Nitrogen and carbon dynamics in grassland soils and plants after application of digestate

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Witzenhausen, den 17. November 2014

Meike Andruschkewitsch

Preface

I submit this thesis to the Faculty of Organic Agricultural Sciences of the University of Kassel to fulfil the requirements for the degree Doktor der Naturwissenschaften (Dr. rer. nat.). This dissertation is based on three articles of mine as first author, which are published by or submitted to international peer reviewed journals. The articles are included in chapter 3, 4 and 5. A general introduction is given in chapter 1. Research objectives are included in chapter 2. In chapter 6, the entire thesis is summarized and general conclusions are drawn.

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Chapter 3: Andruschkewitsch M., Wachendorf C., Wachendorf M. (2013): Effects of digestates from different biogas production systems on above- and belowground grass growth and the nitrogen status of the plant-soil-system. Grassland Science, 59, 183-195.

Chapter 4: Andruschkewitsch M., Wachendorf C., Sradnick A., Hensgen F., Joergensen R.G., Wachendorf M. (2014): Soil substrate utilization pattern and relation of functional evenness of plant groups and soil microbial community in five low mountain NATURA 2000. Plant and Soil, 383, 275-289.

Chapter 5: Andruschkewitsch M., Wachendorf C., Buehle L., Joergensen R.G., Wachendorf M. Shifts of plant functional groups and soil microbial catabolic diversity due to management changes in temperate extensive grasslands. Applied Soil Ecology, submitted.

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List of abbreviations

a.s.l.: Above sea level

AA: Amino acid

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

C: Carbon

C:N_{org}: Carbon to organic nitrogen ratio

C:N_{tot}: Carbon to total nitrogen ratio

CA: Carboxylic acid

CH: Carbohydrate

CLPP: Community level physiological profile

C_{org}: Organic carbon

DF: Discriminant function

DFA: Discriminant function analysis

DM: Dry matter

DON: Dissolved organic nitrogen

E_{plant}: Evenness of plant functional groups

E_{soil}: Evenness of soil microbial substrate utilization pattern

EU: European Union

FM: Fresh matter

H+N: Harvesting with nutrient application

H-N: Harvesting without nutrient application

IFBB: Integrated generation of solid fuel and biogas from biomass

M: Mulching

MBN: Soil microbial nitrogen

MIN: Mineral N fertilizer

N: Nitrogen

N_{min}: Mineral nitrogen

N_{tot}: Total nitrogen

NUE_{min}: Mineral nitrogen use efficiency

PA: Phenolic acid

SCI: Sites of Community Importance

SGD: Digestate from grass silage

SIR: Substrate induced respiration

SOC: Soil organic matter

SOM: Soil organic matter

TN: Total nitrogen

WCD: Whole crop digestate

WHC: Water holding capacity

WRB: World Reference Base for Soil Resources

η^2: Classical eta squared

1 General introduction

This thesis is part of the project "PROGRASS - Securing the conservation of NATURA grassland habitats with a distributed bioenergy production" (www.prograss.eu). The focus of the project is placed on extensively managed conservation grassland habitats which are incorporated in the NATURA 2000 program. These sites have been nominated by the Member States of the European Union as "Sites of Community Importance" (SCI).

In many regions of European countries grassland ecosystems characterize the landscape setting and provide an important contribution to fodder production, soil and water protection and conservation of biodiversity (Schläpfer et al., 1999; Schüpbach et al., 2004; Wrage et al., 2011). Beside these economic and environmental based ecosystem services, grassland areas also offer non-material, cultural services for human society through recreational opportunities and aesthetic experiences (Hopkins, 2009; Lindemann−Matthies et al., 2010). Once, the agricultural grassland management was generally of low intensity, resulting in habitats in which high biodiversity and biomass production were able to co-exist (Hopkins, 2009). Since the mid 20th century, extension and number of these semi-natural, species-rich grassland areas in Europe declined steadily. (Isselstein et al., 2005; Poschlod et al., 2005; Rösch et al., 2009; Beilin et al., 2014). This decrease is caused by the increasing conversion of extensively managed “semi-improved” grassland into intensively managed “improved” grassland accompanied by higher nitrogen fertilizer rates, stocking rates and defoliation frequency (Isselstein et al., 2005). Furthermore, grassland areas are threatened by ploughing up to arable land, afforestation and abandonment due to low profitability and increasing irrelevance of green fodder in animal husbandry (Isselstein et al., 2005; Poschlod et al., 2005; Rösch et al., 2009). The report by the Grasslands Trust (King, 2010) highlighted that Europe’s semi-natural grasslands are still continuing to decline in extent and quality because there is no coherent regulatory and support framework for protection at EU level.

To counteract this ever-increasing loss of species-rich conservation grassland, the PROGRASS project has the main target to deliver a holistic and sustainable approach for obtaining this grassland ecosystems based on a regular, agricultural extensive management and bioenergetic utilization of grassland biomass. To prevent or repress the degradation and loss of these areas a continuous implementation of the traditional management under which the characteristic plant species composition was established is essential (Ellenberg and Leuschner, 2010; Drobnik et al., 2011). Typical traditional managements practices of such

low yield grasslands were grazing by low stocking rates or mowing with a low cutting frequency, partially accompanied by a low manure application rate (Isselstein et al., 2005). Thereby, the first cutting of meadow grassland was often conducted late, which resulted in low crude protein and high crude fibre concentration (White et al., 2004; De Cauwer et al., 2005). This in turn leads to a low nutritional value for animal production (Elsässer, 2003). Therefore, without compensation payments, the traditional management of conservation grassland is usually not profitable (Strijker, 2005). But the regular removal of biomass accompanied by delayed first date of utilization is essential to maintain the biodiverse grassland community. It prevents the invasion of undesirable species and provides a sufficient time period for seed production and reproduction of the characteristic flora and fauna (Ellenberg and Leuschner, 2010). It should be noted that for hay meadow conservation, a permanent grazing with livestock having low requirements of forage quality (e.g. sheep and goats) would not be expedient due to the incompatibility of hoof trampling with many grassland species (Ellenberg and Leuschner, 2010).

As an alternative to animal feeding, the grassland biomass can be used for bioenergy production. However, despite high energy prices and subsidies for bioenergy, conventional methods of bioenergy generation do not achieve an efficient utilization of grass silage or hay (Rösch et al., 2009). The particular problems of using biomass from extensive managed grasslands are mainly their high mineral contents, physiological age and heterogeneity. A thermal utilization of hay is restricted by high mineral contents and nitrogen oxide emission (Prochnow et al. 2009b), which lead to higher efforts in combustion technology and treatments of exhaust gases (Elsässer, 2003). The biogas production from grass silage is restricted by low methane yields (Prochnow et al., 2009a) caused by low protein and high fibre contents. Therefore, Wachendorf et al. (2009) suggest a concept for energy production of heterogeneous and senescent plant biomass within the PROGRASS project, in order to provide a profitable bioenergy utilization of biodiverse semi-natural grasslands. The “integrated generation of solid fuel and biogas from biomass” (IFBB) technique overcomes the restrictions for energy production by generating two material pathways. After hydro-thermal conditioning at 40°C, grass silage is separated with a screw press into a less digestible and lignin-rich solid fraction used as solid fuel for combustion (Hensgen et al., 2012; Richter et al., 2009; Wachendorf et al., 2009), and into an easily digestible liquid fraction used for biogas production (Richter et al., 2010, 2011). A nutrient rich biogas residue remains from

the anaerobic digestion, which is hereinafter referred to as digestate. Digestate application can serve to recycle the nutrients removed by the harvest to the grassland.

However, in terms of a closed nutrient cycle a nutrient recirculation in form of fertilizer application to conservation grasslands, e.g. NATURA 2000 grasslands, poses problems for nature conservation reasons. This is because of the strong relation of the nutrient status of a grassland site and its species composition (Ellenberg and Leuschner, 2010). Semi-natural grasslands often occur on infertile soils and their characteristic plant community composition is sensitive to nutrient application (Čámská and Skálová, 2012). In order to ensure the desired low nutrient status of biodiverse conservation grasslands, it is restricted or completely prohibited by law to add nutrients by fertilizer application. Nevertheless, semi-natural grassland ecosystems include a wide range of different habitats varying in pedologic and climatic site conditions. Therefore the application rates for nitrogen which are tolerated by the plant community vary from 4 to 60 kg N ha^{-1} a^{-1} (Briemle, 1997; Kirkham et al., 2008; Čámská and Skálová, 2012; Samuil et al., 2013). The maintenance of some central European *Arrhenatherion* meadows may even require an additional and regular application of nutrients (Čámská and Skálová, 2012). The N amount returned to the grassland with the digestate produced by the IFBB technique is only 19 to 60% of the N removed with the harvested biomass. This is due to the fact that the N is partly transferred into the liquid phase for biogas production (Hensgen et al., 2012) and to the fact that the N yields from these grasslands are moderate to low. Therefore, the suggested IFBB concept seems to be a suitable approach to maintain nutrient cycles and the plant community structure.

Furthermore, the IFBB digestate is a new product, which differs in its physical and chemical properties from digestates from conventional biogas production systems originated from the whole crop (whole crop digestates: WCDs). During the IFBB process main parts of the fibrous components are reduced prior to the digestion process due to hydrothermal condition and mechanical separation. By screw press separation, up to 80% of the carbon in the original material can be transferred with the coarse particles (>1.5 mm) to the solid fraction (Bühle, 2008). Correspondingly, the liquid fraction shows reduced dry matter (DM) and carbon (C) contents. In general, dry matter content of the raw material is decomposed during digestion processes and 7% dry matter content is a typical value for WCDs (Mokry and Kluge, 2009). The IFBB digestate however shows values of about 2% dry matter content resulting in improved rheological properties. This, in turn, may lead to a more accurate and homogeneous application on the grassland, better runoff from the plant surface and faster

infiltration into the soil. Thereby, the improved rheological properties contribute to reduced ammonia emission risks after digestate application (Gericke et al., 2007; Weiland, 2010) and may reduce emission of volatile, odorous organic substances (Linke et al., 2006). In general digestates provide an enhanced proportion of mineral N and lowered $C:N_{tot}$ ratio in comparison to its raw material, which leads to an increased short–term N availability to plants (Merz and Trösch, 1989; Gutser et al., 2005; De Boer, 2008; Tambone et al., 2010). This effect is likely to be further enhanced due to separation processes (Elsässer et al., 1995). To date, several experiments have been conducted to investigate the effect of digestate application on plants, highlighting a positive effect on yield production and N uptake (Garg et al., 2005; De Boer, 2008; Möller et al., 2008; Terhoeven–Urselmans et al., 2009; Gunnarsson et al., 2010). However, so far no study investigated the interaction of different types of digestate (separated and un-separated), different N application rates and the growth of different grassland species.

The soil microbial community as a major driver of most grassland ecosystem functions is directly affected by digestate application. According to current knowledge, digestates increase soil microbial biomass and activity after application on bare and planted soils (Ernst et al., 2008; Odlare et al., 2008; Terhoeven–Urselmans et al., 2009; Bachmann et al., 2011). Arthurson (2009) reviewed that the amount of metabolically active microorganisms increase after digestate application compared to unfertilized soils due to the input of mineral nutrients and organic material. In particular, the quantity and quality of carbon added to the soil with digestates affect the microbial soil community metabolism (Ernst et al., 2008). The N metabolism of soil microorganism was found to be affected by digestate application. Peters and Jensen (2011) found in an incubation experiment a significant negative correlation between net N mineralization and $C:N_{org}$ ratio of solid fractions from animal slurry separation. Furthermore, soil microorganisms may compete with plants for N (Bardgett et al., 1999; Geisseler et al., 2010) and the N immobilization process is likewise related to the amount and decomposability of C for the microorganisms (Geisseler et al., 2010), and thus may be influenced by digestate composition. Therefore, it might be expected that, because of its reduced C content, the IFBB digestate differs in the effects on soil microbial organisms and their functioning compared to the C richer conventional whole crop digestates.

Beside the direct digestate effects, soil microbial community might be indirectly influenced by changes in plant mediated C dynamics (Bardgett et al., 1999; Dijkstra et al., 2006; Millard and Singh, 2010). Increased plant biomass after digestate application is likely

to influence the soil microbial community considerably by increasing the supply of plant C to the soil with litter, root biomass and root exudation. The root exudates of young seedlings, exuding about 30-40% of their fixed carbon as root exudates, are identified as high quality nutrient source for microorganisms (Whipps, 1990). Furthermore, digestate application induced changes in plant composition and plant species dominance are likely to exert strong selective pressure on the soil microbial community through plant-specific differences in pattern of the root exudation into the rhizosphere (Badri and Vivanco, 2009). The aboveground diversity of plant species and plant functional groups (e.g. graminoids, forbs, legumes) was thereby identified as important factor governing the soil microbial diversity (Loranger-Merciris et al., 2006; Millard and Singh, 2010) and the ecosystem functions performed by the microbial community (Zak et al., 2003). Beside the species diversity of soil microbial community its functional diversity represents a part of the overall microbial diversity in soil, but with more practical and ecological relevance (Zak et al., 1994). However, the microbial functional diversity includes a vast range of soil microbial activities including nutrient transformation, decomposition, suppressing and modification of soil physical processes (Giller et al., 1997; Wardle et al., 1999), which are hard to measure in their full extent. A subset of the microbial functional diversity can be characterized by measurement of the decomposition functions performed by heterotrophic microorgansims. An in-situ approach to measuring this, is the examination of the ability of the microbial community to utilize a number of different C substrates (Campbell et al., 1997; Degens and Harris, 1997). Generally, it is suggested that a functional diverse microbial community is more resistant to stress or disturbance (Degens et al., 2001). In consideration of the supposed mutual influence of plants and soil microbial community, it is important to increase the knowledge of the effects of digestates on both ecosystem components to assess the consequences of digestate application for the ecosystem services. For example a digestate induced reduction in phytodiversity might result in a reduced functional capacity of the soil microbial community.

2 Research objectives

A better understanding of the digestate effects on plant community, soil microbial community as well as nutrient and carbon dynamics is crucial for a sustainable grassland management and the prevention of species and functional diversity loss. The IFBB digestate represents a new type of organic fertilizer, which differs in its composition from conventional digestates originating from whole crop digestion. So far, no information is available on its effects after application on grassland ecosystems. Therefore, the aim of this thesis is to fill gaps in the understanding of the effects of the IFBB digestate on grassland plants and soil microbial community, taking into account the mutual influence of both of these biotic ecosystem components. Two experimental studies were carried out that culminated in three scientific manuscripts.

In the first study (**Chapter 3**), the short-term (intra-seasonal) effects of the IFBB digestate on the growth of different grass species, plant N uptake and the N status of the plant-soil-system including soil microorganisms were investigated for the first time in a 5-month pot experiment under controlled conditions in a greenhouse. To assess the fertilizer value of the IFBB digestate, two additional fertilizer types were included in the study as comparative variants: a conventional whole crop digestate (WCD) and a mineral N fertilizer (MIN). The first study was focussed on nitrogen because of its considerable importance for plant and soil microorganisms and to understand how the variation in IFBB digestate composition, resulting from the separation process, influences the N dynamic to prevent losses to the environment and to ensure an efficient N use in grassland management.

The second study took place under natural conditions at five different German NATURA 2000 grasslands (meadows) and addressed firstly (**Chapter 4**) the general relationship of soil microbial substrate metabolism and the plant community (plant functional groups: graminoides, forbs and legumes) and secondly (**Chapter 5**) the long-term effects of three years of IFBB digestate application on plant functional groups, plant functional group diversity, soil microbial substrate metabolism and catabolic diversity of the grassland systems. The overall aim of the study was to assess the consequences of IFBB-concept implementation on extensive grasslands compared to traditional management (mowing and harvesting) and mulching. The purpose of the scientific manuscript described in Chapter 4 was to investigate the above- and belowground interaction under undisturbed conditions

(without digestate application) initially, to serve as a base for the coming investigations of digestate effects on the five NATURA 2000 grasslands in Chapter 5.

The specific objectives of the studies were:

(i) to investigate the suitability of the IFBB digestate as a fertilizer and to examine digestate effects on grass species and soil microbial community, especially focussing on nitrogen dynamic in the plant-soil system (**Chapter 3**). It was expected that, because of its lower DM and C content, the IFBB digestate has different effects on plant growth and soil microbial organisms compared to a conventional whole crop digestate.

(ii) to investigate the relationship between plant community and functionality of soil microbial community of extensively managed meadows, taking into account temporal variations during the vegetation period and abiotic soil conditions (**Chapter 4**). Plant community was represented by three plant functional groups: graminoides, forbs and legumes. The soil microbial functional diversity was determined by the catabolic response to different carbon substrates.

(iii) to investigate the suitability of IFBB-concept implementation as grassland conservation measure for meadows and possible associated effects of IFBB digestate application on plant and soil microbial community as well as soil microbial catabolic substrate utilization (**Chapter 5**). As comparative meadow conservation measure a control variant without digestate application and a mulch variant (mowing without biomass removal) was implemented in the study.

3 Effects of digestates from different biogas production systems on above- and belowground grass growth and the nitrogen status of the plant-soil-system

Abstract Biogas production from residual biomass (e.g. from extensively managed grassland) can help securing ecosystem services of such vegetation and may contribute to energy production from renewable resources. Proper management of fermentation residues is a major challenge within the technical concepts recently suggested for the conversion of this biomass. A 5-month pot experiment was conducted to investigate the effects of digestates from separated grass silage (liquid fraction) (SGD), produced within the innovative integrated generation of solid fuel and biogas from biomass (IFBB) system and from conventional whole crop digestion (WCD) on grass growth, N uptake and N immobilization. Digestates and a mineral N fertilizer (MIN) as comparative variant were applied at N-rates from 0 up to 20 g Nm^{-2} based on fertilizer mineral N to three different grass species (*Lolium perenne*, *Trisetum flavescens* and *Festuca rubra* subsp. *rubra*). Digestate application increased harvestable biomass constantly with increasing N-rate for *L. perenne*, but not for *Trisetum flavescens* and *F. rubra* subsp. *rubra*. Type of digestate caused species-specific differences in plant growth, as *F. rubra rubra* and *L. perenne* showed higher dry matter (DM) yields of harvestable and root biomass for WCD and *T. flavescens* for SGD application. However, for both digestates, reduced root biomasses were observed compared to the control. The mineral nitrogen use efficiency (NUE_{min}) was over all species 22% higher for harvestable and 33% for stubble biomass after application of SGD compared to WCD, due to greater N uptake related to lower gaseous N losses and favourable mineralization properties. N immobilization measured by soil microbial biomass N (MBN) was influenced by grass species but not by type of digestate or application rate. The lack of effect of digestate application on MBN was attributed to the compensation of the digestate C input by the reduced root biomass production.

3.1 Introduction

The anaerobic digestion process leaves a nutrient-rich fermentation residue (hereafter referred to as digestate), which is usable as organic fertilizer in agriculture. In order to realize the European goal of renewable energy sources covering 20% of the European energy demands by the year 2020 and keeping the competition between food and energy production

from biomass as small as possible, it will be necessary to also use bio waste and landscape conservation material (e.g. municipal green cut, extensive grassland biomass) for bio energy production (Zah et al., 2007). However, such materials are associated with undesirable characteristics for biogas production (e.g. high lignification) (Prochnow et al., 2009a).

To overcome these restrictions, a concept for energy production from heterogeneous and senescent plant biomass was suggested by Wachendorf et al. (2009). Within this integrated generation of solid fuel and biogas from biomass (IFBB) system the grass silage is separated with a screw press prior to anaerobic digestion into a less digestible and lignin-rich solid fraction used as solid fuel for combustion (Richter et al., 2009; Wachendorf et al., 2009; Hensgen et al., 2012) and an easily digestible liquid fraction used for biogas production (Richter et al., 2010, 2011). By screw press separation up to 80% C of the original material can be transferred with the coarse particles (>1.5 mm) to the solid fraction (Bühle, 2008) and the liquid fraction shows reduced dry matter (DM), carbon (C) contents and C:Ntot ratios. Therefore, digestates from separated grass silage (liquid fraction) (SGDs) vary in their physical and chemical composition from conventional whole crop digestates (WCDs) in which the plant material is fermented without being separated previously.

Liquid fractions are also produced in the separation of digestates from whole crop digestion following the digestion process, which is a common method to improve fertilizer properties (Möller and Müller, 2012) further to digestion. The enhanced proportion of mineral N and lowered $C:N_{tot}$ ratio of digestates, in comparison to its raw material, leads to an increased short-term N availability to plants (Merz and Trösch, 1989; Gutser et al., 2005; De Boer, 2008; Tambone et al., 2010). This effect is likely to be further enhanced due to digestate separation (Elsässer et al., 1995). To date, several experiments have been conducted to investigate the effect of digestate application on plants, highlighting a positive effect on yield production and N uptake (Garg et al., 2005; De Boer, 2008; Möller et al., 2008; Terhoeven–Urselmans et al., 2009; Gunnarsson et al., 2010). So far, however, none of these studies investigated the interaction of different types of digestate (separated and unseparated), N application rates and plant species.

N-mineralization processes of digestate organic bound N in soil and thus the supply of mineral N for the plant are influenced by digestate composition (e.g. DM content, particle size distribution, C content, NH_4^+–N content, C:N ratio of the organic matter (C:Norg)) and soil parameters (e.g. soil type, pH value, temperature and water content) (Merz and Trösch, 1989; Gutser et al., 2005; Fangueiro et al., 2010). For example, Peters and Jensen (2011) found a

strong negative correlation between net N mineralization and C:Norg ratio of solid fractions from animal slurry separation in an incubation experiment. Furthermore, soil microorganisms may compete with plants for soil N (Bardgett et al., 1999; Geisseler et al., 2010) and the nitrogen immobilization process is likewise related to the amount and decomposability of C for the microorganisms (Geisseler et al., 2010), and may thus be influenced by digestate composition. According to the current knowledge, digestates increase soil microbial biomass and activity in planted and bare soils (Ernst et al., 2008; Odlare et al., 2008; Terhoeven–Urselmans et al., 2009; Bachmann et al., 2011) indicating an overall positive effect on the soil microbial community.

