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Linkages between extracellular enzyme activities and the carbon and nitrogen content of grassland soils

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ABSTRACT

Important biochemical reactions in soils are catalyzed by extracellular enzymes, which are synthesized by microbes and plant roots. Although enzyme activities can significantly affect the decomposition of soil organic matter and thus influence the storage and cycling of carbon (C) and nitrogen (N), it is not clear how enzyme activities relate to changes in the C and N content of different grassland soils. Here we address whether the activity of C-acquiring (β-1,4-glucosidase, BG) and N-acquiring (L-leucine aminopeptidase (LAP) and β-1,4-N-acetyl-glucosaminidase (NAG)) enzymes is linked to changes in the C and N content of a variety of human-managed grassland soils. We selected soils which have a well-documented management history going back at least 19 years in relation to changes in land use (grazing, mowing, ploughing), nutrient fertilization and lime ($CaCO_3$) applications. Overall we found a positive relationship between BG activity and soil C content as well as between LAP + NAG activity and soil N. These positive relationships occurred across grasslands with very different soil pH and management history but not in intensively managed grasslands where increases in soil bulk density (i.e. high soil compaction) negatively affected enzyme activity. We also found evidence that chronic nutrient fertilization contributed to increases in soil C content and this was associated with a significant increase in BG activity when compared to unfertilized soils. Our study suggests that while the activities of C- and N-acquiring soil enzymes are positively related to soil C and N content, these activities respond significantly to changes in management (i.e. soil compaction and nutrient fertilization). In particular, the link between BG activity and the C content of long-term fertilized soils deserves further investigation if we wish to improve our understanding of the C sequestration potential of human-managed grassland soils.

1. Introduction

Soil ecosystems represent the largest terrestrial reservoirs of carbon (C) and nitrogen (N) and could act either as sources or sinks of C and N depending on the net effect of multiple environmental factors including soil management by humans (Post and Kwon, 2000; Lal, 2004). Changes in the storage and cycling of C and N are strongly dependent on the rate of decomposition of organic detritus returned to soils and the incorporation of C and N into stable soil organo-mineral fractions. Soil organic matter (SOM) decomposition is a predominantly microbial-mediated process whereby soil microorganisms produce a variety of extracellular enzymes, which serve a dual function: (1) to catalyze a set of chemical reactions involved in the degradation of SOM, and (2) to acquire the necessary energy and nutrient resources for the enzyme producers (Sinsabaugh et al., 2009; Brzostek and Finzi, 2011; Wallenstein et al., 2011). Enzyme activities in soils represent key biological processes that link the quality of SOM (e.g. the relative availability of C and N) with the ability of microbes to assimilate nutrients and use C for their own metabolism (Allison et al., 2011). This suggests that shifts in C- or N-acquiring enzyme activities may be linked to changes in the availability and/or storage of C and N within SOM pools.

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Recent studies show that soil enzyme activity is associated with changes in SOM concentration across different terrestrial ecosystems (Sinsabaugh et al., 2008; Trasar-Cepeda et al., 2008; Stursová and Baldrian, 2011; Wallenius et al., 2011). Despite this evidence, our mechanistic understanding of whether shifts in the activity of C- or N-acquiring enzymes might be linked to changes in soil C and N content is still limited. Greater SOM accumulation can lead to increases in the activity of extracellular enzymes 'simply' by providing a wider range of C and N substrates that can be accessed and utilized by a variety of soil microbial groups. In addition, increased stratification of SOM can lead to higher spatial heterogeneity of the soil matrix, which in turn can be associated with a larger number of niches available to microbial enzyme foraging (Allison, 2005).

Changes in microbial enzyme activities in response to land management practices may themselves actively influence SOM concentration and thus soil C and N content. For example, the stimulation or repression of C- or N-acquiring enzymes could influence rates of SOM decomposition (Burns et al., 2013). Furthermore, once released into the soil, these extracellular enzymes may accumulate, adsorb and stabilize onto mineral surfaces thus contributing to increases in the C and N content of SOM (Kandeler et al., 1999; Cotrufo et al., 2013). When enzymes are sorbed to mineral surfaces they become less effective in degrading C and N compounds thus contributing to greater C and N accumulation in mineral soil pools (Allison and Jastrow, 2006; Grandy et al., 2008).

The main goal of our study is to address whether and how changes in soil C and N content correlate significantly with changes in the activity of C- and N-acquiring enzymes in grasslands characterized by very different human management histories. Based on previous studies (Sinsabaugh et al., 2008; Trasar-Cepeda et al., 2008; Stursová and Baldrian, 2011; Wallenius et al., 2011), we hypothesize that soil C and N content should be positively related to the activity of C-acquiring and N-acquiring enzymes in most grassland soils. In particular we hypothesize the existence of a positive feedback between soil C (or N) content and the activity of the C-acquiring (or N-acquiring) enzyme. Because we included in our analyses soil types characterized by very different pH values and because soil pH can strongly influence both enzyme activity (Turner, 2010) as well as soil C and N (Fornara et al., 2011) we also ask whether enzyme activities might relate to changes in C and N content across soils with different pH values.

Secondly, we ask whether and how past and current management practices (i.e. management history) have influenced the relationship between enzyme activity and soil C and N content. Here we compare two sets of grasslands, one of which has been historically managed as permanent grassland and the other, which had been regularly ploughed until the 1960s. We also compare permanent grasslands, which are distributed along a gradient of use intensity (i.e. management intensity) as determined by livestock density, nutrient fertilizer and liming applications (i.e. $CaCO_3$).

Finally, we compare grasslands, which have received long-term applications of N (i.e. nutrient fertilization), asking how the increased availability of N relative to C has ultimately influenced the activity of C- and N-acquiring enzymes as well as the relationship between enzyme activities and the C and N content of these grassland soils.

