCA analysis performed with AWCD values at the end of the incubation period

Substrates	Cd	CG	Cp1	Cp2	Cp3	Cp4
L-Serine	D4	AmA	**0.75**	0.35	0.33	0.39
Glycyl-L-Glutamic Acid	F4	AmA	**0.64**	0.64	0.33	0.25
Putrescine	H4	Amin	**0.88**	0.33	0.28	0.01
Phenylethyl-amine	G4	Amin	**0.88**	0.37	0.22	0.10
2-Hydroxy Benzoic Acid	C3	CbA	**0.87**	0.37	0.21	0.21
D-Galactonic Acid γ-Lactone	A3	CbA	**0.79**	0.11	0.50	-0.05
Itaconic Acid	F3	CbA	**0.76**	0.30	0.37	0.42
α-D-Lactose	H1	Cbh	**0.84**	0.35	0.29	0.26
i-Erythritol	C2	Cbh	**0.84**	0.41	0.24	0.24
D-Xylose	B2	Cbh	**0.83**	0.35	0.23	0.37
β-Methyl-D-Glucoside	A2	Cbh	**0.70**	0.66	0.25	0.13
D,L-α-Glycerol Phosphate	H2	Misc	**0.70**	0.25	0.43	0.47
α-Cyclodextrin	E1	Polym	**0.86**	0.38	0.23	0.24
L-Asparagine	B4	AmA	0.35	**0.77**	0.45	0.25
D-Glucosaminic Acid	F2	CbA	0.19	**0.94**	0.15	-0.15
α-Ketobutyric Acid	G3	CbA	0.45	**0.85**	0.21	0.17
D-Galacturonic Acid	B3	CbA	0.37	**0.77**	0.49	0.12
D-Malic Acid	H3	CbA	0.55	**0.73**	0.24	0.30
D-Mannitol	D2	Cbh	0.32	0.67	0.62	0.17
Glucose-1-Phosphate	G2	Misc	0.39	**0.82**	0.15	0.32
L-Arginine	A4	AmA	0.54	-0.08	**0.73**	-0.32
L-Phenylalanine	C4	AmA	0.30	0.55	**0.70**	0.06
L-Threonine	E4	AmA	0.51	0.51	0.53	-0.04
4-Hydroxy Benzoic Acid	D3	CbA	0.34	0.53	0.61	0.29
D-Cellobiose	G1	Cbh	0.50	0.03	**0.79**	0.16
N-Acetyl-D-Glucosamine	E2	Cbh	0.25	0.35	**0.79**	0.03
Pyruvic Acid Methyl Ester	B1	Misc	0.38	0.45	**0.78**	0.13
Tween 40	C1	Polym	0.26	0.34	**0.85**	0.09
Tween 80	D1	Polym	0.12	0.53	**0.70**	0.12
Glycogen	F1	Polym	0.40	0.15	0.26	**0.85**
		Eigv	10.8	8.1	7.0	2.3
		Var	34.9	26.2	22.7	7.4
		Varacc			83.7	91.2

Component loadings (Cp1, Cp2, Cp3 and Cp4) > 0.7 are in bold. γ-hydroxybutyric acid and water are not included. Substrates ecoplate code (Cd); Chemical guild (CG).

variation in the data set. The first component, Cp1, accounted for 34.9% of the total variance and is characterized by high factor loadings (>0.7) of substrates A2, A3, B2, C2, C3, D4, E1, F3, G4, H1, H2 and H4, and a moderate loading of substrate F4 (Table 3). The second component, Cp2, accounted for 26.2% of the variance and showed large loadings of substrates B3, B4, F2, G2, G3, and H3, and moderate loadings of D2 and F4 (Table 3). The third component, Cp3, explained 22.7% of the variance with high loadings of substrates A4, B1, C1, C4, D1, E2, G1 and moderate loadings of substrates D2, D3 and E4 (Table 3). While the fourth component explained 2.3% of the total variance and only substrate F1 had a high loading (Table 3). This last substrate has a distribution quite similar to that of substrates in Cp1, with the only difference that it shows a slight reactivity at some depths apart from the upper peat sample.