Given the rapidly increasing number of biogas plants and amounts of digestates produced, it is important to understand how the variation in digestate composition, resulting from the separation process, influences plant growth, soil microbial biomass and N dynamic to prevent losses to the environment and to ensure an efficient N use in grassland management. Consequently, an experiment was conducted to investigate the effects of an untreated conventional whole crop digestate and a digestate from separated grass silage (liquid fraction) generated within the IFBB system at different N application rates on the above- and belowground grass growth of different grass species, plant N uptake and on the N status of the plant-soil-system including soil microorganisms.

3.2 Material and Methods

3.2.1 Experimental setup

A 5-month pot experiment with standardized Kick-Brauckmann pots (diameter 21 cm, surface area 346 cm², height 25.5 cm) and a fully randomized design was conducted. The pots were filled with on average 8.9 kg soil DM with a bulk density of 1.15 g cm^{-3} and a gravimetric water content of 11%. Perennial ryegrass (*Lolium perenne* L., cv. Aberavon), a major grass species of intensively managed grassland, was sown and three types of fertilizer were applied once: a digestate from separated grass silage (liquid fraction) (SGD), a digestate from whole crop digestion (WCD) and calcium ammonium nitrate as mineral N fertilizer (MIN) at five N-rates based on the mineral N content: 0, 5, 10, 15 and 20 (g m^{-2}) with four replications. Creeping red fescue (*Festuca rubra* subsp. *rubra* L., cv. Condor) and yellow oatgrass (*Trisetum flavescens* (L.) P.B., cv. Trisett 51) frequently occur in unimproved grasslands of most of the European mountain areas, for which the IFBB system was developed. Therefore, simultaneously to *L. perenne* these species were sown and the two

types of digestate SGD and WCD were applied once at three N-rates based on the mineral N content: 0, 10, and 20 (g m^{-2}) with three replications. The application rates were performed with equal amounts of mineral N in order to exclude the fertilizer short-term effect produced by different mineral N amounts in the digestates. For all grass species the harvestable biomass was assessed at three consecutive cutting dates. Stubble biomass and root biomass were assessed at the third cut for the N-rates 0, 10 and 20. Seeds were sown manually on the soil surface at a rate of 0.42 g per pot for *L. perenne* and at a rate of 0.28 g per pot for *F. rubra rubra* and *T. flavescens*, equivalent to 120 kg ha^{-1} and 80 kg ha^{-1}. The pots were watered biweekly up to 80% of maximum water holding capacity. The position of the pots in the greenhouse was changed weekly. The average temperature in the greenhouse during the experiment was 20.3°C (±4.5), with the average temperature at day and night-time being 22.9°C (±4.2) and 17.7°C (±3.1), respectively.

3.2.2 Soil and fertilizer characteristics

The soil for the pot experiment was an arable soil (Cambisol) from a field near Witzenhausen, Germany, which is used for grass seed cultivation and was collected from 5-20 cm depth. The top 5 cm was previously removed by a tractor bucket, to minimize remaining plant material in the soil. The soil was sieved at 5 mm by a drum sieve (Scheppach rs 400, rpm 45) and had the following characteristics: particle size distribution: 74% sand, 19.5% silt, 6.6% clay; pH (CaCl2): 6.1; P: 7 mg 100 g^{-1} soil (CAL); K: 19 mg 100 g^{-1} soil (CAL); Mg 9 mg 100 g^{-1} soil (CaCl2). The nutrients P, K and Mg were analyzed according to Hoffmann (1991). Organic C and Ntot were determined on dried subsamples (60°C) with an elemental analyzer (vario MAX CHN, Elementar Analysensysteme GmbH, Hanau, Germany) and were 9.06 g kg^{-1} and 0.76 g kg^{-1}, respectively.

The WCD originated from a continuously stirred reactor, which was fed with 47% maize silage, 28% cow dung, 9% grass silage, 9% poultry dung and 7% barley groats. The SGD originated from the IFBB-demonstration plant, which was run with the liquid fraction of grass silage from semi-natural grassland, separated by screw press (perforation of 1.5 mm), and a rest amount (<5%) of separated digestate (pork manure, maize silage), used as inoculum. WCD and SGD were homogenized with a masher (MF-MFAP 2000, Dynamic, Kehl, Germany, rpm 3000–9000) prior to application and analyzing. Calcium-ammonium-nitrate was used as mineral N fertilizer (MIN).

Dry matter and ash content of the digestates was determined after drying at 105°C and 550°C for 3 days, respectively (Table 1). Digestate pH was measured with a standard electrode directly in the substrate. Total N was determined on fresh subsamples by steam distillation on a Büchi 323 (Büchi Labortechnik, Essen, Germany). For mineral N analysis, 5 g of fresh digestate was extracted with 100 ml 0.5 mol l^{-1} K2SO4, shaken for 1 h (200 min^{-1}) and centrifuged at 4000 g. The supernatant was filtered and analyzed for mineral N (ammonium-N and nitrate-N) using a Continuous Flow Analyzer (Evolution II auto-analyzer, Alliance Instruments, Cergy-Pontoise, France). Total C was analyzed on freeze-dried subsamples with an elemental analyzer (vario MAX CHN, Elementar Analysensysteme GmbH). The nutrients of the digestates were analyzed using inductively coupled plasma optical emission spectrometry after ISO 11885 (2009), whereas chlorine was analyzed using liquid chromatography after ISO 10304 (2009). All fertilizers were adjusted with demineralized water to the same amount of water prior to application. MIN-solution pH was measured with a standard electrode and was 7.6.

Table 1: Chemical characteristics of digestates used in the pot experiment. For DM, ash and C n = 2; for N_{tot} n = 5 and for the other parameters n = 1. Standard deviation is given in brackets.

Parameter	Unit	SGD	WCD
DM	% FM	1.41 (±0.02)	11.27 (±0.18)
Ash	% DM	47.40	30.07 (±0.78)
C	% DM	24.76	34.80 (±0.49)
Ntot	% DM	8.85 (±0.63)	6.98 (±0.18)
NH_4^+–N	% DM	6.29	4.64
NO_3^-–N	% DM	n.d.[†]	n.d.
Ca	% DM	4.58	2.14
P	% DM	4.34	1.32
Na	% DM	0.63	0.28
Mg	% DM	4.21	0.83
K	% DM	5.54	5.78
S	% DM	0.57	0.67
Cl	% DM	1.28	0.88
C:Norg	–	9.71	14.62
pH	–	8.10	8.10

[†] n.d. = not detectable

3.2.3 Fertilizer application, sampling and measurement methods

After 40 days of growth and two initial cuts to initiate tillering and reduce the growth of weeds, the fertilizers were applied once to the soil surface. Due to the heterogeneity of digestates, the variation of the mineral N-rates ranged between ± 13% and 19% and was on average ± 16% of the target application rate (Table 2). The cutting dates were 68, 103 and 145 days after sowing. Harvestable biomass was cut at 3 cm height and stubble biomass was separated from the root biomass by cutting exactly at the growth centre. At the final harvest date, root samples were separated from soil samples by collecting with tweezers and washing over sieves (1 mm) with water. Soil samples were sieved (2 mm) and were partly used directly to determine N_{min} content while the rest was stored at 4°C for further measurements. Dry matter yield (DM) of plant fractions (harvestable biomass, stubble biomass and root biomass) was measured after drying for 3 days at 60°C. Total C and total N were analyzed using an elemental analyzer (vario MAX CHN, Elementar Analysensysteme GmbH).

The apparent mineral nitrogen use efficiency (NUE_{min}) refers to the mineral N part in the fertilizer. It was calculated according to the difference method based on Gunnarsson et al. (2010):

$$NUE_{min}\,(\%) = \frac{PFN_{fertilized} - PFN_{unfertilized}}{FN} \text{ x } 100 \qquad \text{(Equ. 1)}$$

where $PFN_{fertilized}$ = amount of N taken up by the fertilized plant fraction, $PFN_{unfertilized}$ = amount of N taken up by the unfertilized plant fraction, FN = amount of mineral N applied with fertilizer. This method is based on the assumption that the N supply by fertilizer does not affect the mineralization of soil organic matter.

For soil N_{min} estimation, 100 g fresh soil was extracted with 400 ml $CaCl_2$ within 2 days after sampling. Ammonium-N and nitrate-N in the extracts were measured with a continuous flow analyzer (Evolution II auto–analyzer, Alliance Instruments). The net mineral N amount, which was not recovered ($N_{unaccounted}$) in the fertilized plant-soil-systems after the third cutting date was calculated as:

$$N_{unaccounted}\,(g\,m^{-2}) = (Nmin_{fertilized} + PN_{fertilized}) - (Nmin_{unfertilized} + PN_{unfertilized} + FN) \qquad \text{(Equ. 2)}$$

where $Nmin_{fertilized}$ = mineral N in fertilized soil, $Nmin_{unfertilized}$ = mineral N in unfertilized soil, $PN_{fertilized}$ = N taken up by the fertilized plant (total harvestable, stubble and root biomass), $PN_{unfertilized}$ = N taken up by the unfertilized plant. The calculated net $N_{unaccounted}$ indicates net N loss via immobilization and gaseous N emissions (negative values) or net N

surplus via mineralization (positive values). This equation is based on the assumption, that plant uptake of dissolved organic N (DON) is negligible compared to plant uptake of mineral N.

For the microbial biomass N (MBN) estimation, first a pre-extraction of the soil was made to minimize the soil N_{min} content (Widmer et al., 1989). Briefly, 25 g fresh soil was pre-extracted with 70 mL 0.05 mol l^{-1} K_2SO_4 by 30 min horizontal shaking at 200 rpm and centrifuging at 2000 *g*. Then MBN was estimated by fumigation-extraction from two portions equivalent to 10 g of the pre-extracted soil. One portion was fumigated for 24 h at room temperature with chloroform. The soil portions were extracted with 40 ml 0.5 mol l^{-1} K_2SO_4 by horizontal shaking for 30 min and filtering. Extractable N_{tot} was measured after combustion at 850°C using a Dimatec 100 automatic analyzer (Dimatec, Essen, Germany). Microbial biomass N (MBN) was calculated as:

$$\text{MBN}\ (\text{mg kg}^{-1}) = \frac{\text{EN}}{k_{EN}} \qquad \text{(Equ. 3)}$$

where EN = total N extracted from fumigated soil – total N extracted from non-fumigated soil and k_{EN} = 0.54 (Joergensen and Mueller, 1996).

Table 2: Applied amounts of fertilizer mineral and total nitrogen (N_{min}, N_{tot}, respectively) and carbon (C_{org}) (g m^{-2}).

	SGD			WCD			MIN		
N-rate	N_{min}	N_{tot}	C_{org}	N_{min}	N_{tot}	C_{org}	N_{min}	N_{tot}	C_{org}
5	5.7	8.0	22.4	5.9	8.9	44.2	5.0	5.0	0
10	11.3	16.0	44.7	11.9	17.9	89.5	10.0	10.0	0
15	17.0	24.0	67.1	17.8	26.8	133.7	15.0	15.0	0
20	22.7	31.9	89.5	23.7	35.7	177.9	20.0	20.0	0

MIN, mineral N fertilizer; SGD, separated grass silage digestate; WCD, whole crop digestate.

3.2.4 Statistical analysis

To reveal the effects of the digestates, the mineral N fertilizer and N-rate on the plant and soil parameters for *L. perenne* analyses of variance (ANOVAs) with a two factorial design were conducted with type of fertilizer (SGD, WCD, MIN) and N-rate (5, 10, 15, 20 for harvestable biomass and 10, 20 for stubble biomass and root biomass) as factors. To reveal

the effects of the digestates, N-rate and grass species on plant and soil parameters, ANOVAs with a three factorial design were conducted with type of digestate (SGD, WCD), N-rate (10, 20) and species (*L. perenne*, *F. rubra rubra* and *T. flavescens*) as factors. Effect sizes were determined by the classical eta squared (η^2), which is defined as the proportion of variation attributable to each factor. Contrasts were defined to examine performance of main factors in specific comparisons. In order to assess the effect of N-rates in comparison with the control, contrasts were conducted. The statistical analyses were performed using R 2.14.1 (R Development Core Team, 2011). Level of significance was set to 0.05.

3.3 Results and Discussion

3.3.1 Plant DM yield

Dry matter yield of the harvestable biomass of *L. perenne* was mainly influenced by fertilizer N-rate (Table 3) and increased from 0.32 in the unfertilized control up to 0.76 kg m^{-2} of the highest N-rate applied (Fig. 1). On average no difference was found between MIN and the digestates. These results are in line with results from a pot experiment of Gunnarsson et al. (2010), in which no significant differences in the aboveground biomass of *L. multiflorum* were measured after digestate and mineral N fertilizer application based on the NH_4^+–N content. DM yields of the harvestable biomass of *T. flavescens* and *F. rubra rubra* ranged from 0.21 to 0.51 and from 0.29 to 0.52 kg m^{-2}, respectively (Fig. 2). *Lolium perenne*, which is cultivated in intensively managed grassland with high N application rates, produced increasing DM yields with increasing digestate N-rate up to the highest rate, while the increase in DM yield was less pronounced for *T. flavescens* and a decrease was observed for *F. rubra rubra*, which are both species cultivated in extensively managed grassland with lower N application rates. Consequently, the grass species explained most of the variation of harvestable biomass (Table 4) after digestate application and the average DM yield was in the order *L. perenne* > *T. flavescens* > *F. rubra rubra*. Despite the strong grass species effect, however, the type of digestate influenced the DM yield, as *F. rubra rubra* and *L. perenne* showed higher values for WCD and *T. flavescens* for SGD. Probably, the hairy species *T. flavescens* was more affected by aboveground tissue injury due to the poorer flow properties of WCD (e.g. larger particle sizes), which also may have affected the root growth of *T. flavescens* (see below).

Although harvestable biomass increased with increasing N-rate, DM yield of stubble biomass of the grass species only changed slightly (Fig. 1, 2) and was not affected by type of

digestate (Table 4). Thus, C input into the soil from stubble breakdown after defoliation is less affected by N application rate and type of digestate.

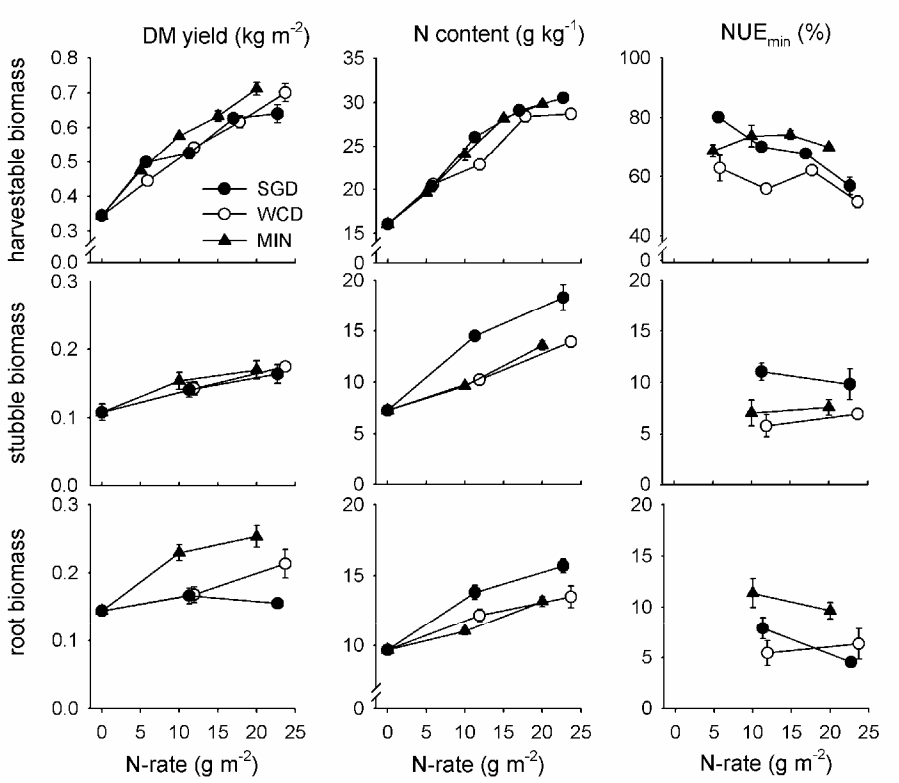


Fig. 1: Mean accumulated DM yield, mean N content and NUE_{min} of harvestable biomass (top row), stubble biomass (middle row) and root biomass (bottom row) of *L. perenne* after 105 days of plant growth since fertilization and three cutting dates in a greenhouse with differing mineral N–rate for SGD, WCD and MIN. DM yield and NUE_{min} for harvestable biomass was calculated as the sum and N content as average of the three cuts, respectively. DM yield, NUE_{min} and N content of stubble and root biomass were assessed at the third cut. Error bars describe standard errors of means (n = 4).

The root DM yield of *L. perenne* ranged from 0.13 to 0.30 kg m^{-2} and was increased after MIN, but not after digestate application (Fig. 1). This finding was supported by several other studies investigating the effect of mineral and organic fertilizer application on grassland (Kandeler et al., 1994; Singh, 1996; Salminen et al., 2001; Van Eekeren et al., 2009). Salminen et al. (2001) found an inhibition of root growth after application of digested material and 7 days of growth in pots, which the authors attributed to the presence of organic acids in the digestates. Therefore, organic acids in digestates may have affected root growth in this experiment. Also at the field level Van Eekeren et al. (2009) measured in a 5-year experiment on permanent *L. perenne* dominated grassland lower root mass in the soil layer 0–10 cm in organic fertilized treatments (150 kg N_{tot} ha^{-1}) than in calcium-ammonium-nitrate fertilized treatments and the control. In contrast to that, other studies observed increased root

biomasses after digestate application for wheat compared to the unamended control (Garg et al., 2005) and for *L. multiflorum* compared to the control and mineral N fertilizer (Gunnarsson et al., 2010).

Root biomass of the investigated species was in the order *T. flavescens* > *L. perenn*e > *F. rubra rubra* and decreased with increasing digestate N-rate for *T. flavescens* and *F. rubra rubra* (Fig. 2), while no difference to the control was detected for *L. perenne*, except for WCD at rate 200. On average over all species application of SGD decreased the root biomass stronger than WCD; however, the opposite was true for *T. flavescens*. This resulted in a significant interaction of species and type of digestate on root DM yield (Table 4).

Table 3: Results of the two factorial ANOVA for effects of N–rate (N) and type of fertilizer (F) and their interaction on plant and soil parameters for *L. perenne* after three cutting dates. HB = harvestable biomass, SB = stubble biomass and RB = root biomass. Significant effects at *P*<0.05. The variance (var.) columns represent the proportion of variance explained by the factor, calculated as classical eta squared (η^2). Coefficient of determination of the model (R^2).

		N			F			NxF			R^2
Parameter		d.f.	*P*	var.	d.f.	*P*	var.	d.f.	*P*	var.	
DM yield	HB	3	<0.001	0.83	2	ns†	0.02	6	<0.05	0.05	0.90
	SB	1	<0.05	0.27	2	ns	0.03	2	ns	0.02	0.32
	RB	1	ns	0.05	2	<0.001	0.59	2	ns	0.07	0.71
N content	HB	3	<0.001	0.91	2	<0.01	0.02	6	ns	0.02	0.95
	SB	1	<0.001	0.38	2	<0.001	0.49	2	ns	0.00	0.88
	RB	1	<0.001	0.28	2	<0.001	0.45	2	ns	0.01	0.74
N yield	HB	3	<0.001	0.97	2	<0.001	0.01	6	<0.01	0.01	0.98
	SB	1	<0.001	0.59	2	<0.01	0.20	2	ns	0.01	0.80
	RB	1	<0.001	0.32	2	<0.05	0.22	2	<0.05	0.09	0.62
NUE_{min}	HB	3	<0.001	0.21	2	<0.001	0.42	6	<0.001	0.18	0.81
	SB	1	ns	0.00	2	<0.01	0.47	2	ns	0.04	0.51
	RB	1	ns	0.05	2	<0.001	0.47	2	ns	0.08	0.60
N_{min}	Soil	1	<0.001	0.16	2	<0.001	0.59	2	ns	0.07	0.82
$N_{unaccounted}$	Soil	1	<0.001	0.44	2	<0.001	0.31	2	ns	0.05	0.81
MBN	Soil	1	ns	0.00	2	<0.01	0.34	2	<0.05	0.23	0.57

† ns = not significant

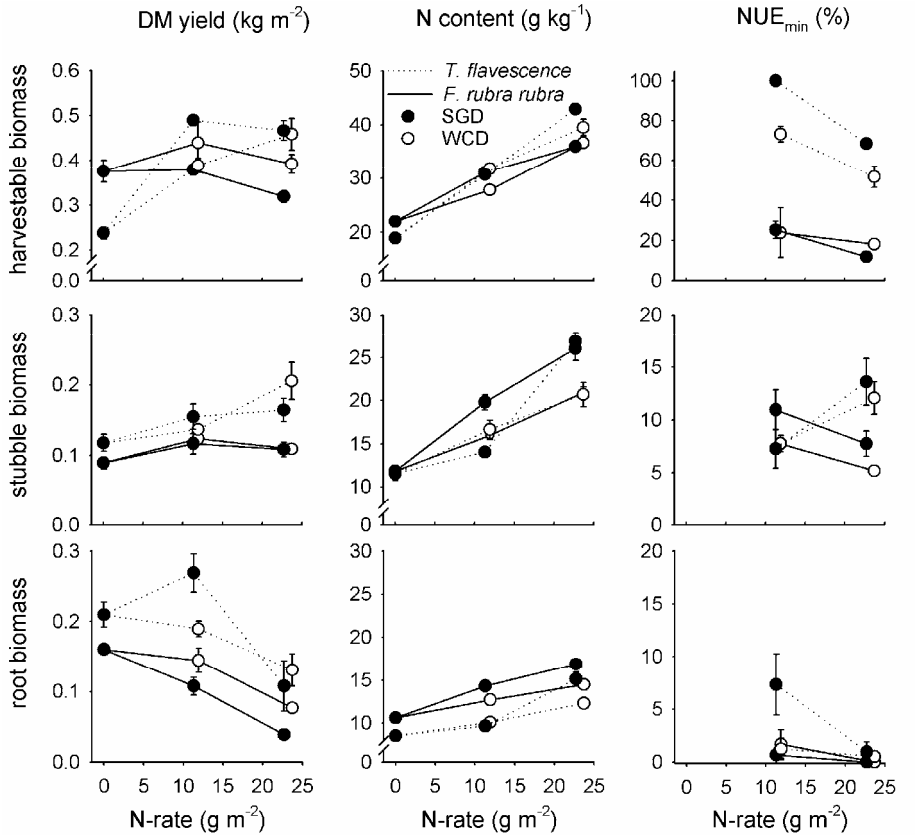


Fig. 2: Mean DM yield, N content and NUEmin of harvestable biomass (top row), stubble biomass (middle row) and root biomass (bottom row) of F. rubra rubra and T. flavescens after 105 days of plant growth since fertilization and three cutting dates in a greenhouse with differing mineral N–rate for SGD and WCD. DM yield and NUEmin for harvestable biomass was calculated as the sum and N content as average of the three cuts, respectively. DM yield, NUEmin and N content of stubble and root biomass were assessed at the third cut. Error bars describe standard errors of means (n = 3).

