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Max-Planck-Institut
für Biogeochemie

**Bachelor Thesis**

# The relation between carbon assimilation in beeches and extracellular soil enzyme activities in a mesocosm experiment


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## Abstract

Increasing atmospheric $CO_2$ levels alter the global ecosystem in many ways. Soils are affected by climate change as the intercept of atmosphere, hydrosphere and lithosphere. Plants increase photosynthesis rates due to elevated $CO_2$. Greater growth induced by a higher supply of carbon causes a depletion of nutrients in soils. Through a higher allocation of sugars belowground by the plants, the microbial biomass in soils can increase. The microbial community, composed of bacteria and fungi, forming symbiosis with plants and also plant roots release extracellular enzymes to decompose soil organic matter and mineralize essential nutrients. These extracellular enzymes are used as soil quality indicators because of their sensitive response to biological changes in the soil system and also as a proxy to assess the nutrient demand of plants and microbes. In this study, the effect of elevated $CO_2$ in the atmosphere to extracellular soil enzymes in planted and bare environments has been examined. For that, I sampled soil of the QUINCY mesocosm experiment and determined the extracellular enzyme activity of four hydrolases (β-glucosidase, N-acetyl-β-D-glucosaminidase, sulfatase, phosphatase) and two oxireductases (peroxidase, phenoloxidase) in four different treatments (elevated $CO_2$, tree; elevated $CO_2$, bare soil; ambient $CO_2$ tree; ambient $CO_2$ bare soil). No statistical significance except for peroxidase activity occurred. However, in the planted environments all enzyme activities responded to $eCO_2$ in terms of increased activity, except for N-acetyl-β-D-glucosaminidase activity, which decreased. The enzyme activities in the bare soils showed no consistent pattern.

# Contents

# List of abbreviations

| | |
|---|---|
| C | carbon |
| N | nitrogen |
| P | phosphor |
| S | sulfur |
| Pg | Peta gram ($10^{15}$) |
| ppm | parts per million |
| $CO_2$ | carbon dioxide |
| $eCO_2$ | elevated $CO_2$ |
| $aCO_2$ | ambient $CO_2$ |
| GPP | gross primary production |
| NPP | net primary production |
| SOM | soil organic matter |
| FOM | fresh organic matter |
| GLU | β-glucosidase |
| N-AC | N-acetyl-β-D-glucosaminidase |
| SULF | Arylsulfatase |
| PHOS | Acid phosphatase |
| PER | Peroxidase |
| PHEN | Phenoloxidase |
| EC | enzyme commission number |
| $C_{mic}$ | microbial biomass [µg/g] |
| TB | dry substance |
| CV | Coefficient of variation |

# List of figures

# List of tables

# 1 Introduction

The thin skin of the earth provides the basis for life on this planet. Both in terrestrial and in aquatic systems soils are essential for numerous species. It is the interface between atmosphere, lithosphere and hydrosphere. Here, diverse exchange processes between the spheres take place. All nutrient cycles cross the soil systems and provide essentials for organisms living in and on soils (Blume et al., 2010b). Plants grow on them, in cooperation and in symbiosis with microbes and higher animals feed on the former. Because of the great importance of this compartment this study was realized.

Since the industrial revolution the ecological balance of this compartment is endangered (Smithson, 2002). The processes of the ecosphere are increasingly altered by anthropogenic global change. Anthropogenic global change contains all changes in the environment caused by human activity, such as sealing of surface, land use changes and generation of energy using fossil fuels. This induces again the climate change, which is caused by an accumulation of greenhouse gases in the atmosphere, produced to a high degree by fossil fuel combustion. Population growth, land use changes and globalization are the main reasons for a rising quantity of greenhouse gases in the atmosphere (IPCC, 2013). The accumulation of greenhouse gases in the atmosphere causes an increasing radiative forcing, thus an ascending temperature through the conversion of short waves to long waves of the sunlight (Lal, 2004; Smithson, 2002). One of the most important greenhouse gases is $CO_2$ (IPCC, 2013). The increase of $CO_2$ of 43% to 400 ppm (2015, Mauna Loa) since 1750 is crucial for the effects of climate change (Foley et al., 2005). Global change has caused a rise in global mean temperature of 0.85 °C in 2012, which causes melting of sea ice, which is again responsible for a higher sea level (Collins et al., 2013).

This increase of $CO_2$ enlarges the pools of carbon on the planet. On a global scale, there are five inter-connected carbon (C) pools. The largest is the ocean containing ca. 40000 Pg C, second comes the geologic and third the pedologic pools (1500-2400 Pg C) followed by biosphere (450-650 Pg C) and atmosphere (ca. 590 Pg C) (Ciais et al., 2013; Lal, 2004). Fossil fuel combustion, respiration, fires and land use changes release 128.7 Pg C per year to the atmosphere, though the increase is extenuated, because 60% of global $CO_2$ emissions are buffered. Half of the buffered C is taken up by oceans and the other half is fixed soils and plants mainly by photosynthesis (Ciais et al., 2013). The pools of the C cycle of terrestrial ecosystems are closely connected in pedosphere, atmosphere and biosphere through photosynthetic activity of plants (Long et al., 2004; Schimel, 1995). Thus, forests play the major role in carbon buffering in terrestrial ecosystems. Overall, forests store $861 \pm 66$ Pg C, growing by $2.4 \pm 0.4$ Pg C per year, which arranges on the one hand a C allocation to the pedosphere and on the other hand an increasing C storage in the biosphere (Pan et al., 2011). Of the total carbon pool in forests, 69% of the carbon is stored in soils while 31% is fastened in plants (Dixon et al., 1994).

Today, plants face an atmospheric carbon concentration that they have never experienced during the last 26 million years (Long et al., 2004). One response of plants to elevated $CO_2$ is an increased photosynthetic activity (Drake et al., 1997; Long et al., 2004), but the high C availability also induces numerous other physiological shifts, such as a decrease in stomatal conductance and transpiration and an increase of respiration (Drake et al., 1997). The reaction of photosynthesis converts $CO_2$ and water with energy from light to oxygen and sugar. Thus, elevated $CO_2$ concentrations supply a higher reactant offer for plants, which increases the gross primary production (GPP) and the buffering effect of forests (Ainsworth and Long, 2005; Melillo et al., 1993). Autotrophic respiration loses part of these C gains, expressed by the net primary production (NPP). The increased photosynthesis delivers nevertheless C, which can also be allocated to plant growth (Waring et al., 1998). This so-called $CO_2$ fertilization effect leads to C allocation aboveground and belowground in ecosystems (Ainsworth and Long, 2005). Plant allocations of C to roots and root activities are often seen, so the greater plant growth happens underground, especially, if soil resources such as water or nutrients are limiting (Schlesinger and Andrews, 2000). A higher priming effect is predicted due to an allocation of C belowground and thus an increased biomass offer in the soils. Hence, a higher abundance and activity of microbes is predicted and consequently a higher respiration. The consumption of C appears higher than the belowground allocation, thus a decrease in soil C storage can be found (Fontaine et al., 2003; Kuzyakov, 2010). Also a stimulation of microbial biomass, thus an immobilization of soil N is expected. Soil microbes influence the land - atmosphere nutrient exchange through their metabolic activity. In the example of C, they both induce an accumulation of C in soils by uptake and also deplete the C pool through respiration (Bardgett et al., 2008).

Biomass from plants, transformed by microbes and soil fauna form the largest source for the soil organic matter (SOM). SOM is the largest determinant in C and nutrient cycles and represents the main nutrient source for plant growth (Fontaine et al., 2003). The bioavailability of C is important because C delivers the energy for enzyme production and growth. SOM mineralization is stimulated by the addition of fresh organic matter (FOM) to the SOM pool. In the pool of SOM the priming effect occurs most commonly, because of the increasing supply of energy and nutrients in SOM and thus increasing respiration (Fontaine et al., 2003; Kuzyakov, 2010).

Greater photosynthesis rates often induce nutrient limitations because of greater uptake rates of nutrients from soil to plant for growth (Oren et al., 2001). If nutrient uptake increases, plants sequester more nutrients, which in turn can result in nutrient depletion of soils (Luo et al., 2004). The recycling of essential nutrients occurs by dead biomass, thus the nutrients are fixed in complex organic structures in biomass and litter, unavailable for plants (Chung et al., 2007). However, SOM mineralization can be fostered by plant mechanisms, such as symbiosis with SOM degrading microorganisms or releasing extracellular enzymes (Dakora and Phillips, 2002).

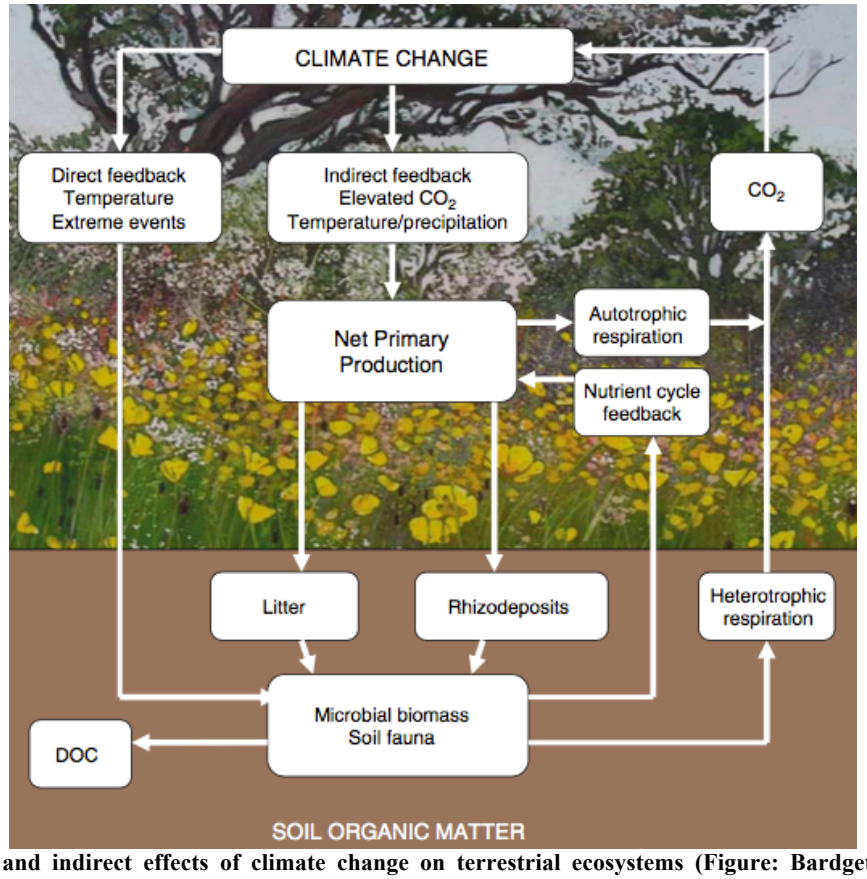


**Figure 1: Direct and indirect effects of climate change on terrestrial ecosystems (Figure: Bardgett et al., 2008). Increasing microbial communities due to elevated $CO_2$ result a higher net primary production, which increases the production of litter and rhizodeposition. Both influence microbial biomass and soil fauna, which are responsible for decomposition processes and heterotrophic respiration. Direct feedbacks are not considered in this study.**

The key process to transform these complex structures into bioavailable products is microbial SOM decomposition (Sinsabaugh et al., 2008). Within this process, enzymes are very important protagonists (Marx et al., 2001) because they catalyze all biochemical reactions, being part of every nutrient cycle. Generally, enzymes are proteins, active microbial biomass and roots in soils release extra- and intracellular enzymes to their environment for mineralizing these nutrients (Dakora and Phillips, 2002). Intracellular enzymes are proteins inside the cells, indicating the direct activity of the microbial biomass. Dead or living cells release extracellular enzymes, which can adsorb on clay or humus particles (Bandick and Dick, 1999; Blume et al., 2010a).

A symbiosis between plants and microorganisms is very common, mostly between plants and mycorrhiza. Almost all higher plants like beeches, oaks and firs are dependent to the finely mycorrhizal network in their roots, which provides nutrients and water for the trees, while the plants provide photosynthesis products for the fungi (Blume et al. 2010a). Thus, the abundance and activity of soil microbes is highest if directly influenced by the presence of live roots, e.g. in the area known as rhizosphere. The higher flux of C to the roots, caused by elevated $CO_2$ in the atmosphere, benefits both, the plant and the symbionts, increasing the rhizosphere and mycorrhizal growth, thus a higher

soil volume is developed and the water and nutrient supply is enhanced (Ainsworth and Long, 2005). Also, heterotrophic microbes proliferate through root exudation of sugars, organic acids and amino acids (Dakora and Phillips, 2002). The rhizosphere releases extracellular enzymes to mineralize the nutrients and make them bioavailable (Blume et al., 2010a). These protein complexes are very sensitive to biological changes in soils and the plant-soil system, therefore the enzymes are also used as soil quality indicators (Bandick and Dick, 1999; Dorodnikov et al., 2009).

With a higher offer of $CO_2$ plants assimilate more C in the rhizosphere, thus they are able to invest the energy-intensive production of enzymes (Meier et al., 2015). The depleting effect of nutrients through greater plant growth induces a higher importance of SOM degrading enzymes, thus the activity of enzymes in nutrient cycles appears stimulated (Allison and Vitousek, 2005; Burns et al., 2013). However, different reactions of enzyme activity with rising $CO_2$ between diverse sites were detected (Burns et al., 2013; Carney et al., 2007; Ochoa-Hueso et al., 2017). Hence, a stimulation of enzyme activities are not only dependent on C as energy supply, but also on nutrients, pH and soil moisture (Chung et al., 2007; Henry et al., 2005).

The pH of soils affects the composition of the active microbial biomass in soils. E.g. roots and fungi prefer acidic environments, while bacteria are commonly discovered in alkaline surroundings (Dakora and Phillips, 2002). The pH also influences the solubility of every nutrient and trace element like zinc, iron and copper (Blume et al., 2010a). Thus, pH accounts the production of SOM degrading enzymes via availability of trace elements, e.g. phenoloxidases have four copper atoms in their reaction center for the reduction of molecules, thus the production of this enzyme depends on the offer of available copper (Sinsabaugh, 2010), and also due to the composition of the microbial biomass (Dakora and Phillips, 2002). Another important factor, controlling microbial biomass and activity is soil moisture. Microorganisms live in a water film around minerals. Too much water means less oxygen for respiration, too less water means no living space for the microbes (Blume et al., 2010a). Additionally, extracellular enzyme activities react to different moistures. In dry soils the enzyme activity is lowest, while with intermediate moisture the conditions for enzyme activity is best (Guenet et al., 2012).

In this work, I examine the effect of increased $CO_2$ assimilation of plants induced by elevated atmospheric $CO_2$ ($eCO_2$) concentrations on soil enzyme activities. Therefore, I assayed six different extracellular enzymes (Table 1). Enzymes are especially important to make nutrients, bound in different complex organic structures in the SOM available for plant. Every enzyme works substrate specific and mineralizes specific chemical bounds (Allison and Vitousek, 2005). Four of the assayed enzymes are hydrolases (EC group 3), which split ester, ether, glycosides, peptides, acidanhydrides and C-C bounds by hydrolysing them. In our case the enzymes only attack esters (EC 3.1) or glycosides (EC 3.2). The other two enzymes belong to group 1, which are oxireductases. This group of enzymes always reduces bonds by using part of it as donor. In our case, peroxidase acts on peroxide as acceptor (EC 1.11) and phenoloxidase acts on diphenols as acceptor (EC 1.10) (NC-IUBMB).

**Table 1: List of assayed enzymes**

| Nomenclature | Enzyme |
|---|---|
| EC 3.2.1.21 | β-Glucosidase (GLU) |
| EC 3.2.1.14 | N-Acetyl-β-D-glucosaminidase (N-AC) |
| EC 3.1.6.1 | Arylsulfatase (SULF) |
| EC 3.1.3.1 | Acid phosphatase (PHOS) |
| EC 1.11.1.7 | Peroxidase (PER) |
| EC 1.10.3.2 | Phenoloxidase (PHEN) |

In the carbon cycle β-glucosidase, Per- and Phenoloxidases are important operators. Microbes, plants and fungi produce GLU. GLU, one of the four assayed hydrolases cut carbohydrates by hydrolyzing glycosides and deliver energy for the microbes. It attacks the β-bound of the molecules. PHEN synthesize lignin and other secondary substances like melanin. Fungi and microbes produce PHEN to mitigate the toxicity of phenolic molecules and metal ions. Laccases are the best researched group of phenoloxidases. It has four copper atoms in its reaction center, so it needs four electrons for reduction. PER are released by white rot fungi and soft rot fungi through excretes or lysis. There is a difference between manganese (Mn) peroxidase and lignin peroxidases. Lignin peroxidases oxidize lignin using hydrogen peroxide as electron acceptor lignin while Mn peroxidases attack lignin indirectly by producing diffusive $Mn^{3+}$. (Eivazi and Tabatabai, 1988; Parham and Deng, 2000; Sinsabaugh, 2010)

The remaining three hydrolases catalyze processes in the different nutrient cycles. N-AC decomposes chitin and murein and is important for the C and N cycling. Its abundance correlates strongly with the abundance of fungal biomass (DeForest et al., 2004). SULF is an important component of the amino acids cysteine and methionine. To make sulfur compounds available for plants, arylsulfatases hydrolyze sulfate esters and their production depends on the offer of $SO_4^{2-}$ (Kertesz, 2000; Scherer, 2009). Another element that is crucial for plant growth is phosphor. In acid soils, acid PHOS are responsible for hydrolyzing esters and anhydrids of phosphor acids. By mineralizing the phosphor it becomes bioavailable for plants (Eivazi and Tabatabai, 1977).

I tested whether soil enzyme activities benefit from plants grown under $eCO_2$. I also compared enzyme activities between planted and bare soil and tested if the differences in enzyme activities are statistically significant.