As suggested by some authors (Fisk et al. 2003) a better interpretation of each Cp can be obtained if the carbon substrates are grouped into general substrate chemical guilds (Table 1). In Table 4 we included the number of substrates of each chemical guild in the main four principal components extracted. Cp1 has the largest diversity of substrates, with aminoacids-amines (4 out of 8 amino-substrates,

Table 4. Number of substrates of each principal component grouped according to chemical guild (Table 1)

Chemical guild	Cp1	Cp2	Cp3	Cp4	Total
Amin/Am A	4	1	3	--	8
Cbh	4	1	2	--	7
Cb A	3	4	1	--	8
Polym	1	--	2	1	4
Misc	1	1	1	--	3
Total	13	7	9	1	30

Amino acids (AmA); Amines (Amin); Carboxylic acids (CbA); Carbohydrates (Cbh); Miscellaneous (Misc); Polymers (Polym).

Table 4), carbohydrates (4 out of 7, Table 4) and carboxylic acids (3 out of 8, Table 4); in Cp2 the most abundant group is carboxylic acids (4 out of 8, Table 4); while Cp3 can be characterized by polymers (Tween 40 and 80) and amino-substrates (3 out of 8, Table 4).

To provide a visual representation of the changes of substrate utilization with depth at the end of the incubation period, a coloured map of relative absorbance for each compound at each depth is given in Figure 3. Values of relative absorbance were calculated with respect to the uppermost peat sample (0-2 cm) that, as already mentioned, showed the highest values for all substrates (except for A4, E2 and F2). The substrates were grouped according to the principal component to which they contribute. Substrates characteristic of Cp1 reacted only in the superficial peat sample; those in Cp2 also reacted in the sample at 46-48 cm; while those in Cp3 are most of the substrates that reacted at greater depths. The only substrate in Cp4 (F1, Glycogen) only showed a significant reaction at the upper peat sample (0-2 cm) and was included in Figure 3 among the substrates of Cp1.

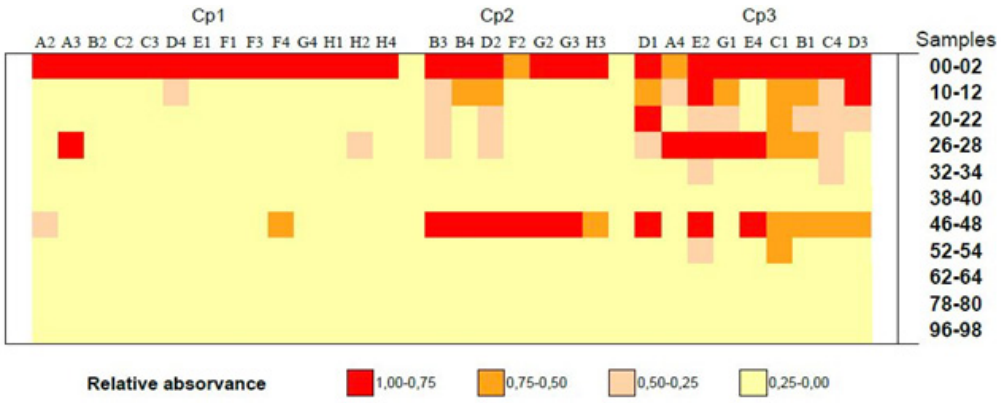

Figure 3. Colour-coded map of relative absorbance (the upper sample taken as reference). The substrates are grouped according to the principal components (compounds with loadings greater than 0.7).

