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Enzyme kinetics inform about mechanistic changes in tea litter decomposition across gradients in land-use intensity in Central German grasslands

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Abstract

Grassland ecosystems provide important ecosystem services such as nutrient cycling and primary production that are affected by land-use intensity. To assess the effects of land-use intensity, operational and sensitive ecological indicators that integrate effects of grassland management on ecosystem processes such as organic matter turnover are needed.

Here, we investigated the suitability of measuring the mass loss of standardized tea litter together with extracellular enzyme kinetics as a proxy of litter decomposition in the topsoil of grasslands along a well-defined land-use intensity gradient (fertilization, mowing, grazing) in Central Germany. Tea bags containing either green tea (high-quality litter) or rooibos tea (low-quality litter) were buried in five cm soil depth. Litter mass loss was measured after three (early-stage decomposition) and 12 months (mid-stage decomposition). Based on the fluorescence measurement of the reaction product 4-methylumbelliferone, Michaelis-Menten enzyme kinetics (V_{\max} : potential maximum rate of activity; K_m : substrate affinity) of five hydrolases involved in the carbon (C)-, nitrogen (N)- and phosphorus (P)-cycle (β -glucosidase (BG), cellobiohydrolase (CBH), cellotriohydrolase (CTH), 1,4- β -N-acetylglucosaminidase (NAG), and phosphatase (PH)) were determined in tea litter bags and in the surrounding soil.

The land-use intensity index (LUI), summarizing fertilization, mowing, grazing, and in particular the frequency of mowing were identified as important drivers of early-stage tea litter decomposition. Mid-stage decomposition was influenced by grazing intensity. The higher the potential activity of all measured C-, N- and P-targeting enzymes, the higher was the decomposition of both tea litters in the early-phase. During mid-stage decomposition, individual enzyme parameters (V_{\max} of CTH and PH, K_m of CBH) became more important.

The tea bag method proved to be a suitable indicator which allows an easy and cost-effective assessment of land-use intensity effects on decay processes in managed grasslands. In combination with enzyme kinetics it is an appealing approach to identify mechanisms driving litter break down.

Keywords

Cellotriohydrolase, cellobiohydrolase, β -glucosidase, phosphatase, N-acetyl-glucosaminidase, tea bag index

1 Introduction

Grassland ecosystems play a significant role in providing ecosystem services such as carbon sequestration, nutrient cycling and fodder production. All these services are linked to litter decomposition and are affected by the intensity of various management practices such as fertilization, mowing and/or grazing (Felipe-Lucia et al. 2020). Soil microorganisms drive litter decomposition by releasing extracellular enzymes which break down macromolecules (e.g. cellulose, chitin, phosphomonoesters) into smaller molecules (e.g. glucose, chitin monomers and phosphates) (Sinsabaugh and Follstad Shah, 2012). The subsequent release of nutrients such as nitrogen (N) and phosphorus (P) is an important function provided by soil organisms (Manzoni et al. 2010) that is related to ecosystem productivity and stability (Ali et al. 2018).

Recent studies conducted under different climatic and soil conditions have shown inconsistent results regarding the effects of grassland land-use intensity on litter decomposition. While Sun et al. (2018) found increased litter decomposition under grazing for an alpine meadow ecosystem in China, Tozer et al. (2020) did not detect a difference between grazed pastures and sites deferred from grazing for 12 months in New Zealand. Tang et al. (2020) reported decreased litter decomposition in salt marsh soils together with a decrease of β -glucosidase activity, most likely due to compaction, but divergent reactions of two N cycle enzymes. Similarly, the effects of mowing on litter decomposition and enzyme activities have been poorly investigated and understood. A possible reason for this may be that mowing frequency often shows a strongly positive correlation with the amount of fertilization. Ochoa-Hueso et al. (2020) reported that enzyme activities related to the C- and N-cycle increased after nutrient additions (mainly N and/or P) and were positively related to litter decomposition.

In order to assess the effects of land-use intensification on litter decomposition the use of standardized litter material of high and low quality along well-defined land-use intensity gradients appears to be a promising approach. High-quality litter has a fast decay rate, which is associated with a high amount of easily degradable organic compounds and high nutrient concentrations, whereas low-quality litter is characterized by low decay rates due to a low proportion of easily degradable organic compounds and low availability of nutrients (Cotrufo et al. 2009). The tea bag method (Keuskamp et al. 2013) provides a standardized approach which uses industrially manufactured and commonly available nylon tea bags containing either green tea (high-quality litter; low C:N ratio) or rooibos tea (low-quality litter; high C:N ratio). The tea bag method can be applied to a large number of sites along gradients of land-use intensity, and has already been used at global scale (Djukic et al. 2018). However, it does not

directly inform about mechanistic changes in litter decomposition driven by microbial processes. Here, enzyme kinetics might be very specific and sensitive measures. Therefore, three enzymes representing distinct steps in cellulose decomposition (van den Brink and Vries, 2011) were used to assess cellulose decay processes: β -glucosidase (BG), cellobiohydrolase (CBH) and cellotriohydrolase (CTH). Cellulose is a major constituent not only of grassland biomass (Kleinebecker et al. 2011) but also of green and rooibos litter (Keuskamp et al. 2013, acid soluble fraction) which decreases during three-months decomposition in both tea types (Duddigan et al. 2020). During tea decomposition microbial metabolites are enriched (Duddigan et al. 2020). An enzyme involved in the decomposition of chitin and peptidoglycan derived from microbial necromass is 1,4- β -N-acetylglucosaminidase (NAG). NAG might become important during later stages of decomposition but can also inform about stoichiometric imbalances most likely in early stages (Mori et al. 2021). Phosphatase (PH) plays an important role in P-cycling and informs about stoichiometric imbalances (Kandeler, 2015).

There is increasing evidence that not only enzyme activities are affected by land-use intensity (Boeddinghaus et al. 2015; Wallenius et al. 2011) but also the affinity of an enzyme towards its substrate (Liu et al. 2020; Tischer et al. 2015) and in consequence the rate of nutrient cycling. Therefore, the enzymatic kinetics as described by the Michaelis-Menten equation (V_{\max} , K_m) can give valuable insights into the regulating mechanisms involved in litter decomposition. The maximum velocity of the enzyme reaction (V_{\max}) is considered as a measure of the enzyme concentration, and the Michaelis-Menten constant (K_m) as a measure of the enzyme's affinity to its substrate (German et al. 2011; Loeppmann et al. 2016; Marx et al. 2001) indicating its behavior under substrate limiting conditions (Hobbie and Hobbie, 2012). In ecological systems, such as soils, V_{\max} and K_m are considered as apparent measures of the catalyzed reaction (Wallenstein et al. 2010; Sinsabaugh et al. 2014), because enzymes catalyzing the same reaction are produced by many different organisms and are in interaction with the soil-humus-matrix. Changes in microbial community structure and/or substrate availability are important drivers of enzyme kinetics. Land-use intensity effects on both are known (Tischer et al. 2015; Le Provost et al. 2021). Thus, it is likely that strong gradients in mowing, grazing and fertilization intensity affect enzyme kinetics, but not necessarily decomposition rate. If an increase in V_{\max} is accompanied by an increase in K_m , the increase in reaction velocity for this specific enzyme (according to the Michaelis-Menten equation) is not that strong as with constant K_m , especially at low substrate concentrations (Dick 2011; Razavi et al. 2015). For example, at a substrate

concentration of $50 \mu\text{M g}^{-1}$ soil an increase in K_m by a factor of four can counterbalance a twofold increase of V_{max} ; i.e. the decomposition rate will not change. It is important to notice that overall decomposition is regulated by interactions of V_{max} and K_m of different enzymes. To determine enzyme kinetics, the measurement of fluorescence based on 4-methylumbelliferone (MUF) (Marx et al. 2001, 2005) has a high standardization potential. The advantage is, that the resulting product – MUF – of the action of multiple enzymes is the same and can be sensitively detected in soil suspensions by fluorescence intensity measurements through high throughput multiplate readers (Kandeler, 2015). This allows the direct comparison across different element cycles which is an important prerequisite for its indicative value. So far studies investigating kinetic enzyme properties are scarce and, to our knowledge, have never been combined with the tea bag approach.

