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Abstract

Evaluating the response of soil organic matter decomposition to warming and changes in rainfall is critical to assess the likelihood of proposed positive feedbacks from the terrestrial to the atmospheric system. The response of soil respiration and extracellular activities (EEAs) to long-term warming and recurrent summer drought was studied in a wet shrubland ecosystem in Wales (UK), after 13 years of climate change simulation in a whole-ecosystem experiment. Over a year soil respiration, temperature and moisture was monitored in the field. During the summer season, coinciding with maximum soil respiration rates, soil inorganic N and P, microbial biomass and the extracellular

activities (EEAs) of a selection of enzymes involved in C, N and P cycling were analysed. Based on previous field measurements of C and N mineralization, we expected a stronger response of C-cycling EEAs, in comparison to N-cycling EEAs, to drought and warming, and a greater sensitivity of C-cycling EEAs to drought than to warming. Drought had a clear impact on soil respiration during the summer season. However, the availability of inorganic N or P was not significantly affected by the treatments. Microbial biomass and C:N ratio also remained unchanged. In contrast to one of our hypothesis, C-cycling EEAs measured under non-optimal conditions that simulated soil environment in the field (pH of 4.1 and with a temperature incubation of 10ºC) showed no significant differences due to long-term warming and recurring drought treatments. Possibly, this assay approach may have obscured treatment effects on the soil enzyme pool. Our results highlight the need for developing methods for the in-situ analysis of EEAs to determine rates of reactions.

Keywords: climate change; soil C; *Calluna vulgaris*; phenol-oxidase, β-glucosidase; microbial C:N

Highlights

- Understanding soil organic matter decomposition is critical to forecast C fluxes
- In a long-term climate change experiment drought stimulated soil respiration
- Summer enzyme activities measured after 14 experimental years were not affected
- In-situ enzyme analysis methods are needed to reconcile field and laboratory data

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48 **term warming and drought in a wet shrubland (NE Wales, UK)**

49

50 ## 1. Introduction

51 Evaluating the response of soil organic matter (SOM) decomposition to warming and
52 changes in rainfall is critical to forecast climate feedbacks under projected climate
53 change scenarios (Christensen et al., 1999; Davidson and Janssens, 2006). The
54 consideration of the long-term acclimation of those processes involved in SOM
55 decomposition, such as enzymatic depolymerisation of organic compounds and
56 microbial respiration, is therefore essential to formulate more realistic models of future
57 C fluxes from soils to atmosphere. For this purpose, long-term climate change
58 experiments are critically needed.

59 Enzymatic depolymerisation is usually considered as one of the rate-limiting steps in
60 SOM decomposition (Burns et al., 2013; Conant et al., 2011). Consequently, several
61 experiments have measured the short- and long-term impact of some of the main
62 climate change drivers - warming and drought - on soil extracellular enzyme activities
63 (EEAs, reviewed by Henry, 2013). Most of these studies have been confined to well-
64 drained mineral soils, where drought often decreases potential EEAs (Sardans and
65 Peñuelas, 2005; Sardans et al., 2008; Steinweg et al., 2012) or enzyme efficiency
66 (Alster et al 2013). In contrast, in wet organic soils drought has been shown to increase
67 the activity of hydrolyzing enzymes, increase the size of the soil dissolved organic
68 carbon (DOC) pool and increase soil CO_2 efflux (Fenner et al., 2005; Fenner et al.,
69 2007; Fenner and Freeman, 2011; Kwon et al., 2013), although this response might be
70 dependent on the drought effect on soil pH (Xiang et al., 2013). In shallower organo-

mineral soils, however, reduced soil moisture does not necessarily lead to an increased enzyme activity, which suggests that oxidase activity has an optimal moisture level (Toberman et al., 2008).