2. Methods

We selected grassland soils, which had a clear history of human management and are established across three European regions. Our aim was to select soils that have been undergoing specific treatments (e.g. nutrient fertilization, grazing, liming) for ≥19 years, as follows.

2.1. Nash's field (England)

Nash's Field is a long-term grassland experiment that was set up in 1991 at Silwood Park, Berkshire, England. This area is characterized by a temperate oceanic climate with average annual temperature of 9.6 °C and annual precipitation of 754 mm (www.climatedata.eu). Nash's Field is a species-poor grassland lying on acid, sandy soil. The experiment is a split-plot design, which includes large (randomly distributed) blocks (each 900 m^2) within which are nested multiple experimental treatments (see Edwards and Crawley, 1999). In our study we specifically tested for the effects of (1) rabbit grazing (±rabbit fencing), (2) liming (±lime, i.e. $CaCO_3$), and (3) nutrient treatments (±N, P, K and Mg fertilizers nutrients) on enzyme activities and soil C and N content. Rabbit grazing was excluded by using 1 m high fences with wire mesh (3 cm diameter mesh size). Each fenced and grazed plot (400 m^2) was divided in two equal plots, one of which was randomly selected to receive lime applications (5 tons $CaCO_3$ ha^{-1}) and the other not. Each of the limed and non-limed plots (144 m^2) was then split into subplots (4 m^2) for the application of five different nutrient combinations (N-only, P-only, N and P together, all nutrients-N, P, K and Mg) plus a control plot without any nutrient treatment. Mineral nutrients have been applied yearly with the following concentrations: 100 kg ha^{-1} of N (as ammonium nitrate- NH_4NO_3), 35 kg ha^{-1} of P (as triple superphosphate), 225 kg ha^{-1} of K (as muriate of potash) and 11 kg ha^{-1} of Mg (as Epsom salts). Total atmospheric N deposition is estimated as ~22 kg N ha^{-1} y^{-1} (www.apis.ac.uk). In summary, the experiment has 4 large blocks (each 900 m^2), 8 rabbit grazing plots (each 400 m^2), 16 liming plots (each 144 m^2), and a total of 80 nutrient plots (each 4 m^2), which were sampled in 2011. Four soil cores (3 cm diameter) were collected from each nutrient plot between 0 and 20 cm soil depth.

2.2. Lautaret (France)

The Lautaret experimental site is located in the Central French Alps on the south-facing slope of the upper valley of the Romanche River (Villar d'Arène, 45°03′N, 6°24′E). Winters are cold and snowy (mean February temperature of −7.4 °C), summers are cool (mean July temperature of 13 °C), while average annual precipitation is 956 mm (see Lavorel et al., 2007, 2011 and Robson et al., 2007 and for more details). The total area is 13 Km^2 and the elevation ranges between 1552 and 2500 m a.s.l. Historical land use trajectories, representing combinations of past and present land use were defined based on historical records and aerial photography (Quétier et al., 2007). Former arable fields (1650–2000 m) were converted to grasslands fifty years ago and are currently used for hay or grazing by sheep or cattle; former (never ploughed) hay meadows (1800–2000 m) are either still mown or grazed by sheep; summer grasslands (>2000 m) have remained under pastoral use over history. In this study we investigated 5 trajectories: three representing previously (until the 1960s) cultivated terraces (1) currently fertilized (mainly animal manure) and mown, (2) mown, (3) unmown and grazed in spring and autumn, and two representing never cultivated (never ploughed) permanent grasslands with a multi-century history of mowing (4) currently mown, and (5) unmown (for > 30 years) and summer grazed. Applications of organic nutrient manure generally occur between May and June each year and on average correspond to the addition of 8 kg N ha^{-1} yr^{-1} (Robson et al., 2007). Here we randomly sampled 2 large grassland replicate plots under each of the five land use trajectories. The plots measured 15 × 15 m and within each we

collected 10 random soil samples between 0 and 20 cm soil depth.

2.3. Agricultural grasslands (Northern Ireland)

We selected 45 permanent grasslands dominated by *Lolium perenne* L. (perennial ryegrass) at three lowland farm sites in County Antrim (54°45′N, 5°55′W; 54°41′N, 6°12′W) and County Fermanagh (54°22′N, 7°43′W), Northern Ireland. The regional climate is temperate and relatively humid influenced by the North Atlantic Drift with mean annual average temperatures and precipitation of 10 °C and 900 mm at the Antrim sites and 8.5 °C and 1300 mm at the Fermanagh site respectively (Sweeney, 1997). These grasslands are grazed on a rotational basis by cattle, fertilized and cut for silage three times a year. The three farms were selected because of their detailed management history, which dates back at least 20 years. We selected the three farms along a gradient of land use intensity based on the density of livestock (cattle) units (i.e. stocking rate) and the application of nutrient fertilizers (N, P, K) and lime ($CaCO_3$). Farm 1 is less intensively used having stocking rates of 0.8 animals ha^{-1}, 163 kg N, P, K ha^{-1} yr^{-1} and 52 kg lime ha^{-1} yr^{-1}; Farm 2 is more intensively used having rates of 1.2 animals ha^{-1}, 130 kg N, P, K ha^{-1} yr^{-1} and 97 kg lime ha^{-1} yr^{-1}; Farm 3 is the most intensively used having rates of 2.05 animals ha^{-1}, 209 kg N, P, K ha^{-1} yr^{-1} and 281 kg lime ha^{-1} yr^{-1}. Synthetic nutrient fertilizer has a macronutrient NPK ratio of 20–10–10 (i.e. 20% N, 10% P_2O_5, 10% K_2O). Ten soil cores (3 cm diameter) were collected from each grassland between 0 and 20 cm soil depth.