In this study we examined 23 agriculturally managed permanent grasslands ranging from non-fertilized pastures with low stocking rates to intensively fertilized hay meadows (Fischer et al. 2010). They represent a gradient in land-use intensity that is typical of Central Germany (Blüthgen et al. 2012). Standardized information on land use exists for more than ten years. The objective was to assess the tea bag method as indicator for land use-driven changes in nutrient cycling in combination with enzyme kinetics (CTH, CBH, BG, NAG, PH) during the short-term (three months) and mid-term stage (12 months) of tea decomposition.

The following questions were asked:

1. Is the tea bag method an early indicator (within three months) of litter decomposition under different land-use intensities in grassland ecosystems?
2. Does high land-use intensity (grazing, mowing and/or fertilization) stimulate decomposition of high- (green tea) and low- quality (rooibos tea) litter?
3. Does the combination of the teabag method with kinetic properties of soil and litter derived enzymes allow a better understanding of mechanisms underlying decomposition at different stages of decay?

2 Material and methods

2.1 Site description

We determined litter decomposition and enzyme kinetics in 23 permanent grassland plots (Fig. A.1) with different land-use intensity (fertilization, mowing, grazing) (Table A.1). All plots are

located in the Hainich-Dün region in central Germany and are part of the Biodiversity Exploratories (www.biodiversity-exploratories.de). The Hainich-Dün exploratory has a size of nearly 1300 km² with an altitudinal gradient from 285 to 550 m above sea level. Climatic conditions within the region are characterized by a mean annual temperature of 6.5 to 8 °C and a mean annual precipitation of 500 to 800 mm. The parent material for soil development is mainly loess over Triassic limestone. The main soil types (Table A.1) are Cambisol, Vertisol and Stagnosol (Fischer et al. 2010). To assess land-use intensity, land managers have been interviewed annually since 2006 about mowing frequency, grazing intensity (mean livestock unit per grazing days per ha) and fertilization (mean kg N per ha) (Vogt et al. 2019). Based on this information, a standardized land-use index (LUI) was calculated for each plot (Table A.1) according to (Blüthgen et al. 2012) using the LUI calculation tool (Ostrowski et al. 2020) implemented in BExIS (<http://doi.org/10.17616/R32P9Q>). To investigate the effects of land-use intensity on litter decomposition average LUI was calculated for the years 2006 to 2016 (three months exposure) the years 2006 to 2017 (12 months exposure), respectively (Table A.1). Basic soil physio-chemical and microbial characteristics available for all 23 grassland plots in the BExIS database (www.biodiversity-exploratories.de) were used to identify further drivers of litter decomposition. In brief the following variables were considered: various resin-bond nutrients like nitrogen (resin-(NO₃-N + NH₄-N)), phosphorous (resin-PO₄-P), calcium (resin-Ca), potassium (resin-K), and magnesium (resin-Mg) (Klaus et al. 2018); total extractable phosphorus (Olsen Pt), as well as its inorganic (Olsen-Pi) and organic (Olsen-Po) fraction (Boeddinghaus et al. 2019); total organic carbon and total nitrogen in the soil as well as the corresponding soil C:N ratio; microbial biomass carbon (MBC) and nitrogen (MBN) as well as microbial C:N ratio; fungal and bacterial phospholipid fatty acids (PLFA) as well as fungal-bacterial PLFA ratio; ergosterol content (Boeddinghaus et al. 2019), and the contents of sand, silt and clay (Fischer et al. 2010).

2.2 Litter decomposition

As a measure of litter decomposition, we used the tea bag method (Keuskamp et al. 2013). The nylon tea bags containing either green tea (*Camellia sinensis*) (C:N ratio: 12.5) or rooibos tea (*Aspalanthus linearis*) (C:N ratio: 66.9, Fig. A.2) (Lipton Unilever L6 125 3 L620 or L6 125 1 L620, respectively) were buried in the topsoil of each grassland (5 cm soil depth) following the protocol of the global litter decomposition study (Djukic et al. 2018). Thus, in each plot, an area of 7 m * 2 m with two subplots in a distance of 4.8 m was marked (Fig. A.1). On each subplot,

four bags of green tea and four bags of rooibos tea were installed between 29th June and 01st July 2016. After three months, as well as after 12 months, four green tea bags and four rooibos tea bags were excavated (e.g. two bags per tea type from each subplot). Adhering soil and fine roots were removed carefully and the fresh weight of all individual bags was measured. We expected that after 12 months of decomposition the remaining tea within one bag might not be enough for all analysis. Therefore, to measure the enzyme kinetics, two composite samples (in total 0.4 g) from two adjacent tea bags (row 1 and row 2) of the same tea type (0.2 g of each bag) were used. These sub-samples were stored at +4 °C until analysis. The fresh weight of the remaining amount of tea in each bag was determined. Then, all bags were dried at 70 °C for 48 h to determine the dry weight (DW) and to calculate mass loss (Djukic et al. 2018) considering the dry weight of tea used for enzyme kinetics.

2.3 Soil sampling

During retrieving of the first tea bags after three months, in direct proximity of two adjacent tea bags ('teasphere') soil samples were taken with a spoon and pooled together. Thus, per plot two soil samples of the 'teasphere' of green and rooibos tea were available, respectively. After sieving (< 2 mm) samples were stored at -20 °C until analysis.

2.4 pH, water content and water-soluble components

We measured pH(H₂O) in the tea samples (tea:solution ratio 1:20 required due to high water absorption of the tea litter) in one mixed sample (0.4 g) per plot by combining 0.1 g of each 70 °C dried green tea bag or rooibos tea bag, respectively. Then 8 ml deionized water was added and samples were shaken. After 30 minutes pH was determined with a pH meter (pH 330/340, WTW, Weilheim, Germany). Soil pH(H₂O) was measured two hours after adding 12.5 ml deionized water to 5 g field fresh soil as described above.

Water content of tea and soil samples was determined gravimetrically after drying about 0.5 g of sample for at least 24 h at 105 °C.

The proportion of water-soluble components in both tea types was determined using a six-day extraction with 100 ml deionized (n=10). Samples were stored in the fridge (about 8°C). Afterwards, the dry weight (70 °C) was measured and mass loss calculated.

2.5 Enzyme kinetics assay

Fluorescent substrates labelled with MUF were used to measure the activity of five extracellular hydrolytic enzymes (EHEs) (Table A.2): Substrates were dissolved in 300 μl dimethyl sulfoxide and diluted in sterile bidistilled water.

The tea or soil suspensions were obtained by mixing 0.4 g tea or 0.5 g soil dw equivalent in 40 ml and 50 ml sterile bidistilled water, respectively. Then the samples were sonicated (Sonopuls Ultraschall-Homogenisator HD 3200, Bandelin electronic GmbH & Co. KG, Berlin, Germany) with 50 J s^{-1} for 96 s or 120 s, respectively. To keep the temperature constant during sonication a cold-water bath was used.