Within this thesis, I will test the following hypotheses:

i. Soils of plants grown under $eCO_2$ exhibit higher potential enzyme activities, because if plants assimilate more C photosynthetically, they are able to invest more C to the roots, where the energy-intensive production of enzymes takes place. Also, symbionts that benefit from the higher energy supply are instigated in producing more enzymes.
ii. An increase in simple degrading carbohydrates through root exudates increases the production of GLU. Greater C assimilation results an increase in SOM, thus a stimulation of PER and PHENOL is expected.
iii. Elevated $CO_2$ does not alter enzyme activities in bare soils. Plants are the connection between soils and atmosphere, so $eCO_2$ should not alter the microbial community of bare soils.
iv. Enzyme activities in the initial substrate are same as in the ambient treatment because there was no C fertilization.

# 2 Materials and methods

Within the QUINCY project a mesocosm experiment was set up. Young European beech trees (*Fagus sylvatica* L.) were planted in airtight chambers, four trees in each chamber and grown under controlled conditions, such as light, temperature and soil moisture. Before the experiment started the trees were cut in most similar habitus and potted in carbonate-free soil in spring. The upper part of the chamber was transparent while the lower part was opaque. During the growing season from July to November the plants developed in an artificial atmosphere. Half of the chambers were treated with an ambient $CO_2$ ($aCO_2$) concentration of 390 ppm $CO_2$, the current concentration in atmosphere, while the other half of the chambers were treated with $eCO_2$ fumigations of 560 ppm $CO_2$, which reflect the projected atmospheric concentration for 2020-2060 (Parry et al., 2007).

## 2.1 Sampling and preparation

The soil used in the experiment comes from a managed common beech forest near Meusebach in Thuringia, Germany (N 50°49’19.5’’, E 11°43’19.5’’). The forest is located on a slope and is a rather shallow soil (personal communication L. Eder). The parent material is lower new red sandstone, precisely the Bernburg-formation from the Lower Trias. The soil is characterized as podsolic cambisol (Bodenatlas). The soil was collected from the upper 20 cm in February 2016 after removal of the organic layers.

After collection the soil was sieved with a 6 mm sieve to remove the residues of the beeches and homogenized for comparable conditions. After two months ca. three year old beeches were potted in ca. 10.5 kg soil. In the early summer (end of May) the trees were set in the chambers, which were closed on 1$^{st}$ of July 2016 (midsummer). $CO_2$ fumigation was executed for 19 weeks, followed by a

destructive harvest. During harvesting all soils were sieved with a 4 mm sieve within 24 hours after collection. Soils used for enzyme assays were stored at -20 °C, and soil used for pH determinations were dried at 40 °C.

## 2.2 pH measurement

The pH-value is defined by the negative decadic logarithm of the hydrogen ions (eq. 1) (Hölting and Coldewey, 2013).

$$pH = -\log a(H^+) \quad (1)$$

About ten grams (10 g ± 0.10 g) of the sieved and air dried soil were weighed in 100 ml polyethylene bottles and 25 ml extraction solution (1 M potassium chloride (KCl), J. T. Baker) was added. Afterwards the samples were stirred in an overhead shaker (GFL®, 3040) for one hour to facilitate an exchange between hydrogen ions from the soil with potassium ions from the solution. Subsequently the suspensions were left upright for one hour to allow soil particles to sediment to the bottom. The supernatant was decanted in beakers. The pH measurements were conducted with the WTW-539 analyzer in combination with a SenTix61-electrode (3 M KCl, WTW).

## 2.3 Determination of soil moisture

Also for a better comparison of the enzyme analyses, the relative differences of the soil-compounds must be determined.

Therefore, five grams (5 ± 0.9 g) of each soil were weighed in beakers and dried at 105 °C for 48 hours. After it cooled down in the exsiccator the dry mass was determined. Equation (2) shows the calculation of the dry mass of the soils.

$$dry\ substance\ (TB)\ [\%] = \frac{dried\ soil\ [g] - wet\ soil\ [g]}{wet\ soil\ [g]} \times 100 \quad (2)$$

## 2.4 Measurement of extracellular enzyme activities

For the measurement of the six different enzyme activities, I used two different measurement-methods, one for the hydrolases and one for the oxireductases. In both principles I created optimum conditions for the enzymes in pH, temperature and substrate availability. Therefor, different substrate solutions for each enzyme were used (Table 2) and the reactions were facilitated by buffers for the standardized method (Table 3) (Marx et al., 2001). All work was carried out under sterile conditions.

**Table 2: Substrates of each enzyme**

| Enzyme | Substrate |
|---|---|
| β-Glucosidase (GLU) | 4-Methylumbelliferyl-β-D-Glucoside |
| N-Acetyl-β-D-Glucosaminidase (N-AC) | 4-Methylumbelliferyl-N-Acetyl-β-Glucosaminide |
| Arylsulfatase (SULF) | 4-Methylumbelliferyl-Sulfate |
| Acid Phosphatase (PHOS) | 4-Methylumbelliferyl-Phosphate |
| Peroxidase (PER) | Tetramethylbenizidin solution with hydrogen peroxide |
| Phenoloxidase (PHEN) | Tetramethylbenizidin solution |

**Table 3: List of buffers and producers**

| Group | Buffer | Producer of chemicals |
|---|---|---|
| Hydrolases | 0,1 M 2-(N-Morpholino)ethansulfonacid | Sigma Aldrich |
| Oxireductases | 50 mM sodium acetate buffer | Merck, Carl Roth GmbH |
| | 205 mM sodium citrat buffer | Carl Roth GmbH |

### 2.4.1 Assay of hydrolases

For the determination of the potential activity of the four hydrolase enzymes I used a microplate fluorimetric assay following Marx et al. (2001). This method benefits from the fluorimetric attribute of Methylumbelliferone (MUF) when a specific enzyme breaks down the chemical bounds of the MUF-substrate (Table 2).

Processing:

For this assay, I added 50 ml of bidistilled to 1 g ± 0.009 g soil and solubilized it with an ultrasonic-disaggregator (Digital Sonifier ®, BRANSON) for one minute and 22 seconds. Under constant stirring 50 µl of the soil suspension was pipetted manually on a microplate. The enzymatic activity of each soil was measured in six replicates per plate (Figure 2a). For the measurement of the phosphatase activity we diluted the suspension 2:1. Subsequently, 50 µl buffer and 100 µl of the particular substrates were added automatically to the wells (freedom eva, TECAN). For calibration purposes, a standard plate with different amounts of MUF standard solution (0, 10, 20, 50, 80, 120 µl) and MES buffer (150, 140, 130, 100, 70, 30 µl) added to the soil suspension, was produced (Figure 2b).

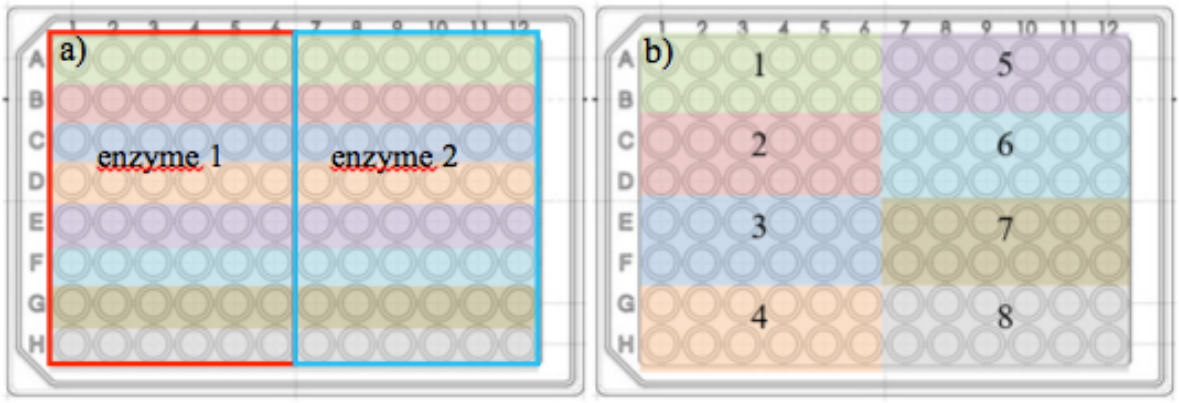


**Figure 2: a) Order of samples and substrates, every color block represents the six replicates of one sample; b) Standard plate, each color is one sample (diagram from J. Heublein, unpublished)**

Measurement:

All plates were pre-incubated at 30 °C with constant shaking for 30 minutes. After 30 minutes the measurement started with an infinite M200 (Tecan) microplate reader and was repeated after 30, 60, 90, 120 and 180 minutes. The reader was connected with the software Tecan i-control 1.5.14.0, in combination with Microsoft Excel 1997.

Principle of measurement:

The Tecan infinite M200 microplate reader first concentrates the light to a specific wavelength using a monochromator and then illuminates the wells through the excitation system. The excitationwavelength was 360 nm (Manual Tecan infinite). The sample emits the light, which a second monochromator detects and separates from the excitation-light. The emission-wavelength was 460 nm (Hesse et al., 2005).

### 2.4.2 Assay of oxireductases

The potential activity of the oxireductases was measured photometrically. After the oxireductases break down the substrate tetramethylbenizidin (TMB), one of the products appears blue by the substrate TMB (Johnsen and Jacobsen, 2008). Peroxidases need additionally hydrogen peroxide for the reaction. The assay followed the principle described by Johnsen and Jacobson (2008), but their implementation was adapted, e.g. they used acetate buffer, while I used potassium acetate buffer. Also instrumentation was different and the preincubation as well as the measurement occurred in Eppendorf tubes (Johnsen and Jacobsen, 2008).

Processing:

I weighed 0.4 g ± 0.009 g soil in beakers and added 50 ml potassium acetate buffer. Then, I also homogenized it just as in 2.4.1 explained. Here, I pipetted 200 µl of the soil suspension in the wells, adding 50 µl potassium acetate buffer for blank samples. For the peroxidases I pipetted 10 µL $H_2O_2$ in

the wells and added afterwards 50 µl of the substrate. To prevent that the substrate is degraded by light, it was stored dark and only added right before the measurement started. For the standards 200 µl potassium actetate buffer, 50 µl substrate and for peroxidase 10 µl $H_2O_2$ was pipetted in one row. The order of soil samples, blanks and standard is shown in figure 3.

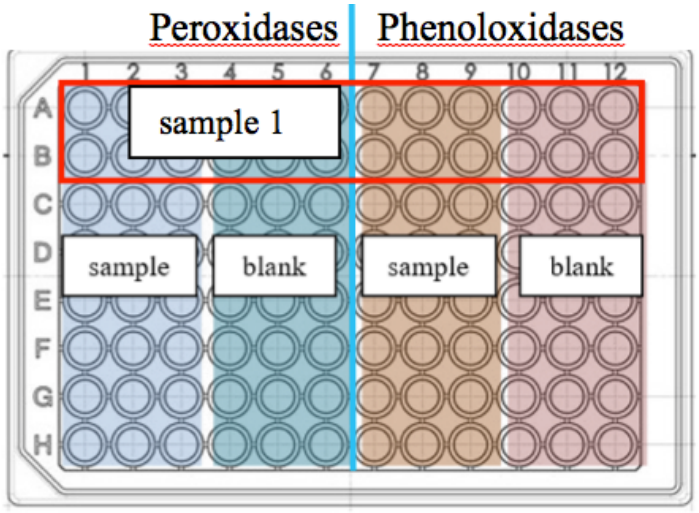


**Figure 3: Layout of the oxireductases, one sample in two rows (diagram from J. Heublein, unpublished)**

Measurement:

Right after finishing the preparations the first measurement started. It was repeated every 15 minutes for one hour. Between measurements the plates were incubated in darkness at 30 °C on shakers. The microplate reader and software are the same as for hydrolases in 2.4.1.

Principle of measurement:

The monochromator-system concentrates the light to the specific wavelength of 630 nm. Different levels of the coloring of the oxidized TMB absorb different wavelength (Manual Tecan infinite). By measuring the intensity of a specific wavelength the percentage of absorbed light can be calculated and the activity of the oxireductases can be determined (Hesse et al., 2005).

## 2.5 Calculation of enzyme activities and statistics

For the calculation of the potential enzyme activity (fluorescence) of the hydrolases the overall slope of the standards (xstd) and the substrate (xsub) concentrations, averaged over the whole measurement period was determined. The mean values of the substrate concentration divided by the mean value of the standard slope, estimated for dry substance (M = mass fresh soil soil; TB = dry substance) yields the mean values of the six replicate measurements of each enzyme (Equation 3) (German et al., 2011).

$$enzyme\ activity\ \left[\frac{nmol}{g\ TB \times h}\right] = \left(\frac{xsub}{xstd} \times \frac{100}{M \times TB}\right) \times 60 \qquad (3)$$

For the calculation of the potential enzyme activity of the oxireductases and also potential enzyme activity the overall gradient of the substrate (xsub) concentrations, averaged over the whole measurement period was determined. The slope of the emission, estimated for dry substance yields the mean values of the six replicate measurements of each enzyme (Equation 4) (German et al., 2011).

$$enzyme\ activity\ \left[\frac{nmol}{g\ TB \times h}\right] = \left(\frac{xsub \times 100}{M \times TB}\right) \times 60 \quad (4)$$

The specific enzyme activity was calculated by dividing the potential enzyme activity per gram soil by microgram microbial biomass per gram dry soil (Equation 5).

$$spec.activity\ \left[\frac{nmol}{g\ TB \times g\ C_{mic} \times h}\right] = \frac{fluorescence}{C_{mic}} \quad (5)$$

The data of $C_{mic}$ was collected by L. Eder (unpublished). One chamber (ES2) was removed because it was an outlier.

Outliers were determined by the coefficient of variation (CV), which is calculated by the standard deviation divided by the mean value of the slopes (Equation 6). When CV among the six replicates was higher than 50% data was identified as outliers and removed.

$$CV\ [\%] = \frac{stddev}{meanslope} \times 100 \quad (6)$$

Further statistical analysis was executed with the software R (R Core Team, 2015). I calculated the mean values and the standard deviation of every treatment and enzyme. Afterwards I tested subsamples for normal distribution by using the Kolmogorov-Smirnov test. To analyze differences in mean values I used the Wilcoxon Rank Sum test if data were not normal distributed or the t-test if the data were normal distributed. Plots were also produced with the software R, using the package ggplot2. All statistical tests and analyses were conducted with a probability of error of $\alpha = 0.05$.

# 3 Results

First, the results from the pH measurement for general characterization of the soils are introduced. Then, I present the effect of $eCO_2$ to soil enzyme activity in planted environments and subsequently the effect of trees to the soil enzyme activity. At last I describe the specific enzyme activity in planted and bare soils. All results are indicated with mean intervals and standard deviation, statistically tested for significance.

## 3.1 pH of the bulk soil

The pH values spread from 3.4 to 3.63, thus the soil was acid (Table 8, Appendix). A slight decrease in pH occurred in planted soils (Figure 4). The pH values in the different treatments ($eCO_2$_tree, $aCO_2$_tree, $eCO_2$_bare, $aCO_2$_bare) appeared not normal distributed (Table 9, Appendix). No significant changes happened in planted soils between different $CO_2$ fumigations ($p = 0.72$), neither in bare soils between the $CO_2$ fumigations ($p = 0.29$). However, within the different treatments significant changes between bare and planted soils occurred. In the ambient $CO_2$ treatment, the decrease from bare ($3.56 \pm 0.04$) to planted ($3.51 \pm 0.04$) soils was significant ($p = 0.009$), in the elevated $CO_2$ treatment the decrease from bare ($3.58 \pm 0.01$) to planted ($3.5 \pm 0.04$) soils was also significant ($p < 0.001$).

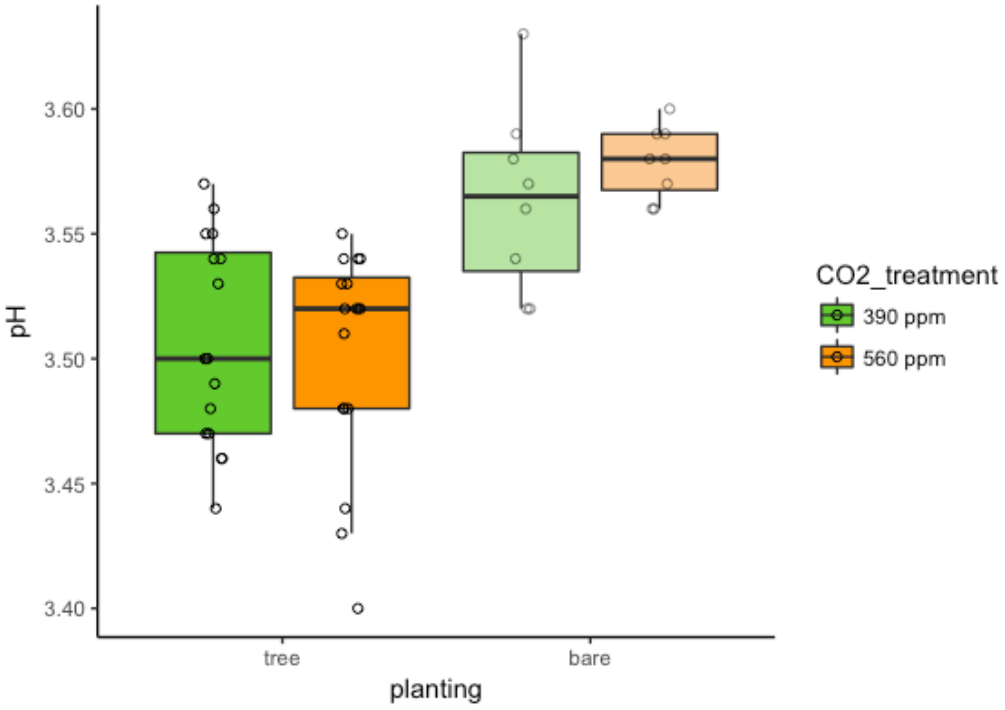


**Figure 4: pH values of all four treatments**

## 3.2 Enzyme activities in the initial substrate

The activity of GLU in the initial substrate was 148.06 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$. N-AC showed an activity of 301.52 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$, while SULF activity appeared with 66.35 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$. PHOS activity was highest again with 2872.62 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$. The oxireductases activity was again lowest with PER 0.66 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$ and PHEN 0.62 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$.

