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# Enzyme properties down the soil profile - A matter of substrate quality in rhizosphere and detritusphere

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ABSTRACT

The decomposition of soil organic matter depends strongly on its availability to microorganisms and their enzymes. The rhizosphere and detritusphere are microbial hot spots due to additional substrate input, leading to high abundance, specific species diversity and functional diversity of microbial communities. However, rhizosphere and detritusphere differ in substrate quality, localization, and duration of input. We hypothesized that the contrasting substrate availability between rhizosphere and detritusphere affects the activity of microorganisms and associated enzymes. Organic carbon (C) from the rhizosphere and detritusphere decreases with soil depth and, consequently, microbial hot spots become rarer and competition for C and nutrients increases. In deeper soil (>40 cm depth) the amount and quality of substrates is expected to decrease and, therefore, the effect of contrasting substrate input to disappear. Plant N uptake is expected to reduce N availability in the rhizosphere of maize compared to the detritusphere and bare fallow. These hypotheses were tested in a factorial field experiment with 1) maize-planted, 2) maize litter-amended, and 3) bare sites. Enzyme kinetic parameters ($V_{max}$, $K_m$, $K_a$), extractable organic C and microbial biomass C were compared in soil affected by rhizosphere and detritusphere throughout the profile to 70 cm depth, to assess microbial C and nutrient limitations. A decrease in enzyme activity with depth due to resource scarcity and lower substrate quality appeared in planted and litter-amended soil. N limitation in planted soil increased the activity and substrate affinity of proteolytic enzymes to provide for microbial N demand through SOM decomposition. This was in line with lower $V_{max}$ ratios ($V_{max}$ for C-cycling enzymes divided by $V_{max}$ for N-cycling enzymes) in planted relative to litter-amended topsoil. The catalytic efficiency of enzymes decreased 2- to 20-fold from top- (<40 cm) to subsoil (>40 cm), irrespective of the substrate input. Substrate quality in the rhizosphere and detritusphere affected enzyme activities only in the topsoil, whereas a sharp decline of C input with depth led to similar activities in the subsoil. Most of the enzyme indexes reflected shifts in allocation of C and nutrients in the rhizosphere and detritusphere. The presented results underline the role of microorganisms as critical links in the C and nutrient transfers in the rhizosphere and detritusphere.



## 1. Introduction

Enzymes in soil catalyze nearly all important transformations in the carbon (C), nitrogen (N), phosphorus (P) and sulfur (S) cycles (Aon et al., 2001; Wallenstein and Burns, 2011). Decomposition of organics is strongly dependent on microbes and enzymes, which are especially abundant in the rhizosphere and detritusphere – two main microbial hot spot environments in soil. The rhizosphere is characterized by high density and quality of substrates for microorganisms (Garbeva et al., 2008; Marschner et al., 2012, 2001), and plants provide a variety of C and energy sources from their roots

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(Gregory, 2006; Paterson, 2003; Paterson et al., 2007). The detritusphere contains large amounts of cellulose, hemicelluloses and lignin, as main components of plant residues (Kandeler et al., 1999; Marschner et al., 2012; Nannipieri et al., 2012).

The microbial C:N:P ratios (ecological stoichiometry) are frequently used to indicate how allocation of C and nutrients by microorganisms influence microbial demands on soil pools (Cleveland and Liptzin, 2007; Heuck et al., 2015; Sinsabaugh et al., 2015). Nowadays it is widely accepted that microbial C:N:P stoichiometry affects microbial mineralization of C sources (Mooshammer et al., 2012). Microbial respiration ($CO_2$) and $N_2O$ production are well known indicators describing microbial activities in soil (Blagodatskaya et al., 2014). In combination with the ratios of commonly measured enzyme activities (Table 1), these indicators provide insights into the microbial community that is investing energy for microbial fitness (Sinsabaugh et al., 2012; 2008; Tapia-Torres et al., 2015). The production of extracellular enzymes is regulated by nutrient availability and energy demand (Sinsabaugh et al., 2009). Thus, enzyme activities are reliable microbial activity indicators and are closely interrelated with soil quality (Bending et al., 2004; Paudel et al., 2011).

Most enzyme studies are restricted to the topsoil, despite the fact that microbial substrate utilization takes place throughout the whole soil profile (Sinsabaugh et al., 1993; Sinsabaugh and Moorhead, 1994; Vranova et al., 2013). Furthermore, only the potential enzyme activity is considered in most studies, whereas rates of enzyme-substrate complex dissociation and enzyme-substrate complex formation are neglected (Koshland, 2002). Therefore, it is of great interest to study how microbial functioning and enzyme systems vary throughout the soil profile.

As interactions between substrate composition, microbial competition, and nutrient availability are complex, we established a factorial field manipulation experiment including maize-planted, maize litter-amended and bare fallow sites. These sites differed (1) in sources of different substrate quality (root-derived vs. litter-derived vs. none) and (2) in the distribution of substrates with depth. Both substrate quantity and quality strongly decrease with soil depth (Fierer et al., 2003a), because most roots are localized in the topsoil, so the rates of C input to subsoil are low (Fierer et al., 2003b). Therefore, the subsoil microbial communities differ in composition and activity from the surface communities (Blume et al., 2002; Fierer et al., 2003a; Fritze et al., 2000).

We combined substrate-induced emission of carbon dioxide ($CO_2$) and nitrous oxide ($N_2O$) with kinetics of the enzymes β-glucosidase (BG), β-cellobiohydrolase (CE), β-xylosidase (BX), acid phosphatase (AP), and leucine- (LE) and tyrosine- (TY) aminopeptidases to disentangle the effects of substrate quality and substrate amount on microbial activity along the depth gradient. Several approaches for integrating the various enzyme activities into unified indexes were compared (Table 1) (Hill et al., 2014; Moorhead et al., 2016, 2013; Nannipieri et al., 2012; Sinsabaugh et al., 2008). These activity indexes of multiple enzymes were related to dissolved organic C (DOC) and extractable nitrogen (EN).

