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Biochemical composition of biomass and its impact on the prediction of the specific methane yield potential

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Dedication

I dedicate this work to:

Whom I owe everything in this life and the life to come, Jesus-Christ;

My wife Anita for the indescribable perseverance and encouragements throughout the years it took to perform and compile this work;

To my children Céleste, Saphir, Gady-Rehema, Stella-Wonder and Sam-Ariel for the sacrifice and the sacrificial willingness to release whenever the duty called;

To all friends in Him (Jesus-Christ), known and unknown, for the faithfulness of holding up the banner of His Grace without fainting.

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List of abbreviations

ADF	acid detergent fiber
ADL	acid detergent lignin
BBCH-Scale	system for a uniform coding of phenological development stages of mono- and dicotyledonous plant species
Bm3	brown-midrib 3 mutant
CDOMD	cellulase digestible organic matter of the dry matter
CSTR	completely stirred tank reactors
DIN EN	Deutsches Institut für Normung e.V. (The German institute for standardization) - European standard
DM	dry matter
DNDF	digestibility of the neutral detergent fiber
EEG	Erneuerbare-Energien-Gesetz (The renewable energy act)
EM	energy maize
Eq.	Equation
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
FM	fresh matter
GE	gross energy
GHG	greenhouse gases
HBT	Hohenheim biogas yield test
HFT	Hohenheimer Futtermitteltest (Hohenheim feed quality test)
HPLC	high performance liquid chromatography
HRT	hydraulic retention time
IEA	International Energy Agency
ICS	International Classification for Standard
ISO	International Organization for Standardization
IVDOM	in vitro digestibility of organic matter
KTBL	Kuratorium für Technik und Bauwesen in der Landwirtschaft (The German association for technology and structures in agriculture)
LAB	lactic acid bacteria
LCA	life cycle analysis
LHV	low heating value

ML	middle lamella
NDF	neutral detergent fiber
NFE	nitrogen free extract
NIRS	near infrared spectroscopy
NSC	nonstructural carbohydrates
OADF	organic acid detergent fiber
ODM	organic dry matter
OLR	organic loading rate
ONDF	organic neutral detergent fiber
OR	organic rest
PW	primary cell-wall
SM	silage maize
SMY	specific methane yield
SRT	sludge retention time
STD	standard deviation of the mean
STP	standard condition of temperature and pressure
StrEG	Stromeinspeisungsgesetz (The power grid access act)
SW	secondary cell-wall
TAC	total anorganic carbon
TDM	total dry matter
TOA	total organic acids
TOC	total organic carbon
VDI	Verein Deutscher Ingenieure (The association of German engineers)
VDLUFA	Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten (The German confederation of agricultural laboratories and research institutes)
VFA	volatile fatty acid
VS	volatile solids (compounds susceptible of being lost during the DM determination)
WSC	water soluble carbohydrates
XA	Ash content
XF	crude fiber content
XL	crude lipid content
XP	crude protein content

List of Symbols

°C	degree Celsius
d	day
g	gram
kg	kilogram
h	hour
ha	hectare
hPa	hectopascal
K	Kelvin
kg	kilogram
L	litre
m^3	cubic meter
Mtoe	million tons oil equivalent
mL	millilitre
m_N^3	normalized cubic meter (biogas volume at 0°C and 1013.25 hPa)
l_N	normalized liter (biogas volume at 0°C and 1013.25 hPa)
ppm	Part per million
kWh	kilowatt-hour
TWh	Terawatt-hour

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1 Introduction and Motivation

Greenhouse gas (GHG) mitigation and energy security are some of the major global challenges in this century. Based on the Intergovernmental Panel on Climate Change report (IPCC 2007), global anthropogenic GHG emissions have been increased steadily and 57% of the total CO_2–equivalent emissions can be related to fossil energy combustion. GHG emissions due to fossil energy combustion are far beyond those due to agricultural or deforestation activities (IPCC 2007). In 2009 the Federal Institute for Geosciences and Natural Resources estimated that global primary energy consumption over the last three decades has increased by about 70% and that conventional crude oil production will reach its apex (“peak oil”) between 2020 and 2025 (Rempel et al. 2009). Andruleit et al. (2012) estimated the current crude oil proportion of global primary energy consumption (PEC) at approximately 34%, and stated that crude oil will continue to be the world’s most important fuel. Nevertheless the authors doubt whether the volumes of crude oil forecast for 2035 can actually be made available to meet the rising energy demand.

Though the predictions by different institutions regarding the development of anthropogenic GHG emissions and fossil fuel availability in the coming three to four decades are conflicting, all experts agree that there is an urgent need to find sustainable fuels to decrease energy related carbon-dioxide emissions and increase energy security for the future generations at the global level. These goals imply adequate policy, low energy consuming technologies, and an increase of renewable energy share in the fuel mix. Achieving these goals, however, is a great challenge since the whole of modern society and its economy rely on low cost energy (Gerling et al. 2006). Energy is the driving force behind our society and industry and the supply of energy is a prerequisite for their functioning (Rempel et al. 2009). Our current high standard of living is not possible without corresponding energy consumption (Kaltschmitt et al. 2006).

In Germany, the federal government has undertaken several measures to tackle these challenges. One of these was to create incentives to favor renewable energy production and technologies. This policy has been in place for over two decades

and started with the Power Grid Access Act (Stromeinspeisungsgesetz -StrEG) in 1991. The above mentioned act was replaced by the Renewable Energy Act (Erneuerbare-Energien-Gesetz "EEG") in 2000, which was amended in 2004, 2009, 2012, and 2014. These developments, together with the implementation of additional EU directives created momentum in the renewable energy sector with both positive results and accelerated production growth. The objective set in 2000, to double the share of renewable energy in the power production from 6.3% to 12% by 2010, was achieved by 2007, with a corresponding reduction of CO_2 emissions of approximately 100 million tons (BMU 2007). The power production from biogas increased drastically from 2.3 TWh in year 2000 to 22.84 TWh in year 2013, which is a 10-folds increase (BMU 2007; Fachverband Biogas e.V. 2013).