3.4. Kinetic of the substrates

AWCD results for all depths and readings presented four main kinetic patterns. The surface sample (0-2 cm) showed an asymptotic (Table 5) evolution leveling off after 100 hours, to reach a maximum absorbance of 1.7 at 200 hours (Figure 4); samples of depths 10-12, 20-22, and 26-28 cm showed an exponential increase (Table 5) but with very low end values of AWCD (0.3-0.5); absorbance in sample at 46-48 cm increased linearly with time (Table 5) to a maximum value of 0.62; while the other samples did not show any reaction or pattern, and AWCD values were very low.

Given that the PCA enabled us to identify three main depth distributions, we also calculated the AWCD for selected substrates representative of each component (those with loadings greater than 0.7; see Table 3. Substrates of Cp1 showed two kinetic patterns (Figure 4): that of the surface sample (0-2 cm) can be approximated to a sigmoidal (Table 5) model; while in the other samples the substrates did not show any activity. Substrates of Cp2 and Cp3 (Figure 4) presented the same kinetic models as those described for the AWCD calculated with all substrates, with slightly higher maximum AWCD values in Cp3 than in Cp2, except for the sample at 46-48 cm of depth for which it was similar (Figure 4).

As mentioned before, AWCD and H index are highly correlated and therefore the latter fits exactly to the same models, providing any additional information on the kinetics of microbial functional diversity in our study case.

Table 5. Statistical summary of the equations of the kinetic patterns

Sample	Equation	r	F	p-Value
AWCD 0-2 cm	Y =0.411 + (0.208 * ln (t))	0.94	73.762	<0.001
AWCD 10-12 cm	Ln (Y) = ln 0.019 + (0.018 * t)	0.94	54.529	<0.001
AWCD 20-22 cm	Ln (Y) = ln 0.014 + (0.019 * t)	0.94	71.770	<0.001
AWCD 26-28 cm	Ln (Y) = ln 0.014 + (0.018 * t)	0.95	90.744	<0.001
AWCD 46-48 cm	Y= 0.019 + (0.003 * t)	0.96	333.774	<0.001
Cp1 0-2 cm	Ln (Y) = 0.487+(-0.509/t)	0.96	122.298	<0.001

Equations (*Y* corresponds to the AWCD of the absorbance at 590 nm, except for the last equation in which it corresponds to the scores of the first principal component; *t* is time in hours).