We hypothesized that the contrasting substrate availability between planted soil and litter-amended soil, reflecting the rhizosphere and detritusphere, respectively, would affect the activity of microorganisms and associated enzymes. The effect of the contrasting substrate availabilities on microbial substrate utilization was predicted to decline with depth due to the lower amount and quality of substrates in the subsoil (>40 cm depth) compared to the topsoil. Furthermore, we hypothesized that lower N contents in the maize-planted soil, due to plant N uptake, would lead to stronger competition between microbes compared to the fallow control. This, in turn, would increase proteases, because of an inversely proportional relationship to low substrate availability (Olander and Vitousek, 2000; Sims and Wander, 2002; Stursova et al., 2006). To our knowledge this is the first study using a broad range of activity indicators to elucidate the tight interactions between microbial activity and contrasting substrate input down the soil profile.

## 2. Material and methods

### 2.1. Study site

The experimental arable field was located in the north-west of Göttingen, Lower-Saxony, Germany (51°33′N, 9°53′E; 158 m NN). The area has a temperate climate with a long-term mean annual precipitation of 645 mm and mean air temperature of 8.7 °C. The dominant soil types are Luvisols occasionally with stagnic properties (Table 2; Kramer et al., 2012; Pausch et al., 2013).

In April 2012 the field was tilled with a chisel plough to a depth of 12 cm and maize was sown at a density of 12 grains $m^{-2}$. Nitrogen fertilizers (ammonium nitrate urea solution: 110 kg N $ha^{-1}$ and diammonium phosphate: 110 kg N $ha^{-1}$) were applied to all treatments, shortly before and after sowing the maize. The corn was not irrigated during plant growth.

In September 2012 corncobs were harvested and maize plants were cut at a height of 10 cm above soil surface. The maize above-ground biomass was hackled to a particle size of 1 $cm^2$ and air-dried to gain litter. In April 2013 the herb layer developed during spring was removed by Glyphosate (4 l $ha^{-1}$). Three weeks later the soil was tilled to a depth of 12 cm, maize sown at a density of 9 grains $m^{-2}$ and fertilized similarly to 2012. In September 2013 maize plants were harvested and removed from the experimental field site.

### 2.2. Treatments

In May 2012 a total of 12 experimental plots (size 5 × 5 m) were conducted and arranged in two adjacent rows separated by a 5 m buffer stripe within and 2 m buffer stripes between rows. Three treatments, each replicated four times, were established differing in resource quality: plant (maize as crop), litter (application of maize litter) and fallow. Maize was removed from the eight plots within the first three weeks after seeding to set up the litter and fallow treatments. For the litter-treated soil four plots received 0.8 kg $m^{-2}$ dry maize litter (equivalent to 0.35 kg C $m^{-2}$, C-content = 44%) approximating the above-ground biomass of maize in June. Litter was grubbed into the first 10 cm of soil on June the 6th 2013. This coincided with the start of the crop growth period to ensure the same conditions for the herbivore and detritivore communities. To accomplish comparable environmental conditions between plots, the litter-amended and the fallow control plots were shaded with blinds (mechanical shading; AGROFLOR Kunststoff GmbH, Wolfurt, Austria). The shading level represented the mean leaf area index of plants during the vegetation period. In addition, plots were regularly weeded to prevent plant carbon input by herbs.

### 2.3. Soil sampling and preparation

We sampled in each plot soil from 0 to 50 cm in 10 cm increments, and from 60 to 70 cm depth, of each plot in July 2013. Each plot was sampled in one position, using a Riverside auger (inner diameter 5 cm, Eijkelkamp, Giesbeek, The Netherlands). The soil samples were frozen at −18 °C until analysis. Prior to analysis the soil samples were thawed at 4 °C. After thawing the soil samples were sieved (<2 mm) and fine roots and other plant debris were carefully removed with tweezers. The soil was then pre-incubated at 22 °C for 72 h. Soil sub-samples from each plot and

**Table 1**
Enzyme indexes: The potential enzyme activity ($V_{max}$), the microbial biomass C content ($C_{mic}$), and the Michaelis-Menten constant ($K_m$) were used to calculate different enzyme indexes for β-glucosidase (BG), β-cellobiohydrolase (CE) and β-xylosidase (BX) represented enzymes in the C-cycle, whereas leucine- (LE) and tyrosine- (TY) aminopeptidases represented N-aquiring enzymes. Acid phosphatase (AP) is responsible for substrate utilization in the P-cycle.

| Enzyme indexes | Description | References |
|---|---|---|
| 1) $V_{max}/C_{mic}$ | Specific enzyme activity (potential activity to microbial biomass) | Trasar-Cepeda et al., 2008; Stone et al., 2014 |
| 2) $V_{max}/K_m$ | Catalytic efficiency of the enzyme | Moscatelli et al., 2012 |
| 3) TAC[a]/TAN[b]; TAC/TAP[c] | $V_{max}$ ratio of C- to N- and C- to P-acquiring enzymes | Sinsabaugh et al., 2008 |
| 4) TAC/(TAC + TAN); TAC/(TAC + TAP) | Proportional enzyme activities of C vs. N and C vs. P acquiring enzyme | Hill et al., 2014; Moorhead et al., 2013 |

[a] Total activity of C-aquiring enzymes ($V_{max}$(BG) + $V_{max}$(BX) + $V_{max}$(CE).
[b] Total activity of N-aquiring enzymes $V_{max}$(LE) + $V_{max}$(TY).
[c] Total activity of N-aquiring enzymes $V_{max}$(AP).

depth were dried at 105 °C for 24 h to determine the moisture content. Moisture contents ranged from 14% for planted to 18% for fallow soil. Prior to incubation the moisture content was adjusted to 60% of the water holding capacity (WHC).