2.4. Soil sampling and analyses

Across all our selected grasslands we collected soil samples using a 3-cm diameter × 20-cm deep soil corer. Fresh soil samples were passed through a 2-mm sieve by hand prior to subsampling and laboratory analysis. Total soil C and N was determined by drying fresh soil samples at 40 °C for 5 days, grinding with a ball mill (Retsch MM 200) and analyzing subsamples by combustion and gas chromatography on a COSTECH Analytical ECS 4010 element analyzer (Costech Analytical, Valencia, California, USA). To assess soil bulk density (BD), two further subsamples were collected using a 8-cm diameter × 20-cm deep PVC soil corer; BD was then calculated as the ratio between the mass of oven-dried soil and volume of fresh soil (g/cm^3). Soil pH was measured using a suspension made of 10 g of air-dried sieved soil in 50 mL of deionized (DI) water (1:5 sample to DI water ratio). For the determination of the soil moisture content (%), a further 10 g of fresh soil was weighed, dried at 105 °C to a constant mass (minimum 48 h) and reweighed. Two g of fresh and sieved soil subsamples (<2 mm) were put into an 8 mL bijou and then frozen to −20 °C for 3 weeks before enzyme analysis.

2.5. Enzyme analysis

The activity of three extracellular enzymes (Table 1) involved in cellulose decomposition (β-1,4 glucosidase (BG)), and N acquisition (L-leucine aminopeptidase (LAP) and β-1,4-N-acetyl-glucosaminidase (NAG)) were assayed according to a modification of the method described by Saiya-Cork et al. (2002). The extracellular enzymes were measured fluorometrically using a 200 μM solution of 4-methylumbelliferone (MUB) or 7-Amino-4-methylcoumarin (AMC) labeled substrates. Three biological buffers (acetate/maleate/bicarbonate) were used depending on the field soil pH (acidic/mildly-acidic and neutral/basic respectively). Using a multichannel pipettor, 50 μL aliquots of 50 mM buffer were dispensed in columns of a 96-well black microplates (Nunk® FluoroNunc™ P8741 Sigma–Aldrich) that served as the blank (buffer + slurry); 200 μL aliquots of 50 mM buffer were dispensed in the reference standard (buffer + standard) and negative control (buffer + substrate) columns (8 analytical replicate per soil per assay each). Two grams of previously defrosted soil were homogenized for 4 min with a Silverston L4R homogenizer (Silverstone Machines Ltd., England) in 250 mL of 50 mM buffer. The soil suspension (soil slurry) was continuously stirred while 200 μL aliquots were dispensed into microplate columns that served as the sample assay (16 analytical replicate soil suspensions for each sample per assay), blank and quench standard (slurry + standard) (8 analytical replicate each). A 50 μl aliquot of fluorescence standard solution (10 μM 4-methylumbelliferone-MUB-, or 7-amino-4-methylcoumarin-AMC-in the case of the LAP assay) was dispensed into microplate columns that served as a reference standard (buffer + standard) and as a quench standard. Finally, sample assay (slurry + substrate) and negative control (buffer + substrate) also received 50 μl of a 200 μM substrate solution (see Table 1) in a final reaction volume of 250 μL. Prepared plates were incubated in the dark at 20 °C for up to 24 h following substrate addition. Fluorescence was measured without addition of NaOH (German et al., 2011; Shaw and DeForest, 2013) using a FLUOstar Omega microplate reader (BMG Labtech) with λ365 nm excitation and λ460 nm emission filters. Following correction of the assay wells' fluorescence measurements for negative controls, blanks and quench standard wells, enzyme activity was expressed as nanomoles of substrate released per hour per gram of dry soil (nmol h^{-1} g^{-1}).

3. Data analysis

Our three grassland sites are located across different geographical areas and under different environmental conditions. Thus we first performed Residual Maximum Likelihood (REML) estimation analyses (which included grassland site as random effect) to test how soil C and N content would vary as a function of fixed key environmental variables (i.e. soil pH, BG and NAG + LAP activities). Second, we performed linear regression analyses to address potential relationships between enzyme activities and the C and N content of our grassland soils. Third, multiple regression analyses were performed within each dataset to search for potential relationships between enzyme activity and other key environmental factors (e.g. soil pH, livestock density, soil C and N content etc.). Finally, we performed site-specific analyses using separate mixed effects ANOVAs, which in turn included as random effects either the four large blocks (in the Nash's Field), or the five land use trajectories (at the Lautaret) or the three farmland sites (in Northern Ireland) and as fixed effects the various treatments applied at each site (e.g. grazing, liming, nutrient fertilization etc.). We tested these fixed effects on multiple response variables (i.e. soil C, soil N, soil pH, and extracellular enzyme activities). We used restricted maximum likelihood (REML) methods to produce final models and checked that these models conformed to modeling assumptions. Significant differences between factor levels (i.e. five nutrient treatments in Nash's Field) were tested using post-hoc Tukey tests. Soil and enzyme data were square-root transformed to improve the normality of errors and homogeneity of variance. We also estimated C- and N-acquiring enzymes activity per unit of soil C and soil N respectively by dividing BG (or NAG + LAP) activity by total soil organic C (and N) content. All analyses were conducted using JMP version 9.0 statistical software (SAS Institute Inc. 2010).

4. Results

Overall our REML analyses showed that soil pH ($F_{1,201} = 21$; $P < 0.0001$) and BG activity ($F_{1,201} = 24$; $P < 0.0001$) were

Table 1
Hydrolytic enzymes (and their substrates) assayed in multiple soil samples collected from three main experimental sites across Europe.