The enzyme assay was conducted in 96-well microplates (flat bottom, black, polystyrene, Greiner bio-one GmbH, Kremsmünster, Austria) with 50 μl of sample suspension and 50 μl of MES buffer (pH 6.5). The pH of the buffer was adapted to the soil pH (Baldrian, 2009; German et al., 2011). Subsequently, 100 μl MUF-substrate solution of different concentration (0, 2.5, 5, 10, 50, 250, 500, 1000 $\mu\text{mol substrate} \cdot \text{l}^{-1}$) was added to the wells. Fluorescence was read with a microplate reader Infinite F200Pro (Tecan Group Ltd., Männedorf, Switzerland) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The time period from substrate addition to fluorescence measurement was kept constant for all samples. Fluorescence measurements were repeated every 30 min. Between the fluorescence reads all plates were stored in the dark at 30 $^{\circ}\text{C}$ and shaken at 150 rpm (Titramax 1000 with Incubator 100, Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). The measurements were stopped when the increase in the fluorescence rate (per 30 min) per well started to decline (Marx et al. 2001, 2005). Enzyme activities ($\text{nmol g}^{-1} \text{h}^{-1}$) were calculated according to German et al. (2011, 2012) based on tea litter mass or soil dw mass, respectively. Then, the Michaelis-Menten function was fitted to the data set using the function *drm* (*drc package*, version 3.0-1, (Ritz et al., 2015) of R version 3.6.2 (R Core Team, 2019)) to obtain the maximum velocity of the enzyme-substrate reaction (V_{max}) and the substrate affinity constant (K_{m}). Decomposition rate (k) and litter stabilization factor (S) were calculated as tea bag index according to Keuskamp et al. (2013) using the three-months mass loss (Table A.3).

2.6 Statistical analysis

To test whether there is a significant correlation between tea mass loss and land-use intensity index (Fig. 2 a-b), Pearson's correlation coefficient was used. The enzyme kinetic data (Fig. 3

a-m and 4 a-h) were tested for significant differences over time and between substrates (soil, tea) using the Wilcoxon signed-rank tests. Principal component analyses (PCA) were used to identify patterns in kinetics of C-cycling or N- and P-cycling enzymes in green and rooibos tea. In ordination biplots the following supplementary variables are shown: mass loss of green and rooibos tea and the LUI components mowing and grazing. Data were centered and standardized by norm and processed with Canoco 5.

We ran linear mixed-effects models (*nlme package*, (Pinheiro et al. 2020)) to find the best set of variables (land-use intensity, enzyme kinetics, basic soil characteristics) that explain overall litter decomposition, in the early-stage (three months) and the mid-stage (12 months). For the selection of the land-use variables a multiple linear regression was calculated. To analyze multivariate dimensions of enzyme kinetic properties, a PCA was run with all enzyme kinetic data for green tea and rooibos tea together. Data were centered and standardized by norm and PCA was performed with the *fviz_pca_biplot* function (*factoextra package*, (Kassambara and Mundt, 2020)). For the three-months data of both tea types, the V_{max} values of all measured enzymes were correlated with the first PCA axis (enzyme dim.1, Fig A.3 a,b) while K_m values of all enzymes were correlated with the second axis of the PCA (enzyme dim.2, Fig. A.3c). Since the 12-months data did not show a clear pattern along the principal components, for the 12-months model individual enzymes were centered and scaled. The same procedure of PCA dimension reduction was done for 21 variables of soil properties, which were reduced to 10 variables (resin-N, resin-Ca, resin- PO_4 -P, resin-Mg, Olsen-Pi, Olsen-Po, soils C:N ratio, MBN, ergosterol, and clay content, Fig. A.4) to avoid multicollinearity and variance inflation (*mctest package*, (Imdad and Aslam, 2018; Imdadullah et al. 2016), *GGally package*, (Schloerke et al. 2018)). The first dimension (soil dim.1, Fig. A.4b) represents the soil nutrients and includes resin-Ca, resin-(NO_3 -N + NH_4 -N), resin- PO_4 -P, as well as the soil organic carbon to nitrogen ratio (C:N ratio). The second dimension (soil dim.2, Fig. A.4c) represents mainly soil microbial properties and includes ergosterol, a fungal marker, and microbial nitrogen (MBN). Additionally, “plot” was used as random factor in the models.

To find the most parsimonious model, we used Akaike information criterion (AIC) and the variance inflation factor (VIF, *car package*, (Fox and Weisberg, 2019)). The final model (Table 1) was selected by comparing second-order Akaike Information Criterion (AICc, *MuMIn package*, (Barton, 2019)) and by checking residual plots and QQ-Norm plots (Fig. A.6, Fig. A.7). Pseudo-R-squared for generalized mixed-effect models (*MuMIn package* (Barton, 2019)) were checked during model selection procedure. An ANOVA was done for every model in order to extract F-values and respective p-values for each factor included in final models. In

order to test for patterns in the model residuals due to the abundance of not considered co-variables, model residuals were plotted against every single variable that was not in the current model (Zuur et al. 2009). If there were patterns in these plots the variable was included in the model. Thereafter, these extended models were checked against the original model with ANOVA function in order to evaluate model-improvement based on Likelihood statistics and to account for any bias caused by the decision based on the AIC (Zuur et al. 2009). To investigate the individual parameters with respective confidence intervals and their proportion of the explanatory power of the “best” model, we ran the *intervals* function of the *nlme package* (Pinheiro et al. 2020; Table A.4 and A.5).

3 Results

3.1 Decomposition patterns in green tea and rooibos tea

3.1.1 Tea mass loss, pH and proportion of water-soluble components

On average, the mass loss (Fig. 1) of green tea was 58.3 % (± 4.4 SD) after three months and increased only slightly to 62.2 % (± 4.9) after 12 months. Mass loss of rooibos tea was 2.5 to 2-fold lower with 23.6 % (± 3.7) after three months and 32.5 % (± 5.5) after 12 months. This factor stayed constant even when considering the average loss of water-soluble components in the first six days which amounted to 33 % in green tea (± 3.6) and 12 % (± 0.5) in rooibos tea. As shown by Blume-Werry et al. (2021) leaching of green and rooibos tea levelled off already after 72 h with a mass loss of 40 and 20 %, respectively, which fits to our own data. Berg (2000) characterised the early phase of decomposition mainly by the loss of water-soluble substances ceasing when nutrient concentrations such as N start to increase. After three months already, an increase in N concentration was detected (Fig. A.2). Thus, when comparing with the concept of Berg (2000), data obtained after three month of tea decomposition characterise the early-stage of phase two of decomposition, e.g. the phase with strong biological degradation after the leaching phase.

Significant positive correlations between mass loss of both tea types and the land-use intensity index (LUI) were observed after three months of decomposition (green tea: $r = 0.42$, $p = 0.04$; rooibos tea: $r = 0.55$, $p = 0.007$) (Fig. 2a). The individual LUI component mowing was positively correlated with green tea ($r = 0.48$; $p < 0.05$) and rooibos tea mass loss ($r = 0.46$, $p < 0.05$). Fertilization correlated only with mass loss of rooibos tea ($r = 0.47$, $p < 0.05$). The

stabilization factor k (Table A.3) had a positive but weaker relationship with LUI ($r = 0.34$, $p < 0.05$). No further correlations between the Tea Bag Index (Table A.3) and LUI components, enzyme or soil variables were detected. After 12 months of exposition in the soil no significant correlations with LUI were detected (Fig. 2b). The average $\text{pH}(\text{H}_2\text{O})$ of both teas was 6.7 ± 0.5 after three months. After 12 months it was slightly higher at 7.5 ± 0.4 (data not shown).

