Department of Environmental Chemistry

-Faculty of Organic Agricultural Sciences-

University of Kassel

Effect of environmental conditions on the N uptake route of soil microorganisms and adaption of a method to determine amino acid oxidase in soil

Dissertation

submitted to the Faculty of Organic Agricultural Sciences of the

University of Kassel

to fulfill the requirements for the degree "Doktorin der Agrarwissenschaften"

(Dr. agr.)

by Dipl. Agr. Biol.

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Witzenhausen, Februar 2015

This work has been accepted by the Faculty of Organic Agricultural Sciences of the University of Kassel as a thesis for acquiring the academic degree of Doktor der Agrarwissenschaften (Dr. agr.).

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Defence day: 28.04.2015

Preface

This work is submitted to the Faculty of Organic Agricultural Sciences at the University of Kassel to fulfill the requirements for the degree “Doktorin der Agrarwissenschaften” (Dr. agr.) and was prepared within the DFG Project: “Nitrogen utilization pathways of soil microorganisms”.

The dissertation is on the basis of two scientific publications as first author, which are submitted in international refereed journals. The papers are included in chapter 3 and 4. The development of a method to measure the activity of the extracellular enzyme L-amino acid oxidase is given in chapter 5.

A general introduction is given in chapter 1. Research objectives are included in chapter 2, and a general conclusion is drawn in chapter 6. Chapter 7 contains a summary of the entire thesis and chapter 8 contains a summary in German language.

The following papers are included in this thesis:

Chapter 3:

Pinggera, J., Geisseler, D., Merbach, I., Joergensen, R. G., Ludwig, B. (2015): Effect of substrate quality on the N uptake routes of soil microorganisms in an incubation experiment. European Journal of Soil Biology (submitted).

Chapter 4:

Pinggera, J., Geisseler, D., Piepho, H.-P., Joergensen, R. G., Ludwig, B. (2015): Effect of substrate quality on the N uptake routes of soil microorganisms in different soil depths. Pedobiologia (submitted).

Chapter 5:

Pinggera, J., Geisseler, D., Joergensen, R.G., Ludwig, B.: Extracellular deamination of amino acids in soil – Adaption of a method to detect amino acid oxidase in soil.

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List of abbreviations

C	Carbon
$CHCl_3$	Chloroform
CH_3OH	Methanol
CO_2	Carbon dioxide
FYM	Farmyard manure
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
L-AAO	L-Amino acid oxidase
LYA	Lucifer Yellow Anhydride
MIT route	Mineralization-immobilization turnover route
N	Nitrogen
NaOH	Sodium hydroxide
NH_4^+	Ammonium
$(NH_4)_2SO_4$	Ammoniumsulfat
NO_3^-	Nitrate
NPK	Nitrogen phosphate potassium
rpm	revolutions per minute
SOC	Soil organic carbon
THAM	Tris (hydroxymethyl) aminomethane
WHC	Water holding capacity

1. General introduction

Nitrogen (N) is an essential nutrient for plants and soil microorganisms. N is generally of limited availability to plants in terrestrial ecosystems, resulting in strong competition between microorganisms and plants (*Vitousek* and *Howarth*, 1991). For understanding and managing ecosystem productivity, it is essential to understand N transformations and the soil microorganisms that perform them. Microorganisms obtain energy and carbon (C) to support their growth through the decomposition of organic material. Moreover they have a need for nutrients, especially N, to build proteins, nucleic acids, and other cellular components.

The first step for the degradation of organic material is made by extracellular enzymes, which are proteins which can catalyze biochemical reactions. Extracellular enzymes break down large polymeric compounds and release soluble amino acids, small peptides or amino sugars. The distribution of total N in humic substances and soils has been reported to consist of about 40% proteinaceous materials (proteins and peptides) (*Schulten* and *Schnitzer*, 1998). Proteins as high molecular weight compounds cannot be utilized directly from the soil microorganisms (*Wanek* et al., 2010). First they have to be depolymerized by extracellular enzymes, more precisely by proteases, which are secreted into the soil by microorganisms such as bacteria and fungi (*Glenn*, 1976; *Fuka* et al., 2007). The final products of protein degradation are free amino acids, which are an important source of N and C for soil microorganisms (*Rothstein*, 2010).

Soil enzymes play a key role in nutrient cycling, they are essential for energy transformation and they regulate ecosystem functions (*Kandeler*, 2007; *Makoi* and *Ndakidemi*, 2008). Microorganisms have to invest energy in the synthesis of enzymes, e.g. extracellular enzymes, to gain nutrients that they need while decomposing substrates of low quality. In organic material with a low NH_4^+ availability, for example in organic material with a wide C to N ratio (C to N ratio > 25), microorganisms must take up additional N from their environment, which is called N immobilization. However, if the organic material is rich in N (C to N ratio < 25), the requirement

of microorganisms for N is easily met and they release inorganic forms of N to the soil solution, which is called N mineralization (*Robertson* and *Groffman*, 2007).

Soil microorganisms are able to take up a wide range of N compounds, for example NH_4^+, NO_3^-, and organic molecules, like amino acids or small peptides (*Merrick* and *Edwards*, 1995; *Marzluf*, 1997). For organic N uptake they have evolved two different mechanisms, the direct route and the mineralization-immobilization-turnover (MIT) route (*Barraclough* et al., 1997). It was reported that both uptake routes were active simultaneously, but the direct route was preferred (*Luxhøi* et al., 2006, *Geisseler* et al., 2009).

In the direct route, microorganisms take up simple organic molecules via different mechanisms directly into the cell. The deamination occurs inside the cell, and only surplus N is released back to the soil solution. Microorganisms provide a wide range of transport enzymes, for example, the uptake of amino acids into cells is catalyzed by functionally specific transport systems. The lack of C, N, or S results in the activation of the synthesis of amino acid transport systems, whereas ammonium and high intracellular concentrations of amino acids result in a repression (*Geisseler* et al., 2010). Thus, under N starvation the direct uptake route should become more important.

In the MIT route all organic N is first mineralized to NH_4^+ before it is taken up by microorganisms. Extracellular enzymes, like L-amino acid oxidases (EC 1.4.3.2; L-AAO) deaminate organic molecules outside the cell, and the N produced is taken up into the cell (*Hadas* et al., 1992). L-amino acid oxidases catalyze the oxidative deamination of L-amino acids to their corresponding oxo acids and ammonia (*Pantoja* et al., 1993, *Pantoja* and *Lee*, 1994). Therefore, L-amino acid oxidase may significantly contribute to the extracellular N mineralization in soil and is a key reaction of the MIT route. In order to assure reliable results, a method for the determination of this enzyme activity of amino acids oxidase should be developed. For the development of a valid enzyme assay it is necessary to consider different factors: moisture level

of the soil (air-dried or field-moist), buffer pH, substrate concentration, amount of soil, incubation time or incubation temperature are factors which should be considered (*Dick,* 2011).

Geisseler et al. (2010) hypothesized that the relative importance of the direct and MIT route during the decomposition of organic material is determined by the form of available N, the source of C, and the availability of N relative to C. Ammonium plays a key role in the regulation of enzyme systems involved in the acquisition of N. At high concentrations of NH_4^+ the enzyme systems for the utilization of alternative N sources are repressed and the MIT route should be the dominant uptake route. In contrast, at low concentrations of NH_4^+, the enzyme systems for the uptake of other N sources are de-repressed and the direct route should be favored.

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2. Research objectives

A better understanding of the mechanisms controlling the different uptake routes of soil microorganisms under different environmental conditions is crucial for understanding mineralization processes of organic material in soil. Quantitative data on the different N uptake routes for different substrates and substrate qualities are scarce and the combined effect of substrates and mineral N on the uptake routes is not sufficiently understood. Thus, this thesis focuses on the investigation of the effect of substrates, mineral N and soil depth on the N uptake routes of soil microorganisms. Furthermore, the development of a method to measure the activity of the extracellular enzyme L-amino acid oxidase should provide information about this key reaction of the MIT route.

The research objectives of this thesis were to:

(i) study the effect of substrate quality of corn with different C to N ratios in the presence and absence of mineral N on the N uptake routes of soil microorganisms in an incubation experiment.

(ii) investigate the effects of soil depth on the microbial community and the N uptake route of soil microorganisms following the addition of corn residues with different C to N ratios in the presence and absence of mineral N.

(iii) develop a method which allows the use of Lucifer Yellow Anhydride (LYA) derivatives of the amino acid lysine for the detection of the extracellular enzyme amino acid oxidase in soil.

3. Effect of substrate quality on the N uptake routes of soil microorganisms in an incubation experiment

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3.1 Abstract

Microorganisms may either directly take up organic N or use the mobilization-immobilization-turnover (MIT) route to satisfy their N demand. However, there is only little information available about the factors controlling these two different uptake routes. The objective was to investigate the effects of substrate quality of corn with different C to N ratios in the presence and absence of mineral N on the N uptake route of soil microorganisms. Soil from the Static Fertilization Experiment Bad Lauchstädt was incubated at 20 °C and 60% of its water-holding capacity. The following treatments were used: no addition (control (C to N ratio = 13), treatment I), addition of corn residues with a C to N ratio of 40 (II), 20 (III), and 40 & $(NH_4)_2SO_4$ (IV). We studied the utilization of added amino acids in order to distinguish between the N uptake routes. The mineralization rate of amino acids increased in the order “addition of corn residues with a wide C to N ratio” (4% of added amino acids) < “corn residues with a small C to N ratio” (27%) < “corn

residues with a wide C to N ratio & $(NH_4)_2SO_4$" and "control treatment" (31% and 32%, respectively) after 21 days of incubation. This means that, under the assumption that all the amino acids were utilized, 96% (treatment II), 73% (III), 69 (IV), and 68% (I) of the added amino acids were taken up directly. These results indicate that the direct route is especially important for substrates with wide C to N ratios in the absence of mineral N (thus for conditions of high C availability). The proportion of added amino acids mineralized increased with time in all treatments, indicating that the MIT route became more important over time. Overall, the results show that the composition of the residue and the presence or absence of mineral N had a marked effect on the N uptake route of soil microorganisms.

3.2 Introduction

Soil microorganisms have evolved two possible mechanisms for their uptake of organic N: the direct route and the mobilization-immobilization-turnover (MIT) route (*Hadas* et al., 1992). In the direct route, simple organic molecules are taken up via various mechanisms directly into the cell. The deamination occurs inside the cell and only the surplus N is released into the soil ammonium (NH_4^+) pool. The advantage of the direct uptake of amino acids is the energy saved that would otherwise be required for synthesizing C structural components (*Geisseler* et al., 2010). In the MIT route, the deamination occurs outside the cell and all N is mineralized to NH_4^+ before assimilation (*Barak* et al., 1990*, Manzoni* and *Porporato,* 2007). An advantage of the MIT route is that only one transporter system for N uptake is required (*Geisseler* et al., 2010).

The first step of both routes is the breaking down of polymeric N compounds by extracellular depolymerases in soil (e.g., protease) and the release of soluble amino acids, small peptides or amino sugars (*Geisseler* et al., 2010). When the MIT route is dominant, protease activity and gross N mineralization rates may be highly correlated since the products of protease activity are the substrates for N mineralization.

The interpretation of gross N mineralization (net mineralization plus immobilization), depends on which N uptake route (either direct uptake of organic N or the MIT route) is dominant. Gross N mineralization is measured by the ^{15}N isotope pool dilution technique and it is defined as the release of NH_4^+ into soil solution. Briefly, the ^{15}N isotope pool dilution method employs the measurement of gross mineralization by enriching the soil NH_4^+ pool with ^{15}N to increase the ^{15}N enrichment above natural abundance. Then the change of size of the NH_4^+ pool and the dilution of the ^{15}N-enrichment (due to release of NH_4^+ in natural abundance coming from the mineralization of soil organic matter) in this pool can be traced (*Kirkham* and *Bartholomew*, 1954; *Murphy* et al. 2003).

If only the MIT route is operative, all the N mineralized by the soil microorganisms passes through the NH_4^+ pool and becomes visible by isotope pool dilution. This implies that gross N mineralization is equivalent to the total amount of bioavailable N. If, however, only the direct route is operative only the surplus N released from cells is determined by isotope pool dilution and this can lead to an underestimation of N bioavailability (*Watkins* and *Barraclough* 1996, *Barraclough*, 1997, *Tahovská* et al., 2013).

One option to distinguish between the N uptake routes is to look at the utilization of amino acids. Amino acids are used as N and C sources by soil microorganisms (*Rothstein*, 2010). Amino acids can be mineralized or taken up directly by soil microorganisms. The importance of the different N uptake routes can be determined measuring gross N mineralization from added amino acids. If the proportion of mineralized amino acid N is high and all the added amino acids were utilized, then the MIT route is likely to be the dominant uptake route. In contrast, if all the added amino acids were utilized, but the proportion of the mineralized amino acid N is low then the direct route must be the dominant one.

Several studies indicated that generally both uptake routes are simultaneously active (*Luxhøi* et al., 2006; *Geisseler* et al., 2009). However, the relative importance of the different uptake routes

may depend on environmental conditions. *Geisseler* et al. (2010) suggested that the most important factors are the form of N available, the source of C, and the availability of N relative to C: (i) Under conditions of high availability of NH_4^+, the direct route may be insignificant, whereas at low availability of NH_4^+, the direct route becomes dominant. (ii) When most of the available C consists of N-containing compounds, such as amino acids and amino sugars, the importance of the direct route should increase, because microorganisms have to meet their C and N demand simultaneously by taking up organic N molecules. However, when carbohydrates or other readily available C sources are available, the MIT route may be favored again. (iii) When the ratio between available C to N is high, N becomes limiting relative to C and N immobilization occurs. Thus, the availability of NH_4^+ decreases and the direct route should therefore be favored over the MIT route. In contrast, when C becomes limiting relative to N, and when microorganisms use large amounts of N containing molecules as C sources, excess N is released as NH_3, which results in a shift from the direct to the MIT route (*Geisseler* et al., 2010).

A short-term microcosm study with application of wheat residues to a silty clay loam soil indicated that 55 and 62% of N was taken up via the direct route from young (C to N ratio of 12) and old wheat residue (C to N ratio of 29), respectively (*Geisseler* et al., 2009). However, quantitative data on the different routes for different substrates and different substrate qualities are scarce (*Geisseler* et al., 2009) and the combined effect of substrates and mineral N on the uptake routes is not sufficiently understood.

Our objective was to investigate the effects of corn residues with different C to N ratios in the presence and absence of mineral N on the N uptake route of soil microorganisms. We hypothesized that the importance of the direct uptake increases with increasing C to N ratios and small availability of mineral N.

3.3 Materials and Methods

3.3.1 Site description

The incubation experiment was carried out with soil from the 'Static Fertilization Experiment' in Bad Lauchstädt (Germany) with an annual mean precipitation of 486 mm and an annual mean temperature of 8.8 °C (average 1896 – 2008). The soil in the long-term trial is a Haplic Chernozem (FAO) with 68% silt, 21% clay, and 11% sand. The crop rotation consists of winter wheat (*Triticum aestivum* L.), sugar beet (*Beta vulgaris* L.), spring barley (*Hordeum vulgare* L.), and potato (*Solanum tuberosum* L.) (*Böhme* and *Böhme*, 2006). For the experiment, soil from three different plots was used: plot 6 treated with 30 t ha^{-1} farmyard manure every second year, plot 13 fertilized with mineral NPK fertilizer (*T. aestivum* 100 kg ha^{-1} N; *B. vulgaris* 170 kg ha^{-1} N, 60 kg ha^{-1} P, 230 kg ha^{-1} K; *H. vulgare* 80 kg ha^{-1} N; *S. tuberosum* 140 kg ha^{-1} N; 60 kg ha^{-1} P, 230 kg ha^{-1} K), and plot 18 as unfertilized control (*Böhme* et al., 2005).

3.3.2 Soil sampling and incubation

Soil samples were collected at the end of October 2011 from the top 20 cm layer of the three plots. The last crop on the plots was sugar beet. The contents of SOC and total N (mean and standard deviation) were 17.9 ± 3.1 g kg^{-1} and 1.4 ± 0.3 g kg^{-1}, respectively.

Samples were stored for three month at 4 °C in the dark before processing. Field-moist samples (< 2 mm) equivalent to 8 g oven dry soil were weighed into 50 mL glass vials. De-ionized (DI) water was added to bring the soil to 60% of its water holding capacity, corresponding to a water content ranged from 266 to 305 g kg^{-1} dry soil. For each treatment (n = 4, see below) and replicate (n = 3), four glass vials (pseudo-replicates) were placed into 1 L glass jars and incubated at 20 °C for 21 days in the dark.

Soil samples were incubated with ground dried corn leaves which differed in their C to N ratios. Residues from young and old corn leaves were mixed to get C to N ratios of 20 and 40.