3.3.2 Plant N status

The N contents and N yields of *L. perenne* were mainly influenced by fertilizer N-rate (Table 3) and increased with increasing N-rate for all plant fractions (Fig. 1). For harvestable biomass, the N contents and N yields ranged from 15.6 up to 31.7 g kg^{-1} DM and from 5.05 up to 19.9 g N m^{-2} (Fig. 1, 3), respectively. On average, SGD showed the highest N contents and N yields for all plant fractions. Differences were significant for the N content of stubble biomass, N content of root biomass and N yield of stubble biomass only. The N yield of root biomass showed on average significantly higher values for MIN (Fig. 3), which is consistent with the higher DM yield of root biomass in the MIN treatment.

Considering all investigated species, N content and N yield of all plant fractions was mainly affected by N-rate and grass species and to a lesser extent by the applied type of digestate (Table 4). The N contents (Fig. 1, 2) and N yields (Fig. 3) increased with

increasing N-rate for all plant fractions, except for N yield of root biomass, which decreased with increasing N-rate for *T. flavescens* and *F. rubra rubra*. The species *F. rubra rubra* and *T. flavescens* showed higher N contents compared to *L. perenne*. This corresponds to the lower DM production of *T. flavescens* and *F. rubra rubra*, which leads to increased concentration of N in the plant biomass. Despite the considerable species effect, SGD produced on average significantly higher N contents and N yields of harvestable biomass (6% and 7%, respectively), stubble biomass (23% and 17%, respectively) and N contents of root biomass (13%) than WCD. This may be due to a smaller proportion of coarse particles of SGD, which is likely to influence the amount of N_{tot} in soil due to improved flow and infiltration properties. According to Merz and Trösch (1989) this in turn leads to an improved N fertilizer value. Furthermore, Peters and Jensen (2011) found a strong negative correlation between net N mineralization and $C:N_{org}$ ratio of solid fraction from animal slurry in an incubation experiment. The lower $C:N_{org}$ ratio of SGD (Table 1) may have therefore promoted rapid N mineralization and increases plant available N in soil.

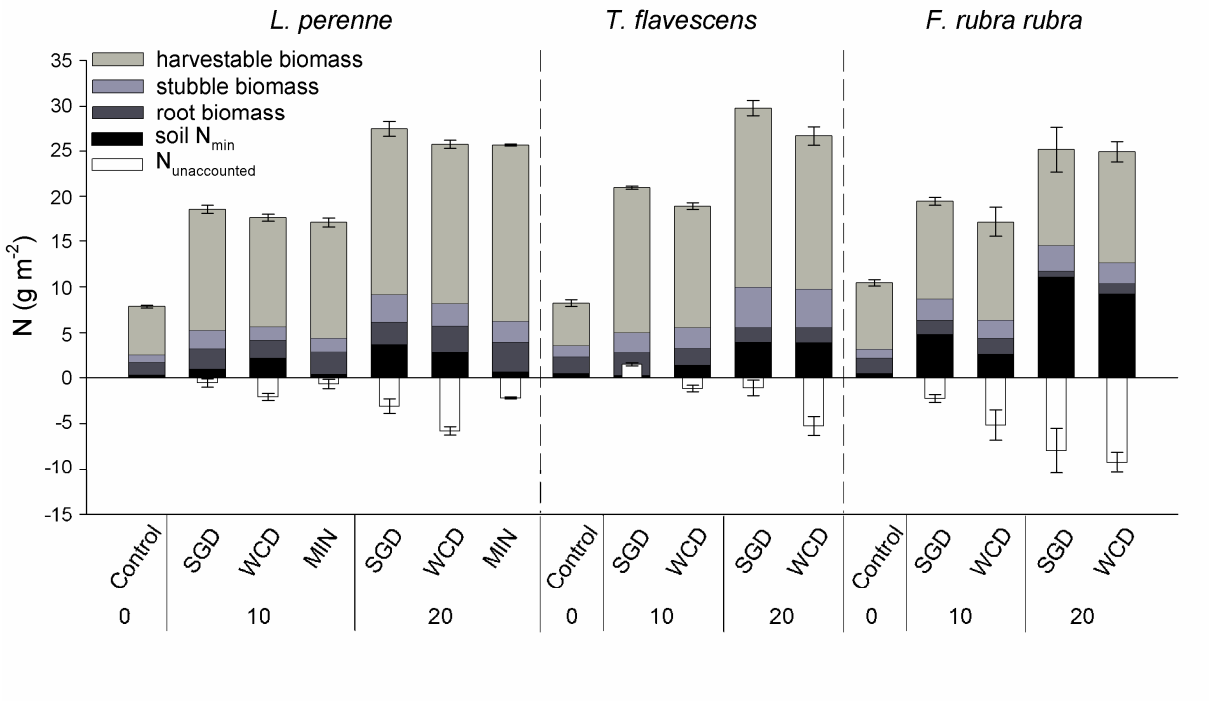


Fig. 3: Mean N yield of different plant fractions of *L. perenne, T. flavescens* and *F. rubra rubra* grown in the greenhouse, mean soil N_{min} and $N_{unaccounted}$, 105 days after a nonrecurring application of SGD, WCD and MIN for the application rates 0, 10 and 20 g NH_4^+–N m^{-2}. Harvestable biomass as the sum of the three cutting dates.

3.3.3 Soil microbial biomass N (MBN)

The MBN content in soil under *L. perenne* was significantly increased by 32% (1.8 g MBN m^{-2}) after MIN application compared to the digestates and the control (Fig. 4). The higher MBN contents for MIN under *L. perenne* may be explained by the higher root biomass production as C resources from root dieback and root exudation can increase soil microbial growth and N immobilization (Dick, 1992). An analysis of the relations of the root DM yield of *L. perenne* and MBN content revealed a significant positive relation (pearson correlation coefficient $r = 0.41$, $P < 0.05$), which supports the hypothesis that the higher MBN content in MIN treatment was an indirect fertilizer effect via the increased root biomass. For the digestates no significant difference in MBN content could be observed compared to the control over all N-rates for *L. perenne*. However, previous studies reported a greater N immobilization after organic fertilizer compared to mineral fertilizer application due to C input to grassland, especially in the long-term (Estavillo et al., 1997; Bittman et al., 2005). However, Estavillo et al. (1997) found a significant treatment effect of cattle slurry on microbial biomass N only for a high application rate of 265 kg N_{tot} ha^{-1} $year^{-1}$ after 2 years of fertilization.

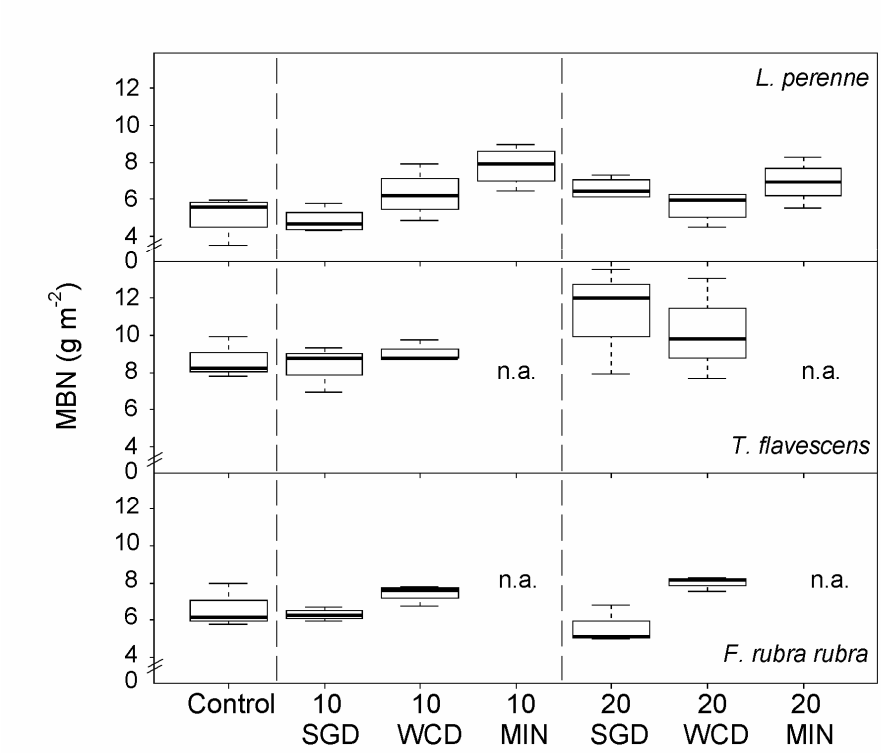


Fig. 4: Boxplots of soil MBN under *L. perenne* (n = 4), *T. flavescens* (n = 3) and *F. rubra rubra* (n = 3), 105 days after a nonrecurring application of SGD, WCD and MIN (0, 10 and 20 g NH_4^+–N m^{-2}). Bold lines represent median, boxes indicate quartiles, and whiskers show minimum and maximum, n.a. = not applied.

Differences were found for MBN among the individual grass species, while no response to the type of digestate or the N-rate was detectable (Table 4). A significantly higher MBN content was detected under *T. flavescens* in the fertilized treatments as well as in the control compared to *F. rubra rubra* and *L. perenne*. This is in line with Bardgett et al. (1999), who found that N addition to grassland did not alter the microbial biomass or activity in consistent way but was largely regulated by the planted grass species. Grass species are known to differ in root growth, quality and quantity of root exudates (Bardgett et al., 1999; Bezemer et al., 2006; Eviner et al., 2006) and may thereby influence the soil microbial biomass more than digestate application. However, although highest MBN was measured under the species that produced the highest root biomasses (*T. flavescens*), no significant relation could be found for MBN and root DM yield after regression analysis. The lack of effect of the digestate application on MBN could be due to lower root development especially for the grass species *T. flavescens* and *F. rubra rubra*, where the C input with the digestates was compensated by decreasing root biomass with increasing digestate application rate. This assumption is supported by a field experiment of Terhoeven-Urselmans et al. (2009) in which digestate application at a rate of 66 kg N_{tot} ha^{-1} led to significantly higher contents of soil ATP, that is, microbial biomass, in an unplanted fallow treatment compared to the unplanted and unfertilized control, while no significant increase for the same soil planted with barley could be detected, as the planted control showed similar soil ATP contents as the amended soil.

3.3.4 Soil N status

Soil N_{min} content under *L. perenne* increased with increasing digestate N-rate (Fig. 3). The highest mean N_{min} content at N-rate 10 was detected for WCD (2.1 g N_{min} m^{-2}) and at N-rate 20 for SGD (3.7 g N_{min} m^{-2}), while MIN was lowest at all N-rates with 0.5 g N_{min} m^{-2}. Contrasts revealed lower soil N_{min} contents for MIN compared to the digestates, which is in accordance with the result from Gunnarsson et al. (2010) of lower soil N_{min} after inorganic fertilizer application compared to digestate application on *L. multiflorum*. Over all grass species, soil N_{min} increased with increasing N-rate from 0.1 up to 14.4 g N_{min} m^{-2} and was strongly affected by the grass species (Table 4), which was mainly due to the higher N_{min} values (up to 14.4 g m^{-2}) under *F. rubra rubra*, caused by low N uptakes (Fig. 3). The values for *T. flavescens* and *L. perenne* were similar in range from 0.3 up to 5.4 g m^{-2}.

The calculated net $N_{unaccounted}$ was negative for all types of fertilizer and N-rates (Fig. 3), except for *T. flavescens* at N-rate 10 for SGD, indicating an overall net N loss via

immobilization in microbial biomass and/or emission from the plant-soil-system via NH_3 and N_2O emissions (Eqn 2), since leaching losses can be excluded in this experiment. For *L. perenne* contrasts revealed on average higher N losses for WCD (−4.0 g N m^{-2}) compared to MIN (−1.5 g N m^{-2}) and SGD (−1.8 g N m^{-2}). For the digestates, gaseous N losses are more likely than N immobilization, because no increase in soil MBN content could be detected for the digestates compared to the control, except for *L. perenne* at N-rate 20 SGD. After application of MIN a significant increase in MBN content was detected, which may have contributed to the net N loss of MIN treatment. The N loss increased over all species with increasing N-rate (Fig. 3) and were on average significantly higher for WCD (−4.7 g N m^{-2}) than for SGD (−2.2 g N m^{-2}), although the grass species again explained most of the variation with the highest N losses for *F. rubra rubra* (Table 4).

3.3.5 Mineral N use efficiency (NUEmin)

The NUE_{min}, which was used in the present study as an indicator for the plant availability of mineral N in the applied fertilizers, was different for the types of digestate and MIN. After MIN application the NUE_{min} of harvestable biomass of *L. perenne* was on average 72% and showed a slight optimum curve with the highest values for N-rate 10 and 15, indicating a constant fertilizer N utilization (Fig. 1). Contrasts revealed a lower NUE_{min} for WCD in comparison to SGD (−16%) and MIN (−19%). The NUE_{min} for stubble biomass and root biomass of *L. perenne* was affected by type of fertilizer (Table 4). While for stubble biomass SGD showed on average significantly higher values than MIN (42%) and WCD (63%), for root biomass MIN showed significantly higher values than SGD (69%) and WCD (78%).

In contrast to the results of the mineral applicated soils, the NUE_{min} after application of the digestates decreased for harvestable biomass of the investigated grass species with increasing digestate N-rate (Fig. 1, 2). This can be explained by the gaseous N losses, measured as negative net $N_{unaccounted}$, especially at high N-rates. This assumption is supported by an experiment of Sowers et al. (1994), in which a low NUE was detected for winter wheat due to excessive N losses after application of 140 kg N ha^{-1} as N fertilizer. Overall, the NUE_{min} was mainly influenced by the grass species (Table 4), pronounced in the order *T. flavescens* > *L. perenne* > *F. rubra rubra*. In general the NUE_{min} depends on the N yield of the control (see Eqn 1). Thus, the difference in the control contributed to the difference in NUE_{min} between *T. flavescens* and *F. rubra rubra* (Fig. 2). The latter is known to grow sufficiently under low N conditions (Gastal et al., 2010). However, on average over all grass

species SGD showed significantly higher NUE_{min} values than WCD for harvestable and stubble biomass (22% and 33%, respectively), which might be caused by lower gaseous N losses in addition to the same reasons as for the improved plant N content and N yield observed of SGD compared to WCD. An indication of an additional N mineralization in SGD treatment is the positive net $N_{unaccounted}$ value for *T. flavescens* at N–rate 10 (Fig. 3) and the NUE_{min} of harvestable biomass of *L. perenne* (Fig. 1) at N–rate 5, which was higher in SGD compared to MIN treatment, while the N loss of SGD and N immobilization in soil microbial biomass in MIN treatment was similar at 2 g m^{-2}.

Table 4: Results of the three factorial ANOVA for effects of N–rate (N), type of digestate (D), species (S) (*L. perenne*, *F. rubra rubra* and *T. flavescens*) and their interactions on plant and soil parameter after three cutting dates. HB = harvestable biomass, SB = stubble biomass and RB = root biomass. Significant effects at *P*<0.05. The variance (var.) columns represent the proportion of variance explained by the factor, calculated as classical eta squared (η^2). Coefficient of determination of the model (R^2).

		N			D			S			NxD			NxS			DxS			NxDxS		
Parameter		d.f.	*P*	var.	d.f.	*P*	var.	d.f	*P*	var.	d.f	*P*	var.	d.f.	*P*	var.	d.f.	*P*	var.	d.f	*P*	var.	R^2
DM yield	HB	1	<0.01	0.04	1	ns[†]	0.01	2	<0.001	0.68	1	ns	0.01	2	<0.001	0.12	2	<0.01	0.05	2	ns	0.00	0.91
	SB	1	<0.05	0.08	1	ns	0.01	2	<0.001	0.40	1	ns	0.02	2	<0.05	0.10	2	ns	0.00	2	ns	0.04	0.66
	RB	1	<0.001	0.14	1	ns	0.01	2	<0.001	0.37	2	<0.05	0.05	2	<0.001	0.19	2	<0.05	0.05	2	ns	0.02	0.82
N content	HB	1	<0.001	0.40	1	<0.001	0.02	2	<0.001	0.48	1	ns	0.00	2	<0.001	0.03	2	ns	0.00	2	<0.01	0.02	0.96
	SB	1	<0.001	0.33	1	<0.001	0.13	2	<0.001	0.35	1	<0.01	0.02	2	<0.01	0.04	2	ns	0.01	2	<0.01	0.04	0.93
	RB	1	<0.001	0.32	1	<0.001	0.16	2	<0.001	0.27	1	<0.05	0.03	2	<0.05	0.05	2	ns	0.01	2	ns	0.02	0.86
N yield	HB	1	<0.001	0.28	1	<0.05	0.02	2	<0.001	0.47	1	ns	0.00	2	<0.001	0.09	2	<0.01	0.04	2	ns	0.00	0.91
	SB	1	<0.001	0.44	1	<0.01	0.06	2	<0.001	0.23	1	ns	0.00	2	<0.01	0.09	2	ns	0.03	2	ns	0.01	0.85
	RB	1	ns	0.03	1	ns	0.00	2	<0.001	0.45	1	<0.05	0.04	1	<0.001	0.19	2	ns	0.04	1	ns	0.01	0.76
NUE_{min}	HB	1	<0.001	0.08	1	<0.001	0.04	2	<0.001	0.75	1	ns	0.01	2	<0.01	0.03	2	<0.01	0.04	2	ns	0.00	0.94
	SB	1	ns	0.01	1	<0.01	0.18	2	ns	0.09	1	ns	0.00	2	<0.001	0.26	2	ns	0.05	2	ns	0.02	0.62
	RB	1	<0.001	0.14	1	ns	0.01	2	<0.001	0.50	1	<0.05	0.05	2	ns	0.19	2	ns	0.04	2	ns	0.01	0.78
N_{min}	Soil	1	<0.001	0.30	1	ns	0.00	2	<0.001	0.40	1	ns	0.01	2	<0.01	0.09	2	ns	0.03	2	ns	0.01	0.83
$N_{unaccounted}$	Soil	1	<0.001	0.31	1	<0.001	0.14	2	<0.001	0.31	1	ns	0.00	2	ns	0.01	2	ns	0.01	2	ns	0.01	0.80
MBN	Soil	1	ns	0.03	1	ns	0.01	2	<0.001	0.57	1	ns	0.02	2	ns	0.03	2	ns	0.03	2	ns	0.03	0.74

[†] ns = not significant

3.4 Conclusions

Overall the digestate from separated grass silage (SGD), produced within the integrated generation of solid fuel and biogas from biomass (IFBB) system, and from whole crop digestion (WCD) increased harvestable biomass. This indicates both digestates as valuable fertilizers in grassland management. However, the results also suggest that an increase in aboveground and belowground biomass with increasing digestate application rate can be expected to be higher for species cultivated at grassland systems with high N status (*L. perenne*) and to be modest or even negative for species cultivated at grassland systems with lower N status (*T. flavescens* and *F. rubra rubra*). Furthermore, SGD and WCD caused species-specific yield effects, which might lead to a shift of species composition in grassland.

The relevance of type of digestate (SGD/WCD) on the N accumulation in the plant biomass was clearly supported by the finding, that over all species SGD showed a higher NUE_{min} of harvestable and stubble biomass attributed to higher plant N uptake, lower gaseous N losses and higher N mineralization compared to WCD. Hence, SGD is suitable as a short-term N fertilizer, which provides the plant with N similar to a mineral N fertilizer. The consequent higher N content of the plant residues (stubble and roots) fertilized with SGD results in favourable litter properties for mineralization processes in soil. However, considering the N status of the plant-soil-system against the background of the two energetic conversion techniques, IFBB-system and whole crop digestion, and closed nutrient cycles, it has to be mentioned that during the IFBB separation process 31% of N harvested with the plant biomass are transferred into the liquid phase (Hensgen et al., 2012), which may be returned to the grassland with the digestate. This is in contrast to the whole crop digestion, where the N would be almost entirely returned to the grassland system. With the high mineral N utilization of SGD, observed especially at low N application rates, N accumulation in the grassland system is not likely to occur, even when taking into account possible N inputs through atmospheric deposition and leguminous N-fixation. This is of advantage for extensively managed and species-rich grassland, which is generally characterized by low N status and its species composition is of high sensibility to N input.