3.1.2 Kinetics of cellulose degrading enzymes

In general, the potential enzyme activities (V_{\max}) were significantly lower in rooibos tea compared to green tea (13 to 14-fold after 3 months and 6 to 14-fold after 12 months), except for CTH (Fig. 3). Interestingly, after three months V_{\max} of CTH was quite similar in rooibos ($226 \text{ nmol g}^{-1} \text{ h}^{-1}$) and green tea ($202 \text{ nmol g}^{-1} \text{ h}^{-1}$). Thereafter, V_{\max} of CTH in green tea increased ($713 \text{ nmol g}^{-1} \text{ h}^{-1}$; Fig. 3j) whereas a significant decrease occurred in rooibos tea after 12 months ($24 \text{ nmol g}^{-1} \text{ h}^{-1}$; Fig. 3k). Also, V_{\max} of CBH was significantly lower (1.5 times) in rooibos tea after 12 months compared to three months. The potential activity of BG in rooibos tea tended to decrease (1277 to $914 \text{ nmol g}^{-1} \text{ h}^{-1}$, Fig. 3b). In contrast, the V_{\max} values for BG in green tea significantly decreased after 12 months compared to three months ($p < 0.01$, 17332 to $7837 \text{ nmol g}^{-1} \text{ h}^{-1}$). After three months, the V_{\max} values in green tea were between 13.5 (CTH) and 60 times (CBH) higher than in the surrounding soil. In rooibos tea the values were 3.0 (BG, CTH) and 4.5 (CBH) times higher in tea bags than in ‘teasphere’ soil.

The K_m values of BG were higher in green tea ($137 \text{ } \mu\text{mol g}^{-1}$) than in rooibos tea ($26 \text{ } \mu\text{mol g}^{-1}$) after three months. For CBH (Fig. 3 g, h) and CTH (Fig. 3 l, m) there were no differences. After 12 months, the K_m values in green tea were four to five times higher for all C enzymes than in rooibos tea. In contrast to the V_{\max} values, after three months the K_m values did not differ significantly between the ‘teasphere’ soil and the respective tea bag. Only the K_m value for BG in green tea was significantly higher ($p < 0.01$, four times) in the tea bag than in the ‘teasphere’ soil and remained at this higher level even after 12 months (Fig. 3c). Also, the K_m value of CBH for rooibos tea showed a significant increase ($139 \text{ } \mu\text{mol g}^{-1}$, $p < 0.01$, Fig. 3h) compared to the surrounding soil, but decreased again after 12 months ($13 \text{ } \mu\text{mol g}^{-1}$). The K_m value of CTH in rooibos tea decreased even stronger (Fig. 3m) between month three ($88 \text{ } \mu\text{mol g}^{-1}$) and 12 ($18 \text{ } \mu\text{mol g}^{-1}$).

PCA (Fig. 4) of V_{\max} and K_m values of C-cycle enzymes in green and rooibos tea indicates that their individual interactions differ depending on tea type and time (Fig. 4 a, b). Often vectors of an individual enzyme parameter in green tea are plotted orthogonal towards the

corresponding value in rooibos tea (e.g. V_{\max} BG and CBH). The potential activity of BG in rooibos tea is negatively correlated with the K_m values of CBH ($r = -0.44$, $p < 0.05$) and by tendency of CTH ($r = -0.40$, $p = 0.058$) after three months (Fig. 4a). Mowing and fertilization intensity are positively correlated with K_m of CBH ($r = 0.50$, $p < 0.05$; $r = 0.53$, $p < 0.05$) and CTH ($r = 0.59$, $p < 0.01$; $r = 0.52$, $p < 0.05$). In green tea the V_{\max} value of BG is highly correlated with the V_{\max} of CBH ($r = 0.96$, $p < 0.001$) and of CTH ($r = 0.64$, $p < 0.001$; Fig. 4a). After three months the separation of sites along PC 1 (32.1%) is mainly driven by high K_m values of CTH (green tea and rooibos tea) as well as high K_m of BG and CBH (rooibos tea) and correlated with the mass loss of rooibos tea as well as the mowing intensity. Axis 2 of the PCA (20.1%) separates sites with high K_m values of BG and CBH in litter of green tea (associated with higher mass loss of green tea) from sites with high V_{\max} values of CBH in rooibos litter. After 12 months (Fig. 4b) the K_m values of CBH were important for the separation of sites along PC 1. The value measured in rooibos litter was positive correlated with PC 1 and the one measured in green tea negative (Fig. 4b). In rooibos litter the K_m of CBH is positively correlated with the K_m of CTH ($r = 0.67$, $p < 0.01$) while in green tea the K_m of CBH is negatively correlated with mass loss ($r = -0.53$, $p < 0.05$). Along PC 2, K_m of BG in rooibos tea (positive loading) and K_m of CTH in green tea (negative loading) mainly contribute to the separation of grassland sites. V_{\max} of BG and CBH of rooibos tea are positively loaded on PC 2 and correlated ($r = 0.75$, $p < 0.01$). Furthermore, the K_m of BG (rooibos tea) is positively correlated with both V_{\max} values (BG: $r = 0.71$, $p < 0.01$; CBH: $r = 0.84$, $p < 0.01$). Along PC 1, V_{\max} of BG is positive correlated with V_{\max} of CBH ($r = 0.64$, $p < 0.01$) and CTH ($r = 0.59$, $p < 0.01$) in green tea (Fig. 4b). There is no separation of sites according to fertilization history in the last decade or the year of sampling (Fig. 4a and 4b).

3.1.3 Kinetics of enzymes involved in the N- and P-cycle

The V_{\max} values of NAG and PH in green tea (Fig. 5a, e) after three months were significantly higher (8 times, $p < 0.01$), than after 12 months. In rooibos tea (Fig. 5f) V_{\max} value of PH was three times higher ($p < 0.01$) in the early-stage of decomposition. The K_m values of NAG (Fig. 5c-d) were twice as high after 12 months than after three months (green tea $p < 0.01$ and rooibos tea $p < 0.05$). Individual patterns of P- and N-cycle enzyme parameters change with tea type and time (Fig. A.5).

3.2 Drivers of overall litter decomposition

The best linear mixed effect model explained 96.7 % of litter mass loss after three months of decomposition (Table 1, Table A.4) and 94.6 % after 12 months (Table A.5). If the random effect (*plot*) was considered, the explanatory power of the model increased to 98.6 % and 95.5 %, respectively. Tea type showed the highest significant influence (see estimates in Table A.4 and A.5) on litter decomposition after three and 12 months. After three months, mowing had the second highest significant estimate (Table A.4). After 12 months mowing was not selected in the model, but grazing showed the 2nd highest estimate (Table A.5). This indicated a differentiated effect of land-use intensity on decomposition. In the three-month model, the V_{\max} values of all enzymes were important for litter decomposition, since enzyme dim.1 has the 3rd highest significant estimate (Table A.4). In contrast, in the 12-months model the V_{\max} values of CTH and PH turned out to be important (with estimates of 1.96 and 1.91, respectively, Table A.5). The higher the V_{\max} values, the higher was litter decomposition. Additionally, it was shown that after 12 months of litter decomposition, the K_m value of CBH was an important predictor. Models without CBH K_m were inferior (Table A.7). After three months the K_m values (enzyme dim.2) did not have any influence. The lower the K_m value of CBH (the higher the substrate affinity), the higher was the litter mass loss after 12 months of decomposition (estimate -1.55, Table A.5). Soil dimension 2 was selected in the three-months model (Table 1), however had only little influence (Table A.4). By tendency litter decomposition after three months was higher in soils with lower fungal biomass and lower microbial biomass N, since both variables contribute with about 65 % to soil dim. 2 (Fig. A.4c). This trend was no longer apparent after 12 months. However, including both soil dimensions improved the quality of the 12 months model (Fig. A.7, Table A.7).