The following treatments were used (each with the three replicates):

- treatment I: control, no addition of residues.
- treatment II: addition of corn residues with a C to N ratio of 40. The amount of N added was 0.1 mg g^{-1} oven dry soil and the amount of C added was thus 4 mg g^{-1}.
- treatment III: addition of corn residues with a C to N ratio of 20. The amount of N and C added were 0.1 mg g^{-1} and 2 mg g^{-1}, respectively.
- treatment IV: addition of corn residues with a C to N ratio of 40 and $(NH_4)_2SO_4$. The amounts added were 0.05 mg organic N g^{-1}, 0.05 mg NH_4^+-N g^{-1} and 2 mg C g^{-1}.

3.3.3 General soil analyzes

The total C and N contents in soil and corn residue samples were analyzed after drying and grinding with a C and N analyzer (Heraeus Elementar Vario EL, Hanau, Germany). Since no carbonates were found in the soil, the total C corresponds to organic C (SOC).

For the extraction of mineral N, 40 mL of 0.5 M potassium sulfate (K_2SO_4) were added to soil corresponding to 8 g oven dry weight [14]. Samples were shaken on a reciprocal shaker for 1 h and the suspension was filtered (folded filter paper, 303, VWR, Radnor, USA) to determine NH_4^+ and NO_3^- concentrations by a continuous flow analyzer (Evolution II auto-analyzer, Alliance Instruments, Salzburg, Austria).

3.3.4 Protease activity

The assay for protease activity was carried out as described by *Ladd* and *Butler* (1972) with a few slight modifications. Protease activity was determined after 3, 7 and 21 days of incubation. Briefly, 1 g of soil was weighed into a test tube and 2.5 mL Tris-buffer (0.2 M, pH 8) and 2.5 mL of a 2% sodium caseinate solution were added. The vials were gently mixed on a vortex, capped, and incubated in a water bath at 40 °C for 2 h. After the incubation, 5 mL of cold (4 °C) 10%

trichloroacetic acid was added to stop enzyme activity. The suspension was filtered (folded filter paper, 303, VWR, Radnor, USA) and 0.5 mL of the supernatant was transferred to a test tube. A volume of 0.75 mL of a citrate-Na_2CO_3 solution and 0.25 mL of three-fold diluted Folin reagent were then added and mixed for 3 seconds on a vortex.

In preliminary tests, the solution became cloudy after addition of Na_2CO_3 and Folin reagent. This affected the absorbance of the samples. Thus, the following modification of the original method was implemented: citrate (0.1 M) was added to the Na_2CO_3 solution (1.4 M, 0.75 mL). Tests showed that the added citrate did not interfere with the color development.

After 30 minutes 0.2 mL of the solution was pipetted into a 96-well plate (Microtest Plate, flat bottom, Sarstedt, Newton, USA) and the tyrosine concentration was determined colorimetrically at 680 nm on a plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The controls ran through the same procedure, one control with added DI-water instead of caseinate and one control without soil.

3.3.5 Gross N mineralization

Gross N mineralization was determined after 3, 7 and 21 days by the ^{15}N pool dilution method described by *Murphy* et al. (2003). Briefly, soil samples corresponding to 4 g oven dry weight were treated with:

a) $(^{15}NH_4)_2SO_4$ (60 atom% ^{15}N) and extracted immediately

b) $(^{15}NH_4)_2SO_4$ (60 atom% ^{15}N) and extracted after 48 h

A total of 10 mg N kg^{-1} dry soil was added with 50 µL of the NH_4^+solution (a + b). The water content changed to 278 – 317 g kg^{-1} dry soil. Immediately after the addition of the labeled ammonium sulfate 30 mL of K_2SO_4 (0.5 M) were added to sample a), placed on an orbital shaker for 1 h at 175 rev min^{-1}, filtered, and stored in a fridge at 4 °C. Sample b) was placed back into the glass jars and incubated for 48 h at 20 °C before undergoing the same treatment as sample a). A

duration of 48 h instead of the more commonly used 24 h was employed because our preliminary experiments indicated that some amino acids were still in solution after 24h and the variability between samples was high. Samples were analyzed for NH_4^+ as described above.

For the determination of atom% ^{15}N in NH_4^+ the solutions were transferred into glass vials with screw caps. Then 0.5 g of MgO was added and the vial immediately covered with PTFE tape. A filter paper disc with a diameter of 5 mm was placed on the tape and 7 µl of $KHSO_4$ (2.5 M) was added. The disc was covered with another piece of PTFE tape, and the vial closed and gently swirled. The vials stood at room temperature for 10 days to complete the diffusion (*Brooks* et al., 1989). Afterwards, the paper discs were removed, dried, and placed into tin capsules for analysis of the atom% ^{15}N using an Elemental Analyzer Delta C Finnigan mass spectrometer.

The ^{15}N pool dilution method makes it possible to determine fluxes of N into and out of the soil NH_4^+ pool. To calculate the gross N mineralization rate the following equation proposed by *Barraclough* (1995) was used by assuming no isotopic discrimination, a uniform distribution of ^{15}N and an equilibrium between N pools:

$$m = \theta * \frac{\log(A_0^*/A_t^*)}{\log(1 + \theta t/A_0)}$$

where *m* is the gross rate of mineralization, superscript * indicates ^{15}N atom excess of the NH_4^+ pool at t=0 (A_0^*) and t=t (A_t^*), θ is the rate at which the ammonium pool changes in size (given by $(A_t-A_0)/t$), and A_0 is the size of the ammonium pool at t=0.

3.3.6 Mineralization of added amino acids

The mineralization rate of added amino acids was determined after 3, 7 and 21 days with the mirror image approach based on *Watkins* and *Barraclough* (1996). Briefly, soil samples corresponding to 4 g oven dry weight were treated with:

a) $(^{15}NH_4)_2SO_4$ + unlabeled glycine and L-leucine

b) $(NH_4)_2SO_4$ + ^{15}N-glycine (98 atom% ^{15}N) and ^{15}N-L-leucine (98 atom% ^{15}N).

A total of 10 mg N kg^{-1} dry soil was added with 50 µL of the ammonium - amino acid mix (c + d). The composition of added N was 50% NH_4^+-N, 25% glycine-N and 25% leucine-N. The samples were extracted after incubation for 48 h at 20°C. The procedure to determine NH_4^+ and atom% ^{15}N in NH_4^+ was the same as described for the gross N mineralization rate.

The calculation for the mineralization rate of added amino acids with the mirror image procedure was described by *Watkins* and *Barraclough* (1996). The change in $^{15}NH_4$ is described by following equation:

$$A_t^* = O^* + \frac{(A_0^* - O^*)}{\left(1 + \frac{\theta t}{A_0}\right)^{(m/\theta)}}$$

where O* is the excess ^{15}N of the added amino acids being degraded. O* is given by

$$O^* = \alpha P^*$$

where P* is the excess ^{15}N of the added amino acids and α is the proportion of the mineralization flux resulting from the degradation of the added amino acids (Table 3.1).

To determine the proportion of excess amino acids in the solution after 48 h of incubation, the solutions with $(^{15}NH_4)_2SO_4$ added (treatment b) and the solutions with $(NH_4)_2SO_4$ + labeled amino acids added (treatment d) were analyzed for ninhydrin-reactive N. The method to measure ninhydrin-N of the microbial biomass is described by *Joergensen* and *Brookes* (1990). Briefly, 0.75 mL of K_2SO_4 (0.5 M) soil extract added with 1.5 mL citrate buffer and 1.25 mL ninhydrin solution were mixed well on a vortex and then placed for 25 min in a boiling water bath. Afterwards the solution was quickly cooled down and 4.5 mL of an ethanol-water mixture (1/1) was added and well mixed on a vortex, then the absorption measured colorimetrically at 570 nm on a plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

Table 3.1: Soil NH_4^+-N concentrations and their ^{15}N isotopic excess after addition of a) $(^{15}NH_4)_2SO_4$ (extracted immediately), b) $(^{15}NH_4)_2SO_4$, c) $(^{15}NH_4)_2SO_4$ + unlabelled glycine + L-leucine and d) $(NH_4)_2SO_4$ + ^{15}N-glycine + ^{15}N-L-leucine (b, c and d extracted after 48 h). Data are mean values of pseudoreplicates with standard errors in brackets (n=3) and were used for the calculation of the mineralization of the amino acids.

		Addition of corn residue with:			
Day of incubation	Treated with:	control	C:N of 40	C:N of 20	C:N of 40 & $(NH_4)_2SO_4$
Ammonium-N (mg kg^{-1} dry soil)					
3	a)	12.5 (0.8)	13.0 (0)	17.8 (1.9)	35.3 (2.5)
	b)	7.2 (0.6)	1.1 (0.7)	3.9 (1.5)	11.2 (4.3)
	c)	15.1 (2.5)	0.9 (0.3)	5.9 (0.7)	15.8 (1.8)
	d)	14.5 (3.3)	0.8 (0.1)	5.6 (0.8)	15.0 (1)
7	a)	15.8 (1.1)	13.9 (0.5)	14.5 (0.5)	18.0 (2.4)
	b)	15.0 (2.6)	1.0 (0.1)	10.1 (1.9)	14.4 (3.8)
	c)	16.8 (4.2)	1.2 (0.1)	17.8 (3)	26.0 (2.9)
	d)	18.5 (2.6)	1.4 (0.1)	16.9 (4)	22.0 (2.5)
21	a)	14.7 (0.8)	13.6 (0.3)	17.9 (1.3)	16.8 (1.5)
	b)	11.5 (1.8)	1.2 (0.1)	14.9 (2.5)	10.7 (1.5)
	c)	20.5 (0.6)	3.0 (0.4)	19.0 (4.9)	20.5 (3.9)
	d)	20.6 (0.6)	2.4 (0)	18.3 (4.3)	23.6 (2.8)
^{15}N (atom% excess)					
3	a)	38.8 (1.5)	35.5 (0.5)	32.2 (2)	20.1 (1.6)
	b)	27.4 (0.9)	1.1 (0)	11.3 (3.5)	10.2 (1.6)
	c)	22.9 (0.7)	2.1 (0.6)	11.5 (1)	11.2 (0.5)
	d)	27.8 (1.7)	2.0 (0.2)	12.8 (1.2)	13.1 (0.7)
7	a)	38.0 (2.1)	38.0 (0.7)	37.1 (0.5)	33.7 (2)
	b)	31.9 (0.9)	3.4 (2.1)	19.3 (3.6)	21.6 (0.5)
	c)	19.5 (1.1)	1.7 (0.1)	15.5 (0.7)	15.7 (0.4)
	d)	28.4 (1.7)	2.8 (0.2)	20.2 (1.7)	20.1 (0.5)
21	a)	38.5 (2)	38.0 (0.5)	33.6 (1)	37.7 (0.5)
	b)	32.2 (0.6)	3.3 (0.7)	20.7 (2)	23.0 (3.7)
	c)	21.4 (0.7)	5.6 (0.6)	14.9 (1.1)	17.9 (0.8)
	d)	27.1 (1.4)	5.7 (0.6)	20.6 (1.5)	22.7 (0.4)

3.3.7 Statistical analyses

Statistical analyses were performed with the SAS program (SAS Institute Inc., 1990). The general linear model (GLM) procedure was used for analysis of variance. Normality of the residuals was evaluated with the Shapiro-Wilk test. Homogeneity of variances was tested with Levene´s test. When necessary, the data were transformed. Mean comparisons were conducted with the Tukey test. Effects were considered significant for $p < 0.05$.

3.4 Results and discussion

Contents of ninhydrin-reactive N components were 0 in treatment I and approximately 0.2 mg kg^{-1} oven dry soil (corresponding to 2% of the amino acids added) in treatments II, III and IV (data not shown). Therefore, with less than 2% of the added amino acids were still in solution after 48 h, suggesting that at least 98% of the added amino acids were either mineralized, taken up by microorganisms as intact molecules or immobilized by soil colloids. The latter, however, is of minor importance because of the following reasons: the amino acids used in our study, glycine and L-leucine, have no net charge at the pH of the soil used, thus they are only weakly adsorbed to cation exchange sites (*Hedges* and *Hare*, 1987). *Rousk* and *Jones* (2010) reported for similar experimental conditions that about 80% of the added amino acids were recovered with a 0.5 M K_2SO_4 solution after shaking the samples for 1 h. However, addition of $HgCl_2$ as a microbial inhibitor resulted in an extraction of 100% of the amino acids, indicating that the reduced extraction efficiency could be attributed to microbial activity and not to sorption by soil colloids (*Rousk* and *Jones*, 2010). In the following discussion, we assume that the proportion of amino acids immobilized abiotically is insignificant, which may result in a slight overestimation of the direct uptake of amino acid utilization.

The results of the mirror image procedure showed that in the control (treatment I), approximately 25% of the amino acids added were mineralized after three days of incubation (Figure 3.1a),

indicating that the remaining 75% of the amino acids added were taken up directly. During the incubation the proportion of amino acid N mineralized increased to about 30% (Figure 3.1a), suggesting that the MIT route became slightly more important with time. However, variability was marked (Figure 3.1a).

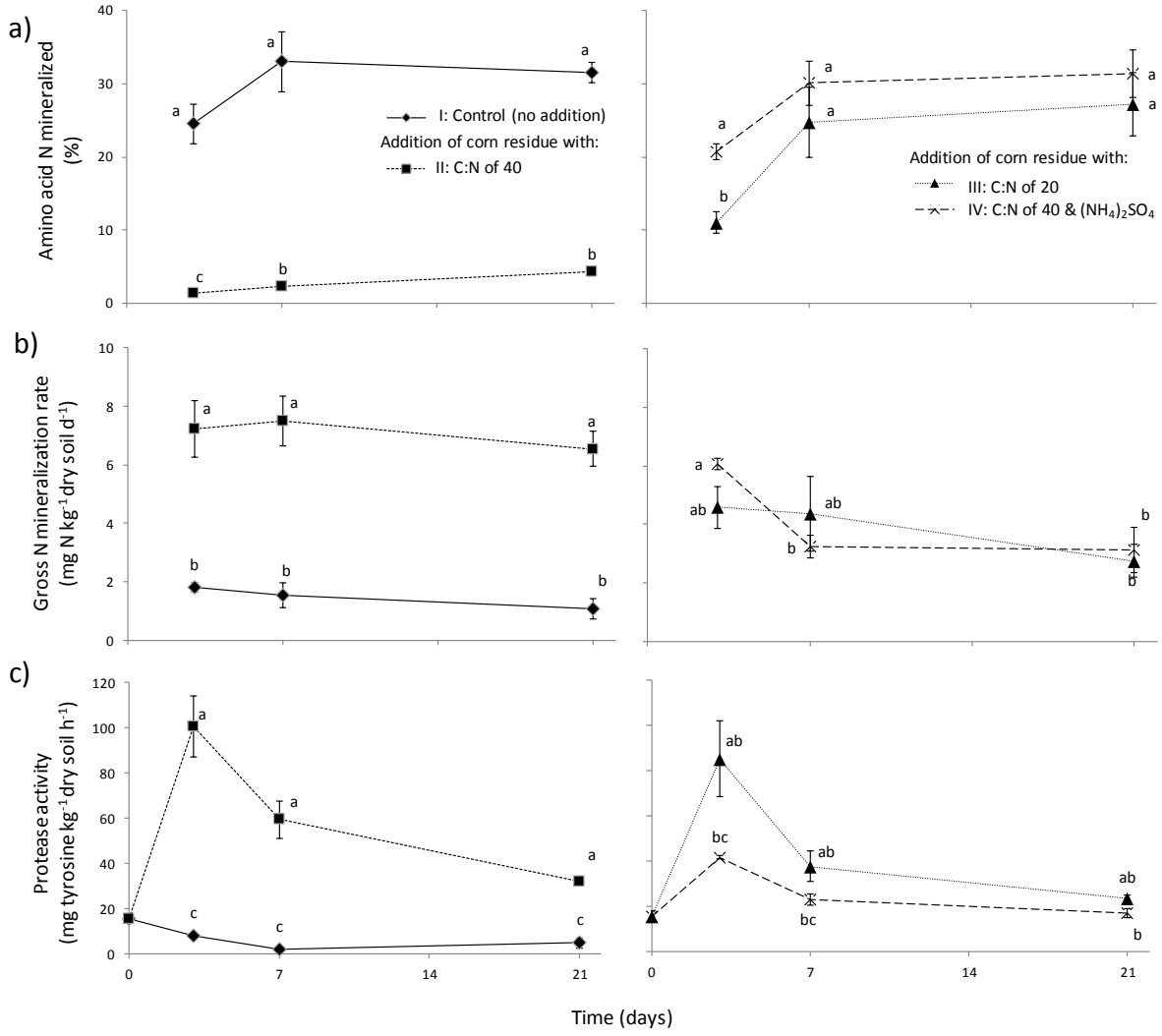

Figure 3.1: Amino acid N mineralization (a), gross N mineralization (b), and protease activity (c) in soils incubated with corn residues of different C to N ratios. Data shown are means ± standard errors (n=3). Lowercase letters indicate significant differences between treatments.