**Table 4: Enzyme activity of the initial substrate nmol*g$^{-1}$ TB*h$^{-1}$ (mean ± standard deviation)**

| GLU | N-AC | SULF | PHOS | PER | PHEN |
|---|---|---|---|---|---|
| **148.06** ± 0.91 | **301.52** ± 9.47 | **66.35** ± 1.71 | **2872.62** ± 181.92 | **0.66** ± 0.02 | **0.62** ± 0.02 |

## 3.3 Effect of the $eCO_2$ treatment to soil enzyme activity in planted environments

Elevated $CO_2$ enrichment in the atmosphere, thus $CO_2$ assimilation in plants resulted a slightly higher enzyme activity for all enzymes, except for N-AC (Table 4).

**Table 5: Mean values with standard deviation of the enzyme activities with two different treatments, ambient and elevated CO2; unit is [nmol * g$^{-1}$ TB*h$^{-1}$]**

| treatment | GLU | N-AC | SULF | PHOS | PER | PHEN |
|---|---|---|---|---|---|---|
| $aCO_2$ | **146.01** ± 38.55 | **218.06** ± 90.92 | **37.21** ± 7.79 | **2011.43** ± 419.04 | **0.64** ± 0.19 | **0.47** ± 0.17 |
| $eCO_2$ | **157.51** ± 42.53 | **209.43** ± 66.72 | **40.13** ± 7.14 | **2755.40** ± 1524.81 | **0.82** ± 0.24 | **0.51** ± 0.3 |

Neither the results of the hydrolases were normal distributed ($p < 0.001$), nor the results of oxireductases ($p < 0.001$) (Table 13, Appendix).

The greatest effect of $eCO_2$ treatment occurred for PHOS (Figure 5 d). The activity in $aCO_2$ treatment resulted in an activity of 2011.43 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$, while $eCO_2$ treatment generated PHOS activities of 2755.40 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$. The difference between the treatments was not significant ($p = 0.17$), due to the great variance.

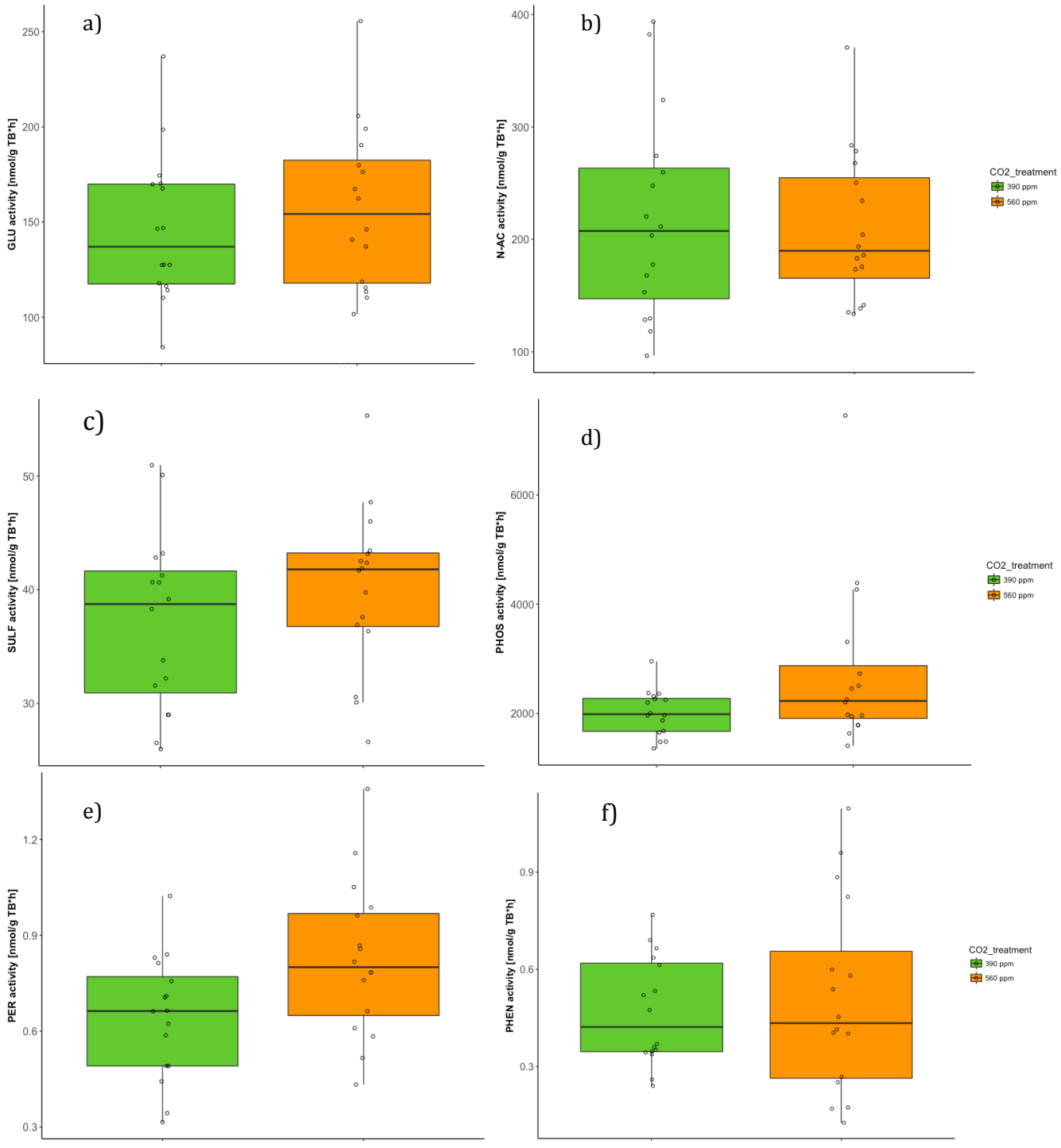


**Figure 5: Potential enzyme activities of all six enzymes in elevated (orange) and ambient (green) $CO_2$ treatment**

Smaller effects appeared with GLU (Figure 5 a). The effect of $eCO_2$ treatment showed a GLU activity of 157.51 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$, with $aCO_2$ insignificant ($p = 0.54$) smaller (146.01 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$ ).

SULF exhibited similar results (Figure 5 c). There was a slight, insignificant ($p = 0.25$) increase of activity with $eCO_2$ treatment from 37.21 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$ to 40.13 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$.

The oxireductases showed very small activities compared to hydrolases. PER demonstrated a slightly higher activity than PHEN (Figure 5 e, f). Within PER, $eCO_2$ treated pots occurred with a higher activity ($aCO_2 = 0.64 \frac{nmol}{g\,TB*h}$, $eCO_2 = 0.82 \frac{nmol}{g\,TB*h}$). The difference between the treatments was significant ($p = 0.04$).

PHEN activity with $eCO_2$ treatment was $0.51 \frac{nmol}{g\,TB*h}$. The achieved activity with $aCO_2$ was $0.47 \frac{nmol}{g\,TB*h}$, so it was an insignificant increase ($p = 0.9$) with almost no effect of $eCO_2$.

A weak negative effect of $eCO_2$ on enzyme activity was observed for N-AC (Figure 5 b). With $eCO_2$ the activity of N-AC decreased very slightly from $218.06 \frac{nmol}{g\,TB*h}$ to $209.43 \frac{nmol}{g\,TB*h}$, but this effect was not significant ($p = 0.96$).

## 3.4 Effect of trees to enzyme activity

In all assays differences between bare and planted soils were detected. The activities were not normal distributed ($p < 0.001$). Furthermore, no coherent effect of trees on the enzyme activities was found, neither within the hydrolases, nor within the oxireductases.

**Table 6: Values of enzyme activities in nmol*g$^{-1}$ TB*h$^{-1}$, mean ± standard deviation, bare soils only.**

| treatment | GLU | N-AC | SULF | PHOS | PER | PHEN |
|---|---|---|---|---|---|---|
| $aCO_2$_bare | **165.56** ± 22.58 | **179.02** ± 23.67 | **43.32** ± 9.55 | **1478.86** ± 269.76 | **0.7** ± 0.13 | **0.35** ± 0.17 |
| $eCO_2$_bare | **128.16** ± 17.93 | **170.91** ± 41.57 | **39.73** ± 8.65 | **1911.07** ± 294.98 | **0.73** ± 0.27 | **0.32** ± 0.17 |

GLU activity of the bare soils in the $aCO_2$ fumigation occurred higher than with trees ($165.56 \frac{nmol}{g\,TB*h}$), but the increase was not significant ($p = 0.15$) (Figure 6 a). Elevated $CO_2$ showed no effect to bare soil enzyme activity. The activity decreased insignificantly ($p = 0.14$) compared to planted soils ($128.15 \frac{nmol}{g\,TB*h}$). The difference between bare soils in ambient and elevated $CO_2$ treatment was significant ($p = 0.005$).

N-AC activity decreased insignificantly without trees in both treatments ($p_e = 0.15$, $p_a = 0.45$) (Figure 6 b). Within the separate treatments, the enzyme activity of the ambient treated soil

(179.02 $\frac{\text{nmol}}{\text{g TB}*\text{h}}$) appeared insignificantly ($p = 0.28$) higher than with elevated $CO_2$ treatment (170.91$\frac{\text{nmol}}{\text{g TB}*\text{h}}$).

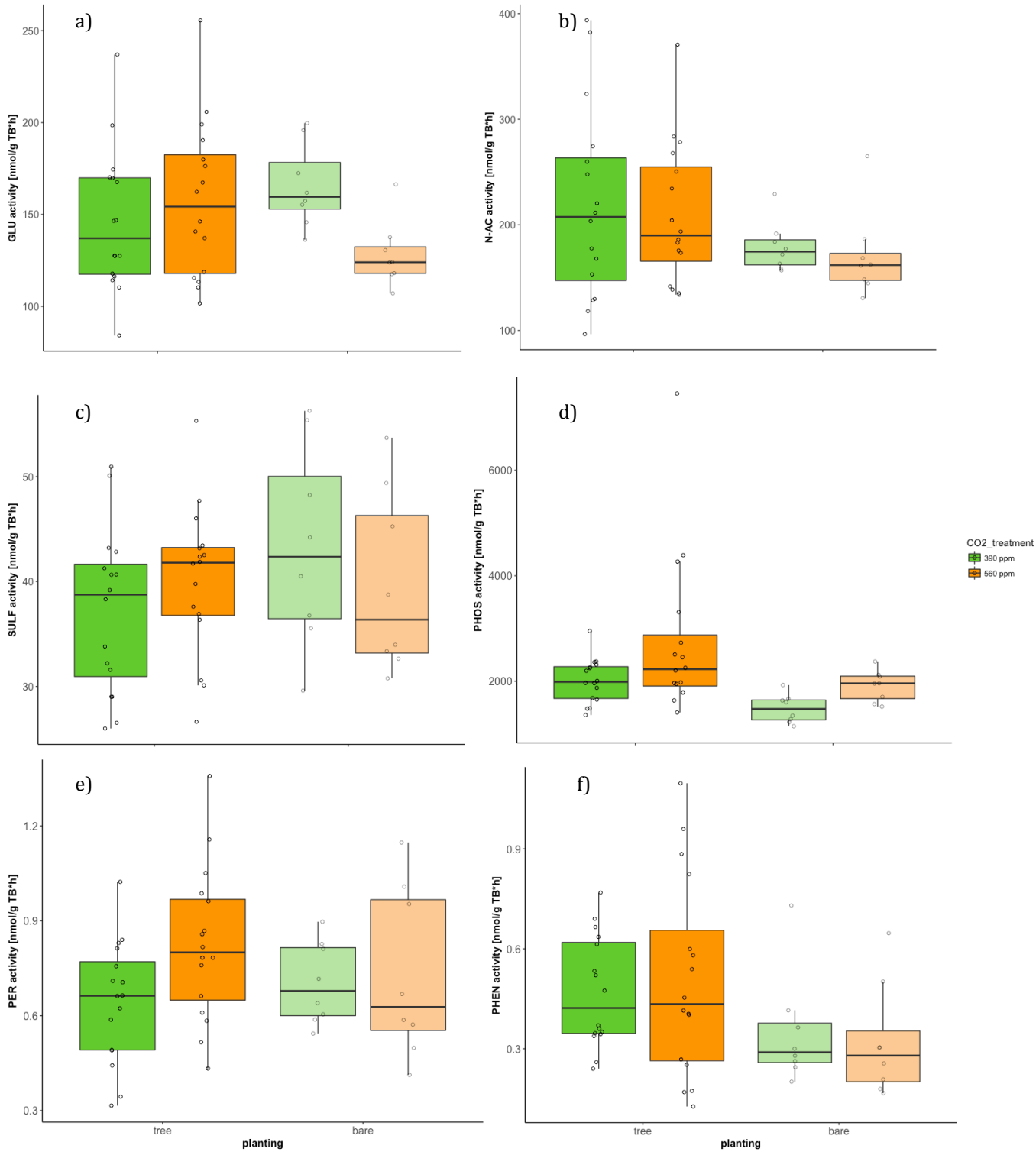


**Figure 6: Potential enzyme activity of all six enzymes in planted and bare soils and elevated (orange) and ambient (green) $CO_2$ fumigation**

The effect of plants with $aCO_2$ treatment increased the activity of SULF (43.32 $\frac{nmol}{g\,TB*h}$) insignificantly ($p = 0.17$ $\frac{nmol}{g\,TB*h}$) (Figure 6 c). In the $eCO_2$ fumigation (39.73 $\frac{nmol}{g\,TB*h}$) a slight decrease ($p = 0.88$) occurred. The decrease from the ambient to the elevated treatment was also insignificant ($p = 0.44$).

PHOS activity decreased without the influence of trees in both treatments (Figure 6 d). With ambient $CO_2$ conditions the activity of bare soil PHOS decreased significantly ($p = 0.002$) to 1478.86 $\frac{nmol}{g\,TB*h}$. This enzyme activity with elevated $CO_2$ fumigation diminished to 1911.1 $\frac{nmol}{g\,TB*h}$, which was an insignificant decrease ($p = 0.11$). In bare soils the difference between the activities with whether ambient or elevated $CO_2$ was significant ($p = 0.015$).

There appeared to be no significant effect ($p = 0.53$) of planting to the activity of PER in the $aCO_2$ treatment (0.7 $\frac{nmol}{g\,TB*h}$) (Figure 6 e). In the $eCO_2$ treatment the activity of PER in bare soils decreased slightly (0.73 $\frac{nmol}{g\,TB*h}$) compared to planted soils ($p = 28$). The difference between bare soils in the two $CO_2$ treatments was very small ($p = 0.88$).

PHEN activities diminished in bare soils, in the $eCO_2$ treatment an activity of 0.32 $\frac{nmol}{g\,TB*h}$ occurred, in $aCO_2$ the activity was 0.35 $\frac{nmol}{g\,TB*h}$ (Figure 6 f). Thus, there were no significant differences between the C treatments ($p = 0.65$), neither between planted and bare soils ($p_e = 0.19$, $p_a = 0.11$).

## 3.5 Specific enzyme activities

The specific enzyme activity underlined the results from 3.1. The specific activities also appeared not normal distributed (Table 16, Appendix). The pattern of the specific enzyme activities in planted soils occurred similar to the absolute enzyme activities, in bare soils the specific activities in ambient $CO_2$ treatments arose clearly higher.

**Table 7: Specific enzyme activities in nmol*g$^{-1}$ TB*g$^{-1}$ $C_{mic}$*h$^{-1}$ with standard deviation.**

| treatment | $GLU_{spec}$ | $N\text{-}AC_{spec}$ | $SULF_{spec}$ | $PHOS_{spec}$ | $PER_{spec}$ | $PHEN_{spec}$ |
|---|---|---|---|---|---|---|
| aCO2_tree | **0.93** ± 0.31 | **1.35** ± 0.57 | **0.24** ± 0.08 | **12.66** ± 3.04 | **0.0042** ± 0.0018 | **0.0030** ± 0.0014 |
| eCO2_tree | **0.94** ± 0.18 | **1.25** ± 0.32 | **0.25** ± 0.06 | **16.03** ± 6 | **0.0052** ± 0.0024 | **0.0032** ± 0.0019 |

| | | | | | | |
|---|---|---|---|---|---|---|
| aCO2_bare | **1.63** ± 0.35 | **1.77** ± 0.4 | **0.43** ± 0.14 | **14.37** ± 2.77 | **0.0068** ± 0.0013 | **0.0035** ± 0.0019 |
| eCO2_bare | **1.14** ± 0.16 | **1.52** ± 0.37 | **0.35** ± 0.08 | **17.05** ± 2.67 | **0.0065** ± 0.0024 | **0.0029** ± 0.0015 |

Elevated $CO_2$ increased the specific enzyme activity of GLU ($eCO_2$_tree = 0.94 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $aCO_2$_tree = 0.93 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$) insignificantly ($p = 0.56$) with growing trees (Figure 7 a). Within the bare soils $GLU_{spec}$ in the ambient $CO_2$ fumigation occurred significantly higher ($aCO_2$_bare = 1.63 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $eCO_2$_bare = 1.14 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $p = 0.007$). The difference in specific activity between planted and bare soils was significant for elevated ($p = 0.03$) for ambient ($p < 0.001$) chambers.