Despite these positive developments, there is still a long way to go, especially in the field of biomass use efficiency. In fact, the amendment of the Renewable Energy Act in 2004 allowed the digestion of crops and brought about not only an increase of biogas plants, but also a considerable need for biomass. To satisfy the hunger for biomass and foster the development of biogas production, an adequate strategy that would maximize the production capacity of biomass on agricultural land was needed. Breeders and agronomists set up an approach that adopted a special energy crop breeding strategy and agronomical techniques whereby the vegetative growth stage of crops could be prolonged, which maximized the capture and conversion of the yearly solar radiation per unit of land into biomass, and consequently increased the dry mass (DM) yield per unit of land.

Since both the biomass breeding strategies and biomass production techniques in this approach follow objectives other than that of the food-feed production pathway (Hahn 2007), the crops produced using this concept present different characteristics than those of the feed-food branch. This overall concept allowed the development of both novel crops and agronomical practices (Cheremisinoff et al. 1980; Scheffer 1998; Becker 2007; Kesten 2007). Still, an ideal energy crop genotype should reveal not only high biomass yielding capacity per unit of land but also a high specific methane yield potential per unit of organic dry matter. To breed for high specific

methane yield potential, one needs reliable predictors for the specific methane yield potential of the energy crop.

This work investigated the influence of biomass biochemical composition on the specific methane yield of energy crops. It assessed the relationship of biochemical composition of energy crops and the specific methane yield potential of these energy crops. Despite the fact that these issues have been studied previously from different standpoints by various authors, a more comprehensive work focusing on maize was still needed. Therefore, the present thesis investigates a much larger number of maize genotypes.

As prerequisite to the issues above mentioned, this work has elucidated the influence of the sample preconditioning methods (ensiling and drying processes) on the measurement of the specific methane yield potential of energy crops. It also investigated the scaling-up of batch fermentation to a semi-continuous flow process. Although ensiled biomass may exhibit different methane yield potentials compared with non-ensiled biomass, batch fermentation processes may be adjusted to extract the full methane yield potential of the samples. In contrast, semi-continuous flow systems are very much affected by operation parameters, which need to be optimized. The knowledge of the degree to which the ensiling process impacts the specific methane yield potential and the energy recovery efficiency in a semi-continuous flow system provide additional information about the influence of the biomass biochemical composition on the specific methane yield potential.

2 State of the art

2.1 Energy crops for biogas production

Energy crops are grown biomass for energy applications. Klass (1998) states that these should be high-yield, low-cash-value species with short growth cycles that grow well in the area in which the biomass energy system is located. Breeders distinguish three types of energy crops: (1) successfully established crops whose breeding objectives match “energy crop” breeding objectives; (2) successfully established crops requiring new breeding objectives for energy production purpose; and (3) crops not successfully established, that show themselves to be promising as energy crop (Becker 2007). Both high biomass yielding capacity and high specific energy yield are required (Meyer et al. 2007).

Whether or not it is necessary to consider all these elements to establish a sustainable biomass energy crop is a critical issue. Elements such as cash-value are dependent on markets, which are often highly volatile. Properly defining an energy crop is beyond the scope of this work. Therefore in the framework of this thesis, a crop is defined as an energy crop only with regard to its ability to be used as feedstock for energy production, and specifically biogas production. Only crop aspects and characteristics relevant for bioprocess engineering considerations are further discussed. Agronomic and sustainability issues related to energy crop production are not part of this thesis and are discussed in details in the literature (EEA 2005, Christen 2007, Frauen 2007, Hahn 2007, Mokry 2007). Crop production topics referring to maize genotypes used in this work are treated by Eder, B (2010).

2.1.1 Biochemical composition of energy crops

Energy crops are mainly composed of carbohydrates, lipids, and proteins in different proportions according to species. These chemical compounds are located either in the protoplasm or in the cell-wall matrix. The protoplasm is mainly made of proteins, lipids, and nonstructural carbohydrates (NSC). While proteins and lipids constitute distinct groups, the nonstructural carbohydrates (NSC), comprise different

compounds that include sugars, starches, fructans, galactans, pectins, β-glucans, etc. The cell-wall matrix is made mainly of structural carbohydrates and represents the fiber fraction of the crop (Van Soest, P. et al. 1991a).

Common energy crops used for biogas production are predominantly made of carbohydrates. Jeroch et al. (1993) estimate the total carbohydrate content in whole-crops to vary between 60% and 80% of the dry matter. A considerable share of carbohydrates in energy crops is made of cell-wall carbohydrates (complex and cross-linked). The share of cell-wall carbohydrates in whole-crop, measured as neutral detergent fiber (NDF), is estimated to range between 30% and 80% (Buxton and Redfearn 1996). Andrieu et al. (1999) analyzed 150 samples of silage maize (including 12 bm3 hybrids) and found that the share of cell-wall (expressed as NDF) varies between 36.5 and 57.5%.

Stems and leaf blades reveal different level of cell-wall content and they are also lignified to different degrees. Stems of most plant species have a greater fiber concentration (NDF) than do leaf blades, and grasses usually contain more fiber than legumes. Higher fiber concentrations in stems occur in part because stems contain more structural and conducting tissues than leaves.