In treatment II (addition of corn residue with a wide C to N ratio) only a small proportion (1.4% after three days) of the added amino acids was mineralized (Figure 3.1a), indicating that the direct uptake route is of great importance in arable soils after application of substrates with wide C to N ratios, supporting our hypothesis. However, the importance of the MIT route slightly increased with incubation time (Figure 3.1a). Similarly, *Geisseler* et al. (2012) reported for incubation experiments with soil and straw that the importance of the MIT route increased with time, with the direct route still being the preferred pathway.

In this treatment, the addition of corn residue resulted in a strong net N immobilization due to the lack of N relative to C (threshold of C:N ≈ 25 to 30 for the N mineralization (*Scheffer* and *Schachtschabel*, 2010; *White*, 2005) (Figure 3.2a, b). The depletion of the mineral soil N pool may result in the de-repression of enzyme systems used for the acquisition of alternative N sources, such as amino acids, and therefore the direct route may be favored over the MIT route (*Geisseler* et al., 2010). The NH_4^+ content in the soil solution increased marginally with time (Figure 3.2b). The NH_4^+ may have either been produced in the MIT route or may have been excreted by microorganisms after direct uptake of amino acids. The latter, however, is unlikely since the corn residue added in treatment II had a wide C to N ratio. Thus, both parameters – mineralization of amino acids added and the NH_4^+ data in this treatment with a substrate with a wide C to N ratio – point to only a small contribution of the MIT route. The simultaneous uptake of added organic N (99 to 96% direct uptake in treatment II) and mineral N (1 to 4% in treatment II) may be due to the coexistence of different microbial communities and soil heterogeneity (*Manzoni* and *Porporato*, 2007). The overall dominance of the direct route (being equal to or greater than 96%) in treatment II is greater than in other studies where a contribution of the direct uptake ranging from 55 to 70% was estimated (*Hadas* et al., 1992; *Luxhøi* et al., 2006; *Geisseler* et al., 2009) and very close to the finding of *Barraclough* (1997), who reported for addition of leucine and glycine to soil that the MIT route was not operative.

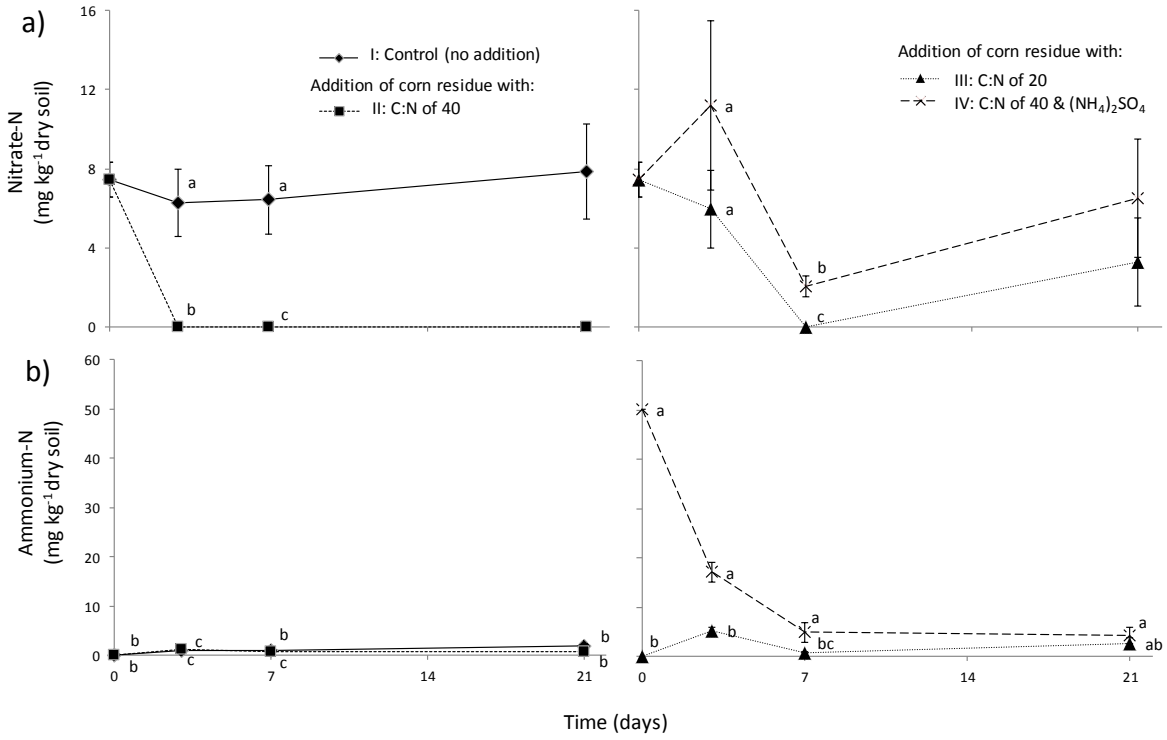

Figure 3.2: Contents of nitrate N (a) and ammonium N (b) of soils incubated with corn residues of different C to N ratios. Data shown are means ± standard errors (n=3). Lowercase letters indicate significant differences between treatments.

Protease activities and the gross N mineralization rates were not closely related in treatment II (Figure 3.1b, c). For instance, the increased protease activity after three days did not lead to an increased gross N mineralization, although the products of protease activity are the substrates for N mineralization. This result further underlines the importance of the direct uptake in treatment II.

In treatment III (addition of corn residue with a C to N ratio of 20) the proportion of added amino acid mineralized was approximately 11% after three days and significantly different from the other treatments (Figure 3.1a). The proportion increased to 27% after 21 days, indicating that approximately 73% of the amino acids added were taken up directly. A comparison of the results of treatments II and III shows that the importance of the MIT route is more pronounced at a narrower C to N ratio. *Geisseler* et al. (2009) reported for wheat residues a similar trend, but absolute values

differed considerably. The mineralization rate of a residue with a narrower C to N ratio (younger residue: 12) was markedly higher than that of a residue with a wider C to N ratio (older residue: 29) and the mineralization rates of the amino acid glycine ranged from 61 to 65% for the younger and from 46 to 56% for the older residues after 31 days of incubation (*Geisseler* et al., 2009).

In treatment IV the proportion of added amino acid mineralized was approximately 21% during the first three days of incubation and increased to 31% after 21 days of incubation (Figure 3.1a). A comparison of treatments II and IV indicates that the importance of the MIT route is markedly more pronounced in the presence of NH_4^+, supporting our hypothesis. The increased concentration of NH_4^+ in treatment IV (50 mg NH_4^+-N kg^{-1} dry soil were initially present, Figure 3.2b) resulted in a shift from 99% - 96% of direct uptake (treatment II) to 79 – 69% of direct uptake in treatment IV. *Geisseler* et al. (2010) suggested that a high concentration of NH_4^+ should result to a shift from the direct to the MIT route. Our data confirm such a shift with an increasing importance of the MIT route at an increased ammonium concentration, whereas the direct uptake still remained dominant. However, more quantitative data on the effect of substrate quality and different ratios of plant residues and mineral N on the N uptake routes in different soils are required, before the conceptual model by *Geisseler* et al. (2010) may be improved for quantitative prognoses.

A comparison of treatments III and IV (same amount of C and N added) shows that the mineralization rate of amino acids is significantly higher after three days of incubation in the presence of NH_4^+, underlining the importance of the presence of ammonium for the MIT route. With increasing incubation time, however, the importance of the MIT route increased in both treatments and differences were no longer significant (Figure 3.1a).

3.5 Conclusion

In conclusion, our results show that the additon of residues with a wide C to N ratio strongly decreased the proportion of amino acid N mineralized. When the same residues were added in

combination with NH_4^+, the effects were much weaker. These results suggest that the availability of N relative to C is a dominant factor determining how soil microorganisms utilize organic N sources. With 1.4 to 33% of the added amino acid N mineralized, the gross N mineralization rate only represented a small and variable proportion of the N available to soil microorganisms in the short term.

3.6 Acknowledgments

We would like to thank *Anja Sawallisch* for technical assistance. This project is financed by the Deutsche Forschungsgemeinschaft ("Nitrogen utilization pathways of soil microorganisms").

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4. Effect of substrate quality on the N uptake routes of soil microorganisms in different soil depths

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4.1 Abstract

A few studies have indicated that substrate quality is an important factor affecting the N uptake route of soil microorganisms, but less is known about the effect of soil depth on the N uptake route at different nutrient availabilities. The objective of this study was to investigate the effects of soil depth on the N uptake route of soil microorganisms in consideration of added corn residue with different C/N ratio in the presence and absence of inorganic N. An incubation experiment with surface soils (0-5 cm, C/N = 10) and subsoils (30-40 cm, C/N = 9) from three German loess sites was carried out for 21 days at 20 °C and 60% of its water-holding capacity. The following treatments were used: no addition (control), addition of corn residues with a C/N ratio of 20, 40, and 40 + $(NH_4)_2SO_4$. To distinguish between the N uptake routes, the mineralization rate of amino acids

was determined using ^{15}N-labeled amino acids. In the control surface soil and subsoil the direct uptake of organic N was favored with no significant ($p < 0.05$) differences between depths despite significantly higher microbial activity, protease activity, gross N mineralization rate and availability of inorganic N in the surface soil, suggesting that N availability relative to C was similar at both depths. Substrate additions resulted in significantly increased protease activities at both depths after 3 to 7 days. Addition of corn residue with a wide C/N ratio resulted in an increased direct uptake (97% and 94% in the surface soil and subsoil, respectively), compared with addition of corn residue with a narrow C/N ratio or addition of corn residue and inorganic N (79 to 91% direct uptake). This suggests that the enzyme system involved in the direct uptake was slightly repressed under conditions of sufficient mineralizable N (C/N of 20) or increased concentrations of NH_4^+. Substrate additions resulted in an initial significantly higher increase in the direct uptake in the surface soil than in the subsoil.

4.2 Introduction

Nitrogen is an essential nutrient for plants and soil microorganisms. The distribution of total N in humic substances and soils has been reported to consist of about 40% proteins and peptides and to about 35% of heterocyclic N compounds (*Schulten* and *Schnitzer*, 1998). Therefore, proteinaceous material plays a central role in N transformation processes. However, proteins cannot be utilized directly by soil microorganisms. They first have to be depolymerized by proteases (*Magasanik*, 1993; *Wanek* et al., 2010), extracellular enzymes, which are secreted into the soil by microorganisms such as bacteria and fungi (*Glenn*, 1976; *Fuka* et al., 2008). Proteases can break down large polymeric compounds and release soluble amino acids or small peptides, which are important sources of N and C for the microorganisms (*Rothstein*, 2010). Protease production is generally induced by the presence of substrate in the medium (*Haab* et al., 1990).

Soil microorganisms are able to take up a wide range of N compounds: NH_4^+, NO_3^-, and organic molecules, such as amino acids or small peptides (*Merrick* and *Edwards*, 1995; *Marzluf*, 1997) and

have developed several mechanisms for N uptake (*Barak* et al., 1990, *Barraclough*, 1997, for a review see *Geisseler* et al., 2010). They can take up simple organic molecules directly into the cell (direct route) or they can first mineralize the molecules to NH_4^+ before uptake via the mineralization-immobilization-turnover (MIT) route (*Hadas* et al., 1992). An advantage of the direct uptake of small organic molecules is energy saving, required for synthesizing C structural components (*Geisseler* et al., 2010). In the MIT route, the deamination of organic material is carried out by extracellular enzymes outside the cytoplasm and all N is mineralized to NH_4^+ before assimilation (*Manzoni* and *Porporato*, 2007).

Microorganisms produce a wide range of transport enzymes for the direct uptake of organic N, which are responsible for the transformations and N uptake processes. For example, the uptake of amino acids into cells is catalyzed by functionally specific transport systems. Laboratory experiments with cultivated microorganisms in growth media indicated that the activation of the synthesis of amino acid transport systems occurs by a lack of C, N, or S and is repressed by ammonium and high intracellular concentrations of amino acids (*Payne*, 1980). Thus, *Geisseler* et al. (2010) suggested that the MIT route is favored in the presence of NH_4^+ at high concentrations and that the direct route may be generally important in aerated soils due to nitrification and plant uptake. The studies available for soil incubations after addition of different plant residues indicate that both uptake routes were active simultaneously, with the direct route being preferred (*Luxhøi* et al., 2006; *Geisseler* et al., 2009). However, the MIT route may be an important alternative when N is severely limiting (*Geisseler* et al., 2012).

Subsoils differ from surface soils in a number of factors: SOC and N contents, bulk density, and activity of soil microorganisms generally decline markedly with depth, with the decline being more pronounced for fungi than for bacteria (*Agnelli* et al. 2004, *Ekschmitt* et al. 2008). In contrast, the changes in the microbial biomass C/SOC ratio and different enzyme activities with depth were variable (*Lavahun* et al., 1996; *Agnelli* et al. 2004; *Enowashu* et al., 2009). However, information on depth related changes of the N uptake route in soils is not available.

Our objective was to quantify the effects of soil depth of three German loess sites on the N uptake route of soil microorganisms, considering corn residue with different C/N ratios in the presence and absence of inorganic N. We hypothesized (i) that N availability is higher in surface soils (0-5 cm) and that the MIT route is thus more important than in subsoils (30-40 cm) and (ii) that addition of corn residue with a wide C/N ratio results in an increased importance of the direct uptake route.

4.3 Material and methods

4.3.1 Experimental sites

The current study was carried out with soil from the no-tillage treatment from three sites located in arable loess-regions of eastern Germany. The sites differ in their soil texture and SOC content (Table 4.1). The crop rotation at these sites consisted of sugar beet (*Beta vulgaris* L.), winter wheat (*Triticum aestivum* L.) and winter wheat. After harvest of the second wheat crop white mustard (*Sinapis alba* L.) was sown as catch crop (*Koch* et al., 2009).

Table 4.1: Soil organic carbon (SOC), total nitrogen (N_{tot}), pH and soil texture are mean values of pseudoreplicates with standard errors in brackets (n=3).

Site	Depth	SOC	N_{tot}	pH	Clay	Silt	Sand
	[cm]	[g kg^{-1} soil]		[$CaCl_2$]	[%]	[%]	[%]
Friemar	0-5	24.0 (0.4)	2.43 (0.06)	6.7 (0.1)	29 (2)	66 (2)	5 (1)
	30-40	6.9 (0.3)	0.72 (0.02)	7.0 (0.1)	37 (7)	57 (5)	6 (2)
Lüttewitz	0-5	18.5 (0.7)	1.84 (0.04)	7.3 (0.0)	17 (1)	79 (1)	4 (0)
	30-40	2.7 (0.2)	0.34 (0.01)	7.1 (0.0)	17 (4)	78 (3)	5 (1)
Zschortau	0-5	14.2 (0.1)	1.40 (0.01)	7.3 (0.0)	13 (0)	59 (1)	28 (1)
	30-40	5.8 (0.5)	0.61 (0.05)	7.3 (0.1)	13 (1)	57 (1)	30 (1)

4.3.2 Soil sampling and incubation

Soil samples were taken in October 2013 from the no-tillage (direct drilling) treatment. The field was divided into three subplots and from each subplot a composite sample was taken at two different depths. The samples were taken from 0 - 5 cm and 30 - 40 cm. Soil samples were sieved (< 2 mm) and stored at 4 °C in the dark before processing. Field-moist samples equivalent to 8 g oven dry soil were adjusted to 60% of its water holding capacity and incubated at 20 °C for 21 days in the dark.

Ground dried corn leaves, which differed in their C/N ratios were added to the soil samples. The following treatments were carried out in triplicate each: (I) control, no addition of residues, (II) addition of corn residues with a C/N ratio of 20; (III) addition of corn residue with a C/N ratio of 40 and (IV) addition of corn residues with a C/N ratio of 40 and $(NH_4)_2SO_4$. To get C/N ratios of 20 and 40 residues from young and old corn leaves were mixed. The amount of 100 mg N kg^{-1} soil was added to the treatments II, III, and IV. In treatment IV, half of the N was added with the residue and the other half with $(NH_4)_2SO_4$**.**

Total C and N contents in soil and corn leaves were analyzed after drying and grinding with a CN analyzer (Heraeus Elementar Vario EL, Hanau, Germany). Since no carbonates were found in the soil, total C corresponds to soil organic C (SOC).

4.3.3 Basal respiration, net-N mineralization and microbial biomass

CO_2 production was determined using NaOH traps (*Coleman* et al., 2002). Cups containing 10 ml 1 M NaOH were added to the 1 L glass jars, which contained 4 glass vials (each containing 8 g of soil). After 3, 7, 14, and 21 days the traps were replaced and the jars aerated. The accumulated CO_2 was determined by titration (*Alef*, 1995).