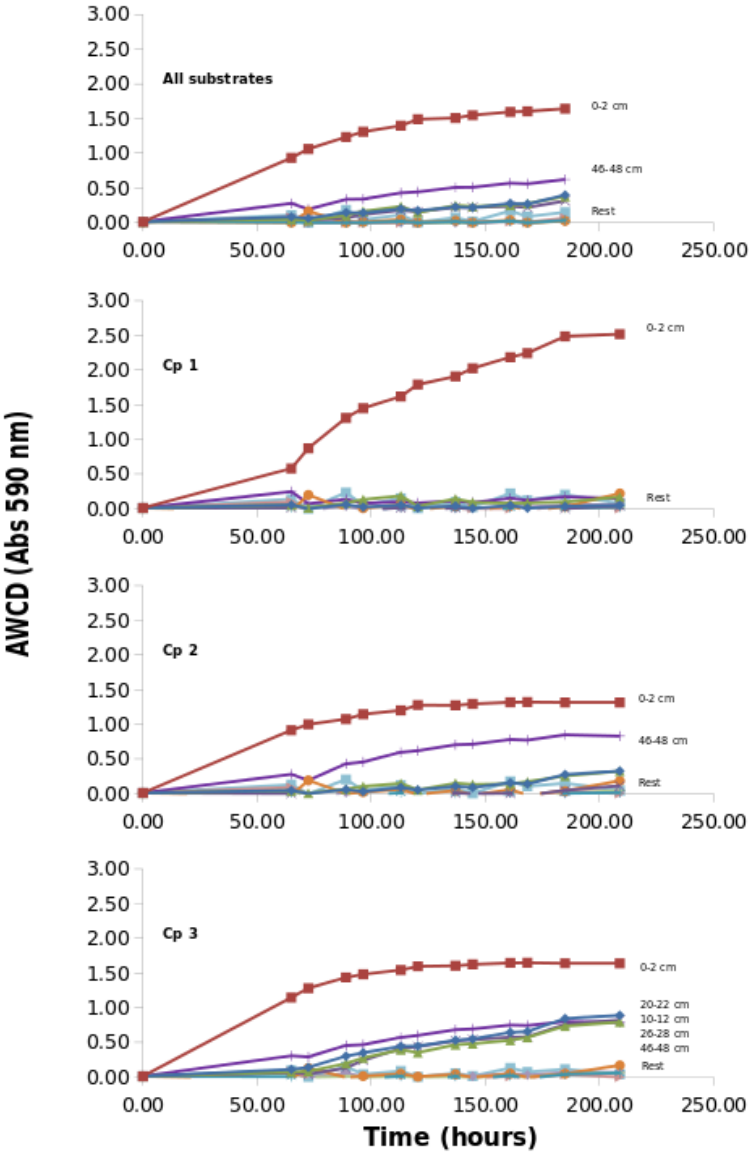

Figure 4. Kinetic curves of AWCD (average well colour development) during the incubation time. Above, AWCD for all substrates. Below, AWCD for substrates representative (loading > 0.7) of each principal component (Cp1, Cp2, Cp3).

4. Discussion

4.1. Physico-chemical properties

The main objective of the properties analyzed was to characterize the chemical environment in the upper meter of the sampled peatland and help determine its possible link to the observed microbial functional diversity. Regarding temperature, we did not find any published data, neither reference values nor records of vertical variation, to compare with ours. Although the measurements were done in only one core and after coring, and thus their interpretation should be taken with care, the data seem to be appropriate because the values show a reasonable depth trend of overall exponential ($r = 0.96$) decreasing temperature.

For the remainder of the properties, a number of publications characterizing the peatlands from Galicia, and more specifically the Xistral Mountains, are available (Martínez Cortizas and García-Rodeja Gayoso 2001; Pontevedra Pombal 2002; Pontevedra Pombal et al. 2006). Some of the studied properties (pH, ash content, bulk density, and organic carbon) can also be found in studies dealing with atmospheric metal pollution (Martinez Cortizas et al. 2002, 2007), in which they are included to ascertain the ombrotrophic nature of the mire. The values and depth records of pHk, BD, ash content, organic C content, and C/N ratios determined are within the range of those provided by the cited authors for peatlands of the Xistral Mountains. Surface N contents given in the literature are lower than those found by us, while pHw values in OBX are higher. The published pH records (see for example Pontevedra Pombal 2002) show lower, more acidic values in the upper 40-50 cm and an increase to higher values right below this depth, while in the OBX core they are almost constant.

Water content values in the OBX core are considerably lower than those given by Pontevedra Pombal (2002) for the upper peat layers. Even more, the typical increase in WC that occurs in other peatlands in the upper 15 cm (Pontevedra Pombal 2002) was found at greater depth (40-50 cm) in the OBX core.

In the core studied by us and in published ones, the depth variation in WC, C and N contents, and C/N ratios, suggests a change in the geochemical environment of the peat deposit that could correspond to the boundary between the acrotelm –the aerobic, acidic, upper section of the peat deposit (with higher susceptibility to oxidative microbial activity)–, and the catotelm – the anoxic, less acidic and thicker section of the peat deposit. The comparison with published records (Pontevedra Pombal et al. 2006) suggests that this boundary is located at different depths in each peat deposit, between 25-60 cm, and the OBX core falls within this range. Nevertheless, some of the observed differences (water content, bulk density and ash content), are probably due to the fact that all records (ours and published ones) represent single measurements in the time domain. Thus, the actual local and temporal variability of these properties has not been assessed and it is likely that variation within the mire is greater (or at least comparable) than between mires.