2.1.2 Plant cell-wall – Composition and Terminologies

Because of its predominant contribution to the total organic dry matter (ODM) of the whole-crop, it is worth understanding the composition of the plant cell-wall and the terminologies used. The plant cell-wall is a strong fibrillar network that gives each cell its stable shape (Cosgrove 2001). Cell-walls enable plants to grow tall, glue cells together and act as a barrier for pathogens entering the cell. Sections through plant cells reveal that the cell-wall is different in shape and chemical composition. Cell-walls are mainly composed of cellulose (30 to 50%), hemicellulose (20 to 30%), pectin (3 to 5%) and lignin mostly incrusted in polysaccharides (Fuchs 2007). That is not to imply that protein and lipids do not participate at all in cell-wall structure, only that their contribution to the overall composition of the cell-wall is low (Himmelsbach 1993).

In general, cell-wall can be classified into two major developmental stages: the primary cell-wall and the secondary cell-wall. These walls are laid down progressively over the growth stages. While cells are dividing and expanding the primary cell-wall (PW) is laid down. Primary cell-wall is considered to be relatively unspecialized and constitutes the outermost layer of the wall of the cell (Wilson 1993). After cell enlargement has stopped, the secondary cell-wall (SW) is laid down inside the PW (Wilson 1993). Secondary walls are often very specialized in structure and composition. Where cells contact, the PW of contiguous cells are separated by special region called the middle lamella (Figure 1). The middle lamella (ML) cements cells together. This region is different from the rest of the wall as it is composed of high pectin content and different proteins (Wilson 1993).

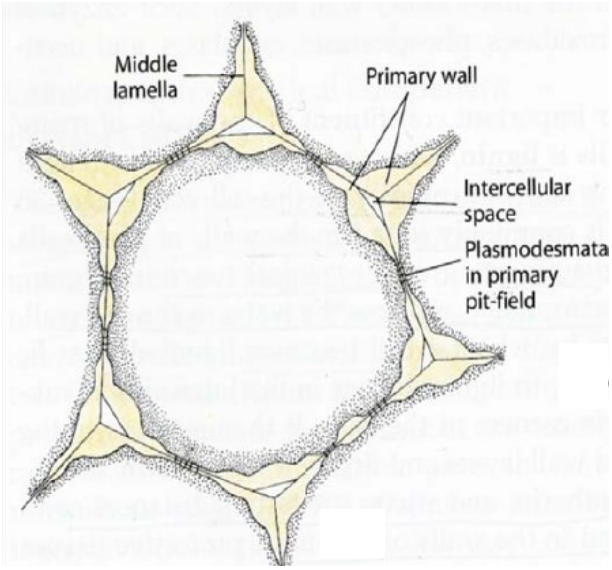

Figure 1: Middle lamella in primary walls cells. (Taiz and Zeiger 2003)

2.1.2.1 Primary cell-wall components

Primary wall consists of the following basic compounds: cellulose, hemicellulose, pectins, structural and non-structural proteins. Cellulose is a homopolymer made of thousands of glucose molecules that are linked together by ß-1,4 glycosidic bonds. The basic unit of cellulose is a disaccharide called cellobiose (Figure 2) that link together to form glucans chains. Glucans chains bond closely to form relatively stiff structures called cellulose microfibrils. Cellulose microfibrils are the major

components of the primary cell-wall. They are formed of several parallel-arranged cellulose molecules tightly associated to each other to hydrogen bounds along the cellulose chains. This structure excludes water and is relatively inaccessible to enzyme attack. Glucan chains, however, contain both crystalline domains as well amorphous sections. The degradation of cellulose is believed to start from the amorphous sections. Cellulases at the surface of fungi and bacteria establish close contact with the substrate and assure the degradation of cellulose. Endoglucanases (Endo-ß-1,4-Glucanase) degrade first the amorphous, water accessible section of the cellulose. The exocellulase attacks the non-reducing ends of the chains to produce tri- and disaccharides (Cellobiose). Cellubiose is cut down to glucose by cellobiase (a ß-1,4-Glucosidase) (Fuchs 2007).

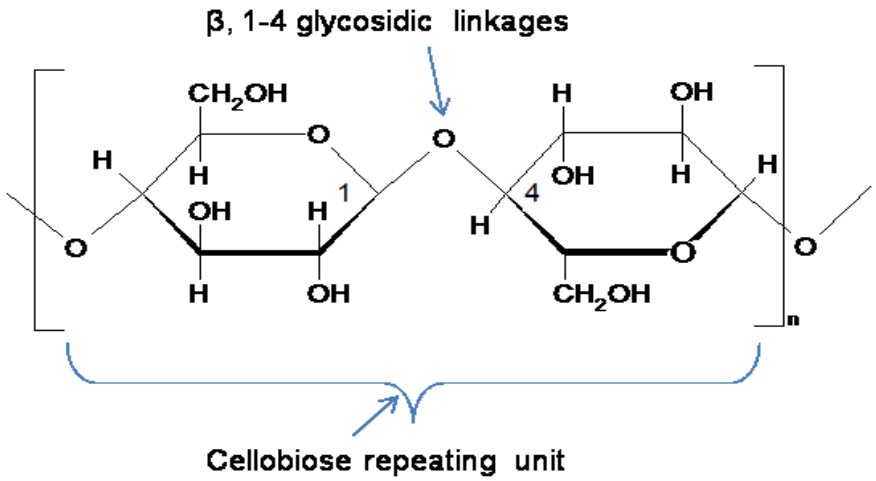

Figure 2: Cellobiose repeating unit of cellulose molecule (Taiz; L. and E. Zeiger 2003).