For the extraction of NH_4^+ and NO_3^-, 40 mL of 0.5 M potassium sulfate (K_2SO_4) were added to soil corresponding to 8 g dry weight (*Kuderna* et al., 1993). Samples were shaken on a reciprocal shaker for 1 h and the suspension was filtered to determine the concentrations by a continuous flow

analyzer (Evolution II auto-analyzer, Alliance Instruments, Salzburg, Austria).

The microbial biomass was determined by using the chloroform fumigation extraction method (*Brookes* et al., 1985; *Vance* et al., 1987). After 0, 3, 7 and 21 days, samples corresponding to 8 g dry weight were fumigated at 25 °C for 24 h with ethanol-free $CHCl_3$. Forty ml of 0.5 M K_2SO_4 were added to extract the fumigated and non-fumigated samples by shaking them on a reciprocal shaker at 175 rev min^{-1} for 1 h. The suspensions were filtered through a filter paper and the organic C and N in the extracts was analyzed by a multi N/C analyzer (Analytik Jena 2100S, Jena, Germany). Microbial biomass C was calculated with a k_{EC} value of 0.45 (*Joergensen*, 1996) and microbial biomass N with a k_{EN} value of 0.54 (*Brookes* et al., 1985).

4.3.4 Protease activity

Potential protease activity was determined after 0, 3, 7 and 21 days of incubation as described by *Ladd* and *Butler* (1972) with a few slight modifications. Briefly, 2.5 mL Tris-buffer (0.2 M, pH 8) and 2.5 mL of a 2% sodium caseinate solution were added to 1 g of soil. The vials were gently mixed, capped, and incubated in a water bath at 40 °C. After 2 h, 5 mL of cold 10% trichloroacetic acid was added to stop enzyme activity. The suspension was filtered and 0.5 mL of the supernatant was transferred to a test tube and 0.75 mL of a Na_2CO_3 solution (1.4 M) and 0.25 mL of three-fold diluted Folin reagent were then added and mixed on a vortex. In contrast to the original method, the Na_2CO_3 solution contained citrate (0.1 M) to prevent the formation of a precipitate, which interfered with the colorimetric analysis. After 30 min 0.2 mL of the solution was pipetted into a 96-well plate (Microtest Plate, flat bottom, Sarstedt, Newton, USA) and the tyrosine concentration was determined colorimetrically at 680 nm on a plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). The controls ran through the same procedure, one control with added DI-water instead of caseinate and one control without soil.

4.3.5 Gross N mineralization

The assay for gross N mineralization was carried out as described by *Murphy* et al. (2003). The gross N mineralization rate was calculated as proposed by *Barraclough* (1995). Gross N mineralization was determined after 3, 7 and 21 days by the ^{15}N pool dilution method. This method allows the determination of fluxes of N into and out of the soil ammonium (NH_4^+) pool. Briefly, soil samples corresponding to 8 g oven dry weight were treated with:

a) $(^{15}NH_4)_2SO_4$ (60 atom% ^{15}N) and extracted immediately

b) $(^{15}NH_4)_2SO_4$ (60 atom% ^{15}N) and extracted after 48 h

With the NH_4^+ a total of 10 mg N kg^{-1} dry soil were added. After addition of labeled ammonium sulfate, 40 mL of K_2SO_4 (0.5 M) were added immediately to sample a), and placed on an orbital shaker for 1 h at 175 rev min^{-1}, filtered, and stored in a fridge at 4 °C. Sample b) was incubated for 48 h at 20 °C, and then underwent the same treatment as sample a). Samples were analyzed for NH_4^+ as described above.

For the determination of atom% $^{15}NH_4^+$ in the solutions, 0.5 g of MgO was added and the vial was immediately covered with PTFE tape. Then a filter paper disc (diameter of 5 mm) was placed on the tape and 7 µl of $KHSO_4$ (2.5 M) was added. With another piece of PTFE tape the disc was covered, and the vial was closed and gently swirled. To complete the diffusion the vials stood at room temperature for 10 days (*Brooks* et al., 1989), then the paper discs were removed, dried, and placed into tin capsules for analysis of the atom% ^{15}N using an Elemental Analyzer Delta C Finnigan mass spectrometer.

4.3.6 Mineralization of added amino acids

After 3, 7 and 21 days the mineralization rate of added amino acids was determined with the mirror image approach based on *Watkins* and *Barraclough* (1996). The procedure for the determination was the same as described for the gross N mineralization rate, but labeled and unlabeled amino acids were added:

c) $(^{15}NH_4)_2SO_4$ + unlabeled glycine and L-leucine

d) $(NH_4)_2SO_4$ + ^{15}N-glycine (98 atom% ^{15}N) and ^{15}N-L-leucine (98 atom% ^{15}N).

With the amino acid mix a total of 10 mg N kg^{-1} dry soil were added. The samples were extracted after an incubation time of 48 h at 20 °C. The calculation for the mineralization rate of added amino acids was carried out as described by *Watkins* and *Barraclough* (1996). Ninhydrin-reactive N was analyzed for the solutions with $(^{15}NH_4)_2SO_4$ added (treatment b) and the solutions with $(NH_4)_2SO_4$ + labeled amino acids added (treatment d) to determine the proportion of excess amino acids in the solution after 48 h of incubation (*Joergensen* and *Brookes*, 1990).

4.3.7 Statistical analyses

The general linear model (GLM) procedure was used for a one-way analysis of variance for each sampling date and depth separately. Response variables were the percentages of amino acid N mineralized, the gross N mineralization rate, protease activity and contents of ammonium and nitrate and the treatment factor had four levels. Normality of the residuals was evaluated with the Shapiro-Wilk test. Homogeneity of variances was tested with Levene´s test. Mean comparisons were conducted with Tukey's HSD test. Effects were considered significant for $p < 0.05$. All of the above analyses were performed with the SAS program version 9.4 (SAS Institute Inc., 2014).

Additionally, a mixed effects model was fitted to study the effect of soil depth on the response variables given above using the mixed models (MIXED) procedure of SAS for each sampling date. The models included the fixed effects for site, treatment and the site-depth and treatment-depth interaction. The residual error was modeled to have a compound symmetry structure for observations down the same soil column (*Piepho* et al., 2004) (implemented using the REPEATED statement and specifying the site-treatment interaction as subject effect). The estimation procedure was restricted maximum likelihood and the denominator degrees of freedom were estimated using the Kenward-Roger method. Studentized residuals were inspected for homoscedasticity and normality. In two cases (gross N mineralization after 21 days, microbial C after 7 days), a square-

root transformation of the response variable was done to achieve normality and homoscedasticity. In cases of significant interactions between treatment and depth, the depth effect for each treatment was inspected (SLICE statement, SAS Institute Inc., 2014).

Correlations of selected data pairs were calculated. Data were tested for normal distribution with the Shapiro-Wilk test. In case of a normal distribution of the data, Pearson's product-moment correlation was calculated, which was done for the pairs microbial biomass C / microbial biomass N as well as microbial biomass C / mineralization of added amino acids. If no normal distribution was given, Spearman's rank correlation was calculated, which was the case for microbial biomass C / CO_2 evolution, as well as mineralization of added amino acids compared with protease activity or inorganic N, respectively. These calculations were performed with the statistical software R (The R Foundation for Statistical Computing, 2014).

4.4 Results

4.4.1 Microbial biomass, basal respiration, protease activity and gross N mineralization

The addition of corn residue resulted in an increase in microbial biomass C in all treatments and soil layers (Fig. 4.1). The increase in microbial biomass C was significant in treatment II and III in both soil layers after day 3. Since day 21, microbial biomass C remained significantly higher after addition of corn residue to the subsoil. In contrast, in the surface soil only treatments with a high C/N ratio (treatment III and IV) were significantly different from the control treatment. In the surface soil microbial biomass C content was initially significantly higher than in the subsoil (Fig. 4.1). Microbial biomass N followed microbial biomass C with C/N ratios between 4 to 7 (data not shown).

During the 21 days, cumulative CO_2 evolution significantly increased with addition of corn residue in the surface soil in the order: corn residue with C/N 40 (mean ± standard error: 1600 ± 20 mg C kg^{-1}) corn residue with C/N 20 (1340 ± 30 mg C kg^{-1}) > addition of corn residue C/N 40 + inorganic N (1310 ± 10 mg C kg^{-1}), exceeding 3.4 times the control treatment (410 ± 30 mg C kg^{-1}).

However, in the subsoil, there was only a slight difference between the treatments with residue addition and the CO_2 evolution was approximately 900 mg C kg^{-1} soil and 12 times higher than that of the control treatment (Fig. 4.1). Approximately 46.5 ± 1.4 % and 41.2 ± 1.1 % of the added C was respired in treatment II, 29.6 ± 0.4 % and 20.8 ± 0.7 % in treatment III and 44.7 ± 1.2 % and 39.4 ± 1.6 % in treatment IV in the surface soil and subsoil, respectively. Microbial biomass C and CO_2 evolution were positively correlated in the surface soil (r = 0.87) and in the subsoil (r = 0.77).

In the control treatment, protease activity remained approximately at the same level during the incubation and was significantly higher in the surface soil (on average 50 mg tyrosine kg^{-1} soil h^{-1}) than in the subsoil (on average 7 mg tyrosine kg^{-1} soil h^{-1}). Addition of substrate with C/N 20 or 40 resulted in marked increases in protease activity from day 3 (Fig. 4.2). Activities did not significantly differ between surface soils and subsoils from day 3 or day 7 (treatment II). Corn residue at C/N 40 + inorganic N (treatment IV) also increased protease activity, but the increase was less pronounced. Gross N mineralization rates slightly decreased in all treatments and in both soil layers during the 21 days and were significantly different between surface soils and subsoils (Fig. 4.3). Gross N mineralization rate was on average 5.6 and 2.7 mg N kg^{-1} soil d^{-1} in the surface soil and in the subsoil, respectively. No clear pattern between the protease activity and gross N mineralization was observed (Fig. 4.2a, 4.3b).

4.4.2 Net N mineralization and amino acid mineralization

Contents of ninhydrin-reactive N components were on average 0.3 mg N kg^{-1} soil (corresponding to 3% of the amino acids added) in all treatments 48 h after the addition of amino acids. Thus, 97% of amino acids added were mineralized or taken up by the microorganisms in the different treatments (data not shown). In the following, percentages of amino acid mineralization or direct uptake refer to this amount of processed amino acids as 100%.

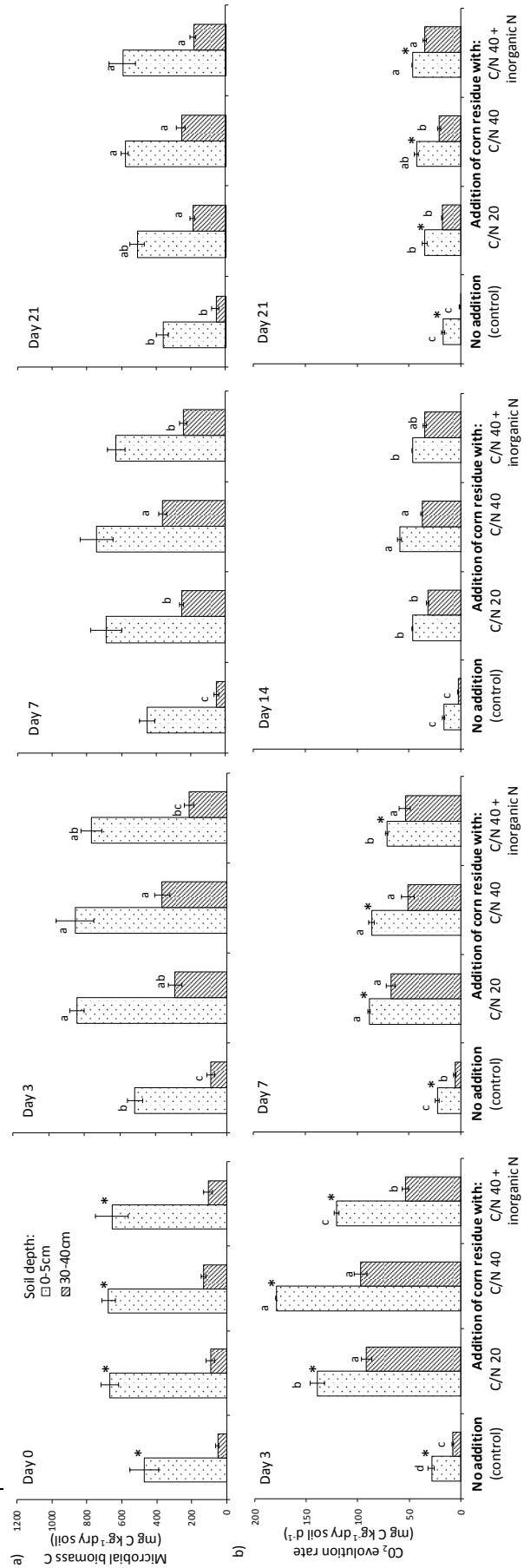

Figure 4.1: Contents of microbial biomass C (a) and CO_2 evolution rate (b) of soils incubated for 21 days with corn residues of different C to N ratios in two different soil depths. Data shown are means ± standard errors (n=3). Treatment means for a common depth sharing no lowercase letters are significantly different according to Tukey's HSD test (5% level). Asterisks show significant differences between surface soils and subsoils for a given treatment.

In the control treatment, net N mineralization occurred at both depths after the initial 7 days (Fig. 4.2). The results of the mirror image procedure showed that in the control treatment 19% and 23% of the added amino acids were mineralized after three days of incubation in the surface soil and subsoil, respectively; thus 81 and 77% of the added amino acids were taken up directly and differences were not significant between both depths (Fig. 4.3). The direct uptake was dominant throughout the 21 days (Fig. 4.3).

Addition of corn residue with C/N 20 (treatment II) resulted in net N immobilization within 7 days in the surface and subsoil; subsequently, net N mineralization took place (Fig. 4.2). There was no significant difference in the direct uptake of organic N between treatment II and the control. After 3 days, 12% and 18% of the added amino acids were mineralized in the surface soil and subsoil, indicating that 88% and 82% were taken up directly (Fig. 4.3). However, the proportion of amino acid N mineralized was significantly higher in the subsoil than in the surface soil after 3 and 21 days.

The combined addition of the corn residue with a wide C/N ratio and inorganic N (treatment IV) resulted in changed patterns of inorganic N dynamics (Fig. 4.2) and protease activity with significant differences between treatment IV and II in the subsoil after day 7 and 21 (Fig. 4.2). However, the combination of corn residue with a wide C/N ratio and inorganic N resulted in no significant changes in the importance of the direct uptake compared with treatment II (Fig. 4.3).

In treatment III, the application of residue with a wide C/N ratio led to a marked net N immobilization in the surface soil and subsoil throughout the incubation (Fig. 4.2). In this treatment, we observed a considerable decrease in the proportion of mineralized amino acids, indicating that 98% and 93% were taken up directly after day 3 (Fig. 4.3). On average, the proportion of amino acid N mineralized in the subsoil was approximately twice as high as in the surface soil.

In the subsoil, there was a marked negative correlation between the direct uptake route and the inorganic N content ($r = -0.69$, $p < 0.05$), whereas in the surface soil, the correlation was less pronounced ($r = -0.39$, $p < 0.05$).