The N immobilization in microbial biomass, measured as MBN, was highly affected by grass species and not by the different C inputs with the types of digestate or application rates. Presumably, the digestate C input was compensated by reduced root biomass. These findings indicate that due to the presence of plants, direct effects of digestate application on soil

microbial biomass and N mineralization-immobilization processes could possibly be masked and become undetectable. However, considerably more research is necessary, especially at the field scale, to provide knowledge about the long-term effects of application of digestates varying in their physical and chemical properties on natural grassland, because shifts in plant composition occur delayed. Further, in terms of the N dynamic of the grassland system an accumulation of N_{org} in soil and mineralization in subsequent years of application should be considered, which can be expected to be higher for WCD compared to SGD.

4 Soil substrate utilization pattern and relation of functional evenness of plant groups and soil microbial community in five low mountain NATURA 2000 grasslands

Abstract

Background and Aims Species rich, semi-natural grassland systems provide several ecosystem functions. The goal was to assess how aboveground composition and evenness affects soil substrate utilization pattern and soil microbial functional evenness.

Methods At five German NATURA 2000 grassland sites, the interactions of plant functional groups (graminoids, forbs and legumes) and belowground microbial functional evenness were investigated in relation to soil properties and sampling date. Functional evenness of soil microorganisms was measured with high spatial resolution by community level physiological profiling (CLPP) using multi-SIR (substrate-induced respiration) at three sampling dates during the vegetation period. Evenness indices were used to compare plant functional group diversity and soil microbial functional diversity.

Results All sites differed in the consistently high soil microbial functional evenness, which was strongly predicted by soil pH, but not by plant functional groups or aboveground plant dry matter production. However, soil microbial functional evenness was particularly decreased by an increasing legume proportion and showed seasonal changes, probably driven by shifts in resource availability and soil water content.

Conclusions Our results suggest that changes in soil chemical properties or in a single key plant functional group may have stronger effects on soil microbial functional evenness than changes in plant functional group evenness.

4.1 Introduction

European semi-natural grassland systems are of high species richness and provide several ecosystem functions such as carbon sequestration, nutrient cycling and biodiversity preservation (Wrage et al., 2011). Despite their ecological and socio-economic relevance, these ecosystems are threatened by plant diversity loss caused by agricultural intensification or abandonment (Poschlod et al., 2005). The plant community directly and indirectly interacts with soil microorganisms, thereby affecting the ecosystem processes mediated by the soil community (Van der Heijden et al., 1998; Millard and Singh, 2010). Nevertheless,

linkages between aboveground plant diversity and soil functions are poorly understood (Loreau et al., 2001), especially under natural and undisturbed conditions. In general, high plant and soil diversity is expected to be associated with high ecosystem complexity, which allows for the provision of ecosystem functions and resilience against disturbances (Schläpfer et al., 1999; Fonesca and Ganade, 2001; Wittebolle et al., 2009).

Soil microbial functional diversity, i.e. the ability of the microorganism to metabolize a set of organic compounds, is suggested to be more relevant to soil functions than microbial community composition (Zak et al., 1994) and has been frequently analyzed by community level physiological profiling (CLPP) (Liu et al., 2008; Zhang et al., 2011; Sradnick et al., 2013). Previous studies indicated a positive relationship between biomass, activity and functional diversity of the soil microbial community and plant diversity (Zak et al., 2003; Liu et al., 2008), but this was rather due to a higher substrate availability mediated by higher plant productivity associated with plant diversity than a direct plant diversity effect per se. The quantity and quality of root exudates vary between plant species and plant life forms such as annuals and perennials (Hertenberger et al., 2002; Marschner et al., 2004; Brimecombe et al., 2007). Plant functional groups (e.g. graminoids, herbaceous plants, legumes) differ in their root exudates (Meier et al., 2008) and chemistry of litter (e.g. C/N ratio of plant tissue) and therefore in resource quality for decomposers (Dijkstra et al., 2006; De Deyn et al., 2008). Consequently, changes in plant community composition may alter activity and structure of soil microbial communities (Johnson et al., 2008; Malchair et al., 2010) and thereby ecosystem functions. For example, N mineralization was related to variations in plant productivity, root N concentration and labile C production of plant species and plant functional groups (Dijkstra et al., 2006). Although, several studies pointed to the importance of plant species diversity and single plant species to the soil microbial community (Eisenhauer et al., 2010; Eisenhauer et al., 2011), others indicate that plant functional groups are main factors influencing soil microbial communities of terrestrial ecosystems (García-Palacios et al., 2011). However, incongruent results were reported as some studies observed a positive relationship of plant functional group richness and soil microbial functional diversity (Stephan et al., 2000), whereas others found no relation (Habekost et al., 2008; Marshall et al., 2011; Zhang et al., 2011).

Plant effects on soil microbial communities are difficult to analyze, as soil factors like pH, SOC content and moisture availability influence soil microbial community and can therefore overlay plant mediated effects (Hertenberger et al., 2002; Marschner et al., 2004;

Bezemer et al., 2006). Harrison and Bardgett (2010) found that abiotic factors like soil type and concentrations of inorganic N and dissolved organic nitrogen were much more important drivers of soil microbial properties than the presence of various plant species. Also temporal dynamics in quantity and quality of plant resources (root exudates and litter) are known to influence soil microorganisms during plant development (Brimecombe et al., 2007), which reduces the validity of conclusions drawn from a single measurement. Habekost et al. (2008) reported seasonal variations in grassland soil microbial community structure, substrate induced respiration and amount of phospholipids fatty acids with higher values in spring compared to late summer.

In the present study, substrate utilization pattern of soil microbial community was measured with high spatial resolution by (CLPP) using multi-SIR (substrate-induced respiration) of five different NATURA 2000 grassland sites at multiple sampling dates during the vegetation season. The functional diversity of soil microbial community was described by the evenness index, which was found to be a key factor of ecosystem stability on the species level by Wittebolle et al. (2009) examining microbial communities. The higher the evenness, the higher is the ecosystem stability to disturbance (Wittebolle et al., 2009). Further we decided using evenness to describe soil microbial diversity for a better comparability with other studies dealing with the substrate utilization approach, as most of them conventionally use the evenness to describe soil catabolic diversity (Bardgett et al., 1999; Degens et al., 2000; Degens et al., 2001; Campbell et al., 2003; Graham and Haynes et al., 2005; Romaniuk et al., 2011; Andersen et al., 2013; Brackin et al., 2013). Although it is generally accepted that diversity is composed of two components (richness and evenness), Degens et al. (2000) recommend using catabolic evenness because it is impractical to measure the immense richness of microbial catabolic functions in soils.

Plant functional group composition (graminoids, forbs, legumes) was examined and plant functional group diversity was calculated by the evenness index with the same high spatial resolution as microbial functional diversity. We used the plant functional group evenness based on the group proportion to the aboveground dry matter (DM) yield, rather than the usually used plant functional group richness (García-Palacios et al., 2011; Marshall et al., 2011; Zhang et al., 2011; Khalsa et al., 2012). As the richness does not provide information about the yield and relative distribution of the groups, the evenness index provides more relevant information for soil-plant interactions by giving the equitability of functional group yields. We hypothesized (1) that in different grassland systems soil microbial utilization

patterns are influenced by plant functional group composition and aboveground plant biomass production as well as by abiotic soil characteristics. We further hypothesized (2) that the higher the plant functional group evenness, the more diverse would be the substrate input to the soil resulting in a more functionally diverse soil microbial community. And we hypothesized (3) that soil utilization pattern and microbial functional diversity are depending on the stage of the vegetation period based on differences in resource supply for the microbial community.

4.2 Materials and methods

4.2.1 Study sites

The experimental sites were part of the EU-project PROGRASS (www.prograss.eu) and were located at the lower mountain region Vogelsberg, Germany. Five experimental sites (I-V, see Tables 5 and 6) were chosen to represent typical grassland vegetation of the region and to display the large diversity of semi-natural grasslands. Site I and II represented Lowland hay meadows with a moderate water and nutrient availability, whereas site I represented a transitional stage to the habitat Mountain hay meadow as our sites lay on the upper altitude level of lowland grassland areas. Site III and IV were typical representatives of semi-natural grasslands of higher mountain altitudes, representing a Mountain hay meadow and a transitional stage of Mountain hay meadow to species-rich *Nardus* grasslands, respectively. Site V represented a wet type of semi-natural grassland (*Molinia* meadow) dominated by *Juncus acutiflorus.* The previous agricultural management of the grassland sites was mowing once to twice per year for hay production without fertilizer application. At the beginning of the PROGRASS project in 2009 at each experimental site, 3 paired plots were established. The paired plots consisted of one harvest treatment without nutrient return (H-N) and one with nutrient return via biogas digestate application (H+N, not considered in this study), each 10 ×10 m. Harvests took place two times annually (July and August), except for the species-rich *Nardus* grassland (site IV), where harvest took place once per year (August) because of its low biomass production. Harvest frequency and time was chosen according to the usual located harvesting regime. Annual DM yield measurement of the plots was carried out by mowing 5 m^2 with a finger-bar mower at a height of 5 cm and subsequent drying the fresh biomass at 105 °C for 3 days.

Table 5: Botanical characteristics and annual DM yields of the study sites. Arithmetic means and standard deviations of the means (n=3).

Site	NATURA 2000 habitat type	Coordinates	Altitude m a.s.l.	DM yields t ha^{-1} a^{-1}	Plant species		Plant functional groups		
					Total number[a]	Dominant species[a]		Composition % DM[b]	Species number[a]
I	Lowland hay meadow (6510)	50° 35' 27.351" N 9° 12' 27.432" E	570	4.7 (0.3)	43 (1.0)	*Festuca rubra, Trifolium pratense, Plantago lanceolata, Holcus lanatus*	Graminoids	65.8 (15.7)	14 (1.5)
							Forbs	24.7 (12.5)	24 (2.7)
							Legumes	9.4 (8.6)	5 (1.2)
II	Lowland hay meadow (6510)	50° 35' 22.1022" N 9° 20' 40.794" E	420	4.2 (0.0)	44 (2.3)	*Festuca rubra, Agrostis capillaries, Plantago lanceolata, Trifolium pratense*	Graminoids	64.3 (18.8)	14 (2.5)
							Forbs	29.0 (17.4)	25 (0.6)
							Legumes	6.7 (6.0)	5 (1.5)
III	Mountain hay meadow (6520)	50° 28' 41.0082" N 9° 15' 20.6706" E	580	4.2 (0.6)	48 (4.4)	*Festuca rubra, Sanguisorba officinalis, Agrostis capillaris, Plantago lanceolata*	Graminoids	62.9 (21.0)	17 (2.1)
							Forbs	35.7 (20.4)	27 (2.6)
							Legumes	1.4 (2.1)	4 (0.6)
IV	Species-rich *Nardus* grasslands (6230)	50° 33' 49.9134" N 9° 15' 45.1476" E	580	3.2 (0.2)	37 (2.9)	*Festuca rubra, Agrostis capillaries, Nardus stricta, Plantago lanceolata*	Graminoids	95.4 (4.2)	18 (1.5)
							Forbs	2.4 (3.2)	13 (3.1)
							Legumes	2.2 (3.3)	6 (1.2)
V	*Molinia* meadow (6410)	50° 33' 55.8684" N 9° 18' 9.7092" E	500	4.8 (1.3)	42 (1.5)	*Juncus acutiflorus, Festuca rubra, Holcus lanatus, Valeriana dioica*	Graminoids	74.6 (24.0)	17 (1.7)
							Forbs	23.7 (23.3)	23 (2.1)
							Legumes	1.6 (2.9)	2 (0.0)

[a] Data collected in June and July 2011 as described by Hensgen et al. 2012, mosses are excluded

[b] Mean values for the subplots and three sampling dates in 2012

The local climate can be characterized as temperate with average temperature (1961-1990) varying with the altitude of the weather station, 8.0 °C (454 m asl) to 6.7 °C, (606 m asl). The average annual precipitation was between 923 and 1211 mm, respectively (PIK, 2009). Soil types were classified according to WRB (IUSS, 2007) and were mainly Cambisols (site I, III and IV), from volcanic bedrock, or Stagnosols (site II and V) (Hensgen et al., 2012)

Table 6: Soil characterization of the study sites from 0-10 cm depth. Arithmetic means and standard deviations of the means, n=3. For texture parameters n=1.

Site	SOC	Total N	C/N	pH	WHC	Water content[a]	Sand[b]	Silt[b]	Clay[b]
	mg g^{-1}	mg g^{-1}		$CaCl_2$	% DM	% WHC	%	%	%
I	44.5 (5.6)	4.5 (0.6)	10.2 (0.2)	5.2 (0.1)	115 (4.8)	57.4 (3.2)	17	57	26
II	42.0 (5.5)	4.2 (0.6)	10.5 (0.2)	4.3 (0.0)	101 (6.4)	66.5 (3.2)	8	69	23
III	59.4 (2.7)	5.6 (0.2)	10.7 (0.2)	4.9 (0.1)	120 (6.5)	61.3 (2.7)	8	65	27
IV	65.4 (3.2)	5.4 (0.3)	12.0 (0.3)	4.3 (0.1)	113 (12.5)	69.1 (2.4)	8	72	20
V	47.9 (9.3)	4.4 (0.9)	10.8 (0.4)	4.6 (0.1)	117 (19.1)	56.5 (4.0)	5	70	25

[a] Means for the three sampling dates

[b] Hensgen et al. 2012

4.2.2 Estimation of plant functional group composition and plant sampling

At each plot 7 subplots (rings having a diameter of 20 cm) were marked along a transect (14.1 m) and soil sampling and plant functional group determination were performed at three sampling dates in 2012. Two sampling dates were set at the beginning of the vegetation period of the Vogelsberg region in the first week of May (2 to 3 May 2012) and the third week of May (18 May), because during that period root growth and exudation rates are considered to be high (Brimecombe et al., 2007). One sampling date was set at the vegetation climax in first week of July (3 to 4 July). Data from plots without digestate application (H-N) are presented.

In May, plant functional group composition was measured at each subplot by estimating visually the DM yields of three plant functional groups (graminoids including sedges and rushes, forbs and legumes) in compliance with proceeding outlined by Davies et al. (1993). In July, plant functional group composition was measured at each subplot by cutting at five

cm height, fractionation and drying at 105 °C for 3 days. Estimation of DM yields of plant functional groups was conducted always by the same person and the accuracy of estimation was checked based on dried subsamples in July (Pearson correlation coefficient r = 0.95).

4.2.3 Plant functional group evenness

To describe the plant functional group diversity, the Shannon evenness index (E_{plant}) was calculated by the formula $E_{plant} = -\sum(p_i \ln(p_i)) / \ln(k)$, where p_i was the DM proportion of the plant functional group of the total DM yield and k the number of functional groups. The evenness index ranges from 0 (no functional diversity) to 1 (all occurring functional groups have the same proportion to the DM yield).

4.2.4 Soil sampling and soil chemical analyses

Soil sampling was performed within the 7 subplots with a soil corer (diameter 2 cm) in 0-10 cm depth. The experimental layout resulted in 315 soil samples (7 sampling replicates per plot, 3 plots per site, 5 sites and 3 sampling dates). Soils were sieved <2 mm and stored at 4°C. Some of samples of site IV and most samples of site V, which were too wet, were carefully dried before sieving at room temperature. Previous investigations revealed a homogenous pH and C/N distribution in the top soil layers of the sites. Consequently soil pH ($CaCl_2$), soil organic C (SOC) and total N (analyzed with an elemental analyzer Vario MAX CHN, Elementar, Hanau, Germany oven dried samples (60°C)) were measured on composite samples of the seven subplots taken in July.

4.2.5 Soil microbial substrate utilization pattern and evenness

Substrate utilization patterns were determined according to the multi-SIR approach using MicroResp™ method after Campbell et al. (2003) for each of the 7 replicates per plot (subplot). Into each 1.1 ml deep-well microtitre plate (Nunc, Thermo electron LED, Langenselbold, Germany), soil was placed volumetrically by a filling-device (MicroResp™) as described in Campbell et al. (2003). The water content was adjusted to 50-70% of the water holding capacity (WHC) with demineralised water. Before measurement the samples were pre-incubated for 7 days at 25°C in the dark and seedlings germinated during incubation were removed.

The carbon substrate utilization patterns were determined by applying four carbohydrates (D-glucose, D-fructose, D-trehalose, L-arabinose), four carboxylic acids (α-ketoglutaric acid, citric acid, malic acid, oxalic acid), eight amino acids (arginine, D-glucosamine, DL-aspartic

acid, L-alanine, L-glutamine, L-leucine, lysine, γ-aminobutyric acid) and one phenolic acid (protocatechuic acid). These substrates were chosen according to their ecological relevance, as most of them are reported as rhizosphere carbon sources (Campbell et al., 1997). The substrates glucosamine, trehalose and α-ketoglutaric acid were identified as key discriminators in studies comparing different ecosystems or management treatments (Campbell et al., 1997; Stevenson et al., 2004; Lalor et al., 2007; Romaniuk et al., 2011). The basal respiration was determined by adding demineralised water. The analysis was started with 22 mg g^{-1} soil water of each substrate in aqueous solutions. Because of low solubility, 6 mg g^{-1} soil water of L-leucine and L-glutamine and 2 mg g^{-1} of protocatechuic acid and aspartic acid were applied. For determining the CO_2 emission a power function was used resulting from a calibration of five different soils according to Sradnick et al. (2013):

$$\mu l\ CO_2 = 63 \times (0.1 + ABS)^3,\ r = 0.98$$

where ABS is the difference in absorption (572 nm) of T1 and T0. The incubation time was 4 h. To describe the soil microbial functional diversity, the Shannon evenness index (E_{Soil}), was calculated by the formula $E_{soil} = -\sum(p_i \ln(p_i)) / \ln(k)$, where p_i was the proportion of the individual substrate respiration to the total substrate respiration and k the number of substrates according to Zak et al. (1994).

4.2.6 Statistical analyses

The respiration rates of the C substrates were standardized by dividing the single substrate respiration by the mean of the 17 substrates to receive a relative measure of its contribution to the mean substrate utilization pattern of the substrates selected in this study. Multivariate outliers of the substrate utilization patterns were removed from the dataset according to Field et al. (2012). To identify site effects on plant functional group evenness we conducted repeated measure with multilevel linear approach followed by orthogonal contrasts, as described by Field et al. (2012), on the pooled data of the seven subplots per plot (arithmetic mean). To identify site and sampling date effects on soil functional evenness, analyses of variance (ANOVA) were conducted with pooled data of the seven subplots per plot and in case of interactions a simple effect analyses was conducted followed by Tukey-tests. Significance level was set at $P<0.05$.

Further multiple stepwise regressions were performed to test for the effect of plant and soil parameter on E_{soil} with the pooled values of the seven subplots per plot. The parameters DM proportion of the individual plant functional groups, site DM yield, soil pH, C/N ratio and water content were included in the preliminary model. Multi-collinearity was tested according to Field et al. (2012) and forbs were excluded from the analyses because parameters associated with forbs were highly correlated with graminoids. Graminoids were kept in the analyses, as they are the dominating plant functional group in grasslands. Further SOC and total N were strongly correlated (Pearson correlation coefficient r = -0.96) and therefore their ratio (C/N) was used instead. Quadratic terms of the parameters were included in the starting model and model reduction was stopped when all parameters were significant at least at the 10% significance level. Model development followed the statistical model selection methods described by Draper and Smith (1998) and obeyed the rules of hierarchy and marginality (Nelder and Lane, 1995). Beta values (*ß*) are the standardized values of the parameter estimates and were used to indicate the relative importance in the model.

To investigate the effects of site and sampling date on the substrate utilization patterns, discriminant function analyses (DFAs) were conducted on the unstandardized and unpooled data set with Statistica. DFAs had to run with unpooled data because of a larger sample size required for multivariate analyses. To describe the discrimination, the substrate specific respiration was correlated to the canonical scores of the significant discriminant functions (DFs). Pearson correlation coefficients were used to express the significance. Further the canonical scores of the Dfs were correlated to the plant and soil parameters to identify their participation to the discrimination. Relation of the evenness indices of plant functional groups and carbon utilization patterns were tested with simple linear regression models on the pooled data set.

4.3 Results

4.3.1 Basal respiration and substrate utilization patterns

The application of C substrates always led to a higher respiration in comparison with the basal respiration. A positive Pearson correlation (r = 0.40) was observed for the SOC content and basal respiration. The contribution of substrate groups to the mean substrate induced respiration was in the order carboxylic acids > carbohydrates > protocatechuic acid > amino

acids (Fig. 5). Discriminant function analyses (Wilks Lamda: 0.015, approx. F = 32.7, $P < 0.001$) identified 4 discriminant functions (DFs) by which the substrate utilization patterns of all of the sites could be separated significantly from each other. DF1 and DF2 together explained 90% of the variance of the sites (Fig. 6a). The substrate utilization patterns from site I and III were identified by DF1 to be distinct from those from site IV and V mainly due to greater utilization of the amino acids aspartic acid, arginine and alanine and lower utilization of two carboxylic acids oxalic and malic acid (Fig. 6a, Table 7). DF2 separated site II from the other sites mainly by having a higher utilization of the carboxylic acids citric acid and α-ketoglutaric acid. DF4 significantly differed site IV from site V (data not shown).

