Biomass, quality traits and methane production of extensive grassland: biodiversity effects and sensor approaches

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

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Witzenhausen, den 09. Oktober 2015

Björn Reddersen

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 Agrarwissenschaften (Dr. agr.).

This dissertation is based on three papers of mine as first author with two of them published by international peer reviewed journals. The papers are included in chapter 4, 5 and 6. A general introduction is given in chapter 2 followed by a summary of the research objectives in chapter 3. A list of the original papers including the chapters in which they appear in this thesis will be given on the following page. A list of other publications descended from this thesis (e.g. contributions to conference proceedings) is given in chapter 9.

The funding for this work came from the Deutsche Forschungsgemeinschaft (DFG), which I like to acknowledge for their continuous effort to support young scientists.

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Last but not least, I will always be grateful for my family who steadily supported me on my journey.

Witzenhausen, October 2015

List of publications

Chapter 4: **Reddersen, B., Fricke, T. and Wachendorf, M. (2014)**: A multi-sensor approach for predicting biomass of extensively managed grassland. In *Computers and Electronics in Agriculture* 109, pp. 247-260.

Chapter 5: **Reddersen, B., Fricke, T. and Wachendorf, M. (2013):** Effects of sample preparation and measurement standardization on the NIRS calibration quality of nitrogen, ash and NDFom content in extensive experimental grassland biomass. In *Animal feed Science and Technology* 183, pp. 77-85.

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Abbreviations

a.s.l.	Above sea level
ADF	Acid detergent fibre
ADL:	Acid detergent lignin
BM	Biomass
CF:	Crude fibre
CFH	Contact field spectroscopy on hay
CL:	Crude lipid
$CH_{4\ area}$:	Area specific methane yield
$CH_{4\ sub}$:	Substrate specific methane yield
DFH	Distance field spectroscopy on hay
DFS	Distance field spectroscopy on silage
DFW	Distance field spectroscopy on standing sward
DIV	Diversity mixture
FGC:	Functional-group composition
FGR:	Functional-group richness
GHG	Greenhouse gas
gr:	Grasses
IFBB:	Integrated generation of solid Fuel and Biogas from Biomass
LAI	Leaf area index
lg:	Legumes
MF:	Mass-flow
NDF_{om}	Neutral detergent fibre based on organic dry matter
NDSI	Normalized difference spectral index
NDVI	Normalized Difference Vegetation Index
NIRS	Near infrared spectroscopy
PC:	Press-cake
PF:	Press-fluid
PM:	Parent-material
QLH	Quarz-cuvette laboratory spectroscopy on hay
RCG	Reed canary grass
REIP	Red edge inflection point
RPD	Ratio of prediction to standard deviation of reference values

RSC	Ratio of prediction to standard error of cross validation
RSMCV	Root mean square error of cross validation
R^2cv	Coefficient of determination for cross validationsh: small herbs
SAVI	Soil adjusted vegetation index
SEC	Standard error of calibration
SECV	Standard error of cross-validation
SR	Simple Ratio vegetation index
SR:	Species richness
STA	Standard mixture
SWWI	Shortwave infrared water index
th:	Tall herbs
TLH	Transport cell laboratory spectroscopy on hay
TLS	Transport cell laboratory spectroscopy on silage
USH	Ultrasonic sward height
VI	Vegetation index
VS:	Volatile solids
WCD	Whole crop digestate
WI	Water Index

1 Summary

Energy policies around the world are mandating for a progressive increase in renewable energy production. Extensive grassland areas with low productivity and land use limitations have become target areas for sustainable energy production to avoid competition with food production on the limited available arable land resources and minimize further conversion of grassland into intensively managed energy cropping systems or abandonment. However, the high spatio-temporal variability in botanical composition and biochemical parameters is detrimental to reliable assessment of biomass yield and quality regarding anaerobic digestion.

In an approach to assess the performance for predicting biomass using a multi-sensor combination including NIRS, ultra-sonic distance measurements and LAI-2000, biweekly sensor measurements were taken on a pure stand of reed canary grass (Phalaris aruninacea), a legume grass mixture and a diversity mixture with thirty-six species in an experimental extensive two cut management system. Different combinations of the sensor response values were used in multiple regression analysis to improve biomass predictions compared to exclusive sensors. Wavelength bands for sensor specific NDVI-type vegetation indices were selected from the hyperspectral data and evaluated for the biomass prediction as exclusive indices and in combination with LAI and ultra-sonic distance measurements. Ultrasonic sward height was the best to predict biomass in single sensor approaches (R^2 0.73 – 0.76). The addition of LAI-2000 improved the prediction performance by up to 30% while NIRS barely improved the prediction performance.

In an approach to evaluate broad based prediction of biochemical parameters relevant for anaerobic digestion using hyperspectral NIRS, spectroscopic measurements were taken on biomass from the Jena-Experiment plots in 2008 and 2009. Measurements were conducted on different conditions of the biomass including standing sward, hay and silage and different spectroscopic devices to simulate different preparation and measurement conditions along the process chain for biogas production. Best prediction results were acquired for all constituents at laboratory measurement conditions with dried and ground samples on a bench-top NIRS system (RPD > 3) with a coefficient of determination $R^2 < 0.9$. The same biomass was further used in batch fermentation to analyse the impact of species richness and functional group composition on methane yields using whole crop digestion and pressfluid derived by the Integrated generation of solid Fuel and Biogas from Biomass (IFBB) procedure. Although species richness and functional group composition were largely insignificant, the presence of grasses and legumes in the mixtures were most determining factors influencing methane yields in whole crop digestion. High lignocellulose content and a high C/N ratio in grasses may have reduced the digestibility in the first cut material, excess nitrogen may have inhibited methane production in second cut legumes, while batch experiments proved superior specific methane yields of IFBB press fluids and showed that detrimental effects of the parent material were reduced by the technical treatment.

2 General introduction

Global energy policies steer for a successive increase in renewable energy production to suffice rising energy demands, to overcome long term shortage of fossil energy resources and to reduce greenhouse gas emissions. The European renewable energy directive (2009/28/EC) (RED) is calling for a contribution of 20% of renewable resources to the total energy mix by the year 2020. In 2014 the proportion of renewable energy in Germany was 12.4% of the total energy mix with a contribution of 60% from biomass (BMWI, 2015). Since 2007, the capacity of biogas plants has been doubled to about 4000MW (BMWI-AGEEstat, 2015) along with an increased cultivation of energy crops, such as rapeseed (bio-diesel), maize (biogas) and miscanthus or short rotation coppice (combustion). The total acreage for biomass supply has been tripled to more than 1.2 million ha. In the course of this development, which is not limited to Germany, concerns have been expressed regarding structural changes in agriculture predominantly through maize monocultures, loss in biodiversity and direct competition with food production (Fargione et al. 2008; Petersen, 2008; Tilman et al. 2009). Therefore, the utilization of extensive grassland from marginal habitats, e.g. abandoned acreage, riparian areas and floodplains for generation of renewable plant resources has been brought attention in an attempt to approach rising demands in cultivation area for energy plants adapted to economic and ecological concerns (Rowe et al. 2009, Tilman et al. 2009, Dale et al. 2010). Effective implementation strategies for the global energy policies require economic calculations on the basis of reliable projections of biomass yield and biomass quality. Although grassland is regarded as a well suited substrate for bioenergy production, i.e. as feedstock for biogas or as solid fuel for combustion (Prochnow et al. 2009a), maize silage still is the most commonly used co-substrate (Weiland et al. 2006, FNR 2009).

Extensive grassland on marginal habitats is characterized by a high spatio-temporal heterogeneity, high species-diversity and variable productivity, which is difficult to assess for quality and yield predictions (Ward et al. 1999, Tockner and Stanford 2002). Direct harvesting of biomass is a widely used most accurate method of determining biomass especially in field trials and wet chemistry is the traditional way to characterize the quality of biomass for anaerobic fermentation. However, these techniques are mostly destructive as well as too expensive and time consuming for larger scale trials in highly variable swards (Haydock and Shaw, 1975; Harmoney et al. 1997; Sanderson et al. 2001). Therefore, non destructive, inexpensive, remote sensing methods with a high sampling rate and flexibility, albeit less in precision, have been developed to estimate forage biomass and quality with varying levels of success (Schellberg, 2008). Remote sensing using hyperspectral reflectance data in the near infrared range has become an important tool to determine aboveground biomass (Mutanga and Skidmore, 2004; Chen et al. 2009, Vescovo et al. 2012, Kawamura et al. 2011).

Using the whole range of the hyperspectral dataset may contain large amounts of redundant information, therefore, univariate and multivariate statistical methods are usually implemented in order to reduce the amount of information and wavebands for the development of a prediction model. Common vegetation indices derived from the hyperspectral dataset are established tools to predict various crop characteristics like grassland biomass (Todd et al. 1998; Boschetti et al. 2007; Numata et al. 2007). However, the prediction accuracy of the various vegetation indices regarding sward biomass is highly site and sward specific (Huang et al. 2004). Saturation effects, complex sward geometry and interfering soil signals may, therefore, limit their applicability as a single predictor (Heege et al. 2008; Huete et al. 1985). The addition of vegetation indices in multi sensor approaches with leaf area index (van Wijk and Williams, 2005) and ultrasonic sward height (Fricke et al. 2013) have been proven beneficial in the assessment of parameters for simple vegetation structures but synergies of a multisensor approach in the assessment of biomass from high diversity extensive grassland using USH, LAI and spectral VIs have yet to be verified.

Regarding remotely sensed quality analyses of biomass for energy production, near infrared spectroscopy is so far the only tool for analysis of large-scale materials and real-time evaluation of multiple constituents like nitrogen, fibre and ash content (Roberts et al. 2004, Windham et al. 1991). The heterogeneous structure of the biomass on a farm scale with mobile spectrometric equipment significantly reduces the prediction quality compared to laboratory scale near infrared or wet chemistry analyses (Starks et al. 2004). Sample preparation and measurement conditions of the calibration set and the predicted samples should match for good results (Stuth et al. 2003). Therefore, forage samples are usually collected from the field, dried, ground and scanned using a bench-top NIRS spectrometer at standardized laboratory conditions. Faster, more direct techniques of data acquisition are required on a farm scale where the rapid demand of biochemical and structural parameters is countered by excessive, time consuming sample preparation. Few studies have been published in recent years to compare the effect of sample preparation and storage conditions of forages on NIRS to determine quality parameters.

High quality feedstock for methane production should contain vast amounts of fermentable carbohydrates, lipids and proteins, and at the same time be poor in hemicelluloses and lignin (El Bassam, 1998). High amounts of indigestible fibre will limit energy availability (Buxton and Redfearn, 1997) and high protein concentrations may lead to inhibiting effects owing to ammonia accumulation (Zubr, 1986). The process of integrated generation of solid fuel and biogas from biomass (IFBB, Wachendorf et al. 2009) is one of several pre-treatment methods, developed in recent years to reduce fibre content and to improve digestibility of the biogas substrate. During the process low digestible fibre content is separated from soluble nutrients, resulting in the press fluid (PF), a substrate optimized for anaerobic fermentation.

The chemical composition of crops is significantly affected by environmental factors (e.g. soil fertility, precipitation and temperature), management (e.g. harvest date, cutting frequency, fertilization) and botanical composition of the sward (McEniry and O'Kiely, 2013). Several recent studies have validated the importance of sward maturity on methane yields, showing that substrate specific methane yield decreases with advancing sward maturity due to an increasing concentration of lignified fibre and hemicellulose with low digestibility (Amon et al. 2007; Prochnow et al. 2005, McEniry and O'Kiely, 2013). Biogas production in batch fermentation procedures is also supposed to be influenced by many factors such as operating temperature, pH value of the digestate, diversity of microorganisms and concentration of trace elements (Raposo et al. 2011; Weiland, 2010). As these process related variables add up to the total variation in the potential methane yield of the substrate, results can differ from calculations based only on chemical composition of the biomass. Only few studies have addressed the influence of botanical composition in species rich grassland on the methane yield in anaerobic digestion batch experiments.

3 Research objectives

The general assumption of this thesis was that high diversity grassland needs reliable non-destructive prediction approaches for biomass and biochemical parameters for proper management in order to be a promising substrate for methane production, as methane yields are affected by changes in biochemical parameters relevant for anaerobic digestions along a SR and functional group gradient. In order to evaluate novel approaches for non-destructive assessment of extensive grassland biomass two experimental studies were carried out:

The first study investigated the performance of multi sensor approaches on three different extensively managed sward types established in the context of a transdisciplinary research network on regional climate adaptation (Roßnagel, 2014). The seed compositions were chosen on the basis of the development and evaluation of a utilization strategy for extensive grassland in river floodplains for bioenergy production. A reed canary grass (*Phalaris arundinacea*) monoculture (RCG), a standard mixture (STA) including seven common species of grasses and legumes and a diversity mixture (DIV) of thirty-six species of grasses, herbs and a legume, which are typical for traditional grassland in river floodplains.

The second and third study were conducted in the framework of the Jena-Experiment with its 82 main plots representing different combinations of functional-group richness (FGR), FGC and SR, all in the context of extensive managed grassland. The experimental design gave a promising base for investigations on the impact of biodiversity on methane yields in anaerobic digestion (c.f. appendix for experimental setup and species list). The experimental set-up was established with the intention to address the criticism provoked by previous diversity studies (Roscher *et al.* 2004). Instead of dividing the species in monocotyledonous and dicotyledonous species, the plant functional groups were chosen more specifically, for example, legumes were regarded as a separate functional group as they can have disproportionate effects on ecosystem processes (Spehn *et al.* 2002). Instead of having one functional group containing all herbaceous species, they were divided into two functional groups, i.e. small herbs and tall herbs. Also, the design of the experiment was aimed at disentangling the effects of SR, FGR and the presence of individual functional groups as much as possible.

The specific objectives of these studies were

(i) to investigate the performance of a multi sensor approach using different combinations of NIRS, ultrasonic distance measurements and LAI in the prediction quality of extensively managed grassland biomass (Chapter 4). While previous studies have shown an improvement of prediction quality with ultrasonic distance measurements by the addition of vegetation indices on binary swards, the performance in more heterogeneous grassland environment remains uncertain. Further, the addition of a third sensor-type might further increase the prediction performance compared to a single or combined two sensor approach.

(ii) to investigate how sample preparation and measurement conditions for extensively managed grassland biomass affect the accuracy of NIRS prediction models for ash content, nitrogen and van Soest fibre fractions as quality parameters for biogas production (Chapter 5). Increased sample preparation and standardized measurement conditions are supposed to increase the prediction performance of NIRS devices but may cause additional costs and effort for quality assessment. Therefore, a balance between preparation effort and prediction accuracy is desired.

(iii) to investigate how SR and functional groups in extensively managed grassland may affect substrate and area specific methane yields of silage and IFBB press fluids in batch experiments (Chapter 6). High fibre contents in extensive grassland biomass are considered detrimental to anaerobic digestion but the floristic and biochemical composition is highly variable. Different variants of species richness and functional groups might therefore affect methane yields. The IFBB process is supposed to enhance the substrate quality for anaerobic digestion. Experiments for diversity effects on methane yields are often on the basis of calculations using the chemical composition of the biomass. Batch experiments consider systemic uncertainties closer to realistic conditions, which might result in more relevant results.

4 A multi-sensor approach for predicting biomass of extensively managed grassland

Abstract Leaf area index (LAI), ultrasonic sward height (USH) and common vegetation indices (VI) derived by spectral radiometric reflection data were collected on an experimental field site with three sward types comprising a pure stand of reed canary grass (*Phalaris aruninacea*), a legume grass mixture and a diversity mixture with thirty-six species in an extensive two cut management system. Sensor measurements and biomass samplings of 0.25 m^2 subplots were conducted biweekly between May and October in 2009 and 2010. Different combinations of the sensor response values were used in multiple regression analysis to improve biomass (BM) predictions compared to exclusive sensors. Wavelength bands for sensor specific NDVI-type vegetation indices were selected from the hyperspectral data and evaluated for the biomass prediction as exclusive indices or in combination with LAI and USH. In the set of tested parameters, ultrasonic sward height was the best to predict biomass in single sensor approaches (R^2 0.73–0.76). Inclusion of LAI improved the model performance and reduced the prediction accuracy by up to 30% for complex swards, while inclusion of vegetation indices resulted only in minor improvements compared to exclusive USH. LAI acted complementary to USH in a combined prediction model, correcting for overestimations of biomass in high swards. Prediction models using exclusive LAI were barely suited to predict biomass accurately (R^2 0.36–0.44) but improved significantly when combined with waveband selected VIs ($R^2 < 0.8$). Combining all three sensors did not significantly improve the model performance.

4.1 Introduction

Aboveground biomass is an important parameter in studies of cultivated and natural vegetation for the development of a sustainable bio energy policy (Jing et al. 2012). Energy policies around the world are mandating for a progressive increase in renewable energy production. Marginal lands such as riparian areas and flood plains with lowered productivity and land use limitations have become target areas for sustainable energy production from energy crops to avoid competition with food production on the limited available arable land resources. Natural flood plains are disturbance-dominated ecosystems that are characterized by a high spatio-temporal habitat heterogeneity, high species-diversity and productivity along with recreational and aesthetic values (Ward et al. 1999; Tockner and Stanford, 2002). They provide a wide range of ecosystem functions, such as filtering pollutants and excess nutrients (Yates and Sheridan, 1983) and reducing erosion rates after flood events due to slowed water downstream, but these functions are challenged by river management, shifting land uses, intensification, and climate change (e.g. Tscharntke et al. 2005, Krause et al. 2011). Krause et al. (2011) identified a loss of 80% of unprotected wetland area for northern Germany over the last 50 years due to intensified management, land use change and abandonment resulting in a loss of biodiversity and other valuable functions of these ecosystems.

For this reason, energy policies are targeting sustainable second generation, non-food energy crops and biofuels which can be designed and managed appropriately to maintain biodiversity, reduce GHG emissions and maintain the basic ecosystem functions of marginal lands (Dale et al. 2010; Rowe et al. 2009, Tilman et al, 2009).

The European Union (EU) Renewable Energy Directive (2009/28/EC) (RED) is calling for 20% of the total energy production to originate from renewable resources by 2020 with biomass being a major contributor. Effective implementation strategies require economic calculations on the basis of accurate projections of expected biomass yields. A broad literature review on five important energy crops revealed that projected biomass yields tend to be overestimated on semi-commercial scale trials or on marginal land, partially due to inappropriate up-scaling from small sized experimental sites (Searle and Malins, 2014). Therefore, biomass projections on the basis of larger commercial scale trials are supposed to be more accurate. Direct harvesting of biomass is currently the most widely used and most accurate method of determining biomass. The main disadvantage to this method is the time involved and consequently, the high cost of each sample. Because this is barely applicable on a larger scale, samples collected only represent a small area out of large and highly variable swards (Haydock and Shaw, 1975; Harmoney et al. 1997; Sanderson et al. 2001). Because the error in yield estimates lies predominately in the variability of the swards and not in the precision of the measurements, it is better to take more samples with less precision than few measured precisely (Haydock and Shaw, 1975). Non-destructive sensing methods with a rapid sampling rate have been developed to estimate forage biomass faster and more efficiently, with varying levels of success (Schellberg, 2008). One rapid, non-destructive method to estimate biomass involves the measurement of the leaf area index (LAI) by remote sensing or optical instruments. The LAI is a dimensionless variable which was first defined by Watson (1947) as the total one sided foliage area per ground surface area. It has been tested for the estimation of grassland and pasture biomass with varying results (Harmoney et al. 1997;

Ganguli et al. 2000; Miller-Goodman et al. 1999). Linear relationships between leaf area index (LAI) and biomass have been identified for Swards of orchardgrass (Dactylis glomerata L.) smooth bromegrass (Bromus inermis L.) and tall fescue grass (Festuca arundinacea L.) (Pearce et al. 1965; Engel et al. 1987; Trott et al. 1988). In contrast to destructive and tedious direct methods for the estimation of LAI by harvesting or litter traps, indirect methods use canopy properties and gap fractions to estimate the proportion of vegetative surface area (Jonckheere et al 2004). Recent studies focus on methods to derive the leaf area index directly from hyperspectral data and vegetation indices with varying results (Darvishzadeh et al. 2008, Stagakis et al 2010). However, it has been shown that a combination of in-situ LAI measurements, e.g. LI-COR LAI-2000, and spectral vegetation indices can provide better estimates for LAI, directly measured by harvest, than LAI or vegetation indices alone (van Wijk and Williams, 2005).

Remote sensing technology using hyperspectral reflectance measurements have become an important approach to estimate aboveground biomass at large special scales. Spectral reflection measurements have been widely used for the characterization of grassland biomass, obtained from hand-held spectral radiometers (Mutanga and Skidmore, 2004; Chen et al. 2009, Vescovo et al. 2012, Kawamura et al. 2011) but may contain large amounts of redundant information. For practical implementation at field scale, hyperspectral measurements are expensive and, therefore, the limitation of wavebands as vegetation indices is desirable. Vegetation indices (VIs) are widely used in remote sensing models for estimation of various crop characteristics (Hatfield and Prueger, 2010, Huang et al. 2012) like grassland biomass (Todd et al. 1998; Boschetti et al. 2007, Numata et al. 2007). However, the performance of VIs is highly site and sensor-specific (Huang et al. 2004). VIs based on NIR/red ratios like the normalized difference vegetation index (NDVI) indicated saturation around a leaf area index of about 2.0-2.5 (Heege et al. 2008), which limits their applicability at higher biomass levels. Modifications have been applied to reduce the saturation effects and the vulnerability to other environmental influences like soil background scattering (Elvidge and Lyon, 1985; Huete et al. 1985; Broge and Leblanc, 2000; Chen et al. 2009). Selection of distinctive narrow bands from hyperspectral data, e.g. according to the NDVI-type formula have shown improvements to traditional VIs (Blackburn, 1998; Thenkabail et al. 2000, Inoue et al. 2008). Other methods utilizing the sward height as a predictor for aboveground biomass have been successfully established in the past. It has been shown that biomass of binary and pure species grassland swards can be predicted well using non-destructive ultrasonic sward height (USH) measurements, reaching R^2 values between 0.75 and 0.82 (Fricke et al. 2011). However, sward geometry, leaf surface and sward density can impact the response signal and may lead to disproportional relationships between plant height and biomass (Hutchings 1990, 1992; Fricke et al. 2011). These limitations in USH might be overcome by a combination with other parameters that are related to sward density and lateral sward geometry, like LAI or spectral vegetation indices. Fricke et al. (2013) have already shown an increased prediction performance of a combined sensor approach using ultrasonic sward height and spectral vegetation indices for commercial binary legume grass swards. However, the increased structural and chemical complexity in swards of higher biodiversity might reduce the performance of spectral vegetation indices as a predictor and, therefore, reduce the synergy effect of a multi sensor approach. The benefit of a multisensor approach in diverse extensive grassland using USH and spectral VIs has yet to be verified.

In the context of a transdisciplinary research network on regional climate adaptation (Roßnagel, 2013), three different extensively managed grassland sward types, adapted to floodplain conditions, were tested for their energy potential with a high resolution covering the extensive spatio-temporal variation in floodplain areas. The main objective of this study was to compare the effectiveness of all possible combinations of three non-destructive sensor methods including ultrasonic sward height, LAI-2000 measurements and traditional spectral vegetation indices for estimating biomass in extensively cut grassland with a complex vegetation structure. Further objective was to evaluate the inclusion of a two band NDVI-type vegetation index according to the normalized difference spectral index (NDSI) formula introduced by Inoue et al. (2008) based on wavelength selection for the common swards.

4.2 Material and Methods

4.2.1 Experimental site

A field experiment was established in autumn 2008 and measurements were conducted in the years 2009 and 2010. The experimental field site was located in a floodplain, 100 meters from the river Werra and 100 m from its tributary Gelster at the city of Witzenhausen (52°21’ N, 9°52’ E, altitude 137 m a.s.l.). The two year average annual rainfall was 863 mm, with an average temperature of 9.0°C. The dominating soil type was a Fluvi-Eutric Cambisol. Prior to the experiment, the area had been used for cultivation of oats and alfalfa and is surrounded by intensified agricultural cropland.