4 Discussion

4.1 Enzyme kinetics during the decomposition process of high- and low-quality litter

To identify suitable functional microbial indicators, it is important to assess their operating ranges (Thiele-Bruhn et al. 2020). So far studies investigating kinetic enzyme properties are scarce and, to our knowledge, have never been combined with the tea bag approach. Only the studies of Ochoa-Hueso et al. (2020) and Oggioni et al. (2020) reported enzyme activities, but not kinetics, in tea bags after three months of decomposition. Litter quality, time of decomposition and habitat (e.g. detritosphere/'teasphere', rhizosphere, bulk soil) are important factors controlling operating ranges of enzymes and their interactions.

4.1.1 Potential enzyme activities

Our data on C-cycling enzymes show that potential enzyme activities were significantly higher in tea litter, especially in green tea, compared to the surrounding ‘teasphere’ soil after three months of decomposition. This indicates that microbial colonization and active microbial decomposition of both tea litters took place. Pioli et al. (2020) and Daebeler et al. (2022) have shown that rooibos as well as green tea litter is colonized after three months by a unique fungal and bacterial community. In our study PH is the only enzyme with similar V_{\max} in both tea types, all other enzymes show higher potential activities in green tea during the early- and mid-stage of decomposition. This is in contrast to the study of Ochoa-Hueso et al. (2020) and Oggioni et al. (2020) who reported higher enzyme activities in green tea also for P-cycle enzymes. One explanation might be that microorganisms in the calcareous grasslands of our study are P-limited. Boeddinghaus et al. (2019) observed a high microbial activity in the same grassland soils when P-rich plant material was available pointing to an intensive turnover of organic P. The V_{\max} of PH decreased after 12 months of decomposition to the level characteristic of rhizosphere soil of different grassland plant species growing on the same experimental sites (Tischer et al. 2019). This might indicate a substantial depletion of organic phosphorus in litter of green and rooibos tea. V_{\max} values of BG in rooibos tea were as high as those measured in the rhizosphere of grassland plant species (Tischer et al. 2019), but potential activities of all other enzymes were higher in tea litter. The potential enzyme activities measured in the early and intermediate phase of rooibos tea decomposition (BG, CBH, NAG, PH) correspond to those detected in decomposing beech litter in forest soils of the same region in Germany (Purahong et al. 2014). This fits to the observation of Pioli et al. (2020) who observed that the fungal and bacterial community in rooibos tea was much more similar to the community in native litter (maybe due to the fact that native litter was mainly classified as low-quality litter).

4.1.2 Substrate affinities

Substrate affinities of C-cycle enzymes in rooibos tea, especially of CBH, were lower than in ‘teasphere’ soil (e.g. higher K_m). In green tea only the K_m of BG was higher than in ‘teasphere’ soil. Tang and Riley (2019) demonstrated the importance of the particle size for the success of enzymes to find adequate binding sites. The substrate affinity, and thus the binding of enzyme to its substrate, increased as well as the maximum depolymerization rate with decreasing particle size (Tang and Riley, 2019). Although not measured in our study, a larger particle size

in tea litter compared to 'teasphere' soil might partly explain the lower substrate affinity detected especially in rooibos tea. This phenomenon was not that pronounced in green tea litter since decomposition progressed faster compared to rooibos litter. It is likely that smaller particles are already present after three months of decomposition of green tea. A decrease in particle size between the early and intermediate phase of rooibos tea decomposition might contribute to the significant decrease of the K_m values for CBH and CTH. A further explanation might be a decrease of in situ substrate availability (Wallenstein and Weintraub, 2008) and thus a lower competition between the MUF-substrate and in situ substrate for enzyme binding sites contributing to lower K_m values (Tischer et al. 2019). An increase of K_m values during the decomposition process, as observed for NAG in both tea litters, might be due to an increase of in situ substrate concentrations (Wallenstein and Weintraub, 2008) associated with microbial colonization. This is also a hint towards an increase in microbial necromass (Fernandez et al. 2016). From other studies it is known that microbial communities continue to change during the decomposition process (Tláskal et al. 2016). For example, changes in fungal and bacterial community composition occurred during 473 days of decomposition of beech litter and were associated with physicochemical shifts of litter properties (Purahong et al. 2016a, 2016b). Thus, it is most likely, that after 12-months of decomposition microbial communities differed even more in rooibos and green tea litter than observed in three months studies (Daebeler et al. 2022) and might have produced less efficient isoenzymes (Khalili et al. 2011; Loepmann et al. 2016). Changes in pH are associated with changes in K_m values (Tischer et al. 2015), with the exception of NAG (Tischer et al. 2019), and often drive soil microbial communities (Bárcenas-Moreno et al. 2016) when gradients in H^+ ion concentration are pronounced. In the present study, the $pH(H_2O)$ in both tea litters was on average 7.5 ± 0.4 after 12 months of decomposition and cannot explain differences in K_m . The mechanisms (e.g. particle size, decrease of in situ substrate availability and/or change in microbial community composition) behind changes in K_m values remain speculative with this approach. However, we could show that frequent and sometimes strong changes in substrate affinity occur. Increases in K_m result in lower reaction velocities (Allison et al. 2018) and thus are an important indicator to be considered in litter decomposition studies.

4.1.3 Interactions between enzymes

Patterns of C-cycle enzymes change with time and litter type. After three months, in rooibos tea V_{max} of BG is negatively correlated with K_m of CBH and by tendency of CTH. This indicates

that in rooibos tea the microbial community produces CTH and CBH isoenzymes which are more efficient at low substrate concentrations and thus quickly deliver cellobiose as substrate for BG, especially at sites with frequent mowing and high fertilization. However, the cellulolytic enzymes are not the rate determining step in rooibos tea decomposition, since no direct linear relationship between them and rooibos decomposition was detected. Even after 12 months no direct relationships appeared and interactions between K_m of CBH and of CTH became more important. Here, it is suggested to include oxidative enzyme activities as well as non-enzymatic reactions such as Fenton reactions (Hammel et al. 2002) in future studies. In contrast, in the cellulose rich green tea (Duddigan et al. 2020, Keuskamp et al. 2013) the V_{max} value of BG was highly correlated with the V_{max} of CBH and of CTH. But again, at sites where microbes produce higher amounts of enzymes involved in the degradation of the cellulose chain, the decomposition rate of the green tea was not highest. Higher decomposition rates occurred at sites with higher K_m values of BG and CBH and thus a microbial community able to produce more substrate for the terminal degradation of cellulose. After 12 months this pattern changed and lower K_m values of CBH as well as high V_{max} of BG and CTH increased green tea mass loss. Thus, land-use intensity shapes the mechanisms driving the individual decomposition of high-quality litter (green tea) and low-quality litter (rooibos tea) in different ways. This might be due to changes in microbial community structure most likely induced by land-use intensity. The microbiome study of Daebeler et al. (2022) identified five microbial orders which might act as indicator species, because they exclusively either colonised green (Enterobacteriales, Hypocreales) or rooibos tea (Cythophagales, Rhizobiales, Helotiales) during three months of decomposition. However, this topic was not investigated in detail in our study and deserves further attention.