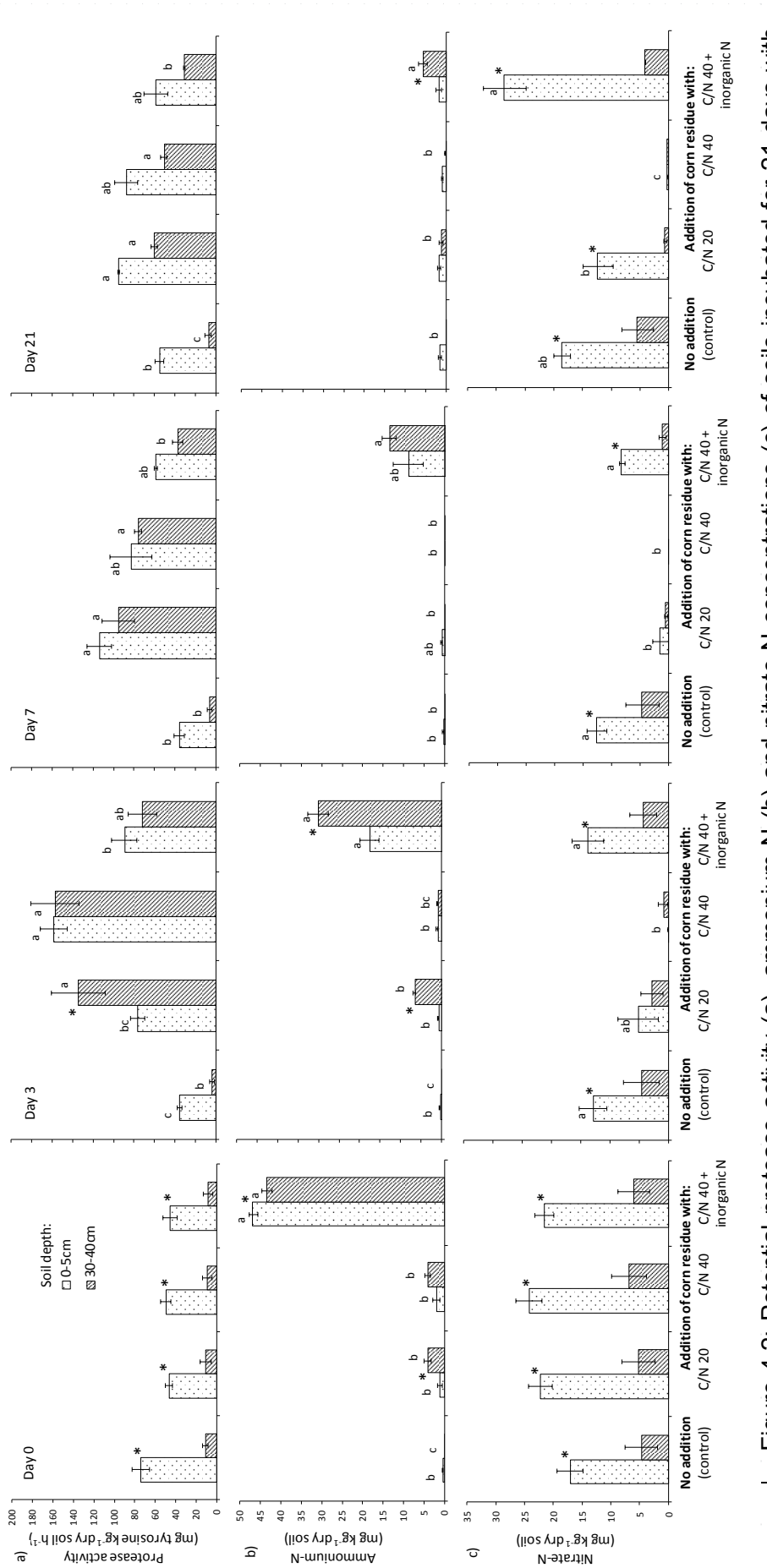

Figure 4.2: Potential protease activity (a), ammonium-N (b) and nitrate-N concentrations (c) of soils incubated for 21 days with corn residues of different C to N ratios in two different soil depths. Data shown are means ± standard errors (n=3). Treatment means for a common depth sharing no lowercase letters are significantly different according to Tukey's HSD test (5% level). Asterisks show significant differences between surface soils and subsoils for a given treatment.

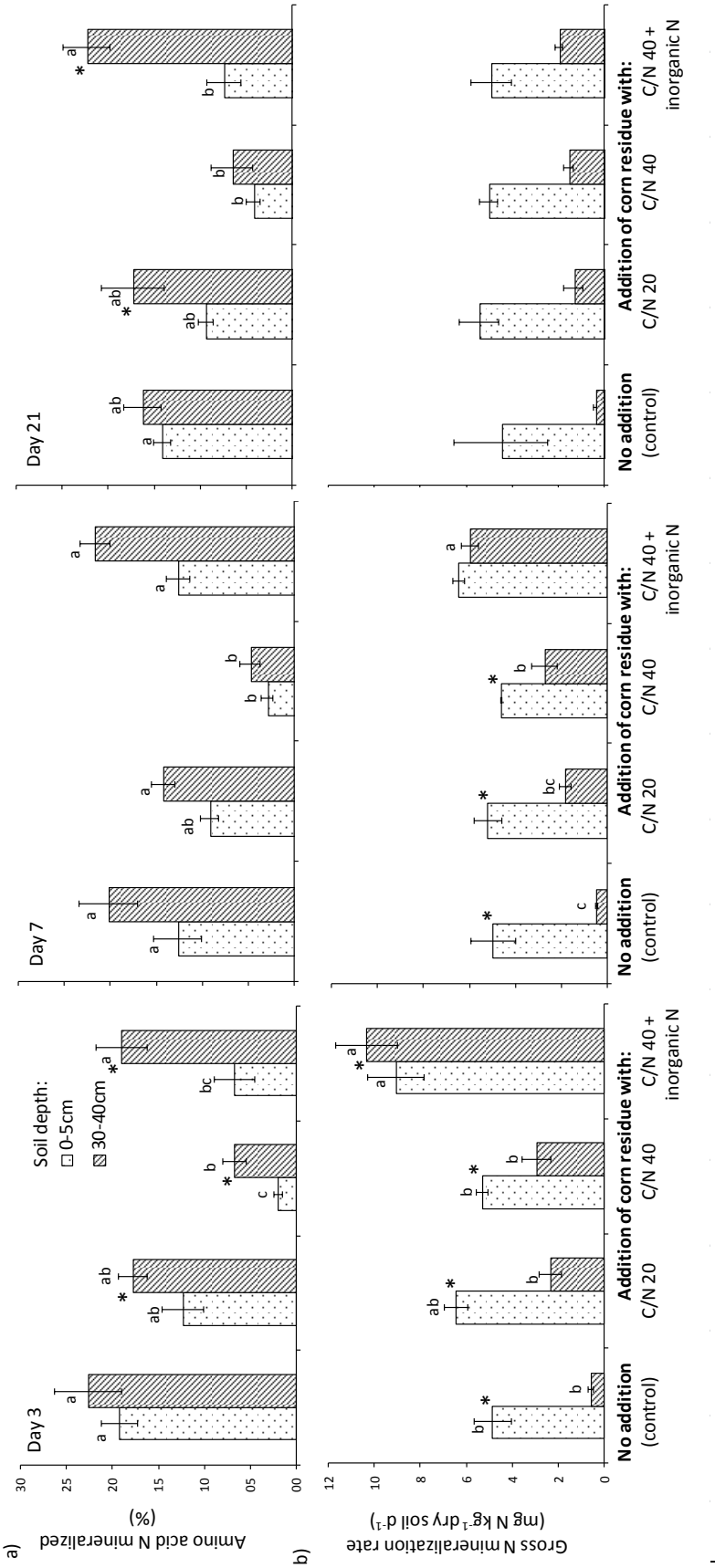

Figure 4.3: Amino acid N mineralization (a) and gross N mineralization (b) of soils incubated for 21 days with corn residues of different C to N ratios in two different soil depths. Data shown are means ± standard errors (n=3). Treatment means for a common depth sharing no lowercase letters are significantly different according to Tukey's HSD test (5% level). Asterisks show significant differences between surface soils and subsoils for a given treatment.

4.5 Discussion

4.5.1 Microbial biomass, carbon turnover, soil protease activity and gross N mineralization

Microbial biomass C and N increased as expected (see e.g., *Nannipieri* et al. 1983) after addition of corn residue in both soil layers independent of the C to N ratio of the substrate and presence or absence of additional mineral N. In the control treatment biological properties such as basal respiration, microbial biomass C and protease activity were markedly higher in the surface soil than in the subsoil due to the much higher presence of soil organic matter. In both soil layers the addition of corn residue resulted in a marked increase in protease activity (Fig. 4.2) due to the induction by the added substrate (*Haab* et al., 1990) and increases were independent of the substrate quality (cf. treatments II and III with C to N ratios of 20 and 40).

The addition of less substrate + NH_4^+ in treatment IV resulted in a lower protease activity than in the other treatments where 100 mg N of corn residues were applied per kg soil, since NH_4^+ is the preferred source of N for soil microorganisms (*Merrick* and *Edwards*, 1995; *Marzluf*, 1997). Therefore it was not necessary for the soil microorganisms to produce more protease for utilizing organic N. Another explanation could be that the amount of protease produced reflected the amount of substrate available and that less protease was produced in treatment IV because of less substrate available. After day 21, protease activity was in both soil layers still higher than before incubation, suggesting that there was still organic material as substrate in the soil or that proteases remained active due to their association with soil colloids (*Burns*, 1982).

When the MIT route is dominant, protease activity and gross N mineralization rates may be highly correlated since the products of protease activity are the substrates for N mineralization. However, no clear pattern between the protease activity and gross N mineralization was observed (Fig. 4.2a, 4.3b). For instance, increased protease activities after 3 to 7 days did not lead to increased gross N mineralization, which would have been the case for a dominance of the MIT route.

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4.5.2 Nitrogen uptake routes of soil microorganisms

The results of the mirror image procedure showed for the control treatments that at both depths approximately 20% of the added amino acids were mineralized; thus approximately 80% were taken up directly. The fact that both uptake routes were simultaneously active may be due to the coexistence of different microbial communities and soil heterogeneity (*Manzoni* and *Proporato*, 2007).

In the control treatments, the marked preference of the direct uptake of organic N occurred in the surface soil and subsoil with no significant differences between depths despite significantly higher microbial activity, protease activity, gross N mineralization rate and availability of mineral N in the surface soil, suggesting that N availability relative to C was similar at both depths. Corn residue at C/N 20 led to net-N mineralization, indicating that there was no lack of N relative to C as was also the case for the control treatment. Overall, a substrate with a narrow C/N ratio did not result in a significant change in the uptake route in the entire incubation period.

In treatment IV, the wide C/N ratio of the substrate was compensated with additional application of inorganic N. This combined addition had the same effect on the importance of the direct uptake as the addition of substrate with a narrow C/N ratio of 20. Addition of corn residue with C/N 40 resulted in marked net N immobilization and thus a depletion of the mineral N pool. *Geisseler* et al. (2010) suggested that this should result in the de-repression of enzyme systems used for the acquisition of alternative N sources and the direct route should be favored over the MIT route as long as N is limiting relative to C. In fact, the addition of corn residue at C/N 40 resulted in an increased direct uptake of generally more than 94%, whereas corn residue at C/N 20 or corn residue at C/N 40 + NH_4^+ reached only 79 or 91% direct uptake, respectively. This suggests that under conditions of sufficient mineralizable N or increased concentrations of NH_4^+, the enzyme system involved in the direct uptake is slightly repressed.

In all treatments with substrate addition, the proportion of amino acid N mineralized was significantly lower in the surface soil after day 3, indicating that the direct uptake route was more

important in the surface soil than in the subsoil. The direct uptake route was negatively correlated with the inorganic N content, in the surface soil (r = -0.39) and in the subsoil (r = -0.69), suggesting that the MIT route generally became more important with increasing inorganic N content. This is in line with *Geisseler* et al. (2010) who suggested that the enzyme systems for the utilization of alternative N sources are repressed at high NH_4^+ availability and therefore the MIT route is more important.

4.6 Conclusion

In the current incubation experiment, the direct uptake route was the dominant N uptake route in all treatments and at both depths. However, corn residue with a wide C/N ratio resulted in a significantly greater importance of the direct uptake than without substrate or after addition of residue with a narrow C/N ratio or inorganic N. The direct uptake route was more important in the surface soil than in the subsoil.

4.7 Acknowledgments

We would like to thank Anja Sawallisch for technical assistance. This project was financed by the German Research Foundation.

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5. Extracellular deamination of amino acids in soil – Adaption of a method to detect amino acid oxidase in soil.

5.1 Abstract

As a large proportion of the organic N input into soil is in form of proteinaceous material, the deamination of amino acids is a key reaction of the mineralization immobilization turnover (MIT) route. Amino acid oxidases from many fungal and bacterial species have been described as cell-surface enzymes. Amino acid oxidase may therefore significantly contribute to the extracellular N mineralization in soil. *Pantoja* et al. (1993) used synthetic fluorescent Lucifer Yellow Anhydride (LYA) derivatives of the amino acid lysine to determine cell-surface enzymatic oxidative deamination of amino acids in sea water. The objective was to adapt the method from *Pantoja* et al. (1993) to determine amino acid oxidase activity in soil. Two arable soils with different soil properties and texture were used to develop a method for soil. Oxidation of LYA-lysine was observed when it was incubated with soil. LYA-lysine and LYA-5-aminovaleric acid was determined by comparison with standard solutions at retention times of 23.6 and 36.7 min, respectively. That means that the detection of amino acid oxidase with synthetic fluorescent Lucifer Yellow derivatives of the amino acid lysine is possible in soil. The effects of different buffer, buffer pH, substrate concentration, incubation time, amounts of soil and incubation temperature on the release of LYA-5-aminovaleric acid were determined. The rate of LYA-5-aminovaleric acid produced showed a broad pH range in both soils. The relationship between the activity of amino acid oxidase and the amount of soil or incubation temperature was linear. However, it was not possible to find the substrate concentration at which the reaction rate is independent on substrate concentration (zero-order kinetics) and therefore a valid soil enzyme method could not be developed.

5.2 Introduction

Organic material serves as main supplier for organic N in soil. The greatest proportion of N containing molecules from plant and microbial residues are proteins with 40% or more of N (*Schulten* and *Schnitzer*, 1998). Soil microorganisms have developed several mechanisms for organic N uptake, namely the direct uptake route and the mineralization-immobilization-turnover (MIT) route (*Barraclough*, 1997). The first step for the degradation of organic material is made by extracellular enzymes. Enzymes are proteins and combined with a substrate they can catalyze a biochemical reaction. Soil enzymes regulate ecosystem functions, are essential in soil for energy transformation and play a key role in nutrient cycling (*Kandeler*, 2007; *Makoi* and *Ndakidemi*, 2008). Extracellular enzymes break down large polymeric compounds and release soluble amino acids, small peptides or amino sugars. In the direct uptake the small organic compounds are taken up directly into the cell and the deamination occurs inside the cell. In contrast, in the MIT route the deamination of small organic compounds occurs outside the cell and only ammonium is taken up by the soil microorganism. Microorganisms can use cell-surface enzymes to oxidize amino acids and amines. The deamination occurs with extracellular enzymes, for example with L-amino acid oxidases (EC 1.4.3.2; L-AAO). L-amino acid oxidases catalyse the oxidative deamination of L-amino acids to their corresponding oxo acids and ammonia (*Pantoja* et al., 1993, *Pantoja* and *Lee*, 1994). L-amino acid oxidases may therefore significantly contribute to the extracellular N mineralization in soil. The deamination of amino acids is a key reaction of the MIT route. *Brearley* et al. (1994) found out that amino acid oxidase had a broad substrate specificity and that 10 of 21 tested L-amino acids and 7 of 20 tested D-amino acids produced significant reaction rates after addition of L-amino acid oxidase. It is reported, that L-amino acid oxidase is produced by red algae, cyanobacteria, Gram negative bacteria, Gram positive bacteria (*Brearley* et al., 1994).

Pantoja et al. (1993) developed a method to determine cell-surface enzymatic oxidative deamination of amino acids in sea water. The technique used synthetic fluorescent Lucifer Yellow Anhydride (LYA) derivatives of the amino acid lysine. The product of the oxidative deamination of

LYA-lysine is LYA-ε-amino-α-ketocaproic acid, which can be determined by HPLC with fluorometric detection. Based on their extensive tests, the authors concluded that the approach allowed selective investigation of cell surface enzymatic oxidation, since neither transport across the cell membrane nor chemical transformation of the probes occurred (*Pantoja* et al., 1993). The fluorescent probes compete effectively with natural amino acids and are similar to the natural compounds (*Pantoja* and *Lee*, 1994).

The objective of the present work was to adapt the method from *Pantoja* et al. (1993) to determine amino acid oxidase in soil.

5.3 Material and methods

5.3.1 Soils

The arable soils used were surface samples (0 – 20 cm) with different soil properties and soil texture (Table 5.1). They were taken from two different sites in central Germany, namely Allerberg and Saurasen. Field-moist soil samples were sieved (< 2 mm) and stored at 4 °C in the dark before processing. Before incubation the samples were kept for 24 h at room temperature.

Total C and N contents were analyzed after drying and grinding with a C to N analyzer (Heraeus Elementar Vario EL, Hanau, Germany). Since no carbonates were found in the soil, the total C corresponds to organic C (SOC).

Table 5.1: Soil organic carbon (SOC), total nitrogen (N_{tot}), pH and soil texture are mean values of three pseudoreplicates.

Sample site	SOC	N_{tot}	pH	Clay	Silt	Sand
	[g kg^{-1} soil]		[H_2O]	[%]	[%]	[%]
Allerberg	2.1	0.1	8.2	13	36	51
Saurasen	0.9	0.1	7.1	16	76	7

5.3.2 Reagents

THAM buffer (0.2 M, pH 7.5): Prepared by dissolving 24.2 g of tris (hydroxymethyl) aminomethane (THAM buffer) in about 800 mL of double deionized (DD) water. The solution was titrated to pH 7.5 with HCl (1 M) and diluted with DD water to 1 L.

Lithium acetate buffer (1 M, pH 5): Prepared by dissolving 10.2 g of lithium acetate dihydrate in 70 mL DD water. The solution was titrated to pH 5.0 with acetic acid and diluted to 100 mL with DD water.