4.2.2 Experimental design

Seed compositions were chosen on the basis of the development and evaluation of a utilization strategy for extensive grassland in river floodplains for bioenergy production. The compositions comprised a reed canary grass (*Phalaris arundinacea*) monoculture (RCG), a standard mixture (STA) including seven common species of grasses and legumes and a diversity mixture (DIV) of thirty-six species of grasses, herbs and a legume, which are typical for traditional grassland in river floodplains. The total study site contained 24 plots divided into three sward seed compositions and two fertilization variants in four-fold replication. The plot size was 4m length and 1.5m width aggregated for each replication in 4 randomized rows. An equivalent of 100 kg N per hectare of chicken manure was manually applied in March 2009 and 2010 on half of the plots. The rest remained unfertilized. All plots were harvested twice per year at the beginning of July and October with a finger bar mover at a cutting height of 5 cm.

4.2.3 Biomass sampling and sensor measurements

Sensor-based measurements were conducted on subplots every two weeks until harvest between May and September with a break of four weeks after the first cutting date. A representative area of 0.25m² in each plot was selected and marked for sensor measurements and biomass sampling. The aboveground biomass of each subplot was clipped manually, 5 cm above soil surface after sensor measurements were taken and afterwards oven dried at 105°C for 48 hours to determine dry matter yields.

4.2.4 Ultrasonic sward height measurements

Measurements of USH were conducted with an ultrasonic distance sensor of type UC 2000-30GM-IUR2-V15 (Pepperl and Fuchs, Mannheim, Germany). The sensor acts both as transmitter and receiver of an ultrasonic signal at 180 kHz within an opening angle of 25° in a sensing distance from 80 to 2000 mm (Fig. 1a). Measurement errors due to temperature are corrected by an integrated thermometer. The ultrasonic echo was converted into an output voltage linear to the measured distance and subsequently transformed by an A/D converter into numerical values, logged on a personal computer and finally converted into sward height values using an empirical linear regression function (EQ 1).

$y = a - 159.03 + 0.08756x$ (EQ 1) Fricke et al. (2011)

Where,

a = mount height of the ultrasonic sensor above soil surface (cm),

x = values from AD/converter (proportional to distance related voltage output),

y = sward height (cm).

A four-legged frame was placed above the subplot. A panel with 5 holes was mounted at 1 or 1.5 m on the frame depending on the sward height, to maintain a distance of at least 20 cm between the top plant organ and the sensor. At each subplot, five measurements were recorded with the ultrasonic sensors placed at five evenly distributed positions on the frame at the centre of the sample quadrate and at four diagonal positions in an equidistant radius of 14.1 cm (c.f. Fricke et al. 2011). The estimated sward height for the plot was calculated as the mean value of the five measurements.

4.2.5 Leaf area index measurements

Leaf area index was measured using a LAI-2000 plant canopy analyzer (Li-COR, Inc., Lincoln, NE). The Instrument was designed to indirectly measure canopy architecture, specifically leaf or folia area index and foliage orientation or mean tilt angle (Fig. 1b). A 45° view restrictor was used to limit the sensor's overall field of view and to mask out the operator. Direct illumination of vegetation can reduce the resulting LAI estimation by 10-50% (Welles and Norman, 1991). Therefore, the area was shaded during measurement using a lightproof umbrella, preventing illumination by direct sunlight. Measurements were made with one reference measurement above the canopy and three measurements on ground level in three replications to account for the spatial variability and leaf orientation of the vegetation.

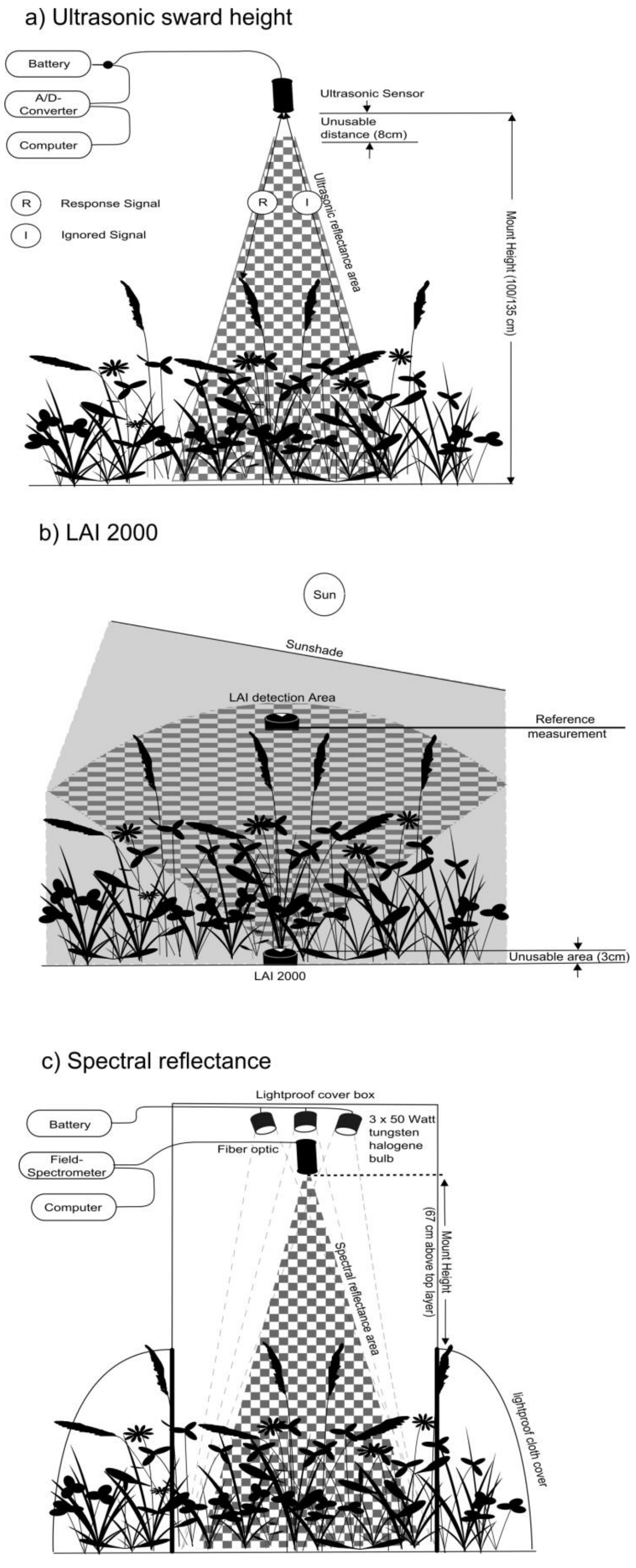

Fig. 1: Operating principles of (a) the ultrasonic sensor, (b) LAI 2000 measurements and (c) hyperspectral reflectance measurements.

4.2.6 Hyperspectral reflectance

Canopy reflectance was assessed using a portable spectrometer of the type FieldSpec Pro 3 (Analytical Spectral Devices, boulder, CO) measuring irradiance from swards in the range from 350 to 2500 nm with a spectral resolution of 3nm (350-1000 nm) and 10 nm (100-2500 nm) in an viewing angle of 25° below the sensor. Measurements were subsequently interpolated by the RS3 software package (Analytical Spectral Devices, boulder, CO) to produce readings at an interval of 1 nm.

The sensor was mounted at a height of 67 cm above the subplot canopy in a lightproof, cubic, cloth covered box, stabilized and leveled out on four telescopic sticks. A lightproof cloth cover shut out incoming sunlight below the cube to prevent atmospheric influences on the reflected spectrum. The sward was artificially illuminated by three 50W tungsten halogen bulbs (Fig. 1c). Spectral calibrations were performed for every plot before measurement using a spectralon panel (Labsphere, Inc., North Sutton, NH) at canopy height. Each spectrum was composed of four measurements representing a total of 40 replicated scans.

4.2.7 Calculation of vegetation indices

Prior to spectral analysis, spectra were smoothed using a Savitzky-Golay smoothing filter (11 point, 5^{th} order filtering operation) (Savitzky and Golay, 1964). The interpolated 1nm spectral channels in the range of 355-2500 nm were used for the selection of wavelengths to calculate common narrow and broad band vegetation indices, as well as for sward-specific wavelength selections fitting best in a regressive prediction of dry matter yield.

Vegetation indices are mathematical transformations of reflectance measurements expressed as ratios, differences or other combinations of different spectral bands, especially in the red and near-infrared region, that are established to obtain information about vegetation and land surface characteristics (Jackson and Huete, 1991). To evaluate the potential of existing vegetation indices (VI) in combination with USH and LAI, a set of VIs recently applied to grassland swards was tested in this study (Biewer *et al*. 2009b; Kawamura *et al.* 2011; Vescovo *et al.* 2012). This selection comprised common red/NIR-based VIs like the simple ratio vegetation index (SR, Jordan 1969), normalized difference vegetation index (NDVI, Rouse *et al.* 1974), red edge inflection point (REIP, Guyot and Baret, 1988) and specific narrow band indices related to the water content of the vegetation like the water index (WI, Peñuelas *et al.* 1997) and the shortwave infrared water index (SWWI, Inoue *et al.* 1993). The soil adjusted vegetation index (SAVI, Huete 1985) was also included, as the soil was sparsely covered in the beginning of the experiment. A set of common broadband VIs (NDVI, SR) was included, which were calculated according to the spectral bands of Landsat Thematic Mapper. Finally, the normalized difference structural index (NDSI), recently published by Vescovo *et al*. (2012), was also included. This index is located on the NIR-infrared shoulder of hyperspectral data and is based on simulated spectral broad bands of Chris Proba (mode 5) with H2 R_{Red} = 656-666 nm, H18 = 745-752 nm and H25 R_{NIR} = 863-881 nm.

All VIs reported here have been calculated from the hyperspectral dataset. The selected VIs were analyzed regarding the prediction of dry matter yield both exclusively and subsequently in combination with USH and LAI to compare their potential for a combined sensor application with improved prediction accuracy.

4.2.8 Regression development and data analysis

Prediction models for biomass with sensor response values were generated for each sensor combination by multiple linear regression using the lm() procedure of the R statistics software package (R Development Core Team, 2013). Dry matter yield was accounted for the dependent variable and USH, LAI and any VI was accounted for the independent variable in a single sensor approach or in any combination of two or three sensors (EQs 2 to 4). The general model included interactions and quadratic terms:

Exclusive single sensor

$$BM = a_0 + a_1 S_x + a_2 S_x^{\ 2} \qquad \text{(EQ 2)}$$

Combination of two sensors

$$\begin{aligned} BM &= a_0 + a_1 S_1 + a_2 S_2 + a_3 S_1 * S_2 + a_4 S_1^2 + a_5 S_2^2 \\ &+ a_6 S_1^2 * S_2 + a_7 S_1 * S_2^2 + a_8 S_1^2 * S_2^2 \end{aligned} \qquad \text{(EQ 3)}$$

Combination of three sensors

$$\begin{aligned} BM &= a_0 + a_1 S_1 + a_2 S_2 + a_3 S_3 + a_4 S_1^2 + a_5 S_2^2 + a_6 S_3^2 \\ &+ a_7 S_1 * S_2 + a_8 S_1 * S_3 + a_9 S_2 * S_3 + a_{10} S_1 * S_2^2 + a_{11} S_1 * S_3^2 + a_{12} S_2 * S_3^2 \\ &+ a_{13} S_1^2 * S_2 + a_{14} S_1^2 * S_3 + a_{15} S_2^2 * S_3 + a_{16} S_1^2 * S_2^2 + a_{17} S_1^2 * S_3^2 + a_{18} S_2^2 * S_3^2 \\ &+ a_{19} S_1 * S_2 * S_3 + a_{20} S_1^2 * S_3 * S_3 + a_{21} S_1 * S_2^2 * S_3 + a_{22} S_1 * S_2 * S_3^2 \\ &+ a_{23} S_1^2 * S_2^2 * S_3 + a_{24} S_1^2 * S_2^2 * S_3^2 + a_{25} S_1 * S_2^2 * S_3^2 + a_{26} S_1^2 * S_2 * S_3^2 \end{aligned} \qquad \text{(EQ 4)}$$

BM = biomass dry matter yield (t ha^{-1})

S_x = Sensor response for USH (cm) (x = 1), LAI (x = 2), and VI (x = 3)

The best fit model was determined by backwards factor selection starting with a full model including all quadratic terms and interactions of the main factors followed by stepwise elimination of non-significant factors at an α-level of 5% according to the rules of hierarchy and marginality (Nelder, 1994; Nelder and Lane, 1995).

For comparison with the vegetation indices, hyperspectral calibration models were developed using the WinISI (version 1.63) calibration software package (Infrasoft International, Port Matilda, Pennsylvania USA). The spectra were reduced for fluorescent light noise at the upper and lower end of the spectrum to 400-2400 nm. Calibration was performed with a modified partial least square regression method (MPLS) (Marten and Næs, 1989) for a first order derivative over a four point interval (Biewer *et al.* 2009b).

The model prediction error for estimating biomass was assessed by using a four-fold cross validation (CV) (Diaconis and Efron, 1983) separately for each sward type and the common swards. Four-fold cross validation split the dataset into four equally sized groups randomly selected along the biomass gradient. Three groups were iteratively used to determine regression parameters and afterwards tested against the remaining data. This procedure is repeated until every group has been left out once. Since the test set or the fourth group has not been used to build the model the calculated root mean square error of cross validation (RMSECV) and the shrinkage between the regression R^2 and coefficient of determination for cross validation (R^2cv) are good indicators of the model robustness and predictive power regarding unknown samples. The ratio of standard deviation and standard error of cross-validation (RSC) as often used in spectral calibration assessment is another stability factor which characterizes the robustness of a calibration equation and provides means for a comparison with other calibrations irrespectively of the units of investigated parameters (Park *et al.* 1997). An RSC value greater than three is considered adequate for analytical purposes in most of the laboratory near infrared applications for agricultural products (Cozzolino *et al*. 2006). RSC values higher than two might already be acceptable for predictions at field scale as variable measurement conditions and sward standardization might reduce the prediction accuracy (Terhoeven-Urselmans *et al.* 2006; Reddersen *et al.* 2013).

4.2.9 Sensor-specific wavelength selection

Most of the existing VIs consider only certain parts of the spectrum, primarily the chlorophyll absorption region (680nm), the near-infrared (NIR) reflectance (800nm) or the green reflectance peak (550nm). In an attempt to use the depth of information included in the large number of bands of hyperspectral data, NDVI-type narrowband normalized difference spectral indices (NDSI) (Inoue *et al.* 2008) were created according to EQ 5:

$$NDSI(b1,b2)=\frac{b1-b2}{b1+b2} \qquad \text{(EQ 5)}$$

All possible two-pair 1nm band combinations in the hyperspectral range from 355 to 2500 nm were used in EQ 5. b1 and b2 were specific narrow band reflection signals with $Wavelength_{b1}$ > $Wavelength_{b2}$. In order to take the limited spectral resolution of existing spectral sensors into account a broadband approach with response signal over a bandwidth of 50nm was also developed using EQ 5.

A total of 2,311,250 indices were used in linear regression models for each sensor combination according to EQ 2-4 to determine the best NDSI index for dry matter yield in the common swards. The capability of each model to explain the variability of biomass was determined by the resulting coefficient of determination of the full model. To avoid confusion with the normalized difference structural index introduced by Vescovo *et al*. (2012), the latter is further indicated as $NDSI_{vesc}$.

4.3 Results

4.3.1 Experimental sward characteristics

Average biomass of swards varied from 0.3 to 14.3 t DM ha^{-1} with an overall mean of 3.7 t DM ha^{-1} for all sampling dates throughout the growing season. Average biomass for the first cutting date at the beginning of July was 7.1 t DM ha^{-1} and 3.6 t DM ha^{-1} for the second cutting date at the beginning of September.

Biomass differed significantly between sward types and years. For an overview Fig 2a shows the temporal variability of biomass as means of both years. Biomass in the year of establishment was 15-25% lower than for the second year. The highest variability in biomass throughout both growing seasons was observed for the standard mixture which also delivered the highest average sward specific values (5.02 t DM ha^{-1}). The main contributors were *Phleum pretense* and *Festuca pratensis* with up to 45% of the total biomass at the first cutting date, followed in the first year by *Papaver rhoeas* and *Matricaria recutia*, which were not part of any seed mixture and invaded the swards from the surrounding areas, with up to 10% of the total biomass in the standard swards. Some plots from the standard mixture reached a very high biomass above 11 t DM ha^{-1} in June and July 2010, few weeks before the first cut.

As a result, lodging occurred and the USH saturated at a maximum sward height of about 1m, although the biomass was still increasing. As this significantly affected the performance of the ultrasonic height measurements; a total of ten estimates, where lodging was apparent, were removed from the dataset for model development. The resulting biomass for common sward and sward specific biomass yields are presented in Tab.1.

Tab. 1: Descriptive statistics of biomass, leaf area index (LAI) and ultrasonic sward height (USH) for the diversity mixture (DIV), standard legume grass mixture (STA), reed canary grass (RCG) and the common swards.

Parameter	Sward type	N[a]	Mean	S.D.[b]	Min	Max
Biomass yield [t DM ha^{-1}]	DIV	167	3.23	1.81	0.54	9.15
	STA	157	4.54	2.25	0.58	10.95
	RCG	167	2.78	1.72	0.28	10.14
	common	491	3.49	2.07	0.28	10.95
LAI	DIV	167	4.06	1.52	0.59	7.84
	STA	157	5.01	1.49	1.28	8.98
	RCG	167	3.59	1.20	0.19	6.35
	common	491	4.20	1.52	0.19	8.98
USH [cm]	DIV	167	53.05	23.13	16.12	103.48
	STA	157	56.01	24.11	18.98	103.38
	RCG	167	40.28	21.25	6.10	123.50
	common	491	49.65	23.79	6.10	123.50

[a] N: Number of samples ; [b] S.D.: standard deviation

Lowest biomass values for single cutting dates were determined for reed canary grass with 5.4 t DM ha^{-1} for the first cut and 3.2 t DM ha^{-1} for the second cut as an average of 2009 and 2010. In some cases, significant weed proportions were identified at an average of 19% of the total biomass (Tab. A.2) for the first cut 2009, dominated by high amounts of *Matricaria recutitia*. The diversity mixture covered the smallest range of biomass between 0.54 t DM ha^{-1} for the first sampling date in May 2009 and 9.15 t DM ha^{-1} for the first cut in 2010. Dominant species were *Festuca pretense, Holcus lanatus, Plantago lanceolata.* The variability of USH was similar for the diversity and standard mixtures, with values between 16 and 104 cm for all sampling dates and an average height of 53 cm for the diversity mixture and 58 cm for the standard mixture (Fig. 2b). RCG did not come up well in the first months of the year of establishment; therefore, the average sward height and biomass was considerably lower in the first year compared to the sward mixtures (34.26 cm).

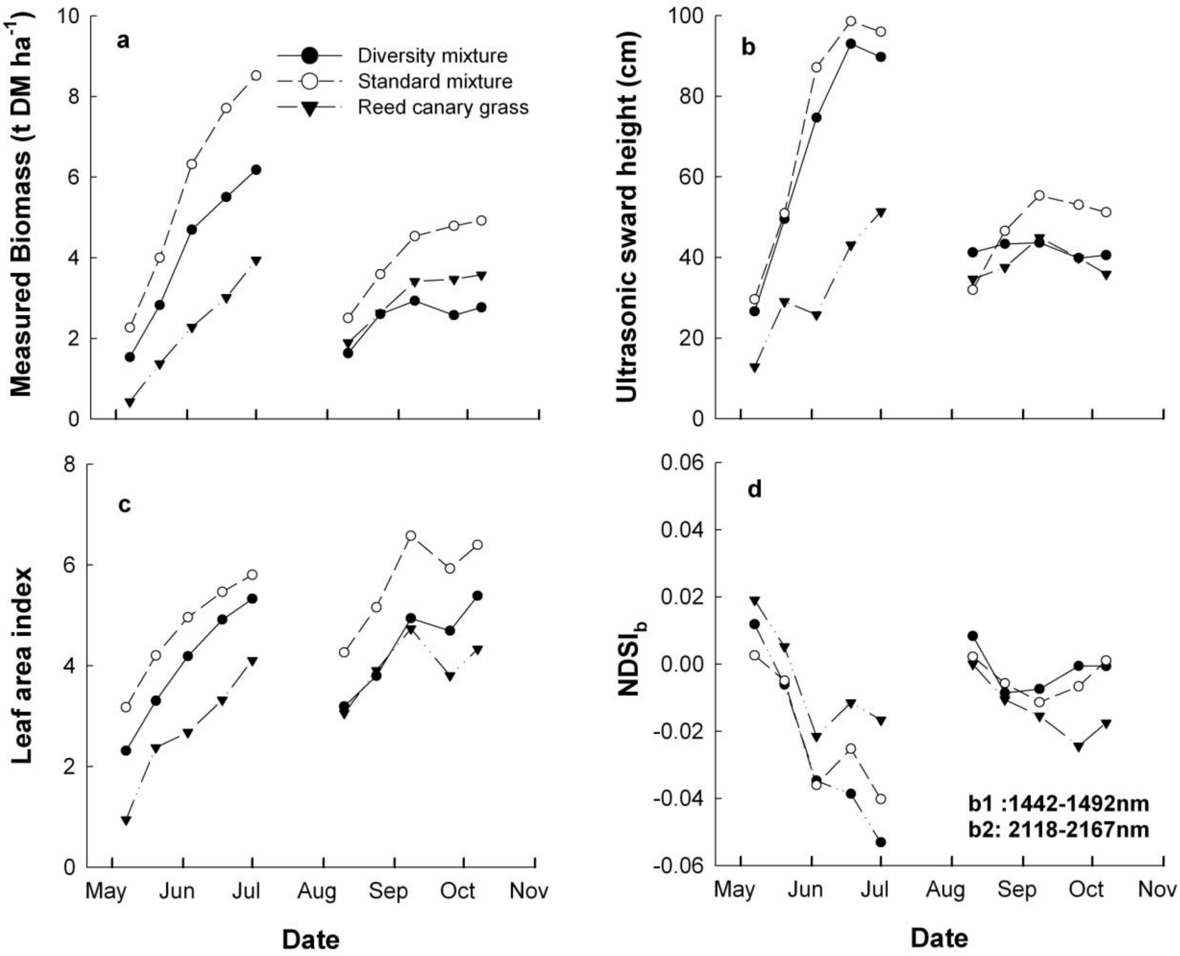

Fig. 2: Temporal variability of biomass (a), ultrasonic sward height (b), leaf area index (c) and the best fit normalized difference spectral index during the vegetation period. Parameters are shown as means of two years for each sampling date. The broadband normalized difference spectral index (NDSIb) is derived from wavelength selection over 50nm wavebands in the spectral range between 355 and 2500 nm. Spectral bands used for NDSI are indicated with b1 and b2

The estimated sward heights increased with accumulation of biomass but the average height for the standard mixture and the diversity mixture stagnated for the last two sampling dates in June and July between 90 cm and 100 cm. USH increase was low in the second cut and the average height remained between 40 and 60 cm for all sward types.

The leaf area index ranged from 0.19 in the RCG swards to 8.89 in the standard mixture with an overall mean of 4.24 for the common swards. The increase in LAI during the early vegetation period was similar to the increase in biomass and indicated saturation effects between values of 4 and 6 in June and July before the fist cut. After the first cut LAI increased again, starting at a lower level and reached a final peak in September. (Fig. 2c).

The best fit NDSI was achieved using a 50nm broadband approach with wavebands of b1 = 2118-2167nm and b2 = 1442-1492nm. As illustrated in Fig. 2d, the NDSI decreased throughout the first growing period and became negative in June, when reflections in the b1 range become lower than b2.

4.3.2 Single sensor approaches

The dataset was separated into subsets for each sward type and regression models were created accordingly. The exclusive use of ultrasonic sward height revealed linear relationships between the measured sward height and the calculated biomass for RCG, for diversity and common swards. In contrast, a quadratic relationship was found for the standard mixture, which is in accordance with findings of Fricke *et al*. (2011) for different mixtures of legumes and grasses. The regression curve leveled off at higher biomass values, up to the point where the lodging effect was observed at biomass values above 11 t DM ha^{-1}. The models indicated adequate R^2-values for all specific sward types (R^2 = 0.74-0.76) which was slightly better than for the common swards (R^2 = 0.73) (Tab. 2). The RMSECV ranged between 0.88 t DM ha^{-1} for reed canary grass and 1.17 t DM ha^{-1} for the standard mixture. The diversity mixture had the best results with an R^2 of 0.76 and a RMSECV of 0.90 t DM ha^{-1}.