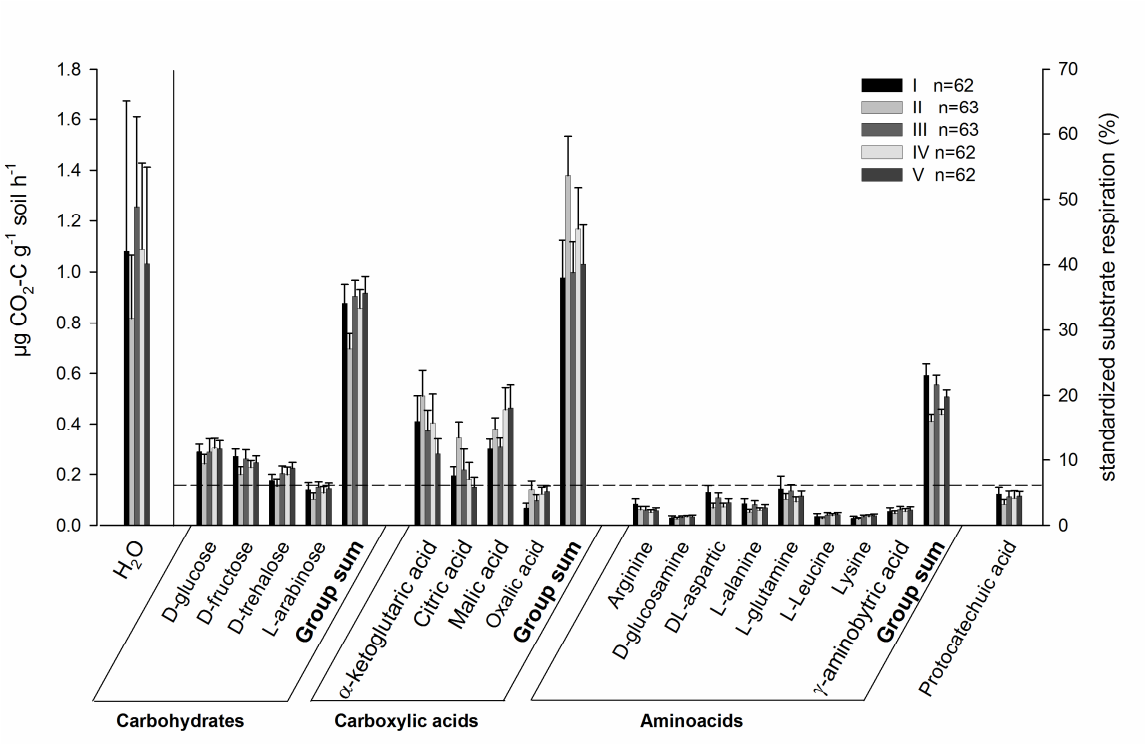


Fig. 5: Basal respiration rate (H_2O) and individual standardized substrate respirations as proportion of the total standardized substrate induced respiration and the substrate group sums (n=5) for the five grassland sites over the sampling period. The dashed line indicates the overall mean of the proportionate substrate respiration. Error bars indicate standard deviations of the means.

Correlation analysis revealed stronger relations for the soil parameters and the DFs than for the plant parameters (Fig. 6a). Key discriminatory substrates of DF1 of the site discrimination correlated strongest to pH, whereas discriminatory substrates of DF2 were strongest correlated with the soil C/N ratio (Table 7). This indicates that soil pH and soil C/N

ratio were the most important factors for site discrimination. However, the plant functional groups correlated with the discriminatory substrates, though to a smaller extend ($r < 0.35$). Graminoids correlated negatively with most of the substrates, the opposite was true for forbs, whereas legumes correlated positively with only a few substrates.

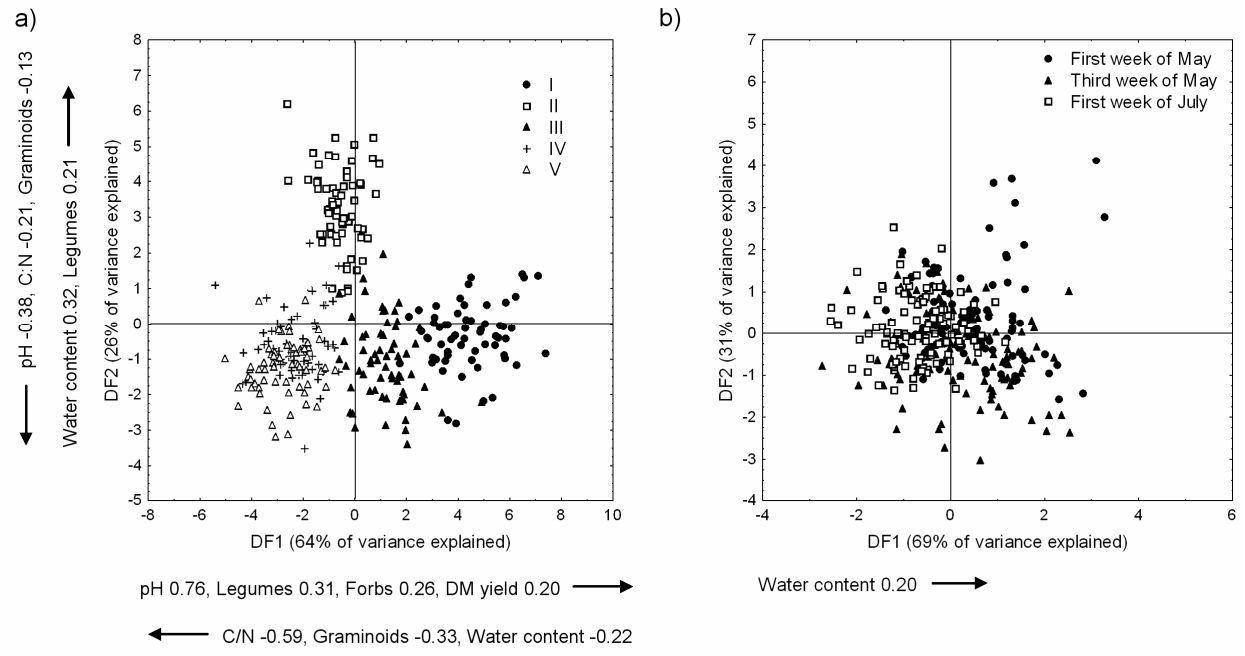


Fig. 6: Discrimination function analysis (DFA) of catabolic response of soil communities to17 C-substrates for five different grassland sites (I-V, for detailed information see table 5 and 6) run for a) site discrimination n=312 and b) sampling date discrimination n=312. Significant Pearson correlation coefficients of soil and plant parameter with the first two DFs are indicated and the direction of the relation is visualised by the arrows.

A discriminant function analyses for the three sampling dates (Wilks Lamda: 0.71, approx. $F = 3.24$, $P<0.001$) revealed one DF (DF1) separating significantly the substrate patterns measured in May from those measured in July (Fig. 6b). This separation was mainly caused by higher utilization of malic acid and lysine and lower utilization of glutamine in May (Table 7). DF1 weakly correlated with the soil water content (Fig. 6b).

Table 7: Pearson correlation coefficient between substrate utilization of individual substrates and the discriminating canonical discriminate functions of the sites and of the sampling dates to soil and plant parameters (n=313). Substrates are sorted by the values of DF1 site discrimination. Pearson correlation coefficients r>50 are written in bold. Substrate groups are abbreviated as follows carbohydrates CH, carboxylic acids CA, amino acids AA and phenolic acid PA.

Substrates	Substrate groups	Site discrimination		Sampling date discrimination				Plant functional group composition			
		DF1	DF2	DF1	Water content % WHC	pH	C/N	Graminoids % DM	Forbs % DM	Legumes % DM	DM yield t ha^{-1} a^{-1}
DL-aspartic a.	AA	**0.71*****	-0.23***	0.05	-0.06	**0.65*****	-0.47***	-0.32***	0.28***	0.21***	0.23***
Arginine	AA	**0.68*****	0.09	0.19***	0.07	**0.51*****	-0.49***	-0.29***	0.23***	0.25***	0.22***
L-alanine	AA	**0.62*****	-0.24***	0.21***	-0.06	**0.58*****	-0.40***	-0.31***	0.29***	0.16**	0.22***
D-fructose	CH	**0.62*****	-0.17**	0.14*	0.08	**0.57*****	-0.42***	-0.26***	0.25***	0.09	0.27***
L-glutamine	AA	**0.58*****	-0.08	-0.15**	-0.02	**0.52*****	-0.44***	-0.28***	0.27***	0.10	0.21***
Protocatechuic a.	PA	**0.51*****	-0.24***	0.00	0.08	0.49***	-0.30***	-0.19***	0.17**	0.12*	0.19***
D-glucose	CH	0.47***	-0.14*	0.26***	0.22***	0.42***	-0.27***	-0.13*	0.13*	0.05	0.21***
α-ketoglutaric	CA	0.45***	**0.58*****	0.24***	0.26***	0.07	-0.31***	-0.23***	0.17**	0.24***	-0.09
L-arabinose	CH	0.43***	-0.26***	0.03	0.05	0.44***	-0.29***	-0.24***	0.24***	0.06	0.17**
γ-aminobutyric a.	AA	0.40***	-0.16**	0.10	0.05	0.37***	-0.28***	-0.28***	0.28***	0.09	0.13*
L-leucine	AA	0.31***	-0.32***	0.20***	0.02	0.35***	-0.17**	-0.24***	0.25***	0.03	0.14*
Citric a.	CA	0.28***	**0.68*****	0.19***	0.27***	-0.05	-0.36***	-0.30***	0.26***	0.22***	-0.05
D-glucosamine	AA	0.28***	-0.21***	0.26***	0.13*	0.27***	-0.15**	-0.20***	0.19**	0.08	0.07
D-trehalose	CH	0.25***	-0.26***	0.17**	0.15**	0.33***	-0.21***	-0.18**	0.19***	0.00	0.20***
Lysine	AA	0.16**	-0.25***	0.31***	0.11	0.21***	-0.09	-0.19***	0.20***	0.02	0.06
Malic a.	CA	-0.17**	0.20***	0.35***	0.49***	-0.18**	0.03	0.08	-0.07	-0.04	0.03
Oxalic a.	CA	-0.25***	0.41***	0.03	0.35***	-0.33***	-0.01	-0.17**	0.17**	0.05	-0.06

$P < 0.05$; ** $P < 0.01$, ***$P < 0.001$

4.3.2 Plant functional group and soil microbial functional evenness

Plant functional group evenness (E_{plant}) ranged widely from 0.0 to 1.0 between the subplots. The repeated measure (multilevel linear model approach) revealed that type of grassland had a significant effect on the evenness of plant functional groups $\chi^2(4)=50.65$, $P<0.001$. Orthogonal contrasts revealed that evenness was significantly higher for the hay meadows (site I, II and III), with relatively high amounts of forbs and legumes (Table 5), compared to the *Nardus* and *Molinia* grassland (site IV and V), $b=0.43$, $t(8)=14.5$, $P<0.001$. *Molinia* grassland was significantly higher compared to *Nardus* grassland $b=-0.14$, $t(8)=-7.5$, $P<0.001$. The *Molinia* meadow showed a mediate mean value of 0.48 and the *Nardus* grassland showed with 0.20 the lowest mean value, because of high grass proportion of 95% of the DM yield.

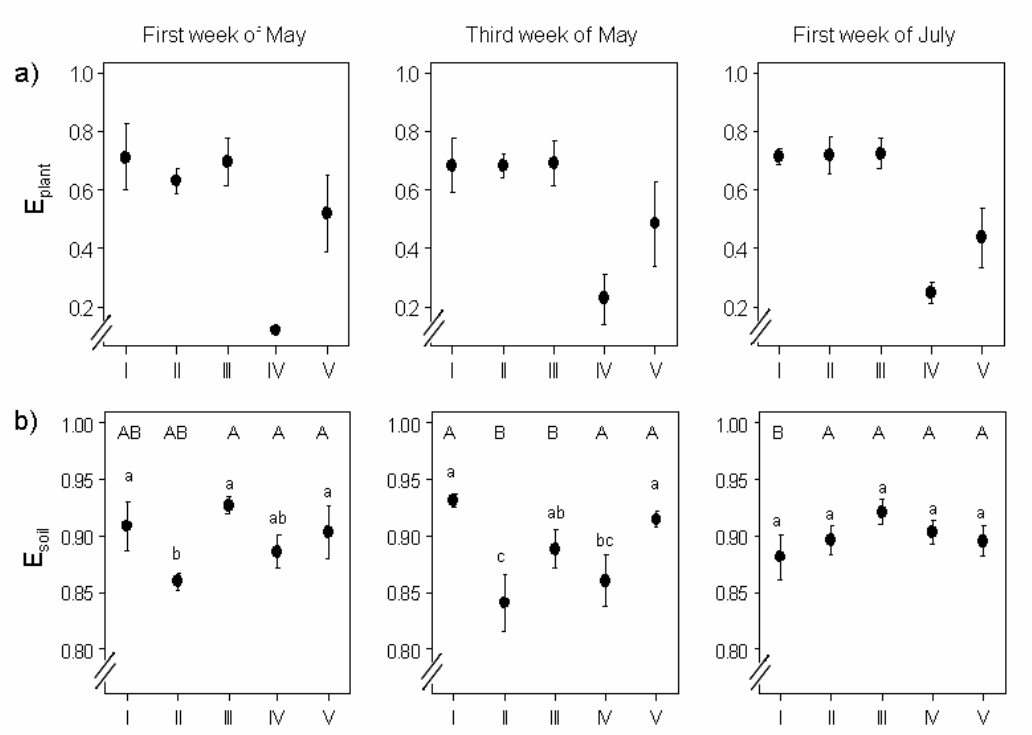


Fig. 7: Evenness indices of a) plant functional groups and b) soil substrate utilization patterns of the sites (I-V) for the three sampling dates. Statistics of a) are given in the text. For b) lower case letters indicate significant differences between the sites at the same sampling, upper case letters indicate significant differences between the sampling dates for each site (Tukey-test, *P*<0.05). Error bars indicate standard deviations of the means (n=3).

The values of microbial functional diversity measured by the evenness index of the substrate respiration (E_{soil}) ranged from 0.79 to 0.95 between the subplots. ANOVA revealed a significant site × sampling date interaction effect on E_{soil} $F(8,30) = 6.56$, $P<0.001$, n=45.

This indicates that the sampling date effects on E_{soil} differed between the grassland sites. Simple effect analyses and Tukey tests revealed a decrease of E_{soil} for site II (not significant) and III (significant) from first week of May to third week of May and an increase again to the first week of July (Fig. 7). At site I E_{soil} decreased from May to July. No significant sampling date effect was found for site IV and V. At no sampling date, a significant linear regression analyses of E_{plant} and E_{soil} could be detected (Fig. 8).

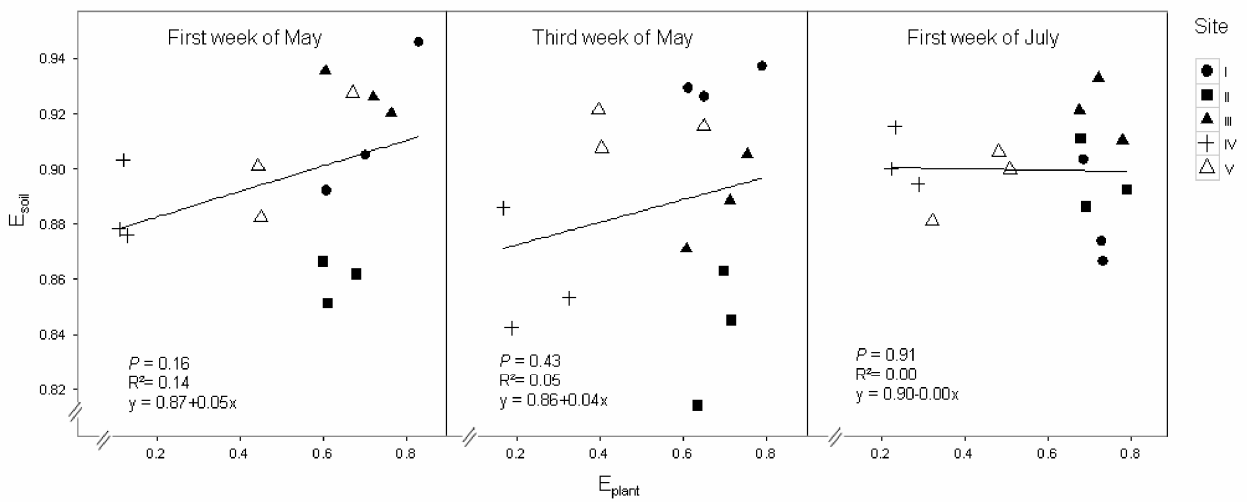


Fig. 8: Linear regressions for E_{plant} and E_{soil} for the three different sampling dates (n=15; n=15; n=15).

4.3.3 Multiple stepwise regressions on soil microbial functional evenness

The results of the multiple stepwise regression models, which tested the effects of soil and plant parameters on E_{soil}, are presented in table 8. In May soil parameters contributed more to the models than plant parameters, whereas in July the plant parameter legumes and DM yield were identified as important drivers of E_{soil}. The models achieved high coefficients of determination (R^2 = 0.68-0.92). The water content was found to have quadratic effects on E_{soil} in May, which was mainly negatively expressed for the measured range (Fig. 9). Increasing soil pH and soil C/N ratio increased E_{soil} in May. However, a linear Pearson correlation indicated no relation of SOC and E_{soil}. At each sampling date an increasing proportion of legumes decreased E_{soil}. Similarly an increasing proportion of graminoids decreased E_{soil} at the third week of May. As a consequence of the negative correlation of graminoids and forbs, an increasing forb proportion leads to an increase of E_{soil}. The total DM yield production was found to affect the E_{soil} in the form of an extremely low optimum curve in July (Table 8).

Table 8: Coefficients of determination and parameter estimates for the multiple stepwise regressions of E_{soil} and the soil and plant parameters (n=15) for each of the three sampling dates. Beta coefficients (ß) are the standardized values of the parameter estimates and indicate the relative importance in the model.

	Unit	First week of May		Third week of May		First week of July	
R^2		0.87		0.92		0.68	
R^2 adj.		0.79		0.85		0.59	
P		0.001		0.002		0.005	
df[a]		9		7		11	
		Estimates P	*ß*	*Estimates P*	*ß*	*Estimates*	*ß*
Intercept		1.555		9.996		0.776	
Water content	% WHC	-0.028 .	-6.17	-0.078 *	-12.55	-	-
pH		0.058 **	0.73	-2.924 *	-27.07	-	-
C/N		-	-	0.025 .	0.46	-	-
Graminoids	% DM	-	-	-0.002 **	-0.82	-	-
Legumes	% DM	-0.011 **	-1.93	-0.004 *	-0.45	-0.005***	-0.88
DM yield	t ha^{-1} a^{-1}	-	-	-	-	0.072 *	3.66
Water content^2	% WHC	0.000 .	6.03	0.001 *	12.02	-	-
pH^2		-	-	0.319 *	27.74	-	-
Leg^2		0.001 **	1.67	-	-	-	-
DM yield^2	t ha^{-1} a^{-1}	-	-	-	-	-0.009 *	-3.95

'.' $P < 0.1$; * $P < 0.05$; ** $P < 0.01$, ***$P < 0.001$

[a] Degrees of freedom

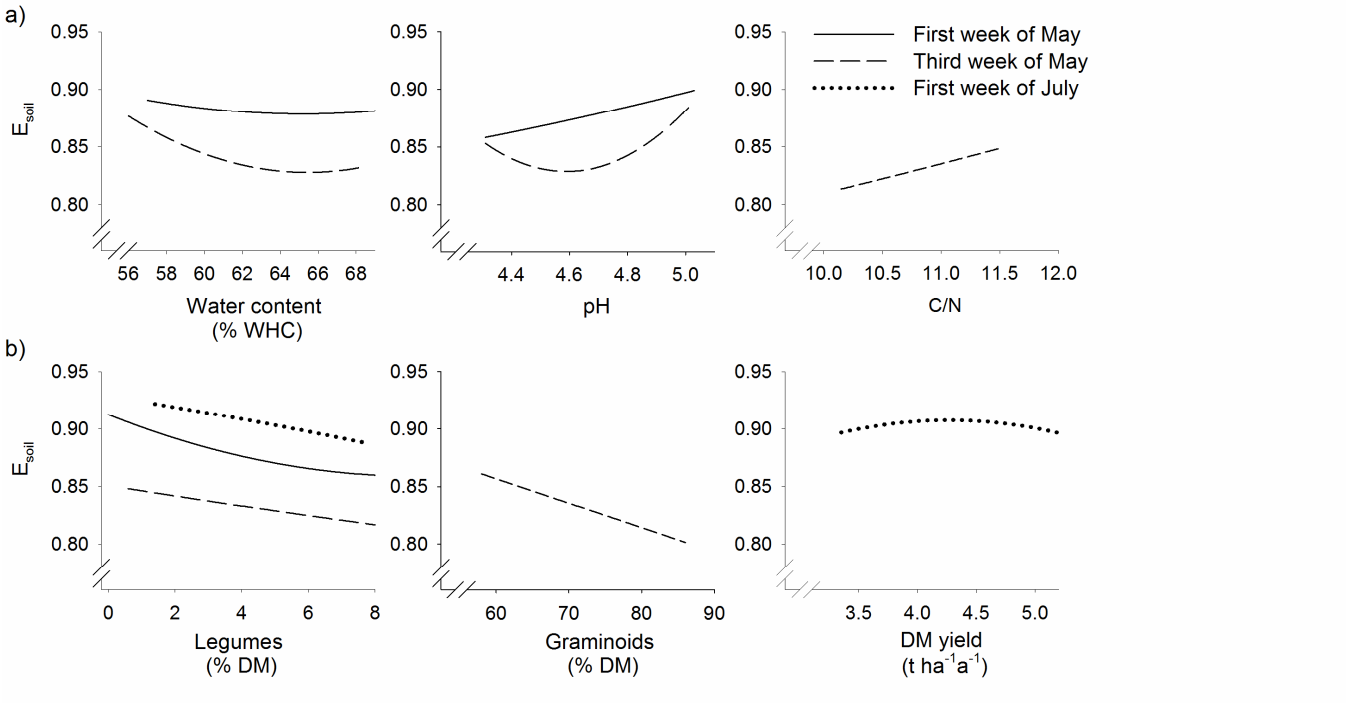


Fig. 9: Predictions of E_{soil} at the three different sampling dates for a) soil parameters and b) the plant functional groups legumes and graminoids and DM yield identified from the multiple stepwise regression models stated in table 8 (n=15) (forbs were excluded from the models due to a strong negative inter-correlation with the graminoids). For each parameter presented all the other variables in the model were set to their mean value.

