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Contrasting response of summer soil respiration and enzyme activities to long-term warming and drought in a wet shrubland (NE Wales, UK)

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

48

49 1. Introduction

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

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

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

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

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

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

98 **2. Material and methods**

99 Experimental set up and field measurements

100 Whole ecosystem warming and summer drought treatments were established during
101 1999 in an upland Atlantic shrubland dominated by *Calluna vulgaris* (L.) located in NE
102 Wales (UK, 53º 03’ 19”N, 03° 27’ 55”W). Mean annual air temperature at the site is 8.2
103 ºC, rainfall is 1700 mm, and potential evapotranspiration is 302 mm. The soil at the site
104 is an organic-rich humo-ferric Podzol, and has been classified as a Ferric stagnopodzol
105 in the Hafren Series in the Soil Survey of England and Wales (Cranfield University,
106 2014). The ecosystem has remained unmanaged and undisturbed over at least the last 25
107 years, and has moved from a “mature” to “degenerate” phase of shrubland succession
108 (Domínguez et al., in press).

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

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

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

128 Soil sampling and chemical analyses

129 In July 2012 (mid-summer, within the experimental drought period) a composite soil
130 sample (0-10 cm depth) was obtained from each experimental plot by mixing three
131 subsamples collected with a cylinder auger at three different locations within each of the
132 plots to conduct enzyme assays. Therefore, there were three replicates per treatment for
133 the subsequent soil analyses. Sampled soils had a high organic matter content (SOM >
134 30 %), and included decomposing debris. Soils were transported to the lab in a
135 refrigerated container, and kept between 2 and 4 ºC until enzyme assays were
136 completed, within the following 72 hours. Prior to analysis roots were removed, and
137 soils were sieved to < 2 mm.

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

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

148 Enzyme assays and microbial biomass analysis

149 In each sample the potential activity of six different hydrolytic enzymes involved in C,
150 N and P cycling was assayed using 4-methylumbelliferone (MUF) or 7-amino-4-methyl
151 coumarin (AMC) linked-substrates: α-glucosidase, β-glucosidase, N-acetyl- β-D-
152 glucosaminidase, cellobiohydrolase, acid phosphatase and leucine-aminopeptidase. The
153 activity of these enzymes has been found to be particularly sensitive to increased
154 oxygen availability during drying events in organic-rich soils (Fenner et al., 2011;
155 Freeman et al., 2004). A protocol modified from that proposed by Freeman et al. (1995;
156 1997) for peatland soils was used. With the objective of assessing whether the
157 respiration response was related to increases in EEA reaction rates, measured under pH
158 and temperature conditions similar to those occurring in the soil environment in the
159 field, a buffer solution with a pH similar to that of bulk soil (50 mM acetate buffer
160 solution, pH 4.6) was used, and incubation temperature was set to 10 ºC, which is
161 similar to the average soil temperature during the summertime in all the treatments.
162 Substrate concentrations and incubation times were selected based on previous analysis
163 of substrate saturation curves determined for each enzyme at the same pH and
164 temperature conditions in a set of soil samples from the site (Appendix, Table A1), to
165 ensure that each hydrolytic enzyme was assayed under saturating conditions. Seven mL
166 of substrate + buffer solution were added to 1 g of fresh soil and incubated in the dark.
167 Then, soil suspension was transferred to centrifuge tubes, centrifuged for five minutes,

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

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

185 Data analysis

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

191

192 **3. Results and discussion**

193 The drought treatment induced a decline in soil moisture, which was not limited to the
194 experimental rainfall reduction period (June-September), but persisted throughout the
195 year (Fig. 1). In contrast, warmed soils were wetter than control soils, likely due to an
196 increase in bryophyte abundance in the warming treatment after the natural drought of
197 2005 that changed soil water dynamics (Robinson et al., 2016). This increase in soil
198 moisture in warmed soils could enhance water-excess conditions, which restricts
199 oxygen diffusion to decomposition reaction sites and limits SOM decomposition
200 (Fenner and Freeman 2011; Freeman et al. 2001). However, soil respiration rates were
201 slightly higher under warming in comparison to the control treatment, although this
202 increase was not statistically significant. The long-term warming effect on respiration
203 found for this organo-mineral soil was therefore subtle, much lower than that reported
204 for deeper organic soils in North Wales (Kim et al., 2012).