The plots of fit between the predicted and measured biomass values showed an underestimation for prediction models using LAI and VIs at higher levels of biomass yield, which was observed to a lesser extent for USH (Fig. 3). Leaf area index showed a weak linear relationship with the measured biomass. Regression analysis for the common swards showed, that the LAI accounts only for 41% of the variance in the biomass (Tab. 2). The relation between the measured biomass and the LAI 2000 measurements were linear for the standard sward and the diversity mixture, as well as for the common swards. Reed canary grass showed a quadratic relationship between biomass and the leaf area index, which also delivered the best regression results with a R^2 of 0.46 and a RMSECV of 1.28 t DM ha^{-1}. Biomass of the diversity mixture and the standard mixture could not be predicted adequately using exclusive LAI measurements.

The ability of the investigated existing VIs to predict the biomass of extensive grassland swards was low. All tested VIs showed very low correlations to the biomass yield (R^2 between 0.01 and 0.34) and were not adequate as stand-alone predictors (Tab. 2). The performance of the individual vegetation indices differed between the swards. While the soil adjusted vegetation index correlated slightly with the biomass for the diversity mixture (R^2 =

0.33; RMSECV = 1.50 t DM ha^{-1}) and the standard mixture (R^2 = 0.31; RMSECV = 1.91 t DM ha^{-1}), there was almost no correlation for RCG (0.09).

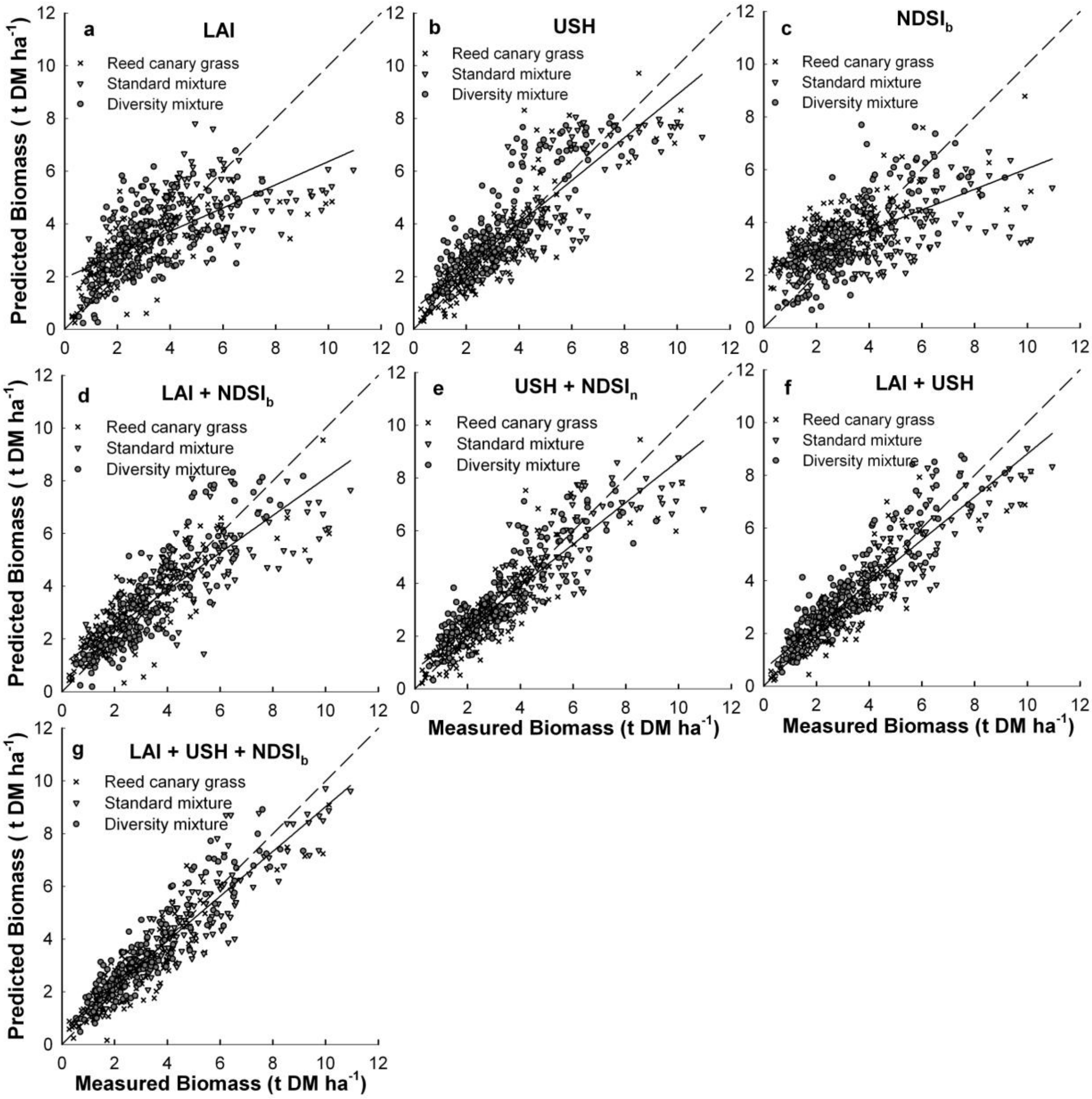

Fig. 3: Plots of fit between measured and predicted biomass for exclusive ultrasonic sward height (USH), leaf area index (LAI) and the best fit normalized difference spectral index (NDSI), as well as and for two sensor and three way combinations of USH, LAI and the best fit vegetation index for the respective sensor combination in context of the common swards.

The water index (WI) showed the best results for reed canary grass (R^2 = 0.34, RMSECV = 1.44 t DM ha^{-1}), but performed weak for the diversity mixture (R^2 = 0.12; RMSECV = 1.75 t DM ha^{-1}). Broad and narrowband vegetation indices for NDVI and SR did not show any significant relationship to the common swards (R^2 = 0.06; RMSECV = 2.02 t DM ha^{-1}).

Tab. 2: Regression and cross validation (CV) statistics for single sensor approaches on the common sward and sward-specific relationships between measured biomass as dependent variable using ultrasonic sward height (USH), leaf area index (LAI) and vegetation indices. Broadband (b) and narrowband (n) versions of the same vegetation index are differentiated by subscript lowercase letters. The best fit model for each sensor combination is indicated in bold.

	Common Swards (N^f = 501)					**Diversity mixture (N = 167)**					**Standard mixture (N = 157)**					**Reed Canary Grass (N = 167)**				
Sensors/VI's	R^2 [a]	SE [b] (t ha^{-1})	Rcv^2 [c]	RMSECV [d] (t ha^{-1})	RSC [e]	R^2	SE (t ha^{-1})	Rcv^2	RMSECV (t ha^{-1})	RSC	R^2	SE (t ha^{-1})	Rcv^2	RMSECV (t ha^{-1})	RSC	R^2	SE (t ha^{-1})	Rcv^2	RMSECV (t ha^{-1})	RSC
LAI	0.44	1.55	0.43	1.55	1.33	0.38	1.42	0.38	1.45	1.25	0.37	1.81	0.36	1.81	1.24	0.46	1.28	0.41	1.28	1.34
USH	0.73	1.08	0.73	1.08	1.91	0.76	0.89	0.76	0.90	2.01	0.74	1.15	0.73	1.17	1.93	0.75	0.86	0.74	0.88	1.96
Hyperspectral	0.71	0.85	0.56	1.05	1.48	0.87	0.57	0.76	0.76	2.04	0.89	0.66	0.63	1.20	1.65	0.70	0.71	0.45	0.96	1.34
SR_b	0.06	2.01	0.05	2.01	1.03	0.29	1.54	0.27	1.57	1.15	0.16	2.08	0.13	2.10	1.07	n.s.	n.s.	n.s.	n.s	n.s
SR_n	0.06	2.01	0.05	2.01	1.03	0.29	1.53	0.28	1.56	1.16	0.15	2.09	0.12	2.11	1.07	0.03	1.71	<0.01	1.40	1.23
$NDVI_b$	0.04	2.03	0.03	2.03	1.02	0.25	1.57	0.24	1.61	1.12	0.15	2.08	0.14	2.09	1.08	n.s.	n.s.	n.s.	n.s	n.s
$NDVI_n$	0.05	2.02	0.04	2.02	1.02	0.26	1.56	0.25	1.59	1.13	0.16	2.08	0.14	2.08	1.08	n.s.	n.s.	n.s.	n.s	n.s
REIP	0.09	1.98	0.09	1.97	1.05	0.1	1.72	0.07	1.79	1.01	n.s	n.s	n.s	n.s	n.s.	0.17	1.58	0.12	1.59	1.08
SAVI	0.17	1.89	0.16	1.91	1.08	0.33	1.49	0.31	1.50	1.2	0.31	1.89	0.30	1.91	1.18	0.09	1.66	0.04	1.71	1.01
WI	0.20	1.86	0.19	1.86	1.11	0.12	1.70	0.11	1.75	1.03	0.26	1.94	0.25	1.96	1.15	0.34	1.4	0.31	1.44	1.20
SWWI	0.08	1.98	0.07	1.99	1.04	n.s. [g]	n.s.	n.s.	n.s	n.s	0.16	2.07	0.15	2.1	1.07	0.22	1.52	0.19	1.56	1.10
$NDSI_n$	0.32	1.70	0.32	1.71	1.21	0.53	1.21	0.53	1.24	1.45	0.36	1.81	0.34	1.84	1.22	0.44	1.29	0.43	1.32	1.31
$NDSI_b$	**0.39**	**1.62**	**0.38**	**1.62**	**1.28**	**0.58**	**1.18**	**0.57**	**1.18**	**1.53**	**0.41**	**1.74**	**0.40**	**1.75**	**1.29**	**0.56**	**1.16**	**0.54**	**1.35**	**1.28**
$NDSI_{vesc}$	0.01	2.06	0.01	2.05	1.01	n.s.	n.s.	n.s.	n.s	n.s	0.04	2.22	0.02	2.25	1	0.06	1.68	0.01	1.69	1.02

[a] R^2: Regression coefficient of determination; [b] SE: Standard error of regression; [c] R^2cv: Cross validated coefficient of determination; [d] RMSECV: root mean square error of cross validation; [e] RSC: quotient of standard deviation omeasured values and RMSECV; [f] N: number of values incorporated in the regression models (only 473 samples were included in hyper spectral regression models); [g] n.s.: no significant contribution of vegetation indices

The best results for a hyperspectral approach using a MPLS regression for the complete spectral range from 355 to 2500 nm was achieved for the first derivative with 4 point gap and 4 point smoothing interval. The regression results were on par or even better than the results for ultrasonic sward height (R^2 between 0.70 and 0.87) (Tab. 2). However, R^2 reduction by 0.25 in the 4 fold cross validation indicated an overfitting of the models, especially for reed canary grass and the standard mixture, which compromised the good calibration results.

4.3.3 Multiple sensor approaches

The combination of LAI and USH improved prediction accuracies for all sward types and reduced the standard error for the common swards by 18% compared to exclusive ultrasonic sward height and by 43% compared to exclusive use of LAI. The performance for the sensor combination was similar for all sward types with the lowest improvement for reed canary grass (Tab. 3). The sensor combination reached RSC values above 2 for all sward types and the common swards indicating an adequate performance for field level analysis.

Performance of combinations of LAI with existing vegetation indices showed a high variation. Highest R^2 values were reached with SAVI (R^2 = 0.65; RMSECV = 1.25 t DM ha^{-1}), which also delivered best results for the diversity (R^2 = 0.71; RMSECV = 1.02 t DM ha^{-1}) and the standard mixture (R^2 = 0.67; RMSECV = 1.34 t DM ha^{-1}). However, a combination of LAI and broad- or narrowband approaches for NDVI and simple ratio performed better for reed canary grass (R^2 = 0.64-0.66; RMSECV = 1.06-1.09 t DM ha^{-1}) according to the RSC. None of the tested vegetation indices reached adequate prediction results on a field level according to the RSC.

Prediction accuracy for a combination of USH and published Vis improved only slightly compared to exclusive USH. Highest increase in R^2 was reached with REIP for the common swards (R^2 = 0.77 from 0.73), as well as for the standard mixture (R^2 = 0.78 from 0.74) and reed canary grass (R^2 = 0.81 from 0.75). The normalized difference spectral index on the NIR shoulder ($NDSI_{vesc}$) introduced by Vescovo *et al.* (2012) reached higher results for the diversity mixture than the REIP (R^2 = 0.81 from 0.76). Unlike the combination of LAI with VIs, performance of USH with different VIs were quite similar, except for RSC, where the addition of a vegetation index to the prediction model did not significantly improve the accuracy of the prediction compared to a two sensor combination of LAI and USH (Tab. 2).

Tab. 3: Regression and cross validation (CV) statistics for dual sensor approaches on common sward and sward-specific relationships between measured biomass as dependent variable and sensor models using exclusive sensors and combinations of ultrasonic sward height (USH), leaf area index (LAI) and Vegetation indices. Broadband (b) and narrowband (n) versions of the same vegetation index are differentiated by subscript lowercase letters. The best fit model for each sensor combination is indicated with bold letters and numbers.

	Common Swards (N^f = 501)					**Diversity mixture (N = 167)**					**Standard mixture (N = 157)**					**Reed Canary Grass (N = 167)**				
	R^{2a}	SE^b	Rcv^{2c}	$RMSECV^d$	RSC^e	R^2	SE	Rcv^2	SECV	RMSECV	R^2	SE	Rcv^2	SECV	RSC	R^2	SE	Rcv^2	RMSECV	RSC
Sensors/VI's		($t\ ha^{-1}$)		($t\ ha^{-1}$)			($t\ ha^{-1}$)		($t\ ha^{-1}$)			($t\ ha^{-1}$)		($t\ ha^{-1}$)			($t\ ha^{-1}$)		($t\ ha^{-1}$)	
LAI+USH	**0.82**	**0.89**	**0.80**	**0.89**	**2.32**	**0.84**	**0.73**	**0.82**	**0.73**	**2.46**	**0.84**	**0.93**	**0.79**	**0.97**	**2.33**	**0.79**	**0.79**	**0.79**	**0.84**	**2.06**
LAI+NDVIb	0.52	1.44	0.50	1.44	1.44	0.67	1.07	0.60	1.13	1.60	0.56	1.52	0.53	1.57	1.44	0.65	1.04	0.42	1.06	1.62
LAI+NDVIn	0.52	1.44	0.50	1.44	1.44	0.67	1.06	0.61	1.11	1.63	0.56	1.52	0.53	1.57	1.44	0.64	1.06	0.43	1.09	1.58
LAI+SRb	0.53	1.43	0.51	1.43	1.45	0.67	1.05	0.60	1.07	1.69	0.57	1.51	0.53	1.57	1.43	0.66	1.03	0.42	1.08	1.59
LAI+SRn	0.52	1.43	0.5	1.43	1.45	0.68	1.05	0.60	1.08	1.68	0.57	1.51	0.53	1.57	1.43	0.66	1.02	0.43	1.09	1.58
LAI+REIP	0.47	1.51	0.45	1.52	1.36	0.46	1.34	0.44	1.38	1.31	0.38	1.79	0.34	1.79	1.26	0.58	1.15	0.43	1.19	1.45
LAI+SAVI	0.65	1.23	0.6	1.25	1.66	0.71	0.98	0.66	1.02	1.77	0.67	1.32	0.64	1.34	1.69	0.59	1.12	0.50	1.22	1.40
LAI+WI	0.54	1.42	0.51	2.02	1.02	0.42	1.39	0.39	1.44	1.26	0.55	1.53	0.53	1.56	1.45	0.58	1.13	0.53	1.14	1.51
LAI+SWWI	0.47	1.51	0.45	1.52	1.36	n.s.[g]	n.s.	n.s.	n.s	n.s	0.46	1.67	0.44	1.68	1.34	0.52	1.21	0.46	1.21	1.42
LAI+$NDSI_{vesc}$	0.49	1.49	0.45	1.49	1.39	0.52	1.28	0.46	1.32	1.37	n.s.	n.s.	n.s.	n.s	n.s	0.50	1.24	0.4	1.26	1.36
LAI+NDSIn	0.57	1.36	0.54	1.36	1.52	0.70	1.00	0.66	1.00	1.81	0.61	1.41	0.6	1.44	1.57	0.63	1.06	0.54	1.14	1.51
LAI+NDSIb	**0.71**	**1.13**	**0.66**	**1.13**	**1.83**	**0.8**	**0.83**	**0.8**	**0.84**	**2.15**	**0.7**	**1.28**	**0.6**	**1.37**	**1.65**	**0.74**	**0.91**	**0.63**	**0.94**	**1.82**
USH+NDVIb	0.76	1.02	0.75	1.03	2.01	n.s.	n.s.	n.s.	n.s	n.s	0.75	1.14	0.74	1.17	1.92	0,76	0,85	0,75	0,90	1,91
USH+NDVIn	0.76	1.03	0.75	1.04	1.99	n.s.	n.s.	n.s.	n.s	n.s	0.76	1.14	0.73	1.17	1.93	0,76	0,85	0,75	0,88	1,97
USH+SRb	0.76	1.02	0.75	1.02	2.03	n.s.	n.s.	n.s.	n.s	n.s	n.s.	n.s.	n.s.	n.s	n.s	0,76	0,85	0,75	0,87	1,97
USH+SRn	0.76	1.01	0.75	1.02	2.03	n.s.	n.s.	n.s.	n.s	n.s	0.75	1.14	0.74	1.16	1.95	0,76	0,85	0,75	0,87	1,97
USH+REIP	0.77	1.00	0.76	1.00	2.06	0.78	0.86	0.77	0.90	2.01	0.78	1.06	0.77	1.08	2.08	0,81	0,77	0,73	0,87	1,98
USH+SAVI	0.75	1.04	0.74	1.05	1.97	n.s.	n.s.	n.s.	n.s	n.s	0.76	1.12	0.74	1.20	1.88	0,78	0,82	0,73	0,85	2,03
USH+WI	0.73	1.07	0.72	1.09	1.9	0.78	0.87	0.75	0.93	1.94	0.77	1.11	0.73	1.14	1.99	0,77	0,84	0,71	0,88	1,95
USH+SWWI	0.74	1.07	0.72	1.08	1.92	n.s.	n.s.	n.s.	n.s	n.s	n.s.	n.s.	n.s.	n.s	n.s	0,79	0,81	0,71	0,85	2,03
USH+NDSIvesc	0.75	1.03	0.75	1.04	1.99	**0.8**	**0.81**	**0.78**	**0.82**	**2.20**	0.77	1.09	0.76	1.13	2.00	**0,79**	**0,79**	**0,77**	**0,83**	**2,07**
USH+NDSIn	**0.80**	**0.94**	**0.79**	**0.94**	**2.20**	0.79	0.83	0.78	0.84	2.15	**0.78**	**1.06**	**0.78**	**1.09**	**2.07**	0,77	0,83	0,77	0,86	2,00
USH+NDSIb	0.79	0.94	0.79	0.95	2.18	0.78	0.85	0.77	0.86	2.11	0.78	1.07	0.77	1.10	2.04	0,78	0,82	0,76	0,90	1,91

[a] R^2: Regression coefficient of determination; [b] SE: Standard error of regression; [c] R^2cv: Cross validated coefficient of determination; [d] RMSECV: root mean square error of cross validation; [e] RSC: quotient of standard deviation of measured values and RMSECV; [f] N: number of values incorporated in the regression models; [g] n.s.: no significant contribution of vegetation indices

4.3.4 Sensor optimized band selection

To determine the best performing sensor combination on the basis of the NDVI formula (b_2–b_1)/ (b_2+b_1), a wavelength selection was conducted using the common sward dataset in combination with regression functions EQ 2–EQ 4. Regression analysis was conducted using all possible band combinations in the common sward dataset, separately for 1 nm and 50 nm intervals. The best waveband combination for the full model was then used to develop individual regression models for the different sward types and the common swards obeying the rules of hierarchy and marginality. The best fit wavelength band combinations for the 1 nm bandwidths were about the same as for the 50 nm bandwidths for each sensor combination (Tab. 4). The bands for the exclusive vegetation index and in combination with LAI were located in the short wave infrared region on the slopes of the 2000 nm water absorption band and in the vicinity of the 1400 nm water absorption bands (exclusive VI: b_2 = 2140nm; b_1 = 1455 nm; LAI +VI: : b_2 = 1868nm; b_1 = 1421 nm).

Tab. 4: Best fit waveband combinations (b1, b2) for the normalized difference spectral index (NDSI) for the respective sensor combination as an exclusive parameter or in combination with ultrasonic sward height (USH) and leaf area index (LAI).

Sensor combination	Narrowband [1 nm]		Broadband [50 nm]	
	b2	b1	b2	b1
Exclusive NDSI	2178	1431	2117-2167	1442-1492
NDSI + LAI	1844	1403	1836-1886	1393-1443
NDSI + USH	1237	714	1198-1248	687-737
NDSI + LAI + USH	1778	1764	1732-1782	1720-1770

The best fit wavelength bands for the sensor combination of USH and VIs were located on the ascending slope of the 2_{nd} water absorption band (b_2 = 1258 nm) and in the red-edge spectral region (b_1 = 712), whereas for the three sensor combination these bands were close to each other on the reflection peak at 1700 nm (b_2 = 1709nm; b_1 = 1670 nm). For all sensor combinations sensor-specifically selected wavelength bands were superior to published vegetation indices both in terms of R^2 and standard error.

Although the predictive power of models with NDSI indices was better than traditional VIs, the overall performance of exclusive vegetation indices was poor with a maximum R^2 of 0.39 and RMSECV of 1.62 t DM ha^{-1} for the common swards. The broadband approach using 50 nm bandwidth generally delivered better results than the 1nm bandwidth with a reduction of RMSECV by 5%. The performance of LAI significantly improved from an R^2 of 0.44 to 0.71 by the addition of the broadband NDSI, determined for this sensor combination. The highest improvement was achieved for the diversity mixture (R^2 = 0.8 from 0.38; RMSECV 0.84 t DM ha^{-1} from 1.45 t DM ha^{-1}) (Tab. 2).

The addition of NDSI did barely improve the predictive power of the USH measurements similar to the existing vegetation indices. The best results for the common swards were achieved for the narrow band NDSI selected for this sensor combination (R^2 = 0.80; RMSECV = 0.94). Due to the low improvement of the inclusion of VIs and because wavebands were selected on the basis of the common swards, sward-specific regression models for the diversity mixture and reed canary grass had better results for $NDSI_{vesc}$ than NDSI (Tab. 2). The addition of a wavelength selected VI for the three sensor combination did not significantly improve the accuracy of the prediction (Tab. 3) compared to a two sensor combination of LAI and USH (Tab. 2), whereas it reduced the variance of the residuals and approximated the predicted biomass closer to the measured values especially at higher levels of biomass (Fig. 3).

The interpretation of the complex regression models particularly for the sensor combinations (Tab. 2-3) is difficult, as the models contain various interactions and quadratic as well as cubic terms. Thus, the main features of the models are presented graphically (Fig. 4) by forming predictions from the models to represent each of the salient features of the models, following the procedure proposed by Connolly and Wachendorf (2001). Predictions for significant interactions of sensors are plotted as two lines (one each for a high or low level of one sensor, i.e. approximately its mean±standard deviation) or as three lines with an additional line for the mean for the one sensor. Values for the other sensor are varied continuously in the range of highest observed frequency of values. The range chosen for prediction from the sensor data was selected to exclude values close to the observed minimum and maximum of values, and predictions outside the range of the observed data were excluded. Biomass increased with increasing LAI (Tab. A.3; Fig. 4a), but the slope increased with decreasing NDSI values, i.e. the stronger the reflectance in the near infrared was. For this sensor combination maximum accuracy was achieved with such wavelengths resulting in a negative relationship between NDSI and biomass. In the best fit sensor combination of $NDSI_n$ with USH wavelengths were selected which created a positive relationship between NDSI and biomass (Fig. 4b).