In a long-term (13-year-old) field experiment assessing the impact of warming and summer drought in a wet shrubland, drought was shown to provoke a progressive stimulation of soil respiration in the organo-mineral soil, without signs of attenuation in a decadal time-scale, and with several indications of the increase in respiration having a heterotrophic origin (Domínguez et al., 2015; Sowerby et al., 2008). Analyses of soil EEAs during the first two years of climate manipulation revealed no impact of warming or drought in EEAs, which was in line with a modest change in C mineralization (Sowerby et al., 2005), and no change in N mineralization (Emmett et al., 2004). The aforementioned progressive increase in soil respiration and a progressive increase in DOC concentration within the drought plots (Sowerby et al., 2010) suggest that the activity of soil enzymes involved in C-cycling may have changed among treatments with time. In some organic-rich soils the increase in CO_2 efflux in response to drought has been shown to be related to a general activation of hydrolases, due to the release of inhibition by phenolic compounds (Fenner and Freeman, 2011; Freeman et al,. 1997). Therefore, stimulation of the activity of other hydrolases, such as amino-peptidase and acid phosphatase, might be also expected in the drought treatment.

In this work, respiration of this wet shrubland soil was monitored over a year, after 13 years of climate change simulation. Soil EEAs, microbial biomass and inorganic N and P were also measured during the summer season. In agreement with the previously described stronger response of field soil respiration to drought than to warming, and with the relative insensitivity of N mineralization to air temperature increase reported for *Calluna vulgaris* shrublands (Beier et al., 2008) we hypothesized that: 1) drought

would have a greater long-term impact on soil EEAs and microbial biomass than warming, and 2) enzymes involved in C-cycling would show a clearer increase in activity than enzymes involved in N cycling in the drought treatment..

2. Material and methods

Experimental set up and field measurements

Whole ecosystem warming and summer drought treatments were established during 1999 in an upland Atlantic shrubland dominated by *Calluna vulgaris* (L.) located in NE Wales (UK, 53º 03' 19"N, 03° 27' 55"W). Mean annual air temperature at the site is 8.2 ºC, rainfall is 1700 mm, and potential evapotranspiration is 302 mm. The soil at the site is an organic-rich humo-ferric Podzol, and has been classified as a Ferric stagnopodzol in the Hafren Series in the Soil Survey of England and Wales (Cranfield University, 2014). The ecosystem has remained unmanaged and undisturbed over at least the last 25 years, and has moved from a "mature" to "degenerate" phase of shrubland succession (Domínguez et al., in press).

The experiment had a randomized block design with three replicate plots of 4 × 5 m allocated to the control, drought and warming treatments, respectively. Automated retractable roofs were used in the field to manipulate air temperature and rainfall (see Beier et al., 2004 for a full description). Briefly, the warming treatment consisted of a passive night-time system that used reflective aluminium curtains to cover vegetation at night, resulting in reflection of long-wave radiation and in a reduction of heat loss, which produced an increase of 0.2-2.0 ºC in mean monthly air temperature. The drought treatment consisted of waterproof polyethylene curtains triggered by a rain sensor that on average excluded 54 % of the rainfall between June and September (experimental drought period). Control, warming and drought plots received, on average, 1357, 1212

and 743 mm of rainfall, respectively, during the studied year (2102). There was no drought × warming treatment.

During 2012, as for most preceding years, soil respiration was measured fortnightly in three plots per treatment (three measurements per plot) using a LI-8100 automated soil CO_2 flux system (LI-COR, Lincoln, Nebraska USA), using 5 cm high collars permanently inserted 1 cm into the soil. Soil temperature and moisture were continuously recorded at 0–5 cm depth with Reference Thermistor sensors (Probe 107, Campbell Scientific, Logan, UT, USA) and a Time Domain Reflectometer (TDR; CS616, Campbell Scientific, Logan, UT, USA), respectively.

Soil sampling and chemical analyses

In July 2012 (mid-summer, within the experimental drought period) a composite soil sample (0-10 cm depth) was obtained from each experimental plot by mixing three subsamples collected with a cylinder auger at three different locations within each of the plots to conduct enzyme assays. Therefore, there were three replicates per treatment for the subsequent soil analyses. Sampled soils had a high organic matter content (SOM > 30 %), and included decomposing debris. Soils were transported to the lab in a refrigerated container, and kept between 2 and 4 ºC until enzyme assays were completed, within the following 72 hours. Prior to analysis roots were removed, and soils were sieved to < 2 mm.