N-$AC_{spec}$ showed again a slight increase in the ambient $CO_2$ fumigation ($aCO_2$_tree = 1.35 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $eCO_2$_tree = 1.25 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$), though the increase appeared insignificant ($p = 0.96$) (Figure 7 b). The bare soils of N-$AC_{spec}$ occurred in ambient $CO_2$ insignificantly higher (1.77 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $p = 0.16$) than the specific activity in bare soils with elevated $CO_2$ (1.52 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$). The increase from planted to bare soil in the elevated $CO_2$ treatment was insignificantly ($p = 0.12$), while in the ambient $CO_2$ treatment the specific activity from planted to bare soil increased significantly ($p = 0.03$).

$SULF_{spec}$ arose slightly higher for elevated $CO_2$ fumigation ($eCO_2$_tree = 0.24 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $aCO_2$_tree = 0.25 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; $p = 0.78$) (Figure 7 c). In bare soils the specific activity appeared increased in both treatments, in the ambient the specific activity increased significantly (0.43 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, $p < 0.001$), in the elevated the growth in activity occurred also significantly (0.35 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, $p = 0.003$). Between the ambient and elevated fumigations in the bare soils, the increase appeared insignificantly ($p = 0.23$).

$PHOS_{spec}$ occurred with 16.03 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$ in the elevated $CO_2$ fumigation and tree growth (Figure 7 d). It appeared insignificantly higher ($p = 0.1$) than with ambient $CO_2$ treating (12.66 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$). The specific phosphatase activity with elevated $CO_2$ increased without the rhizosphere of trees (17.05 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, $p = 0.35$), such as the specific activity in an ambient $CO_2$

atmosphere without planting increased (14.34 $\frac{\text{nmol}}{\text{g TB}*\text{g C}_{\text{mic}}*\text{h}}$, p = 0.26). The growth between elevated and ambient bare soil activities was insignificant (p = 0.08).

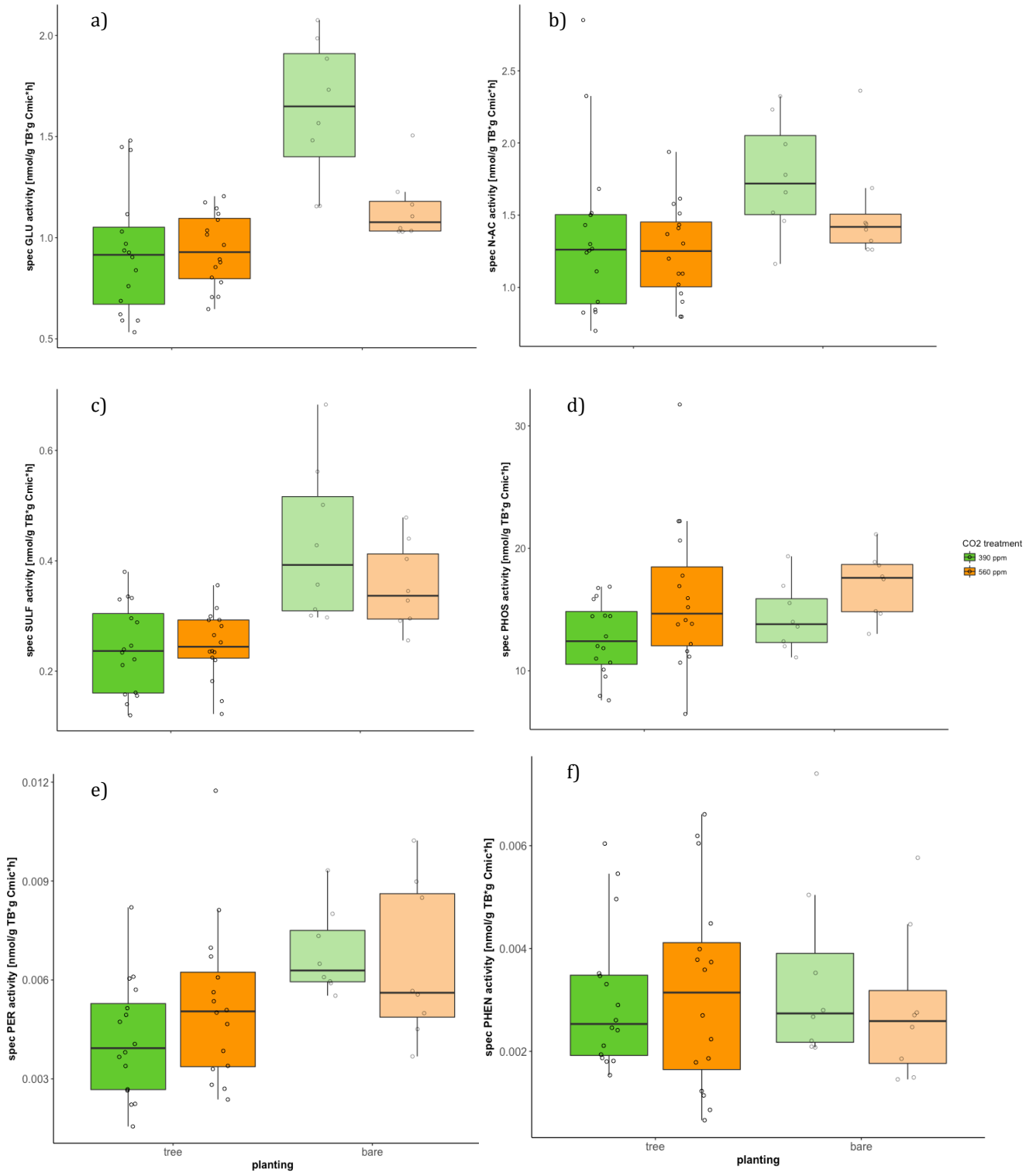


**Figure 7: Specific enzyme activities of all six enzymes in planted and bare soils and elevated (orange) and ambient (green) treatment**

$PER_{spec}$ was in the planted, elevated $CO_2$ treatment with 0.0052 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$ and ambient with 0.0042 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$ (p = 0.21). In the elevated $CO_2$ gassing the specific activity increased again very slightly without trees (0.0065 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, p = 0.24), such as in the ambient $CO_2$ treatment the specific activity increased (0.0068 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, p = 0.002). The rise in specific activity in bare soils from ambient to elevated $CO_2$ treating appeared insignificantly (p = 0.44).

$PHEN_{spec}$ showed the same pattern as the other enzymes. Soils under elevated $CO_2$ treatment and with plants reached a mean value of 0.0032 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$, while ambient $CO_2$ treatment and planting reached a mean value of 0.003 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$ (p = 0.9). In bare soils the specific activity was highest without $CO_2$ addition (0.0035 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$), which was insignificantly higher than with elevated $CO_2$ fumigation (0.0029 $\frac{nmol}{g\,TB*g\,C_{mic}*h}$; p = 0.44). In the elevated $CO_2$ treatment, the difference in the values occurred insignificantly (p = 0.88), as well as in the ambient treatment (p = 0.45).

# 4 Discussion

Elevated atmospheric $CO_2$, caused by global change alters the soils in numerous ecosystems in many ways. Caused by the greater photosynthetic rate of the plants, a depletion of nutrients in soils happens, thus, the nutrient cycles are altered. A direct reaction to these changes is expected in the extracellular enzyme activities (Chung et al., 2007). Many studies examined extracellular enzyme activities in many ecosystems under different treating. E.g. Allison and Vitousek (2005) assayed the activities of GLU and PHOS in rainforest volcanic tephra substrate without any treatment and with addition of several complex nutrients. Hereby in the untreated samples GLU came up with an activity of ca. 2500 $\frac{nmol}{g\,TB*h}$, while PHOS showed an activity of ca. 30000 $\frac{nmol}{g\,TB*h}$. In a fire-adapted scrub oak ecosystem in Florida PHEN activity was ca. 400 $\frac{nmol}{g\,TB*h}$ (Carney et al., 2007), while the activity of PHEN in a temperate beech forest near Vienna with ca. 1500 $\frac{nmol}{g\,TB*h}$ occurred (Kaiser et al., 2010). PER activity appeared increased with ca. 4500 $\frac{nmol}{g\,TB*h}$ and N-AC activity was highest with ca. 250000 $\frac{nmol}{g\,TB*h}$ (Kaiser et al., 2010). Obviously, the extracellular enzyme activities show a large variety, depending on the climate zone, plant species and soil, soil moisture, pH and nutrient availability. Also, elevated atmospheric $CO_2$ concentrations, due to climate change alter the soil in many ways. In my study the focus was on the effect of $CO_2$ assimilation in plants to the extracellular enzyme activity.

## 4.1 Relationship between enzyme activities in plantings and in different $CO_2$ treatments

With elevated $CO_2$ fumigation I expected to instigate the active microbial biomass to invest the higher energy supply into the production of more extracellular enzymes, because of the greater plant growth and consequently a higher need for nutrients. The production of enzymes would allow breaking the complex organic molecules down and making bounded nutrients bioavailable. The potential enzyme activities of planted soils and elevated $CO_2$ fumigation confirmed this hypothesis, except for N-AC. The differences between the $CO_2$ treatments were not significant except for PER (Table 14, Appendix). The increasing allocation of C to the roots with elevated $CO_2$ and the increasing pool of SOM stimulates roots and microbes to produce extracellular enzymes (Allison and Vitousek, 2005; Bardgett et al., 2008). Previous studies demonstrate an increase with $eCO_2$ in all assayed enzyme activities (Bhattacharyya et al., 2013). Other studies depict a substrate specific increase of enzymes and others show that the enzyme activity is dependent on the composition of the microbial community (Carney et al., 2007; Dorodnikov et al., 2009). Thus, many parameters influence the activity of soil enzymes, which were not part of this study. However, the stimulation effect of $eCO_2$ fumigation is represented in the results, although it is not significant. The period of the experiment was rather short, so the tendency of the $eCO_2$ effect was not intensified yet, in case that the effect was real.

The decrease in N-AC activity in the $eCO_2$ treatment appears surprisingly, because plants need a higher N supply for the increasing growth belowground. Consequently, plants produce more enzymes to break N-compounds down, decreasing the production if the N supply is guaranteed (Allison and Vitousek, 2005; Dakora and Phillips, 2002). For example, Guenet et al. (2012) found an increase in N-AC activity after elevated $CO_2$ treatment. Differences in the content of bioavailable N in the environments might be one reason for the differences between Guenet et al. and my research. That would implicate that the soils used in this experiment contain enough bioavailable N that the production of other nutrient breaking enzymes appeared more important for the plants and microbes in order to increase growth. Also the period of $CO_2$ fumigation was much shorter than in Guenet et al., where the experiment length was ten years (Guenet et al., 2012). Nevertheless, the activity of N-AC was the greatest after PHOS. Allison and Vitousek (2005) showed that PHOS increased as reaction to C and N addition and decreased with P addition. These results suggest that the soils were not limited in C and N but limited in P. PHOS is positively correlated with P in soils, so PHOS activity rises when P supply is rather restricted, consequently a high P availability would inhibit PHOS production (Olander and Vitousek, 2000). The indication of a high N supply in the soils is contrary to the theory that C mineralizing enzymes can also release N, thus the production of N-AC is not as important as I suspected (Olander and Vitousek, 2000). All C degrading enzymes increased in the $eCO_2$ treatment, thus the C mineralizers could have released N and the production of N-AC was inhibited.

Nevertheless, the increase of all C degrading enzymes support the first suggestion that there was no limitation in N and C because Phillips et al. (2012) showed, that in N limited soils the sequestration of C is restricted (Phillips et al., 2012).

SULF showed only a small activity compared with the other hydrolases. However, a very slight rise in the elevated $CO_2$ treatment was observed. That could be traced back to the increasing pool of SOM because of the greater belowground allocation of C by plants, which stimulates the production of SULF (Scherer, 2009). The small reaction and overall number of activity might be implicated with the acidic pH of the soils. Arylsulfatases show an optimum at the pH of 5.8 while our soils exhibited with a pH 3.4 - 3.63 (Acosta-Martinez and Tabatabai, 2000). Additionally, in acidic soils a shift from bacterial to fugal biomass was determined (Dakora and Phillips, 2002), thus SULF, mainly generated by bacteria, appears disadvantaged (Kertesz, 2000). If there is a shift to more fungi, the decrease of N-AC appears illogical, because fungi mainly release this enzyme (DeForest et al., 2004).

Under $eCO_2$ the pools of labile and active C increase, thus GLU as cellulose degrading enzyme reacts to $eCO_2$, but insignificantly. That confirms my hypothesis, that with $eCO_2$ GLU accelerates the decomposition of labile C compounds in the rhizosphere (Bhattacharyya et al., 2013; Fang et al., 2015). Additionally, this enzyme also provides energy for microbes, thus with an increasing microbial biomass the production of GLU increases (Eivazi and Tabatabai, 1988). PER shows as the only enzyme a significant increase of activity in the $eCO_2$ treatment (Table 14, Appendix). It is responsible for depolymerizing lignin and is preferred generated by fungi (Sinsabaugh, 2010). This, together with the decreasing pH with increasing $CO_2$ substantiates the assumption that a shift in the microbial community to more fungi happened. Mycorrhiza as a fungus benefits from the higher $CO_2$ supply in the atmosphere through a higher photosynthesis rate of the plants in terms of a higher carbohydrate accommodation by roots, as in my first hypothesis assumed (Meier et al., 2015). A higher abundance of ectomycorrhiza is coupled with a high turnover of C and other nutrients (Read and Perez-Moreno, 2003). PHEN shows barely any increase in $eCO_2$, although it participates in the same nutrient cycle. Additionally, the total enzyme activity is lower than the activity of PER. This might be induced by limited copper sources (Sinsabaugh, 2010).

## 4.2 Enzyme activities in bare soils

The rhizosphere as the interface between roots and soils contains the microbial biomass in biofilms (Nannipieri et al., 2003) and regulates the release of extracellular enzymes (Dakora and Phillips, 2002). Theoretically the microbial biomass is able to fix $CO_2$, however only in combination with the energy of light (Lange et al., 1994). Thus, in our experiment, the atmosphere should not affect the microbial biomass in bare soils, since the connection through plants is missing (Long et al., 2004).

Some of the enzymes in bare soils reacted as I hypothesized and decreased. In the $aCO_2$ treatment N-AC, PHOS and PHEN activities and in the $eCO_2$ treatment GLU, N-AC, PHOS and PHEN activities

occurred lower than the enzyme activities in planted bulks. In the case of N-AC and PHEN the differences between $aCO_2$ and $eCO_2$ was insignificant (Table 14, Appendix), which reinforces my hypothesis that plants connect the atmosphere with the pedosphere and changes in the composition of atmosphere gases are imparted through the rhizosphere (Long et al., 2004; Meier et al., 2015). In $eCO_2$ PHOS activity is significantly higher than in $aCO_2$ (Table 14, Appendix) but still lower than in $aCO_2$ with trees. Microbial community, particularly cyanobacteria, which are mostly independent from the rhizosphere might benefit from $eCO_2$ in the atmosphere, which would lead them to a greater extracellular enzyme production (Allison and Martiny, 2008; Lange et al., 1994). This assumption can be neglected, because the activity of autotrophic microorganisms is coupled with light (Blume et al., 2010a). Nevertheless, some chemo-lithotrophic bacteria could still benefit from $eCO_2$, independent from light-energy (Blume et al., 2010a).

Unexpected results were detected in the bare soils within the enzyme activities of GLU, SULF and PER. The determined values appeared greater than the activities in planted environments but only insignificant. These results attenuate my preceding hypothesis and cannot be explained with previous theories. PER activity is highest in the $eCO_2$ tree treatment, but second comes $eCO_2$ bare and third $aCO_2$ bare. The differences in PER activity between $aCO_2$ and $eCO_2$ bare soil treatment are insignificant (Table 14, Appendix), which imply that $eCO_2$ in combination with trees has the greatest influence ($p = 0.005$) to extracellular enzymes but the other treatments only a slight influence (Figure 6 e). So, my first hypothesis, that $eCO_2$ in the atmosphere increases the production of C degrading enzymes caused by a greater photosynthesis rate can be partly confirmed. Additionally, the increased activity of PER in $eCO_2$ proves my second hypothesis that enzymes, degrading C compounds of the SOM would occur increased because of a higher C allocation to woody tissues under $eCO_2$ (Oren et al., 2001). Fungi mainly produce PER, thus, this outcome supports the suggestion of a shift to a higher abundance of fungi (Baldrian, 2006).

The results of SULF show a high variety and no significant changes in enzyme activity between the different treatments. But in mean values, the SULF activity in the bare $aCO_2$ treatment is highest, second comes $eCO_2$ planted and $eCO_2$ bare (Table 6). Thus, there is barely any effect of trees and $CO_2$ treatment to the soil enzyme SULF. It has been observed before, that SULF increases with the presence of living roots (Kertesz, 2000) but in this experiment the effect did not appear. A factor of $CO_2$ acclimation of plants is the size of the pots they are growing in. In small pots, roots are restricted in growth and the sink for C compounds is limited (Arp, 1991). But this explanation would apply to all enzymes, so it can be obviated. The disruption caused by soil collection might have disturbed the balance of SULF producers. In the planted environments, the rhizosphere of the trees could have regulated the production but in bare soils no regulation took place, thus at harvesting the SULF producing microbes were still confused.