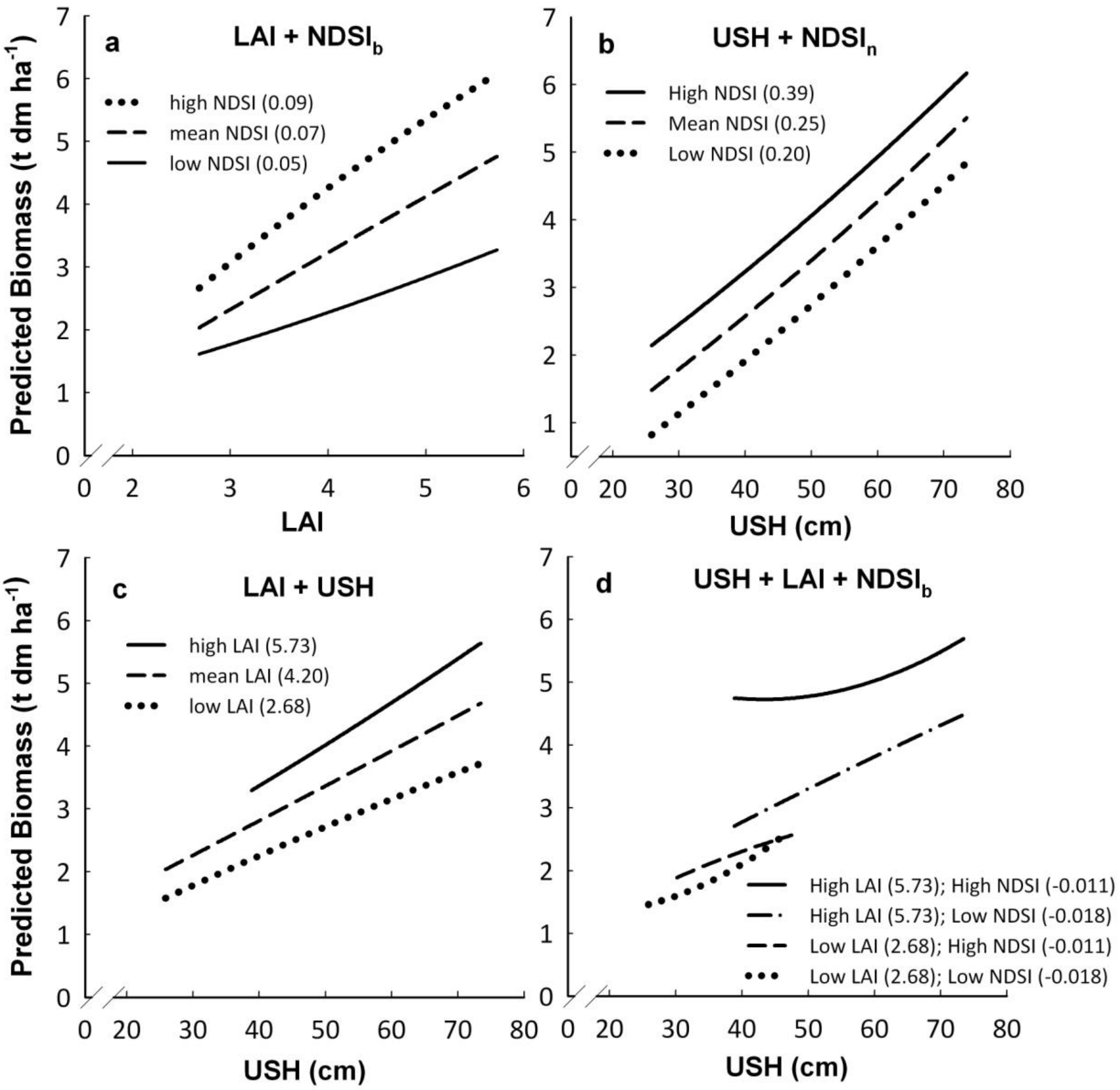

Fig. 4: Best fit Predictions of biomass (BM) in common swards based on ultrasonic sward height (USH), leaf area index (LAI) and two band normalized difference spectral index (NDSI) in the range of mean values ± standard deviation. Parameter value combinations that were not supported by the range of measured sensor values were excluded from the graphs. The wavebands for the development of the vegetation indices are selected separately for the basis of each sensor combination. Broadband vegetation indices, indicated by the subscript letter b are selected on the basis of 50nm bands from the hyper spectral range between 355 and 2500 nm. Narrowband indices indicated by the subscript letter n are selected on the basis of 1 nm bands.

Biomass increased almost linearly with increasing USH and no interaction was found between the two sensors. For the LAI x USH sensor combination a positive interaction between both sensors was detected, meaning that biomass increased all the more with increasing USH, the higher LAI of the swards (Fig. 4c). As combinations of low USH and high LAI values did not exist in the dataset, care was taken not to exceed the scope of validity when determining the range of predictions. The investigation of the three-sensor combination resulted in an extremely complex model (Tab. A.5), including various terms, i.e. thirteen two-way interactions and four three-way interactions containing different quadratic variables.

As all of them were significant, they were maintained in the final model, but could be displayed quite efficiently in a single diagram (Fig. 4d). Similarly to the USH x $NDSI_b$ model, wavelength selection for $NDSI_b$ resulted in a positive relation to biomass. While biomass increased strongly with increasing VI values at high levels of LAI, the effect of VI was negligible at low levels of LAI. It can further be concluded that at all levels of LAI the VI effect is enhanced at low levels of USH.

4.4 Discussion

It is well documented, that many VIs tend to asymptotically saturate at high LAI or biomass values (e.g. Thenkabail *et al.* 2000, Mutanga and Skidmore, 2004, Heege *et al.* 2008). NDVI already saturates at LAI values around 2-3 (Heege *et al.* 2008). In our study, this LAI level was exceeded for all sampling dates and swards, except for the first sampling dates of each growth period which rendered NDVI unsuitable to deliver appropriate prediction accuracy for biomass. The same can be assumed for broadband and narrowband simple ratio NIR/red VIs, which had quite similar results to broadband and narrowband NDVI. The large variation in R^2 and SE values, observed for the remaining VIs, indicates that the VIs respond differently to a variation in biomass. Although the accuracy of all published VIs was low, VIs located on the short wave infrared band region (WI, SWWI), that are related to the canopy water content, gave better results than most VIs in the VIS/NIR region. This is in accordance with findings of Psomas *et al*. (2011) on species rich grassland and of Fricke *et al*. (2013) on binary legume grass mixtures and can be explained by the close correlation between canopy water content and green biomass (Asner, 1998; Anderson *et al.* 2004). In the set of traditional VIs, SAVI best predicted biomass of diversity mixture and standard mixture, but had a lower accuracy for RCG. Soil adjusted vegetation index is more resistant to gaps in the canopy structure than the NDVI or simple ratio vegetation index (Huete, 1985). Those were numerous in the early succession stages, especially for measurements taken in May and June 2009, where the canopy was not fully developed. NDSI wavebands, selected for the common swards, improved the prediction accuracy for all sward types compared to traditional VIs. While various authors found better performances for narrowband VIs (Blackburn. 1998; Thenkabail *et al.* 2000; Mutanga and Skidmore, 2004), in this study the results for 50nm broadband NDSI were better than for 1nm narrowbands. Highest R^2-values, which were comparable to the LAI prediction accuracy, were centered at the 3rd water absorption band in the short wave infrared region at 1450nm and the region at 2100nm which is associated with cellulose, lignin and other structural compounds of the vegetation (Elvidge, 1990; Roberts *et al*. 1993). Regions with higher R^2 values were also found on the slopes of the first water absorption band between 900 and 1100nm and on the descending slope of the 4^{th} water absorption band between 1650 and 1800 nm, supporting the close relationship between canopy water content and biomass.

The vertical structure of mixed and homogenous grassland swards is usually characterized by a decrease in bulk density with increasing sward height (Clark *et al.* 1974; Delagarde *et al.* 2000) but the center of gravity for the bulk mass is higher in complex swards (Sanderson *et al*. 2006). Therefore, a saturation effect can occur for the prediction of biomass with USH when the biomass density decreases disproportionately at the upper canopy layers compared to the lower layers. A saturation effect was observed for the standard mixture, inflicted by lodging swards at late sampling dates in the early summer. Although lodging swards were eliminated from the model dataset, there was still a quadratic relationship between USH and biomass in the standard mixture, indicating a saturation effect at higher biomass values. This effect was primarily observed in the sampling periods in June and July, when flowers of *Phleum pratense* were overgrowing the bulk biomass by 10 to 15 cm. The swards of the second growth period from mid to late summer had a higher proportion of legumes, dominating the lower and mid layers of the sward, which appeared to have a more evenly distributed sward density along the height gradient. The grass fraction, especially *P. pretense* was still the major component in the upper layers, but only towered a few centimeters above the other species; thus, biomass prediction for the second growth period was more accurate compared to the first growth period.

A quadratic relationship between biomass and USH in legume grass mixtures was also observed by Fricke *et al*. (2011) who reached R^2 values up to 0.85 in binary swards. Fricke *et al*. (2011) suggested a significant impact of weed proportion on the biomass predictions, because some weeds may develop faster and gain greater heights than sown species. While there were some occasional weed proportions above 10% of the aboveground biomass, mainly constituted by *P. rhoeas* and *M. recutia*, those species were usually lower than the top canopy layer which is primarily measured by USH. However, the leaf angle, canopy structure and movement of plant organs affect the deflection of the ultrasonic signal and may lead to the detection of subordinate layers instead (Hutchings, 1991, 1992). Linear relationships between biomass and USH with maximum R^2 value were observed for RCG and the diversity mixture which indicated a consistent increase of biomass along the height gradient. A linear trend between sward height and biomass was also reported by Hutschings *et al*. (1990) and Trott *et al*. (2002), who investigated biomass-height relationships on permanent grassland.

Biomass predictions using exclusive leaf canopy analyzer measurements delivered poor results compared to USH. A possible reason was pointed out by Harmoney *et al*. (1997), who compared the ability of LAI-2000 measurements to determine the biomass yield of grassland swards with sward height estimations using the visual obstruction method. Hence, the wide viewing angle of the optic instrument can detect light passing through vegetation surrounding the clipped areas, which distorts the LAI readings for unknown sward properties. The better results for reed canary grass compared to the multiple species mixtures are supporting this. The structural appearance of the RCG sward was more homogenous during the vegetation period, except for the efflorescence in June and July when the sward significantly increased in height while the rate of biomass increase had not changed. This is also indicated by the low standard deviation of LAI for RCG compared to the mixtures. The diversity mixture has the highest spatio-temporal variability in the sward structure due to different maturity stages of the species in the composition. As a result, the prediction model is compromised. Another problem arises at high levels of LAI. As pointed out by Gower *et al*. (1999), the estimation of LAI with the gap-fraction method, as used by the LAI-2000, reaches an asymptotic saturation as the LAI approaches 5-6. This range was reached for the standard and diversity mixture in the late first and second growing period in June/July and August/September (Fig 2c), resulting in an underestimation at these dates. Good predictions were achieved at the early samplings in May and June when the vegetation height and density was low. However, LAI 2000 measurements can be difficult on pasture plots with low sward conditions. Below-canopy measurements are needed, which might not be feasible in early succession stages because of the sensor height which is 3 cm (Welles and Norman 1991). The combination of USH and LAI increased the prediction accuracy significantly for all swards while reducing the prediction error by 20% compared to exclusive USH and 50% compared to exclusive LAI. The inclusion of LAI seems to compensate for the saturation effect in a pure USH model in lodging swards like the standard mixture and for single emerging flowers like in RCG. The influence of LAI was less at lower sward heights, where the estimates of a pure USH model were more accurate. On the other hand, sward height compensates for saturation effects of

LAI-2000 measurements in dense swards with a high lateral variability like the diversity mixture and the standard mixture. The prediction accuracy of an exclusive LAI model was also positively affected by the addition of spectral VIs and in most cases increased significantly. All prediction models incorporated significant interaction terms between simple or squared LAI and VI values, which show a distinctive synergy between the two sensors in relation to sward biomass. However, the model performances had a high variation depending on the individual VI and sward type. High R^2 values for the NDSI waveband combination were found in the short wave infrared region around the 3rd and 4th water absorption bands at 1450nm and 1800nm and in the 2100nm region similar to the exclusive NDSI approach. Only small improvements were achieved with waveband combinations in the red edge region, which coincides with the low improvement of an inclusion of REIP into the exclusive LAI model. Exclusive LAI prediction accuracies increased significantly when paired with better performing exclusive vegetation indices in the red/NIR region like SR, NDVI and SAVI. Exceptions were combinations of SR and NDVI in the RCG mixture which had no significant correlation to biomass but still delivered R^2 values of more than 0.6 in the combined sensor approach.

The addition of traditional VIs did not significantly improve the prediction accuracy of pure USH. Wavelength selected NDSIs in combination with USH showed a similar pattern for regions of higher relevance as for exclusive NDSI and in combination with LAI. However, the highest R^2 values for this sensor combination were found in a small band in the red edge region in combination with the second water absorption band at 1200nm. The significance of the red edge is also reflected in the higher accuracy of the USH x REIP prediction model compared to the other traditional VIs, especially in RCG. The variation of R^2 values for all waveband combinations was generally low, which indicates a strong dominance of sward height as a declaring variable in the USH x NDSI sensor combination. This is particularly shown in the diversity mixture, where, out of the traditional VIs, only REIP and WI had a significant contribution in the prediction model. Regarding the low improvement for the USH x VI sensor combination compared to exclusive USH and the three sensor combination compared to USH x LAI, USH seems to detect most of the variability in the swards that is covered by the VIs. Therefore, the inclusion of VIs could not contribute much additional information to the USH x LAI model but significantly increased the number of terms. As a result, redundant information of VIs increased the R^2 values of the three-sensor combinations slightly, especially in RCG, but created an overfitting of models. This is also indicated by the cross validation results, which remained in a similar margin as the two sensor combination.

The usefulness of integrated sensor combinations as a tool for biomass mapping in precision agriculture or quality analysis is not only dependent on the accuracy of the prediction but also on the effectiveness of the sampling procedure for each sensor and the vulnerability to environmental conditions. Data acquisition with sensors mounted on tractors and harvesters, while standard on the go management procedures are conducted, are preferable to static measurements. On the go measurements with USH have been shown to give good results in the center of the legume grass swards (Fricke *et al*. 2011) and spectral sensors are already established tools in precision farming. However, the results in this study have shown that USH is vulnerable to lodging swards, which might occur in high swards or after heavy rainfall. Even plant movement during a slight breeze may obstruct USH measurements. Gap fraction derived LAI measurements are difficult to implement for on-the go applications, as sensors need to be inserted regularly at the canopy basis which may create additional gaps in the sward and may cause dirt and damage on the sensor. Thus, technical developments are necessary to exploit the full benefit of combined sensor measurements.

4.5 Conclusions

It was shown that the combination of multiple sensors can significantly improve the prediction accuracy for biomass of extensively managed grassland in floodplains with minor dependence on sward maturity and sward diversity. The results from this study suggest that exclusive ultrasonic height measurement is a well suited predictor for biomass for various grassland communities. However, the sensor has weaknesses at high levels of biomass and sward height. The combination of USH with LAI can increase the prediction accuracy, minimize the weaknesses of the single sensors and reduce the prediction error by 30%.

This study has also shown that vegetation indices and hyperspectral data may not be well suited to predict biomass. Further, sensor-specific NDVI-type vegetation indices derived by wavelength selection are superior to many traditional VIs, especially in combination with LAI measurements. However, a combination of all three sensor types does not significantly improve the prediction performance of an USH and LAI approach and may lead to an overfitting of the model.

5 Effects of sample preparation and measurement standardization on the NIRS calibration quality of nitrogen, ash and NDFom content in extensive experimental grassland biomass

Abstract Near infrared spectroscopy (NIRS) is a common method to analyze the quality of grassland biomass. However, the effort required for sample preparation and measurement can restrict the sampling rate and prediction quality. To assess suitable approaches for animal feeding and bioenergy recovery of grassland biomass, NIRS calibrations for N, ash and ash-free neutral detergent fibre content (NDFom) of hay, silage and standing swards of botanically diverse grassland communities were developed. Seven methods, representing different combinations of measurement conditions and sample preparation, were applied on silages, hay and standing sward and compared using a bench-top system (FOSS XDS Rapid Content Analyzer) and a field spectrometer system (ASD Fieldspec 3). Considering standard error of cross validation (SECV) and its inverse ratio to sample standard deviation (RPD) as the main parameters for comparison, best results were acquired for all constituents at laboratory measurement conditions with dried and ground samples on the bench-top system (RPD > 3) with a coefficient of determination R2 < 0.9. The lower degree of standardization of measurement conditions on the field spectrometric methods lead to lower RPD values and a higher SECV compared to the laboratory approaches. Predictive accuracy of calibrations for nitrogen and NDFom using hay of greater particle size were still acceptable for a farm-scale application in combination with a field spectrometer (RPD > 2). Calibration of ash content was challenged by high ash contamination during sampling and open canopies in the field which resulted in lower calibration quality especially on silages and standing sward. Predictions for silages and standing sward were poor probably due to moisture and structural heterogeneity and, thus, only suitable for a crude high-low differentiation.

5.1 Introduction

The chemical composition of grassland used in animal feeding and bioenergy production varies as a function of several factors including herbage species and nutrient availability (White et al. 2004; Khalsa et al. 2012), stage of maturity (Waramit et al. 2011; McEniry and O'Kiely 2013), harvesting method (Hughes et al. 2012; Meehan et al. 2012) and storage (Beck et al. 2009). Wet chemistry is the traditional way to characterize forages for their nutritive value. However, these techniques are often destructive, expensive and time consuming and are not really adapted to real-time feedstuff analysis. A fast and accurate prediction of nutritive parameters is a key to optimize feeding regimes for both ruminant livestock and bioenergy conversion. In this context, near infrared spectroscopy (NIRS) is an advantageous technique for many applications because it can provide a rapid, non-destructive analysis for multiple parameters. Near infrared reflectance spectroscopy (NIRS) has been first addressed as a useful tool for evaluation and quantification of chemical composition of forages by Norris et al. (1976). Nowadays NIRS is routinely used in the feedstuff industry as a tool to determine feedstuff composition for quality control (Cheli et al. 2012) and is the only tool for analysis of large-scale materials and real-time evaluation of multiple constituents (Roberts et al. 2004). Statistical procedures are used to develop and quantify relations between the spectral reflectance data and reference values obtained by wet chemistry. NIRS calibrations for various biochemical, abiotic and structural substrate characteristics in bioenergy production and animal feeding of grassland biomass have been successfully developed. Nitrogen belongs to the most frequently measured constituents in forages and feedstuffs (Roberts et al. 2004). Besides nitrogen, the most common parameter to be estimated by near infrared spectroscopy is fibre with ADF and NDF being the most frequently reported fibre components. NDF content has been reported on dried samples of botanically complex grassland and forage samples (Garcia Ciudad et al. 1993), grass silage (Park et al. 1998), as well as on fresh pastures samples (Alomar et al. 2009). Ash content is often considered as a problematic parameter in NIRS. Although minerals do not absorb in the near infrared region, NIRS is able to detect endogen ash content in forages associated in complexes with organic compounds. High amounts of soil contamination may include characteristic wavelength of the silica reflectance spectra (Windham et al. 1991) and strongly affects the baseline of the spectrum in the 1100–1300 nm zone (Paul 1988).

Robust and accurate prediction of unknown samples depends upon a calibration set that is consistent with the chemical and structural parameters of the target population. Sample preparation and measurement conditions of the calibration set and the predicted samples should match for good results (Stuth et al. 2003). Therefore, forage samples are usually collected from the field, dried, ground to a small particle size and scanned using a bench-top NIRS spectrometer at standardized laboratory conditions. Faster, more direct techniques of data acquisition are required on a farm scale where the rapid demand of biochemical and structural parameters is countered by excessive, time consuming sample preparation. Determination of standing forage quality with portable spectrometers reduces laborious sampling and sample preparation and provides real time data acquisition often at a cost of prediction quality due to the heterogeneous nature of the samples (Starks et al. 2004). Relatively few studies have been published in recent years to compare the effect of sample preparation and storage conditions of forages on NIRS to determine quality parameters. Alomar et al. (2003) studied the effect of heat- and freeze-drying on silage samples in preparation for NIRS. Tyson et al. (2010) and Gherardi Hein et al. (2010) compared different particle sizes of eucalyptus parts for NIRS calibrations of carbohydrate content and showed that extensive sample preparation and standardization of measurement conditions does not always improve prediction accuracy. Tyson et al. (2010) reached better results on intact eucalyptus pulp than for milled samples. Terhoeven-Urselmanns et al. (2008) reached a higher calibration quality for fresh samples of Chinese cabbage than for dried samples.

The purpose of this study is to compare the effects of different methodical approaches in NIRS on calibration quality of highly diverse European grassland communities cultivated in field experiment which comprised 82 different plant species combinations of up to 4 functional groups (i.e. grasses, legumes, tall and small herbs) and up to 60 grassland species. Seven NIRS methods were tested on Nitrogen, ash and NDFom content for a variety of sample conditions (standing sward, silage, hay) of grassland biomass which differed in the intensity of sample preparation (chopping and milling) and measurement standardization (field spectroscopy, mobile plant probe, laboratory spectroscopy). Method specific calibrations were used to test the following hypotheses:

I) NIRS calibration for high-diversity European grassland communities can be developed in a sufficient accuracy for application in animal feeding and bioenergy production with cross validation errors not exceeding 1/3 of the standard deviation of wet chemistry reference values.

II) Predictive accuracy of NIRS calibrations increases with increasing sample preparation from the measurement of undisturbed swards in the field to dried and milled samples analysed in the laboratory.

III) Incident radiation and distance between sensor and substrate during spectroscopic measurement affect the calibration quality with distant spectral data acquisition being less powerful than close spaced configurations of substrate, light source and sensor, respectively.

5.2 Material and Methods

5.2.1 Study Area and Sample Origin

This study was carried out within the Jena biodiversity grassland experiment based on a pool of 60 mesophyllic grassland species from Molinio-Arrhenateretea meadows. The experimental design comprised a gradient of 1, 2, 4, 8, 16 and 60 species with a gradient of up to four functional groups containing grasses, small herbs, tall herbs and legumes covering a wide range of grassland vegetation structures for broad-based calibrations. Spectral data were collected on the experimental field-site and on hay and silage samples of the 3x3m core areas of the 82 big plots located on the floodplains of the river Saale in Jena (Thuringia, Germany, 50°55’N, 11°35’E, 130m a.s.l). Plots were unfertilized and mowed twice per year (end of May and end of September). Additional information about the plot design can be found in Roscher (2004).

Field spectroscopy on standing sward canopy was conducted in 2008 and 2009 right before the first and the second cut 3 cm above soil surface from three randomly placed 50x50 cm squares in the core area of each plot. Afterwards, biomass for NIRS measurements and chemical analysis was harvested separately from each plot. Randomized subsamples for wet chemistry were taken from the 50x50 cm squares formerly used for spectral measurement. Biomass for hay and silage preparation was harvested from the surrounding 3x3 m core area of each plot and dried in a forced-air oven at 65°C for 48 hours. Remaining biomass was chopped to an average length of 1 cm for silage preparation and randomly collected subsamples were ensiled without additives in 2l glass vessels. Dried and ensiled material was stored at room temperature at dry and dark conditions until start of NIRS measurements in 2010.

5.2.2 Reference Analysis

Dried fresh matter was analysed for nitrogen, ash and ash-free neutral detergent fibre (NDFom) content to develop a constituent reference dataset for all methods.
Nitrogen (N) concentration was determined using an elemental analyser (vario MAX CHN, Elementar Analysesysteme GmbH, Hanau, Germany). Approximations for sample ash content were determined by combustion in a muffle furnace at 550°C for 12h.
Due to limited laboratory resources a subdivision of only 100 samples was analysed for NDFom content according to Van Soest et al. (1991), assayed without heat stable amylase and exclusive residual ash. Samples were selected by spectral Mahalanobis distance to obtain a representative cross section for the whole dataset. Hay samples were ground through a 1-mm screen with a FOSS sample mill (CyclotecTM 1093, Haan, Germany) and calibrated with a XDS-Rapid Content Analyzer NIRS system (Foss NIRSystems, Hillerød, Denmark). With the resulting calibration model (R^2=0.96; SECV: 25.8 g/kg DM) NDFom content of the remaining samples was predicted.