### 2.4. Substrate-induced respiration

The substrate-induced respiration (SIR) method is generally used to measure microbial biomass by amendment with easily available C. We determined the $CO_2$ efflux following the addition of glucose and mineral salts (Anderson and Domsch, 1985; Anderson and Joergensen, 1997). Production of $N_2O$ was also measured as an indicator of N sources for nitrification and denitrification. In total, 25 g samples of soil were incubated in flasks for 4 h after addition of the substrate. The SIR substrate mixture contained glucose (10 mg $g^{-1}$) and mineral salts (1.9 mg $g^{-1}$ $(NH_4)_2SO_4$; 2.25 mg $g^{-1}$ $K_2HPO_4$; and 3.8 mg $g^{-1}$ $MgSO_4 \cdot 7H_2O$) (Blagodatsky et al., 2000). Glucose is an important components of root exudates (Derrien et al., 2004; Whipps and Lynch, 1983). The amount of mineral salts was selected so that the added substrate did not change soil pH (change < 0.1) (Blagodatskaya et al., 2007). Gas samples (15 ml) were taken hourly and the $CO_2$ as well as $N_2O$ concentrations were analysed by gas chromatography (GC 6000 VEGA series 2, Carlo Erba instruments, UK). The slopes of measured hourly $CO_2$ and $N_2O$ concentrations were corrected by the specific gas flux (according to the gas constant, air pressure and temperature) and multiplied by the headspace volume (1098 $cm^3$) to obtain the individual flux rates for each soil sample. Microbial biomass C ($C_{mic}$) was determined using the individual flux rate (Anderson and Joergensen, 1997) and calculated according to the equation by Anderson and Domsch (1978, 2010):

$$C_{mic}\left(\mu g\ g^{-1}\ soil\right) = \left(\mu l\ CO_2\ g^{-1}\ soil\ h^{-1}\right) \times 40.04 \quad (1)$$

### 2.5. Dissolved organic carbon and extractable nitrogen

Moist soil (7.5 g) was extracted with 30 ml of 0.05 M $K_2SO_4$ for 1 h by overhead shaking (40 rpm) (Bruulsema and Duxbury, 1996). The soil suspension was centrifuged for 10 min at 2500*g*. The supernatant was then filtered through Rotilabo-rondfilters (type 15A, Carl Roth GmbH & Co.KG). The organic C and N content of the $K_2SO_4$ extracts were measured using a multi N/C analyzer (multi N/C analyzer 2100S, Analytic Jena).

### 2.6. Enzyme assays

We used 4-methylumbelliferone-β-D-cellobioside, 4-methylumbelliferone-β-D-glucoside, 4-methylumbelliferone-phosphate, 4-methylumbelliferone-7-β-D-xyloside, L-leucine-7amino-4-methylcoumarin hydrochloride and L-tyrosine-7amino-4-methylcoumarin to determine the enzyme activities of β-cellobiohydrolase (exo-1,4-β-glucanase, EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), acid phosphatase (EC 3.1.3.2), β-xylosidase (EC 3.2.2.27) and leucine-/tyrosine-aminopeptidase (EC 3.4.11.1), respectively. β-glucosidase (BG), β-cellobiohydrolase (CE) and β-xylosidase (BX) represented enzymes in the C-cycle, whereas leucine- (LE) and tyrosine- (TY) aminopeptidases represented N-cycling enzymes. Acid phosphatase (AP) is responsible for substrate utilization in the P-cycle.

Fluorogenic substrates enable direct quantitative comparison of the activity of enzymes responsible for various functions (Marx et al., 2001; Nannipieri et al., 2012). This is because the fluorogenic compounds (MUF or AMC) are enzymatically released in amounts equimolar to the number of bonds broken (corresponding to enzyme function). Enzyme activity is therefore expressed in the same units for various different enzymes, based on calibration by MUF or AMC. This standard analysis of enzyme kinetics is based on the assumption that the binding of substrate to one enzyme binding site does not affect the affinity or activity of an adjacent site. That is, neither substrate nor product acts as an allosteric modulator to alter the enzyme velocity.

Half a gram of soil was added to 50 ml sterile water in autoclaved jars and dispersed by an ultrasonic disaggregator (50 J $s^{-1}$ for 120 s; De Cesare et al., 2000). Aliquots of 50 µl were withdrawn while stirring the suspension and dispensed into 96-well microplates (Brand pureGrade, black). Fifty microliter of buffer was added (0.1 M MES buffer, pH 6.1 for carbohydrases and phosphatase, 0.05 M TRIZMA buffer, pH 7.8 for leucine-/tyrosine-aminopeptidase) (Marx et al., 2005, 2001). Finally, 100 µl of substrate solution was added at a series of concentrations (20, 40, 60, 80, 100, 200, 400 µmol substrate $g^{-1}$ soil). Plates were kept at 22 °C, agitated and measured fluorometrically (excitation 360 nm; emission 450 nm) after 1 h, 2 h, and 3 h incubation using an automated fluorometric plate reader (Wallac 1420, Perkin Elmer, Turku,

**Table 2**
Selected soil properties of the study site (adapted from Loeppmann et al., 2016; data from Kramer et al., 2012; Pausch and Kuzyakov, 2012).

| Horizon | Depth [m] | Texture clay/silt/sand [% (w/w)] | pH [$CaCl_2$] | Bulk density [g $cm^{-3}$] |
|---|---|---|---|---|
| Ap1 | 0–0.25 | 7/87/6 | 6.0 a | 1.38 a |
| Ap2 | 0.25–0.37 | 7/88/5 | 6.2 a | 1.61 b |
| Btw1 | 0.37–0.65 | 7/88/5 | 6.6 b | 1.55 c |
| Btw2 | >0.65 | 7/88/5 | 7.0 c | 1.68 b |

Finland). Fluorescence was converted into an amount of MUB (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin) by reference to the fluorescence of standard solutions, which had been prepared in sub-samples of the various soil suspensions. Each field replicate was measured as an analytical triplicate.

The kinetic parameters $V_{max}$ and $K_m$ were estimated using a non-linear regression model (Michaelis-Menten kinetics) (Marx et al., 2001). $V_{max}$ is the decomposition rate at saturating substrate concentrations; $K_m$ reflects the enzyme's affinity for the substrate. The $K_m$ corresponds to the weighted sum of rate constants for the dissociation of the enzyme-substrate complex divided by the rate constant for its formation (Koshland, 2002).

$V_{max}$ and $C_{mic}$ were used to determine the specific enzyme activity (Table 1; Index 1) (Nannipieri et al., 2012; Stone et al., 2014). Furthermore, we determined the catalytic efficiency (Table 1; Index 2) for each treatment and enzyme (Gianfreda et al., 1995; Koshland, 2002; Moscatelli et al., 2012). It reflects the total enzyme catalytic process combining enzyme-substrate complex dissociation and the rate of enzyme-substrate complex formation (Cornish-Bowden, 1995; Koshland, 2002). These parameters were selected to compare the catalytic properties of each enzyme with different substrate input (litter, rhizodeposits) (Cervelli et al., 1973; Esti et al., 2011).