4.4 Discussion

4.4.1 Substrate utilization patterns of soil microbial community

All five NATURA 2000 grassland sites differed in their substrate utilization patterns based on the discrimination function analysis, however species-rich *Nardus* grassland (site IV) and *Molinia* meadow (site V) showed marginal differences. Despite the small gradient in soil pH the utilization of the substrates most responsible for site discrimination was most strongly associated to soil pH and to a lesser extent to the plant functional group proportions and site DM production. This is in accordance to previous studies which revealed that even within a small range soil pH can substantially influence the carbon utilization pattern of grassland soils (Degens et al., 2001; Grayston et al., 2004). Corresponding with our results Sradnick et al. (2013) reported a higher utilization of key discriminating substrates mainly amino acids and carbohydrates with increased pH in an arable soil (pH 6-7). Considering recent studies of Brackin et al. (2013) and Stevenson et al. (2004) it is probable that pH is also the main factor discriminating substrate utilization pattern of different ecosystems with high distinctions in plant community composition. They demonstrated that grassland and arable soils have a relatively higher response to easily metabolised high-energy substrates (carbohydrates and amino acids), whereas more acidic forest soils responded to a larger extent to less energy-rich carboxylic acids. By comparing three semi-natural Irish grasslands, Liliensiek et al. (2012) found that pH strongly affected the microbial community, but within each soil, plant species composition was the main influencing factor. However, we could not detect strong correlations between individual plant functional groups and the ability of the soil microorganism to use specific substrates within sites (data not shown). Rather, the intensity and direction of the correlations was highly dependent on sampling date. Meier et al. (2008) found under alpine meadow communities higher fluxes of phenolic components into the soil from woody herb roots (*Acomastylis rossii*) than for grass roots (*Deschampsia caespitose*). Corresponding to that DF1 of site discrimination was associated with protocatechuic acid utilization and separated forb rich sites (I and III) from sites with lower forb proportion (IV) (Fig. 6a). However, only a weak positive across site correlation of protocatechuic acid and forb proportion could be observed in this study (Table 7). Although the range of measured soil parameters was low, our data suggest that the aboveground plant functional group composition of species-diverse low mountain grasslands is marginal responsible for the substrate utilization pattern of soil microbial communities compared to soil parameters.

Despite temporal complexity, some general conclusions can be drawn from the entire dataset. In accordance with previous studies (Buyer and Drinkwater, 1997; Grayston et al., 2001; Papatheodorou et al., 2012) substrate utilization patterns were found to depend on the sampling date, with dates in May significantly differing from dates in July. This discrimination was mainly caused by the higher utilization of the substrates lysine and malic acid, whereas the latter is known to favour the growth of rhizobacteria when released from plant roots (Rudrappa et al., 2008). This suggests that soil microbial community was altered by plant life cycle from the beginning to the climax of the vegetation period, reflecting possible changes in substrate availability due to a shift in quantity and quality of root exudates (Brimecomp et al., 2007). For example an experiment under controlled conditions revealed direct effects of plant age on composition of root exudates and root released substrates correlated with the functional capacity of the rhizosphere microorganism to metabolize these compounds (Chaparro et al., 2013). The sampling date discrimination was weakly associated with differences in soil water content (Fig. 6b), whereas samples in July were on average drier than in May (data not shown).

4.4.2 Evenness of plant functional groups and soil microbial substrate utilization pattern

The aboveground functional evenness significantly differed between the sites, with highest values for the meadow sites (I, II and III). Overall all sites, the soil microbial catabolic evenness showed high values as previously shown by other field studies on permanent grasslands in Europe and East Africa (Graham and Haynes, 2005; Murugan et al., 2014). This further supports the growing body of evidence that soils under diverse semi-natural grassland are in general of high microbial functional diversity and, as previously found, more diverse than agricultural and forest soils (Degens et al., 2001; Graham and Haynes, 2005; Murugan et al., 2014). Also recent findings of seasonal dependency of the soil microbial functional diversity described by evenness index (Andersen et al., 2013) could be confirmed in this study.

We found no significant relationship between E_{plant} and E_{soil} during the entire sampling period. Based on previous studies (Johnson et al., 2008; Meier et al., 2008; Malchair et al., 2010) we hypothesized that a high evenness of the chosen plant functional groups (graminoids, forbs, legumes) would increase the evenness of substrate utilization pattern by providing a more heterogeneous resource pattern for the soil microbial community. Possibly,

focussing on other aboveground diversity measurements like diversity indices including the species level (Eisenhauer et al., 2010; Eisenhauer et al., 2011) or plant functional traits (De Deyn et al., 2008) might have more power in assessing plant and soil diversity relations by supplying a broader range of information. However, E_{soil} was influenced by discrete plant functional group abundance. For example, an increase in graminoids, and in particular legumes, decreased E_{soil}. Hedlund (2002) and Habekost et al. (2008) found that the presence of legumes has a discriminating influence by increasing gram negative bacteria and decreasing fungal biomass, which might therefore have a negative effect on the catabolic diversity.

The hypotheses that plant biomass production and soil microbial functional diversity are positively related is frequently postulated as a consequence of better nutrient supply for plants mediated by diverse microbial communities (Van der Heijden et al., 1998; Eisenhauer et al., 2012). In addition, high plant biomass production influences carbon-limited microbes by increasing resource exudation (Zak et al., 2003; Liu et al., 2008). However, this assumption could not be confirmed in this study. Even aboveground plant DM yield explained most variation of E_{soil} in July (Table 8), the relationship followed rather a weak optimum curve than a linear function. The aboveground DM yield of the investigated semi-natural grassland sites showed relative small variation. Possibly larger gradients would result in a positive linear relation of soil catabolic diversity and aboveground plant biomass, similar to which was found in a grassland study with a broad range of 13-140 g DM m^{-2} (Liu et al., 2008).

In May, increases in water content decreased E_{soil} (Table 8), indicating that the chosen range for the optimal water content during the SIR measurements was too broad. An increase in water content may have led to limiting conditions for microbial catabolic processes and therefore decreased E_{soil} (Gömöryová et al., 2013). The decrease of E_{soil} with decreasing soil pH (5.2 to 4.3) is in accordance to results of an artificial acidification experiment with grassland soil of Degens et al. (2001). Kemmit et al. 2006 observed similarly to the respiration response of most of the amino acids used in this study a reduced metabolism with decreasing pH, whereas other substrates were unaffected, leading to an unbalanced substrate utilization and hence decreased E_{soil}. In addition to differences in microbial biomass and community structure, a possible explanation for reduction of amino acid respiration would be the favoured adsorption to soil exchange sites of minerals under acidic conditions (Strahm and Harrison, 2007; Rothstein, 2010). However, Sradnick et al. (2013) found no correlation

between soil pH and microbial functional diversity (Shannon diversity index) for an arable soil.

Previous studies reported an increasing catabolic evenness with increasing organic matter content in the soil due to differing land uses (e.g. permanent grassland versus arable land) and application of organic fertilizer (Degens et al., 2000; Brackin et al., 2013; Sradnick et al., 2013). Contrary to these results, no correlation of SOC and only a weak positive relation of the soil C/N ratio and E_{soil} were found in this study, probably because soils of the investigated semi-natural grassland sites differed only little in C/N ratio (10.2-12.0) and SOC content (42-65 mg C g^{-1}). Within these levels, variation does not lead to changes in the catabolic diversity of the soil microbial soil community, although the substrate utilization pattern might differ. Accordingly, Zak et al. (2003) hypothesized a weaker effect of plant community diversity on soil microbial communities in SOC rich soils (130 mg g^{-1}) compared with low SOC soils (4-5 mg g^{-1}). Due to the small range in SOC content and pH of the investigated soils, our findings can not be extrapolated to soils with a broader range of these parameters. Furthermore, influence of nutrient and water regime on substrate utilization patter should be systematically investigated.

4.5 Conclusions

The low mountain semi-natural grasslands investigated here, showed consistently high soil microbial functional evenness and all differed in their response pattern to the selected carbon substrates. This indicates the high functional range of soil microbial communities of different NATURA 2000 grassland types, despite similar agricultural management and geography. Our data suggest that aboveground plant functional group evenness and soil microbial functional evenness are not linked to each other. Although the ranges of soil properties were low, abiotic soil factors, especially soil pH, are the main factors in influencing the soil substrate utilization pattern and determining soil microbial functional evenness, whereas plant functional group composition, plant group evenness and aboveground plant dry matter production are less important. However, a single plant functional group may play a key role, as for instance an increasing legume proportion consistently decreased soil microbial functional evenness. Furthermore, soil microbial functional evenness depends on the sampling date, probably driven by changes in source availability. Thus the temporal dependency of the substrate utilization pattern, leads to the conclusion that data of future studies, which focus on

the functionality of the soil microbial community, should base on multiple sampling dates through the vegetation period.

Our study emphasizes the role of abiotic soil factors and key plant functional groups rather than plant functional group evenness as important determinants of soil microbial catabolic responses. Such considerations are crucial in further completing our understanding of plant-soil diversity interactions and, hence, ecosystem functioning of biodiverse semi-natural grasslands.

5 Shifts of plant functional groups and soil microbial catabolic diversity due to management changes in temperate extensive grasslands of a lower mountain range

Abstract Over the last decades species rich, semi-natural grasslands are increasingly threatened by agricultural intensification, abandonment or afforestation. At five German NATURA 2000 grassland sites, we assessed the effects of digestate application and mulching as alternative managements to harvesting without nutrient application on plant functional groups (graminoids, forbs and legumes) and soil microbial substrate utilization pattern three years after implementation. Substrate utilization pattern were measured using multi-SIR (substrate-induced respiration) at three sampling dates during the growing season. Evenness indices were used to estimate plant functional group diversity and soil microbial catabolic diversity. Digestate application and mulching increased the aboveground plant dry matter production. No shifts in plant functional group composition and evenness were observed after digestate application, whereas mulching tended to show contrasting responses depending on the grassland type. Only small and transient shifts in soil microbial substrate utilization pattern and catabolic evenness were induced by digestate application. SOC, total N, C/N and soil pH value were not affected by any treatment. Our results provide evidence that moderate dose digestate application may serve as an ecologically more suitable management alternative compared with mulching for keeping semi-natural grassland meadows under agricultural management and without substantially influencing plant functional group composition nor soil microbial substrate catabolism.

5.1 Introduction

European semi-natural grasslands are grasslands accompanied with a more or less extensive agricultural management and remaining relatively ‘unimproved’ in agricultural terms (Hopkins, 2009). However, these systems are of high ecological and socio-economic relevance, as they provide several ecosystem functions, such as biomass production, carbon sequestration, as well as biodiversity preservation (Schläpfer et al., 1999; Schüpbach et al., 2004; Wrage et al., 2011). They have established under long-time extensive management forms such as grazing with low stocking rates or mowing with a low cutting frequency, which are accompanied by low manure application rates or even no nutrient return (Isselstein et al., 2005). Over the last decades, these ecosystems are increasingly threatened by agricultural

intensification, abandonment or afforestation (Poschlod et al., 2005; Beilin et al., 2014). To ensure their preservation, remaining areas were assigned to the Habitats Directive (Council Directive 92/43/EEC), which includes a network of protected sites across Europe (NATURA 2000) and a strict system of species protection. For preservation of the characteristic plant species composition, the former extensive management has to be continued or reintroduced (Drobnik et al., 2011). Unfortunately, nowadays such management forms are hardly profitable to farmers (Strijker, 2005), as they result in lower biomass yields and low forage quality because first cutting of extensive meadows is often conducted late resulting in low crude protein and high crude fibre concentration (White et al., 2004; De Cauwer et al., 2005).

In order to provide an approach for profitable utilization of the remaining semi-natural grassland material, Wachendorf et al. (2009) suggested a new concept for bioenergy production of heterogeneous and senescent plant biomass. The integrated generation of solid fuel and biogas from biomass (IFBB)-concept overcomes the given restrictions for bioenergy production from these biomasses, such as low methane yields (Prochnow et al., 2009a) and high mineral contents (Prochnow et al., 2009b), by generating a solid and a liquid material pathway (Richter et al., 2009; Richter et al., 2010; Richter et al., 2011). The remaining biogas residue (hereinafter referred as digestate) from the liquid pathway, can be applied to grasslands (Hensgen et al., 2012). However, the effect of this digestate on the grassland plant and soil community under natural conditions is yet to be investigated (Andruschkewitsch et al., 2013).

Semi-natural grasslands frequently exist on infertile soils and their characteristic plant community composition is sensitive to nutrient application (Čámská and Skálová, 2012). Therefore, fertilization of conservation areas e.g. NATURA 2000 grasslands is restricted or completely prohibited. Nevertheless, maintenance of these ecosystems may require the return of nutrients (Čámská and Skálová, 2012), because the development of the vegetation was associated with occasional organic fertilizer application. Further, a moderate return of nutrients would increase biomass yields, which may enhance profitability of grassland farming. Due to different grassland types and site characteristics, N application rates tolerated by the plant community widely range from 4-60 kg N ha^{-1} a^{-1} (Briemle, 1997; Kirkham et al., 2008; Čámská and Skálová, 2012; Samuil et al., 2013). The N returned with the IFBB digestate is 19 to 60% of the harvested amount, and therefore lower as in mulched systems

because N is partly transferred into to the solid fraction, which finally constitutes a solid fuel for combustion (Hensgen et al., 2012).

Mulching, i.e. cutting plant biomass and leaving it on the site, is frequently applied in temperate grassland conservation because it is easier to implement as harvesting or grazing. However, mulching is not profitable to farmers (Blumenstein et al., 2012) and the absence of nutrient removal is occasional associated with undesired ecological effects such as eutrophication (Laser, 2002; Briemle, 2005) and formation of a less decomposable biomass layer (Uhlířova et al., 2005). This may result in plant community changes and loss of plant species diversity.

On the other hand a modification of the former grassland management by mulching or fertilizer application results in alteration of abiotic and biotic soil properties (Brodie et al., 2003; Liliensiek et al., 2012) and might therefore also alter soil microbial functioning. Soil microbial communities are major drivers of most grassland ecosystem functions and highly linked to plant community (Bardgett et al., 1999; Dijkstra et al., 2006) with higher microbial diversity under unimproved and species diverse grassland compared to improved and less diverse grassland (Loranger-Merciris et al., 2006; Millard and Singh, 2010). However, belowground ecosystem components are often neglected in conservation research. For arable soils, it was shown that digestates increased soil microbial biomass and activity (Terhoeven-Urselmans et al., 2006; Odlare et al., 2008), whereas effects on grassland soils are rarely investigated (Andruschkewitsch et al., 2013). Particularly effects of application of digestate and mulching on both, the plant biomass and diversity, as well as soil functional diversity, have not simultaneously been investigated before.

A study was conducted on five different NATURA 2000 grassland sites to elucidate the effects of IFBB digestate on plants and soil microbial community. The treatments comprised harvesting with and without digestate application and a mulching treatment. The harvest treatment without digestate application represents the actual conservation management and the treatment with digestate with a return of 50% of the harvested N is based on the data of Hensgen et al. (2012), who showed a maximum transfer of 50-60% of N from European semi-natural grassland biomasses into the liquid phase, converted after fermentation to digestate. In consideration that variation in soil microbial community occur during the growing season (Brimecombe et al., 2007; Habekost et al., 2008; Andruschkewitsch et al., 2014), the analysis was based on multiple sampling dates.

We hypothesized that, due to greater net nutrient outputs, IFBB digestate application has minor effects relatively to mulching on (1) aboveground plant dry matter yield, plant functional group composition and diversity and on (2) soil microbial carbon substrate utilization pattern and catabolic diversity of semi-natural, extensive managed meadows. Furthermore, we hypothesized that (3) digestate effects are strongest shortly (14 days) after application.

5.2 Material and Methods

5.2.1 Study sites

The experimental sites were part of the EU project PROGRASS (www.prograss.eu) and were located at the lower mountain region Vogelsberg, Middle Germany. Five experimental sites (I-V) with different NATURA 2000 habitat type were chosen to represent typical vegetation communities of conservation grasslands of a lower mountain range of a temperate region (Table 9). Over the past decades, the sites were managed extensively by mowing with a low cutting frequency one or two times per year and without fertilizer application. This resulted in agricultural unimproved grassland sites of high species richness but relatively low biomass productivity. The local climate is characterized as temperate with a long-time (1961-1990) average temperature varying with the altitude, 8.0°C (454 m a.s.l.) to 6.7°C, (606 m a.s.l.). The average annual precipitation was between 923 and 1211 mm, respectively (PIK, 2009). Soil types were classified according to WRB (IUSS, 2007) and were mainly Cambisols (site I, III and IV), from volcanic bedrock, or Stagnosols (site II and V). Soil texture in the upper soil layer was silty loam and the pH ($CaCl_2$) ranged from 4.3 (sites II and IV) to 5.2 (site I) (Hensgen et al., 2012).

5.2.2 Experimental setup and sampling

At each site, an experimental area of 700 m² was selected, where plant community was characteristic for the NATURA 2000 habitat type and homogenously distributed. In 2009, three treatments (100 m²) were established at each site: a control representing the actual conservation treatment harvesting without nutrient application (H-N), IFBB technique implementation including harvesting and digestate application (H+N) and a mulching treatment including cutting and spreading chopped biomass (M). H-N and H+N were established in triplicates and M with one repetition. Aboveground biomass was harvested and mulched two times per year during the first two weeks of July and September. The

species-rich *Nardus* grassland site IV was harvested only in July because of its low biomass production. Harvest frequency and time was chosen according to the usual located harvesting regime. Annual dry matter (DM) yield of the plots was carried out by mowing 5 m^2 with a finger-bar mower at a height of 5 cm and subsequent drying the fresh biomass at 105°C for 3 days.

The nutrient recirculation on H+N plots started in 2010. It was based on recycling 50% of N removed by the harvest, leading to variable P and K application rates (Table 9). The IFBB digestate was simulated by pressing a conventional biogas digestate (swine slurry and maize silage as co-ferment) with a screw press (AV, Anhydro, Kassel, Germany) as original material from the IFBB prototype plant was not available. The separated digestate was analyzed for N, P, K and applied uniformly with watering cans once per year in the first week of May.

At each single plot of the five investigated sites, 7 subplots (diameter 20 cm) were marked along a transect (14.1 m). Soil was sampled and plant functional group was determined in the subplots at three consecutive dates in 2012: directly before digestate application (day 0), day 14, and day 56 after digestate application, resulting in 147 samples per site. At the first two sampling dates, plant functional group composition at each subplot was determined by estimating visually the DM proportion of the plant functional groups, i.e. graminoids including sedges and rushes, forbs and legumes in compliance with proceeding outlined by Davies et al., 1993). Estimation was conducted always by the same person and its accuracy was checked at the last sampling date 56 after digestate application, based on the weighted proportion of dried subsamples of plant functional groups ($r = 0.95$, $P<0.001$). Plant functional group composition was measured by cutting at 5 cm height, manual fractionation and drying at 105°C for 3 days. To describe the plant functional group diversity, the Shannon evenness index (E_{plant}) was calculated by the formula $E_{plant} = -\sum(pi \ln(pi)) / \ln(k)$, where pi was the DM proportion of the plant functional group to the total DM yield and k the number of functional groups. The evenness index ranges from 0 (total dominance of one functional group) to 1 (all occurring functional groups have the same proportion to the DM yield). We rather used the plant functional group evenness based on the group proportion to the aboveground plant DM yield than the usually used plant functional group richness (Zhang et al., 2011; Khalsa et al., 2012). As the richness does not provide information about the yield and relative distribution of the groups, the evenness index provides more relevant information for soil-plant interactions by giving the equitability of functional group yields.

Table 9: Botanical characteristics and nutrient inputs of the H+N treatment at the study sites. Arithmetic means and standard deviations of the means in brackets; H-N = harvest without nutrient return (n=3), H+N = harvest with nutrient return via digestate (n=3) and M = mulching (n=1).

Site nr.	NATURA 2000 habitat type	Altitude m a.s.l.	Treatment	Plant species		Nutrient input H+N [b]		
				Total number[a]	Dominant species[a]	kg N ha^{-1} a^{-1}	kg P ha^{-1} a^{-1}	kg K ha^{-1} a^{-1}
I	Lowland hay meadow (6510)	570	H-N H+N M	43 (1.0) 42 (5.1) 35 (-)	*Festuca rubra, Trifolium pratense, Plantago lanceolata, Holcus lanatus*	35 (4)	3 (0)	28 (3)
II	Lowland hay meadow (6510)	420	H-N H+N M	44 (2.3) 44 (1.5) 47 (-)	*Festuca rubra, Agrostis capillaries, Plantago lanceolata, Trifolium pratense*	47 (9)	4 (1)	37 (7)
III	Mountain hay meadow (6520)	580	H-N H+N M	48 (4.4) 54 (7.0) 48 (-)	*Festuca rubra, Sanguisorba officinalis, Agrostis capillaris, Plantago lanceolata*	32 (8)	3 (1)	25 (6)
IV	Species-rich *Nardus* grasslands (6230)	580	H-N H+N M	37 (2.9 34 (1.0) 32 (-)	*Festuca rubra, Agrostis capillaries, Nardus stricta, Plantago lanceolata*	30 (6)	3 (0)	24 (5)
V	*Molinia* meadow (6410)	500	H-N H+N M	42 (1.5) 44 (2.3) 38 (-)	*Juncus acutiflorus, Festuca rubra, Holcus lanatus, Valeriana dioica*	46 (10)	4 (1)	37 (8)

[a] Plant species data collected in June and July 2011 as described by Hensgen et al. (2012), mosses are excluded

[b] Mean of 2010-2012

Soil samples were taken with an auger of a diameter of 2 cm within the subplots at each date at 0-10 cm depth, sieved < 2 mm and stored at 4°C. Some of samples of site IV and most samples of site V, which were too wet, were carefully dried before sieving at room temperature. Soil pH-CaCl2 (1:2.5 w/w) was measured on composite samples of the moist soil of the seven subplots for each sampling date. Soil organic C (SOC) and total N were determined on composite samples of dried soil (60°C) for each subplot with an elemental analyser (Vario MAX CHN, Elementar, Hanau, Germany) at the latest sampling date.