Nitrate and ammonium concentrations were determined in 0.5 M K_2SO_4 soil extracts spectrophotometrically. Dissolved organic carbon (DOC) was also determined in these extracts using a TOC-V-TN analyzer (Shimadzu Corp., Kyoto, Japan). Molybdate reactive P was determined colorimetrically (Murphy and Riley, 1962) in Mehlich-3 soil extracts (Mehlich, 1984), using a microplate reader (Biotek, Winooski, VT, USA). Water-soluble

soil phenolics were determined spectrophotometrically using the Folin-Ciocalteau's reagent, following the procedure described by Toberman et al. (2008). Total C and N content of bulk soil was analysed by dry combustion in a Leco CN-2000 Analyser (Leco Corp., St. Joseph, MI, USA). Organic matter content was estimated by combustion of the samples at 375 ºC for 16 h.

Enzyme assays and microbial biomass analysis

In each sample the potential activity of six different hydrolytic enzymes involved in C, N and P cycling was assayed using 4-methylumbelliferone (MUF) or 7-amino-4-methyl coumarin (AMC) linked-substrates: α-glucosidase, β-glucosidase, N-acetyl- β-D-glucosaminidase, cellobiohydrolase, acid phosphatase and leucine-aminopeptidase. The activity of these enzymes has been found to be particularly sensitive to increased oxygen availability during drying events in organic-rich soils (Fenner et al., 2011; Freeman et al., 2004). A protocol modified from that proposed by Freeman et al. (1995; 1997) for peatland soils was used. With the objective of assessing whether the respiration response was related to increases in EEA reaction rates, measured under pH and temperature conditions similar to those occurring in the soil environment in the field, a buffer solution with a pH similar to that of bulk soil (50 mM acetate buffer solution, pH 4.6) was used, and incubation temperature was set to 10 ºC, which is similar to the average soil temperature during the summertime in all the treatments. Substrate concentrations and incubation times were selected based on previous analysis of substrate saturation curves determined for each enzyme at the same pH and temperature conditions in a set of soil samples from the site (Appendix, Table A1), to ensure that each hydrolytic enzyme was assayed under saturating conditions. Seven mL of substrate + buffer solution were added to 1 g of fresh soil and incubated in the dark. Then, soil suspension was transferred to centrifuge tubes, centrifuged for five minutes,

and 300 μL-aliquots of the supernatant solution were transferred to 96-well plates for measurement of fluorescence using an excitation wavelength of 330 nm and an emission wavelength of 450 nm (Cary Eclipse Fluorescence Spectrophotometer, Agilent, Santa Clara, CA, USA). Addition of NaOH to improve fluorescence emissivity conditions was not necessary because of the high sensitivity of the equipment.

Extracellular phenol oxidase activity was measured following the procedure proposed by Toberman et al. (2008) previously optimized for soils collected from the same location. Homogenates of 1 g of soil in 9 ml of ultra-pure water were prepared by gentle mixing in a vortex to minimise cell disruption. Aliquots of 300 μL of these homogenates were diluted with 450 μL of ultra-pure water, then 750 μL of 10 mM dihydroxy phenylalanine (L-DOPA) were added to the homogenates, and then they were incubated during 9 min at 10 ºC, followed by centrifugation for 5 minutes. Absorbance of the supernatant (three aliquots of 300 μL) was measured at 460 nm, and phenol oxidase activity calculated using Beer-Lambert´s Law, with a molar absorption coefficient for the L-DOPA product 3-dihydroindole-5,6-quinone-2-carboxylate (diqc) of 3.7×10^4 (Mason, 1948). Microbial biomass C and N was estimated using the chloroform fumigation-extraction method (Vance et al., 1987).