Cellulose microfibrils are bound together by hemicelluloses into a network. Contrary to cellulose, which is made of only one type of monosaccharide and one type of glycosidic linkage, hemicellulose is a general term used for heteropolymers containing combinations of different monosaccharides and/or glycosidic linkages. They are named after the main sugar component (Fuchs 2007). Hemicelluloses form shorter chains than cellulose and are branched. They are not crystalline, but rather water soluble and relatively easily degraded. Because of the different types of sugars and glycosidic linkages that compose hemicelluloses, their enzymatic degradation requires a wide range of hydrolytic activities (Rooke and Hatfield 2003). The cellulose-hemicellulose network is embedded in Pectins (a highly hydrated

polysaccharide gel phase). Apart from these main components, cell-wall also possess several types of structural and non-structural proteins (Brett and Waldron 1996). In the cell types with only a PW, the PW plus ML is thin and neither layer becomes lignified. Because of this special feature, this type of cell creates little problem for either digestion or physical breakdown (Wilson 1993).

2.1.2.2 Secondary cell-wall

Secondary wall is comprised of several layers. It is very different in structure from the primary cell-wall. After the plant expansion ceases, phenolic sub-units of lignin start infiltrating the space between cellulose microfibrils where they become cross-linked. As lignin forms, water is displaced from the cell-wall to form a hydrophobic matrix. Lignification is initiated in the ML and PW and proceeds throughout the SW as cells age (Wilson 1993). Secondary cell-walls become lignified to various degrees.

2.1.2.3 Terminologies

The cell-wall matrix or the fiber fraction of the crop has been expressed in the past as crude fiber content of the Weender system (Henneberg und Strohmann, 1860) as implemented in VDLUFA (1988) and now properly expressed by the neutral detergent fiber (NDF) content of the crop. The use of the crude fiber value of the Weender system as an expression for the cell-wall content of a crop is misleading especially in the context of this work. In fact, during the determination of the crude-fiber fraction (Weender) most of the lignin and hemicellulose is extracted and included into the nitrogen free extract fraction (Van Soest 1967) while the NFE fraction is supposed to represent only the nonstructural carbohydrates component. This anomaly was corrected by Van Soest (1967) so that the absolute content of the cell-wall can only be accurately estimated by the NDF content.

Nevertheless, following considerations should be taken into account while interpreting NDF value as crop cell-wall content: during the determination of the NDF fraction, pectin and biogenic silica are dissolved in the neutral detergent reagent so that NDF value does not include these compounds. At the same time silicaceous soil minerals (namely earth impurities) are comprised in the NDF value (Van Soest et al. 1991b, VDLUFA 1988). Hence, the NDF value of a crop expresses its content in hemicelluloses, cellulose, lignin, lignin-N-compounds and earth impurities. It does not include the pectin and biogenic silica fraction of the crop. A partial amendment can be made by considering the mineral free NDF-value, also called the organic neutral detergent fiber (ONDF). Furthermore, it is necessary to note that in opposition to the chemical composition of a crop which provides an exact value of a specific chemical compound, the NDF value represents rather a group of heterogeneous substances of different structures and more probably with different behavior concerning their biodegradability in AD process. This means that though the NDF value represents to a certain extent the cell-wall matrix of the crop, its use as a qualitative and objective parameter for the comparison of different crops and crop varieties is limited.

Additionally, the Van Soest system allows differentiating cross-linked fibers into two subclasses: acid detergent fiber (ADF) and acid detergent lignin (ADL). ADF value expresses cellulose, lignin and lignin-N-compounds, but also acid-insoluble ash and all silica (biogenic and earth impurities). As for the NDF value, to avoid biases by comparing different crops or crop varieties using this fraction it is also important to use at least the mineral free ADF-value also called organic acid detergent fiber (OADF). The amendment remains, however, partial since the structure of fibers in two different crops are not necessarily the same even if they display the same absolute content of NDF or ADL. The ADL fraction represents the crude lignin content. While ADF is only to a very limited degree undigested, ADL is known to be not digested at all. Because of all these considerations caution should be taken when comparing results of different works. In practice, one uses the following equations to estimate specific fiber types, although this approach delivers some inconsistencies (Van Soest and Robertson 1985):

$$\mathbf{NDF - ADF = Hemicellulose} \qquad \text{(Equation 1)}$$

$$\mathbf{ADF - Lignin = Cellulose} \qquad \text{(Equation 2)}$$

To our knowledge, studies dealing with the assessment of the influence of the biochemical traits on the methane yield potential of energy crops have not yet taken into account these methodological inconsistencies and the way to palliate them, especially with respect to the use of biochemical crop traits as predictors of specific methane yield potential.

2.2 Energy value of crops

In general the chemical composition of a feedstock determines its energy yield potential, as each chemical compound possesses a specific gross energy. However, for both animal nutrition and biogas technology the knowledge of the absolute chemical energy content of an energy crop (whole-crop) is of limited value. For instance, although crops (whole-crop) contain almost the same amount of gross energy as cereal grains per unit of organic dry matter, their energy values (as feedstuff) are lower and much more variable than that of cereal grains (Barrière et al., 2004). The difference is mainly due to both high cell-wall content and limited digestibility of whole-crop.

Therefore in order to evaluate the energy value of an energy crop in biogas technology, one needs in addition to the absolute gross energy content, further information about the cell-wall content and its biodegradability. In fact, while the "protoplasm chemical energy" made of lipids, proteins, and NSC (including pectin) will be readily mobilized, the "cell-wall biochemical energy" made of structural carbohydrates will be only partially mobilized. Although pectin is found in the cell-wall matrix, and can be considered to belong strictly to the cell-wall chemical energy group, the calculation of the cell-wall value excludes the pectin content. One argues that pectin is readily digested and hence different from other cell-wall constituents (Van Soest et al. 1991a). Accordingly pectin content is considered together with

non-structural carbohydrates so that the NSC value includes pectin. This is also sound at the bioprocess engineering standpoint.