4.2 Drivers of overall litter decomposition in the early- and mid-stage

Despite different enzymatic interactions in green and rooibos tea (chapter 4.1.3) it was possible to identify those with contributions to the mass loss of green as well as rooibos tea (overall litter decomposition). First of all, tea type, and thus litter quality, was the main driver of overall litter decomposition in the early- and mid-stage with higher decomposition of high-quality litter (green tea). This was not surprising and fits very well to studies using the tea bag approach (Djukic et al. 2018; Houben et al. 2018; Ochoa-Hueso et al. 2020; Pioli et al. 2020; Wang et al. 2019) as well as to studies using the classical litterbag approach with plant material of different origin like litter of deciduous versus coniferous tree species (Krishna and Mohan, 2017; Rawat

et al. 2020), grass versus fern litter (Potthast et al. 2010) or grassland root litter versus forest root litter (Solly et al. 2014). Early stage litter decomposition of green as well as of rooibos tea significantly increased with increasing land-use intensity index (summarizing mowing, fertilization and grazing intensity) indicating that high land-use intensity promotes initial decomposition rates. This direct relationship disappeared after 12 months of decomposition. Also, Solly et al. (2014) did not detect a direct relationship between the LUI and the mass loss of on-site collected fine roots after 12 months of decomposition on the same grassland plots (average root mass loss 20%, $n = 50$). They concluded that land-use intensity indirectly influenced root decomposition by changing the quality of root litter (lignin/N ratio).

Interestingly, in our study in the early phase (three months) the decomposition of green as well as of rooibos tea was significantly higher in plots with a high mowing intensity during the last 10 years. Running the same model with fertilization instead of mowing turned out to be non-significant, although mowing and fertilization intensity are highly correlated in the studied grasslands. This might be explained by the fact that cutting back the above-ground biomass more frequently forces the plants to produce regularly new biomass, above- as well as below-ground (Dickinson and Polwart, 1982). Especially in the uppermost 15 cm of the soil long-term mowing significantly increased the belowground net primary production over years (Xu et al. 2014). Increases in root biomass and in root exudation in turn, as well as more frequent changes in root exudate patterns, might favour growth of soil microorganisms (Eisenhauer et al. 2017). As a result, the plants and microorganisms have an increased demand for nutrients. Since many nutrients are present in the litter, it is conceivable that microorganisms and partly plants invest into the production of a broad suite of extracellular enzymes, which will break down the organic material to cover their needs (Wallenstein and Burns, 2011). Gilmullina et al. (2020) reported that mowing even enhanced soil extracellular enzyme production per microbial biomass unit when compared to grazing. This fits well to the fact that V_{\max} values of all enzymes (enzyme dim. 1) in the tea litter drive decomposition in our model. Soil dimension 2, that is mainly soil content of fungal biomass and nitrogen in microbial biomass, was negatively correlated with overall litter decomposition. In this context the findings of Tláskal et al. (2016) are interesting. They reported that after two months of *Quercus petraea* litter decomposition not only the amount of fungal ergosterol was highest but also the abundance of OTUs belonging to bacteria which are able to decompose fungal mycelia. Tláskal et al. (2016) conclude that in the early-stage of decomposition a large part of the bacterial community did not invest energy into the breakdown of plant polymers but uses fungal necromass as C and N source. Thus, mining for

N from tea litter might be not that important at sites with a higher level of microbial biomass N and/or other available N sources.

In our model of overall mid-stage litter decomposition grazing intensity in the past 11 years becomes an important land-use characteristic. High grazing intensity reduced overall decomposition. Already Shariff et al. (1994) found lower litter decomposition rates on heavily grazed areas compared to moderately grazed areas. Olofsson and Oksanen (2002) reported higher shrub litter decomposition on lightly grazed areas than on heavily grazed areas too, and argued that some plants attacked by grazing produce repellents which could reduce decomposition rates. Substances that inhibit litter decomposition can be released into the soil via the plant roots (Hobbie, 1992). Since tea bags contain litter of shrubs (*Camellia sinensis*, *Aspalanthus linearis*) and were placed in the upper 5 cm of densely rooted topsoil, this mechanism might contribute to the lower decomposition in highly grazed plots. Our results showed that after 12 months individual enzymes (V_{\max} of CTH and PH, K_m of CBH) are important to explain overall litter decomposition. The importance of the potential enzyme activity of PH might be related to P-limitation in some of our grassland soils as discussed above. At sites where microorganisms produce more efficient CBH isoenzymes, e.g. with lower K_m values, faster overall litter decomposition was observed. The fact that V_{\max} of the endocellulase CTH is an important variable driving mid-stage decomposition might be explained by enhanced decomposition of cellulose between month three and 12. Snajdr et al. (2011) reported for *Quercus petraea* leaves a shift towards cellulose degradation after four months with highest activities of endocellulase, endoxylanase and NAG after 12 months. In the first four months a fast removal of hemicelluloses occurred associated with highest activities of BG, CBH and xylanase (Snajdr et al. 2011). It is recommended to combine in future studies tea bags and enzyme kinetics with measures on litter chemistry and microbial community structure to further improve mechanistic understanding.

5 Conclusion

Tea bags containing either green tea or rooibos tea proved to be a suitable indicator to assess land-use effects on overall litter decomposition in real-world grassland ecosystems particularly in the early-stage (three months), since direct correlations with LUI are obvious. Linear mixed models helped to identify those LUI components which mainly contributed to the explanation of overall litter mass loss. That is mowing intensity after three months and grazing intensity after 12 months. To evaluate mid-stage decomposition (12 months) it is recommended to

combine the tea bag method with measurements of enzyme kinetics inside the tea litter. Individual enzyme properties (V_{\max} of CTH and PH, K_m of CBH) became more important in explaining overall litter decomposition over time compared to the early-phase where the potential activity of all enzymes together enhanced litter mass loss. The enzyme kinetics can inform about rate-limiting processes such as acquisition of phosphate (V_{\max} PH) and cellulose degradation (V_{\max} of CTH, K_m of CBH). Interactions between V_{\max} and K_m values of cellulose degrading enzymes show distinct temporal dynamics in rooibos and green tea. These different interactions might become more pronounced in the later stage of decomposition (> one year) and deserve further assessment, e.g. by designing studies for structural equation modelling including measures on microbial community structure, tea chemistry and oxidative enzyme activities

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Data availability

This work is based on data elaborated by the ESCAPE II project of the Biodiversity Exploratories program (DFG Priority Program 1374). The datasets are publicly available in the Biodiversity Exploratories Information System (<http://doi.org/10.17616/R32P9Q>). Dataset IDs of soil variables: 14686, 18787, 19286, 20251, 20447, 20447, 20037, 19853. However, to give data owners and collectors time to perform their analysis the Biodiversity Exploratories' data and publication policy includes by default an embargo period of three years from the end of data collection/data assembly which applies to the remaining datasets (IDs: 26966, 25946, 25166, 21049). These datasets will be made publicly available via the same data repository.

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model) and 2006 to 2017 (12 month model) (Blüthgen et al., 2012, Vogt et al., 2019). *Soil dim.1* and *soil dim.2* represent the first and second axis of a dimensional extraction using PCA. *Soil dim.1* contains information on the nutrient situation and *soil dim.2* on the soil microbial conditions. The variables *enzyme dim.1* and *enzyme dim.2* also derive from another PCA, where *enzyme dim.1* comprises the V_{max} values and *enzyme dim.2* the K_m values of the five investigated EHEs. Since a dimensional reduction was not possible for the 12-month data, the individual enzymes were included in the model (K_m of cellobiohydrolase ($CBH K_m$), V_{max} of celotriohydrolase ($CTH V_{max}$), V_{max} of phosphatase ($PH V_{max}$)).