205 In contrast to warming, drought had a significant year-round effect on soil CO_2 efflux,
206 enhanced during the summer season when increases in soil CO_2 efflux were up to 50 mg
207 $C\text{-}CO_2\ m^{-2}\ h^{-1}$ (repeated measures ANOVA: drought effect $p = 0.044$ - Tukey post-hoc
208 test, compared to control-; time × treatment effect: $p = 0.0005$).

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

216 The lack of treatment effects on soil ammonium found here contrasts to the 70%
217 decrease observed in the drought plots one year after treatment initiation (summer
218 2000), which was interpreted as a consequence of a temporal shift in community
219 composition (indicated by change in microbial C:N) towards increased fungal
220 dominance, that enhanced the decomposition of substrates with higher C:N ratios
221 (Jensen et al., 2003). In our study we did not find such pattern, likely because summer
222 2000 was a specially wet season (rainfall of 340 mm for the June-August period, a 67 %
223 greater than rainfall for the same period in 2012), when changes in soil N mineralisation
224 between drought and control treatments might be particularly enhanced given the high
225 sensitivity of N mineralization to water-excess conditions (Emmett et al., 2004).

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

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

241 not significantly greater in the drought plots (Domínguez et al., 2015), and C
242 translocation belowground was reduced by 40 % in the drought treatment, as found in a
243 ^{14}C pulse-labelling experiment (Gorissen et al. 2004). Another possible explanation is
244 that the in-situ response of soil respiration to the treatments is related to changes in
245 other C-processing enzymes, not analysed in this work.

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

266 enzyme pool (analysed using the optimized approach), despite clear in-situ effects of
267 these climate change drivers on C and N mineralization (Bell et al., 2010; Jing et al.,
268 2014; Steinweg et al., 2013).

269 **4. Conclusions**

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

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286

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420 **Figure captions**

421

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

425

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

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439 **Table 1** Soil pH, DOC, C:N, available N and P, and microbial biomass and C:N ratio
440 (mean ± standard error) in the control, drought and warming treatments. There were no
441 significant differences among treatments for these variables (linear mixed models,
442 treatment effect non-significant). SOM = soil organic matter; DOC = dissolved organic
443 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

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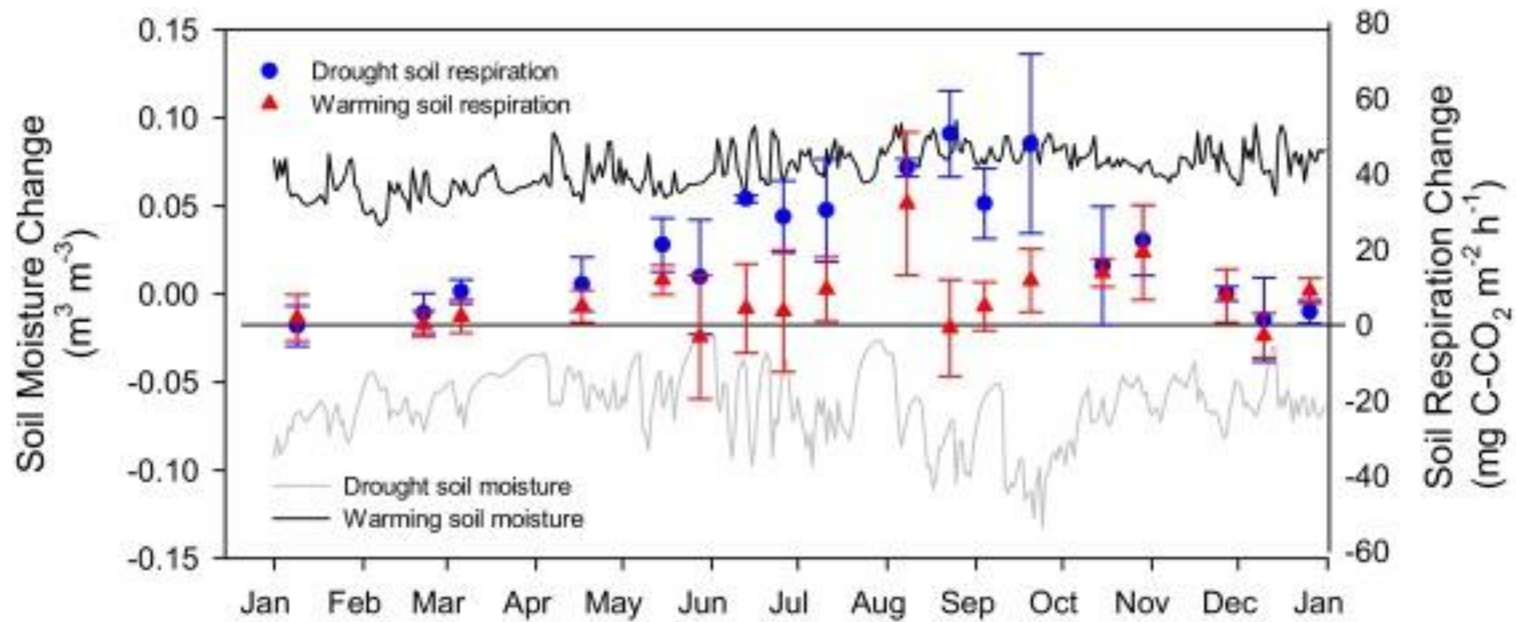