GLU activities of bare soils appear with a insignificant higher activity in the $aCO_2$ treatment (Table 14, Appendix). The enzyme activity of the different $aCO_2$, bare soil samples were not measured on one day, thus a failure in the methods can be eliminated. However, finding an explanation for the outcome is difficult. Maybe the soils were not completely homogenized, so for the enzyme analysis of the other treatments I took drier soil but for $aCO_2$ I took soil with better moisture. This is unlikely, just because the moisture of the bare soil chambers was lower than in planted environments (Table 10, 11, Appendix). Another possibility is the seasonal difference between harvesting. The first chambers were harvested in the beginning of November and the last not until December. Previous studies show that GLU activity in a temperate beech forest is highest in June and November, thus a increase in glucose production is usual in late autumn but not in the winter (Kaiser et al., 2010). If the $aCO_2$, bare chambers were harvest right in the beginning and the other chambers only later on, the seasonal difference might play a little role. Dead cells can also release extracellular enzymes. Remains from roots and other living organisms might have released enzymes, independent from changes in the atmosphere. Also, extracellular enzymes complex with humic colloids and organic matter, thus there were already an amount of enzymes in the soils before the experiment started (Bandick and Dick, 1999). These enzyme activities appear independent from the experiment but can not eliminated.

## 4.3 Enzyme activities after the treatment compared to enzyme activities in the initial substrate

The enzyme activities in the initial substrate of N-AC, SULF, PHOS and PHEN appeared higher than the enzyme activities of the experiment. GLU and PER activities were in the variance of the results of the experiment (Table 6). Thus, the results of GLU and PER support my hypothesis, that the enzyme activities of the initial substrate achieve similar values as the planted, $aCO_2$ treated soils. The decrease of the other four enzyme activities after the experiment diminishes my hypothesis (Figure 8).

One possible reason for the decrease might also be the chamber effect. When the space for root growth and consequently for symbionts is limited the production of extracellular enzymes becomes reduced (Arp, 1991). Before soil collection there was no limitation of space, thus previous roots and microbes had enough space for growth. Additionally, the disturbance by soil sample taking and thus higher priming might be an explanation for this occurrence. Abiotic changes might stimulate the microbial biomass; so they decompose a higher amount of SOM, thus produce more enzymes to catalyze the reactions (Kuzyakov, 2010). The time of the year might also be responsible for the differences in the enzyme activity. The enzyme production is linked to the microbial community and follows a seasonal pattern (Kaiser et al., 2010). Soil sampling in this experiment was in February thus in early spring and harvesting followed from November until December, which is late autumn. Kaiser et al (2010) detected a first peak in the PHEN activity in early spring but N-AC reacted barely until May.

In a Mediterranean climate (mean temperature: 15 °C, mean precipitation: 94 cm) the activity of PHOS and N-AC was highest in winter (Waldrop and Firestone, 2006). The cycle of activity might be shifted backwards in a Mediterranean climate zone because in Mediterranean regions the climate of winter corresponds mostly the climate of spring in regions of beech origin in temperature and moisture. In Erfurt, representative for Thuringia, the mean temperature in February was over 0 °C by night and over 5 °C by day. In March the daily mean temperature was 3.5 °C (accuweather.com). The precipitation was around 35 $l*m^{-2}$ in February and 42.6 $l*m^{-2}$ in March (wetterkontor.de). These climatic conditions lead to the assumption that plants prepared for budding and first enzymes were released. It is striking that C complex degrading enzymes except for PHEN showed same activities in the initial substrate as in the experiment but other nutrient degrading enzymes appear higher. That would confirm my prevenient consideration, because phosphor is mainly needed for buds, while N is higher in the beginning of the leave growing period than in midsummer (Stolle, 1956).

For more specific explanations more data, e.g. on microbial community composition and microbial biomass of the initial substrate as well as of the soils after the experiment are missing. Information about the supply of nutrients in the soils would also help for a better understanding of the outcomes.

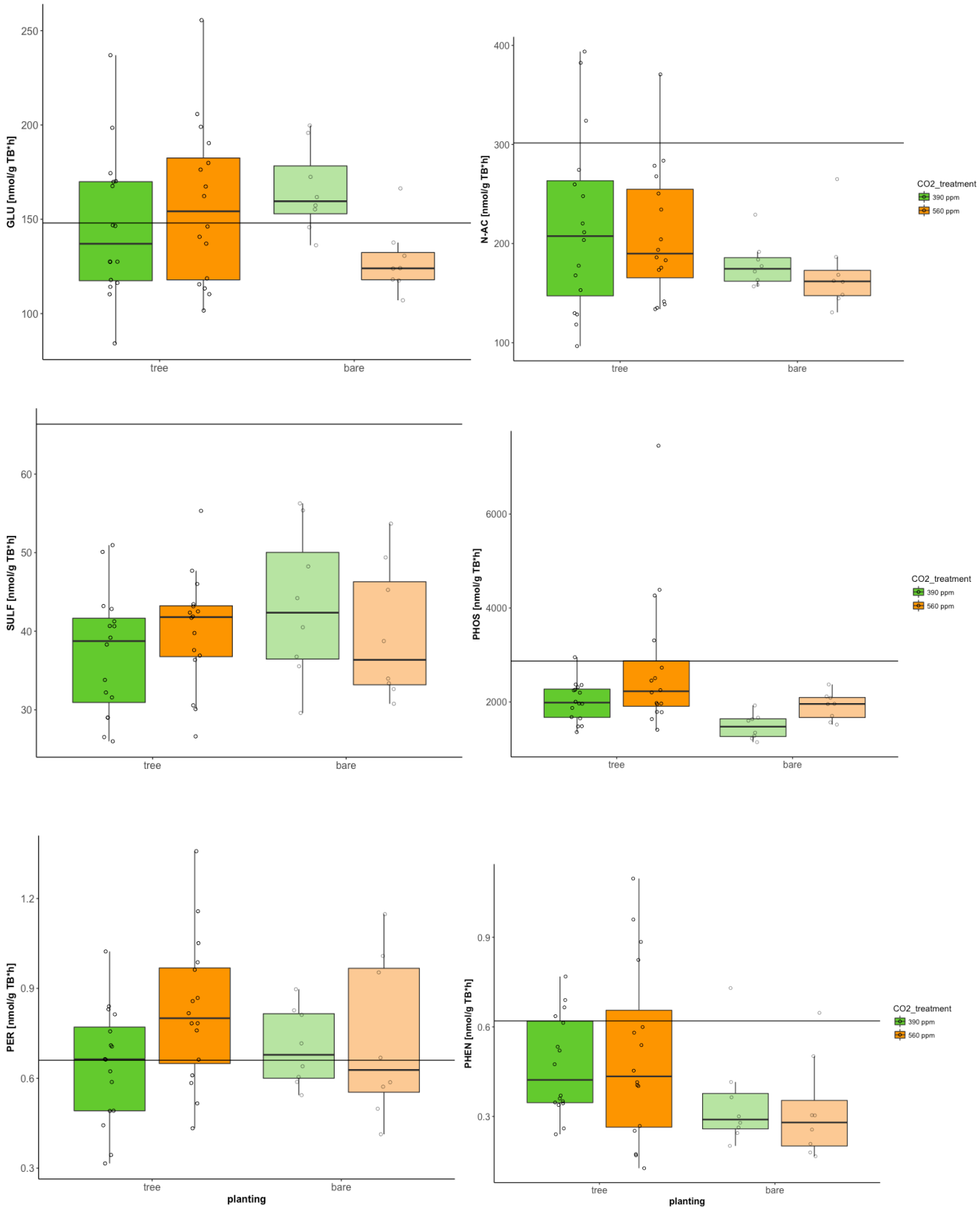


**Figure 8: Potential enzyme activities of all six enzymes in planted and bare soils and elevated (orange) and ambient (green) $CO_2$ treatment, black line shows the enzyme activity of the initial substrate**

## 4.4 Shift in specific enzyme activities

The specific enzyme activities describe the potential enzyme activity per unit of microbial biomass ($C_{mic}$). The microbial biomass in all treatments is not normal distributed ($p < 0.001$) and in mean values, a slight increase in the $eCO_2$ treatment is detected ($p = 0.78$). In the bare soils, the $C_{mic}$ content of the soil in $eCO_2$ treatment is insignificantly higher than in $aCO_2$ treatment ($p = 0.08$). The shift to a higher $C_{mic}$ with plants and $eCO_2$ can be ascribed to the higher photosynthetic activity of the beeches and the belowground allocation of C (Ainsworth and Long, 2005; Drake et al., 1997). The variation of the $C_{mic}$ values is very great; the median of the microbial content is lower than in planted soils, thus the result is not statistically robust.

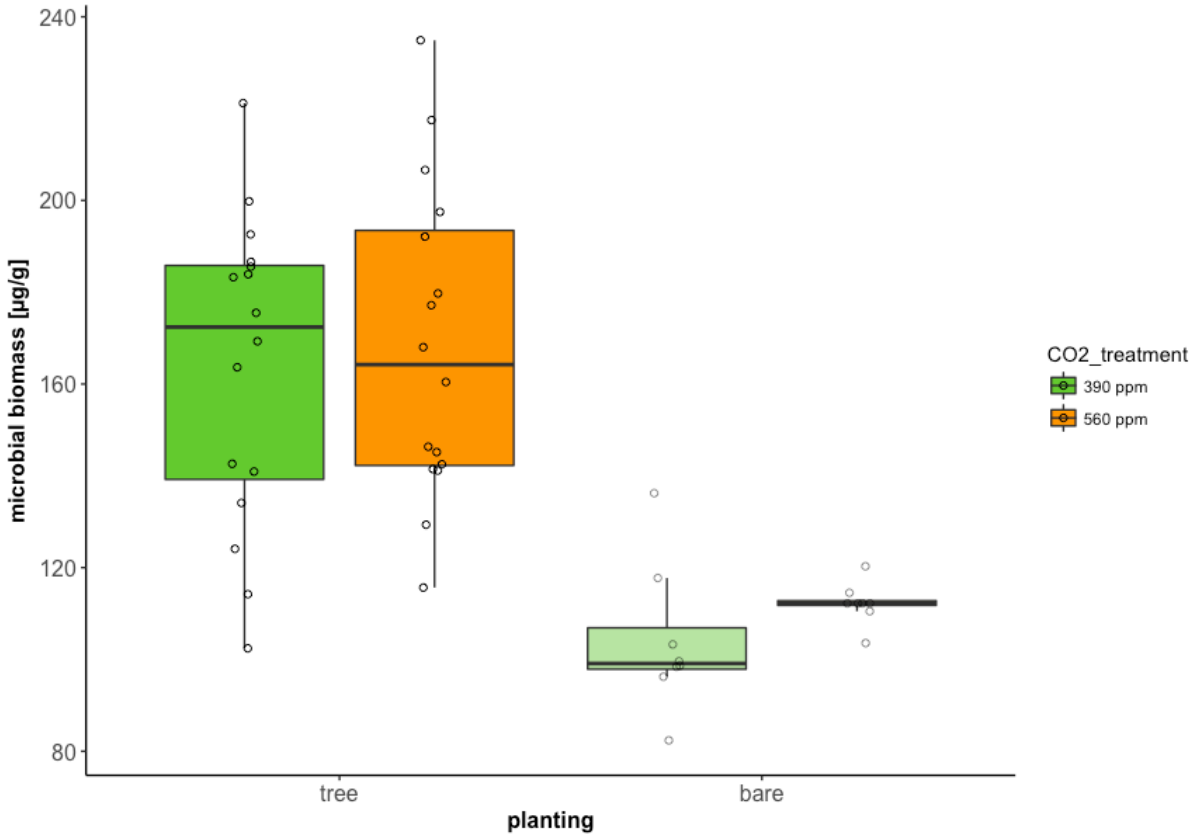


**Figure 9: $C_{mic}$ in all four treatments**

Trees in enriched $CO_2$ atmosphere allocate more C in the soil because of a greater photosynthesis rate (Ainsworth and Long, 2005; Drake et al., 1997). Because of this higher energy supply for microbes the microbial biomass and thus respiration are stimulated as a response (Bardgett et al., 2008; Carney et al., 2007). The increased microbial community composed of several bacteria and fungi, releases in return a higher number of extracellular enzymes (Burns et al., 2013). Thereby, the enzyme activity increases parallel with $C_{mic}$. The correlation of $C_{mic}$ over the potential enzyme activities should occur positively because with a higher amount of microbial biomass in soils more enzymes become released and consequently the enzyme activity increases. This pattern occurred in the results of planted soils in

the case of GLU, N-AC, SULF and PHOS in correlation with $C_{mic}$ (Figure 10). Hereby, $eCO_2$ treated soils with trees correlated best with $C_{mic}$. The relation of $C_{mic}$ and the activities of PER and PHEN in planted soils was negative in both $CO_2$ treatments, thus with a higher amount of $C_{mic}$ in soils the enzyme activities of the oxireductases decreased (Figure 10). For GLU and N-AC the relation of the activity in the $aCO_2$, bare treated soils appeared negative, while both $aCO_2$ treatments of SULF were also negative. The correlations of $C_{mic}$ with PER was positive in $aCO_2$ and $eCO_2$, bare soils. But all correlations are insignificantly (Table 17, Appendix), thus it is statistically not robust. Though in all cases the hydrolase enzyme activities in $eCO_2$ treated and planted soils correlated positively with $C_{mic}$. Nevertheless, the variance of the values and consequently the coefficient of determination of the linear regression appear vague (Table 18, Appendix). The best correlation was found for GLU in $eCO_2$ and planted treatments, which reinforces again my previous assumption that the relation of $C_{mic}$ and enzyme activity reacts parallel. Additionally, N-AC, PHOS and PER activities correlate best in the same treatment. Only SULF and PHEN show best correlations in $aCO_2$ and bare environment (Table 18, Appendix).

The specific enzyme activity reflects the result of the potential activity in planted environments, thus the increasing effect of $eCO_2$ combined with trees appears. The bare soil specific activities occur proportionally high, because the potential activities are mostly insignificant lower (Table 17, Appendix), while the microbial biomass in the bare soils is significantly lower (Table 17, Appendix). Nevertheless, the correlation of enzyme activities in $eCO_2$, planted environments with $C_{mic}$ occurred. The activity of the oxireductases was highest with low $C_{mic}$ except for the activity of PER in $aCO_2$, bare treated soil. Maybe, the more microbes are in soils the less they invest in enzymes as the oxireductases particularly if copper is limiting (Baldrian, 2006) and other enzymes decompose SOM for nutrients.

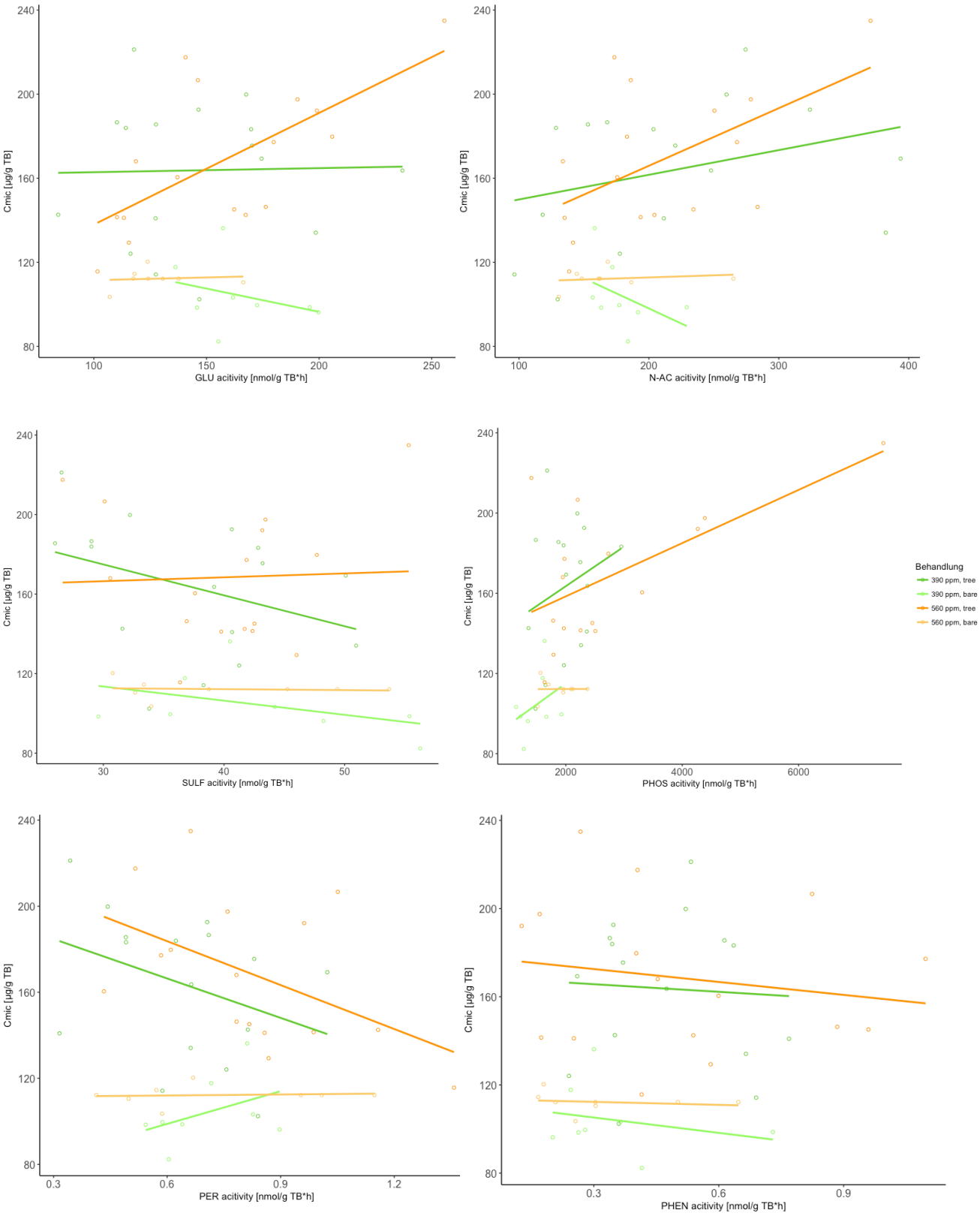


**Figure 10: $C_{mic}$ over potential enzyme activites**

# 5 Conclusion

In the experiment an increase of extracellular soil enzyme activity through $eCO_2$ in the atmosphere was detected in all planted environments, except for N-AC activity. Nevertheless, these results were statistically insignificant except for the increase in PER activity. Thus, my first two hypotheses, that the enzyme activity increases because of the chain of cause and effects and that the activity of enzymes, which take part in the C cycle increase, seems to be plausible.