4.2. Microbial functional diversity

Regarding microbial activity, the maximum values of absorbance we obtained are similar or higher to those given for different experiments in the literature (between 1.6-2.7) (Campbell et al. 1997; Preston-Mafham et al. 2002), although most publications do not provide the raw results (e.g. Fisk et al. 2003). AWCD values, integration of the overall microplate absorbance at the end of the incubation period, are comparable to those found in the literature: 0.4-1.1 at 150h (Campbell et al. 1997), 1.5 at 100h (Stefanowicz 2006) and 0.97 at 70h (Fisk et al. 2003). But maximum values of the H index were lower than those in the literature: 3.8-4.20 (Lupwayi et al. 1998), 4.1-4.39 (Gómez et al. 2000); and similar to those found by Yao et al. (2006) 3.1-3.3, although this index is used less often and there is not much literature available for comparison. Our results for both AWCD (0.77) and H (2.96) are also comparable with those found for the epipedon of a mineral soil from NW Spain studied by Fontúrbel et al. (2012). Despite AWCD and H providing mostly

the same information in our experiment (they were highly correlated), according to Harch et al. (1997) they strictly provide information about different components of the functional diversity. However, in accordance with Jost (2006), in order to compare diversity indices we should use the transformed values since the parameter obtained (D) allows a sensible, stable and easy to interpret general measure of similarity. In our study this parameter shows that the uppermost peat section has a much greater diversity than the others. Despite the recommendation of Jost (2006), transformed index usage is not common in Ecoplates methodology.

The relative variation between duplicates (e.g. between 0-2 cm and 0-2 cm *r*, see **Table 2**) analyses found in our experiment may be due to i) spatial heterogeneity, as suggested by Barkovskii et al. (2009) who found that there are differences in organic-matter-driven diagenesis depending on the bacterial fraction, or ii) it may be because the inoculation was not equally successful.

The PCA results showed that there were four patterns of substrate degradation. As a synthesis it can be said that i) some of the most simple C-sources (those grouped in Cp1) showed maximum reaction at the surface of the mire and no reaction at the other depths; ii) four of the carboxylic acids-substrates (Cp2) were preferentially degraded at the surface of the peatland and right above the limit of the acrotelm/catotelm boundary; iii) the more complex C-sources (polymer-substrates in Cp3) and some amino-substrates showed reactivity to a greater depth; and iv) no reaction was found below 52-54 cm. No comparable experiments were found in the literature, and thus, to our knowledge, these are the first results on vertical variations of microbial functional diversity in peatlands using sole-C-source utilization profiles. Fisk et al. (2003) is the only publication we were able to find dealing with an experiment of microbial functional diversity in peatlands, but they compared reactions on superficial samples in different locations and mires and not with depth, and their results are not directly comparable because they used a different type of Biolog microplate.

The degradation of N-containing substrates at greater depth is also consistent with findings by different researchers (Bridgham et al. 1998; Aerts et al. 1999), who showed that N cycling in peatlands is quite active due to its nature as limiting nutrient. A similar reasoning can be applied to the significant consumption of the more complex substrates (polymers Tween 40 and 80) in the upper half meter of the peat deposit. Research on the depth changes in C-groups and molecular composition of the peat (Pontevedra Pombal 2002; Buurman et al. 2006; Kaal et al. 2007; Barkovskii et al. 2009) showed an exponential decrease of polysaccharides with depth; thus, it is likely that the most labile compounds (including polysaccharides) are preferentially degraded in the upper part of the peat deposit while the more complex and recalcitrant ones are still degraded at greater depth. As for the preferential degradation of some carboxylic acids at the acrotelm/catotelm boundary, we do not have an explanation and it can only be speculated that oxygen availability and temperature (which showed a secondary maximum at this depth) may have played a role.