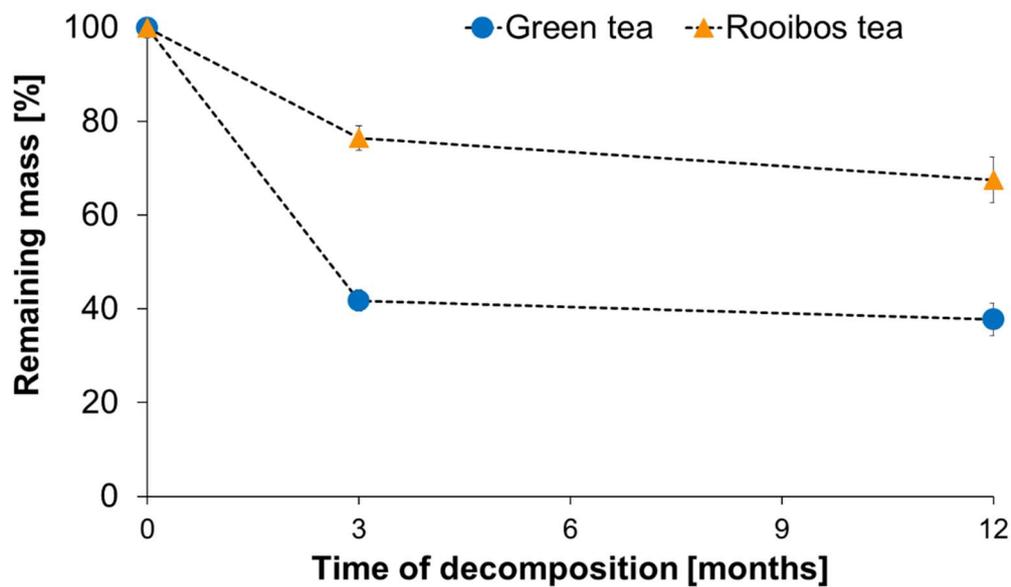


Fig. 1: **Tea mass loss over time.** Remaining tea mass [%] of green tea (open circles) and rooibos tea (filled triangles) after three- and 12-months decomposition in the topsoil of 23 grassland sites (mean values, bars represent standard deviation).

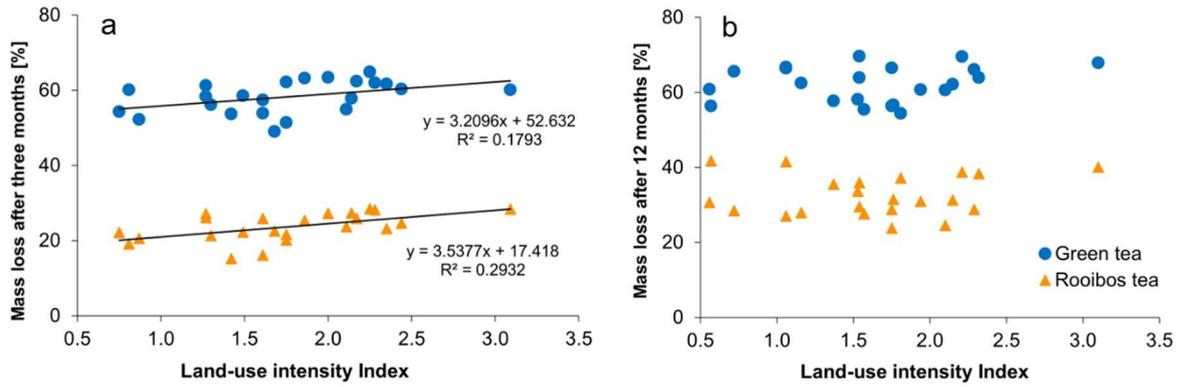


Fig. 2: **Mass loss versus land-use intensity.** Mass loss [%] of green tea (open circles) and rooibos tea (filled triangles) after three months (a) and 12 months (b) in relation to the land-use intensity index of the respective year ((a) 2016 and (b) 2017). Significant correlations (Pearson, $p < 0.01$) after three months ($n = 46$) and non-significant relationships ($p > 0.05$) between tea mass loss and LUI after 12 months ($n = 44$) are shown.

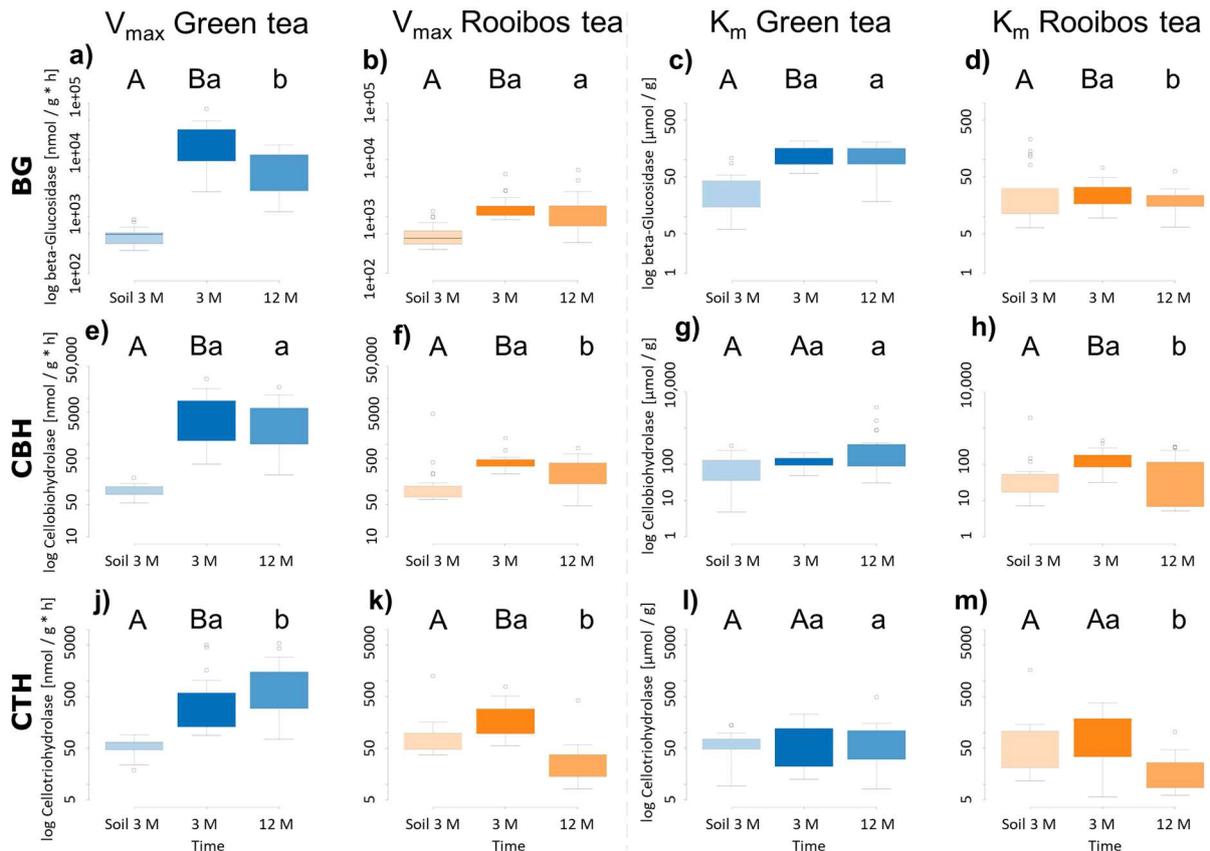


Fig. 3: **C enzyme kinetics.** V_{max} (a, c) and K_m (b, d) values of β -glucosidase (BG), V_{max} (e, g) and K_m (f, h) of cellobiohydrolase (CBH), and V_{max} (j, l) and K_m (k, m) of cellotriohydrolase (CTH) in soil and tea bags after three months ($n=23$) and after 12 months ($n=22$). Different capital letters indicate significant differences between soil and tea bags after three months.