Also, $C_{mic}$ was affected by $eCO_2$; the increase from bare soils to planted environments appeared statistically significant. However, the correlation between enzyme activities and $C_{mic}$ appeared only for hydrolases in $eCO_2$ atmosphere. $C_{mic}$ correlated most precisely with the hydrolase activities in the $eCO_2$ and planted environments. The activities of the oxireductases correlated negatively, but with a high variance.

Unexpected results occurred in bare soils. The activity of GLU increased in $aCO_2$ atmosphere significantly, what is controversial to previous theories. For a deeper process understanding of this outcome, regular measurements of soils enzyme activity each month and an elucidation of the composition of the microbial community could be conducted. A repetition of this experiment with a longer growing period would give information whether the effect of $eCO_2$ was an accident or predicted. Furthermore, a determination of nutrient content in soils and microbial biomass would allow additional insight.

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http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/ (20.6.17)

http://www.accuweather.com/de/de/erfurt/99084/march-weather/171707?monyr=3/1/2016 (10.7.17)

http://www.wetterkontor.de/de/wetter/deutschland/monatswerte.asp?y=2016&m=2 (10.7.17)

# Appendix

*1. Protocols for the enzyme assays:*

## Determine microbial enzyme activity in soils

***Part 1: C, N, P and S cycle via microplate fluorimetric assay***

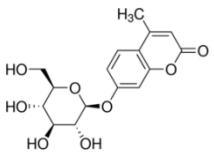
**Content:**



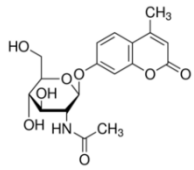
## 1. Basic principles of the analytical procedure

The active microbial biomass in soils produces different kinds of intracellular and extracellular enzymes to catalyze catabolic and anabolic reactions. Hydrolytic exoenzymes allow these microbes to open up potential energy and nutrient resources for microbial or plant uptake. By studying the activity of extracellular enzymes involved in the C, N, P and S cycling it is possible to examine functional diversity in soils.

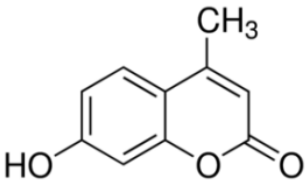
We measure the activity of β-glucosidase, N-acetyl-glucosaminidase, phosphatase and sulfatase by using the "Multisubstratassay" of Marx et al. (2001). This method uses the fluorescent compound 4-methylumbelliferone (MUF) which is bonded to enzyme specific substrates (Abb. 1). In this bonded form MUF is inactive. Only the spun-off MUF is fluorescent and the fluorescence intensity is directly proportional to the amount of spun-off MUF. By using a computerised microplate fluorimeter (e. g. Microplate Fluorescence Reader FLx800 ® from Bio-Tek Instruments inc.) it is possible to quantify fluorescence emission due to release of MUF product for several enzymes over time. In comparison with standard concentrations it is possible to quantify the activity of soil microbial enzymes.



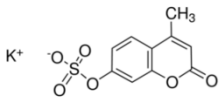

4-Methylumbelliferyl β-D-glucopyranoside β-glucoside



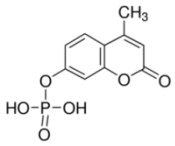

4-Methylumbelliferyl N-acetyl-β-D-glucosaminide



4-methylumbelliferone



4-Methylumbelliferyl sulfate



4-Methylumbelliferyl phosphate

Abb. 1: 4-methylumbelliferone basic structure (left) and methylumbelliferone-associated substrates (right)

## 2. Devices and consumables

| devices | consumables | chemicals | solutions |
|---|---|---|---|
| • balance<br>• autoclave<br>• ultrasonic disaggregator<br>• incubator (30°C)<br>• stopwatch<br>• magnetic stirrer<br>• Microplate Reader (360/460 nm) with PP-Microplates (black, 96 wells)<br>• electronic pipette (50-1000 µl; optional)<br>• multichannel pipette (5-50 µl)<br>• pipettes (10-100 µl; 100-1000 µl)<br>• Weighing boats (for weighing substrate) | • 150 ml beaker (1 per sample)<br>• 50 ml volumetric pipette<br>• 2 x 10 or 50ml graduated pipettes<br>• 1 box 200 µl pipette tips<br>• 5 ml pipette tips<br>• 1 ml pipette tips<br>• 10ml volumetric flasks<br>• 50ml volumetric flasks<br>• sterile 10 ml PP-tubes (to store substrate solutions) | • MES hemisodium salt (sigma aldrich M8902-100G)<br>• 4-Methyl-umbelliferone / MUF (sigma aldrich M1381-25G)<br>• Dimethyl sulfoxide / DMSO (sigma aldrich D8418-250ML)<br>• Methanol | • 0.1M MES-Buffer<br>• 10 mM MUF-stuck solution<br>➔ 10 µM MUF-working solution<br>• 10 mM substrat-stuck solution<br>➔ 1 mM Substrat-working solution |

## 3. Procedure

# 4. Preparations (some days in advance)

### 4.1 soil sieving

- carefully sieve fresh (or - 20 °C stocked) soil samples to < 2 mm; remove roots and stones ( > 2 mm)

### 4.2 prepare buffer and standard solutions

→ *doesn`t need to be sterile*

- **0,1 M MES-Buffer** (1 liter):
  - weigh in the corresponding amount of MES-buffer (20.673g for MES-Buffers with molar mass of 206.73 g/mol) in a 1 liter volumetric flask and fill it up with ddi. water.
  - autoclave buffer solution (program 9 – liquids)
- **10 mM MUF-stuck solution:**
  - dissolve 17.62 mg 4-Methylumbelliferone with 5 ml Methanol in a 10 ml volumetric flask. After it is completely dissolved, fill the flask up to 10 ml with ddi. water. (volume contraction!)
- **MUF-working solution** (stable for 2 months in the fridge):
  - 100 µM MUF: Combine 500 µl of MUF-stuck solution with 49.5 ml MES-Buffer in a 50 ml volumetric flask (make 2 of them)
  - 10 µM MUF: Combine 400 µl of MUF-stuck solution with 3600 µl MES-Buffer in blue sterile PP tubes, transfer 500 µl in a 50 ml volumetric flask and fill it up with 49.5 ml MES-Buffer (make 2 of them)

## 4.3 autoclave consumables and solutions

→ *The following consumables and solutions have to be autoclaved in advance. Attention: Solids and liquids need to be autoclaved separately.*

- solids (programme 1): beaker, 50 ml volumetric pipette, pipette tips
- liquids (programme 9): ddi. water (1-2 liter), MES-buffer solution

### 4.4 prepare substrate solutions

→ *sterile production*

- **10 mM Substrate-stuck solution** (stable for 6 months at -20 °C):
  - Use 1/10000 of the substrate molar mass as initial weight (see Tab. 1) and pre-dissolve each substrate with 300 µl of Dimethyl sulfoxide (DMSO) in sterile PP-Tubes. Add 9.7 ml sterile ddi. water afterwards.

| Substrat | Used short cut | Molar Mass [g/mol] | Weighted sample |
|---|---|---|---|
| 4-Methylumbelliferyl-β-D-Glucoside | β-Glu | 338.31 | 0.033831g |
| 4-Methylumbelliferyl-N-Acetyl-β-D-Glucosaminide | N-Ac | 379.36 | 0.037936g |
| 4-Methylumbelliferyl-sulfate | Sulf | 294.32 | 0.0294g |

| | | | |
|---|---|---|---|
| 4-Methylumbelliferyl-Phosphate | Phos | 300.11 | 0.030011g |

Tab. 1: MES Substrate Solutions

- **1 mM Substrate-working solution** (stable for 3 weeks in the fridge):
  - For each enzyme substrate you need an aliquot of 1 ml of the associated substrate-storage-solution.
  - Mix it with 9 ml sterile MES-Buffer in sterile PP-Tubes

➔ 10ml working solution are sufficient for 32 samples.

# 5. Processing

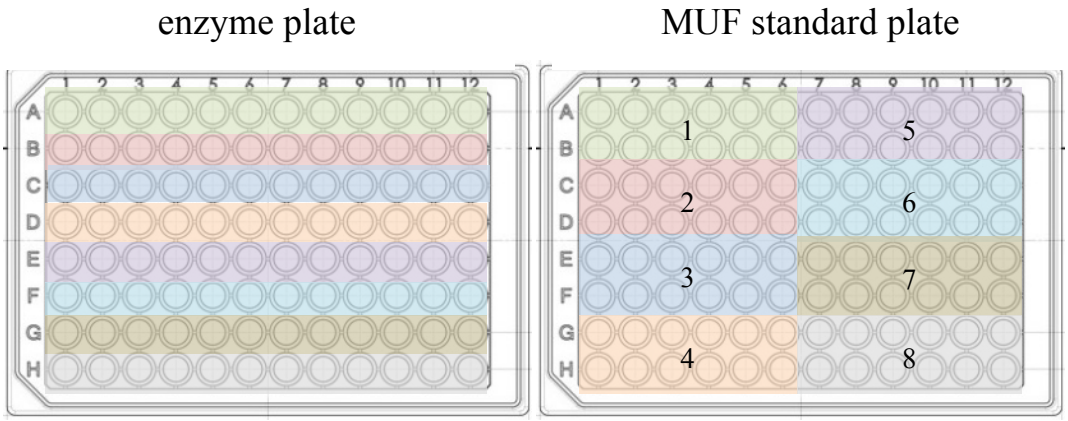
## Sample preparation

- weigh 1g of soil sample into a 150 ml beaker
- add 50 ml sterile bidest. water via volumetric pipette (for larger amount of soil, use 100 ml sterile water)
- solubilise each sample for 1 min 2s via ultrasonic disaggregator → avoid contamination among samples by rinsing the rod of the ultrasonic device with bidest. water and dried with a paper towel after each sample
- run the ultrasonic disaggregator with a low energy input (35%) and put the rod approximately 1-1,5 cm in the soil solution.

## Pipet the soil suspension

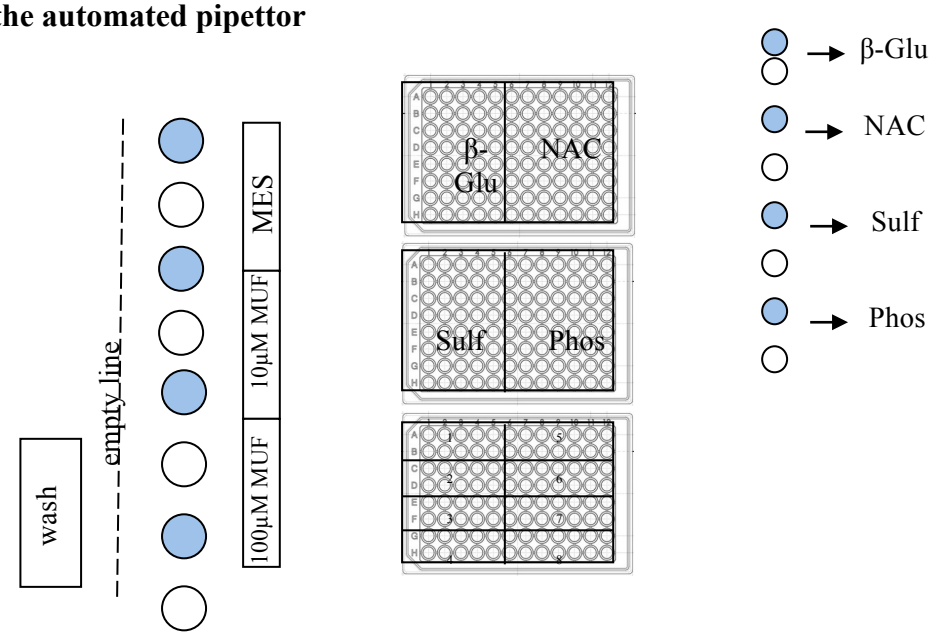
On each microplate we can analyze 8 soil samples. The soil suspension has to be continuously stirred while an aliquot of 50 µl is transferred via multichannel pipette into rows of 12 wells each. To avoid contamination among samples the magnetic stir bar has to be rinsed with ddi. water and dried with a paper towel after each sample.

For the MUF standard plate only half of the row is pipette with one sample but two rows, one below the other.




Sometimes the phosphatase activity for some samples is too high for detection in the later phase of measurement. Therefore it is recommended to dilute the soil solution for this enzyme 1:2 after pipettes all other positions. Add 48.5 ml ddi. water to the soil suspension while the beaker is still on the stirrer. After dilution pipette the part of the microplate where the phosphatase activity will be measured.

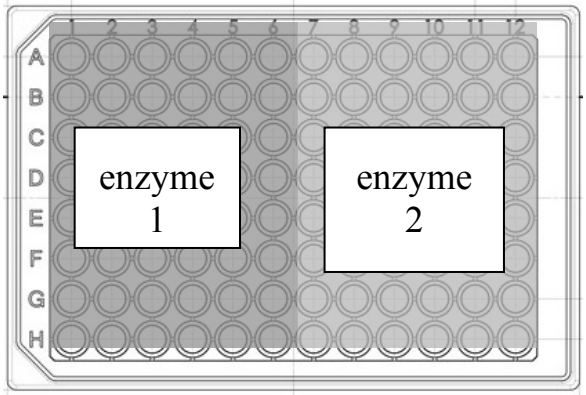
**Positions in the automated pipettor**

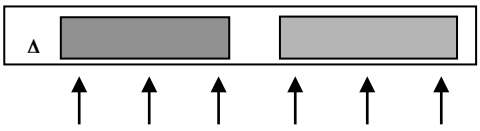


## Pipet MES-Buffer and enzyme substrate solutions

On each enzyme plate we can analyze the activity of two different enzymes with 6 replicates for each sample. The additional MUF standard plate serves as a reference without any enzyme addition.

MES-Buffer and enzyme substrate solutions are pipette automatically. The enzyme plates are pipette with 50 µl MES- Buffer and 100 µl substrate solution in each well.

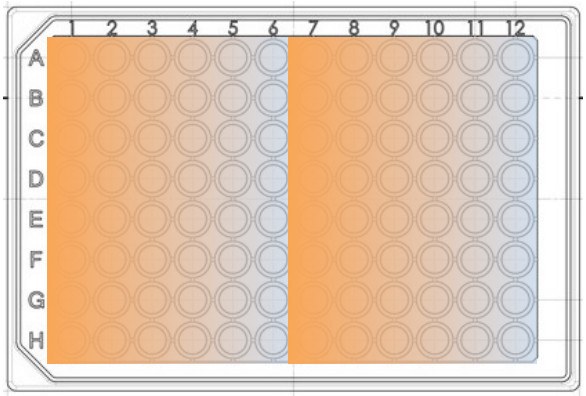


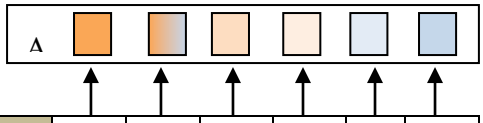



| | | | | | | |
|---|---|---|---|---|---|---|
| soil in µl | 50 | 50 | 50 | 50 | 50 | 50 |
| buffer in µl | 50 | 50 | 50 | 50 | 50 | 50 |
| substrate in µl | 100 | 100 | 100 | 100 | 100 | 100 |

The MUF standard plate is pipette with different concentrations of MUF-standard and MES-buffer solution in the following order:

- MUF - standard: 0, 10, 20, 50, 80, 120 µl
- MES - buffer solution: 150, 140, 130, 100, 70, 30 µl



| | | | | | | |
|---|---|---|---|---|---|---|
| soil in µl | 50 | 50 | 50 | 50 | 50 | 50 |
| std. in µl | 0 | 10 | 20 | 50 | 80 | 120 |
| buffer in µl | 150 | 140 | 130 | 100 | 70 | 30 |

## Sample preincubation and measurement in the microplate reader

To avoid measurement overlapping we can measure at most 6 microplates. Therefore there is a cut-off of 16 samples for the analysis of 4 enzymes.

Micro-titer plates have to be incubated in the dark at 30°C while they are shaken on a micro-titer plate shaker at 300 rpm. To ensure a linear incline of the microbial activity the microplates should be pre-incubated before the first measurement for 30 min. To avoid drying-out of the wells the microplates should be covered with Parafilm or another microplate as a lid.

The fluorescence is measured after 0, 30, 60, 120 and 180 min with 360 nm excitation and 460 nm emission using a microplate reader (Infinite 200, Tecan, Crail-sheim, Germany). Open the software i-control 1.5 on the desktop. Open script: …

*Infos zur Messung bzw. Start der Messung?*

## 5. Remarks

Aufgrund der sehr geringen eingewogenen Probenmenge muss unbedingt auf eine möglichst sorgfaltige Homogenisierung der Bodenprobe geachtet werden. Der Boden sollte gut durchmischt d.h. < 2 mm gesiebt vorliegen. Auf die Ultraschallbehandlung und auf das Rühren während des Pipettierens kann nicht verzichtet werden. Ein Ultraschallbad bringt schlechtere Ergebnisse.

## 6. References

Herold, N. et al. (2014): Applied Soil Ecology 73 (2014) Soil property and management effects on grassland microbialcommunities across a latitudinal gradient in Germany. Applied Soil Ecology 73, 41– 50.