LYA-lysine: The production of LYA-lysine was adapted from *Pantoja* et al. (1993). Briefly, 236 mg N-α-t-Boc-L-lysine and 107 mg LYA were added to 5 ml of a 1 M lithium acetate buffer. The mixture was heated to 105 °C under stirring and reflux. After 6.5 h, first 10 mL of boiling DD water was added and then 2.5 g of potassium chloride. After reaching room temperature the mixture was placed in the fridge at 4 °C overnight. After filtration the product was washed with 2 x 10 mL of 10% aqueous potassium thiocyanate and 2 x 10 mL of ethanol. Then it was dried in vacuo at least for 24 hours. A yellow powder was left and it was stored in a desiccator before processing. For the incubation with soil, LYA-lysine was dissolved in THAM buffer.

LYA-5-aminovaleric acid: The production of LYA -5-aminovaleric acid was similar to that for LYA-lysine, excepting that 112.5 g monoamine 5-aminovaleric acid were added to the mixture instead of N-α-t-Boc-L-lysine and that the refluxing time was continued for 12h (*Pantoja* et al., 1993).

KH_2PO_4 (0.05 M, pH 4.5): Prepared by dissolving 6.8045 g potassium dihydrogen phosphate in 1 L DD water. For degasing and sterilization the eluent was filtered over 0.2 µm polyamide filters.

Methanol (90%): Prepared by addition of 100 ml DD water to 900 ml methanol (CH_3OH). For degasing and sterilization the eluent was filtered over 0.2 µm polyamide filters.

5.3.3 Assay of amino acid oxidase activity

Soil amino acid oxidase activity was assayed by placing 1 g of field-moist soil into a 70 mL plastic beaker, and then adding 3 ml of 5 µM LYA-lysine dissolved in THAM buffer (0.2 M, pH 7.5).

The mixtures were placed in a shaking (200 rpm^{-1}) water bath at 37 °C for 3 h. After incubation, the enzyme reaction was stopped with an ice bath and the suspension was filtered with a 0.2 µm polyamide filter to remove microorganisms. To 1 mL filtrate 0.2 µL H_2O_2 (30%) was added to oxidize LYA-ε-amino-α-ketocaproic acid to LYA-5-aminovaleric acid. This method was based on the oxidative decarboxylation that α-keto acids undergo when exposed to mild oxidants. LYA-lysine and the secondary product, LYA-5-aminovaleric acid, were monitored by HPLC.

5.3.4 Effect of different properties on the amino acid oxidase activity

The above procedure was modified to test the effects of different buffers (phosphate and THAM buffer), buffer pH, substrate concentrations, incubation temperatures and incubation times on amino acid oxidase activity.

To determine the buffer solution for the optimal activity of amino acid oxidase two different buffer solutions were tested, namely phosphate buffer (0.1 M) and THAM buffer (0.2 M). The activity was assayed with 2.5 µM of LYA-lysine in the presence of 9.2 units of commercial amino acid oxidase from the venom of the snake *Crotalus adamanteus* (EC 1.4.3.2) and the buffer solution. After 2, 4, 6, 8 and 24 h of incubation at 20 °C the concentration of LYA-5-aminovaleric acid was determined in both buffer solutions.

To determine the optimal pH for amino acid oxidase activity in soils, the activity was assayed with 1 g of soil at 5 µM of LYA-lysine in the presence of THAM buffer at pH values ranging from 6 to 8.4.

Substrate concentrations of 0.001, 0.005, 0.01, 0.02, 0.05, 0.1, 0.25, 0.5, 1, 5 and 10 mM were tested. The effect of reaction time was evaluated by having the samples reacting for 2, 4, 6 and 8 hours. The effect of varying the amount of soil was determined by using 0.2, 0.5, 1 and 2 g of soil. The temperature dependence of L-amino acid oxidase activity was evaluated via conducting the reactions at temperatures of 4, 20 and 37 °C using the assay described above.

5.3.5 Quantitative analysis of the probes

The determination of the compounds was adapted from *Pantoja* et al. (1993). Compounds were measured with the HPLC system consisting of a Dionex (Germering, Germany) P 580 gradient pump, a Dionex Ultimate WPS – 3000TSL analytical autosampler with in-line split-loop injection and thermostat and a Dionex RF 2000 fluorescence detector set at 550 nm emission and 424 nm excitation wavelength with medium sensitivity.

LYA-lysine and LYA-5-aminovaleric acid were determined with fluorometric detection using a HPLC. Samples were stored in the autosampler at 4 °C prior to measurements. A 250 x 4.6 mm Hypersil ODS C-18 (5 µm) column was used with mobile phase of aqueous phosphate buffer (0.05 M KH_2PO_4, pH 4.5) and methanol (90%). With 1 ml/min flow velocity the following gradient was used: CH_3OH increasing from 0 to 14% in 17 min, 14% CH_3OH for 5 min, 14 – 20% in the next 5 min, 20% for 2 min, 20 – 50% in the next 3 min, 50 – 100% in the next 5 min, 100% CH_3OH for 5min, 100 – 20% in the next 5 min, 20 – 0% in 5 min and 0% CH_3OH for 5 min.

Results were quantified by comparison with standard solutions of LYA-lysine and LYA-5-aminovaleric acid of known concentration.

5.4 Results and discussion

After the incubation of LYA-lysine and soil, peaks with retention times of 23.6 min and 36.7 min were measured with HPLC. By comparison with the standard solutions we identified the peak of 23.6 min as LYA-lysine and the peak of 36.7 min as LYA-5-aminovaleric acid. Oxidation of LYA-lysine was observed when it was incubated with soil, suggesting that amino acid oxidase deaminated LYA-lysine to LYA-ε-amino-α-ketocaproic acid. Through the addition of H_2O_2 LYA-ε-amino-α-ketocaproic acid was oxidized to LYA-5-aminovaleric acid. This indicates that the detection of amino acid oxidase with synthetic fluorescent Lucifer Yellow derivatives of the amino acid lysine is possible in soil.

5.4.1 Buffer pH

The activities of phosphate and THAM buffer were similar, but THAM buffer showed in all times a higher activity (Figure 5.1).

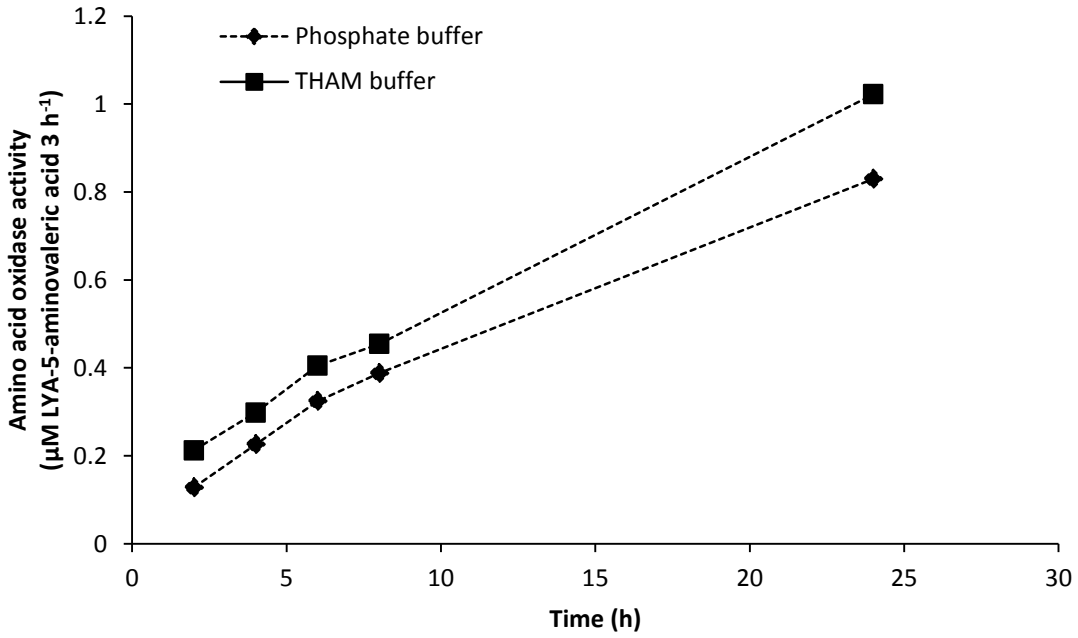

Figure 5.1: Effect of phosphate and THAM buffer on amino acid oxidase activity in two arable soils during 24 hours.

The rate of LYA-5-aminovaleric acid produced showed a broad pH range in both soils with an optimum around pH 7.5. This value agrees with the range (6.5-9) reported by *Geueke* (2002), *Brearley* et al. (1994) and *Braun* et al. (1992). In soil from Allerberg the activity of amino acid oxidase decreased with pH values higher than 7.5, whereas soil from Saurasen showed a broad pH optimum (Figure 5.2).

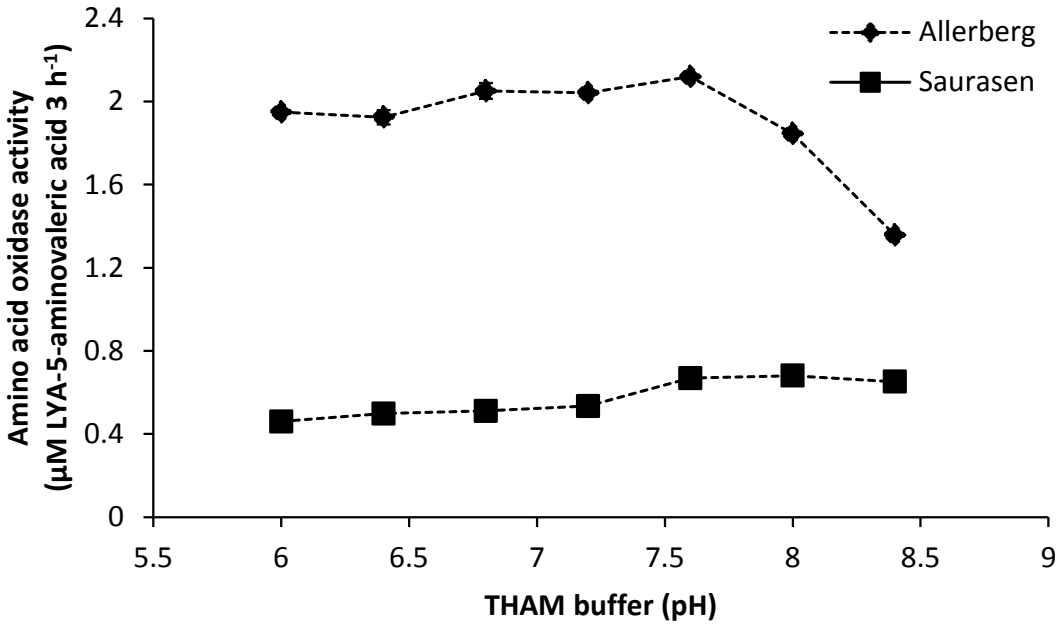

Figure 5.2: Effect of THAM buffer pH on amino acid oxidase activity in two arable soils.

5.4.2 Substrate concentration and amount of soil

A wide range of substrate concentrations were used (0-10 mM) to determine the concentration at which the reaction rate is independent on substrate concentration (zero-order kinetics). *Pantoja* et al. (1993) used a substrate concentration of 100 nM to 5 µM LYA-lysine in sea water. Measuring the potential amino acid oxidase activity eliminates possible effects of interactions between soil colloids and the substrate on the measured enzyme activity. However, it was not possible to find this concentration. The reaction rate increased with increases in the substrate concentration and showed that after addition of 10 mM LYA-lysine the increase of LYA-5-aminovaleric acid was still linear (Figure 5.3). We do not expect that after addition of LYA-lysine with higher concentrations the zero-order kinetics can be fulfilled, because we detected after the incubation with high concentrations still high concentrations of LYA-lysine in solution. That means that the solution is saturated with the substrate and that there should be enough substrate for further enzyme activity. We assume that at high concentrations the detection of the fluorescent compounds is problematic.

Therefore, we diluted 10 mM LYA-lysine after incubation with soil to 100 µm LYA-lysine to determine the substrate concentration with HPLC. A clear peak was visible. However, after calculation of the substrate concentration we still found a linear relation between the substrate and product.

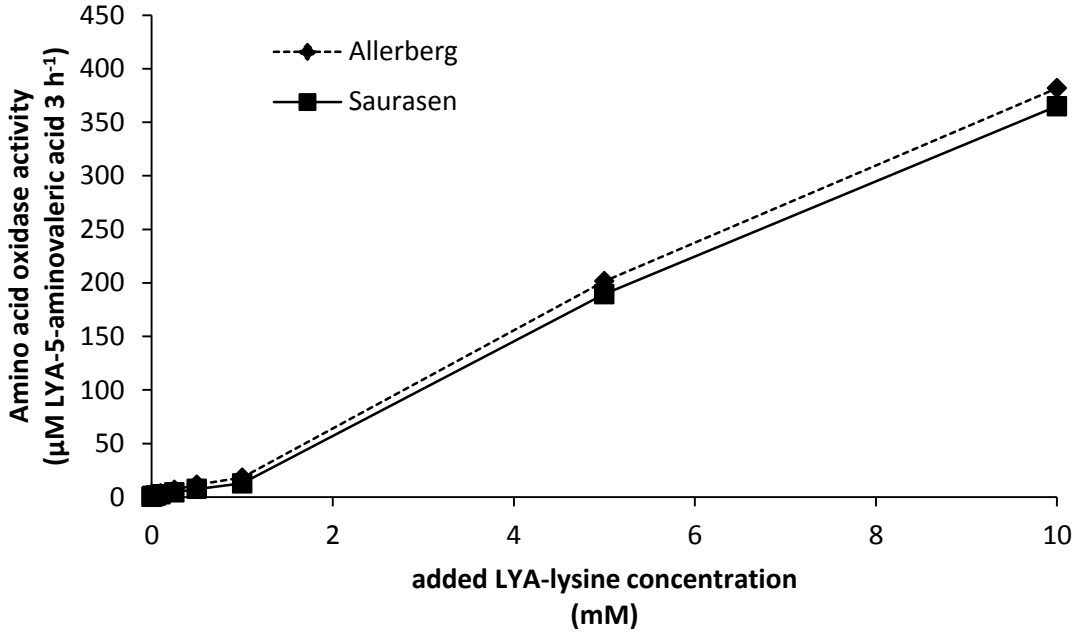

Figure 5.3: Effect of substrate concentration on release of LYA-5-aminovaleric acid in two arable soils.

5.4.3 Amount of soil and incubation time

Under the conditions selected and tested, amino acid oxidase activity in soil was linear with increasing amount of soil used and showed that 1 g of soil was satisfactory for measuring the activity of this enzyme (Figure 5.4).

The relationship between the incubation time and the activity of amino acid oxidase was linear in the tests with different buffers without soil. This relationship is usually linear in enzyme catalyzed reactions, as long as the enzyme is stable and retains its full activity (*Dick*, 2011). After addition of LYA-lysine to soil, the production of LYA-5-aminovaleric acid still increased with increasing time (Figure 5.5).

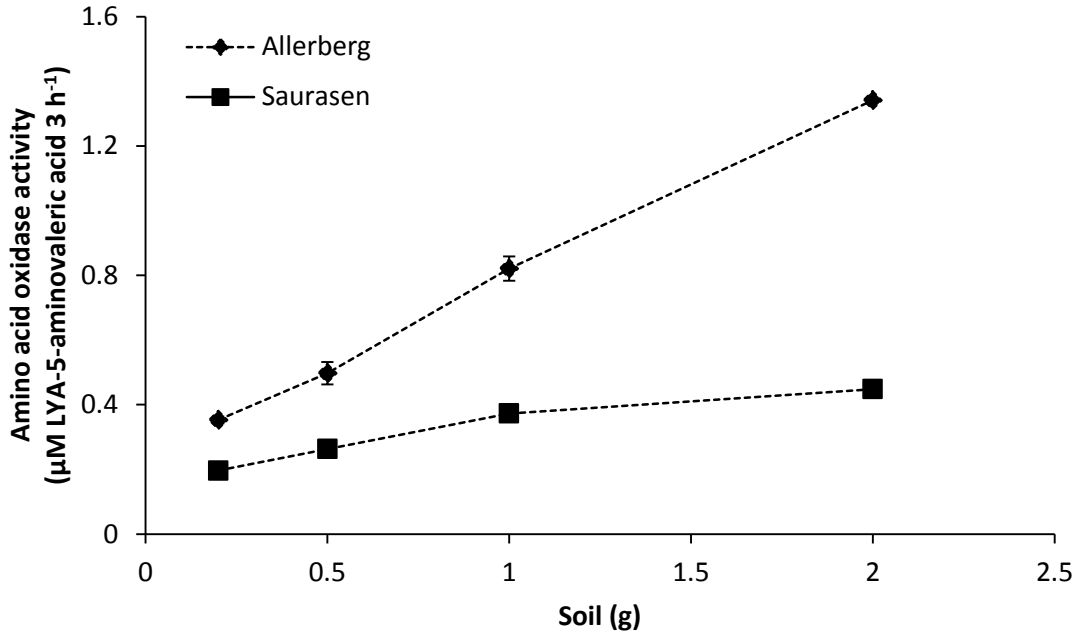

Figure 5.4: Effect of amount of soil on release of LYA-5-aminovaleric acid in two arable soils.