5.2.3 Sample preparation and spectral data collection

Spectral data were collected in the field and laboratory on standing sward, hay and silages of the various grassland vegetation. The seven methods applied were coded by three letters representing the measurement set-up, the spectroscopic device and the sample condition.
Prior to harvest, spectral data were collected on each subsample spot at a distance of 0.67 m above the canopy layer of the standing swards in an area of 0.07 m² with a lightproof hardware device connected to a portable spectrophotometer (ASD Fieldspec®3 Analytical spectral Device, Inc., Boulder, CO, USA) (method DFW). During measurements plants were provided with constant illumination by three 50W tungsten halogen bulbs (Tab. 5). Four repeated measurements in the range of 350-2500 nm were taken at three spots representing the average species composition of each plot. Spectral data were recorded as 1/R (R=reflectance). The spectral device was calibrated with a white spectralon calibration panel right before each measurement.

NIRS reflectance spectra of hay were obtained with a XDS-laboratory spectrometer and an ASD Fieldspec3 field spectroscopy system. For each system two methodical approaches were applied to record data: The distance field spectroscopy on hay (DFH) represents a low level of sample preparation and method standardisation. Hay samples were cut to a maximum particle size of 10 cm and measured with the field spectrometer at a distance of 67 cm above the sample surface in a topless non-reflecting box which was covered by a lightproof fabric tissue. The measured sample surface covered a total area of 0.07m² and was constantly illuminated by three 50W tungsten halogen bulbs. Spectra of samples were recorded twice as an average of 40 measurements. As a second approach we conducted contact field spectroscopy on hay (CFH) by replacing the lightproof hardware device from DFH with the ASD plant probe foreoptic accessory to collect spectral data. The plant probe is designed with an inbuilt 6W halogen light source to record spectra in a 0.5 cm² area in direct contact with the material. Twenty spectra as an average of 40 measurements each were recorded equally distributed throughout the box. Spectra were recorded at 20 equally distributed spots within the box as an average of 40 replicated measurements at each spot. In both methods samples were re-mixed after each recorded spectrum. The spectral device was then calibrated according to manufacturer's recommendations with a white spectralon calibration panel.

Tab. 5: NIRS-measurement set-up and sample condition of the applied methods

Methodcode	NIRS-device	Measurement set-up	Material	Particle size
[a]DFW	ASD Fieldspec 3	Fibre optic; Distance	Fresh	Un-harvested field canopy
[b]DFS	ASD Fieldspec 3	Fibre optic; Distance	Silage	1 cm
[c]DFH	ASD Fieldspec 3	Fibre optic; Distance	Hay	10 cm
[d]CFH	ASD Fieldspec 3	Plant probe; Contact	Hay	10 cm
[e]TLS	FOSS XDS RCA	Transport cell	Silage	1 cm
[f]TLH	FOSS XDS RCA	Transport cell	Hay	0.6 cm
[g]QLH	FOSS XDS RCA	Quarzt cuvette	Hay	0.1 cm

[a]DFW: distance field spectrometry on standing sward; [b]DFS: distance field spectrometry on silage; [c]DFH: distance field spectrometry on hay; [d]CFH: contact field spectrometry on hay; [e]TLS: transport cell laboratory spectrometry on silage; [f]TLH: transport cell laboratory spectrometry on hay; [g]QLH: quartz cuvette laboratory spectrometry on hay

The combination of the laboratory spectroscopy system and the transport cell filled with coarsely ground samples of hay (method TLH) represented a further increase in sample pretreatment and measurement standardization. Samples were milled through a 6 mm screen with a Retsch cutting mill (SM 100, *Retsch* GmbH, Haan, Germany) and measured twice on the Foss XDS Rapid-Content-Analyzer laboratory NIRS system equipped with the transport cell (measured area 40 cm²).

The most intensive sample preparation was represented by measurements with the XDS spectrometer using finely ground samples of hay in a circular quartz cuvette (method QLH). Samples were ground through a 1mm screen with a FOSS sample mill (CyclotecTM 1093, Retsch GmbH , Hahn, Germany). Since this method is identical with the reference method for NDFom content, calibration quality of this method was compared with other methodical approaches only on the basis of samples, for which wet chemistry reference values were available to avoid circular calibration.

Silage samples remained untreated prior to measurements and were measured immediately after being excavated from the glass vessels to minimize moisture losses and deterioration. Spectra were recorded in two methodical approaches identical to methods TLH and DFH and were coded as TLS and DFS accordingly.

5.2.4 Calibration Development and Statistics

Calibration development was conducted using WinISI III (version 1.63) calibration software package (Infrasoft International, Port Matilda, PA, USA). Due to technical reasons in sample preparation, spectra for the first harvest 2008 were excluded from calibration process of hay and silage samples. The second harvest 2008 was excluded from the standing sward method due to methodical inconsistencies between the years 2008 and 2009. Spectra were averaged plotwise for each method to reduce random noise effects and additionally smoothed using Savitzky-Golay smoothing filter (11 point 5th order filtering operation) (Savitzky and Golay, 1964). For spectral data acquired by the XDS spectrometer the full VIS-NIR region from 400-2500nm was used for calibration development. Fluorescent light noise from artificial illumination at the upper and lower end of the spectrum from the Fieldspec3 spectra was visually identified and the spectra were subsequently trimmed to 420-2400 nm. To facilitate calculations spectral data were reduced according to Azzouz et al. (2003), keeping the first of eight data points, leaving 256 data points for calibration. Those treatments showed good results on most constituents in earlier studies (Perbandt et al. 2010a, 2010b). Calibration was performed with a modified partial least square regression method (MPLS) (Martens and Næs, 1989) with four cross validation segments. The number of terms was used as recommended by the WinISI software. SNV and first polynomial detrend transformation was used for scatter and slope correction (Barnes et al. 1989). We tested numerous combinations of pre-processing associated with the regression model combining first and second order derivatives and 4 to 12 points smoothing operations to get best results for calibration. A first order derivative over a 4 point interval with an additional 4 point smoothing operation appeared to provide the best calibration results. All spectra from each method were subjected to this mathematical treatment to facilitate direct method comparison. H-outlier and samples for which the predicted value heavily exceeds the reference value (T-outliers) were removed from the calibration set according preset limits of the WinISI software.

For CFH the influence of repeated measurements on the calibration quality was measured. Separate calibrations were carried out based on 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 20 recorded spectra for each sample. Spectra for calibration were selected equally distributed in a 5x4 spots matrix of the sample box.

Prediction accuracy of calibrations was assessed by cross-validation. For method comparison three parameters were used: The coefficient of determination of the calibration (R^2), standard error of cross validation (SECV) and the residual predictive deviation (RPD) of the cross-validation which is the standard deviation of reference values divided by the SECV (Williams and Sobering, 1996). These parameters are widely used in NIRS to compare calibration performance.

5.3 Results

5.3.1 Reference Data

As a consequence of the high plant diversity gradient in the experimental design and sampling at two very different stages of growing season nitrogen, ash and NDFom content showed a large variation in the sample set (Tab. 6). Mean ash content is slightly higher than ash content found in other studies on extensive grassland (e.g. Richter et al. 2010). Heavy rainfall may have caused considerable plant contamination with soil right before the second cut 2008 and thus increased the overall ash content of samples taken.

Tab. 6: Descriptive statistics of reference data parameters for nitrogen, ash and NDFom.

Constituent	N	Mean g/kg DM	Min g/kg DM	Max g/kg DM	[a] S.D. g/kg DM
N	245	19.3	9.5	38.4	5.4
Ash	245	100.2	56.4	411.5	32.5
[b]NDFom	245	424.4	211	672.3	81.8

[a]S.D.: standard deviation of reference values; [b]NDFom: neutral detergent fibre content assayed without heat stable amylase and exclusive residual ash

Contaminations could not be removed entirely in the sample preparation process and therefore were incorporated in the reference dataset. Remarkably low NDFom values below 300 g/kg DM were reached on samples from the second cut 2009 containing only plants of the small herbs and tall herbs fraction.

5.3.2 Calibration results

The calibration and cross validation statistics are listed in Tab. 7. As anticipated the calibration quality increased from low to high levels of method standardization. Calibrations developed on silage samples and standing sward were of low quality. Especially determination coefficients for ash content did not exceed a R^2 of 0.7. High R^2 on ash, above 0.9, could only be reached for laboratory spectroscopy on hay in method TLH (R^2: 0.9) and QLH (R^2: 0.93). Predictive accuracy in cross validation was also the lowest for ash in combination with method DFS (RPD: 1.27; SECV: 14.3 g/kg) and only slightly higher in combination with the TLS approach on silages (RPD 1.42; SECV 11.5 g/kg) and with standing sward, method DFW (RPD: 1.33; SECV: 12.3 g/kg). DFW also delivered the lowest values for nitrogen (RPD: 1.31; SECV: 3.9 g/kg) and NDFom (RPD: 1.37; SECV: 29.8 g/kg). The wide gap between R^2 (0.72) and 1-VR (0.42) for nitrogen with the DFW method indicates inconsistent results among the single cross-validation groups which suggest a lack of robustness in the calibration model. The same applied for method DFS with R^2 0.73 and 1-VR 0.49. Predictive accuracy for silage material could only be slightly improved with the transition from the field spectroscopic approach (DFS) to the laboratory spectrometric system (TLS).

The spectroscopic approaches on dried material delivered better results than on silage samples or standing sward. Comparing methods DFS and DFH as well as TLS which shared the same measurement design but differed in sample characteristics revealed a decrease in SECV of 30% for nitrogen, 35% for ash and almost 50% for NDFom in the laboratory approach. The error for DFS and DFH on NDFom only decreased slightly. Method CFH which is characterized by an identical sample condition and spectrometric system as DFH but has a narrower distance of measurement resulted in a lower cross validation error for nitrogen of 2.2 g/kg (CFH) compared to 2.4 g/kg (DFH) and for NDFom (0.73 g/kg; 0.77 g/kg). The error for ash increased from 9.4 g/kg (DFS) to 10.8 g/kg (CFS). The best values were achieved with method QLH, where the highest level of sample standardization occurred. Significantly higher predictive accuracy was achieved for all constituents with method TLH which is probably due to the increased heterogeneity of the unground material in the latter method. Best results were acquired for nitrogen (RPD: 5.58; SECV: 0.9 g/kg) with a low coefficient of variation (SECV/mean) of <0.05 of mean reference values. Ash (RPD: 3.07; SECV: 7.4 g/kg) and NDFom (RPD: 3.42; SECV: 25 g/kg) also delivered good results.

Tab. 7: NIRS calibration and cross-validation statistics of nitrogen, NDFom and ash content for standing sward, hay and silage.

Constituent	Method code	N	Mean g/kg DM	[h] S.D. g/kg DM	[i]SEC g/kg DM	R²	[j]SECV g/kg DM	[k]1-VR	[l]Slope	[m]RPD
N	[a]QLH	234	19.1	5.2	0.8	0.98	0.9	0.97	0.97	5.58
	[b]TLH	235	19.2	5.3	1.9	0.87	2.1	0.84	0.86	2.53
	[c]CFH	231	19.2	5.4	1.8	0.88	2.2	0.83	0.81	2.4
	[d]DFH	225	19.2	5.2	2.2	0.82	2.4	0.79	0.88	2.18
	[e]TLS	230	19	5.1	2.6	0.73	3	0.65	0.73	1.69
	[f]DFS	227	18.7	4.8	2.5	0.73	3.4	0.49	0.69	1.4
	[g]DFW	150	19.3	5.0	2.7	0.72	3.9	0.42	0.62	1.31
Ash	QLH	224	96.9	22.6	5.9	0.93	7.4	0.89	0.91	3.07
	TLH	220	96.5	22.5	7.3	0.9	8.2	0.87	0.88	2.73
	CFH	214	94.8	18.1	9.2	0.74	10.8	0.65	0.79	1.68
	DFH	220	93.9	17.2	7.6	0.8	9.4	0.70	0.74	1.83
	TLS	217	93.2	17.5	11.7	0.55	12.3	0.50	0.47	1.42
	DFS	220	94.1	18.2	13.2	0.47	14.3	0.37	0.47	1.27
	DFW	148	88.9	15.3	9.0	0.65	11.5	0.44	0.24	1.33
[n]NDFom	QLH	143	416.8	85.3	23.6	0.92	25	0.92	0.91	3.42
	TLH	229	431.2	76.2	27.8	0.87	29.6	0.85	0.87	2.58
	CFH	234	427.1	77.3	39.0	0.75	40.6	0.73	0.80	1.9
	DFH	230	429.1	79.5	35.5	0.8	38.3	0.77	0.74	2.08
	TLS	231	426	76.5	42.1	0.7	52.8	0.53	0.67	1.45
	DFS	231	425.9	76.2	43.9	0.67	45.5	0.65	0.68	1.68
	DFW	142	419.4	68.3	38.5	0.68	49.8	0.53	0.54	1.37

[a]QLH: quartz cuvette laboratory spectroscopy on hay; [b]TLH: transport cell laboratory spectroscopy on hay; [c]DFH: distance field spectroscopy on hay; [c]CFH: contact field- spectroscopy on hay; [e]TLS: transport cell laboratory spectroscopy on silage; [f]DFS: distance field spectroscopy on silage; [g]DFW: distance field spectroscopy on standing sward; [h]S.D.: standard deviation of reference value; [i]SEC: standard error of calibration; [j]SECV: standard error of cross validation; [k]1-VR: coefficient of determination of cross validation; [l]slope of reference values vs. NIRS; [m]RPD: S.D./SECV; [n]NDFom: neutral detergent fibre content assayed without heat stable amylase and exclusive residual ash

Significantly higher predictive accuracy was achieved for all constituents with method TLH which is probably due to the increased heterogeneity of the unground material in the latter

method. Best results were acquired for nitrogen (RPD: 5.58; SECV: 0.9 g/kg) with a low coefficient of variation (SECV/mean) of <0.05 of mean reference values. Ash (RPD: 3.07; SECV: 7.4 g/kg) and NDFom (RPD: 3.42; SECV: 25 g/kg) also delivered good results.
Quality assessment of hay with the contact probe (CFH) produced differing results depending on the number of replicates in the measurement. As expected the RPD increased with measurement replicates (Fig. 5) and a maximum RPD within experimental boundaries was reached at 19 measurements for nitrogen (RPD: 2.18) and 20 measurements for ash (RPD: 1.83) and NDFom (RPD: 2.08). Calibration statistics for the different numbers of measurement are shown in Tab. 8.

To determine the optimal measurement number a logarithmic regression was fitted to the RPD values and the regression value at 20 measurements was set to 100% of maximum achievable prediction quality within the experimental design.

Tab. 8: NIRS calibration statistics for increasing measurement number for nitrogen, ash and NDF content

Constituent	Parameter	Number of Measurements										
		1	3	5	7	9	11	13	15	17	19	20
N	R^2	0.74	0.77	0.81	0.79	0.8	0.81	0.80	0.81	0.81	0.83	0.82
	[a]RPD	1.66	1.91	2.12	2.01	2.06	2.10	2.06	2.12	2.13	2.18	2.18
	[b]SECV (g/kg DM)	3.11	2.74	2.42	2.61	2.55	2.49	2.56	2.47	2.48	2.40	2.41
Ash	R^2	0.51	0.66	0.74	0.76	0.76	0.78	0.79	0.83	0.83	0.83	0.80
	RPD	1.26	1.49	1.52	1.66	1.67	1.69	1.78	1.89	1.85	1.93	1.83
	SECV (g/kg DM)	13.64	11.62	11.29	10.37	10.36	10.19	9.70	9.14	9.32	8.95	9.42
[c]NDFom	R^2	0.66	0.74	0.75	0.75	0.80	0.79	0.79	0.79	0.79	0.80	0.80
	RPD	1.56	1.81	1.86	1.81	2.06	1.98	1.99	1.99	2.01	2.02	2.08
	SECV (g/kg DM)	49.37	43.31	41.98	42.76	37.55	40.24	39.93	40.08	39.55	39.59	38.32

[a]RPD: ratio of standard deviation of reference dataset to standard error of cross validation; [b]SECV: standard error of cross validation; [c]NDFom: neutral detergent fibre content assayed without heat stable amylase and exclusive residual ash

As 100% could not be achieved due to the logarithmic type of regression, the number of measurements for optimal quality/effort ratio within experimental boundaries was defined as 95% of that for 20 measurements (Fig. 5). Optimal measurement numbers for nitrogen and NDFom were reached at 10 and 11 measurements respectively. The regression for ash content followed a steeper trend, hence, the optimal quality/effort ratio was attained at 13 measurements.

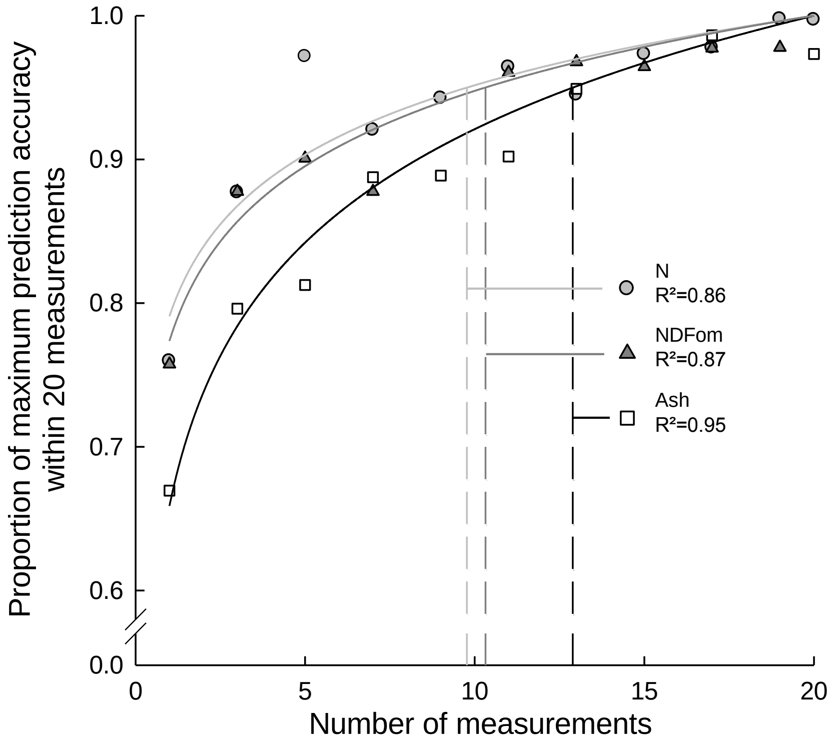

Fig. 5: Increasing prediction accuracy by number of repeated sample measurements for N, ash and NDF reported on an ash free basis, assayed without heat stable amylase (NDFom) of grassland hay. Maximum prediction accuracy within the experiment boundaries is determined by the value at 20 measurements resulting of logarithmic regression of calibrations' RPD values with corresponding number of repeated measurements per sample (N: y = 7.4632Ln(x) + 0.7483; NDFom: y = 6.978Ln(x) + 0.79089; ash: y = 11.722Ln(x) + 0.65469). Respective measurement numbers at 0.95 of maximum quality for each constituent are indicated with dashed vertical lines.

5.4 Discussion

The RPD value is often used for determining prediction accuracy of calibrations as it is independent of the scale of the reference parameter.

Many differing RPD classifications can be found in literature referring to different NIRS methods and substrates like soil properties (Chang et al. 2001) or a classification for cereal grains (Williams and Sobering, 1993) which is also often used for grassland applications. The often cited Williams and Norris (2001) consider RPD values greater than 3 as adequate for analytical purposes in most of NIRS applications for agricultural products. In this study RPD values greater than 3 were only reached for the approach of highest standardization (QLH) on all three constituents. A RPD value >5 for N further indicates excellent predicting capabilities. While this classification is suitable for a high degree of standardization measurement, sample conditions at field or crop scale reduce prediction power ,therefore, lower RPD levels may still indicate good calibration results (Perbandt et al. 2010b; Teerhoven-Urselmann et al. 2008). In that case an acceptable RPD value would be 2 or higher (Cohen et al. 2005). In this study calibrations for nitrogen and NDFom developed on hay samples in combination with the field spectrometric device can therefore be regarded as acceptable for prediction of unknown samples, whereas calibrations for silages and standing sward and field spectrometric calibrations for ash content are at best suitable for differentiating high and low values.

Slope of reference vs. NIRS values suggest an overestimation of samples low in ash and NDFom and an underestimation of samples with high contents which was also observed in de Boever et al. (1995) on two different compound feeds for cattle and Kjos (1991) on Norwegian forage samples. This effect was more prominent for fresh silage and standing sward than for hay samples.

The poor cross validation statistics of standing sward measurement may be partly explained by varying soil reflectance due to open canopies. As some plots were scarcely populated, soil reflectance on standing sward measurements has to be taken into account as it significantly alters the plant spectrum.

Many studies use selected wavebands to reduce data or to circumvent water absorption bands of wet samples. Biewer et al. (2009a) on the other hand received better calibration results for legume-grass swards with the full spectrum instead of selected wavebands. They also reached higher RPD values for nitrogen and ash content using a similar portable spectrometric device, which could have been due to structural and chemical heterogeneities in the different plant communities of the present experiment challenging the calibration process much stronger. Heterogeneity lessened when the samples were dried, ground and homogenized which resulted in better quality parameters for methods of higher standardization level.

Murray and Cowe (2004) recommended omitting the longer wavelengths in favor of the Herschel infrared region (780-1100 nm) for extremely wet samples as silages. Beyond 1400 nm, water in wet tissue will be the largest absorber compared with other major constituents (Murray and Cowe, 2004). Cozzolino et al. (2006) found RPD values >2 using the Herschel region for calibrations of crude protein content on fresh silage samples but results for NDFom were only slightly better than in the present study. Starks et al. (2004) developed calibrations for nitrogen and NDF for remotely sensed data of the standing sward of Alicia Bermuda grass using a portable spectroradiometer covering wavebands in the 368-1100 nm region. The SECV for NDF in that study was lower than observed in method DFW and SECV for nitrogen was on the same level. As spectroscopic devices covering a wider range of wavebands are usually more expensive, calibrations using reduced wavebands could probably be more efficient for wet samples.

As for silages, other studies have shown that accurate prediction models for nitrogen are possible (Park et al. 1998). However, RPD for ash was also below 3 and therefore only acceptable for on-farm samples. Unlike the latter study, silage samples from this study were not ground before measurements increasing the material's heterogeneity and, thus, contributing to the significantly lower calibration quality for those parameters.

Further, calibration quality may have been reduced due to the fact, that reference data in this study were acquired from dried and ground material with a higher grade of standardization than fresh silages used for spectral data assessment. Vranić et al. (2005) found lower SECV values for calibrations on fresh silages, referring to reference values recalculated on a fresh matter basis compared to a dry matter basis. Particularly NDFom values could have been affected by the drying process. Regarding undried samples calibrations of fibre content Alomar et al. (2003) found a significant increase of 10% in NDF content of pasture samples after aerobic oven drying at 60°C for 48h and suggested the use of freeze drying in reference methods for calibration of undried samples. However, the level of increase differed among substrates. Burrit et al. (1988) for example found a significant increase in NDF of up to 93% at drying temperatures of 40°C in extrusa samples of esophageal fistula forage collected in the wet season (January to April) in northeast Brazil, whereas samples from the dry season showed no significant increase in NDF content. Thus, regarding the wide range of NDFom content and differing sampling conditions temperatures of 65°C in the reference sample preparation procedure may have increased the NDFom content of some samples and therefore decreased the calibration quality.

In contrast to wet matter samples, calibration and cross validation results for hay seem to be in line with findings of other studies. Our results for nitrogen on ground hay samples (QLH) are similar to the findings of Garcia Ciudad et al. (1999) for NIRS calibrations of dried

and ground samples of high diversity grassland. SEC values on ash content with laboratory approaches (QLH: 5.9 g/kg DM) and (TLH: 7.3 g/kg DM) were consistent with results of Redshaw et al. (1986) on hay of legume grass mixtures and higher than findings of Vasquez de Aldana (1996) on pastures from different sites of semi arid grassland communities (SEC: 4.6 g/kg DM; R^2:0.88). Garcia Ciudad et al. (1993) reported calibration results for NDF of hay with an SEC of around 20 g/kg DM for selected wavelengths on botanically complex grassland in central western Spain which confirms this study's findings.