We integrated the activities of enzymes involved in the same process as indicators of organic matter degradation and nutrient transformation. It is assumed that the sum of major C-acquiring enzyme activities is a better indicator of total C-acquisition than BG alone (Bell et al., 2014; Nannipieri et al., 2012). Thus, enzyme activity ratios (Table 1; Index 3 and Index 4) were used as a tool for examining relative allocation to energy versus nutrient acquisition (Stone et al., 2014).

We translated the enzyme activity proportions (TAC/(TAC + TAP)) and (TAC/(TAC + TAN)) (Sinsabaugh et al., 2008) into vector lengths and directions (angles) that provide clear metrics of relative C limitation vs. nutrient limitation (Moorhead et al., 2016, 2013). The angle quantifies the relative P vs. N limitation

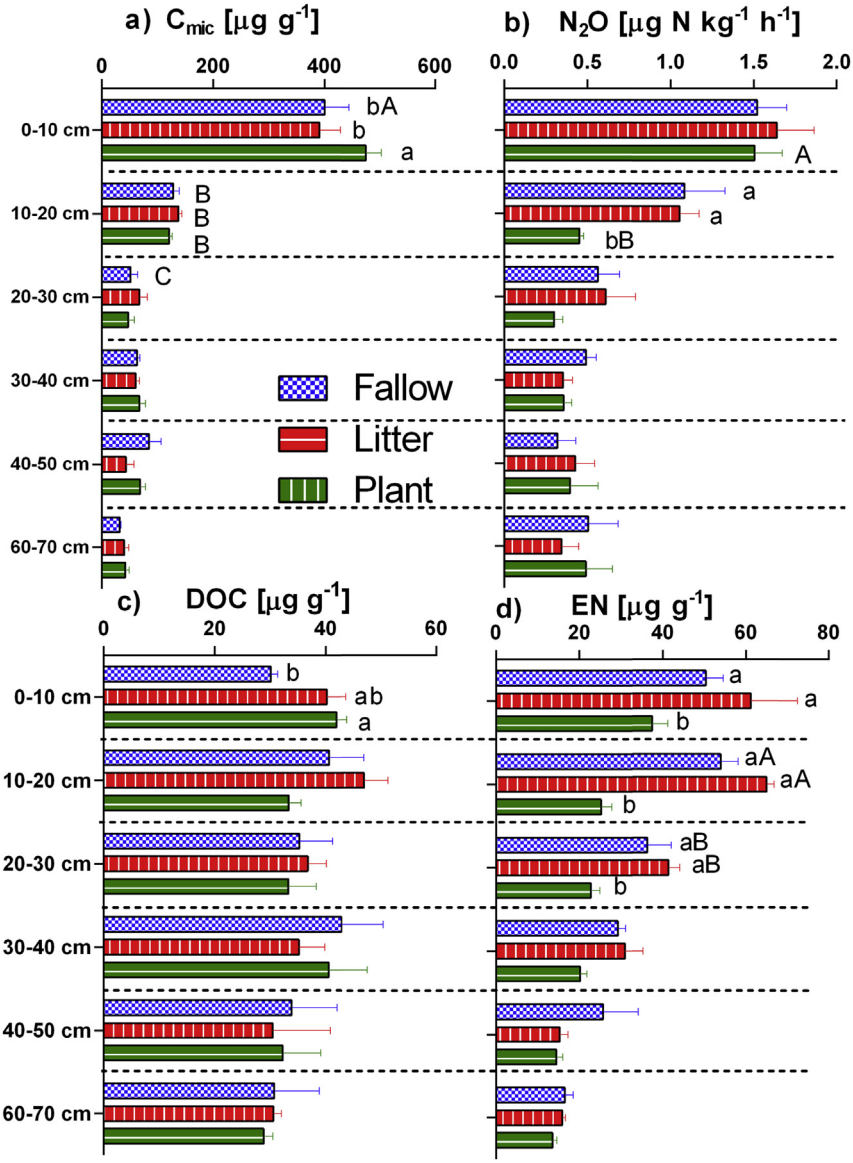


**Fig. 1.** a) microbial biomass C ($C_{mic}$), b) $N_2O$, c) dissolved organic C (DOC), and d) extractable N content (EN) with depth for bare fallow (Fallow), litter-amended (Litter) and planted soil (Plant). Significant differences between the treatments are indicated by lower case letters. Capital letters are used to show significant differences with depth ($P < 0.05$) by comparing the upper layer with the layer below (e.g. 0–10 vs. 10–20 cm). Additional information on comparison of topsoil vs. subsoil layer are given in Supplementary Table 4.

(Supplementary Figure 1b; Moorhead et al., 2016). Enzyme activity toward P acquisition is reflected by the steepness of the vector angle. With increasing enzyme production toward C acquisition relative to N and P, the vector length increases (Supplementary Figure 1a). The increasing vector length is interpreted as a relative increase in C limitation, and increasing vector angle as a relative increase in P vs. N limitation (Moorhead et al., 2013).

Vector length was determined as the square root of the sum of the squared values of x and y. Relative C- vs. P-acquiring enzyme activities were represented by x and the relative C- vs. N-acquiring activities by y (Moorhead et al., 2016).

$$\text{Vector length} = \text{SQRT}\left(x^2+y^2\right) \quad (2)$$

The vector angle was calculated as the arctangent of the line extending from the plot origin to point (x, y) (Moorhead et al., 2016):

$$\text{Angle} = \text{DEGREES}(\text{ATAN2}\ (x,\ y)) \quad (3)$$

### 2.7. Statistics

The means of four field replicates with standard errors are presented in tables and figures. The Shapiro-Wilk test was applied to test for normality. We used Pearson correlation coefficients to interpret the degree of linear relationships. Multiple t-tests were applied to characterize the effects of contrasting substrate input (litter, rhizodeposits) and soil depths (each layer was tested separately). When significant effects were identified, a multiple post-hoc comparison using the Holm-Sidak test ($P < 0.05$) was performed. The kinetic parameters were fitted by minimizing the least-square sum using GraphPad Version 6 software (Prism, USA). The three analytical replicates of enzyme activity curves were used for each of the four replicated soil samples at each depth. Parameter optimization was restricted to the applied model equation as indicated by maximum values of $r^2$. Outliers were identified by the ROUT method, based on the False Discovery Rate (FDR), where Q was specified as the maximum desired FDR (Motulsky and Brown, 2006).