5.2.3 Soil microbial substrate utilization pattern

Substrate utilization patterns were determined according to the multi-SIR approach using MicroResp™ method after Campbell et al. (2003). Into each 1.1 ml deep-well microtitre plate (Nunc, Thermo electron LED, Langenselbold, Germany); soil was placed volumetrically by a filling-devise (MicroResp™) as described in Campbell et al. (2003). The water content was adjusted to 50-70% of the water holding capacity (WHC) with demineralised water. The wells were pre-incubated for 7 days at 25°C in the dark and seedlings germinated during incubation were removed. The respiration responses were detected by applying aqueous solutions of different carbons substrates into individual chambers of the well and sealing the well with a colorimetric CO_2 trap accordingly to Campbell et al. (2003).

The carbon substrate utilization patterns were determined by applying four carbohydrates (D-glucose, D-fructose, D-trehalose, L-arabinose), four carboxylic acids (α-ketoglutaric acid, citric acid, malic acid, oxalic acid), eight amino acids (arginine, D-glucosamine, DL-aspartic acid, L-alanine, L-glutamine, L-leucine, lysine, γ-aminobutyric acid) and one phenolic acid (protocatechuic acid) into individual chambers of the well. These substrates were chosen according to their ecological relevance, as most of them are reported as rhizosphere carbon sources (Campbell et al., 1997). The substrates glucosamine, trehalose and α-ketoglutaric acid were identified as key discriminators in studies comparing different ecosystems or management treatments (Campbell et al., 1997; Stevenson et al., 2004; Lalor et al., 2007; Romaniuk et al., 2011). The basal respiration was determined by adding demineralised water in one chamber of the well. The analysis was conducted for the most substrates with 22 mg g^{-1} soil water. Because of its low solubility, L-leucine and L-glutamine were applied with 6 mg g^{-1} soil water and protocatechuic acid and aspartic acid with 2 mg g^{-1}. The colour development of the CO_2 trap was measured at 572 nm (FLUORstar, BMG, Offenburg, Germany) immediately before sealing the well and 4 h after incubation (25°C) in the dark. For

determining the basal respiration a power function was used resulting from a calibration of five different soils according to Sradnick et al. (2013):

µl CO2 = 63 x (0.1 + ABS)3, r = 0.98

where ABS is the difference in absorption of T1 and T0. To describe the soil microbial functional diversity, the evenness index (E_{soil}), was calculated by the formula $E_{soil} = -\sum(pi\ ln(pi)) / ln(k)$, where pi was the proportion of the individual substrate respiration to the total substrate respiration and k the number of substrates according to Zak et al. (1994). It is important to note, that carbon substrate utilization patterns are only indicators of functional diversity based on the ability of the soil microbial community to utilize a selected range of substrates.

5.2.4 Statistical analyses

We used two-way analysis of variance (ANOVA) on DM yield, SOC, total N (TN) and C/N, using site and treatment (H+N and H-N) as main factors. Because of the single repetition of the mulching, this treatment was not included in statistical analyses to avoid an unbalanced statistical design. However, descriptive results were included in the discussion, giving suggestions for further experiments and management applications. We used three-way ANOVA on soil pH, basal respiration and the microbial functional evenness indices with site, treatment and sampling date as independent factors. Sampling date was treated as independent factor, as for each sampling date soil was destructively sampled within the subplot. When interactions were detected, simple effects ANOVAs were conducted to receive detailed information. All ANOVAs were conducted with the pooled values of the seven subplots per plot (arithmetic mean). To identify site effects on plant functional groups and their evenness (E_{plant}), we conducted a repeated measure analysis with multilevel linear model approach, using site and treatment as main factors and sampling date as random factor (Field et al., 2012), on the pooled data of the seven subplots per plot (arithmetic mean). The analyses started with creating a baseline model without any predictor other than the intercept, after that main effect models were created. The analysis ended up with an interaction model including all main effects and their interaction.

The respiration rates of the substrates were standardized by dividing each substrate respiration by the mean of the 17 substrates to receive a relative measure of its contribution to the mean substrate utilization pattern. Multivariate outliers of these patterns, comprising only 0.01% of the data, were removed from the dataset according to Field et al. (2012). To

investigate the effects of site, sampling date and treatment on the substrate utilization patterns, MANOVA was conducted on the unstandardized data. Significant main effects and three-way interaction identified by MANOVA were followed up by discriminant function analyses (DFA) with Statistica. To describe the discrimination, the substrate specific respiration was correlated to the canonical scores of the significant discriminant functions (DFs). Pearson correlation coefficients were used to express the significance.

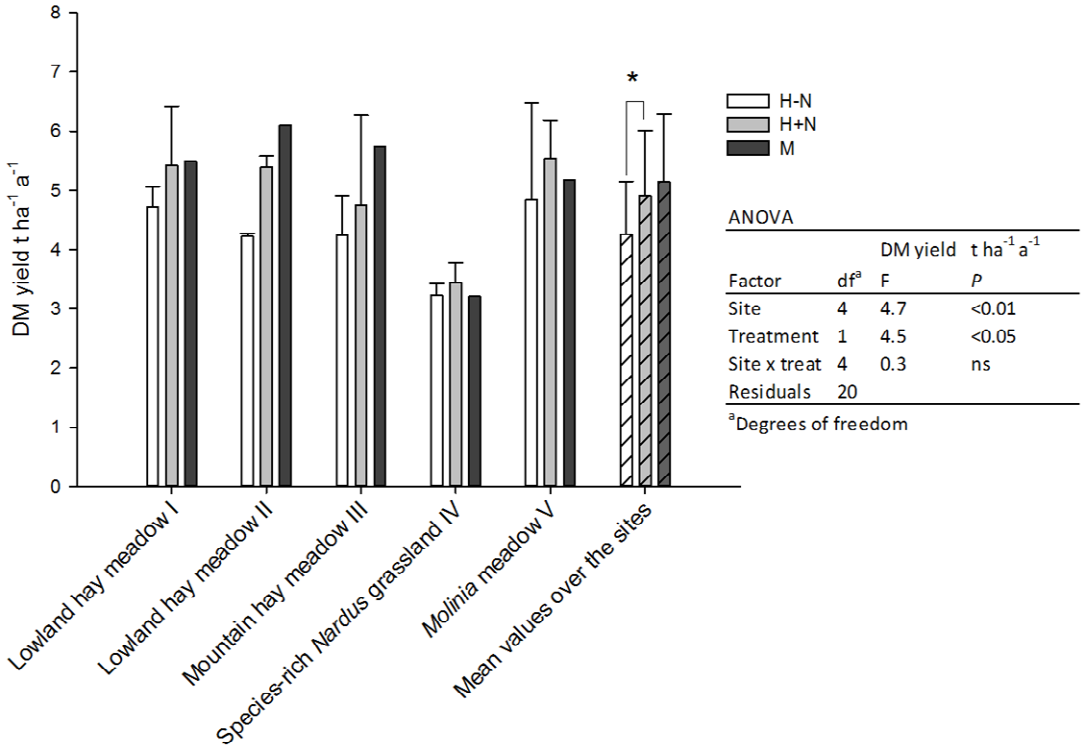
Analyses were performed with R, if not stated otherwise. Statistical treatment comparisons were conducted for H-N and H+N, values for M were represented graphically due to the single repetition. The significance level was set at $P < 0.05$.

5.3 Results

5.3.1 Aboveground plant dry matter yield, functional group composition and diversity

DM yields of the sites varied in a typical range for extensive grasslands from 3.2 to 6.1 t ha^{-1} a^{-1} (Fig. 10). On average over all sites the treatments H+N and M increased the annual DM yield by 14 and 19%, respectively, compared with H-N. Thereby the highest increase was observed at the Lowland hay meadow II, which received the highest nutrient amounts.

The mean values of plant functional group diversity measured by the evenness index (E_{plant}) ranged from 0.20 to 0.75 with significant differences for the sites but not for the treatments H-N and H+N (Table 10). The Lowland and Mountain hay meadows showed highest values for E_{plant} (sites I-III), while the species-rich *Nardus* grassland (site IV) showed lowest values (Table 10), because of its high graminoid proportion of 95% of the DM yield. M treatment tended to affect plant functional group composition and plant functional group diversity of the sites. On site I, mulching led to decreased graminoid and legume proportions in comparison with H-N, while forb proportion was increased. Because of higher legume and forb proportions on the M plot at site IV, E_{plant} was increased. On the opposite M decreased E_{plant} at site V.



ANOVA

		DM yield	t ha^{-1} a^{-1}
Factor	df[a]	F	*P*
Site	4	4.7	<0.01
Treatment	1	4.5	<0.05
Site x treat	4	0.3	ns
Residuals	20		

[a]Degrees of freedom

Fig. 10: DM yields of five grassland sites and their means after harvest without nutrient return (H-N), harvest with nutrient return (H+N) and mulching (M). ANOVA results of the DM yields with the factors grassland site (I-V) and treatment (H-N and H+N). Mulching (M) was not statistically evaluated. Error bars indicate standard deviations of the means.

Table 10: Plant functional proportion of the DM yield and evenness index of plant functional group diversity of the sites (I-V) for the treatments averaged over the sampling dates. Standard deviations of the means are given in brackets; H-N = harvest without nutrient return, H+N = harvest with nutrient return and M =mowing and spreading chopped biomass. Summary of multilevel linear model analyses with the treatments H–N and H+N.

		Graminoids %			Forbs %			Legumes %			E_{plant}		
Site number /treatment		H-N n=9	H+N n=9	M n=3	H-N n=9	H+N n=9	M n=3	H-N n=9	H+N n=9	M n=3	H-N n=9	H+N n=9	M n=3
I		65.6 (8.6)	63.9 (10.7)	58.1 (3.0)	24.9 (5.7)	25.7 (10.8)	37.1 (2.6)	9.6 (4.1)	10.3 (4.8)	4.8 (2.2)	0.70 (0.08)	0.66 (0.06)	0.68 (0.03)
II		64.3 (5.0)	70.5 (7.3)	69.9 (4.7)	29.0 (5.1)	21.8 (6.4)	27.6 (5.4)	6.7 (2.0)	7.7 (3.3)	2.5 (1.7)	0.68 (0.06)	0.64 (0.09)	0.60 (0.04)
III		62.9 (14.4)	62.8 (5.1)	63.4 (5.1)	35.7 (13.5)	34.9 (5.0)	34.9 (4.0)	1.4 (1.4)	2.3 (0.9)	1.6 (1.1)	0.70 (0.06)	0.70 (0.06)	0.75 (0.04)
IV		95.4 (2.3)	93.6 (6.7)	87.6 (9.0)	2.4 (1.8)	2.8 (5.5)	4.2 (2.3)	2.2 (1.4)	3.7 (3.6)	8.2 (6.8)	0.20 (0.08)	0.20 (0.13)	0.37 (0.18)
V		74.6 (10.6)	76.7 (10.1)	92.2 (0.4)	23.7 (11.0)	22.2 (10.4)	7.5 (0.7)	1.6 (1.1)	1.2 (0.6)	0.3 (0.3)	0.48 (0.12)	0.47 (0.10)	0.31 (0.06)
	df[a]	L.Ratio	*P*		L.Ratio	*P*		L.Ratio	*P*		L.Ratio	*P*	
Baseline Model	5												
Site Model	9	39.1	<0.001		38.9	<0.001		25.9	<0.001		50.7	<0.001	
Treatment Model	10	0.3	ns		1.0	ns		2.5	ns		1.4	ns	
Interaction Model	14	2.9	ns		3.0	ns		1.9	ns		1.5	ns	

[a] Degrees of freedom

5.3.2 Soil chemical parameters, substrate utilization pattern and functional diversity

Soil pH, SOC, TN and C/N differed significantly between the sites and were not affected by any treatment (Tables 11 and 12). However, soil pH was marginally affected by sampling date (Table 11). The mean basal respiration ranged from 1.40 to 2.87 µg CO_2-C g^{-1} h^{-1}, with significant differences between the sites (Table 11). Basal respiration and SOC content were positively correlated ($r = 0.49$, $P<0.001$). No differences were observed for the treatments H+N and H-N (Table 11). However, a site × sampling date interaction was detected, caused by a significant increase in basal respiration until day 56 after digestate application for site II. The mulching treatment did not lead to differences in basal respiration (Fig. 11). No differences in standardized utilization of single substrates or substrate groups could be detected for the treatments, averaging sites and sampling dates (Fig. 11). The contribution of substrate groups to the mean respiration decreased in the order carboxylic acids > carbohydrates > protocatechuic acid > amino acids.

Table 11: Basal respiration and soil pH of the study sites from 0-10 cm depth for the three treatments and averaged over the sampling dates. Arithmetic means and standard deviations of the means; H-N = harvest without nutrient return, H+N = harvest with nutrient return and M = mulching. Summary of ANOVA results with the factors site (I-V), treatment (H–N and H+N) and sampling date (0, 14 and 56 days after digestate application) and their interactions.

		Basal respiration µg CO_2-C g^{-1} h^{-1}			pH -		
Site number /treatment		H-N n=9	H+N n=9	M n=3	H-N n=9	H+N n=9	M n=3
I		2.03 (0.59)	1.88 (0.30)	2.14 (0.38)	5.19 (0.06)	5.19 (0.11)	5.28 (0.04)
II		1.53 (0.19)	1.40 (0.44)	1.40 (0.15)	4.38 (0.04)	4.39 (0.08)	4.36 (0.06)
III		2.34 (0.46)	2.34 (0.55)	2.87 (0.40)	4.86 (0.05)	4.84 (0.15)	4.92 (0.07)
IV		2.04 (0.27)	2.13 (0.31)	1.76 (0.38)	4.29 (0.05)	4.36 (0.13)	4.36 (0.02)
V		1.93 (0.42)	1.97 (0.17)	1.81 (0.25)	4.61 (0.10)	4.64 (0.11)	4.79 (0.14)
Factor	df[a]	F	*P*		F	*P*	
Site	4	16.2	<0.001		227.9	<0.001	
Treatment	1	0.2	ns		0.6	ns	
Sampling date	2	0.0	ns		3.3	<0.05	
Site x treat	4	0.4	ns		0.6	ns	
Site x date	8	3.7	<0.01		0.3	ns	
Treat x date	2	1.9	ns		0.8	ns	
Site x treat x date	8	2.1	ns		0.1	ns	
Residuals	60						

[a] Degrees of freedom

Table 12: Soil parameters SOC, TN and C/N assessed at sampling date 56 days after digestate application from 0-10 cm depth for the three treatments. Arithmetic means and standard deviations of the means; H-N = harvest without nutrient return, H+N = harvest with nutrient return and M = mulching. Summary of ANOVA results with the factors site (I-V), treatment (H–N and H+N) and sampling date (0, 14 and 56 days after digestate application) and their interactions.

		SOC			TN			C/N		
		mg g^{-1}			mg g^{-1}			-		
Site number /treatment		H-N n=3	H+N n=3	M n=1	H-N n=3	H+N n=3	M n=1	H-N n=3	H+N n=3	M n=1
I		45.50 (5.62)	42.74 (0.90)	48.13 (-)	4.47 (0.63)	4.16 (0.04)	4.54 (-)	10.18 (0.20)	10.27 (0.16)	10.60 (-)
II		44.04 (5.51)	41.10 (1.28)	44.21 (-)	4.19 (0.60)	3.97 (0.21)	4.33 (-)	10.52 (0.24)	10.36 (0.26)	10.20 (-)
III		59.36 (2.70)	58.18 (10.71)	59.45 (-)	5.56 (0.16)	5.48 (1.08)	5.56 (-)	10.67 (0.19)	10.63 (0.17)	10.68 (-)
IV		65.42 (3.18)	64.59 (9.22)	57.42 (-)	5.45 (0.31)	5.43 (0.76)	4.56 (-)	12.01 (0.30)	11.91 (0.52)	12.59 (-)
V		47.90 (9.34)	46.78 (6.42)	38.77 (-)	4.45 (0.93)	4.35 (0.64)	3.38 (-)	10.81 (0.44)	10.77 (0.13)	11.48 (-)
Factor	df[a]	F	*P*		F	*P*		F	*P*	
Site	4	14.1	<0.001		6.9	<0.01		33.4	<0.001	
Treatment	1	0.6	ns		0.4	ns		0.2	ns	
Site x treat	4	0.0	ns		0.1	ns		0.1	ns	
Residuals	20									

[a] Degrees of freedom

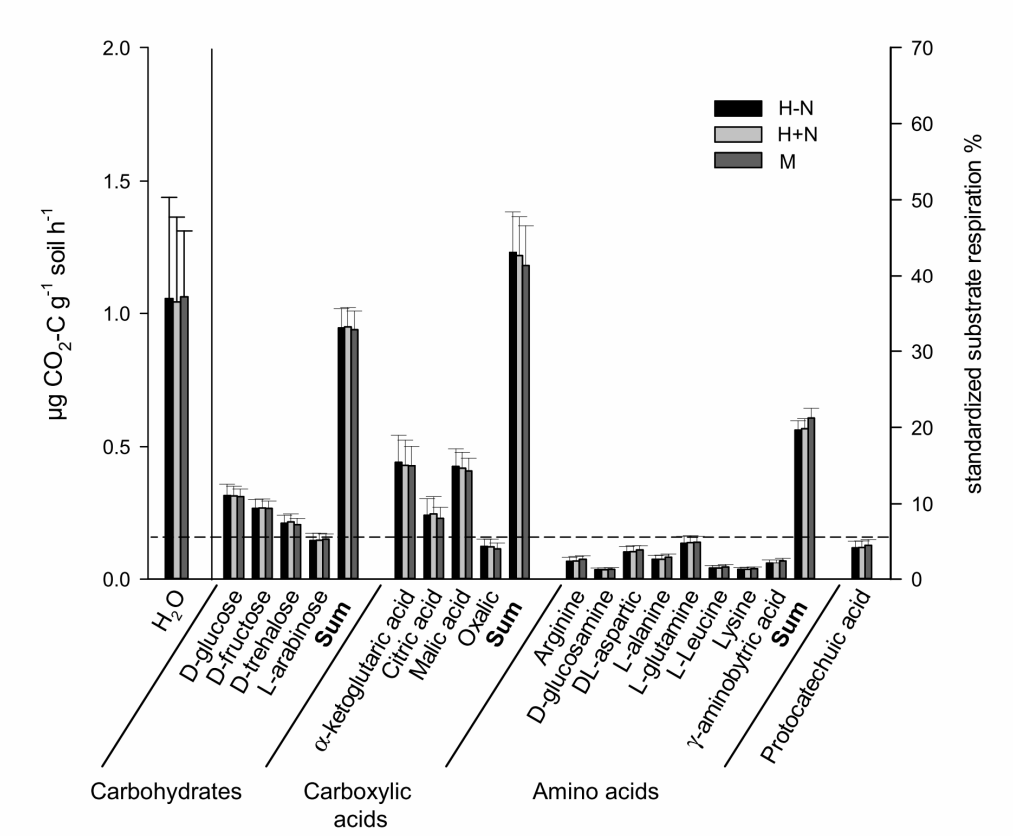


Fig. 11: Basal respiration rate (H_2O) and individual standardized substrate utilization as proportion of the total standardized substrate utilization and the substrate group sums for the five grassland sites depending on the treatment summarized for the entire sampling period. The dashed line indicates the overall mean of the proportional substrate respiration. Error bars indicate standard deviations of the means; H-N = harvest without nutrient return, H+N = harvest with nutrient return and M = mulching.

There were significant main effects identified by MANOVA for site and sampling date and a site × treatment × sampling date interaction for the unstandardized substrate utilization pattern (Table 13). All of the sites significantly differ in their substrate utilization pattern identified by discriminant function analyses (Wilks Lamda: 0.015, approx. F = 65.8, $P < 0.001$). Day 0 and day 14 samples differed from the day 56 samples (Wilks Lamda: 0.791, approx. F = 4.41, P < 0.001). Discriminant function analyses on treatment effects conducted for each site and sampling date individually, revealed that H+N significantly differed from H-N at all sampling dates at site I, while differences for site II and IV occurred at day 0 and for site III at day 14 (Table 14). Site V showed no differences in substrate utilization pattern between the treatments. Correlation analyses of the DF1 of the significant DFAs for treatment effects revealed that the most powerful key discriminatory substrates were associated to the amino acid group (Fig. 12). However, dependent on sampling date and

study site, the higher utilization of amino acids was attributed to H-N or H+N. The M treatment tended to increase the utilization of amino acids over all grassland sites (Fig. 11).

Table 13: Summary of MANOVA results for unstandardized substrate induced respiration pattern (µg CO_2-C g^{-1} h^{-1}) of the 17 substrates with the factors site (I-V), treatment (H-N and H+N) and sampling date (0, 14 and 56 days after digestate application) and their interactions.

Factor	df[a]	F	*P*
Site	4	2316	<0.001
Treatment	1	576	ns
Sampling date	2	1154	<0.001
Site x treat	4	2316	<0.001
Site x date	8	4664	<0.001
Treat x date	2	1154	<0.001
Site x treat x date	8	4664	<0.001
Residuals	592		

[a] Degrees of freedom

Table 14: Discriminant function analysis of the substrate induced respiration pattern by the treatments H-N and H+N separately for each grassland site (I-V) and sampling dates (0, 14 and 56 days after digestate application).