2.2.1 *In-vivo* and *In-vitro* estimates of digestibility

To have access to further information required for the evaluation of the energy value, namely the degree to which the crop is mobilized for milk or meat production, one conducts *in-vivo* digestibility experiments. *In-vivo* digestibility can vary according to whether cattle or sheep are used in trials (Aerts et al. 1984, Barrière et al. 2004), with the level of feed intake (Woods et al 1999) and physiological status of the animal. It remains nevertheless the most reliable parameter for the determination of the energy value of whole-crop because of the natural milieu (rumen) in which it is determined. Still the factors evoked above set a limit as to the accuracy with which *in-vivo* digestibility can be predicted from any analysis of whole-crop (Tilley and Terry 1963). To mitigate the drawbacks of the *in-vivo* methods, plant breeders have increasingly used *in-vitro* methods to estimate the digestibility of whole-crop. Furthermore, *in-vivo* digestibility techniques are laborious and require a large quantity of forage.

In-vitro digestibility techniques can be distinguished by two categories, namely the rumen liquor based techniques and the enzymatic techniques. Aufrère (1982) states that the methods involving the use of the rumen liquor are more accurate for the prediction of the *in-vivo* digestion than the enzymatic ones. The most used rumen liquor methods are: the two-stage dry matter disappearance method of Tilley and Terry (1963), and the digested neutral detergent fiber (NDF) method (Van Soest and Wine, 1967, Goering and Van Soest, 1970). In the method of Tilley and Terry (1963), a sample of dried forage is digested anaerobically with rumen micro-organisms at 39°C in the dark for 48h, followed by a pepsin digestion at 39°C for 48h. At the end of the incubation time, the dry weight of the residue is determined and from this weight is subtracted the weight of residue found in the blank (which represents undigested food particles and microorganisms derived from the rumen liquor) to obtain the accurate weight of the undigested residue. The *in-vitro* digestibility of dry matter (IVDM or IVDOM when expressed on organic dry matter

basis) is calculated as the percentage dry matter disappearance. The NDF method of Van Soest and Wine (1967b) differs from the Tilley and Terry method in that a neutral detergent extraction replaces the pepsin treatment (Meyer at al. 1971).

The rumen liquor methods, however, are more costly for laboratories running larger number of samples, as animals to provide rumen liquor have to be kept. Therefore, several authors proposed different enzymatic methods based on the use of cellulase preparations (Aufrère, 1982). The most used are: the Jones and Hayward (1975) and the Boever et al. (1986) methods. The Jones and Hayward (1975) method is performed in two steps, namely the pepsin pretreatment in a diluted chlorhydric acid solution for 24h followed by a cellulase digestion for 48h. Both steps take place at 40°C. The Boever et al. (1986) method is performed in three 3 steps, namely the digestion in a pepsin solution at 40°C for 24 h followed by a starch hydrolysis in the same solution at 80°C for 45 min and finally a digestion using cellulase (from *Trichoderma viride*) at 40°C for 24 h. The result of the *in-vitro* digestibility is then referred to as the cellulase digestible organic matter of the dry matter (CDOMD), and in Germany widely referred to as the enzymatic digestibility of ODM (according to its German terminology - Enzymlösliche organische Substanz). This is the expression mainly used in this thesis. These *in-vitro* estimates of digestibility for whole-crop are used commonly as predictors for specific methane yield potential.

2.2.2 Plants' cell-wall and digestibility

As previously mentioned the "protoplasm chemical energy" is mobilized with ease, provided a certain exposition of the protoplasm (e.g. chopping or chewing) and minimum retention time in rumen or the digester are available. The availability of the "cell-wall chemical energy" is rather very much subject to digestibility. Hence the classification of whole-crops with respect to their digestibility is actually the ranking of their cell-wall's digestibility.

In general, cell-wall digestibility varies among species, within the crop's organs and throughout its maturation stages. Buxton and Redfearn (1996) indicated that the differences in cell-wall digestibility between leaves and stems are normally less in

grasses than in legumes. Grasses have a larger share of NDF that is potentially digestible. Furthermore, it is generally agreed that as crops age, lignification of the cell-wall impacts its digestibility. The digestibility of stems declines more rapidly with increasing plant maturation than the digestibility of leaf blades. The digestibility also declines down stems (Buxton and Redfearn 1996). Twenty to thirty percent of cell-wall polysaccharide fibers are incrusted with lignin (Van Soest and Robertson 1985). In maize stems, lignification of the stem is limited to very strict zones of cell-wall (Van Soest and Robertson 1985).

Despite these general trends, the literature makes reference to inconsistencies between cell-wall (and/or lignin) content and cell-wall digestibility. For example, Jung and Buxton (1994) found no reliable negative correlations between lignin concentrations and cell-wall digestion for forages of similar maturity. The authors could not elucidate the exact causes of this effect. Andrieu et al (1999) found that the relationship between DNDF (digestibility of NDF) and different fiber fractions (NDF, ADF, ADL) were highly significant for perennial forages. However, these relationships were either not significant (for NDF) or imprecise (for ADF and ADL) when “whole-crop” maize samples were considered. Barrière et al. (2004) showed that the correlation between NDF content and NDFD was close to zero, indicating that no significant relationship existed between cell-wall digestibility and cell-wall content when maize plants were harvested at a similar stage of maturity.

These studies show that although cell-wall content and cell-wall digestibility are important parameters for the categorization of biomass crops, these parameters cannot be used as accurate predictors of their energy values, especially within a species.

2.2.3 Anaerobic biodegradability

Anaerobic degradation is a biological process where organic compounds are converted to their most oxidized (CO_2), and most reduced (CH_4) states (Angelidaki, 2002). There are two main methods to evaluate the recovery efficiency or anaerobic biodegradability of energy crops. The first method is based on the measurement of substrate depletion, namely the fraction of the substrate that disappears from the digester. In energy crop based anaerobic digestion systems, the substrate fed to the digester is measured in organic dry matter (also called volatile solids). The degradability is then expressed in percentage as quotient of output to input (corrected with respect to the blank). The second method is based on the determination of the energy conversion efficiency, corrected of the process inherent losses (as adopted by Amon et al., 2003b, Amon et al. 2007a). In both cases, the biodegradability expressed in percentage as quotient of output to input can be compared, in absolute terms, to digestibility values (percentage value of the forage fraction that disappears from the gut).