## 3. Results

### 3.1. Microbial biomass C and $N_2O$ production

Microbial biomass C ($C_{mic}$), determined by the SIR method, declined sharply with depth (Fig. 1b) and was higher in planted soil than in litter-treated ($P < 0.01$) and fallow soils ($P < 0.05$) in the upper 10 cm (Fig. 1a). $C_{mic}$ was strongly correlated to SOC content for litter-treated ($r^2 = 0.98$, $P < 0.05$) and fallow soil ($r^2 = 0.98$, $P < 0.05$), whereas $C_{mic}$ was not significantly correlated to SOC content in planted soil ($r^2 = 0.31$) (not shown). Total organic C and N content at each depth were similar between the treatments (Fig. 2). Furthermore, SIR-derived microbial biomass was closely correlated to $N_2O$ production, irrespective of substrate quality (planted, $r^2 = 0.96$, $P < 0.001$; litter, $r^2 = 0.92$, $P < 0.01$; fallow, $r^2 = 0.89$, $P < 0.05$) (not shown). However, when comparing the specific enzyme activities (Table 1; Index 1) of leucine aminopeptidase (LE) and tyrosine aminopeptidase (TY) with $N_2O$, the relationships between either LE or TY and $N_2O$ production shifted to a negative correlation (r between −0.21 and −0.62) with depth.

The highest $N_2O$ production was measured in the top 10 cm with similar intensities for all treatments (Fig. 1b). $N_2O$ emission in planted soil dropped by 67% from 0 to 10 to 10–20 cm depth. $N_2O$ production at 10–20 cm in planted soil was much lower (58%) than that of the litter-amended and fallow soil at corresponding depth.

### 3.2. Dissolved organic carbon and extractable nitrogen

Dissolved organic C (DOC) content was significantly ($P < 0.05$) higher in the surface layer of planted compared to fallow soil, indicating the importance of labile C in the rhizosphere (Fig. 1c). Extractable nitrogen (EN) content was reduced in planted soil compared to litter-amended soil by 39, 61, and 45% at 0–10, 10–20, and 20–30 cm depths, respectively. EN content declined from 10 to 20 to 20–30 cm depth for litter-amended and fallow soil by 37 and 33%, respectively (Fig. 1d).

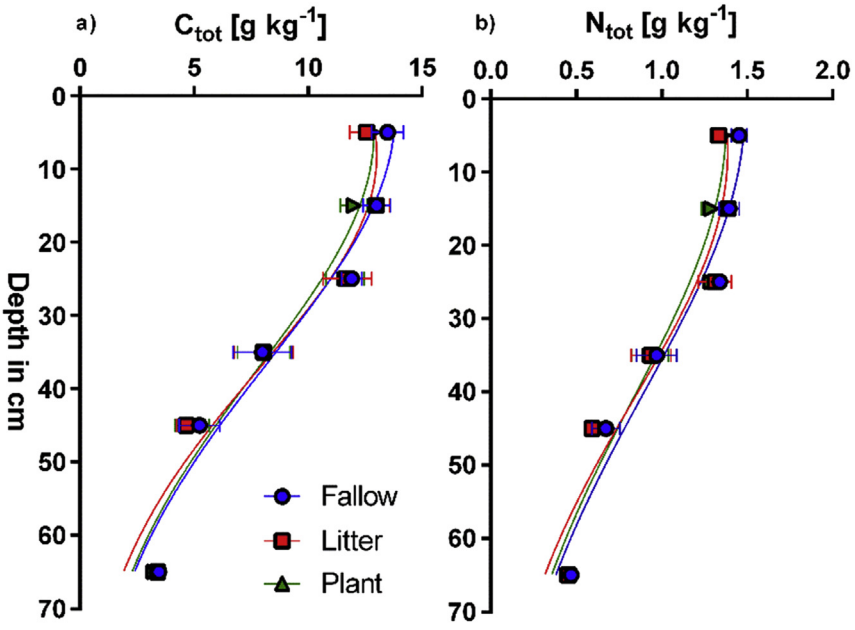


**Fig. 2.** Total carbon ($C_{tot}$) and nitrogen ($N_{tot}$) content with depth for bare fallow (Fallow), litter-amended (Litter) and planted (Plant) soil.

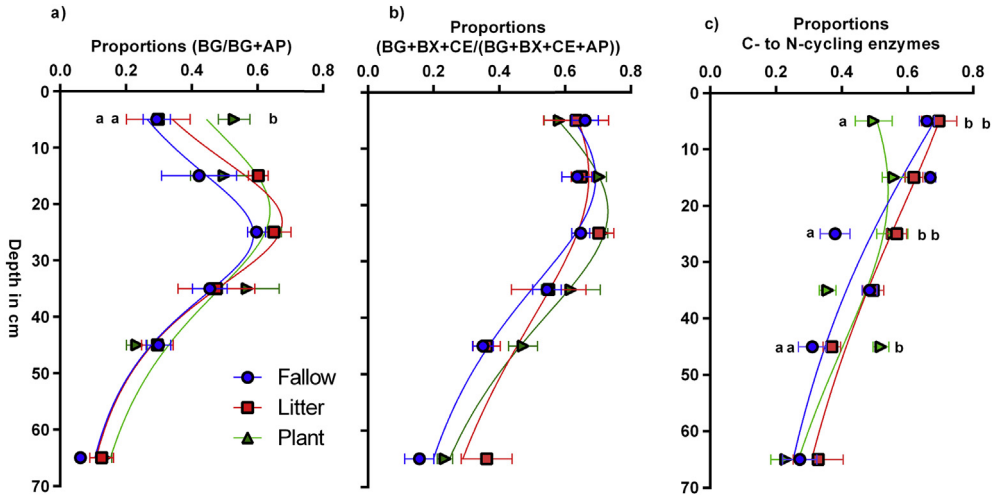


**Fig. 3.** Proportions of C- to P- and C- to N-cycling enzymes with depth for fallow (Fallow), litter-amended (Litter) and planted soil (Plant). a) Proportions of β-glucosidase (BG) to acid phosphatase (AP) $V_{max}(BG)/[V_{max}(BG)+V_{max}(AP)]$); b) Proportions of β-glucosidase (BG), β-xylosidase (BX), β-cellobiohydrolase (CE) to acid phosphatase (AP), and c) Proportions of BG, BX, AP to leucine- (LE) and tyrosine- (TY) aminopeptidase (Table 1; Index 4). Significant differences between the treatments are indicated by lower case letters ($P < 0.05$).