Taken together, the patterns of microbial functional diversity and the records of the physico-chemical properties and elemental composition suggest that the acrotelm/catotelm boundary is an effective one for oxidative degradation of organic substrates. Almost all substrates were degraded in the upper sample, where higher temperature and, likely, highest oxygen availability may have promoted a maximum in microbial activity in the peat column. But the change of geochemical environment (oxygen availability, temperature, nutrients, organic matter quality, etc.) with depth results in increasing limitations to bacterial growth. On the other hand, studies using more sophisticated techniques (like PLFAs, T-RFLP, FISH) also found that microbial communities and associated decomposition processes of all peatlands types are vertically stratified, as redox conditions and carbon quality change (Williams and Crawford 1983; Sundh et al. 1997; Morales et al. 2006; Dedysh et al. 2006). This all supports the suggestion of Andersen et al (2013) that the vertical stratification of microbial

communities in peatlands arises primarily from energy constraints.

Thus, our results are consistent with previous knowledge on physico-chemical and geochemical properties and the functioning of peatlands. Nevertheless, as we commented in the methods section, no assessment of the density of the inoculum was done. This may have implications on the results that can be obtained using Ecoplates, as differences in carbon consumption at different depths with different, standardized, microbial biomass are expected. On the other hand, our aim was to more closely reflect the conditions of the natural environment. As found by Barkovskii et al. (2009), there can be large differences in bacterial fractions and composition at short distances.

4.3. Kinetics of microbial functional diversity

AWCD overall results showed that substrate degradation followed four kinetic patterns: asymptotic, exponential, linear and no reaction with time. In the literature, the general kinetic pattern is sigmoidal (Campbell et al. 1997; Preston-Mafham et al. 2002; Stefanowicz 2006) and therefore we believe our results should also agree with a sigmoidal evolution. The lack of coincidence is possibly due, on one hand, to the absence of measurements in the first 50 hours.

The kinetics of substrates grouped based on the results of the PCA showed certain differences respect the general model, including two main features: intensity of substrate utilization and total time of reaction. The results for the Cp1-substrates in the upper sample fit to the expected sigmoidal model, thus indicating that the asymptotic model of the superficial sample obtained with the pooled substrates (overall AWCD) is in fact dominated by the kinetics of Cp2- and Cp3-substrates, and not by Cp1-substrates. The results also suggest that full substrate reaction may have only been reached in the uppermost peat sample, with larger values for Cp1- than Cp3- and Cp2-substrates both in intensity (i.e higher overall AWCD, 2.7 vs 1.4-1.7) and utilization time (asymptotic behavior at ~200 hours and ~100 hours respectively). Campbell et al. (1997) also studied the kinetic of the reaction depending on the chemical composition of the substrates, but their results are hardly comparable since, as we have found in our study, substrates of the same chemical group may show different behaviour and therefore integrated results may not be representative. Previous PCA analysis seems to be a proper statistical strategy to overcome this problem.

5. Conclusions

Our analysis of microbial functional diversity using sole C-source utilization profiles applied to a peat core (OBX) sampled in the Tremoal do Pedrido raised bog showed that there are significant variations with depth in the degradation of Ecoplates carbon sources. These variations were reflected both by the AWCD and the Shannon-Wiener index (H) of diversity (which were highly correlated) and more accentuated on the “effective number of species” (D).

The degradation patterns we observed indicate that some of the most accessible C-sources may be rapidly used at the surface of the mire while N-compounds and the most complex substrates are still degraded at great depth. Some differences were observed, both in intensity of substrate degradation and total time of reaction, when substrates were grouped according to the results of the principal components analysis.

The results suggest that there can be a “stratification” of the microbial communities, so that microorganisms which preferentially utilize simple sources may concentrate in the surface of the mire while those able to degrade more complex compounds (which are left) are still active in the deeper sections of the acrotelm.

It also seems that oxygen availability and temperature at specific depths may influence the biodegradation of carboxylic acids. We did not find substrate utilization below the acrotelm/catotelm boundary, suggesting that this boundary is an effective one for oxidative degradation of the peat organic matter. Although this is consistent with previous research on peat organic chemistry we do not actually know if the position of the boundary is a seasonal feature (as it may be expected).