Because of both longer retention time and different microbial flora, the recovery efficiency (or anaerobic biodegradability) of anaerobic digestion systems is expected to be higher than rumen digestibility. A review of different works on anaerobic digestion by Hobson and Wheatley (1993) notes both higher cellulolytic activity in digesters than in the rumen of animals, and different microorganisms in digesters, which differ greatly from those found in the rumen.

2.2.4 Predictors of the specific methane yield potential of energy crops

It has long been known that the chemical elemental composition of a feedstock can be used to predict stoichiometrically the theoretical methane yield potential of a feedstock (Buswell and Boruff, 1932; Boyle, 1977). The ratio of CH_4 to CO_2 depends on the oxidation state of the carbon present in the organic substrate (Angelidaki, 2002). In practice, the digestion of complex organic substrates results in biogas with a significantly low CO_2 content because of the relatively high solubility of CO_2 in the

digester aqueous phase, as well as other possible chemical bonds with carbon (e.g. with cations) (Weiland 2001). The equations developed by Buswell and Boruff (1932) and Boyle (1977) assume a complete degradation of the feedstock without considering either inherent energy losses or the partial biodegradability of cell-wall fractions during the anaerobic digestion process. With respect to inherent conversion losses, the literature gives different figures ranging from 3% to 10% (Braun 1982, Angelidaki and Sanders 2004, Scherer 2007). For energy crops that need first to be almost totally hydrolyzed and acidified, an upper limit of 10% given by Angelidaki (2002) seems be adequate. By subtracting the process conversion losses from the calculated stoichiometric value, one can predict the "theoretical maximum specific methane potential". The "theoretical maximum specific methane potential" can also be predicted from the calorimetric lower heating value after deducing the conversion losses (Amon, 2003b). This term gives information to the full potential, but remains nevertheless an academic reference value, because of the partial biodegradability of cell-wall.

Therefore, models involving both chemical composition and degradability have been developed to predict the specific methane potential of energy crops. The predictors used in most models (Baserga 1998; Keymer and Schilcher, 1999; Weißbach 2010) include, on the one hand, the chemical composition of the Weender analysis (Henneberg und Strohmann, 1860) as implemented in VDLUFA (1988) and the Van Soest cell-wall fractions, and on the other hand, the ruminal digestibility. In some cases, the gross energy recovery efficiency is considered instead of the ruminal digestibility (Amon 2003b and 2007b).

Amon et al. (2007b) found that crude fat and crude protein values contribute most to the methane energy value, and used these to predicted the specific methane yield potential of maize based on XP, XL, XF, and NFE with a very high coefficient of determination ($R^2 = 0.968$). It is worth mentioning, however, that the traits used in this model were measured based on % DM content. Kaiser (2007) developed a model XP, XL, organic rest, hemicellulose, cellulose and ADL and predicted the specific methane yield potential of maize with a high coefficient of determination ($R^2 = 0.88$). Based on these results, it is to be expected that the biochemical composition

and the *in-vitro* estimates of digestibility are sufficiently robust to accurately predict the specific methane yield potential. However, the results of validation by other authors (e.g. Czepuck et al., 2006) are conflicting. Mittweg et al. (2012) evaluated the models of Baserga (1998), Amon et al. (2007b), and Weißbach, (2010) and found that Amon's model was the most suitable for maize, showing the lowest bias to the measured values. Grieder et al (2011) found poor performance for the three models ($R^2 < 0.04$) and suggested a model based on the NIRS spectra.

2.2.5 Determination of the specific methane yield in batch systems

Although batch-tests are known to be cumbersome, tedious, and time consuming, the specific methane yield potential (also called biochemical methane potential) can only be correctly determined using batch-tests. Some batch-tests allow determination of the specific methane yield potential with an acceptable repeatability (coefficient of variation ranging between 1.8 and 2.8%) (Ohl, 2011; Mittweg et al., 2012). The specific methane yield potential expresses the ultimate biochemical specific methane production for indefinite degradation time. In practice the degradation time is definite and the methane potential is estimated by extrapolation of a methane time degradation curve (Angelidaki and Sanders, 2004). This helps predict the methane yield to be expected under practical conditions.

The VDI 4630 guideline (2006) recommends 6 systems that can be used for the assessment of the specific methane yield potential of energy crops. The basic approach is to incubate a small amount of the sample with an anaerobic inoculum and to measure the methane generated, usually by simultaneous measurements of gas volume and gas composition. The choice of the system is determined by the type of the substrate to be tested. Nevertheless, all systems dealing with energy crop should fulfill following functions:

- gas tightness (leak test should be carried out using biogas or a synthetic gas of similar composition);

- constant temperature (using a heater, thermostat and a fan for equipment running in incubation chambers or water baths with the water level in the bath being higher than the fill levels in the fermentation vessels);
- Mixing device, especially for substrate producing floating layer or scum (e.g. agricultural substrates).

Three key criteria which secure the results of an anaerobic batch-test are: the correct determination of the DM and ODM; the quantity and quality of the inoculum; and the computation procedure. The most important aspects are briefly presented below.