Data analysis

Repeated measures ANOVA was applied to test for significant differences in field soil respiration rates among treatments and over time. Linear mixed models were applied to microbial biomass, enzyme data and soil chemistry data, previously log-transformed to meet normality, with treatment as fixed factor and block as random factor, using SPSS v 21. Significance level was fixed to $p \leq 0.05$.

3. Results and discussion

The drought treatment induced a decline in soil moisture, which was not limited to the experimental rainfall reduction period (June-September), but persisted throughout the year (Fig. 1). In contrast, warmed soils were wetter than control soils, likely due to an increase in bryophyte abundance in the warming treatment after the natural drought of 2005 that changed soil water dynamics (Robinson et al., 2016). This increase in soil moisture in warmed soils could enhance water-excess conditions, which restricts oxygen diffusion to decomposition reaction sites and limits SOM decomposition (Fenner and Freeman 2011; Freeman et al. 2001). However, soil respiration rates were slightly higher under warming in comparison to the control treatment, although this increase was not statistically significant. The long-term warming effect on respiration found for this organo-mineral soil was therefore subtle, much lower than that reported for deeper organic soils in North Wales (Kim et al., 2012).

In contrast to warming, drought had a significant year-round effect on soil CO_2 efflux, enhanced during the summer season when increases in soil CO_2 efflux were up to 50 mg C-CO_2 m^{-2} h^{-1} (repeated measures ANOVA: drought effect $p = 0.044$ - Tukey post-hoc test, compared to control-; time × treatment effect: $p = 0.0005$).

As with the results obtained two years after treatment initiation, and in contrast to our first hypothesis, extracellular enzyme activities did not significantly differ among treatments after 13 years of climate manipulation (Fig. 2), neither on a dry soil basis nor when calculated as substrate used per microbial biomass unit (mass-specific activity, data not shown). Likewise, microbial biomass, microbial C:N ratio, water-extractable phenolics and soil nitrate, ammonium and available phosphate were similar among treatments (Table 1).

The lack of treatment effects on soil ammonium found here contrasts to the 70% decrease observed in the drought plots one year after treatment initiation (summer 2000), which was interpreted as a consequence of a temporal shift in community composition (indicated by change in microbial C:N) towards increased fungal dominance, that enhanced the decomposition of substrates with higher C:N ratios (Jensen et al., 2003). In our study we did not find such pattern, likely because summer 2000 was a specially wet season (rainfall of 340 mm for the June-August period, a 67 % greater than rainfall for the same period in 2012), when changes in soil N mineralisation between drought and control treatments might be particularly enhanced given the high sensitivity of N mineralization to water-excess conditions (Emmett et al., 2004).

Despite field measurements suggesting enhanced SOM mineralization (greater soil respiration - Fig.1 -, and progressive increases in DOC concentration in soil water from the drought plots, Sowerby et al 2010), treatments had no effect on enzyme activities, measured under non-optimal conditions to simulate field soil environment (pH of 4.1 and temperature incubation of 10 ºC). A possible explanation for these results is that the increases in soil respiration were simply caused by increases in microbial biomass or changes in the efficiency in the use of C substrates. In the laboratory analysis, however, we did not detect any change in microbial biomass among treatments, nor a change in the C:N ratio in microbial biomass, which could have indicated a shift in the composition of the microbial community and, possibly, a change in its substrate use efficiency.

In addition, the increases in soil respiration could be also due to an increase in the autotrophic component, or in the supply of labile C compounds to microorganism from plant roots, stimulating microbial respiration. However, there are indications that respiration changes are likely driven by heterotrophic processes, as root biomass was

not significantly greater in the drought plots (Domínguez et al., 2015), and C translocation belowground was reduced by 40 % in the drought treatment, as found in a ^{14}C pulse-labelling experiment (Gorissen et al. 2004). Another possible explanation is that the in-situ response of soil respiration to the treatments is related to changes in other C-processing enzymes, not analysed in this work.