Interpretation of results must be cautious because the use of Ecoplates implies selecting a very specific part of the microbial population (metabolically active and aerobic bacteria capable of growing at specific lab conditions). Also, adding more uncertainty to the results is the fact that Ecoplates allow the characterization and not only the comparison of microbial communities, because of the concentration and type of carbon substrates (as also discussed by Bossio and Scow 1995, and Preston-Mafham et al. 2002). Therefore a study using a selection of peat representative organic compounds, such as those characterized by pyrolysis-GC/MS in previous studies (Buurman et al. 2006), as carbon substrates and at different lab conditions could allow better information to be obtained on the microbial activity in peatlands. Further research is needed to determine the influence of others factors such as inoculum density, incubation time, temperature and spatial and seasonal variability within the mire. Otherwise it could be interesting to combine Ecoplates with other techniques, to provide information on the relative contribution of different microorganisms to the dynamics of organic matter in peatlands.

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SUPPORTING MATERIAL

1. PHYSICO-CHEMICAL CHARACTERIZATION

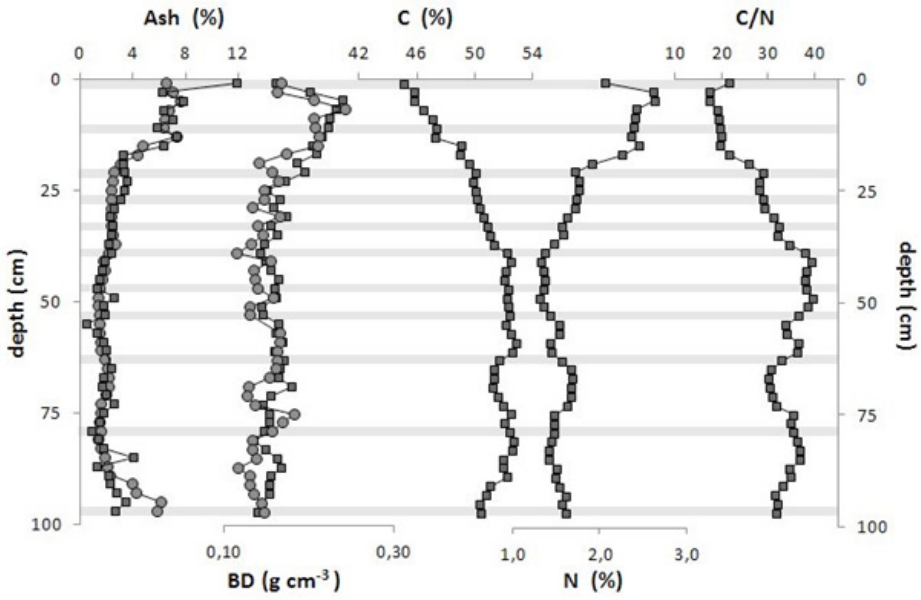

Figure 1. Supporting Material Depth records of: ash content (Ash); bulk density (BD); carbon (C) and nitrogen (N) contents; carbon/nitrogen ratio (C/N). Circles OBX core, squares TPD core. The shadows indicate the depths at which the samples were taken for Ecoplates.

2. RESULTS OF ABSORBANCE VALUES AT THE END OF THE INCUBATION PERIOD FOR EACH SAMPLE AND SUBSTRATE

In the upper peat sample, almost all substrates had high reactivity (i.e absorbance), except the blank (A1) and γ-hydroxybutyric acid (E3), that did not react, and D,L-a-Glycerol phosphate (H2), that showed low absorbance. Samples corresponding to depths 20-22 cm and 26-28 cm present high to medium absorbance values in some compounds (A3, A4, B1, C1, D1, D2, E2, E4, F1 and G1). Samples of depths 32-34 cm and 38-40 showed almost no substrate utilization. In samples 46-48 cm and 52-54 cm there was a relatively high activity in A2, B1, B3, B4, C1, C4, D1, D2, D3, E2, E4, E4, F2, F4, G2, G3, and H3. At greater depth (most until 48 cm) eleven substrates (B1, B3, B4, C1, C4, D1, D3, E2, E4, G1) reacted, with average absorbance ranging from 1.02 to 2.12 and only C1 and E2 showed a reaction until 54 cm. Below 54 cm, peat samples showed no activity, except F1.