The organic dry matter (ODM) content is determined according to the DIN – EN 12879 (2001) at 550±25°C for at least 30 min, while the DM determination is performed according to the DIN EN 12880 (2001) at 105±5°C. Apart from these norms, additional precautions have to be taken for the drying of energy crops. For starchy substrate such as maize, VDLUFA recommends that the drying be performed in two stages; first at 40°-60°C followed by a 3-hours drying at 105°C (VDLUFA 1988). This avoids starch to swell and incrust water. If water is incrusted in swelling starch, it will not be removed from the substrate when the drying temperature reaches 105°C. In fact, starch can bind physically water several times its weight.

In addition to a proper DM and ODM determination, further care must be taken for silages by correcting the loss of volatile solids that occur during the DM determination. To measure the fraction of these volatile compounds, the DIN 38414-19 (1999) should be followed or an analysis using the HPLC should be performed. Further details for the correction of the dry solid losses and its impact on the determination of methane yields are given in the literature (Weißbach and Kuhla, 1995; Weißbach, 1994; Mukengele and Oechsner, 2007).

Sample preconditioning techniques and/or substrate handling operations (Scholwin and Gattermann, 2006) can have also influence on the determination of the specific methane yield potential. Whole-crop samples are heterogeneous materials

consisting of leaves, stalks, and grains. Therefore, they need to be homogenized prior to being used for digestion trials. Homogenization processes, however, vary from one laboratory to another. The most commonly used operations are mechanical: chopping or blending, drying, and milling.

The drying-milling procedure can potentially have an impact on the determination of the specific methane yield potential. In fact, the respiration process continues during the drying procedure. Prolonged respiration can lead to depletion of the energy content. Furthermore, milling increases the specific surface area of the substrate. Since mechanical pretreatments do not destroy extensively plant cell-wall, it is generally agreed that the specific surface area increase can cause the biogas production rate to increase, but not the specific methane yield potential. Reports in the literature, however, are conflicting. For example, Schumacher (2008) found that the use of milling had neither positive nor negative impact on the specific methane yield potential of maize whole-crop, but that milling caused the specific methane yield potential of straw to increase of 15%. The drying-milling procedure shows a great advantage for homogeneous samples.

The non-drying-chopping procedure has also been shown to have some drawbacks. Because of the large number of whole-crop samples used in anaerobic digestion trials, non-dried samples have to be deep frozen prior to being used. This procedure can also cause damage to the samples and hence has an impact on the specific methane yield potential of the samples. For instance, from the food processing field, it is known that freezing-thawing causes dehydration damage, drip loss, tissue fractures, and mechanical damage from ice crystals during freezing (Kidmose and Martens 1999). Drip losses can lead to energy loss through leaching. The drip losses can also be fostered by the chopping process and lead to lower specific methane yield potential of the samples. Investigations with silages showed that the re-incorporation of the expressed juice, or the recovery of the total energy, is not always guaranteed (Porter, 1992). Therefore, the use of these different preconditioning procedures can lead to conflicting results when the specific methane yield potential of energy crops is determined.

To secure the comparability of batch-tests, the quality and activity of the inoculum should also be taken care of. The results of a batch-test can be affected by the microbial activity of the inoculum (KTBL, 2010). For the digestion of substrates of agricultural origin, the VDI 4630 Guideline recommends to use an inoculum from a full-scale agricultural biogas plant fermenting the same substrate. The proportion between the test-substrate and the inoculum is of 1:2 on ODM basis to avoid acidogenesis shock that may impede the process. Moreover, the biogas yield of the test-substrate should be higher than 80% of the total biogas produced (test-substrate + inoculum) (VDI-4630, 2006). In fact, an inoculum with an intensive gas production can jeopardize the results as the test-substrate will stop producing gas, while the inoculum still produces its own gas. Hansen et al. (2004) found that when the inoculum itself produced a significant amount of methane, the detection limit of the process was limited. This means also that when the test-substrate has a very low biogas yield potential, the uncertainty of the results increases because the difference between the test-sample and the control samples might not be significant. If the potential is low, the ratio of test-substrate to inoculum should be increased. Furthermore, it is recommended that the biological activity of the inoculum be also proven by fermenting a substrate whose biogas potential is known in parallel with the test-substrate. This increases the reliability of the digestion test (VDI-4630, 2006).

The last criterion that needs to be observed in order to increase the comparability of batch-test is the computation procedure. The biogas volume measured has to be corrected with respect to the moisture content in the gas and the results should be expressed at STP (standard temperature and pressure) conditions. As recommended by the norms DIN 38414-S8 (1985) and VDI 4630 (2006), the specific methane yield potential is expressed in l_N or m_N^3 of CH_4 per weight of ODM of the substrate.

2.3 Maize as energy crop

2.3.1 Growth pattern

Zea mays L. ssp. mays, commonly referred to as maize or corn, belongs to the grass tribe Andropogoneae of the family Gramineae (Poaceae), and to the Order of Poales (Strable and Scanlon, 2009). It has a rush development of roots and leaf systems. In a short time (about 12 weeks) after sowing, it develops to a plant of 2 to 3 m height. In the following 2 to 3 months it produces 400 to 600 grains (Zscheischler et al. 1990). From emergence to physiological maturity, maize plants undergo several growth stages that are mainly divided into two categories: vegetative growth and reproductive growth. Figure 3 shows the different maize growth stages (UIE, 2010). Vegetative stages are represented by “V”, with the numbers indicating the number of leaves that are completely developed. The reproductive stages are represented by “R”.

The vegetative growth phase starts with the plant emergence (VE) and ends when the tassel is completely extended before the silks are totally visible (VT). Each leaf arises from a node and is separated by an internode. This architectural disposition results in a leaf arrangement that is capable of maximizing sunlight exposure. The upper leaf surface is pubescent and adapted for solar energy absorption. The lower leaf surface is glabrous and has numerous stomata that favor carbon dioxide absorption. The result is a photosynthetically efficient plant capable of high total dry-matter production (Stoskopf 1985). With a leaf index of eight, 95% of the usable light can be captured. From the time that the plant forms 10 leaves, it begins a rapid and steady increase in nutrient and dry matter accumulation which will continue until far into the reproductive stages (Ritchie et al. 1993). During the vegetative growth period a considerable share of assimilates (water soluble carbohydrates) is kept in the stalk (up to 40% of the stem total solids) (KWS 2007). When the tassel is completely extended (before the silks are totally visible) the vegetative growth ends. This means that from this point on dry matter yield (in term of further vegetative growth) will not take place anymore and the plant has reaches its full height.