### 3.3. Soil enzymes

#### 3.3.1. Enzyme indexes

It is assumed that the sum of major C-acquiring enzyme activities is a better indicator of total C-acquisition than BG alone. Therefore, we compared proportional enzyme activities (Table 1; Index 4) between the treatments and with depth. When we reduce the information on TAC by using only BG as C-acquiring enzyme ($V_{max}(BG)/[V_{max}(BG)+V_{max}(AP)]$) and compare it with the proportions of all measured C-acquiring enzymes (TAC/(TAC + TAP)), lower values were obtained when only BG was used as a representative for C-cycling hydrolases in the surface layer of litter-amended and bare fallow soil (Fig. 3a, b). This was constant with specific enzyme activities of CE and BX, which were respectively 88 and 69% lower for planted than for litter-treated soil at 0–10 cm depth, reflecting strong cellulolytic decomposition of plant litter (Supplementary Table 1). In deeper soil layer this effect disappeared.

Potential ($V_{max}$) and specific ($V_s$) enzyme activities of LE in the upper 10 cm were higher for planted than for litter-amended and fallow soil (Supplementary Table 1), indicating higher production of proteolytic enzymes. This was confirmed by the sum of N-degrading enzyme activities in the upper 20 cm, which was 41 (0–10 cm) and 43% (10–20 cm) higher in planted soil than in litter-amended soil (Fig. 4). The higher proteolytic activity in planted soil was corroborated by lower proportional enzyme activities (TAC/(TAC + TAN)) and lower $V_{max}$ ratio of C- to N-cycling enzymes (TAC/TAN; Fig. 4). In the upper 20 cm, a higher activity ratio ($P < 0.05$) of

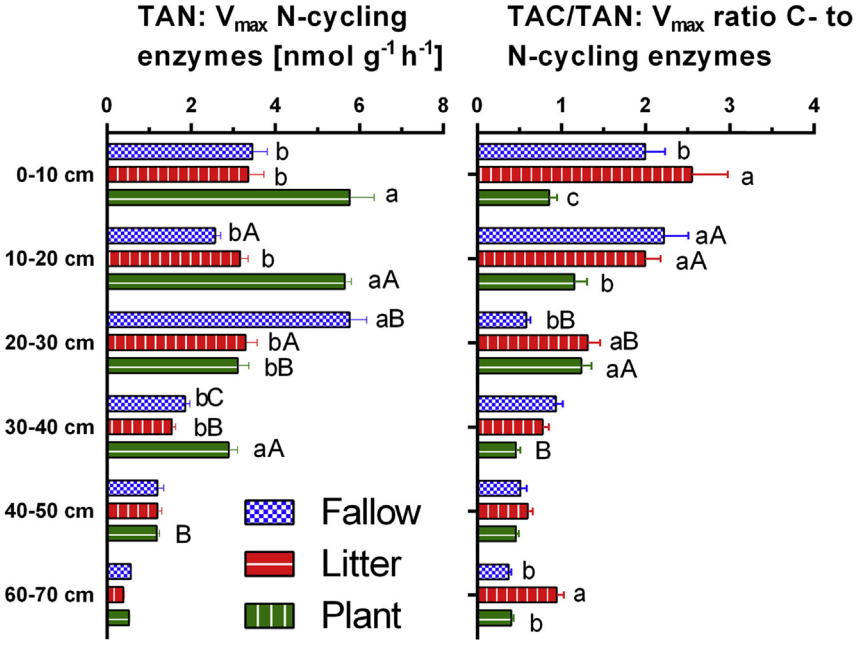


**Fig. 4.** Total activity of N-cycling enzymes (TAN; left); $V_{max}$ ratio between C-and N-cycling enzymes (TAC/TAN) with depth for bare fallow (Fallow), litter-amended (Litter) and planted soil (Plant). Further description of the indexes is presented in Table 1. Significant differences between the treatments are indicated by lower case letters. Capital letters are used to show significant differences with depth ($P < 0.05$) by comparing the upper layer with the layer below.

C- to N-cycling enzymes was determined for litter-treated than for planted soil (Fig. 4). Vector length (Equation (2)) and angle (Equation (3)) did not show evidence for N limitation in planted soil, but rather C limitation (30–40 cm) (Supplementary Figure 1).

### 3.3.2. Catalytic efficiency

At each depth, the sum of specific enzyme activities, the sum of catalytic efficiencies, and the proportional enzyme activities (TAC/(TAC + TAN)) were computed and tested for correlation to EN and DOC content (Supplementary Table 3). The sum of catalytic efficiencies was better correlated to EN and DOC across all treatments and depths than the sum of specific enzyme activities. Furthermore, the sums of catalytic efficiencies were strongly correlated with the EN for all soils throughout the profile (Supplementary Table 3), with the strongest relationship for planted soil (e.g. $r^2 = 0.97$, $P < 0.01$). The mean catalytic efficiency of enzymes decreased 2- to 20-fold from top- (<40 cm) to subsoil (>40 cm), reflecting the lower substrate quality with increasing depth (Fig. 5). The catalytic efficiency of TY was higher in planted than in litter-amended topsoil. This indicated highly efficient action of specific aminopeptidases with strong affinity to the substrate in the upper 40 cm of planted soil.

## 4. Discussion

The contrasting substrates (rhizodeposits vs. litter) affected soil microbial activity indicators only in the upper 40 cm, reflecting strong dependence of microbial activities on C and N sources (Šnajdr et al., 2008).