Marx, M.-C., Wood, M., Jarvis, S.C., (2001): A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology and Biochemistry 33, 1633–1640

Summarized by Jessica Heublein and Theresa Klötzing 2016

Most recent amendment October 2016

# Determine microbial enzyme activity in soils

***Part 2: Peroxidases and Phenoloxidases***

**Content:**



## 1. Basic principles of the analytical procedure

Peroxidases and Phenoloxidases are required for decomposition of lignin and play an important role in the carbon cycle of the soil. The activities of peroxidases and phenoloxidases can be determined photometrically by addition of the substrate TMB (tetramethylbenzidine).

Colour reaction: The result of the colorless leuco TMBH2 (which is here used as a substrate) catalyzed by peroxidases and phenoloxidases, is a cyan dye tetramethylbenzidine (TMB). Unlike phenoloxidases, peroxidases catalyze this reaction only under the influence of hydrogen peroxide. Phenoloxidase doesn´t need $H_2O_2$ to catalyze it.

## 2. Devices and consumables

| devices | consumables | chemicals | solutions |
|---|---|---|---|
| • balance<br>• autoclave<br>• pH-meter<br>• ultrasonic device<br>• incubator (30°C)<br>• stopwatch<br>• magnetic stirrer<br>• Microplate Reader (360/460 nm) with PP-Microplates (transparent, 96 wells)<br>• electronic pipette (50-1000 µl)<br>• multichannel pipette (5-50 µl)<br>• pipettes (10-100 µl; 100-1000 µl)<br>• Weighing boats | • 150 ml beaker (1 per sample)<br>• 50 ml volumetric pipette<br>• 50ml Falcon-tubes<br>• schott flasks (250 ml, 500 ml, 1L)<br>• glas bowl<br>• volumetric flasks | • Substrat TMB (Tetramethylbenzidin, sigma aldrich Nr. 860336)<br>• Dimethyl sulfoxide / DMSO (sigma aldrich D8418-250ML)<br>• 0.3% $H_2O_2$ | • 50mM sodium acetate buffer<br>• 205mM sodium citrate buffer<br>• 1M citric acid<br>• 12% acetic acid |

## 3. Procedure

# 4. Preparations (some days in advance)

### 4.1 soil sieving

- carefully sieve fresh (or - 20 °C stocked) soil samples to < 2 mm; remove roots and stones ( > 2 mm)

### 4.2 prepare acids and buffer solutions

→ *doesn`t need to be sterile*

- **12% acetic acid:**
  - add 12 g acetic acid to 88g $H_2O$ in a 100ml volumetric flask (or 30g acetic acid to 220g $H_2O$ for 250ml)

- **1M citric acid:**
  - weigh in the corresponding amount of citric acid (10.507 g for 50ml or 21.014g for 100ml) in a volumetric flask and fill it up with ddi. water.

- **50mM sodium acetate buffer** (136.08g/mol), pH5.0:
  - weigh in the corresponding amount of sodium acetat (6.804g for 1L, 13.608g for 2L) in a volumetric flask and fill it up with ddi. water.
  - Fill the buffer into a big glas bowl, put it on the magnetic stirrer and adjust the pH-value to 5.0 by dropping 12% acetic acid in while checking the pH via pH-meter

- **205mM sodium citrate buffer**, pH4.0:
  - weigh in the corresponding amount of sodium citrate (6.029g for 100ml or 30.15g for 500ml) in a volumetric flask and fill it up with ddi. water.
  - Fill the buffer into a big glas bowl, put it on the magnetic stirrer and adjust to pH-value 4.0 with 1M citric acid

### 4.3 autoclave consumables and solutions

→ *The following consumables and solutions have to be autoclaved in advance. Attention: Solids and liquids need to be autoclaved separately.*

- solids (programme 1): beaker, 50 ml volumetric pipette, pipette tips
- liquids (programme 9): *ddi. water (1-2 liter),* sodium acetate buffer, sodium citrate buffer
  - store them in the fridge at 2 – 8 °C

# 5. Processing

### 5.1 Sample preparation

- Weigh 0,4g of soil sample into a 150 ml beaker
- add 50 ml sterile sodium acetate buffer via volumetric pipette
- solubilise each sample for 1 min via ultrasonic emulsifier → avoid contamination among samples by rinsing the rod of the ultrasonic device with ddi. water and drying with a paper towel after each sample
- run the ultrasonic emulsifier with a performance of 35% and put the rod approximately 1-1,5 cm in the soil solution.

## 5.2 Preparation of the substrate (TMB)-solution (MG=240,35g/mol)

Substrat TMB (Tetramethylbenzidin, $C_{16}H_{20}N_2$) has to be **stored in the fridge** at 2 – 8 °C

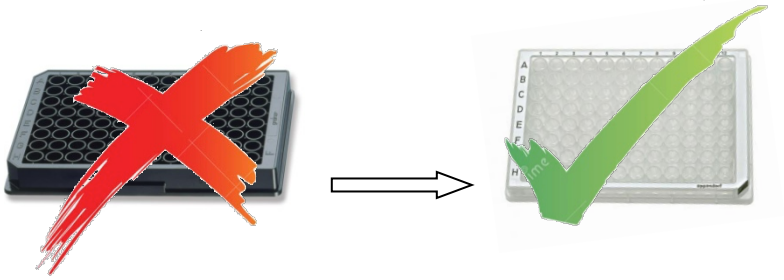
→ *you have to prepare the substrate solution directly before you start to pipette the soil solution into the microplates*

- **60 mM Substrate-stock solution**:
  - dissolve 0.04326 g TMB in 1.5 ml DMSO
  - after it is completely dissolved, add 1.5 ml sterile $H_2O$ (Careful: TMB has to be dissolved in DMSO before you add the water, otherwise the TMB won´t dissolve completely)

- **12 mM Substrate-working solution**:
  - add 12 ml of citrate buffer to the complete stock solution (sufficient for 3 plates)

Because Peroxidases and Phenoloxidases are measured colormetrically not fluorimetrically,

so we have to use transparent microplates!



## 5.3 How to pipette for Phenoloxidase

### 5.3.1 Pipette the solubilised soil solution

On each microplate we can analyse 8 soil samples in theory. For each set of samples, one row of the microplate has to function as a negative control. If there is a free row in the microplate left, also prepare a standard soil.
The soil suspension has to be continuously stirred while an aliquot of 200µl is transferred via multichannel pipette into one row of 12 wells. The same procedure follows for all samples. In the last row pipette only 200 µl of sodium acetate buffer into all 12 wells as negative control.

To avoid contamination among samples the magnetic stir bar has to be rinsed with ddi. water and dried with a paper towel after each sample.

### 5.3.2 Pipette acetate buffer and substrate (TMB) solution

Pipette 50 µl sodium acetate buffer in each "blank" well (7-12).

Pipette 50 µl substrate in the "sample" wells (1-6) of the first microplate, note time and start the measurement, then pipette substrate in second microplate…

.

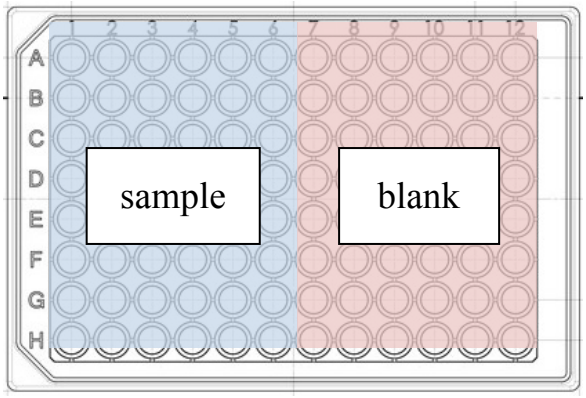
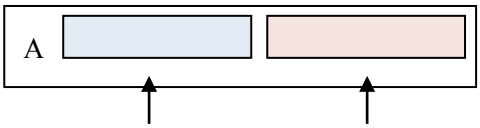
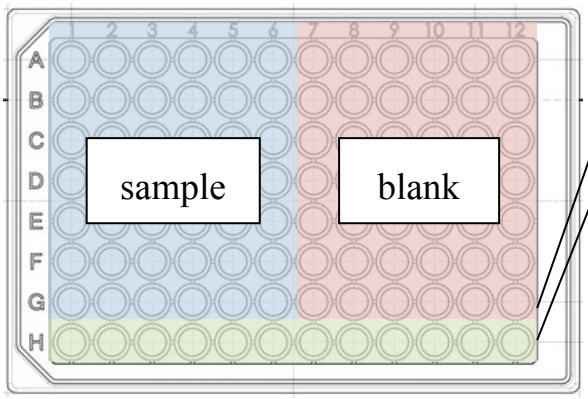



**Plate 1:** A-H: soil sample 1 - 8




| | | |
|---|---|---|
| soil in µl | 200 | 200 |
| buffer in µl | - | 50 |
| substrate in µl | 50 | - |



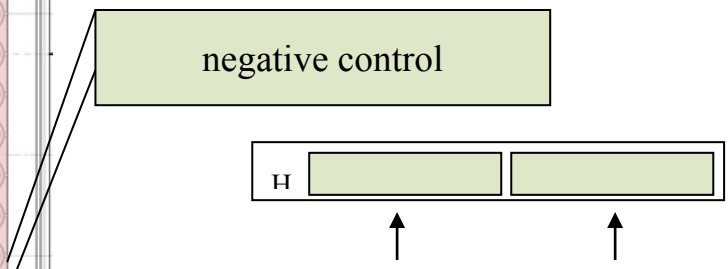




**Plate 2:** A-G: soil sample 9 – 15; H: negative control (buffer and substrate only)

| | | |
|---|---|---|
| soil in µl | - | - |
| buffer in µl | 200 | 200 |
| substrate in µl | 50 | 50 |

## 5.4 How to pipet for Peroxidase and Phenoloxidase

### 5.4.1 Pipette the solubilised soil solution

On each microplate we can analyse 4 soil samples in theory. For each set of samples, one row of the microplate has to function as a negative control. If there is a free row in the microplate left, also prepare a standard soil.

The soil suspension of one sample has to be continuously stirred while an aliquot of 200µl is transferred via multichannel pipette into two rows of 12 wells. The same procedure follows for all samples. In the last row pipette only 200 µl of sodium acetate buffer into all 12 wells as negative control.

To avoid contamination among samples the magnetic stir bar has to be rinsed with ddi. water and dried with a paper towel after each sample.

## 5.4.2 Pipet acetat buffer and substrate (TMB) solution

Pipette 50 µl sodium acetate buffer in each well on the side of the blank (for the soil) (well 4-6; well 10-12)

Pipette 50 µl substrate in all "sample wells" (well 1-3; well 7-9) of the first microplate, note time and start the measurement, then pipette substrate in second microplate…

**Per-& Phenoloxidase** **Phenoloxidase**

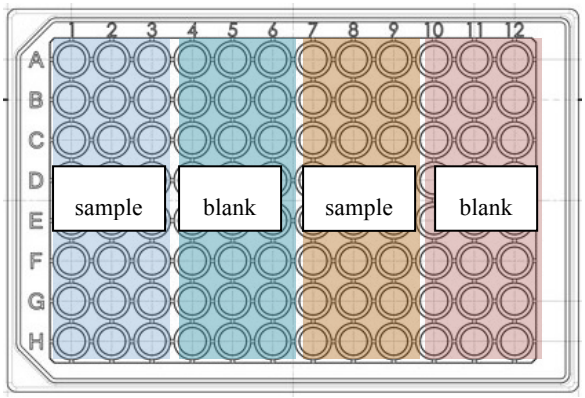
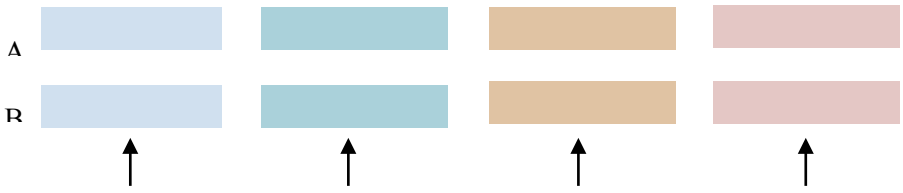
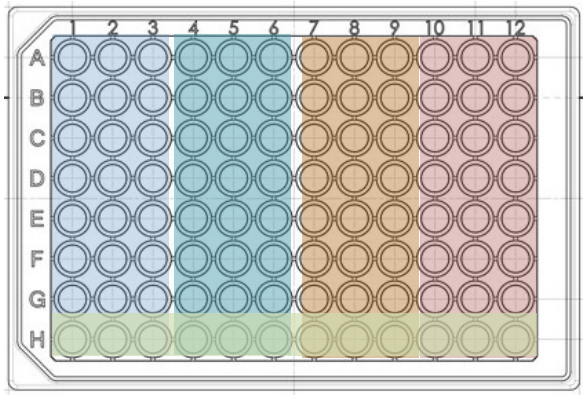



**Plate 1:** A-H: soil sample 1 - 4




| | | | | |
|---|---|---|---|---|
| soil [µl] | 200 | 200 | 200 | 200 |
| Buffer [µl] | - | 50 | - | 50 |
| $H_2O_2$ [µl] | 10 | 10 | - | - |
| Substrate[µl] | 50 | - | 50 | - |

**Per-& Phenoloxidase** **Phenoloxidase**




negative control

**Plate 2:** A-G: soil samples, H: negative control (buffer and substrate only)

| | H | | | |
|---|---|---|---|---|
| soil [µl] | - | - | - | - |
| Buffer [µl] | 200 | 200 | 200 | 200 |
| $H_2O_2$ [µl] | 10 | 10 | - | - |
| Substrate[µl] | 50 | 50 | 50 | 50 |

## 5.5 Measurement

For the measurement use the Tecan microplate reader. Open the software i-control 1.5 on the desktop. Open scripts: phenol-peroxidase.

The measurement of the microplates takes place at point 0 and another measurement follows after every 15 minutes (point 0, 15, 30, 45, 60 minutes).

## 6. Remarks

## 7. References

Summarized by Jessica Heublein and Theresa Klötzing 2016

Most recent amendment September 2016

*2. pH*

**Table 8: Table of pH in every single sample, EL and EH are $eCO_2$, planted soils, AL and AH are $aCO_2$, planted soils ES are $eCO_2$ bare soils and AS are $aCO_2$, bare soils**

| sample | pH | sample | pH |
|---|---|---|---|
| EL2a | 3,53 | AS1a | 3,56 |
| EL2b | 3,48 | AS1b | 3,52 |
| EL2c | 3,48 | AS1c | 3,58 |
| EL2d | 3,4 | AS1d | 3,57 |
| EH2a | 3,55 | AS2a | 3,59 |
| EH2b | 3,54 | AS2b | 3,63 |
| EH2c | 3,53 | AS2c | 3,52 |
| EH2d | 3,54 | AS2d | 3,54 |
| EL3a | 3,48 | ES1a | 3,57 |
| EL3b | 3,54 | ES1b | 3,58 |
| EL3c | 3,44 | ES1c | 3,56 |
| EL3d | 3,51 | ES1d | 3,6 |
| EH3a | 3,52 | ES2a | 3,58 |
| EH3b | 3,52 | ES2b | 3,56 |
| EH3c | 3,52 | ES2c | 3,59 |
| EH3d | 3,43 | ES2d | 3,59 |
| AL2a | 3,48 | | |
| AL2b | 3,54 | | |
| AL2c | 3,55 | | |
| AL2d | 3,47 | | |
| AH2a | 3,53 | | |
| AH2b | 3,46 | | |
| AH2c | 3,49 | | |
| AH2d | 3,44 | | |
| AL3a | 3,5 | | |
| AL3b | 3,46 | | |
| AL3c | 3,5 | | |
| AL3d | 3,47 | | |
| AH3a | 3,56 | | |
| AH3b | 3,55 | | |
| AH3c | 3,57 | | |
| AH3d | 3,54 | | |

**Table 9: Test of normal distribution and Wilcoxon Rank Sum test**

| | p norm | p-value |
|---|---|---|
| **$aCO_2$, tree** | $2{,}576*10^{-14}$ | 0.7192 |
| **$eCO_2$, tree** | $2{,}587*10^{-14}$ | 0.2886 |
| **$aCO_2$, bare** | $2{,}266*10^{-7}$ | $9.534*10^{-5}$ |
| **$eCO_2$, bare** | $2{,}264*10^{-7}$ | 0.009085 |

*3. Soil moisture*

**Table 10: Dry substance [%] of every single sample**

| sample | %TS | sample | %TS |
|---|---|---|---|
| EL2a | 81,89 | AS1a | 91,92 |
| EL2b | 86,69 | AS1b | 92,99 |
| EL2c | 87,59 | AS1c | 94,76 |
| EL2d | 88,10 | AS1d | 93,22 |
| EH2a | 92,00 | AS2a | 92,96 |
| EH2b | 90,11 | AS2b | 89,17 |
| EH2c | 88,34 | AS2c | 85,75 |
| EH2d | 88,83 | AS2d | 92,39 |
| EL3a | 89,33 | ES1a | 91,72 |
| EL3b | 86,49 | ES1b | 93,35 |
| EL3c | 90,50 | ES1c | 90,87 |
| EL3d | 86,92 | ES1d | 95,26 |
| EH3a | 84,18 | ES2a | 88,01 |
| EH3b | 82,61 | ES2b | 87,99 |
| EH3c | 89,09 | ES2c | 86,98 |
| EH3d | 90,21 | ES2d | 89,78 |
| AL2a | 90,96 | 2016/1 | 83,68 |
| AL2b | 89,06 | 2016/2 | 83,97 |
| AL2c | 86,79 | | |
| AL2d | 81,27 | | |
| AH2a | 91,37 | | |
| AH2b | 91,26 | | |
| AH2c | 85,70 | | |
| AH2d | 89,46 | | |
| AL3a | 88,07 | | |
| AL3b | 84,04 | | |
| AL3c | 87,27 | | |
| AL3d | 83,23 | | |
| AH3a | 88,35 | | |
| AH3b | 91,88 | | |
| AH3c | 93,18 | | |
| AH3d | 90,40 | | |