Enzymes and their function in soils	Final product	E.C.	Substrate
β-1,4-glucosidase (BG) Hydrolysis of cellulose	Glucose (sugar)	3.2.1.21	4-MUB-β-D gluco (pyrano)side
β-1,4-N-acetyl-glucosaminidase (NAG). Hydrolysis of chitin.	N-acetyl-β-D-glucosamine (sugar)	3.2.1.14	4-MUB-N-acetyl-β-D glucosaminide
L-Leucine aminopeptidase (LAP). Hydrolysis of amino acid residues (N- terminus of peptides and proteins)	Leucine (other amino-acids)	3.4.11.1	L-Leucine-7-amido-4 methylcoumarin

E.C. = Enzyme Commission number; 4-MUB = 4-methylumbelliferyl.

significantly related to soil C after we controlled for grassland site identity. Similarly soil pH ($F_{1,201} = 5.7$; $P = 0.03$) and NAG + LAP activity ($F_{1,201} = 35$; $P < 0.0001$) were significantly related to soil N across our grassland sites. Results from linear regression analyses show significant positive relationships between extracellular enzyme activities and soil C and N content (Fig. 1) across our study sites.

4.1. Enzyme activity and soil C and N content across a wide range of soil pH values

The positive relationship between enzyme activity and soil C and N content was not affected by changes in soil pH and remained significant between BG and soil C (Fig. 2a–c) as well as between NAG + LAP and soil N (Fig. 2d–f) across our grasslands. This suggests that regardless of variation in soil pH the activity of C-acquiring and N-acquiring enzymes remains significantly related to changes in soil C and N content. In different multiple regression analyses where we included both soil pH and enzyme activity as predictor variables we found that BG and NAG + LAP activities in the Lautaret grasslands remained significantly related to soil C and N content respectively (Table 2).

4.2. Enzyme activity and soil C and N content across grasslands with different histories of soil management

We compared soil enzyme activities between permanent grasslands (never ploughed) and grasslands that underwent regular cultivation and ploughing until the 1960s in the Central French Alps. We found a positive relationship between BG activity and soil C content of both permanent and historically ploughed grasslands (Fig. 3a). Grassland soils that were historically ploughed and received nutrient additions have a significantly higher soil C content compared to permanent grassland soils ($P < 0.0001$). Similarly we found a positive relationship between NAG + LAP activity and soil N content of permanent grasslands but not between NAG + LAP and soil N of historically ploughed (and nutrient-fertilized) grasslands (Fig. 3b).

4.3. Enzyme activity and soil C and N content in agricultural grasslands

We found that soil enzyme activities were neither related to soil C nor to soil N content in intensively managed agricultural grasslands (Table 3). In multiple regression analyses where enzyme activities were included with soil bulk density and livestock density (i.e. number of cattle per hectare) we found that only soil bulk density and livestock densities were significantly (negatively) related to soil C and soil N (Table 3).

4.4. Enzyme activity and soil C and N content under N fertilization

We found that the addition of N fertilizer to soils (Nash's field and Lautaret) had a significant positive effect on BG activity (Fig. 4a) and that long-term N fertilization was also positively related to soil C content (Fig. 4b). We did not find any significant effect of N fertilization on NAG + LAP activity (Fig. 5a) but N fertilization had a positive effect on total soil N (Fig. 5b). N fertilization had a positive significant effect on BG activity per unit of soil C ($P < 0.004$) (Fig. 6a) and a negative significant effect on NAG + LAP activity per unit of soil N (Fig. 6b). Finally, we found that the activities of C- and N-acquiring enzymes were positively related (Fig. 7) and that these activities were affected by N fertilization. In particular BG activity relative to NAG + LAP increased with increases in N fertilization whereas NAG + LAP activity relative to BG increased with decreases in N fertilization.

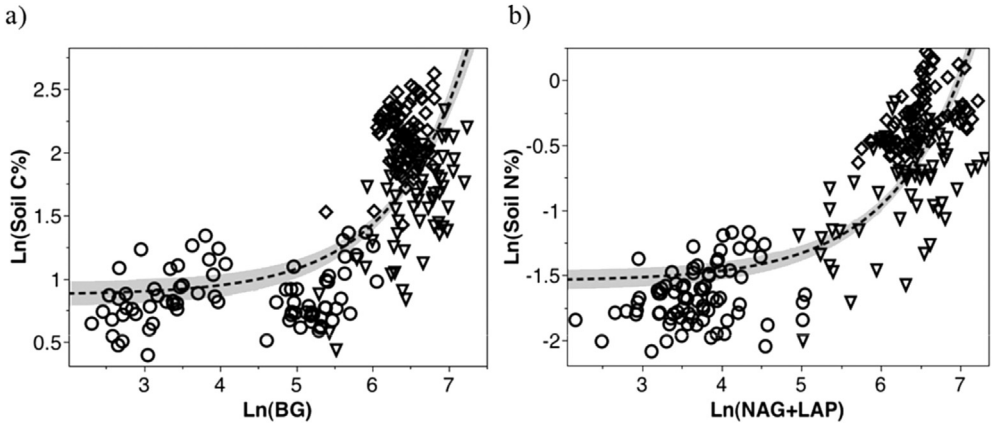

Fig. 1. Relationships between extracellular enzyme activities (nmol h^{-1} g^{-1}) and soil C (a) and N (b) content. BG = β-1,4 glucosidase; NAG = β-1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Best fit exponential curves of our ln–ln regressions: Ln (Soil C%) = $0.87 + 0.001^{Ln(BG)}$ ($R^2 = 0.52$, $P < 0.0001$); Ln (Soil N%) = $-1.54 + 0.001^{Ln(NAG+LAP)}$ ($R^2 = 0.64$, $P < 0.0001$). Symbols represent three different grassland sites: circles = Nash's Field (England); triangles = agricultural grasslands (Northern Ireland); diamonds = Lautaret (France).