5.5 Conclusions

This study showed that a broad-based calibration model of highly diverse European grassland communities using NIRS is possible and may provide acceptable prediction accuracy for application in animal feeding and bioenergy production.

Calibration quality increased with sample preparation and measurement standardization. Best results were achieved on a laboratory NIRS device in combination with dried and ground samples. The field spectrometric approaches on dried samples, representing a lower degree of sample preparation and measurement standardization are still able to deliver an acceptable predicting capability for nitrogen, ash and NDFom content depending on the level of desired accuracy. While slightly increasing the predictive accuracy for calibrations of nitrogen and NDFom, the lower distance to the sample of the contact measurement compared to the distance measurement did not increase the accuracy of calibrations for ash content. However, calibration models developed in this study are barely suitable for prediction of silages or standing swards although a differentiation of low and high values may be possible. For this material NDFom and ash content and, to a lower extent, nitrogen content was noticeably underestimated by NIRS at higher values.

6 Effects of species richness and functional groups on methane yields from anaerobic digestion in batch fermenters: results from experimental grassland silages and press-fluids

Abstract This study examines the influence of species richness and functional groups on the substrate- specific methane yield from batch experiments on silages and press fluids and the related area-specific gross energy yield of Central European grassland communities along a well-defined diversity gradient (1-60 species) and across different combinations of functional groups (legumes, small herbs, tall herbs and grasses).

Overall, species richness and the presence of most functional groups showed only minor or no influence on the methane production, As an exception, grasses had a positive effect on the substrate specific methane yields. Methane yields of grassland silages from a first cut were substantially higher than from the second cut in autumn. Increased biomass production on high diversity populations lead to an increased area specific methane yield compared to low diversity populations. Effects of biodiversity on the substrate specific methane yields of whole crop digestion were even lower for pressfluids which were produced by mechanical dewatering after hydrothermal conditioning.

6.1 Introduction

Semi natural grasslands are among the world's greatest hotspots of biodiversity on a smaller scale (Wilson et al. 2012) but due to changes in land use in the last decades, leading to either intensification or abandonment of the target areas, biodiversity is expected to decline (e.g. Poschlod et al. 2005 and Kleijn et al, 2011).

Conservation of those habitats is dependent on continuous human intervention, traditionally grazing and mowing for animal husbandry. In developed countries forage quality of semi-natural grassland cut is decreasingly suitable for animals with high milk and meat performance, leading to farmers abandoning their grassland in favour of high productive meadows (Isselstein et al. 2005). This leads to a surplus of permanent grassland that requires other means of management to maintain and protect species richness (Mitchley 2001).

Revenue from bioenergy production of waste biomass is an economic incentive for farmers to manage low value grassland and maintain biodiversity. According to Prochnow et al. (2009a) grassland is a well suited substrate for many ways of bioenergy production, like a feedstock for biogas production or solid fuel for combustion. Out of the pool of substrates used for biogas production in Germany, grass silage is already among the most commonly used co-substrates after maize (Weiland, 2006). An ideal feedstock for methane production should be rich in fermentable carbohydrates, lipids and proteins, and at the same time be poor in hemicelluloses and lignin (El Bassam, 1998). Also, the ratio of fibre to protein, often expressed by the carbon to nitrogen ratio (C/N), is an important factor for high methane yields, as too high fibre contents will limit energy availability (Buxton and Redfearn, 1997) and too high protein concentrations may lead to process failure owing to ammonia accumulation (Zubr, 1986).

The chemical composition of crops is significantly affected by environmental factors (e.g. soil fertility, precipitation and temperature), management (e.g.harvest date, cutting frequency, fertilization) and botanical composition of the sward (McEniry and O'Kiely 2013). Several recent studies have validated the importance of sward maturity on methane yields, showing that substrate specific methane yield decreases with advancing sward maturity due to an increasing concentration of lignified fibre and hemicellulose with low digestibility (Amon et al. 2007; Prochnow et al. 2005, McEniry and O'Kiely 2013).

The process of integrated generation of solid fuel and biogas from biomass (IFBB, Wachendorf et al. 2009) is one of several pre-treatment methods, developed in recent years to reduce fibre content and to improve digestibility of the biogas substrate. In train of the process low digestible fibre content is separated from soluble nutrients, resulting in the press fluid (PF), an optimized substrate for anaerobic fermentation.

Only few studies have addressed the influence of botanical composition in species rich grassland on the methane yield in anaerobic digestion. Khalsa et al. (2013) had issued the impact of species richness and functional group compositions on the methane yield potential based on the chemical composition of the biomass according to VDI 4630 (2004), corrected for empiric digestibility values of the respective functional group compositions. It was concluded that substrate specific methane yields ($CH4_{sub}$) decline with increasing species richness (SR) with antagonistic impacts of grasses and legumes. On the other hand, area specific methane yields ($CH_{4\ area}$) increase due to a higher biomass production on high diversity swards. However, accordance of calculations based on chemical composition and actual methane yields from batch digestion experiments have yet to be validated.

The aim of this study was to systematically approach the relationships of biodiversity patterns in respect to functional group composition (presence/absence or abundance) and SR with substrate specific methane yield ($CH_{4\ sub}$) and area specific methane yield ($CH_{4\ area}$) derived from anaerobic digestion. The approach was conducted on silage samples and IFBB press fluids from the field site of the Jena Experiment (Roscher et al. 2004) digested in batch fermentation experiments.

6.2 Materials and methods

6.2.1 Experimental design

In May 2002 an experimental site with semi-natural mesophilic grassland was established in the floodplain of the River Saale (near Jena, Thuringia, Germany, 50°55' N, 11°35' E, 130m a.s.l.). Mean annual air temperature in the Jena area is 9.3°C with an annual precipitation of 587 mm (Kluge et al. 2000). The site had originally been grassland and was converted into arable land around 1960. Soil conditions resemble Eutric Fluvisol (FAO, 1994) and soil texture changes from sandy loam to silty clay with increasing distance to the river (Fig. 6).

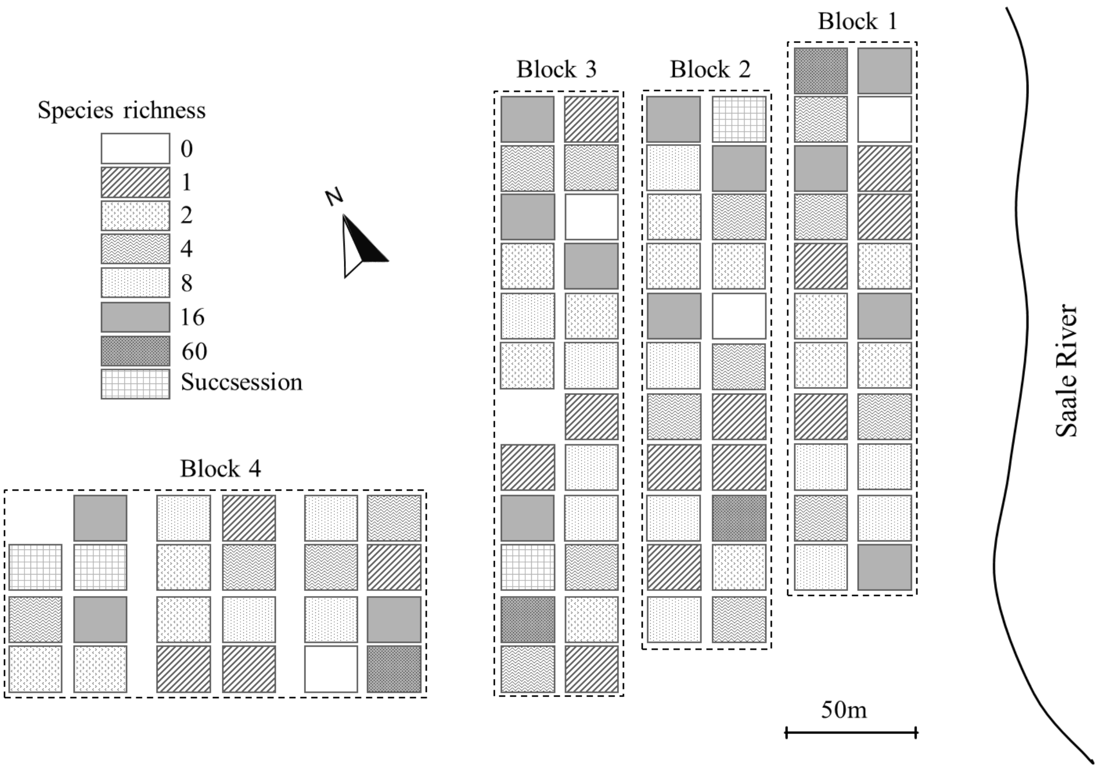

Fig. 6: Map of the Jena-Experiment showing the different species richness treatments and their distribution across the four blocks (Khalsa, 2013).

Sixty plant species were used to create a gradient in plant species richness (SR) (1, 2, 4, 8, 16, and 60) and in functional group richness (FGR) (1, 2, 3, and 4). Functional groups were defined, according to the morphological, phenological and physiological traits of the plant species, as grasses (n = 16), small herbs (n = 12), tall herbs (n = 20) and legumes (n = 12) (for detailed list of plant species see Roscher et al. (2004). Eighty-two plots (20 x 20m) were established on four blocks accounting for the differences in soil texture. 16 possible combinations of SR and FGR were realized and replicated over the four blocks. The location of the mixtures within each block was fully randomized. Management of the site was two cuts per year (late May and late August) with no additional fertilization. Plots were weeded twice per year to maintain the original species diversity. The experimental setup is described in full detail in Roscher et al. (2004)

6.2.2 Biomass sampling

Aboveground biomass for batch experiments was harvested twice per year in 2008 and 2009, in late May and late August right before the maintenance cut. Due to technical problems in silage production PF from 2008 was not considered in the analysis and comparisons for press fluid and whole crop digestion where made only on the basis of the first cut in 2009. Aspects regarding only whole crop digestion where made on the basis of the whole dataset containing samples of two cuts in the years 2008 and 2009.

Three randomly placed samples of 20 x 50 cm were cut by hand at 3 cm stubble heights. Biomass was separated into target species, dead plant material and weeds, dried (70°C, 48 h) and weighed. Total biomass (t DM ha^{-1}) was derived from an average of the three samples. Annual biomass was calculated as the sum of biomass from the first and second cut. Additional 300g aboveground biomass was sampled from each plot (n = 82) for both years and both cuts (total n = 164) for forage quality analysis.

The rest of the biomass on the 3 x 3m core area of each plot was harvested (3cm above soil surface), chopped at a mean length of 1cm and ensiled without additives (>90 days) in 2l glass vessels. Additional biomass of the first cut per plot and year was ensiled in 50 l polyethylene barrels for hydrothermal conditioning. The low pH and the bacterial activity during ensiling enhance the disintegration of the plant material and thereby promote the mass-flow (MF) of minerals and nutrients from the parent material (PM) into the liquid phase, also referred to as press fluid (PF), during hydrothermal conditioning.

6.2.3 Hydrothermal conditioning and mechanical dehydration

Each sample of the first cut went through a hydrothermal conditioning process at the end of the ensiling period according to the procedure of integrated generation of solid fuel and biogas from biomass (IFBB) (Wachendorf et al. 2009). The hydrothermal conditioning was conducted in a modified concrete mixer, which contained a mixture of PM and water in a proportion of 1:4. The material was heated by gas burners and kept at a constant temperature of 60°C while being continuously stirred for 15 minutes. Afterwards, the mash was separated into the PF and a solid phase, also referred to as press cake (PC), by mechanical dehydration using a screw-press (Type Av, Anhydro Ltd., Kassel, Germany). The conical screw had a pitch of 1:6 and a rotational speed of 6 revolutions min^{-1}. The cylindrical screen encapsulating the screw had a perforation of 1.5 mm.

Samples of PM before and after hydrothermal conditioning, as well as PF and PC, were analysed for DM content after 48 h drying at 105°C.

6.2.4 Chemical composition analysis

The silage and IFBB press cake (PC) was analyzed for C, H and N using an elemental analyser (vario MAX CHN, Elementar Analysensysteme GmbH, Hanau, Germany). Ash content was determined by combustion at 500°C in a muffle furnace. Neutral detergent fibre (NDF), acid detergent Fibre (ADF) ,acid detergent lignin (ADL), crude Fibre (CF), crude lipids (CL), the macronutrients phosphorus (P) and volatile compounds like ethanol, butyric-, lactic- and acetic acid as well as the pH value were determined according to standard methods (Naumann and Bassler, 1976). Hemicelluloses were calculated as the difference between NDF and ADF, cellulose content was calculated as the difference between ADF and ADL. ADL was assumed to approximately matching lignin content. Concentrations of phosphorus in PC and silage were predicted with a near-infrared-spectrometer (XDS Rapid Content Analyser, FOSS NIRSystems Inc., Laurel, USA) using calibration equations developed on similar biomass (R^2: 0.81).

The concentration of chemical compounds (represented by Z; g kg^{-1} DM) in the PF was calculated from the concentration of Z in the silage (SIL), PC and the DM concentration of silage after hydrothermal conditioning (SILC) and the PC, the PF after mechanical dehydration according to:

$$Z_{PF} = \frac{DM_{SILC} \cdot Z_{SIL} - Y \cdot DM_{PC} \cdot Z_{PC}}{X \cdot DM_{PF}} \qquad \text{(EQ 6)}$$

X and Y are quantities of the PF and the PC as a proportion of the silage after hydrothermal conditioning, respectively, which were calculated by:

$$X = \frac{DM_{PC} - DM_{SILC}}{DM_{PC} \cdot DM_{PF}} \qquad Y = 1 - X \qquad \text{(EQ 7)}$$

6.2.5 Digestion Experiments

Determination of substrate specific methane yields ($CH4_{sub}$) for PF and silage was performed in a batch process in accordance to VDI 4630 (2004) for whole crop digestion tests in 20L gas proof polyethylene containers. Digesters were filled with 8 kg fresh matter (FM) of inoculum containing digested slurry from a biogas plant and filled up with 4 kg of water and a feedstock of 400g FM of silage for whole crop digestion or 4kg FM of press fluid. Two Digesters were filled with 8 kg inoculum and 4 kg of water to monitor the biogas potential of the inoculum. Electrical stirrers mixed the material every 3 h for 15 min. The experiments were maintained in a mesophile temperature range of 37°C, with a fluctuation of ±1°C through a warmed water basin equipped with a heating unit, a circulating pump and a temperature sensor. The fermentation time was 14 days for press fluid and 35 days for silage to reach the abortion criterion for the fermentation period suggested in VDI 4630. Further details on the biogas sampling procedure and measurements of methane yield were described in Richter et al. (2009). To account for a possible loss of volatile organic matter during the drying process (Buffière et al. 2008), the DM content in the PM and PF was corrected for volatile compounds using determined concentrations of volatile compounds according to Weißbach et al. (2008).

The area specific methane yield ($CH_{4\ area}$) was calculated as the product of substrate specific methane yield and the biomass yield of each plot. The energy content of methane was calculated with 37.78 MJ per Nm³. The annual yield is the sum of both cuts both cuts as an average of two years.

6.2.6 Statistical analysis

The Jena experiment was designed to vary SR, FGR and functional group composition (FGC) as orthogonally as possible. While a fully balanced design is not possible as e.g. the lowest

SR cannot be combined with highest FGR, it can still be statistically accessed by analysing the dependent variable in an analysis of variance (ANOVA) with sequential sum of squares (Schmid et al. 2007). In this type of analysis variables that are fitted before others take up all the variation they can explain, ignoring the possibility that the later variables might also explain some of this variation (Hector et al. 2010). The characteristics of this type of analysis can then be used to identify effects that are independent of the variables fitted before.

In the resulting model block effects were fitted first to take account for the gradient in soil conditions, block wise weeding and mowing. It can therefore be assumed that the variance explained by variables fitted after the block effect is independent of the block effect.

SR was fitted first after block effects as this parameter is a major factor in the experimental design. To test the effects of SR the log linear contrast of 1 to 16 species was used. The 60 species plots were used as a reference for maximum diversity but were not included in the statistical analysis. The presence/absence of functional groups was fitted after SR to test for their individual effects. For silages, all values used in the ANOVA were mean values of two years separated for each cut but for press fluids only data of 2009 was regarded due to inconsistencies in the batch fermentation process between samples of both years.

Multiple regression analysis was conducted on all plots to estimate the influence of functional group abundance and chemical constituents on $CH_{4\ sub}$ by selecting the terms for inclusion in the model depending on standard statistical model selection methods (Draper and Smith 1998) This implies that effect terms with $p < 0.05$ were included according to the rules of hierarchy and marginality (Nelder, 1994 and Nelder and Lane, 1995). All statistical analyses were done in R 2.14.1 (R Development Core Team, 2012).

6.3 Results and Discussion

6.3.1 Biomass yield and chemical composition of the substrate

The dry matter content of biomass is an important factor for the silage fermentation process, which finally determines the silage quality as a substrate for biogas production. Optimal dry matter concentrations range between 30 and 40% (DLG Praxishandbuch Futterkonservierung, 2006). Concentrations below 30% may result in an undesirable excess fermentation and might increase the amounts of lactic and acetic acids in the forage and also increase the probability of butyric acid production and, therefore, lower the energetic value of the biomass. Dry matter concentrations above 40% might reduce the pre-fermentation effect of the ensiling and the silage will likely have a decreased digestibility of fibre and starch contents. The dry matter content in this study decreased slightly along the diversity gradient and in the presence of legumes in the functional group mixtures. The presence of grasses had a counteracting effect in the first cut and increased the dry matter content. However, this effect was not significant on the second cut (Tab. 10). The overall dry matter concentration in the ensiled biomass was around the lower end of the optimal range and below (Tab. 9), especially for the first cut and legume monocultures. 8 silos of the first cut and 43 silos of the second cut of both years could not be used for batch experiments due to either degraded silos or low biomass yield, especially on low species communities. As a consequence, those plots were not considered for sample characteristics and statistical analysis. As sample losses were evenly distributed throughout the biodiversity pattern of FGR, FGC and SR, it was not regarded as being detrimental to the orthogonal experimental design.

The ideal substrate for biogas conversion has a high concentration of easy degradable compounds (Klimiuk et al, 2010). High concentrations of lignocellulosic structures in the biomass might reduce the degradability and may result in a lower biogas yield (Klimiuk et al, 2006; Triolo et al. 2012, Li et al; 2013). Lignin concentrations above 100 g kg^{-1} are considered detrimental for methane production (Triolo et al, 2012). This critical value was surpassed for the average lignin concentrations in the small herbs monocultures. Grass monocultures had the highest concentrations of hemi-cellulose and cellulose while having the lowest concentrations of lignin. In particular, the concentration of hemicellulose was more than two times as high as in any other functional group monoculture (Tab. 9). The high cellulose to lignin ratio in grasses might be beneficial compared to the other functional groups, as it has been shown that fermentation of grasses results in a higher digestibility of cellulose per given amount of lignin compared to legumes (Tomlin et al. 1965). SR had no significant effect on the concentration of fibre fractions.

Regarding the essential macronutrients a ratio of 600:15:5:1 for C:N:P:S should be sufficient for anaerobic fermentation (Weiland, 2010). The mean C content was 456 g kg^{-1} oDM with slightly lower concentrations in the second cut. The abundance of legumes had a positive effect on the C content of the 2^{nd} cut biomass but did not affect significantly the first cut material (Tab. 10).

Nitrogen ranged between 11 and 49 g kg^{-1} at an average of 19 g kg^{-1} with small differences between cutting regimes. The N content was negatively correlated to SR in the second cut and declined from 22 g kg^{-1} to 17 g kg^{-1} from 1 to 60 species, whereas the first cut was not significantly affected by SR. The presence of legumes increased the nitrogen content in both cuts, whereas the presence of grasses had a decreasing effect on the first cut biomass only (Tab. 10). This was also reflected in the mean values of the functional group monocultures which contained the highest amount of N for legumes and the lowest amount for grasses (Tab. 9). Depending on favourable environmental conditions, Weiland (2010) suggests a C/N ratio between 15:1 and 30:1 for an optimal anaerobic fermentation process. At higher ratios, excess carbon cannot be converted efficiently by bacteria after available nitrogen resources are depleted. Lower ratios may lead to nitrogen toxicity and inhibit the fermentation process. The minimum C/N ratio was reached for most functional groups and diversity levels (Fig. 7a). However, grass monocultures on the first cut, rich in cellulose, vastly exceeded the suggested range, while the average C/N ratio of legume monocultures was located at the lower end of the suggested range for an optimal conversion (Fig 7b).

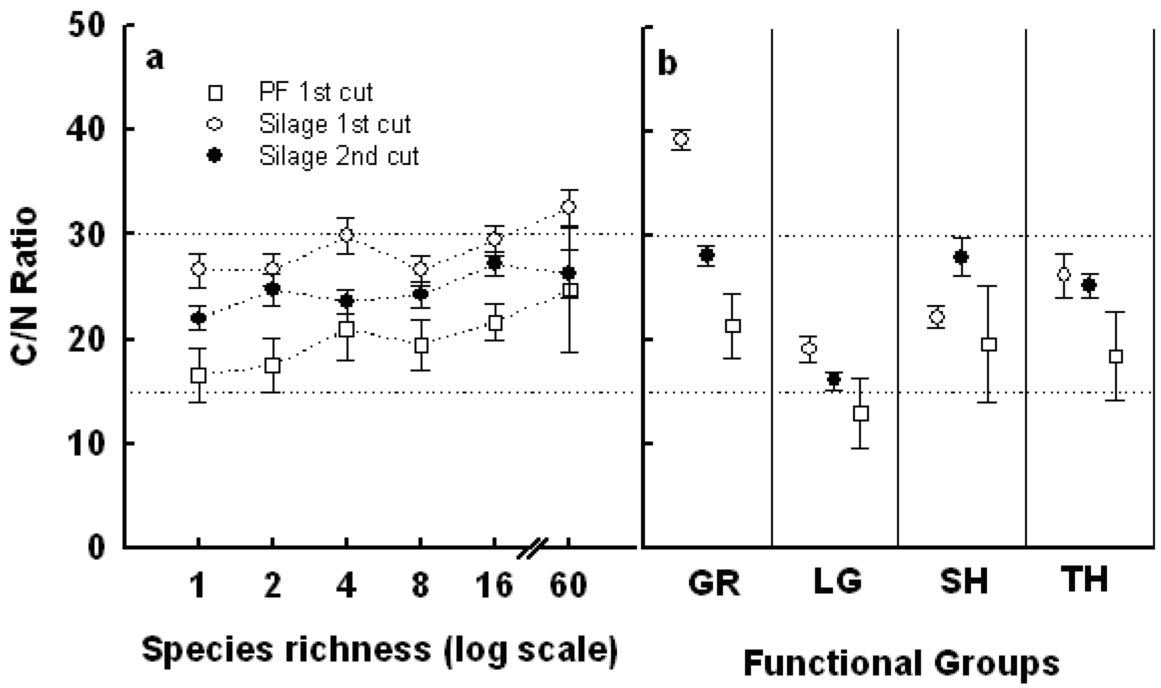

Fig. 7: Mean C/N Ratio of silage from the first and second cut averaged over 2008 and 2009 and press fluid from the first cut biomass 2009 as means of species richness (a) and functional groups monocultures (b).

Phosphorus content showed a wide variation between 1.8 and 7 g kg^{-1} oDM and decreased significantly at higher C/N ratios in the first cut biomass ($p < 0.01$). It was also negatively affected by the abundance of grasses and positively affected by the abundance of legumes in the first cut material (Tab. 10). However, the correlation between abundance of functional groups and phosphorus content was not significant for the second cut, although the C/N ratio was still positively affected by the abundance of grasses. Similar to the P concentrations, the concentrations of S were positively influenced by the presence of legumes and negatively influenced by the presence of grasses. Additionally a negative correlation between species richness and S concentration has been observed.

Tab. 9: Mean values of organic dry matter, carbon, nitrogen, hydrogen, phosphorus and fibre fractions (hemicellulose, cellulose and lignin) of silages, including standard deviation (SD) in functional group monocultures as well as the total mean. Values are means of 2008 and 2009.