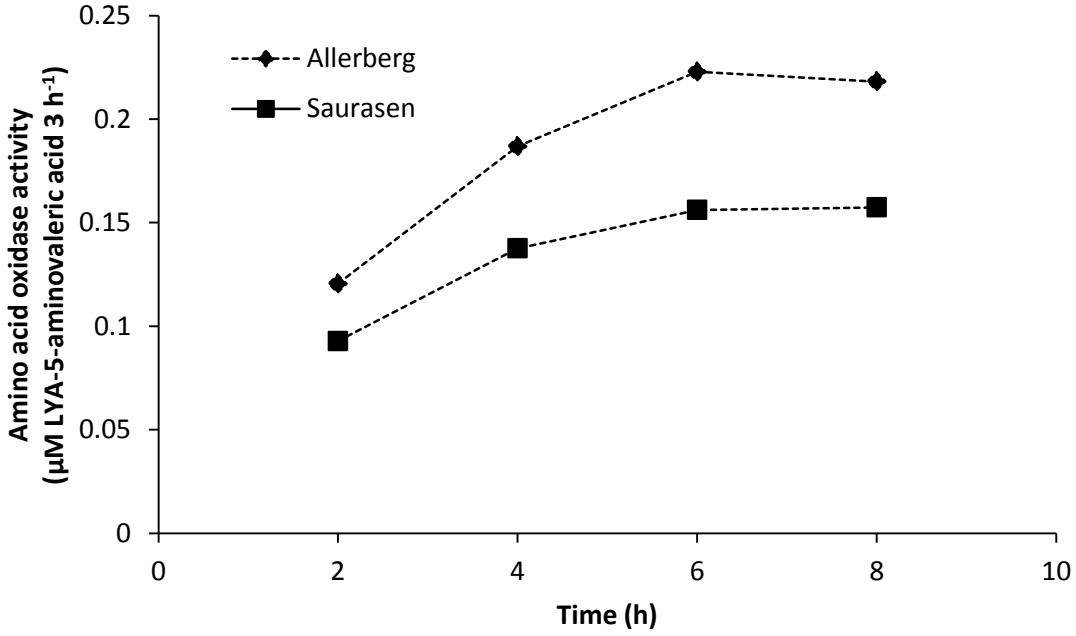

Figure 5.5: Effect of incubation time on release of LYA-5-aminovaleric acid in two arable soils.

5.4.4 Temperature of incubation

Activity of amino acid oxidase increased with increasing reaction temperature (Figure 5.6). In general, enzyme-catalyzed reactions proceed at faster rates with increasing temperature until a temperature is reached above which the enzyme activity decreases due to denaturation. Optimal amino acid oxidase activity is descripted by *Brearley* et al. (1994) at approximately 50 °C. The activity of amino acid oxidase was assayed at 37 °C because this temperature has been used extensively for assay of other enzymes in many biological materials, including soils (*Acosta-Martinez* and *Tabatabai*, 2000; *Dick*, 2011). Thus, 37 °C was used for the reaction temperature for the subsequent experiments in this study.

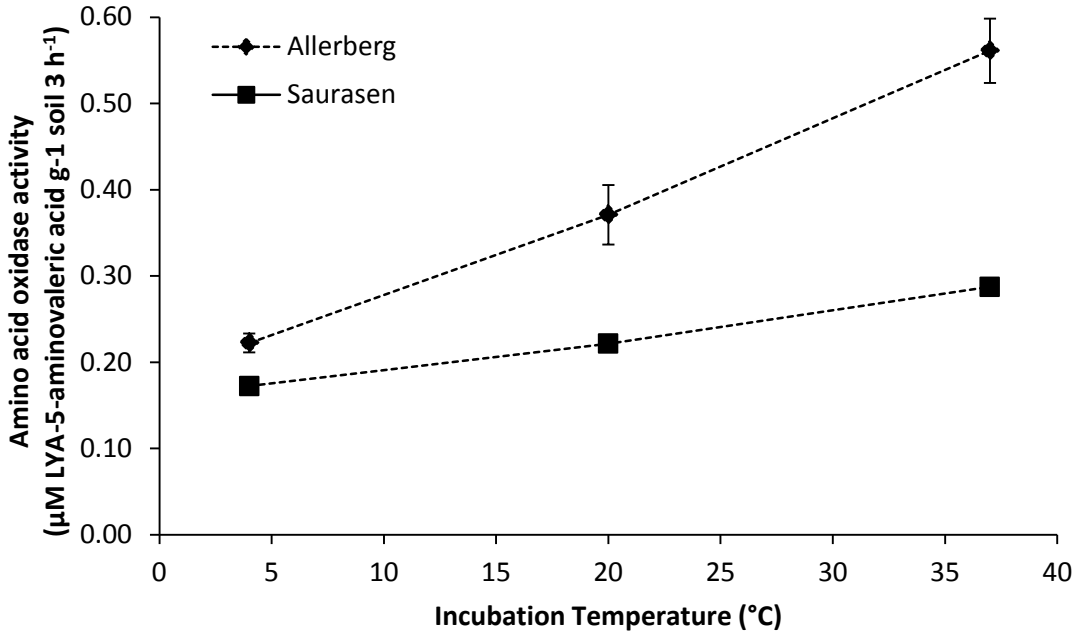

Figure 5.63: Effect of incubation temperature on release of LYA-5-aminovaleric acid in two arable soils.

5.5 Conclusion

LYA-lysine and LYA-5-aminovaleric acid was determined by comparison with standard solutions at retention times of 23.6 and 36.7 min, respectively. This indicates that the detection of amino acid

oxidase with synthetic fluorescent Lucifer Yellow derivatives of the amino acid lysine is possible in soil. The results suggested that the method is sensitive for detection of amino acid oxidase activity using 1 g of soil and 3 h of reaction time at 37 °C. However, it was not possible to find the substrate concentration at which the reaction rate is independent on substrate concentration (zero-order kinetics) and therefore we were not able to develop a valid soil enzyme assay.

5.6 Acknowledgments

We would like to thank *Anja Sawallisch* and *Gabriele Dormann* for technical assistance. This project is financed by the Deutsche Forschungsgemeinschaft ("Nitrogen utilization pathways of soil microorganisms").

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6. General conclusion

In general, the results of the present work indicate that the direct uptake of organic N is the preferred uptake route of soil microorganisms in arable soils. After addition of corn residue with a wide C to N ratio the direct uptake route was significantly ($p<0.05$) more important than after the addition of corn residue with a small C to N ratio or additional NH_4^+. The results indicated that the MIT route also contributed to the N uptake by about 30%. Addition of corn residue with a wide C to N ratio led to the immobilization of N and resulted in the depletion of the mineral soil N pool. The lack of N induced the de-repression of enzyme systems used for the acquisition of alternative N sources such as amino acids, and the direct route was favored over the MIT route. In contrast, addition of corn residue with a small C to N ratio led to the mineralization of N in the soil solution. If the mineral N level in the soil solution was sufficient, the enzyme systems used for the acquisition of alternative N sources were repressed and the MIT route became more important. An additional presence of NH_4^+ resulted in an increased importance of the MIT route, suggesting that the enzyme systems are repressed through the high level of mineral N in the soil solution.

The direct uptake route was more important in the surface soil than in the subsoil for all treatments. Higher organic material content in the surface soil probably resulted in an adaption of the microorganisms to the direct uptake route. Moreover, the content of microbial biomass C and N was higher in the surface soil, suggesting a large requirement of nutrients and energy, both of which the direct uptake of organic material provides.

The detection of the extracellular enzyme amino acid oxidase via synthetic fluorescent Lucifer Yellow derivatives of the amino acid lysine is possible in soil. However, it was not possible to find the substrate concentration at which the reaction rate is independent of substrate concentration (zero-order kinetics) and therefore we were not able to develop a valid soil enzyme assay.

7. Summary

Soil microorganisms have developed two different mechanisms for N uptake: the direct route and the mineralization-immobilization-turnover (MIT) route. In the direct uptake route, small organic molecules, such as amino acids, are taken up directly into the cell via different mechanisms. The deamination occurs inside the cell and only the surplus N is released back to the soil solution. In contrast, in the MIT route, organic material is deaminated outside the cell with extracellular enzymes, like amino acid oxidase. The deamination of amino acids is a key reaction of the MIT route and the quantitative detection of the enzyme amino acid oxidase (EC 1.4.3.2; L-AAO) may significantly contribute to the extracellular N mineralization in soil. However, which uptake route is more dominant depends on the form of available N, the source of C and the availability of N relative to C. The objectives of the present thesis were:

(i) to study the effect of substrate quality on the N uptake routes of soil microorganisms in an incubation experiment.

(ii) to investigate the effects of soil depth on the microbial community and on the N uptake route of soil microorganisms following the addition of corn residues with different C to N-ratios in the presence and absence of mineral N.

(iii) to develop a method which allows the use of Lucifer Yellow Anhydride (LYA) derivatives of the amino acid lysine for the detection of the extracellular enzyme amino acid oxidase in soil.

For objective I we incubated soil samples from the long term trial in Bad Lauchstädt with added corn residues with different C to N ratios and mineral N for 21 days at 20 °C and 60%water holding capacity (WHC). The following treatments were used: no addition of residues (control, treatment I),

addition of corn residues with a C to N ratio of 40 (II), 20 (III), and 40 with further added $(NH_4)_2SO_4$ (IV). After 3, 7 and 21 days of incubation, the extent of added amino acid mineralization, gross N mineralization rates and protease activity were determined. Under the assumption that all added amino acids were taken up or mineralized, the direct uptake route was more important in soil amended with corn residues with a wide C to N ratio. The mineralization rate of amino acids increased in the order “addition of corn residues with a wide C to N ratio” (5%) < “corn residues with a small C to N ratio” (27%) < “corn residues with a wide C to N ratio & (NH4)2SO4 and control treatment” (31% and 32%, respectively) after 21 days of incubation, suggesting that 95% (treatment II), 73% (III), 69% (IV), and 68% (I) of the added amino acids were taken up directly. In all treatments the proportion of the added amino acids that were mineralized increased with time, indicating that the MIT route became more important over time.

To investigate the effects of soil depth on the microbial community and on the N uptake route of soil microorganisms after the addition of corn residues with different C to N ratios in the presence and absence of mineral N (objective II), we used soil samples from three long-term arable sites in central Germany. Soil samples were taken in two soil depths (0-5 cm; 30-40 cm) and incubated for 21 days at 20 °C and 60% (WHC) with corn residues with different C to N ratios and mineral N. The following treatments were applied: no addition of residues (control, treatment I), addition of corn residues with a C to N ratio of 20 (II), 40 (III), and 40 together with $(NH_4)_2SO_4$ (IV). After 3, 7 and 21 days of incubation, we determined the extent of added amino acids mineralization via ^{15}N-labeled amino acids as well as the gross N mineralization rate, potential protease activity and the contents of ammonium, nitrate and microbial biomass C and N. Under the assumption that all amino acids were taken up or mineralized, the direct uptake was favored in the control surface soil and subsoil with no significant ($p < 0.05$) differences between depths despite significantly higher microbial activity, protease activity, gross N mineralization rate and availability of mineral N in the surface soil, suggesting that N availability relative to C was similar in both depths. The addition of corn residue resulted in a marked increase of protease activity in both depths due to the induction from

the added substrate. Addition of corn residue with a wide C to N ratio resulted in a significantly greater share of the direct uptake (97% and 94%) than without the addition of residues (85% and 80%) or addition of residue with a small C to N ratio (90% and 84%) or mineral N (91% and 79% in the surface soil and subsoil, respectively), suggesting that under conditions of sufficient mineralizable N (C to N ratio of 20) or increased concentrations of NH_4^+, the enzyme system involved in the direct uptake is slightly repressed. Substrate additions resulted in an initially significantly higher increase of the direct uptake in the surface soil than in the subsoil.

As a large proportion of the organic N input into soil is in form of proteinaceous material, the deamination of amino acids is a key reaction of the mineralization immobilization turnover (MIT) route. Therefore the enzyme amino acid oxidase (EC 1.4.3.2; L-AAO) may significantly contribute to the extracellular N mineralization in soil. To determine the amino acid oxidase activity in soil (objective III), we adapted the method to determine amino acid oxidase in sea water from Pantoja et al. (1993). Soil from two different arable sites in central Germany and synthetic fluorescent Lucifer Yellow Anhydride (LYA) derivatives of the amino acid lysine were used to determine the activity of amino acid oxidase in soil. Briefly, 3 mL of LYA-lysine (5 µM) solved in THAM buffer was incubated with 1 g of soil for 3 h in a shaking water bath at 37°C. After incubation the solution was placed in an ice bath to reduce the enzyme activity to a minimum and the solution was immediately filtered with a 2 µm filter to remove microorganisms. The product of LYA-lysine and amino acid oxidase is LYA-ε-amino-α-ketocaproic acid, which was oxidized to LYA-5-aminovaleric acid via H_2O_2 addition, as it is not stable for detection. The determination was performed via fluorometric detection using a high performance liquid chromatography (HPLC). LYA-lysine and the oxidation product LYA-5-aminovaleric acid were determined by comparison with standard solutions at retention times of 23.6 and 36.7 min, respectively. Thus, the determination of amino acid oxidase activity via synthetic fluorescent Lucifer Yellow derivatives of the amino acid lysine is possible in soil. To develop a sensitive method we tested the effects of different buffer solutions, buffer pH values, incubation temperatures, incubation times, amounts of soil and substrate concentrations on

the release of LYA-5-aminovaleric acid. As buffer solutions, phosphate buffer (0.1 M) and THAM buffer (0.2 M) were tested. Measured enzyme activities were slightly higher in solutions with THAM buffer. To find the optimum pH value of the THAM buffer solution, different pH values were tested. The rate of LYA-5-aminovaleric acid production showed a broad pH range with an optimum around pH 7.5. With increasing incubation temperature, time or amount of soil the production of LYA-5-aminovaleric acid increased, indicating an increased enzyme activity. Different substrate concentrations (0-10 mM) were tested to find the concentration at which the reaction rate is independent on substrate concentration (zero-order kinetics). However, it was not possible to find this concentration. At high concentrations the detection of the fluorescent compounds is problematic. Therefore, we diluted solutions with high concentrations after incubation and measured the product LYA-5-aminovaleric acid with HPLC. However, after the calculation of the actual concentration of LYA-5-aminovaleric acid we still could not obtain zero-order kinetics of the substrate. Furthermore, after the incubation with high concentrations of LYA-lysine, we still found these high concentrations also in the solution, indicating that the solution was saturated with the substrate and the enzyme was unable to metabolize all the available substrate to LYA-5-aminovaleric acid. Overall, it was possible to determine the activity of amino acid oxidase in soil, but because it was not possible to find the substrate concentration at which the reaction rate is independent on substrate concentration, it was not possible to develop a valid soil enzyme assay.

8. Zusammenfassung

Mikroorganismen erhalten durch den Abbau von organischem Material Energie, Kohlenstoff (C) und Nährstoffe. Ein besonders wichtiger Nährstoff ist Stickstoff, der für den Aufbau von Proteinen, Nukleinsäuren und andere Zellbestandteile benötigt wird. Bodenmikroorganismen haben zwei verschiedene Wege zur N-Aufnahme von organischem Material entwickelt: den direkten Aufnahmeweg, bei dem kleine organische Moleküle direkt in die Zellen aufgenommen werden und den indirekten Weg, bei dem die Aminogruppe des organischen Materials außerhalb der Zelle mit Hilfe von extrazellulären Enzymen abgespalten und dann in die Zelle aufgenommen wird.

Um die Mineralisationsprozesse von organischem Material im Boden besser zu verstehen, ist es wichtig, die Kontrollmechanismen dieser N-Aufnahmewege zu kennen. In dieser Arbeit werden die Auswirkungen von Substratzugabe unterschiedlicher Qualität, mit und ohne Zugabe von mineralischem Stickstoff, in unterschiedlicher Bodentiefe auf den N-Aufnahmeweg untersucht. Des Weiteren soll eine Methode zur Messung der Aktivität des extrazellulären Enzyms L-Aminosäureoxidase entwickelt werden, das eine wichtige Rolle bei der indirekten N-Aufnahme spielt.

Die vorliegende Arbeit ist in folgende Abschnitte unterteilt:

(i) Bestimmung der Auswirkungen von Pflanzenstreu mit unterschiedlicher Substratqualität in An- und Abwesenheit von mineralischem Stickstoff auf die N-Aufnahmewege der Bodenmikroorganismen in einem Inkubationsversuch.