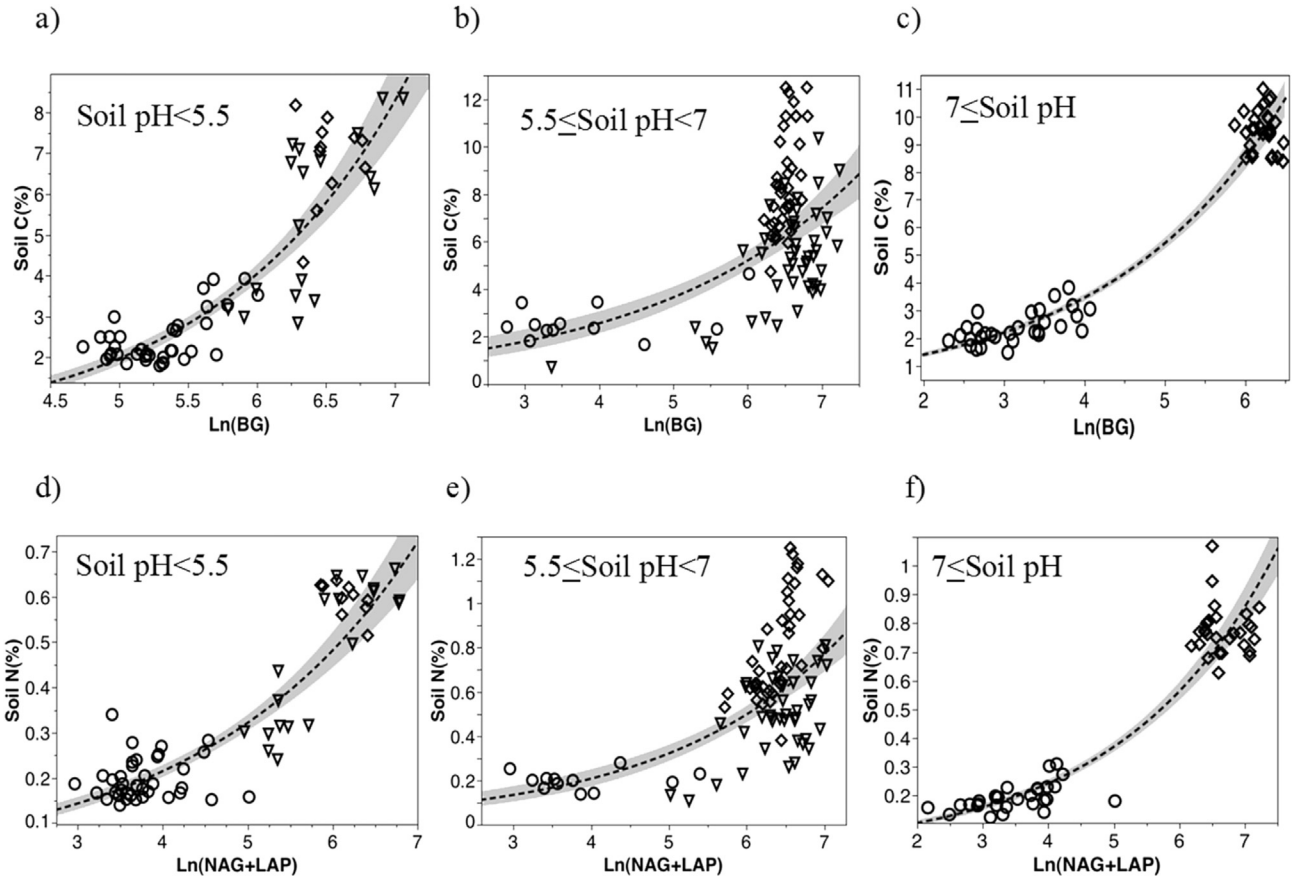

Fig. 2. Relationships between extracellular enzyme activities (nmol h^{-1} g^{-1}) and soil C (a, b, c) and N (d, e, f) content across a range of soil pH values (3.83 ≤ pH ≤ 7.74). BG = β-1,4 glucosidase; NAG = β -1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Symbols as in Fig. 1. Dashed lines represent best fit exponential curves of our ln–ln regressions. Statistical details: (a) Ln (Soil C%) = $-2.9 + 0.716^{Ln(BG)}$ ($R^2 = 0.80$, $P < 0.0001$); (b) Ln (Soil C%) = $-0.47 + 0.35^{Ln(BG)}$ ($R^2 = 0.46$, $P < 0.0001$); (c) Ln (Soil C%) = $-0.55 + 0.45^{Ln(BG)}$ ($R^2 = 0.95$, $P < 0.0001$); (d) Ln (Soil N%) = $-3.1 + 0.4^{Ln(NAG+LAP)}$ ($R^2 = 0.80$, $P < 0.0001$); (e) Ln (Soil N%) = $-3.31 + 0.43^{Ln(NAG+LAP)}$ ($R^2 = 0.53$, $P < 0.0001$); (f) Ln (Soil N %) = $-3.1 + 0.42^{Ln(NAG+LAP)}$ ($R^2 = 0.92$, $P < 0.0001$).

Table 2
Relationships between BG and NAG + LAP activities and soil C and N content. BG = β-1,4 glucosidase; NAG = β -1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Results are from multiple regression analyses using data from never ploughed and ploughed (until 1960) permanent grasslands in the Central French Alps.