	Grasses		Legumes		Small herbs		Tall herbs		Common swards	
Parameter	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
oDM (g kg^{-1} FM)										
1st cut	308.32	± 73.33	248.94	± 50.86	232.0	± 53.85	247.76	± 66.50	248.68	± 64.61
2nd cut	330.82	± 109.62	249.74	± 66.49	348.8	± 129.33	298.92	± 92.68	317.75	± 107.48
Carbon (g kg^{-1} DM)										
1st cut	455.08	± 5.34	463.67	± 6.19	456.0	± 8.10	456.24	± 4.56	457.52	± 6.57
2nd cut	448.35	± 8.81	459.18	± 5.63	444.1	± 14.94	436.34	± 40.40	448.74	± 18.55
Nitrogen (g kg^{-1} DM)										
1st cut	11.71	± 1.14	25.85	± 6.76	21.21	± 3.33	18.75	± 4.57	17.92	± 5.74
2nd cut	16.27	± 2.20	29.45	± 5.09	16.61	± 3.54	17.74	± 2.83	19.69	± 5.54
Hydrogen (g kg^{-1} DM)										
1st cut	55.49	± 0.54	56.05	± 0.80	54.7	± 0.88	54.59	± 1.36	54.82	± 4.52
2nd cut	55.74	± 1.15	55.93	± 1.19	53.0	± 1.74	51.55	± 5.57	54.08	± 2.75
Phosphorus (g kg^{-1} DM)										
1st cut	2.46	± 0.33	3.07	± 0.78	3.25	± 0.72	3.29	± 0.60	2.94	± 0.57
2nd cut	3.75	± 0.69	3.23	± 0.63	4.50	± 0.99	4.25	± 1.06	3.70	± 0.85
Hemicellulose (g kg^{-1} DM)										
1st cut	232.31	± 53.10	89.27	± 49.75	83.8	± 38.43	100.84	± 59.52	132.95	± 69.78
2nd cut	210.44	± 61.62	56.62	± 33.62	82.7	± 54.91	58.35	± 58.51	101.63	± 70.81
Cellulose (g kg^{-1} DM)										
1st cut	314.95	± 40.93	235.52	± 74.29	210.9	± 86.53	267.08	± 67.40	270.31	± 63.85
2nd cut	269.39	± 33.46	207.38	± 46.30	226.7	± 59.07	205.85	± 41.88	233.38	± 47.63
Lignin (g kg^{-1} DM)										
1st cut	49.86	± 16.21	83.34	± 37.95	109.5	± 65.46	72.10	± 21.02	74.32	± 33.29
2nd cut	45.16	± 14.71	90.77	± 29.52	108.0	± 35.54	78.89	± 24.05	82.02	± 31.17

Tab. 10: Analysis of variance of dry matter, carbon, nitrogen and phosphorus concentration in the silage (in g kg^{-1} DM). Shown are the effects of species richness (SR) and the presence/absence of individual functional groups on the dependent variables. All values are means of both years. Arrows indicate and increase (↑) or a decrease (↓) with the presence of the individual functional group.

Factor	DF	Dry matter		Carbon		Nitrogen		Phosphorus	
		F	P	F	P	F	P	F	P
Concentration in the 1st cut parent-material									
Block	3	6.78	<0.001 ↓	2.50	0.07	0.39	0.76	5.04	0.003 ↑
Log(SR)	1	15.18	<0.001 ↓	0.04	0.84	0.11	0.74	2.04	0.16
Legumes	1	14.37	<0.001 ↓	3.57	0.06	7.70	0.007 ↑	5.06	0.03 ↑
Grasses	1	16.66	<0.001 ↑	1.66	0.20	20.60	<0.001 ↓	17.09	<0.001 ↓
Tall herbs	1	0.41	0.52	1.46	0.23	1.84	0.18	11.55	0.001 ↑
Small herbs	1	0.55	0.46	2.04	0.16	0.44	0.51	2.33	0.13
Residuals	61								
Concentration in the 2nd cut parent-material									
Block	3	3.44	0.03 ↓	1.49	0.23	1.45	0.24	1.34	0.27
Log(SR)	1	0.05	0.83	1.25	0.27	7.07	0.01 ↓	3.05	0.09
Legumes	1	9.13	0.00 ↓	5.98	0.02 ↑	45.35	<0.001 ↑	4.76	0.04 ↓
Grasses	1	2.72	0.11	0.01	0.93	1.89	0.18	2.72	0.11
Tall herbs	1	1.14	0.29	0.35	0.56	0.35	0.56	0.31	0.58
Small herbs	1	7.07	0.01 ↑	0.00	0.98	2.40	0.13	0.11	0.75
Residuals	40								

The IFBB process is supposed to mobilize minerals detrimental for the combustion of biomass while keeping the structural components in the solid phase in order to improve its quality as a solid fuel. As a side effect, a liquid phase (PF) with low contents of fibrous fractions is produced, which may provide an enhanced substrate for biogas production.

Fibre-fractions, including ligno-cellulose structures with low digestibility, were almost entirely removed in the IFBB process and were not considered for statistical analysis of PF. However, a certain mass flow of organic compounds into the press fluid is desired as a feedstock for anaerobic microbes, which is needed to cover to electricity demands of the procedure. Across the different diversity levels in the experimental layout, the mean mass flow (MF) of organic dry matter and C content was around 30% (Fig. 8). This matches the results of other studies on the mass flow of dry matter content after hydrothermal conditioning of extensive grassland biomass at the respective temperature (Richter et al. 2011; Wachendorf, 2009).

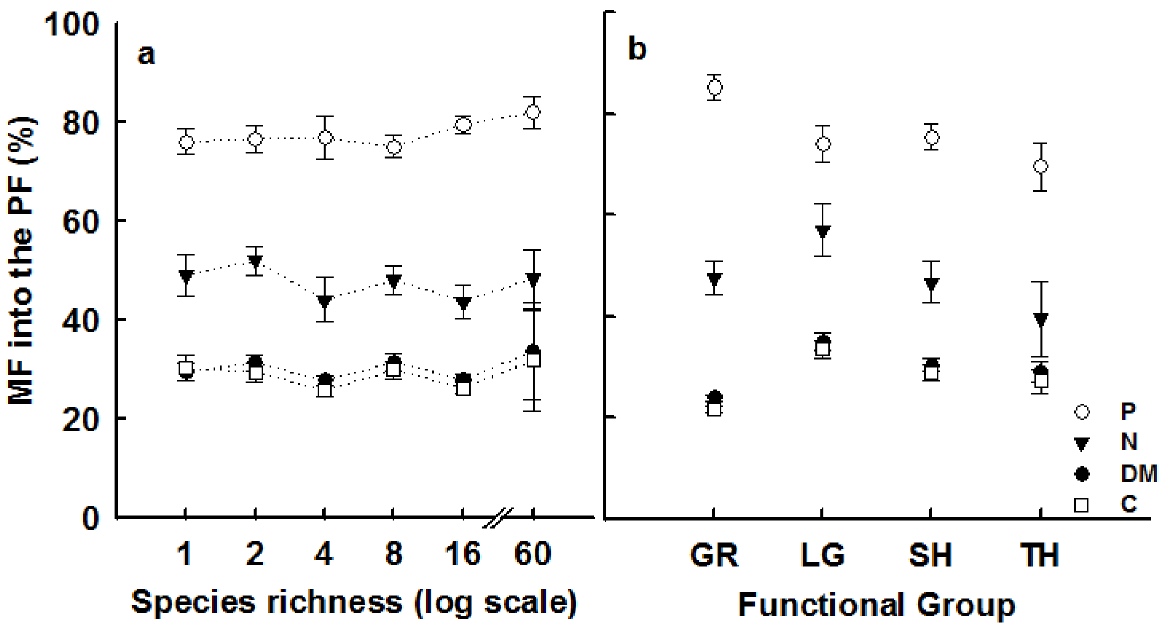

Fig. 8: Mass-flow into the press fluid for dry matter, phosphorus, nitrogen and carbon as means of species richness and functional groups. Values are derived from the first cut material 2009

Tab. 11: Analysis of variance of dry matter, carbon, nitrogen and phosphorus concentration and mass-flow into the press fluid (in g kg^{-1} DM) as means of the first cut material in 2009. Shown are the effects of species richness (SR) and the presence/absence of individual functional groups on the dependent variables. Arrows indicate an increase (↑) or a decrease (↓) with the presence of the respective functional group.

		Dry matter		Carbon		Nitrogen		Phosphorus	
Factor	DF	F	P	F	P	F	P	F	P
Mass-flow into the press-fluid									
Block	3	1.52	0.22	2.15	0.10	0.32	0.81	6.84	<0.001 ↓
Log(SR)	1	0.52	0.48	0.37	0.55	2.10	0.15	0.10	0.75
Legumes	1	8.97	0.004 ↑	10.77	0.002 ↑	1.49	0.23	0.25	0.62
Grasses	1	11.72	0.001 ↓	13.35	<0.001 ↓	0.02	0.89	0.17	0.68
Tall herbs	1	0.05	0.83	0.06	0.81	0.27	0.61	0.11	0.74
Small herbs	1	1.12	0.29	1.30	0.26	2.73	0.10	5.11	0.03 ↓
Residuals	60								
Concentration in the press-fluid									
Block	3	0.74	0.53	0.41	0.75	1.36	0.26	2.22	0.10
Log(SR)	1	1.02	0.32	0.69	0.41	5.02	0.03 ↑	2.49	0.12
Legumes	1	1.06	0.31	0.49	0.49	4.95	0.03 ↑	0.65	0.43
Grasses	1	3.76	0.06	-	0.99	1.84	0.18	1.22	0.27
Tall herbs	1	1.36	0.25	1.12	0.30	1.24	0.27	0.83	0.37
Small herbs	1	0.05	0.82	0.28	0.60	0.12	0.73	0.22	0.65
Residuals	60								

The presence of legumes increased the MF of C and DM into the PF while the presence of grasses had a decreasing effect. Around 50% of N content was transferred into the PF, resulting in an increased C/N ratio compared to the parent material (Fig. 7). As a result, the mean C/N ratio for grass monocultures in PF was located in the range of an optimal anaerobic fermentation procedure (Weiland, 2010) (Fig. 7b). The nitrogen mass flows were the highest for legumes which further decreased the C/N ratio in the PF below the optimal range. This might have lead to an excess in nitrogen within the fermentation process, which can inhibit microbial growth due to ammonia toxicity and restrain the fermentation process (Chen et al. 2008).

Most effects on the chemical composition of the parent material regarding species richness and functional groups were eliminated during the IFBB conversion process, except an increased concentration of nitrogen due to the presence of legumes (Fig. 8 and Tab. 11), which is in consistence with the findings of Khalsa et al. (2013) for the PC of both cuts.

6.3.2 Methane yields and diversity effects

Biogas production in batch fermentation procedures is supposed to be influenced by many factors such as operating temperature, pH value of the digestate, diversity of microorganisms and concentration of trace elements (Rapozo et al, 2012; Weiland, 2010). The actual methane yield is further influenced by retention time, type of digestion system and substrate quality of the feed. Factors related to the digestion system like temperature and slurry composition were kept constant to reduce the variability of the methane yield to the biomass composition of the substrate.

Kinetics of methane production related to PF and PM followed mostly monophasic curves, starting with a steep increase of methane production before slowing down to a plateau state after which no significant gas production had been observed (Fig. 9). This state with a daily biogas production rate of less than 1% of the total volume is defined by VDI4630 as a termination criterion for batch fermentation tests. The termination criterion was achieved after 20 days for PM and only 12 days for PF, and there was hardly any difference among diversity levels and most functional groups. The time period was delayed for grass monocultures of PM from the first cut to 22 days probably due to the increased C/N ratio of the biomass. The termination criterion in the legumes monocultures of the PF was delayed to 13 days. As Zubr (1986) found best results at a C/N ratio of 25, i.e. at the upper end of the suggested range by Weiland (2010), the fermentation process might have been delayed in the legume monocultures due to an excess of nitrogen after IFBB processing, potentially leading to ammonia toxicity.

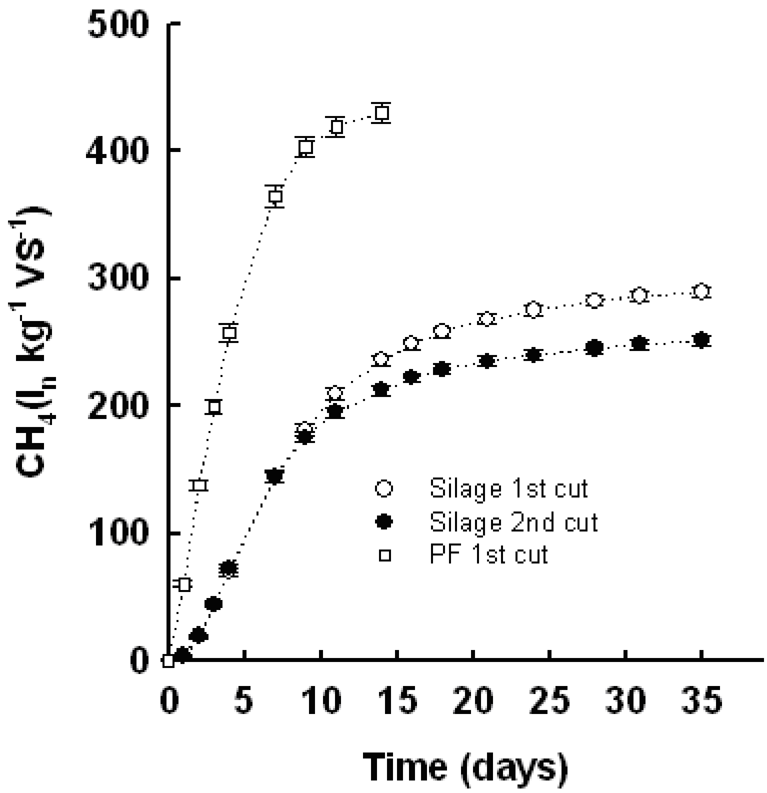

Fig. 9: Cumulated daily methane yield of batch fermentation of silage taken from the first and second cut material of 2008/2009 and press fluid taken from the first cut biomass 2009.

Methane yields after 35 days for whole crop digestion (WCD) and 14 days for PF were considered for direct comparisons among different treatments. The substrate specific CH_4 yields ($CH_{4\ sub}$) of PM varied from 123 to 512 l_n kg^{-1} VS, with an overall mean of 289 l_N kg^{-1} VS for the first cut after a 35 days fermentation period. $CH_{4\ sub}$ yields of the second cut were significantly lower with a mean value of 251 l_N kg^{-1} VS ($P < 0.001$) and a range of 111 to 367 l_N kg^{-1} VS. Maximum values did not reach the level of the first cut, but were more in line with values reported in literature for fresh grass and grass silage (Prochnow et al. 2009b; Murphy et al. 2011). Neither the P content of PF nor PM did correlate with the resulting $CH_{4\ sub}$, which suggests that despite the low C/P ratio P was not a limiting factor in the digestion PM. Among the fibre fractions only the hemicellulose-lignin ratio showed a significant relationship with $CH_{4\ sub}$. In accordance with findings of Alaru et al (2011) hemicellulose-lignin ratio was positively related to $CH_{4\ sub}$ with $R^2 = 0.46$.

The range of $CH_{4\ sub}$ yields for PF was similar to the 1st cut PM (198 to 588 l_N kg^{-1} VS) with a higher average yield of 429 l_N kg^{-1} VS. Statistical analysis revealed no significant differences between SR levels and $CH_{4\ sub}$ both for PM and PF. However, $CH_{4\ sub}$ of PF was positively influenced by the presence of grasses.

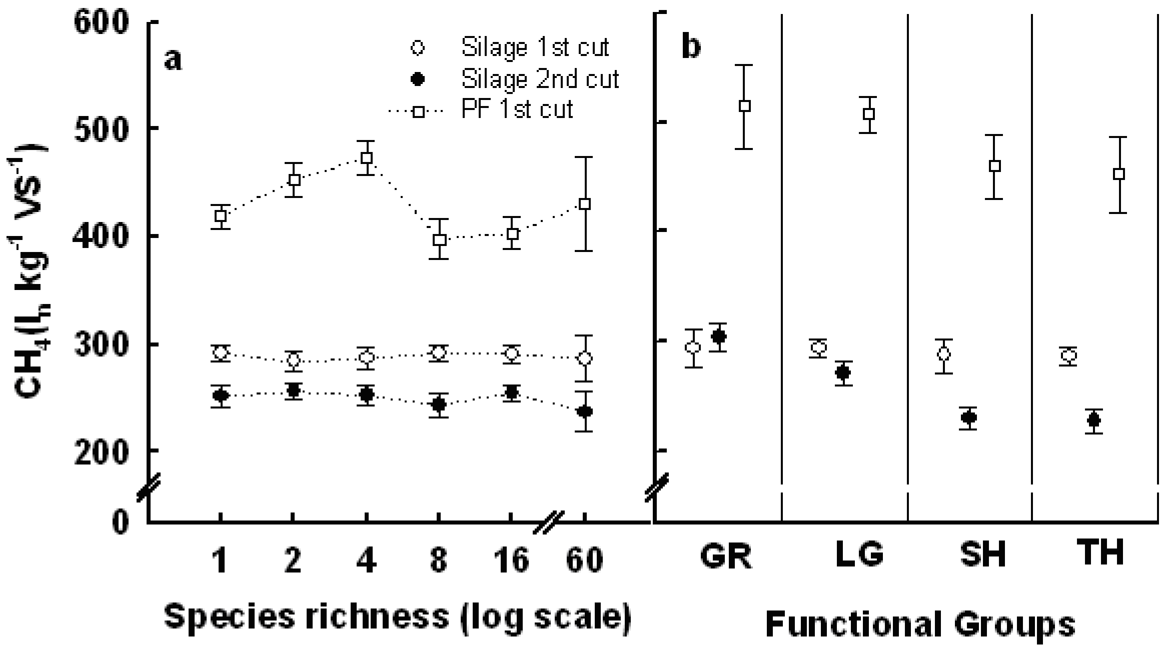

Fig. 10: Average substrate specific methane yield of grassland silage as means of the first and second cut 2008/2009 and pressfluid as means of the first cut 2009. Values are presented as means for species richness (a) and functional group monocultures (b).

While having no effect in the first cut, the presence of grasses also increased $CH_{4\ sub}$ of the PM in the second cut, whereas the presence of herbs reduced $CH_{4\ sub}$ (Tab. 12). This is partially supported by findings of Khalsa et al (2013) who observed an increase in $CH_{4\ sub}$ yield due to the presence of grasses on both cuts. Unlike the other functional groups, the mean values of crude lipids and crude fibre in grass monocultures varied much between the cuts. This resulted in higher fibre content, especially cellulose, and a lower concentration of lipids in the first cut, as well as high amounts of hemi-cellulose and the least amount of proteins, factors that are detrimental to methane production.

Sward biomass yields were investigated by Khalsa (2013) and varied widely at an average of 3.0 t oDM ha^{-1} on the first cut, ranging from 0.2 t oDM ha^{-1} in the scarcely populated functional group monocultures to 7.5 t oDM ha^{-1} in the all functional group mixtures.

Tab. 12: Analysis of variance of $CH_{4\ sub}$ yield of parent material (PM) and press fluid (PF) (in l_N kg^{-1} VS). Shown are the effects of species richness (SR) and the presence/absence of individual functional groups on the dependent variables. All values for the parent material are means of either the first or second cut material of both years. All values for press fluid are means of the first cut 2009. Arrows indicate and increase (↑) or a decrease (↓) with the presence of the respective functional group.

		PM 1st cut		PM 2nd cut			PF 1st cut (2009)		
Factor	**DF**	**F**	**P**	**F**	**P**		**F**	**P**	
Block	3	0,995	0,402	6,918	<0.001	↓	1,334	0,272	
Log(SR)	1	0,768	0,384	0,893	0,350		3,288	0,075	
Legumes	1	1,108	0,297	0,802	0,376		1,885	0,175	
Grasses	1	0,685	0,411	5,494	0,024	↑	4,705	0,034	↑
Tall herbs	1	0,107	0,745	7,545	0,009	↓	0,471	0,495	
Small herbs	1	0,803	0,374	3,480	0,069		0,003	0,960	
Residuals	61								

The average biomass yields leveled off for the second cut to 1.6 t oDM ha^{-1}, ranging from 0.3 t oDM ha^{-1} in the monocultures to 6.6 t oDM ha^{-1} in the all functional group mixtures. The mean $CH_{4\ area}$ yield of the PM was estimated at 879 m^3 ha^{-1} and 392 m^3 ha-1 for the first and second cut with wide ranges of 42 to 2278 m^3 ha-1 and 57 to 1516 m^3 ha-1 respectively.

In compliance with Khalsa et al. (2014), who found that $CH_{4\ area}$ was indirectly affected by SR through a positive correlation between SR and the biomass yield, strong positive relations between $CH_{4\ area}$ and biomass yield (R^2 = 0.91) could be observed, increasing from one to sixty species by 943 m^3 ha^{-1} and by 247 m^3 ha^{-1} in the first and second cut respectively. $CH_{4\ area}$ yield had a mean of 656 m^3 ha^{-1} with a wide range from 131 to 1654 m^3 ha^{-1}, which was largely due to the variance in biomass yield of 0.5 to 7.6 t DM ha^{-1}. Contrarily, biomass of the first cut 2009 was not affected by an increase in SR, therefore, $CH_{4\ area}$ for PF analyzed in this study remained unaffected by SR (Fig. 11). As the biomass yield between the two cuts was significantly different with 3.2 t DM ha^{-1} in the first cut and 1.4 t DM ha^{-1} in the second cut, $CH_{4\ area}$ yield in the first cut (867 m^3 ha^{-1}) was almost double the value of the second cut (445 m^3 ha^{-1}).

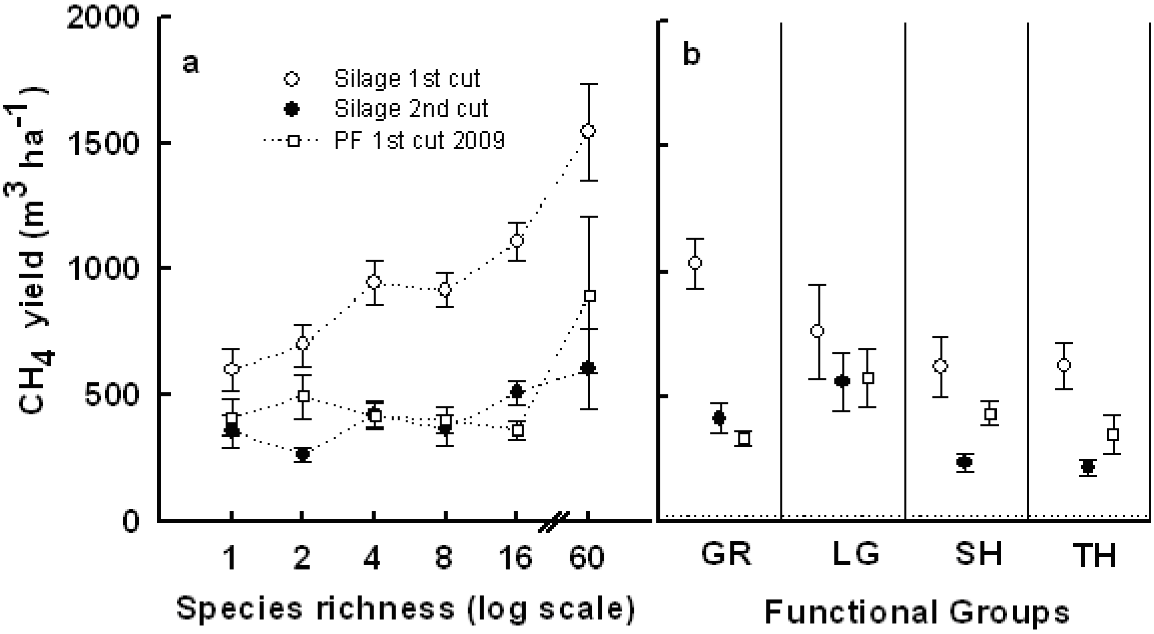

Fig. 11: Average area specific methane yield of grassland silage as means of the first and second cut 2008/2009 and pressfluid as means of the first cut 2009. Values are presented as means for species richness (a) and functional group monocultures (b).