Fig. 1

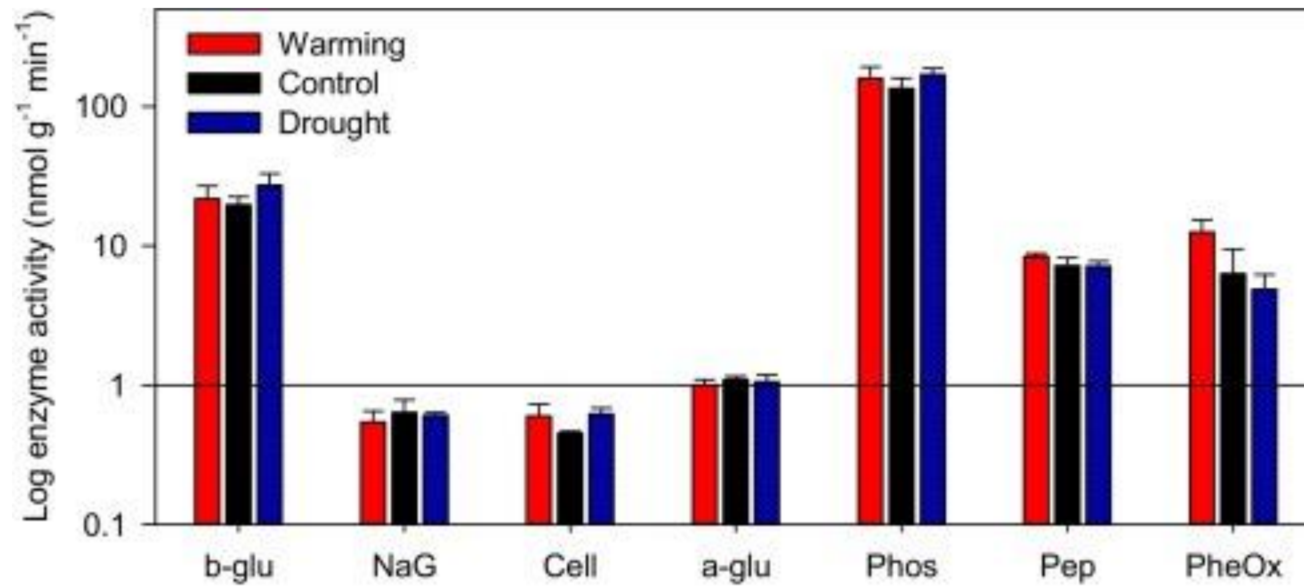

Fig. 2

Appendix

Table A1

Analysed hydrolytic enzymes, with indication of substrate concentration used and incubation times.

Enzyme	**Substrate**	**Substrate concentration (mM)**	**Incubation time**
α-glucosidase	4-Methylumbelliferyl α-d-glucopyranoside	400	90 min
β-glucosidase	4-Methylumbelliferyl β-d-glucopyranoside	200	45 min
N-acetyl-β-d-glucosaminidase	4-Methylumbelliferyl N-acetyl-β-d-glucosaminide	300	4 h
Cellobiohydrolase	4-Methylumbelliferyl β-d-cellobioside	200	4 h
Acid phosphatase	4-Methylumbelliferyl phosphate	200	45 min
Leucine-aminopeptidase	l-Leucine-7-amido-4-methylcoumarin hydrochloride	400	45 min