It is important to note that it is not possible to conclude that there were no treatment effects on the soil enzyme pool because the assays were not run under optimal conditions. Possibly, if assays were conducted under those conditions that maximise hydrolytic enzyme activities (typically, at 20-30 ºC and using pH buffer with pH< 5), differences in EEAs might be significant. With optimal pH and temperature conditions, enzyme assays give information about the size of the pool of active enzymes, which is determined by the balance between the rates of enzyme production by microbes and the rates of enzyme degradation in the soil environment. The non-optimised approach, in contrast, attempts to mimic the soil environment in order to estimate enzyme reaction rates at natural pH and temperature conditions (German et al., 2011; Burns et al., 2013). These two approaches might produce very different results. Therefore, in our study the potential effect of the treatments on enzyme activity might be obscured by the use a non-optimised enzyme assay. We expected, however, that if treatment provoked a large effect on the soil enzyme pool this would be detectable with our assay conditions, given that several works with wet organic soils have shown that enzyme assays conducted at similar conditions (pH and temperature set to represent field conditions) can detect significant changes in enzyme reaction rates in response to a range of factors, such as simulated drought (Fenner and Freeman 2001; Freeman et al., 1997), CO_2 enrichment (Fenner et al., 2007) or increased oxygen availability (Freeman et al., 2004). In any case, several works have reported no effects of climate change treatments on soil

enzyme pool (analysed using the optimized approach), despite clear in-situ effects of these climate change drivers on C and N mineralization (Bell et al., 2010; Jing et al., 2014; Steinweg et al., 2013).

4. Conclusions

Under field conditions, recurrent summer droughts had a profound effect on soil respiration in wet organo-mineral soils, producing larger increases in CO_2 emissions than long-term warming, which suggested enhanced C mineralization in the drought treatment. Treatments had no effect on C-cycling enzyme reaction rates, measured under non-optimised pH and temperature conditions that simulated the soil environment in the field. Therefore, significant effects on the soil enzyme pool cannot be completely excluded, because the assays were not run under optimal conditions. Our results highlight the need for developing and applying methods for in-situ analysis of EEAs to advance our understanding of the impact of these drivers on SOM decomposition.

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420

421 **Figure captions**

422

423 **Fig. 1** Soil respiration change (symbols, mean ± standard error, right axis) and soil
424 moisture change (difference from control treatment; lines, left axes) in the experimental
425 drought and warming plots.

426

427 **Fig 2** Extracelullar enzyme activities in the soils from the experimental treatments
428 (mean + standard error). b–glu: β–glucosidase; NaG: N–acetyl–β–D–glucosaminidase ;
429 Cell: cellobiohydrolase; a–glu: α–glucosidase; Phos: acid phosphatase; Pep: leucine–
430 aminopeptidase; PheOx: phenol–oxidase.

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Table 1 Soil pH, DOC, C:N, available N and P, and microbial biomass and C:N ratio (mean ± standard error) in the control, drought and warming treatments. There were no significant differences among treatments for these variables (linear mixed models, treatment effect non-significant). SOM = soil organic matter; DOC = dissolved organic carbon.

	Treatment		
	Control	Drought	Warming
pH	4.14 ± 0.02	4.02 ± 0.06	3.97 ± 0.24
C:N	25.0 ± 0.4	26.2 ± 0.6	26.5 ± 0.3
NH_4^+ (mg kg^{-1})	13.9 ± 0.7	13.1 ± 4.3	18.5 ± 1.0
NO_3^- (mg kg^{-1})	25.6 ± 0.1	23.1 ± 2.9	27.6 ± 3.2
P (mg kg^{-1})	33.7 ± 17.4	44.8 ± 10.6	48.0 ± 2.8
DOC (mg kg-1)	59.1 ± 3.5	65.1 ± 33.5	63.1 ± 21.8
Phenolics (mg kg^{-1})	7.41 ± 0.86	8.27 ± 2.15	6.83 ± 1.63
Microbial biomass (mg kg^{-1})	2680 ± 404	3204 ± 441	2815 ± 230
Microbial biomass (mg g SOM^{-1})	7.12 ± 0.49	7.48 ± 0.20	6.51 ± 1.24
Microbial C:N	6.65 ± 0.73	6.11 ± 0.65	6.61 ± 1.02