### 4.1. Enzyme indexes

This research provides insights into distinct profiles of soil enzyme indexes as influenced by the rhizosphere and the detritusphere. We compared the applicability of several indexes proposed as indicators of microbial nutrient limitation (Allison and Vitousek, 2005; Moorhead et al., 2016; Sinsabaugh and Follstad Shah, 2012). Proportional activities of C- versus N-cycling enzymes (Table 1; Index 4) and the relative $V_{max}$ of these enzymes (Table 1; Index 3) showed similar patterns with depth. Rhizodeposition may have

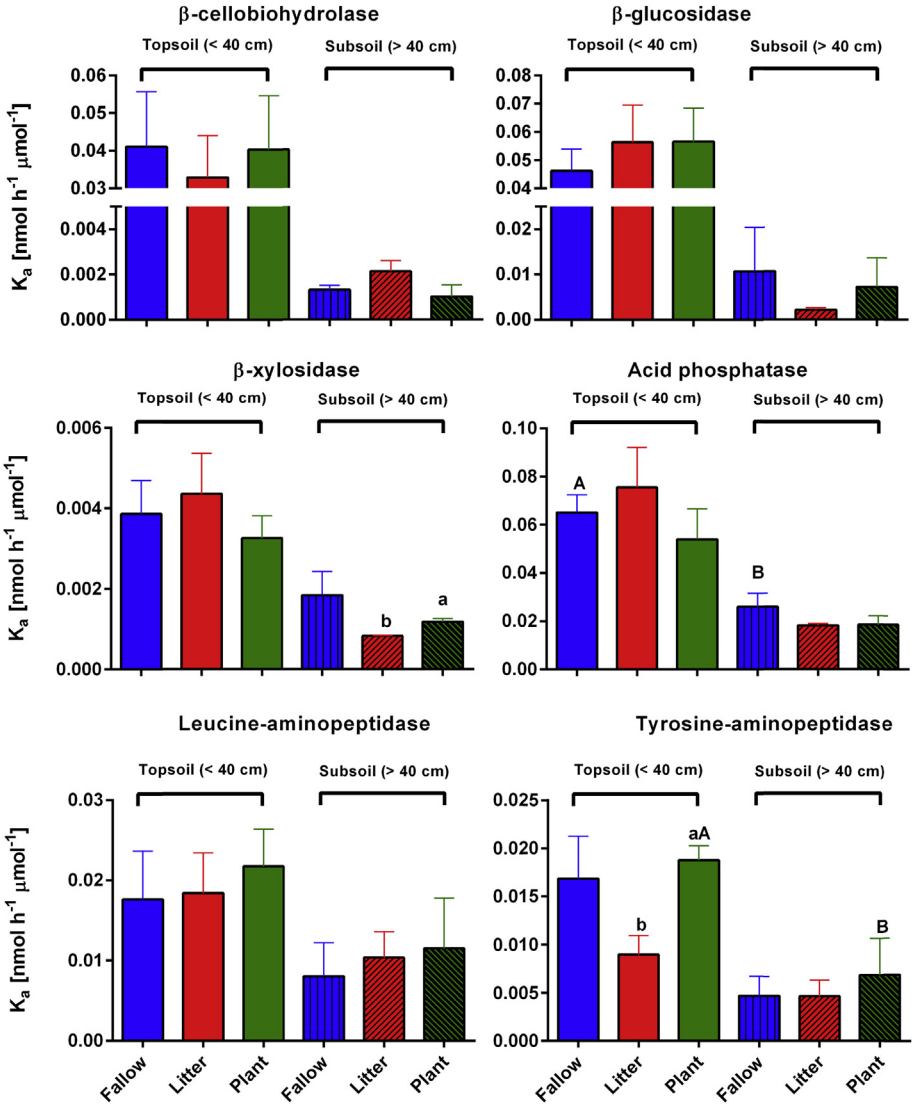


**Fig. 5.** Mean catalytic efficiency ($K_a$) of C-, N- and P-cycling enzymes for top- (<40 cm) and subsoil layer (>40 cm) in bare fallow (Fallow), litter-amended (Litter), and planted soil (Plant). Significant differences between the treatments are indicated by lower case letters. Capital letters are used to show significant differences with depth ($P < 0.05$).

decreased the relative $V_{max}$ and the proportional activity of C- to N-cycling enzymes compared to litter-amended and fallow topsoil, reflecting increased microbial N acquisition in planted soil, due to mining of additional N from SOM (Kuzyakov, 2002; Luo et al., 2006). This suggested that enzyme production was induced by resource limitation.

Specific enzyme activities ($V_s$) of acid phosphatase increased almost 3-fold from 0 to 10 to 10–20 cm in planted and bare fallow soil. Higher phosphatase activities in soil enhance the mineralization of organic phosphates when P is limited (Olander and Vitousek, 2000).

Vector length as a measure of C limitation, and vector angle as a measure of P vs. N limitation, did not show any pattern between the treatments in the upper 30 cm (Supplementary Figure 1). However, lower proportions calculated as $V_{max}(BG)/[V_{max}(BG)+V_{max}(AP)]$ relative to TAC/(TAC + TAP), demonstrated that the use of one single enzyme biased the assessment of substrate utilization. When three C-cycling enzymes were considered, the activities of BX and CE counterbalanced the low activities of BG in the detritusphere. Therefore, artificial enzyme indexes, which do not consider the great redundancy and complex interactions in the suite of soil enzymes, fail to adequately reflect the biological background and mechanisms.

Under natural conditions, enzymes interact with each other in order to utilize the complex substrate structures. The activities of cellulolytic, proteolytic and chitinolytic enzymes are usually assigned to the C and N cycles, respectively. A single enzyme, such as BG, for example, is responsible for terminal steps in the decomposition of both cellulose and bacterial/fungal peptidoglycan (Beier and Bertilsson, 2014; Humann and Lenz, 2009; Park and Uehara, 2008), and hence participates in both C and N elemental cycles. Thus, the interpretation of multiple enzyme indexes requires a certain degree of caution.