Legume presence had a significant positive effect in the second cut and was also beneficial in the first cut (Tab. 13), which was also reflected in the mean values of the functional group monocultures, where legumes had the highest $CH_{4\ area}$ in the second cut and the second highest in the first cut. This can also be attributed to the positive effect of legumes in functional group mixtures on the area specific dry matter yield (Khalsa et al, 2013). High biomass yields for grasses in the early summer resulted in a positive impact of grasses on the 1st cut $CH_{4\ area}$ yields. However, the lower grass biomass production in late summer reduced the positive impact for the annual yields.

Similar to $CH_{4\ area,}$ the annual $CH_{4\ area}$ had a wide range of 378 to 2595 $m^3\ ha^{-1}$ with a mean of 1292 $m^3\ ha^{-1}$. It was significantly affected by SR ($p < 0.001$) and doubled (979 to 2027 $m^3\ ha^{-1}\ a^{-1}$) from 1 to 60 species. The presence of legumes also had a strong effect ($p < 0.001$) on annual $CH_{4\ area}$ yield, which is in line with the patterns found in the functional group monocultures, where legumes had the second highest annual $CH_{4\ area}$ yield after grass monocultures. (Fig. 11b).

Tab. 13: Analysis of variance of $CH_{4\ area}$ yield of parent material (PM) and press fluid (PF) (in $m^3\ ha^{-1}$). Shown are the effects of species richness (SR) and the presence/absence of individual functional groups on the dependent variables. All values for the parent material are means of either the first or second cut material of both years. All values for press fluid are means of the first cut 2009. Arrows indicate and increase (↑) or a decrease (↓) with the presence of the respective functional group.

Factor	DF	PM 1st cut			PM 2nd cut			PF 1st cut (2009)		
		F	P		F	P		F	P	
Block	3	1,888	0,141		2,805	0,052		2,768	0,049	↓
Log(SR)	1	50,897	<0.001	↑	4,583	0,038	↑	1,911	0,172	
Legumes	1	3,971	0,051		13,502	<0.001	↑	6,948	0,011	↑
Grasses	1	6,211	0,016	↑	1,348	0,252		2,221	0,141	
Tall herbs	1	0,828	0,367		2,133	0,152		0,046	0,830	
Small herbs	1	0,993	0,323		0,118	0,733		1,978	0,165	
Residuals	60									

6.4 Conclusions

This study showed that diversity effects in extensive diverse grasslands can barely be connected to specific methane yields based on batch fermentation experiments. Impacts of species richness on substrate specific methane yields were almost not existent or disguised by wide error margins between replicates. Moreover, species effects were masked by species-specific chemical characteristics in a complex way. Among the functional groups presence of grasses and legumes in the mixtures were most determining factors influencing methane yields, but differed between cuts. While high lignocellulose content and a high C/N ratio in grasses may have reduced the digestibility in PM of the first cut material, excess nitrogen may have inhibited methane production in second cut legumes. Batch experiments proved the superior specific methane yields of IFBB press fluids and showed that detrimental effects of the parent material were reduced by the technical treatment. Due to a positive correlation between SR and the biomass yield, strong positive relations between $CH_{4\ area}$ and biomass yield (R^2 = 0.91) was found. Legume presence had a significant positive effect on $CH_{4\ area}$ and grasses were also beneficial if their contribution to the yield was high.

7 General discussion and conclusions

7.1 Current state of the extensive grassland in Germany

In 2014, around 4.6 million hectare (27.7%) of the total agricultural area in Germany was used as permanent grassland compared to 5 million hectares in 2003 (29.4%) when the European Union acknowledged its ecological value within the midterm reform of the Common Agricultural Policies (CAP). 4% of this area is regarded as low yielding grassland and grassland set aside for nature conservation or rough grazing (Statistisches Bundesamt, 2015). As part of the cross compliance policies, especially European council regulation (EC) nos. 796/2004 and 73/2009, the member states were obliged to define maintenance standards for permanent grassland for the good agricultural and environment conditions (GAEC) to ensure the ratio in relation to the total agricultural area. The total loss should not exceed 10% compared to the reference year 2003 and corrective measures have to be applied if the decrease of permanent grassland is more than 5%. In contrast to most EU member states Germany monitored the development of grassland conversion on a federal state level. This implied that up to the 5% threshold per federal state farmers had no further obligations to meet to plough up their land. Favouring a „first-come, first-served“- land conversion with farmers being afraid of future constraints if they wait too long (Osterburg et al. 2010), the 5% threshold was exceeded in four federal states by 2010. As a result, authorization obligations were implemented for further land conversion and compensation areas demanded for lost grassland. The main driving forces for the decrease of permanent grassland are a lowered grazing livestock and the Renewable Energy Act favouring the cultivation of maize as a feedstock for biogas plants (Rösch et al. 2009). Further, decoupling direct payments from production to farmers in context with the 2003 reforms were detrimental to shepherds without own grazing grounds and resulted in a reduction of sheep and goat livestock in Germany (Huyghe et al. 2014), often grazing on less favourable areas. The low or varying nutritive value of extensive grassland became less attractive for an increasing demand of high yielding dairy cows due to decreasing raw milk prizes.

Grass is already a common feedstock for anaerobic co-digestion in Germany (Weiland, 2006; Prochnow et al. 2009a), granting an incentive for an alternative utilization of the biomass. However, biomass conversion from low yielding grassland is often not financially attractive for farmers. Even though natural handicap payments are given for management of grassland under adverse conditions, permanent grassland is still converted into arable land in favour of more lucrative energy crops. With more than 50% of the converted grassland, forage maize for ruminants or energy production is the most common cultivated follow up crop (Nitsch et al. 2012). This development leads to further abandonment of low yielding and high nature value (HNV) grassland and a surplus of unutilized biomass. By January 2015, in the framework of the "Greening" policies, nationwide authorization constraints were implemented on the conversion of permanent grassland and individual farm quotas were registered, including a new 5% threshold compared to the reference year 2012 for the preservation of permanent grassland.

7.2 Alternative utilization concepts

Direct payments for extensive grassland are connected to a minimum maintenance defined by the "good agricultural and environment condition" (GAEC) standards. High biodiversity levels can only be retained by continuous maintenance, especially in areas threatened by farm abandonment (Heinsoo et al. 2010). Unfortunately, minimum GAEC standards for permanent grassland, e.g. annual mulching, can be considered sufficient to keep the areas open but are not sufficient to prevent a loss in biodiversity on the long run (Kollmar et al. 2009). Alternative utilization or additional concepts of grassland in regions with low nutritive value and difficult to conserve by traditional grazing could promote a sustainable management that meets socio-economic aspects and reduce environmental impacts. About 200.000 ha of grassland are situated in less favourable areas, including 17.500 ha completely taken out of production (Statistisches Bundesamt, 2015). On the one hand, this structurally and biochemically versatile biomass represents an attractive source for the generation of biofuels and biochemical, but the energetic exploitation of highly diverse grassland biomass is supposed to be difficult in conventional conversion systems. Sustainable management of extensive grassland usually requires 1-2 cuts per year with late harvest periods (Prochnow et al. 2005). Extensive grassland with high fibre content, esp. at later maturity stages, has been considered as a promising resource for thermal conversion into energy via combustion (Prochnow et al. 2009b, Rösch et al. 2009), but contains high amounts of elements that contribute to ash formation or become volatile, create harmful corrosion and emission problems (van Loo and Koppejan, 2008).

Although grass is a common co-substrate for biogas production, high concentrations in crude fibre are hardly biodegradable in anaerobic conditions, making the material economically unfeasible for animal feeding or biomethanization (Prochnow et al. 2005, Richter et al. 2011). Adequate methods for pre-treatment and enhancement of the biomass are therefore desirable to increase the energetic value of grass. However, results from chapter 6.3.1 indicate that differences in fibre content and biochemical composition between different sward-compositions were largely masked by the wide substrate specific variance of methane yields in the fermentation process. This might impede the development of strategies for substrate specific enhancement.

Concepts for green biorefineries have been developed in recent years throughout Europe to enhance the biomass value through cascade utilization of the whole biomass, making it more attractive for farmers (Xiu and Shahbazi 2015). Most concepts involve the separation of the biomass into a liquid fraction (pressfluid) and a solid fraction solid fraction (presscake). Regarding the conversion into energy, the system for Integrated Generation of Solid Fuel and Biogas from Biomass (IFBB) utilizes the pressfluid as a feedstock for biogas production and the presscake for combustion (Richter and Wachendorf, 2010). This system inherits the advantage of lower contents of indigestible fibre and as a result higher substrate specific methane yields of the pressfluid (see chapter 6.3.2) and lower emissions rates of the presscake because of partial displacement of detrimental minerals and nitrogen (Wachendorf et al. 2009).

7.3 Optimizing site specific management

Extensive grassland on marginal habitats is characterized by a high spatio-temporal heterogeneity, high species-diversity and variable productivity (Ward et al. 1999, Tockner and Stanford 2002). Although chapter 6 has shown that species richness and functional group composition in diverse mixtures have little effect on the methane yield in batch experiments, harvesting at later maturity stages results in significantly lower methane yields which is also supported by other studies (Amon et al 2007, Prochnow et al. 2009a). C/N ratios out of the perfect range for anaerobic fermentation (15-30:1), e.g. due to high fractions of legumes or grasses in the mixture can also have negative impacts on the fermentation kinetics and methane yields (Weiland et al. 2006). The access to accurate information on the botanical composition can, therefore, be helpful to evaluate the potential of extensive grassland as a substrate for bioenergy provision. Non-destructive methods with a high spatial resolution or sampling rate like remote sensing are recommended to estimate to estimate biomass productivity and quality to derive suitable management practices. In specific remote sensing methods through the calculation of univariate or multivariate vegetation indices like the Normalized Difference Vegetation Index (NDVI) or structural parameters like the Leaf Area Index (LAI) or sward height by Airborne Laser Scanning (ALS) can be used to monitor the vegetation status, estimate biomass and improve management practices (Wei 2010, Zlinszky et al. 2014). The results on multi sensor approaches in chapter 4 indicate that, unlike spectral vegetation indices, sward height is a good predictor for biomass yield in swards with high diversity.

Remote sensing methods with a high spatial and temporal resolution are required to precisely determine biochemical characteristics and productivity of biomass from large scale fields with more than a few hectares (Dusseux et al. 2011). Usually, two different approaches can be distinguished with respect to remote sensing. The first approach comprises point measurement devices like spectrometers. In combination with GPS coordinates these devices create dense discrete measurements on the field that can be interpolated to retrieve a continuous coverage. Although these approaches area very time consuming for data collection on a larger scale compared to aerial and satellite imaging spectral reflection measurements obtained from hand-held spectral radiometers, they are widely used for the characterization of grassland biomass (Mutanga and Skidmore, 2004; Chen et al., 2009) but may contain large amounts of redundant information. In Chapter 4 and 5 multivariate regression and calculation of vegetation indices were used to reduce redundancies in the spectral dataset and create calibration models for biomass (chapter 4) and biochemical parameters relevant for biogas production (chapter 5). Unfortunately, both attempts to create broad based calibration models using NIRS were barely suitable for prediction of vegetation parameters of the sward. But prediction accuracy increased to an acceptable level for field scale after being chopped up to 10 cm stalk length which makes NIRS sensors suitable as a utility for rapid quality assessment on harvesters. The second approach comprises imaging devices like cameras or multi and hyperspectral sensors that record data along a scan line or in a larger measurement array. These sensors are usually attached to aircraft or satellites. Coarse resolution sensors on satellites are currently not suitable to detect the subtle changes of extensive grassland on a mesoscale (e.g. Modis), while high resolution sensors are not suitable to cover intra-annual variability due to a low revisit frequency (e.g. Ikonos, Hyperion) (Lecerf et al. 2005).

However, the launch of high spatial and temporal resolution sensors like Sentinel-2 and Venµs in 2016 might enhance the prospection of grassland at field scale.

7.4 Conclusions

The results from this thesis indicate the difficulties of NIRS-derived prediction models in heterogeneous swards where high structural and biochemical variability is detrimental for accurate predictions. Tested broad based calibration models using the whole hyperspectral dataset or common vegetation indices derived from hyperspectral NIRS measurements were insufficient for an implementation in precision agriculture in extensive grassland when applied on whole crop or biomass with a low degree of standardization. On the other hand, sward height measurements using remote sensing techniques appear to be a promising tool to determine biomass yield. The close connection of biomass and area specific methane yield makes sward height a viable parameter for an approximate assessment of area specific methane yield in extensive grassland. The combination with gap fraction LAI measurements can further improve the prediction accuracy of sward height especially in tall swards that tend collapse due to its own weight. However, an implementation of a combined mobile sensor approach is difficult without a redesign of the measurement principle of the LAI sensor.

It has also been shown that biochemical parameters and species composition of diverse sward mixtures are largely insignificant to the substrate specific methane yield, which is positive for energetic conversion, as it reduces expenses for contingent substrate specific pre-treatments prior to anaerobic fermentation. Among the functional groups legumes and grasses were the most determining factors but their influence differed between cuts and had an influence on the C/N ratio, which in turn had an effect on the substrate specific methane yields. However, batch experiments proved the superior specific methane yields of IFBB press fluids and showed that detrimental effects of the parent material were reduced by the technical treatment.

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Appendix

Tab. A.1 List of plant species used in the Klimzug-Experiment and the respective sward type and functional group they were attributed to. Total number of species in each functional group is indicated.

Grasses	Legumes	Herbs
	Diversity Mixture	
Alopecurus pratensis	Lotus pedunculatus	Achillea millefolium
Anthoxanthum odoratum	N=1	Achillea ptarmica
Cynosurus cristatus		Anthriscus sylvestris
Deschampsia caespitosa		Bistorta officinalis
Festuca nigrescens		Cardamine pratensis
Festuca pratensis		Centaurea jacea
Holcus lanatus		Cirsium oleraceum
Poa palustris		Crepis biennis
Poa pratensis		Filipendula ulmaria
Scirpus sylvaticus		Galium album
Trisetum flavescens		Geranium pratense
N=11		Heracleum sphondylium
		Leontodon autumnalis
		Pimpinella major
		Plantago lanceolata
		Prunella vulgaris
		Ranunculus acris
		Rumex acetosa
		Sanguisorba officinalis
		Selinum carvifolia
		Silaum silaus
		Silene dioica
		Silene flos-cuculi
		Succisia pratensis
		N=24
	Standard Mixture	
Festuca pratensis	Trifolium repens	
Phleum pratense	Trifolium hybridum	
Poa pratensis	N=2	
Alopecurus pratensis		
Agrostis stolonifera		
N=5		
	Reed canary grass	
Phalaris arundinacea		
N=1		

Tab. A.2: Fractions of the functional groups grasses, herbs and legumes in the 1st cut biomass. Values are shown as means of 2009 and 2010.

Functional group	RCG[a]		STA[b]		DIV[c]	
	N[d]	% DM[e]	N	% DM	N	% DM
Grasses	5	78.6	4	60.3	10	55.5
Herbs	9	19.1	7	11.5	16	44.0
Legumes	1	0.3	2	29.7	2	4.0

[a] RCG = Reed canary grass (*Phalaris arundinacea*); [b] STA = Standard mixture; [c] DIV = diversity mixture; [d] N = Number of species; [e] DM = Dry matter

Tab. A.3: Selected prediction model equations of measured biomass (BM[a]) for single sensor approaches using sward height (USH[b]), leaf area index (LAI[c]) and the best fit vegetation index. Broadband normalized difference spectral index ($NDSI_b$) is based on sensor specific wavelength selections of 50nm bandwidth.

Sensor	Equation
Common swards	
USH	BM = -0.192 + 0.074*USH
LAI	BM = -0.299 + 0.902*LAI
$NDSI_b$	BM = 2.894 - 48.903*NDSI[d]
Diversity mixture	
USH	BM = -0.385 + 0.068*USH
LAI	BM = 0.230 + 0.737*LAI
$NDSI_b$	BM = 2.688 - 47.049*NDSI
Standard mixture	
USH	$BM = 1.077 + 0.122*USH - 0.000334*USH^2$
LAI	$BM = -2.482 + 2.074*LAI - 0.123*LAI^2$
$NDSI_b$	BM = 2.878 + 39.777*NDSI
Reed canary grass	
USH	BM = -0.049 + 0.070*USH
LAI	$BM = 0.686 + 0.071*LAI + 0.129*LAI^2$
$NDSI_b$	BM = 1.992 - 51.210*NDSI

[a]BM: Biomass ($t*ha^{-1}$) as dependent variable; [b]USH: Ultrasonic sward height as independent variable ; [c]LAI: Leaf area index as independent variable; [d]NDSI: Normalized difference spectral index

Tab. A.4: Selected prediction model equations of measured biomass (BM[a]) for dual sensor approaches using sward height (USH[b]), leaf area index (LAI[c]) and the best fit vegetation index. Broadband normalized difference spectral index ($NDSI_b$) is based on sensor specific wavelength selections of 50nm bandwidth. Narrowband NDSI ($NDSI_n$) is based on 1nm wavelengths.

Sensor	Equation
Common swards	
USH + LAI	BM = -0.429 + 0.255*LAI + 0.534*USH – 0.00027*USH² + 0.0000695*LAI*USH²
LAI + $NDSI_b$	BM = -1.395 + 3.098*LAI -14.869*NDSI - 0.235*LAI² + 386.633*NDSI² - 29.872*LAI*NDSI + 4.481*LAI²*NDSI – 18.205*LAI*NDSI²
USH + $NDSI_n$	BM = -3.222 + 0.0628*USH + 11.37*NDSI + 0.00022*USH²
Diversity mixture	
USH + LAI	BM = 0.202 - 0.003*LAI + 0.027*USH + 0.007 *USH²
LAI + NDSI	BM = 2.620 + 0.670*LAI – 26.525*NDSI + 0.049*LAI² - 0.862*NDSI²
USH + $NDSI_{vesc}$	BM = -2.488 + 0.033*USH + 100.5*NDSI + 0.00033*USH² +750.3*NDSI²
Standard mixture	
USH + LAI	BM = 0.584 -1.747*LAI + 0.078*USH + 0.360*LAI² - 0.001*USH² + 0.049*LAI*USH - 0.010*LAI²*USH + 0.000048*LAI²*USH²
LAI + NDSI	BM = 0.759 + 4.009*LAI – 189.222*NDSI - 0.388*LAI² .123*LAI²
USH + $NDSI_b$	BM = -3.919 + 0.09606*USH + 10.689*NDSI
Reed canary grass	
USH + LAI	BM = -0.304 + 0.208*LAI + 0.059*USH - 0.000823*LAI² - 0.00037*USH²
LAI + $NDSI_b$	BM = 10.151 - 1.588*LAI - 182.898*NDSI + 0.2663*LAI² + 786.833*NDSI² + 4.979*LAI²*NDSI + 287.921*LAI*NDSI² - 96.044*LAI²*NDSI²
USH + $NDSI_{vesc}$	BM = -3.311 + 0.119*USH + 87.784*NDSI - 1.299*USH*NDSI

[a]BM: Biomass ($t*ha^{-1}$) as dependent variable; [b]USH: Ultrasonic sward height as independent variable ; [c]LAI: Leaf area index as independent variable; [d]NDSI: Normalized difference spectral index; $NDSI_{vesc}$: Normalized difference structural index according to Vescovo et al. (2011)

Tab. A.5: Selected prediction model equations of measured biomass (BM[a]) for three sensor approaches using sward height (USH[b]), leaf area index (LAI[c]) and the best fit vegetation index. The vegetation index NDSI[d] is based on sensor specific wavelength selections of 50nm bandwidth.

Sensor	Equation
Common swards	
USH + LAI + $NDSI_b$	$BM = -6.44 – 0.0383*USH + 4.39*LAI – 847.4*NDSI + 0.0081*USH^2 + 0.6941*LAI^2$ $- 21530*NDSI^2 - 0.1182*USH*LAI – 49.93*USH*NDSI + 913.1*LAI*NDSI$ $- 0.00772*USH*LAI^2 - 347*USH*NDSI^2 + 37690*LAI*NDSI^2 - 0.00185*USH^2*LAI$ $- 2.08*USH^2*NDSI + 10.46*LAI^2*NDSI + 0.000233*USH^2*LAI^2$ $+ 109.6*USH^2*NDSI^2 - 1774*LAI^2*NDSI^2 - 1215*USH*LAI*NDSI$ $– 0.565*USH^2*LAI*NDSI + 0.051*USH^2*LAI^2*NDSI - 3288*USH^2*LAI*NDSI^2$ $+ 2.782*USH^2*LAI^2*NDSI^2$
Diversity mixture	
USH + LAI + $NDSI_n$	$BM = 1.443 + 0.022*USH + 1.447*LAI + 362.7*NDSI - 0.358*LAI^2 + 17050*NDSI^2$ $- 0.00166*LAI^2*USH – 17*LAI^2*NDSI – 3892*LAI*NDSI^2$
Standard mixture	
USH + LAI + $NDSI_n$	$BM = -2.102 - 0.124*USH – 2.172*LAI – 253.9*NDSI - 0.00126*USH^2$ $+ 0.697*LAI^2 + 0.0598*USH*LAI + 4.885*USH*NDSI – 0.0163*USH*LAI^2$ $-0.000055*USH^2*LAI^2 + 19.55*LAI^2*NDSI -0.303*USH*LAI^2*NDSI$
Reed canary grass	
USH + LAI + $NDSI_{vesc}$	$BM = 1.963+ 0.663*USH – 6.533*LAI -120.2*NDSI – 0.0235*USH^2 + 1.168*LAI^2$ $+117.7*NDSI^2 - 18.89*USH*NDSI – 0.0181*USH*LAI^2 + 50.15*USH*NDSI^2$ $+186.2*LAI*NDSI + 0.00641*USH^2*LAI – 0.000374*USH^2*LAI^2$ $–0.822*USH^2*NDSI – 3.183*USH^2*NDSI^2 – 18.16*LAI^2*NDSI$ $–339.6*LAI^2*NDSI^2 + 13.17*USH*LAI^2*NDSI^2 - 0.199*USH^2*LAI*NDSI$ $+0.0127*USH^2*LAI^2*NDSI$

[a]BM: Biomass ($t*ha^{-1}$) as dependent variable; [b]USH: Ultrasonic sward height as independent variable ; [c]LAI: Leaf area index as independent variable; [d]NDSI: Normalized difference spectral index; $NDSI_{vesc}$: Normalized difference structural index according to Vescovo et al. (2011)

Tab. A.6: List of plant species used in the Jena-Experiment and the respective functional group they were attributed to. Total number of species in each functional group is indicated

Grasses	**Small herbs**	**Tall herbs**	**Legumes**
Alopecurus pratensis	Ajuga reptans	Achillea millefolium	Lathyrus pratensis
Anthoxanthum odoratum	Bellis perennis	Anthriscus sylvestris	Lotus corniculatus
Arrhenatherum elatius	Glechoma hederacea	Campanula patula	Medicago lupulina
Avenula pubescens	Leontodon autumnalis	Cardamine pratensis	Medicago × varia
Bromus erectus	Leontodon hispidus	Carum carvi	Onobrychis viciifolia
Bromus hordeaceus	Plantago lanceolata	Centaurea jacea	Trifolium campestre
Cynosurus cristatus	Plantago media	Cirsium oleraceum	Trifolium dubium
Dactylis glomerata	Primula veris	Crepis biennis	Trifolium fragiferum
Festuca pratensis	Prunella vulgaris	Daucus carota	Trifolium hybridum
Festuca rubra	Ranunculus repens	Galium album	Trifolium pratense
Holcus lanatus	Taraxacum officinale	Geranium pratense	Trifolium repens
Luzula campestris	Veronica chamaedrys	Heracleum sphondylium	Vicia cracca
Phleum pratense	n = 12	Knautia arvensis	n = 12
Poa pratensis		Leucanthemum vulgare	
Poa trivialis		Pastinaca sativa	
Trisetum flavescens		Pimpinella major	
n = 16		Ranunculus acris	
		Rumex acetosa	
		Sanguisorba officinalis	
		Tragopogon pratensis	
		n = 20	

Fig. A.1 Top-view on the Jena-Experiment site (© www.the-jena-experiment.de).