Independent variables	Soil C%			Independent variables	Soil N%		
	Estimate	t ratio	P		Estimate	t ratio	P
Intercept	−15.1	−12.9	<0.0001	Intercept	−0.58	−7.2	<0.0001
Soil pH	1.6	11.8	<0.0001	Soil pH	0.03	2.9	0.004
Ln (BG)	1.93	18.8	<0.0001	Ln (NAG + LAP)	0.15	18.8	<0.0001

5. Discussion

Overall our results confirm our hypothesis that the activity of both C- and N-acquiring extracellular enzymes is positively related to the C and N content of very different grassland soils. This agrees with the findings from previous studies, which show positive relationships between enzyme activity and soil organic matter (SOM) concentration (Sinsabaugh et al., 2005, 2008). The positive relationship between enzymes activity and soil C and N content suggests the existence of direct and/or indirect links between these microbial functions and variation in the C and N content of SOM in the grasslands analyzed. It is not clear however, whether it is the greater activity of BG (or NAG + LAP) that ultimately causes increases in soil C (or N), or whether it is the greater size of the SOM pool which provides more C and N substrates that, in turn, ultimately promote greater microbial enzyme activity. To date, there have been few experimental studies that have specifically addressed potential 'cause-and-effect' relationships between enzyme activity and changes in soil C and N content (see for example Trasar-Cepeda et al., 2008; Wallenius et al., 2011). It is accepted that soil enzyme activities are closely related to changes in key soil physicochemical properties as well as changes in microbial biomass and SOM content (Caldwell, 2005; Sinsabaugh et al., 2008; Allison et al., 2011; Stursová and Baldrian, 2011).

In our study we demonstrated that increased activity of BG is associated with higher soil C content and furthermore that this is true across soils with very different pH values and management histories. In terms of management history our evidence is that BG activity and soil C are positively related either across permanent grasslands or across historically ploughed grasslands. This relationship is particularly significant in ploughed grasslands, which had also received N additions (Fig. 3a). We are not sure whether increases in BG activity could be related to positive N-induced effects on grass yields (and thus to higher cellulose pools) because

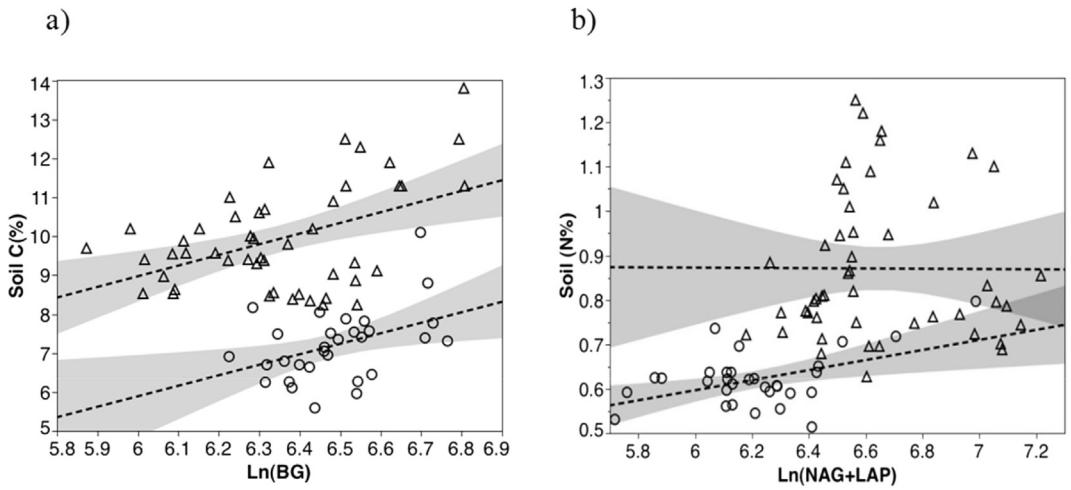

Fig. 3. Relationships between extracellular enzyme activities (nmol h^{-1} g^{-1}) and soil C (a) and N (b) content of permanent grasslands (white circles) and historically ploughed grasslands (white triangles) in Lautaret (France). Ploughed grasslands also received N additions whereas permanent grasslands did not. BG = β-1,4 glucosidase; NAG = β-1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Dashed lines indicate best linear fits. (a) Soil C% = −10.3 + 2.7*Ln BG; R^2 = 0.19 (permanent grasslands) and Soil C % = −7.5 + 2.7*Ln BG; R^2 = 0.20 (ploughed grasslands); (b) Soil N% = −0.08 + 0.11*Ln (NAG + LAP), R^2 = 0.23 (permanent grasslands) and Soil N% = 0.89−0.003*Ln (NAG + LAP), R^2 = 0.00001 (ploughed grasslands).

Table 3
Relationships between BG and NAG + LAP activities and soil C (%) and N (%) content. BG = β-1,4 glucosidase; NAG = β −1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Results are from multiple regression analyses using data from lowland intensively used agricultural grasslands (Northern Ireland). Only soil bulk density and livestock density (i.e. number of cattle per hectare) are significantly (negatively) related to soil C and soil N content.

Independent variables	Soil C%			Independent variables	Soil N%		
	Estimate	t ratio	P		Estimate	t ratio	P
Intercept	20	4.46	<0.0001	Intercept	1.67	11.1	<0.0001
Soil Bulk density	−8.7	−6.7	<0.0001	Soil Bulk density	−0.67	−6.5	<0.0001
Livestock density	−1.88	−5	<0.0001	Livestock density	−0.16	−5.8	<0.0001
Ln (BG)	−0.11	−0.18	0.85	Ln (NAG + LAP)	−0.12	−1.38	0.17