### 4.2. Top-vs. subsoil

The decrease of microbial biomass C down the soil profile is connected with decreasing availability and quality of organics (Blume et al., 2002; Fierer et al., 2003a, 2003b; Trumbore, 2000). Roots provide easily available C to the microbial community (Nguyen, 2003), which mobilizes nutrients from sources unavailable to plants (Kuzyakov and Xu, 2013). At the same field site more than 50% of the roots were distributed in the upper 10 cm of the Ap horizon and the weighted average root biomass C declined with depth, from 104 kg C $ha^{-1}$ at the 0–10 cm depth to 15 kg C $ha^{-1}$ at the 40–50 cm (Pausch et al., 2013). About 20% of the C assimilated by maize is transferred to below-ground pools at this arable field site (Pausch et al., 2015). The C transferred below-ground by the roots was immediately utilized by microbes in the upper 10 cm, as recently shown by increased specific growth rates (Loeppmann et al., 2015). The effect of diminishing substrate availability with depth on EN and $N_2O$ production was significant only in the upper 30 cm, which reflected that the arable topsoil under maize cultivation is a hot spot for microbial decomposition. Consequently, substrate quality plays an important role in controlling the vertical distribution of enzymes.

In deeper soil layers, the amount and quality of substrate were reduced, which was reflected in the differences between top- (0–40 cm) and subsoil (>40 cm) for most of the measured indicators of microbial activity, especially in the presence of plants. For example, all potential enzyme activities declined from top-to subsoil (Supplementary Table 1), as frequently shown before (Gelsomino and Azzellino, 2011; Šnajdr et al., 2008; Steinweg et al., 2013). However, the catalytic efficiency down the soil profile was not considered in most of these studies. The catalytic efficiency of enzymes ($K_a$) describes the specific rate of catalytic reaction, considering the enzyme affinity to the substrate ($K_m$). The $K_a$ decreased by 2- to 20-fold from top- (<40 cm) to subsoil, irrespective of the substrate input (Fig. 5). The variation of $K_m$ implied that enzyme-specific efficiencies of substrate utilization are strongly dependent on the affinity to the substrate. Nevertheless, the decrease of $K_a$ from top-to subsoil indicated that the driving forces were substrate quantity and quality.

### 4.3. Rhizosphere vs. detritusphere

Microbial biomass C and $N_2O$ production in the upper 20 cm showed significant effects of substrate input (rhizodeposits in the rhizosphere and maize-litter in the detritusphere). Particularly, the decrease of $N_2O$ production in planted soil from 0 to 10 to 10–20 cm may be defined as greater N limitation, which reflected maize as a sink for N. This was in line with lower EN contents in planted soil compared to litter-amended and bare fallow soil (Fig. 1d). Correspondingly, a weak relationship ($r^2 = 0.31$) between $C_{mic}$ and SOC was determined in planted soil, which may be explained by co-limitation of nutrients (e.g. N) in the rhizosphere. However, the strong correlations between $C_{mic}$ and $N_2O$ production, indicated that N sources for nitrification or denitrification were not the limiting factor for $N_2O$ production. Eventually, $O_2$ limitation occurred during SIR, which was proportional to the size of the microbial biomass, and mainly controlled the $N_2O$ efflux from the soil. Since $N_2O$ production is mediated by both biotic and abiotic processes and by oxygen availability, the link between soil organic matter degradation and $N_2O$ production is not always straight forward (Blagodatskaya et al., 2014). Moreover, the $N_2O$ reflected the total mineralized N, which strongly varies depending on the substrates used by microorganisms (Zhu et al., 2013).

In the presence of plants, EN, $N_2O$, $V_{max}$ ratio and proportional activity of C- to N-cycling enzymes were lower than in litter-amended soil at 0–10 and 10–20 cm depths. However, the catalytic efficiency of tyrosine aminopeptidase strongly increased in planted compared to litter-amended topsoil. This suggested strong effects of N limitation on the decomposer community in the presence of plants (Vitousek and Howarth, 1991). N limitation induced a shift in the catalytic properties of proteolytic enzymes (leucine and tyrosine aminopeptidases) which was in accordance with previous studies (German et al., 2011; Rejsek et al., 2008; Sims and Wander, 2002). This reflected higher investment in N-releasing enzyme production in planted soil than in litter-amended and fallow soil (Phillips et al., 2011; Stursova et al., 2006). It also confirmed the production of proteolytic enzymes with high substrate affinity (revealed by low $K_m$) by competitive microorganisms (Supplementary Table 2) and reutilization of microbial residues for maintenance when nutrients are limited (Bradford, 2013).

Cellulolytic specific enzyme activity was up to 10-fold higher in the litter-amended than in the planted surface layer (Supplementary Table 1). Maize litter may stimulate the decomposition of lignocellulosic materials by fungal communities and their enzymes (Kramer et al., 2012; Moll et al., 2015).

The C:N or the lignin:N ratio of plants is often used as a measure of litter quality and a predictor of decomposition rate, but the role of N in the regulation of litter decomposition is too complex to be characterized by measures of total N concentration (Sinsabaugh et al., 1993; Tian and Shi, 2014). Instead, we determined the activity ratio between C- and N-cycling enzymes (Fig. 4b), which was lower for planted than for litter-treated soil throughout the soil profile. This can be explained by relatively greater access to readily utilized labile root C sources, because N-limitation is defined with reference to relative N vs. C availability, suggesting better nutrient supply for microbes in the detritusphere (Šnajdr et al., 2011).

Similarly, the idea of a "better" N supply for microbes in the detritusphere is based on C:N enzyme ratios and remains a relative concept. The recalcitrant substrates (e.g. lignin and tannin) in the detritusphere may affect organic N mineralization (Valenzuela-Solano and Crohn, 2006).

## 5. Conclusions

The availability of C and nutrients in soil and especially in the rhizosphere and detritusphere strongly affected the microbial biomass and the catalytic efficiency ($K_a$) of hydrolytic enzymes with depth-dependent contrasting patterns. Dissolved N is decisive for enzyme activities, and decreases with depth. In particular, under root-induced N limitation, proteolytic enzymes had increased activity and affinity to substrate, which reflected the energy investment of microorganisms for nutrient acquisition. Enzymes' catalytic efficiency decreased 2- to 20-fold from top- (<40 cm depth) to subsoil. The contrasting input and quality of substrates in rhizosphere and detritusphere influenced microbial decomposition only in the topsoil (0–40 cm), whereas in the subsoil (>40 cm depth) the effects of contrasting substrate input disappeared. Proportions of multiple enzyme activities as well as catalytic efficiencies reflected both stoichiometric and C-quality effects on decomposer communities.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.08.023.

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