(ii) Untersuchung der Auswirkung von unterschiedlichen Bodentiefen und Zugabe von Streu mit unterschiedlichem C/N-Verhältnis auf die mikrobielle Gemeinschaft und den N-Aufnahmeweg von Bodenmikroorganismen in drei Ackerböden.

(iii) Anpassung einer Methode zur Aktivitätsbestimmung des extrazellulären Enzyms Aminosäureoxidase in Boden mit Hilfe von Lucifer Yellow Anhydrid (LYA) Derivaten der Aminosäure Lysin.

Der Abbau von proteinhaltigem organischem Material beginnt mit Protease, einem extrazellulärem Enzym das Proteine katalysiert. Durch die biochemische Reaktion werden größere polymere Bestandteile in Aminosäuren, kleine Peptide oder Aminozucker zerlegt. Die Bodenmikroorganismen haben nun die Möglichkeit, diese Bestandteile mit Hilfe von Enzymen weiter zu zerlegen (indirekter Weg) oder sie direkt in ihre Zelle aufzunehmen (direkter Weg). Beim indirekten N-Aufnahmeweg wird der ganze organische Stickstoff vor der Aufnahme in die Zelle zu NH_4^+ mineralisiert. Der direkte Weg setzt voraus, dass Mikroorganismen in der Lage sind, organische Bestandteile im Ganzen durch ihre Zellwand zu transportieren. Dieser Transport kostet Energie und erfolgt über verschiedene Transportprozesse. Zum Beispiel wird durch den Mangel an Kohlenstoff, Stickstoff oder Schwefel das Transportsystem für Aminosäuren aktiviert, bei hohen Ammonium- oder Aminosäure-Gehalten aber unterdrückt. Nachdem die Aminosäuren aufgenommen wurden, können diese zum Aufbau von z.B. Proteinen genutzt werden. Hat der Organismus aber genügend Stickstoff und es mangelt zum Zellaufbau an Kohlenstoff, wird die Aminogruppe mit intrazellulären Enzymen abgespalten und zurück in die Bodenlösung entlassen. Eine unserer Hypothesen ist daher, dass sich bei Streuzugabe mit einem engen C/N-Verhältnis der überschüssige Stickstoff in der Bodenlösung sammelt und durch den dann hohen Ammoniumgehalt die Transportsysteme für Aminosäuren unterdrückt werden. In diesem Fall würde der indirekte Aufnahmeweg an Bedeutung gewinnen, ebenso wie bei der Zugabe von mineralischem Stickstoff. Im Gegensatz dazu herrscht bei Streuzugabe mit einem weiten C/N-Verhältnis Stickstoffmangel und die Transportsysteme für Aminosäuren werden aktiviert, wodurch der direkte Aufnahmeweg eine größere Rolle spielt.

Im Folgenden sind die einzelnen Teilabschnitte dieser Arbeit näher erläutert:

(i) Im ersten Teil wurden Bodenproben vom Oberboden (0-20 cm) des Langzeitdüngeversuchs in Bad Lauchstädt entnommen und mit getrockneten und gemahlenen Maisblättern mit unterschiedlichem C/N-Verhältnis und mineralischem Stickstoff für 21 Tage bei 20 °C inkubiert. Die folgenden Behandlungen wurden durchgeführt: (I) keine Zugabe (Kontrolle), (II) Zugabe von Maisresten mit einem C/N-Verhältnis von 40, (III) Zugabe von Maisresten mit einem C/N-Verhältnis von 20 und (IV) Zugabe von Maisresten mit einem C/N-Verhältnis von 40 und Ammoniumsulfat ($(NH_4)_2SO_4$). Die Stickstoffzugabe war bei den Behandlungen (II), (III) und (IV) gleich und betrug 0,1 mg N g^{-1} trockener Boden. Nach 3, 7 und 21 Inkubationstagen wurde der Anteil von zugegebenen Aminosäuren, die innerhalb von 48 h mineralisiert wurden, bestimmt, außerdem die Brutto-N-Mineralisationsrate und die Enzymaktivität des Enzyms Protease. Unter der Annahme, dass alle zugegebenen Aminosäuren aufgenommen oder mineralisiert wurden, ist bei der Mineralisation von Maisresten mit einem weiten C/N-Verhältnis der direkte Aufnahmeweg wichtiger als bei den anderen Behandlungen. Nach 21 Inkubationstagen stieg die Mineralisationsrate und damit auch die Bedeutung der indirekten Aufnahme in folgender Reihenfolge: Zugabe von Maisresten mit einem C/N-Verhältnis von 40 (5% der zugegebenen Aminosäuren wurden mineralisiert) < Zugabe von Maisresten mit einem C/N-Verhältnis von 20 (27% der zugegebenen Aminosäuren wurden mineralisiert) < Zugabe von Maisresten mit einem C/N-Verhältnis von 40 & $(NH_4)_2SO_4$ und keine Zugabe von Pflanzenresten (31% bzw. 32% der zugegebenen Aminosäuren wurden mineralisiert). Somit wurden 95% der zugegebenen Aminosäuren in der Behandlung (II), 73% in der Behandlung (III), 69% in der Behandlung (IV) und 68% in der Kontrollbehandlung (I) direkt aufgenommen. In allen Varianten nahm der Anteil der mineralisierten Aminosäuren mit der Zeit zu, der indirekte Aufnahmeweg wird also mit der Zeit wichtiger. Nach der Zugabe von Maisresten nahm auch die Enzymaktivität des extrazellulären Enzyms Protease zu, d.h. die Mikroorganismen bildeten vermehrt ein Enzym

zum Proteinabbau, um an Nährstoffe zu gelangen. Auch die Brutto-N-Mineralisationsrate ist nach Zugabe von organischem Material durch dessen Abbau höher als ohne Zugabe von organischem Material.

(ii) Um den Effekt der Bodentiefe auf die mikrobielle Gemeinschaft und auf die N-Aufnahmewege der Bodenmikroorganismen unter Berücksichtigung von zugegebenen Maisresten mit unterschiedlichem C/N-Verhältnis zu untersuchen, wurden Bodenproben von drei Ackerflächen in den Tiefen 0-5 cm und 30-40 cm entnommen. Zusammen mit getrockneten und gemahlenen Maisblättern wurde der Boden bei 20 °C für 21 Tage inkubiert. Die Behandlungen des Bodens waren: (I) keine Zugabe (Kontrolle), (II) Zugabe von Maisresten mit einem C/N-Verhältnis von 20, (III) Zugabe von Maisresten mit einem C/N-Verhältnis von 40 und (IV) Zugabe von Maisresten mit einem C/N-Verhältnis von 40 & $(NH_4)_2SO_4$. Nach 3, 7 und 21 Inkubationstagen wurde der Anteil der mineralisierten zugegebenen Aminosäuren bestimmt, außerdem die Brutto-N-Mineralisationsrate, die Enzymaktivität der Protease, Basalatmung, Ammonium- und Nitratgehalte sowie der Kohlenstoff- und Stickstoffgehalt der mikrobiellen Biomasse. Unter der Voraussetzung, dass alle zugegebenen Aminosäuren aufgenommen oder mineralisiert wurden, war die Mineralisationsrate im Oberboden niedriger als im Unterboden. Bei der Zugabe von Substrat mit einem weiten C/N-Verhältnis lag der direkte Aufnahmeweg im Durchschnitt bei 97% im Oberboden und bei 94% im Unterboden, während bei der Zugabe von Maisstreu mit einem engen C/N-Verhältnis der Anteil der direkten Aufnahme im Durchschnitt bei 79% im Oberboden und 91% im Unterboden lag. Insgesamt führte die Zugabe von Substrat zu einem Anstieg des direkten Aufnahmewegs. Vor allem direkt nach der Substratzugabe führte diese zu einem signifikanten Anstieg des direkten Aufnahmewegs im Oberboden im Vergleich zum Unterboden. Die Zugabe von Maisstreu mit einem weiten C/N-Verhältnis (40), führte im Vergleich zur Zugabe von Maisstreu mit einem engen C/N-Verhältnis (20) zu einer Zunahme des direkten Aufnahmewegs.

Da ein großer Anteil organischer Stickstoff in Form von proteinhaltigem Material in den Boden kommt, spielt die Desaminierung von Aminosäuren durch extrazelluläre Enzyme eine wichtige Rolle. So katalysiert die L-Aminosäureoxidase (EC 1.4.3.2) spezifisch die oxidative Desaminierung der L-Aminosäuren, d.h. die Bildung der entsprechenden α-Ketosäuren, H_2O_2 und Ammonium. Es ist anzunehmen, dass die L-Aminosäureoxidase so wesentlich zur extrazellulären N-Mineralisation beiträgt. Durch die Bestimmung dieser Enzymaktivität können Aussagen über den indirekten N-Aufnahmeweg getroffen werden.

(iii) Um die Aktivität des Enzyms messen zu können, wurde die von *Pantoja* et al. (1993) entwickelte Methode zur Messung von Aminosäureoxidase in Meerwasser auf das Medium Boden übertragen. *Pantoja* et al. (1993) nutzte dazu die Derivate der Aminosäure Lysin und des fluoreszierenden Lucifer Yellow Anhydrid (LYA). Durch die Aktivität der Aminosäureoxidase wird LYA-Lysin in LYA-ε-Amino-α-Ketocapronsäure umgewandelt. Da diese Säure beim Messen nicht stabil bleibt, wird sie durch die Zugabe von Wasserstoffperoxid (H_2O_2) zu LYA-5-Amino-Valeriansäure oxidiert. Das Messen erfolgt durch fluorometrische Detektion mittels Hochleistungsflüssigkeitschromatographie (HPLC). Standardlösungen bekannter Konzentration von LYA-Lysin und LYA-5-Amino-Valeriansäure dienten zur genauen Bestimmung als Referenz. Die Retentionszeit betrug für LYA-Lysin 23,6 min und für LYA-5-Amino-Valeriansäure 36,7 min.

LYA-Lysin wurde in THAM-Puffer gelöst und in einer Konzentration von 5 µM zu 1 g feldfrischem Boden gegeben. Diese Lösung wurde während der Inkubation in einem Wasserbad bei 37 °C für 3 h geschüttelt. Nach der Inkubation wurden die Proben in einem Eiswasserbad abgekühlt, um die Enzymaktivität stark zu verlangsamen und anschließend mit einem 2 µm Filter gefiltert, um die Mikroorganismen aus der Lösung zu entfernen. Nach der Zugabe von H_2O_2, um LYA-ε-Amino-α-Ketocapronsäure in LYA-5-Amino-Valeriansäure zu

oxidieren, wurden die Proben mittels HPLC getrennt und detektiert.

Es war möglich, die Aktivität von L-Aminosäureoxidase mit Hilfe des synthetisch fluoreszierenden Derivats der Aminosäure Lysin in Boden zu detektieren. Um eine Methode zur qualitativen und quantitativen Bestimmung der Enzymaktivität zu entwickeln, müssen nach *Dick* (2011) verschiedene Faktoren berücksichtigt werden. Demnach spielt es eine Rolle, ob das Substrat oder Produkt gemessen wird, außerdem spielen die Substratkonzentration, die Bodenmenge, die Inkubationszeit sowie die Inkubationstemperatur eine wichtige Rolle, schließlich ist noch zu beachten, auf welchem Wege die Inkubation gestoppt werden kann und bei welchem pH-Wert eine optimale Reaktion abläuft. Um diese Faktoren zu testen und eine geeignete Methode zu entwickeln, wurden Bodenproben (0-20 cm) von zwei unterschiedlichen Standorten mit unterschiedlicher Textur entnommen und mit LYA-Lysin inkubiert. Es wurden zwei unterschiedliche Pufferlösungen getestet: Phosphat-Puffer (0.1 M) und THAM-Puffer (0.2 M). Im Vergleich zum Phosphat-Puffer konnten nach der Inkubation mit THAM-Puffer leicht höhere Enzymaktivitäten gemessen werden. Um den optimalen pH-Wert des Puffers für die Enzymaktivität zu finden, wurde der THAM-Puffer mit unterschiedlichen pH-Werten eingestellt und mit LYA-Lysin inkubiert. Die Produktionsrate von LYA-5-Amino-Valeriansäure zeigte ein weites pH-Spektrum mit einem Optimum um den pH-Wert 7,5. Mit ansteigender Inkubationstemperatur, Inkubationszeit oder Bodenmenge stieg die Produktionsrate von LYA-5-Amino-Valeriansäure und somit die Aktivität der Aminosäureoxidase. In der Methode, die von *Pantoja* et al. (1993) entwickelt wurde, lagen die zugegebenen Substratkonzentrationen zwischen 100 nM und 5 µM. Um die Konzentration zu finden, bei der die Produktionsrate von LYA-5-Amino-Valeriansäure unabhängig von der Konzentration des zugegebenen Substrates ist, wurde Substrat in unterschiedlichen Konzentrationen (0-10 mM) zugegeben und die Aktivität der Aminosäureoxidase bestimmt. Aber auch durch die Zugabe von hohen Substratkonzentrationen war es nicht möglich, die maximale Produktionsrate zu finden. Die Messung mittels HPLC gestaltete sich bei hohen

Konzentrationen schwierig, da die Peaks nicht mehr eindeutig voneinander getrennt werden konnten. Je höher die zugegebene Substratkonzentration war, desto größer war auch die Produktionsrate von LYA-5-Amino-Valeriansäure, obwohl noch eine große Menge an LYA-Lysin in der Lösung war. Die Lösung hätte somit eigentlich mit LYA-Lysin gesättigt sein und die Produktionsrate mit einer noch höheren Substartkonzentration nicht weiter steigen sollen. Auch das Verdünnen einer 10 mM LYA-Lysin Lösung nach der Inkubation führte zu keinen Ergebnis, da nach der Rückrechnung die Produktionsrate weiterhin anstieg. Zusammenfassend kann gesagt werden, dass es zwar möglich war, die Enzymaktivität der Aminosäureoxidase im Boden nachzuweisen, aber nicht möglich die Substratkonzentration zu finden bei der die Aminosäureoxidase ihre maximale Produktionsrate erreicht und somit unabhängig von der zugegebenen Substratkonzentration wird. Daher war es nicht möglich eine gültige Methode zur Enzymaktivitätsbestimmung zu entwickeln.

Danksagung

Mein Dank gilt Herrn Professor Dr. Bernard Ludwig für die stetige Unterstützung und Motivation, sowie die Möglichkeit meine Dissertation in diesem interessanten Forschungsthema anzufertigen.

Des Weiteren möchte ich mich bei Herrn Professor Dr. Rainer Georg Jörgensen für seinen fachlichen Beistand und seine hilfreichen Vorschläge bedanken.

Für das Heranführen an das Thema und für die Unterweisung in die Arbeitsmethoden möchte ich mich ganz besonders bei Herrn Dr. Daniel Geisseler bedanken.

Ich möchte mich, in fachlicher und persönlicher Hinsicht, bei all meinen Kolleginnen und Kollegen bedanken die mich nicht nur tatkräftig unterstützt haben, sondern mich stets aufbauten und für die erforderliche Abwechslung sorgten. Sie gaben mir mit Ihrem fundierten Fachwissen viele Anregungen für meine wissenschaftliche Arbeit. Danke an: Dr. Rouven Andruschkewitsch, Dennis Grunwald, Dr. Michael Kaiser, Dr. Deborah Linsler, Anja Nüsse, Dr. Christiane Piegholdt, Dr. Christel Ross, Svendja Vormstein. Außerdem danke ich Anja Sawallisch, die mir stets Ansprechpartner war und mein Forschungsprojekt durch ihre Ideen, ihre Anregungen und ihre konstruktive Kritik bereicherte.

Bedanken möchte ich mich auch für die technische Hilfe bei Gabi Dormann, Sabine Ahlers, Elsar Zwicker und bei meinen unermüdlichen Hiwis Oliver Schäfer und Sven Thorwirth.

Außerdem möchte ich mich bei Dr. Deborah Linsler nicht nur für die vielen fachlichen Ratschläge, sondern vor allem auch für ihre jahrelange Freundschaft mit vielen spontanen Aktionen, die mein Leben bereicherten, bedanken.

Mein besonderer Dank gilt meiner Familie, die mich in den Höhen und Tiefen meines Lebens stets unterstützt und mich zu dem geprägt hat was ich heute bin.

Bei der Deutschen Forschungsgemeinschaft möchte ich mich für die Finanzierung dieser Arbeit bedanken.

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig, ohne unerlaubte Hilfe Dritter angefertigt und andere als die in der Dissertation angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen sind, habe ich als solche kenntlich gemacht. Dritte waren an der inhaltlich-materiellen Erstellung der Dissertation nicht beteiligt; insbesondere habe ich hierfür nicht die Hilfe eines Promotionsberaters in Anspruch genommen. Kein Teil dieser Arbeit ist in einem anderen Promotions-oder Habilitationsverfahren verwendet worden.

Witzenhausen, Februar 2015

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(Johanna Pinggera)