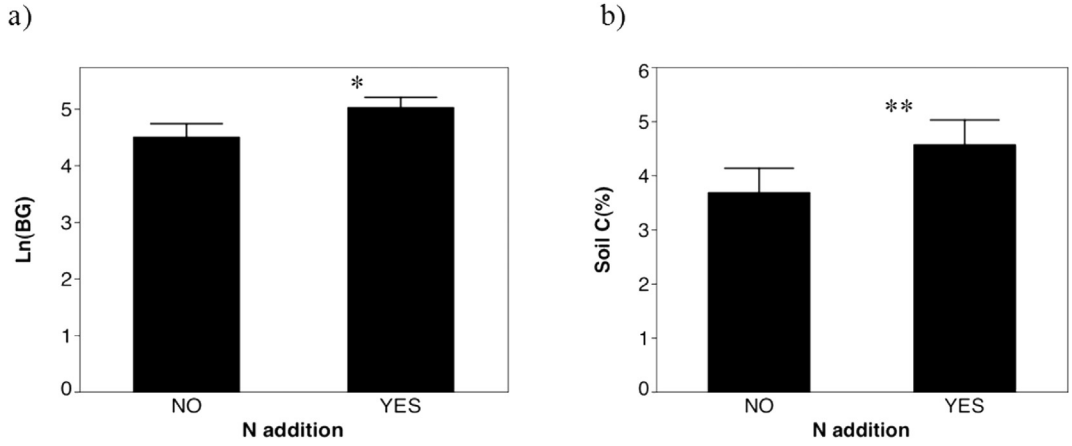

Fig. 4. Relationship between N fertilization and (a) BG activity (nmol h^{-1} g^{-1}) and (b) soil C content of grassland soils sampled within the Nash's Field (England) and Lautaret (France) experiments. BG = β-1,4 glucosidase (ns = not significant, *<0.01, **<0.001, ***<0.0001).

most of aboveground biomass produced every year in these grasslands is removed by humans.

In grassland soils, cellulose represents a significant proportion of plant detritus returned to the soil, and the production of BG is therefore very important for catalysis of the final step in cellulose depolymerization, which is the hydrolysis of cellobiose to glucose (Sinsabaugh and Follstad Shah, 2011). Greater BG activity could be related to plant litter inputs to soils and thus to the pool of cellulose-rich substrates available for decomposition. A recent study shows, for example, how the addition of plant biomass to soils from different cover crops is positively related to both BG activity and soil C sequestration (Peregrina et al., 2014). Once BG has been produced and released into the soil, it could potentially be disabled through binding to mineral and organic colloids (Allison and Jastrow, 2006) thus contributing (together with other microbial 'waste' products) to the accumulation of C in soils (Cotrufo et al., 2013; Cenini et al., 2015).

Our findings show however that in more intensively managed

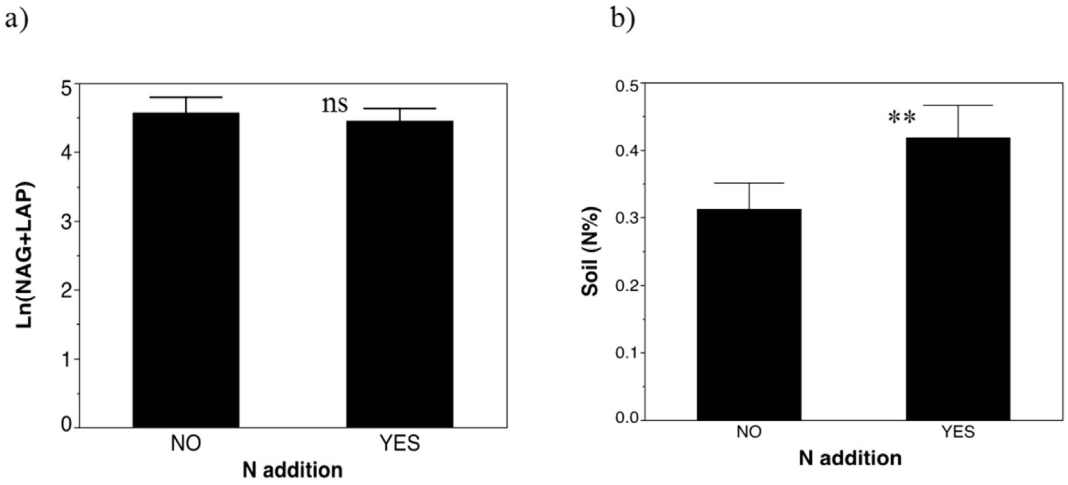

Fig. 5. Relationship between N fertilization and (a) NAG + LAP activity (nmol h^{-1} g^{-1}) and (b) soil N content of grassland soils sampled within the Nash's Field (England) and Lautaret (France) experiments. NAG = β-1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase (ns = not significant, *<0.01, **<0.001, ***<0.0001).

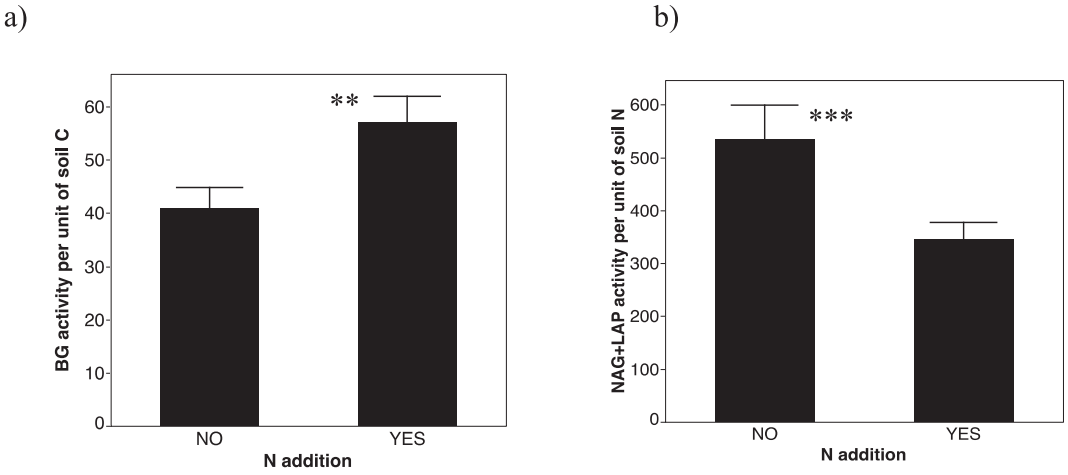

Fig. 6. Relationship between N addition and (a) the activity of BG (nmol h^{-1} g^{-1}) and (b) NAG + LAP (nmol h^{-1} g^{-1}) per unit of soil C and N respectively. BG (and NAG + LAP) activity per unit of soil C (or N) was calculated dividing BG (and NAG + LAP) activity by total soil organic C (and N) content. BG = β-1,4 glucosidase, NAG = β-1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. (ns = not significant, *<0.01, **<0.001, ***<0.0001).