	I		II		III		IV		V	
Days after application	Squared distance	*P*	Squared distance	*P*	Squared distance	*P*	Squared distance	*P*	Squared distance	*P*
0	11.52	<0.01	17.28	<0.001	2.60	ns	7.53	<0.05	4.51	ns
14	19.86	<0.001	2.59	ns	6.39	<0.05	5.66	ns	2.26	ns
56	9.72	<0.01	4.41	ns	5.02	ns	3.01	ns	3.86	ns

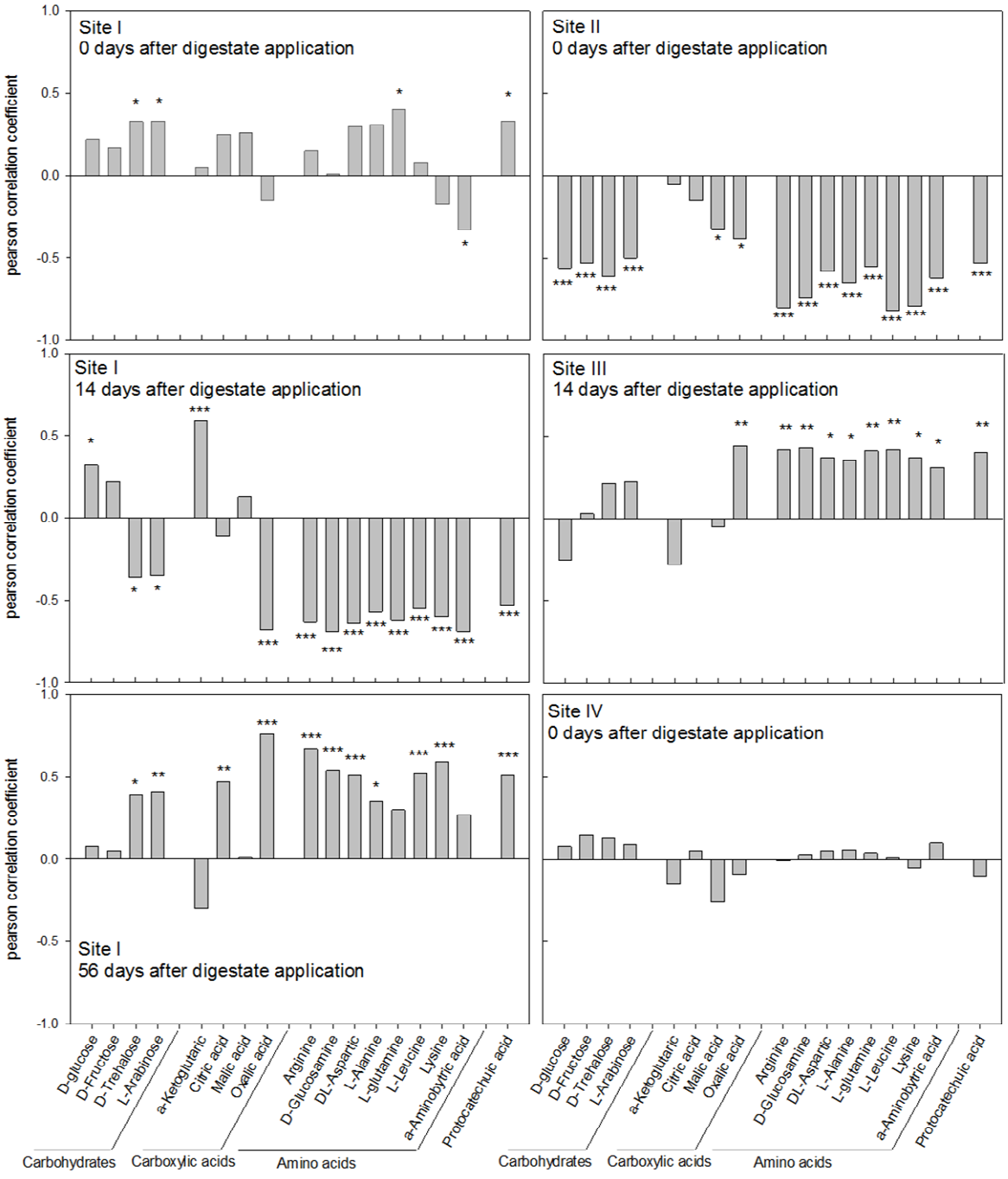


Fig. 12: Pearson correlation coefficient between substrate utilization of individual substrates and the significant discriminating canonical discriminate function (DF) 1 of the treatments (H-N and H+N) identified by discriminant function analyses (DFAs; see table 14). Negative correlation coefficients are associated with higher substrate utilization for H-N, positive correlations with higher substrate utilization for H+N. Asterisks indicate significant correlation.

The microbial functional diversity measured by evenness (E_{soil}) describes the uniformity of the utilization of carbon substrates by the soil microbial community and ranged from 0.82 to 0.94, and the site-specific average increased in the order II < IV < V < I < III (Fig. 13). Significant site × treatment × sampling date interaction were caused by a lower E_{soil} for treatment H+N for site I and a higher E_{soil} for site III in comparison with H-N at day 14. The effect of the M treatment on E_{soil} varied considerably between the sampling dates for the single grassland sites, however, on average the M treatment tended not to differ from the other treatments.

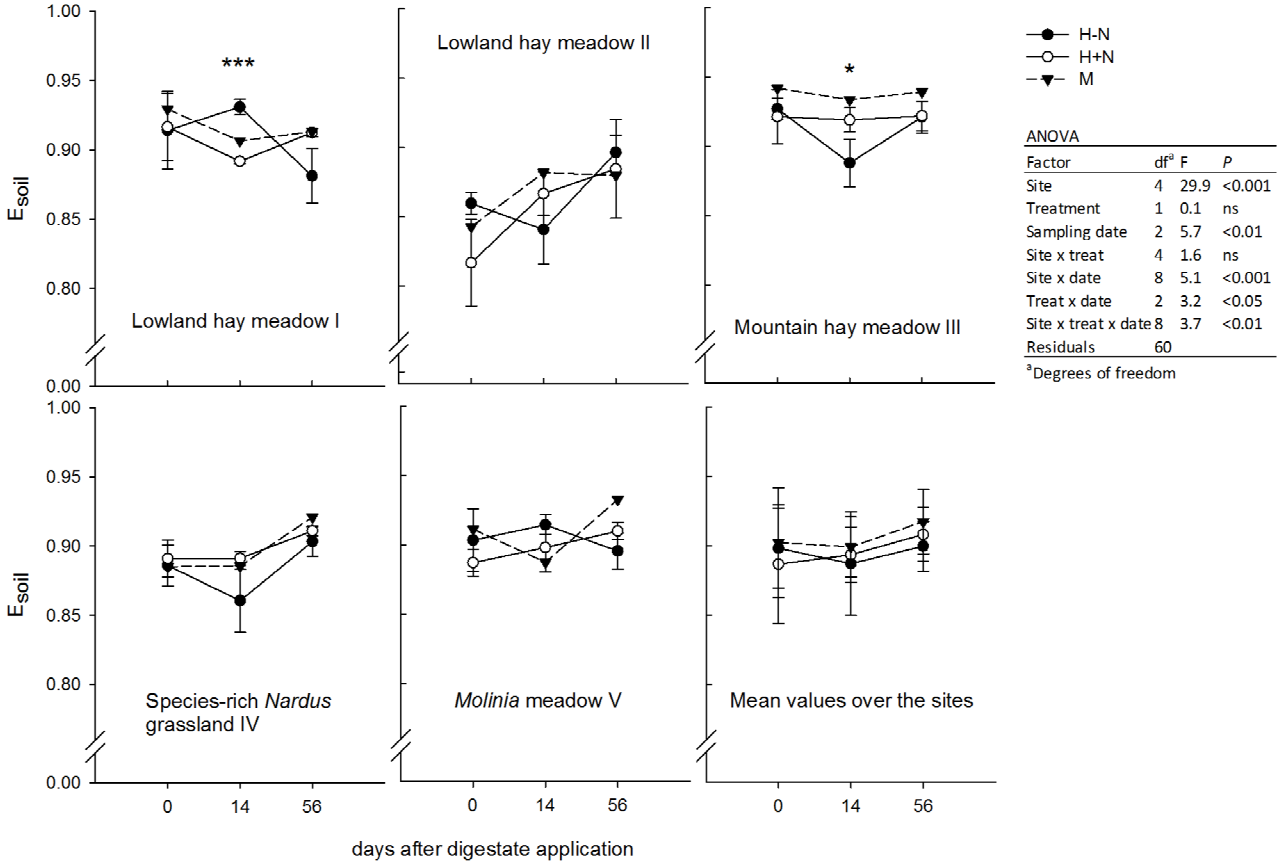


Fig. 13: The evenness indices of soil microbial functional diversity (E_{soil}) of five grassland sites depending on treatment and sampling date and the mean values over all sites. Error bars indicate standard deviations of the means. High evenness values indicate little variation in catabolism of substrates, whereas low evenness values indicate large variation in catabolism of substrates. Asterisks indicate significant differences between harvest without nutrient return (H-N) and harvest with nutrient return (H+N) within one sampling date identified by simple effects ANOVAs. Mulching (M, dashed line) was not statistically evaluated.

5.4 Discussion

5.4.1 Response of aboveground plant parameters to applied treatments

Nutrient return with the IFBB digestate (H+N) and mulching (M) resulted on average in higher aboveground biomass productivity in comparison with the actual harvesting without nutrient return (H-N). This is in accordance to studies reporting increases in semi-natural grassland productivity after mulching (Mašková et al., 2009; Doležal et al., 2011) and nutrient supply (Briemle, 1997; Lepš, 2004). We showed that both alternative treatments (H+N and M) have the potential to alter the biomass production of semi-natural grasslands within three years of application, with mulching tending to increase the biomass production stronger than digestate application. In consequence, we suggest that IFBB-concept implementation is of potentially lower ecological impact than mulching, which might promote eutrophication, nitrate leaching and gaseous emissions (Larsson et al., 1998; Ruhe et al., 2001). However, this might not be the case for nutrient-poor grassland types with a low biomass yield potential (about 3 t^{-1} DM ha^{-1} a^{-1}). Briemle (1997) reported for *Mesobrometum* grassland after 10 years of mulching lower biomass yields compared with mowing and NPK application. In our study, mulching did not increase biomass production of the nutrient-poor species-rich *Nardus* grassland compared with harvesting without nutrient application.

Despite the significant changes in aboveground DM production, there was no effect of H+N on the composition of plant functional groups and their evenness, indicating no changes in plant competition at the functional group level after three years. In contrast, mulching resulted in contradictory results concerning plant functional group composition and evenness (site I, IV and V). This is in coincidence with Kahmen and Poschlod (2008), which investigated the response of plant functional traits (e.g. life from, plant height) to management treatments. They stated to the limitations in finding plant functional responses among treatments over a broad range of grassland types. However, studies focussing on a single grassland type (*Arrhenaterion*), have clearly pointed to the sensitiveness of plant functional groups to different management treatments. Čámská and Skálová (2012) revealed that nitrophilous species and tall graminoids increased after harvesting and application of 56 kg N ha^{-1} a^{-1} over a period of seven years. Laser (2002) reported lower yield proportions of legumes and other dicotyledonous species of higher light requirement after mulching due to litter accumulation and reduced light intensity in the sward. With respect to the measured aboveground plant functional group three-year response to mulching treatment, the results of

our study coincide with other studies, observing that changes in plant species composition and diversity may occur within 2 to 5 years of mulching or nutrient application (Jacquemyn et al., 2002; Laser, 2002; Gaisler et al., 2013). On the other hand, Vanderpoorten et al. (2004) noted that it might take decades of changes in mowing regimes to appear in plant community shifts. Thus, we cannot exclude that in case of H+N shifts in plant functional group composition and evenness may occur after a longer application period.

In this study, shifts in plant functional group composition and evenness were detectable only for mulching compared with mowing, indicating possible changes in ecosystem conditions. Mulching is generally known to be especially problematic for grassland types with high aboveground productivity e.g. wetlands (Laser, 2002), where residual biomass increases nutrient availability and support growth of nitrophilous species (Gaisler et al., 2013). Negative effects on community composition and diversity depend on litter turnover, which may take four weeks (Moog et al., 2002) up to one growing season (Mašková et al., 2009) according to prevailing climate conditions, biomass quantity and quality. In our study litter accumulation was evident as un-decomposed plant residues were apparent on the mulching plots after one growing season. With respect to plant functional group composition the implementation of the IFBB-concept with digestate application would therefore provide a better alternative to traditional management on semi-natural grassland than mulching. However, an exception might be low productive grasslands of 2 to 4 t DM ha^{-1} a^{-1} (Gaisler et al., 2013), like the nutrient-poor species-rich *Nardus* grassland in this study (site IV), which was even positively influenced in terms of plant functional group evenness by mulching.

5.4.2 Response of microbial and abiotic soil parameters to applied treatments

Basal respiration differed between the grassland sites according to their SOC content, but was not altered by any treatment (Fig. 11). In accordance to this, Bardgett et al. (1999) found in a microcosm experiment that the short-term activity of soil microorganisms in N-limited upland grasslands was more regulated by plant species traits than by a direct effect of N application. At the field scale, stronger gradients in management intensity than in our study may be necessary to cause measurable effects. For example, Grayston et al. (2001) observed that the microbial biomass and activity was enhanced in temperate improved grasslands systems compared with un-improved grasslands systems. However, it remains unclear whether phyto-sociological or nutrient status-related factors cause these differences.

Congruent to results from investigations of the H-N treatment plots (Andruschkewitsch et al., 2014), all of the sites significantly differ in their substrate utilization pattern of selected substrates identified by discriminant function analyses. Digestate application did not alter the substrate utilization pattern over the grassland sites and sampling dates but it induced small and transient differences on the site level. Only the Lowland hay meadow (site I) showed differences at all three sampling dates. This suggests that the catabolism of the soil microbial community was persistently influenced by digestate application only at this site. The individual substrates responsible for the digestate treatment discriminations on the site level were more frequently assigned to the amino acid group, which is similar to results found after cattle slurry application to grass-clover and maize monoculture soils (Murugan et al., 2014). Marshall et al. (2011) found that the amino acid induced respiration was decreased in mineral NPK-fertilized grassland soil. They suggested that the reason was an increased utilization of the more easily accessible fertilizer N than the bound amino acid N. However, in our study a lower utilization of amino acids was associated with either the H-N or the H+N treatment, depending on grassland site and sampling date. Mulching tended to increase the utilization of amino acids over the sites, possibly reflecting an increased N-demand of the soil microorganisms caused by increased aboveground biomass production and plant-soil competition for N (Harrison et al., 2008). Therefore, the amino acid catabolism of soil microbial community seems to be most sensitive to changes of grassland management. Analyses of soil inorganic N and dissolved organic N might be useful to further elucidate differences in substrate utilization pattern (Jones et al., 2004).

Previous studies have reported that, depending on application rate and experiment duration, organic fertilizers may reduce the soil microbial catabolic diversity of arable soils in comparison with unfertilized plots (Romaniuk et al., 2011). Nevertheless, overall our results indicate that both management treatments did not result in any distinct and lasting changes in soil catabolic evenness over the entire sampling period. On the site level, however, digestate application and mulching resulted in transient and inconsistent differences in catabolic evenness in comparison with the actual conservation management treatment (H-N) throughout the sampling period. The observed temporal variation in the responses to the management treatments of both, substrate utilization pattern and catabolic evenness of the soil microbial community, could neither be explained by variation in the plant functional group composition and their evenness nor in soil chemical parameters. However, the increase of aboveground plant biomass production by both management treatments was likely accompanied by changes

of the phenological plant development, root/shoot ratio and root system architecture (Fan and Harris, 1996; Bardgett et al., 1999, Cleland et al., 2006; Mašková et al., 2009). For example, the root biomass development of typical grass species from extensive semi-natural grassland systems (*T. flavescens* and *F. rubra rubra*), have been observed to decrease by digestate application in a pot experiment (Andruschkewitsch et al., 2013). In addition, root exudation also depends on active phases of root growth; during which the release of exudates is high (Brimecombe et al., 2007). Both in turn may have resulted in spatial and temporal differences in root exudation between the treatments and thereby altering soil microbial community (Badri and Vivanco, 2009).

Taken together, the results indicate that catabolic response and evenness of soil microbial community is resilient to changes in management treatment and is not strongly linked to aboveground DM yield, but rather depends on temporal shifts in root development and exudation pattern throughout the growing period. These effects are likely to be most evident in ecosystems where the soil catabolic evenness is as high (Degens et al., 2000; Degens et al., 2001) and aboveground biomass is small relative to the root biomass, The latter account for more than 80-90% of plant carbon stocks in grasslands (Jackson et al., 1996). Unfortunately, for reasons of experimental design it was not possible to consider belowground biomass repeatedly over the sampling period. Additional information on the belowground biomass might help to explain plant-soil interactions in response to the management treatments.

5.5 Conclusions

The grassland management treatments harvesting with moderate dose digestate application (H+N) and mulching (M) increased the aboveground plant DM production in comparison with harvesting without nutrient application (H-N) of different NATURA 2000 grasslands. Further H+N did not lead to shifts in plant functional group composition (graminoids, forbs, legumes) and evenness, whereas M tended to show contrasting responses depending on the grassland type. We found that the H+N treatment induced only small and transient shifts of the microbial substrate utilization pattern, whereas tendencies for an increased utilization of amino acids were observed for M. Digestate application and mulching did not lead to consistent temporal effects or lasting changes on soil catabolic evenness, which indicates a high functional resilience of the grassland soil community. The soil chemical parameters SOC, total N, C/N and soil pH value were not affected by any treatment.

The implementation of the IFBB-concept with recycling 50% of the harvested N by digestate application may serve as an alternative conservation management to harvesting without nutrient return. It keeps semi-natural grassland meadows under profitable agricultural management by enabling bioenergy production from the harvested material and thereby having no substantial ecologically negative effects. Mulching should be considered with more caution, due to a stronger impact on aboveground plant community composition (at the plant functional group level) and on soil microbial substrate catabolism. Studies with an extended observation period are necessary to confirm these results for nature conservation purposes in the long term.

6 Synthesis and general conclusions

The evaluation of effects of the IFBB digestate on (1) soils planted with different grass species in comparison to conventional whole crop digestate and mineral N fertilizer and on (2) different semi-natural grasslands increased the understanding of plant and soil microbial response to the implementation of the IFBB concept to grasslands. Based on the research objectives highlighted in Chapter 2, the following synthesis and conclusions can be drawn:

(i) The application of IFBB digestate from separated grass silage (SGD), whole crop digestate (WCD) and mineral N fertilizer (MIN) to pots planted with different grass species (*L. perenne*, *F. rubra rubra* and *T. flavescens*) revealed inconsistent effects for grassland species. In general, harvestable biomass yield was increased by both digestates. However, the increase was higher for the species from cultivated grassland systems with high N status (*L. perenne*) and was modest or, at highest application rates, even negative for the species cultivated at grassland systems with lower N status (*T. flavescens* and *F. rubra rubra*). This was particularly true for the belowground biomass yields. The type of digestate (SGD/WCD) affected the N accumulation in the plant biomass as over all species SGD showed a higher mineral nitrogen use efficiency (NUE_{min}) of harvestable and stubble biomass attributed to higher plant N uptake, lower gaseous N losses and higher N mineralization compared to WCD. This was probably due to better rheological properties and lower C/N_{org} ratio of SGD. Therefore, SGD is suitable as a short-term N fertilizer, which provides the plant with N similar to a mineral N fertilizer. The N immobilization in microbial biomass, measured as MBN, was highly affected by grass species but not by the type of digestate or application rates.

(ii) The five investigated low mountain semi-natural grasslands showed consistently high soil microbial functional diversity (described by evenness of catabolic response to different carbon substrates) and all differed in their response pattern to the selected carbon substrates. The data further suggest that the evenness of aboveground plant functional groups (graminoides, forbs, legumes) and soil microbial functional evenness are not linked to each other. Although the ranges of soil properties were low, abiotic soil factors, especially soil pH, were identified as the main factors influencing the soil substrate utilization pattern and determining soil microbial functional evenness, whereas

plant functional group composition, evenness and aboveground plant dry matter production were less important. However, a single plant functional group may play a key role, as for instance an increasing legume proportion consistently decreased soil microbial functional evenness. Furthermore, soil microbial functional evenness depends on the sampling date, probably driven by temporal changes in source availability.

(iii) The three-year implementation of the management treatments of low dose IFBB digestate application (H+N) and mulching (M) at five low mountain semi-natural grasslands increased the annual aboveground plant DM production in comparison with harvesting without nutrient application (H-N). H+N did not lead to shifts in plant functional group composition (graminoids, forbs, legumes) and evenness, whereas M tended to show contrasting responses depending on the grassland type. We found that the H+N treatment induced only small and transient shifts of the microbial substrate utilization pattern, whereas tendencies for an increased utilization of amino acids were observed for M. Digestate application did not lead to consistent temporal effects or lasting changes on soil microbial catabolic evenness. The soil chemical parameters SOC, total N, C/N and soil pH value were not affected by any treatment.

Overall, these results indicate that the digestate generated during the IFBB process stands out from digestates of conventional whole crop digestion on the basis of higher nitrogen use efficiency and that it is useful for increasing harvestable biomass and the nitrogen content of the biomass, especially of *L. perenne*, which is a common species of intensively used grasslands. This may offer to farmers the possibility to apply organic fertilizer to their intensively managed grassland in the form of an internally produced digestate from species-rich grassland biomass from less productive, extensively managed sites. Thus, a basic requirement in organic farming according to the conservation of biodiversity can be met in a whole farm approach, without having to relinquish high animal forage benefits. However, a medium application rate of IFBB digestate (50% of nitrogen removed with harvested biomass, corresponding to 30-50 kg N ha^{-1} a^{-1}) may be a possibility for conservation meadow management without changing the functional above- and belowground characteristic of the grasslands, thereby offering an ecologically worthwhile alternative to mulching. Overall, the soil microbial biomass and catabolic performance under planted soil was marginally affected by digestate application but rather by soil properties and partly by grassland species, legume

occurrence, and probably temporal variation in root development and exudation. The investigated extensively managed meadows revealed a high soil catabolic evenness, which was resilient to medium IFBB application rate after a three-year period of application. However, soil properties and/or occurrence of legumes may possibly change due to longer application periods and higher application rates. In addition to changes in plant community, soil microbial biomass and its catabolic performance are likely to respond, which could result in a differentiation from the original plant and soil functional characteristic of conservation grassland.

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