**Table 11: Mean values and standard deviation of the dry substance for each treatment**

| | $eCO_2$_tree | $aCO_2$_tree | $eCO_2$_bare | $aCO_2$_bare |
|---|---|---|---|---|
| **MW [%TS]** | 87,68 | 88,27 | 90,50 | 91,65 |
| **SD** | 2,83 | 3,38 | 2,87 | 2,86 |

*4. Tables of enzyme activties*

**Table 12: Potential enzyme activities of all samples as mean values of the six repetitions [nmol*g$^{-1}$ TB*h$^{-1}$]**

| sample | GLU | N-AC | SULF | PHOS | PER | PHEN |
|---|---|---|---|---|---|---|
| **AH2a** | 127,51 | 96,54 | 38,31 | 1652,05 | 0,59 | 0,69 |
| **AH2b** | 127,58 | 153,08 | 25,99 | 1873,82 | 0,49 | 0,61 |
| **AH2c** | 237,01 | 247,84 | 39,18 | 2373,19 | 0,66 | 0,47 |
| **AH2d** | 114,20 | 128,50 | 29,01 | 1963,17 | 0,62 | 0,34 |
| **AH3a** | 198,51 | 382,31 | 50,96 | 2262,01 | 0,66 | 0,67 |
| **AH3b** | 146,83 | 129,78 | 33,80 | 1480,41 | 0,84 | 0,36 |
| **AH3c** | 116,28 | 177,64 | 41,26 | 1967,96 | 0,76 | 0,24 |
| **AH3d** | 127,38 | 211,47 | 40,67 | 2361,81 | 0,32 | 0,77 |
| **AL2a** | 110,25 | 167,90 | 29,01 | 1485,34 | 0,71 | 0,34 |
| **AL2b** | 84,18 | 118,27 | 31,58 | 1360,05 | 0,81 | 0,35 |
| **AL2c** | 170,20 | 220,31 | 43,20 | 2249,74 | 0,83 | 0,37 |
| **AL2d** | 174,45 | 393,70 | 50,10 | 2006,36 | 1,02 | 0,26 |
| **AL3a** | 169,84 | 203,56 | 42,84 | 2953,70 | 0,49 | 0,64 |
| **AL3b** | 167,63 | 259,73 | 32,21 | 2198,32 | 0,44 | 0,52 |
| **AL3c** | 117,83 | 274,39 | 26,53 | 1679,56 | 0,34 | 0,53 |
| **AL3d** | 146,47 | 323,92 | 40,64 | 2315,33 | 0,71 | 0,35 |
| **EH2a** | 115,53 | 141,61 | 46,02 | 1789,98 | 0,87 | 0,58 |
| **EH2b** | 101,62 | 138,65 | 36,36 | 1635,90 | 1,36 | 0,41 |
| **EH2c** | 113,34 | 135,16 | 39,77 | 2507,99 | 0,86 | 0,25 |
| **EH2d** | 110,31 | 193,66 | 42,37 | 2254,07 | 0,99 | 0,17 |
| **EH3a** | 176,34 | 283,64 | 36,91 | 1783,90 | 0,78 | 0,88 |
| **EH3b** | 179,87 | 267,87 | 41,88 | 1977,02 | 0,58 | 1,10 |
| **EH3c** | 162,31 | 234,33 | 42,53 | 2455,93 | 0,82 | 0,96 |
| **EH3d** | 146,18 | 186,05 | 30,10 | 2203,98 | 1,05 | 0,82 |
| **EL2a** | 167,39 | 204,27 | 41,71 | 1966,18 | 1,16 | 0,54 |
| **EL2b** | 118,67 | 133,84 | 30,57 | 1949,49 | 0,78 | 0,45 |
| **EL2c** | 205,80 | 183,17 | 47,70 | 2730,19 | 0,61 | 0,40 |
| **EL2d** | 140,72 | 173,43 | 26,62 | 1409,12 | 0,52 | 0,40 |
| **EL3a** | 199,04 | 250,48 | 43,17 | 4268,11 | 0,96 | 0,13 |
| **EL3b** | 137,10 | 175,65 | 37,60 | 3311,03 | 0,43 | 0,60 |
| **EL3c** | 255,59 | 370,65 | 55,32 | 7454,75 | 0,66 | 0,27 |
| **EL3d** | 190,40 | 278,45 | 43,44 | 4388,79 | 0,76 | 0,17 |
| **AS1a** | 195,83 | 229,15 | 55,38 | 1223,80 | 0,64 | 0,73 |
| **AS1b** | 161,79 | 156,80 | 44,22 | 1146,35 | 0,83 | 0,36 |
| **AS1c** | 155,28 | 183,84 | 56,27 | 1279,82 | 0,60 | 0,42 |
| **AS1d** | 199,73 | 191,68 | 48,25 | 1347,28 | 0,90 | 0,20 |
| **AS2a** | 136,23 | 171,89 | 36,76 | 1602,95 | 0,72 | 0,24 |
| **AS2b** | 157,36 | 158,35 | 40,50 | 1635,87 | 0,81 | 0,30 |
| **AS2c** | 145,81 | 163,22 | 29,60 | 1667,25 | 0,54 | 0,26 |
| **AS2d** | 172,47 | 177,24 | 35,55 | 1927,53 | 0,59 | 0,28 |
| **ES1a** | 118,07 | 144,62 | 33,37 | 1703,84 | 0,57 | 0,17 |
| **ES1b** | 166,37 | 186,49 | 32,64 | 1956,43 | 0,50 | 0,30 |
| **ES1c** | 123,86 | 168,42 | 30,77 | 1565,96 | 0,67 | 0,18 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **ES1d** | 107,05 | 130,59 | 33,97 | 1519,12 | 0,59 | 0,26 |
| **ES2a** | 137,64 | 161,35 | 53,69 | 2373,34 | 0,41 | 0,50 |
| **ES2b** | 124,10 | 162,36 | 49,39 | 1961,21 | 0,95 | 0,65 |
| **ES2c** | 130,62 | 265,05 | 45,26 | 2119,71 | 1,01 | 0,21 |
| **ES2d** | 117,54 | 148,39 | 38,75 | 2088,92 | 1,15 | 0,30 |

**Table 13: Values of normal distribution of the enzyme activities**

| treatment | Glu | N-Ac | Sulf | Phos | Per | Phenol |
|---|---|---|---|---|---|---|
| **$aCO_2$, tree** | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $1{,}52*10^{-6}$ | $6{,}197*10^{-6}$ |
| **$eCO_2$, tree** | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $1{,}495*10^{-7}$ | $4{,}429*10^{-5}$ |
| **$aCO_2$, bare** | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | 0,00015 | 0,00442 |
| **$eCO_2$, bare** | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | $2{,}2*10^{-16}$ | 0,00059 | 0,00598 |

**Table 14: p-values of the enzyme activities between all four treatments**

| treatment | Glu | N-Ac | Sulf | Phos | Per | Phenol |
|---|---|---|---|---|---|---|
| **tree** | 0,539 | 0,956 | 0,254 | 0,171 | 0,043 | 0,897 |
| **bare** | 0,005 | 0,279 | 0,442 | 0,015 | 0,879 | 0,645 |
| **$eCO_2$** | 0,136 | 0,153 | 0,881 | 0,106 | 0,383 | 0,192 |
| **$aCO_2$** | 0,153 | 0,452 | 0,172 | 0,002 | 0,528 | 0,106 |

*5. Tables of $C_{mic}$ and specific activities*

**Table 15: $C_{mic}$ and specific enzyme activities [nmol*$g^{-1}$ Cmic*$g^{-1}$ TB*$h^{-1}$]**

| sample | $C_{mic}$ | $GLU_{spec}$ | $N\text{-}AC_{spec}$ | $SULF_{spec}$ | $PHOS_{spec}$ | $PER_{spec}$ | $PHEN_{spec}$ |
|---|---|---|---|---|---|---|---|
| **AH2a** | 114,219 | 1,12 | 0,85 | 0,34 | 14,46 | 0,0051 | 0,0060 |
| **AH2b** | 185,568 | 0,69 | 0,82 | 0,14 | 10,10 | 0,0026 | 0,0033 |
| **AH2c** | 163,643 | 1,45 | 1,51 | 0,24 | 14,50 | 0,0041 | 0,0029 |
| **AH2d** | 183,913 | 0,62 | 0,70 | 0,16 | 10,67 | 0,0034 | 0,0019 |
| **AH3a** | 134,091 | 1,48 | 2,85 | 0,38 | 16,87 | 0,0049 | 0,0050 |
| **AH3b** | 102,416 | 1,43 | 1,27 | 0,33 | 14,45 | 0,0082 | 0,0035 |
| **AH3c** | 124,095 | 0,94 | 1,43 | 0,33 | 15,86 | 0,0061 | 0,0019 |
| **AH3d** | 140,925 | 0,90 | 1,50 | 0,29 | 16,76 | 0,0022 | 0,0055 |
| **AL2a** | 186,607 | 0,59 | 0,90 | 0,16 | 7,96 | 0,0038 | 0,0018 |
| **AL2b** | 142,617 | 0,59 | 0,83 | 0,22 | 9,54 | 0,0057 | 0,0025 |
| **AL2c** | 175,506 | 0,97 | 1,26 | 0,25 | 12,82 | 0,0047 | 0,0021 |
| **AL2d** | 169,314 | 1,03 | 2,33 | 0,30 | 11,85 | 0,0060 | 0,0015 |
| **AL3a** | 183,272 | 0,93 | 1,11 | 0,23 | 16,12 | 0,0027 | 0,0035 |
| **AL3b** | 199,803 | 0,84 | 1,30 | 0,16 | 11,00 | 0,0022 | 0,0026 |
| **AL3c** | 221,194 | 0,53 | 1,24 | 0,12 | 7,59 | 0,0016 | 0,0024 |
| **AL3d** | 192,599 | 0,76 | 1,68 | 0,21 | 12,02 | 0,0037 | 0,0018 |
| **EH2a** | 129,354 | 0,89 | 1,09 | 0,36 | 13,84 | 0,0067 | 0,0045 |
| **EH2b** | 115,662 | 0,88 | 1,20 | 0,31 | 14,14 | 0,0117 | 0,0036 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **EH2c** | 141,142 | 0,80 | 0,96 | 0,28 | 17,77 | 0,0061 | 0,0018 |
| **EH2d** | 141,477 | 0,78 | 1,37 | 0,30 | 15,93 | 0,0070 | 0,0012 |
| **EH3a** | 146,342 | 1,20 | 1,94 | 0,25 | 12,19 | 0,0054 | 0,0060 |
| **EH3b** | 177,172 | 1,02 | 1,51 | 0,24 | 11,16 | 0,0033 | 0,0062 |
| **EH3c** | 145,166 | 1,12 | 1,61 | 0,29 | 16,92 | 0,0056 | 0,0066 |
| **EH3d** | 206,630 | 0,71 | 0,90 | 0,15 | 10,67 | 0,0051 | 0,0040 |
| **EL2a** | 142,517 | 1,17 | 1,43 | 0,29 | 13,80 | 0,0081 | 0,0038 |
| **EL2b** | 168,024 | 0,71 | 0,80 | 0,18 | 11,60 | 0,0047 | 0,0027 |
| **EL2c** | 179,732 | 1,15 | 1,02 | 0,27 | 15,19 | 0,0034 | 0,0022 |
| **EL2d** | 217,494 | 0,65 | 0,80 | 0,12 | 6,48 | 0,0024 | 0,0019 |
| **EL3a** | 192,118 | 1,04 | 1,30 | 0,22 | 22,22 | 0,0050 | 0,0007 |
| **EL3b** | 160,435 | 0,85 | 1,09 | 0,23 | 20,64 | 0,0027 | 0,0037 |
| **EL3c** | 234,890 | 1,09 | 1,58 | 0,24 | 31,74 | 0,0028 | 0,0011 |
| **EL3d** | 197,491 | 0,96 | 1,41 | 0,22 | 22,22 | 0,0038 | 0,0009 |
| **AS1a** | 98,614 | 1,99 | 2,32 | 0,56 | 12,41 | 0,0065 | 0,0074 |
| **AS1b** | 103,274 | 1,57 | 1,52 | 0,43 | 11,10 | 0,0080 | 0,0035 |
| **AS1c** | 82,379 | 1,89 | 2,23 | 0,68 | 15,54 | 0,0073 | 0,0050 |
| **AS1d** | 96,235 | 2,08 | 1,99 | 0,50 | 14,00 | 0,0093 | 0,0021 |
| **AS2a** | 117,745 | 1,16 | 1,46 | 0,31 | 13,61 | 0,0061 | 0,0021 |
| **AS2b** | 136,220 | 1,16 | 1,16 | 0,30 | 12,01 | 0,0060 | 0,0022 |
| **AS2c** | 98,424 | 1,48 | 1,66 | 0,30 | 16,94 | 0,0055 | 0,0027 |
| **AS2d** | 99,610 | 1,73 | 1,78 | 0,36 | 19,35 | 0,0059 | 0,0028 |
| **ES1a** | 114,524 | 1,03 | 1,26 | 0,29 | 14,88 | 0,0050 | 0,0015 |
| **ES1b** | 110,500 | 1,51 | 1,69 | 0,30 | 17,71 | 0,0045 | 0,0028 |
| **ES1c** | 120,299 | 1,03 | 1,40 | 0,26 | 13,02 | 0,0056 | 0,0015 |
| **ES1d** | 103,567 | 1,03 | 1,26 | 0,33 | 14,67 | 0,0057 | 0,0025 |
| **ES2a** | 112,223 | 1,23 | 1,44 | 0,48 | 21,15 | 0,0037 | 0,0045 |
| **ES2b** | 112,223 | 1,11 | 1,45 | 0,44 | 17,48 | 0,0085 | 0,0058 |
| **ES2c** | 112,223 | 1,16 | 2,36 | 0,40 | 18,89 | 0,0090 | 0,0019 |
| **ES2d** | 112,223 | 1,05 | 1,32 | 0,35 | 18,61 | 0,0102 | 0,0027 |

**Table 16: Test of normal distribution of $C_{mic}$ and specific enzyme activities**

| | $C_{mic}$ | $GLU_{spec}$ | $N\text{-}AC_{spec}$ | $SULF_{spec}$ | $PHOS_{spec}$ | $PER_{spec}$ | $PHEN_{spec}$ |
|---|---|---|---|---|---|---|---|
| $aCO_2$, tree | $2.2*10^{-16}$ | $1.846*10^{-8}$ | $4.604*10^{-10}$ | $4.943*10^{-5}$ | $2.2*10^{-16}$ | 0.00031 | 0.00031 |
| $eCO_2$, tree | $2.2*10^{-16}$ | $1.491*10^{-9}$ | $4.725*10^{-11}$ | $4.747*10^{-5}$ | $2.2*10^{-16}$ | 0.00031 | 0.00032 |
| $eCO_2$, bare | $2.2*10^{-16}$ | $5.58*10^{-7}$ | $2.667*10^{-8}$ | 0.002718 | $2.2*10^{-16}$ | 0.02199 | 0.02235 |
| $aCO_2$, bare | $2.251*10^{-7}$ | $1.119*10^{-7}$ | $1.016*10^{-7}$ | 0.001848 | $2.2*10^{-16}$ | 0.02169 | 0.02225 |

**Table 17: p-values between all four treatments of $C_{mic}$ and specific enzyme activities**

| | $C_{mic}$ | $GLU_{spec}$ | $N\text{-}AC_{spec}$ | $SULF_{spec}$ | $PHOS_{spec}$ | $PER_{spec}$ | $PHEN_{spec}$ |
|---|---|---|---|---|---|---|---|
| **tree** | 0.7804 | 0.5641 | 0.9556 | 0.7804 | 0.1016 | 0.2099 | 0.8965 |
| **bare** | 0.08086 | 0.006993 | 0.1605 | 0.2345 | 0.0829 | 0.4418 | 0.4418 |
| **$eCO_2$** | 0.0001252 | 0.03243 | 0.12 | 0.002785 | 0.3503 | 0.2381 | 0.881 |
| **$aCO_2$** | 0.00012 | $8.158*10^{-5}$ | 0.03243 | 0.0009436 | 0.2636 | 0.001656 | 0.4523 |

**Table 18: Coefficient of determination of the correlations of $C_{mic}$ with each enzyme activity**

| enzyme | treatment | $R^2$ | enzyme | treatment | $R^2$ |
|---|---|---|---|---|---|
| **GLU** | $aCO_2$, tree | 0.004886 | **PHOS** | $aCO_2$, tree | 0.06031 |
| | $aCO_2$, bare | 0.09558 | | $aCO_2$, bare | 0.1222 |
| | $eCO_2$, tree | 0.4385 | | $eCO_2$, tree | 0.3528 |
| | $eCO_2$, bare | 0.01041 | | $eCO_2$, bare | 0.00003 |
| **N-AC** | $aCO_2$, tree | 0.1005 | **PER** | $aCO_2$, tree | 0.1219 |
| | $aCO_2$, bare | 0.1757 | | $aCO_2$, bare | 0.1647 |
| | $eCO_2$, tree | 0.2847 | | $eCO_2$, tree | 0.2339 |
| | $eCO_2$, bare | 0.03248 | | $eCO_2$, bare | 0.008078 |
| **SULF** | $aCO_2$, tree | 0.129 | **PHEN** | $aCO_2$, tree | 0.003198 |
| | $aCO_2$, bare | 0.1762 | | $aCO_2$, bare | 0.05783 |
| | $eCO_2$, tree | 0.001681 | | $eCO_2$, tree | 0.02922 |
| | $eCO_2$, bare | 0.008007 | | $eCO_2$, bare | 0.02762 |

# Statement of authorship

I hereby declare that I am the sole author of this bachelor thesis and that I have not used any sources, than those, listed in the references. It has not been accepted in any previous application for a degree.

Ruth Adamczewski

Jena, 24.07.2017

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