grasslands (i.e. those with increased livestock numbers and increased soil compaction) the activity of BG and NAG + LAP was not significantly correlated to soil C and N content. Enzyme activity and thus the storage of soil organic C (SOC) is affected by both soil texture and mineral composition and it appears that in the Northern Ireland grasslands we investigated, increases in both soil bulk density and animal stocking rates have significantly negatively affected soil C and N content. Previous studies have shown how increased soil compaction can lead to negative effects on microbial activity (Welbaum et al., 2004). Compaction increases soil aggregation, which may prevent enzymes from accessing C and N substrates inside aggregates (Sollins et al., 1996).

Our results also show that BG activity responds positively to long-term N additions to grassland soils (Fig. 4a) and that soils receiving N additions have significantly greater soil C content (Fig. 4b). We explain this positive N-induced effect on BG activity based on the fact that the activity of C-acquiring (or N-acquiring enzymes) tends to respond to changes in environmental substrate availability (Chróst, 1991; Sinsabaugh et al., 1993; Allison and Vitousek, 2005). The work of Mergel et al. (1998), for example, showed that N fertilization stimulated the production of C-containing compounds in soils, compounds which derive from plant root decomposition and from root exudates. Root exudation can also enhance the decomposition of stable forms of organic matter, stimulate mineralization of C-containing compounds and contribute to the incorporation of soil C forms into stable soil fractions. We expected that long-term N additions to soils would have contributed a reduced C *vs*. N availability for microbial growth, thus stimulating the production of C-acquiring rather than N-acquiring enzymes. Our investigation provides evidence of this as fertilized soils showed significantly higher BG activities and lower NAG + LAP activities. This result also agrees with the results of other researchers, which showed that increased inorganic N availability has negative effects on the activity of N-acquiring enzymes (Stursová et al., 2006). As further support for the positive N-induced effect on BG, we found that the BG activity per unit of soil C was significantly higher in soils receiving chronic N additions (Fig. 6a). Nutrient fertilization is a very common practice across human-managed grasslands and its cascading effects on both microbial activity and on soil C accumulation still need to be clarified.

In our study we did not find any linkages between N additions, NAG + LAP activity and soil N content. On one side N fertilization reduced NAG + LAP activity and also the activity of NAG + LAP per unit of soil N. On the other side N fertilization was positively related

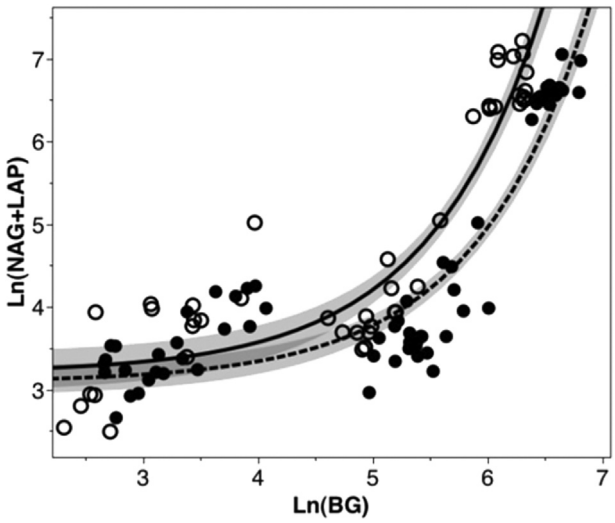

Fig. 7. Relationship between ln (BG) (nmol h^{-1} g^{-1}) and ln (NAG + LAP) (nmol h^{-1} g^{-1}) in soils which have received N additions (filled circles) and soils which did not receive any N addition (open circles). BG = β-1,4 glucosidase, NAG = β-1,4-N-acetylglucosaminidase; LAP = leucine aminopeptidase. Best fit equations: Ln (NAG + LAP) = 3.2 + $0.006^{Ln(BG)}$ (R^2 = 0.87, P < 0.0001; white circles); Ln (NAG + LAP) = 3.1 + $0.004^{Ln(BG)}$ (R^2 = 0.87, P < 0.0001; filled circles).

to soil N content. This apparent mismatch between low activity of N-acquiring enzymes and high soil N may be simply due to the fact that N is found in a diversity of compounds (e.g. amino sugars, polypeptides, humus), which are degraded by a diverse range of N-acquiring enzymes. The addition of inorganic forms of N to soils through fertilization may as a consequence provide N forms available to be incorporated in SOM, thus explaining the positive relationship between N addition and soil N content (see Fig. 5b).

6. Conclusion

Overall, our study shows that the activity of C- and N-acquiring enzymes is positively related to changes in soil C and N content across very different grassland systems. This positive relationship may be explained by the fact that increased accumulation of SOM may provide a variety of C and N substrates as well as a range of spatial niches which support greater microbial enzyme foraging and activities. Thus our evidence is that BG activity may represent a good indicator of both soil organic matter quality and C sequestration trends as was found in recent studies (Stott et al., 2010; Cenini et al., 2015). We suggest however, that human-induced changes in grassland management (e.g. N fertilization, livestock density, soil compaction etc.) significantly influence enzyme activity, which in turn could have consequences for the C and N content of SOM. Thus the activity of C- and N-acquiring enzymes may not be simply a reflection of SOM concentration itself, and that changes in these enzyme activities could actively influence the accumulation of SOM, and also, consequently, the C and N content of human-managed grassland soils.

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