Different small letters indicate significant differences between tea bags after three and 12 months. Significance was tested with Wilcoxon signed-rank test ($p < 0.05$). Circles above and below box plots show outlier-suspected values.

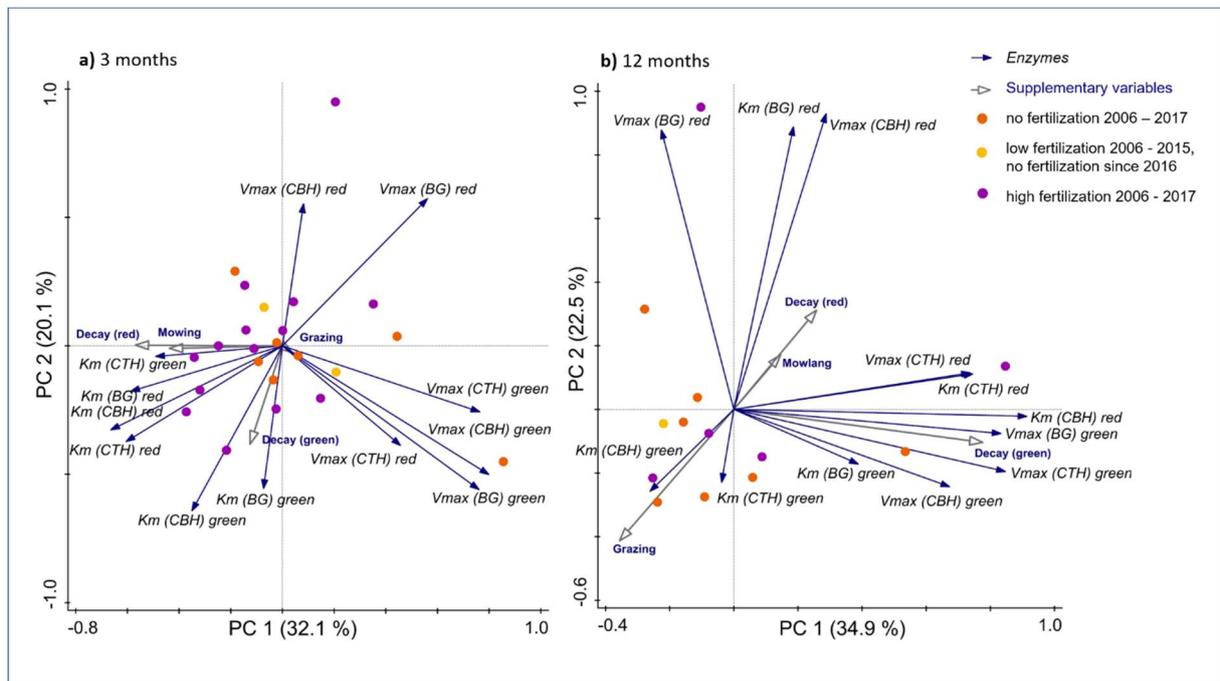


Fig. 4: Principal component analysis (PCA) of C enzyme kinetic patterns. PCA of the V_{\max} and K_m values of the carbon cycle enzymes (BG, CBH, CTH) in litter of green or rooibos tea after a) three months decomposition and b) 12 months decomposition in the topsoil of 23 grassland sites. Grazing and mowing intensity during the last 10 to 11 years, respectively, as well as mass loss of green tea (decay green) and rooibos tea (decay red) are shown as supplementary variables. Orange circles are plots with no fertilization, yellow circles are plots with low fertilization and purple circles are plots with high fertilization.

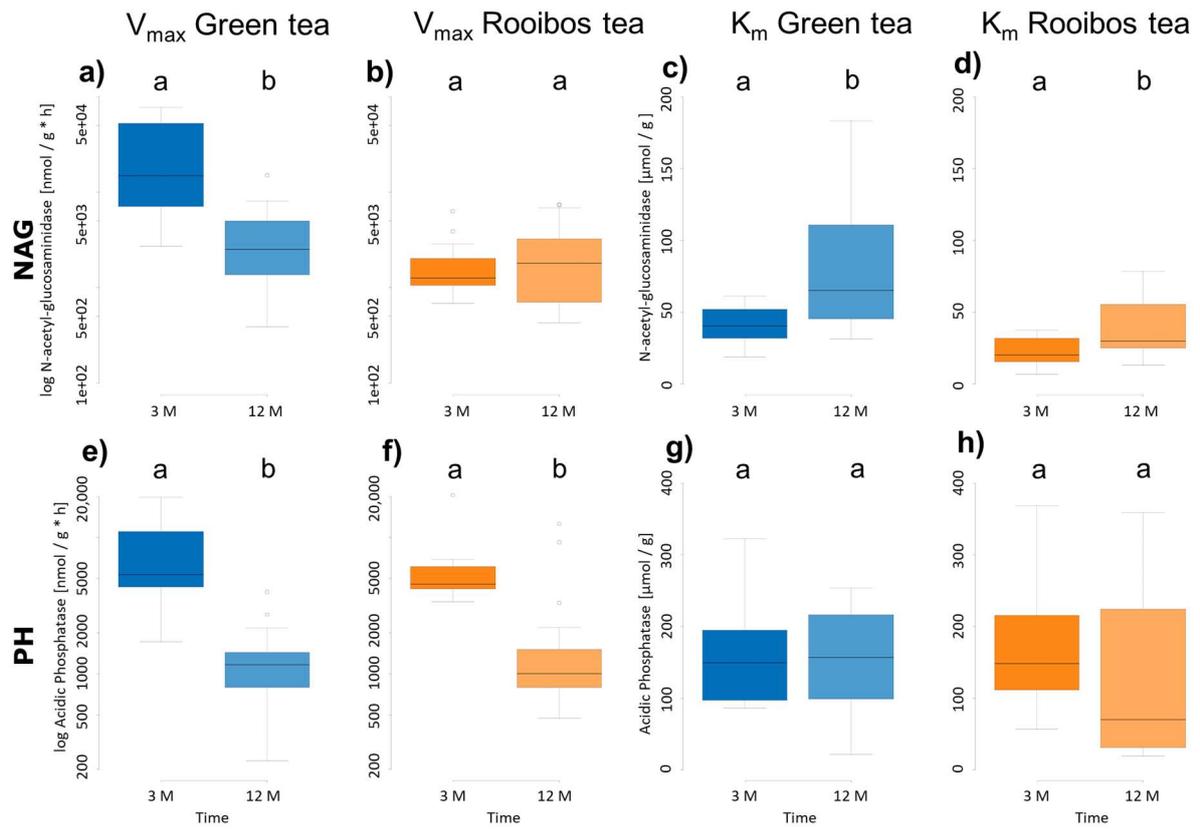


Fig. 5: **N and P enzyme kinetics.** V_{max} (a, c) and K_m (b, d) values of 1,4- β -N-acetylglucosaminidase and V_{max} (e, g) and K_m (f, h) phosphatase in tea bags after three months (n=23) and after 12 months (n=22). Different small letters indicate significant differences between tea bags after three and 12 months after Wilcoxon signed-rank test ($p < 0.05$). Circles above and below box plots show outlier-suspected values.