Table. Supporting material 1.
Average absorbance values at the end of the incubation period for each sample

Samples	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	D1	D2	D3	D4
00-02	0.0	1.4	1.6	1.8	2.2	2.5	2.0	2.4	2.6	2.5	2.0	2.6	2.2	2.6	2.2	1.5
00-02r	0.0	1.2	1.4	0.6	1.9	2.1	1.9	2.4	2.6	2.4	1.7	2.2	2.1	2.4	1.9	1.3
10-12	0.0	0.0	0.0	0.5	1.3	0.1	0.7	1.3	1.8	0.1	0.0	1.1	1.5	1.3	1.9	0.5
16-18	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.7	0.0	0.1	0.1
20-22	0.0	0.0	0.0	0.6	1.3	0.1	0.7	0.7	2.1	0.1	0.1	1.6	1.9	0.9	1.1	0.1
20-22r	0.0	0.0	0.0	0.2	1.1	0.0	0.3	0.5	1.8	0.2	0.0	1.7	1.7	1.5	0.3	0.0
26-28	0.0	0.0	1.4	2.1	1.2	0.0	0.6	0.0	1.8	0.1	0.0	1.2	1.0	1.0	0.2	0.0
32-34	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.9	0.0	0.2	0.1	0.1
38-40	0.0	0.0	0.0	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1
46-48	0.0	0.7	0.0	0.0	1.3	0.1	2.1	2.2	1.7	0.3	0.1	1.9	2.1	2.2	1.3	0.1
52-54	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.1	1.5	0.1	0.0	0.1	0.1	0.0	0.1	0.1
62-64	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.1	0.1
78-80	0.0	0.0	0.0	0.0	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
96-98	0.0	0.0	0.0	0.0	0.1	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
96-98r	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.0
Samples	E1	E2	E3	E4	F1	F2	F3	F4	G1	G2	G3	G4	H1	H2	H3	H4
00-02	2.7	2.4	0.2	2.6	2.4	1.6	1.8	2.0	2.5	1.3	1.1	2.4	2.6	0.5	1.9	1.1
00-02r	2.5	2.3	0.1	2.5	0.4	0.7	1.4	1.9	2.5	0.8	1.1	1.6	2.5	0.3	1.3	0.6
10-12	0.0	2.6	0.1	0.2	0.5	0.1	0.3	0.2	1.3	0.0	0.1	0.0	0.0	0.1	0.0	0.0
16-18	0.1	0.1	0.1	0.4	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0
20-22	0.1	1.1	0.1	0.5	0.7	0.2	0.5	0.3	2.2	0.1	0.1	0.1	0.3	0.1	0.1	0.0
20-22r	0.1	0.1	0.0	0.1	0.1	0.0	0.2	0.1	0.0	0.1	0.2	0.0	0.0	0.0	0.0	0.0
26-28	0.0	2.4	0.1	2.4	0.0	0.0	0.1	0.2	2.5	0.0	0.0	0.0	0.0	0.1	0.0	0.1
32-34	0.1	0.9	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0
38-40	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0
46-48	0.2	2.0	0.1	2.2	0.1	2.2	0.1	1.1	0.2	1.1	1.1	0.1	0.0	0.0	1.2	0.0
52-54	0.1	1.1	0.0	0.1	0.2	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.1
62-64	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.0	0.0	0.0	0.0
78-80	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0
96-98	0.3	0.2	0.2	0.2	0.8	0.1	0.2	0.1	0.1	0.2	0.1	0.1	0.0	0.1	0.0	0.0
96-98r	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0

Substrates code (A1, A2 ... H4) according to (Table 1); Duplicates (r).