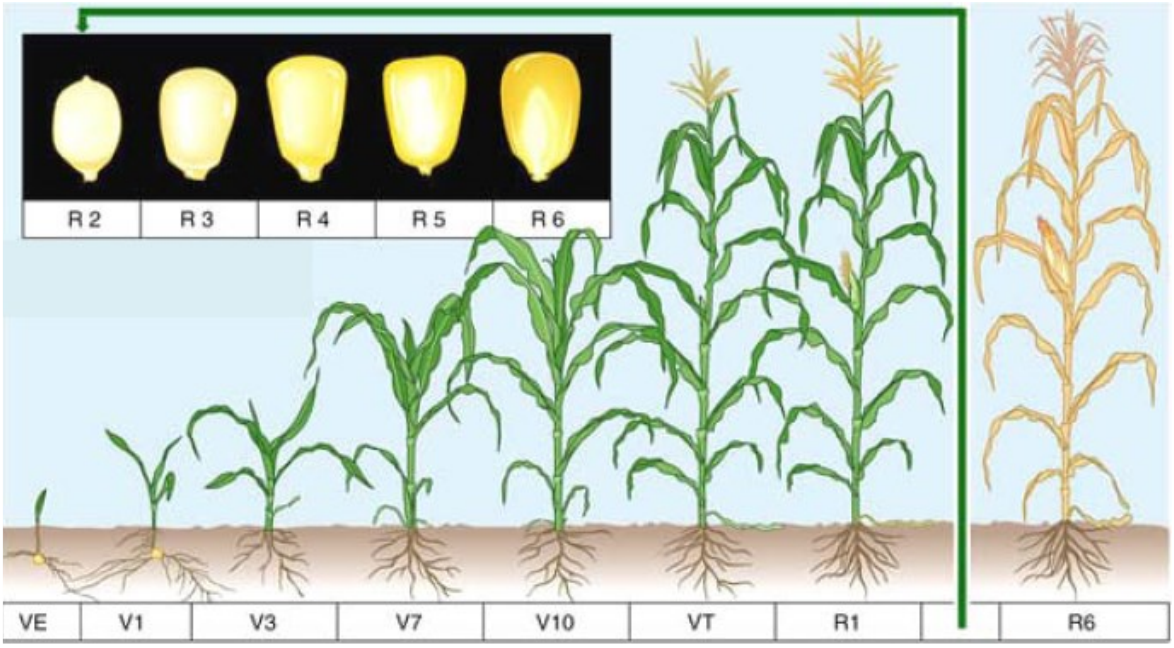

Figure 3: Maize growth periods. (UIE 2010).

The reproductive growth phase (R) begins with the full appearance of silks outside the husks. After the time of silk formation (R1), the growth is predominantly impregnated by the dry matter increase in grains (Zscheischler et al. 1990). The Kernels are white (R2), resembling a blister in shape, while starch has begun to accumulate. In the milk stage (R3), kernels are in a rapid rate of dry matter accumulation with a moisture content of approximately 80%. They are yellow on the outside with a milky white inner fluid. With the accumulating starch, the fluid in the endosperm thickens to a pasty consistency (R4). This is called the dough stage. At the end of the dough stage, the total dry matter of the whole-crop is between 30-35%. Maize may be harvested for conservation as silage at this stage (Zscheischler et al. 1990).

After this stage, kernels become dented (R5). This occurs as the moisture content of the kernel begins to decrease at a faster pace. At the beginning of this stage, the kernel will have about 55% moisture. The starch in the kernel continues to evolve from the pasty consistency of the dough stage to a much harder texture. The starch will begin to harden in the kernel beginning at the top where a small hard white layer of starch was formed and work down towards the cob. The physiological maturity

(R6) is reached when all kernels on the ear have attained their maximum dry matter accumulation, the hard starch layer has advanced completely to the cob and a black layer is formed. All normal maize plants follow this general pattern however at different time intervals according to breed, location and environmental conditions.

For the use of maize crop as feedstock for biogas production, a few key transformations take place during the above described growth process. The first key transformation is the shift in different carbohydrate fractions. The chemical composition of the crop does not change as such (e.g. from carbohydrate to lipid), but water soluble carbohydrates (WSC), which are primarily stored in the stalk, are progressively transported in the generative parts where they are converted into starch as the crop matures. This relocation starts taking place at flowering. The husk and the cob serve also as intermediate reservoir for starch (KWS 2007). Hence, with progressing maturity, vegetative fractions are impoverished of sugars while cobs get replenished with nutritive substances. From the beginning of reproductive growth to the end of the dough-ripeness, the share of the vegetative fraction (in % of the total dry matter) decreases from 93% to 46%, while the cob share increases from 7% to 51% (Jeroch et al. 1993).

The second major change is the decrease in the cell-wall content, although these continue lignifying as the crop ages. The crude fiber content remains more or less constant. The crude protein content remains also more or less unchanged (KWS, 2007). However, the entire growth process is accompanied by a steady increase of the total dry matter content of the whole-crop (Figure 4). Because of this precise growth pattern, the dry matter content is generally used as a quality criterion for maize whole-crop. Nevertheless, if the starch and dry matter accumulation in the kernel do not proceed as above described, using the dry matter of the whole-crop as quality parameter can be misleading.

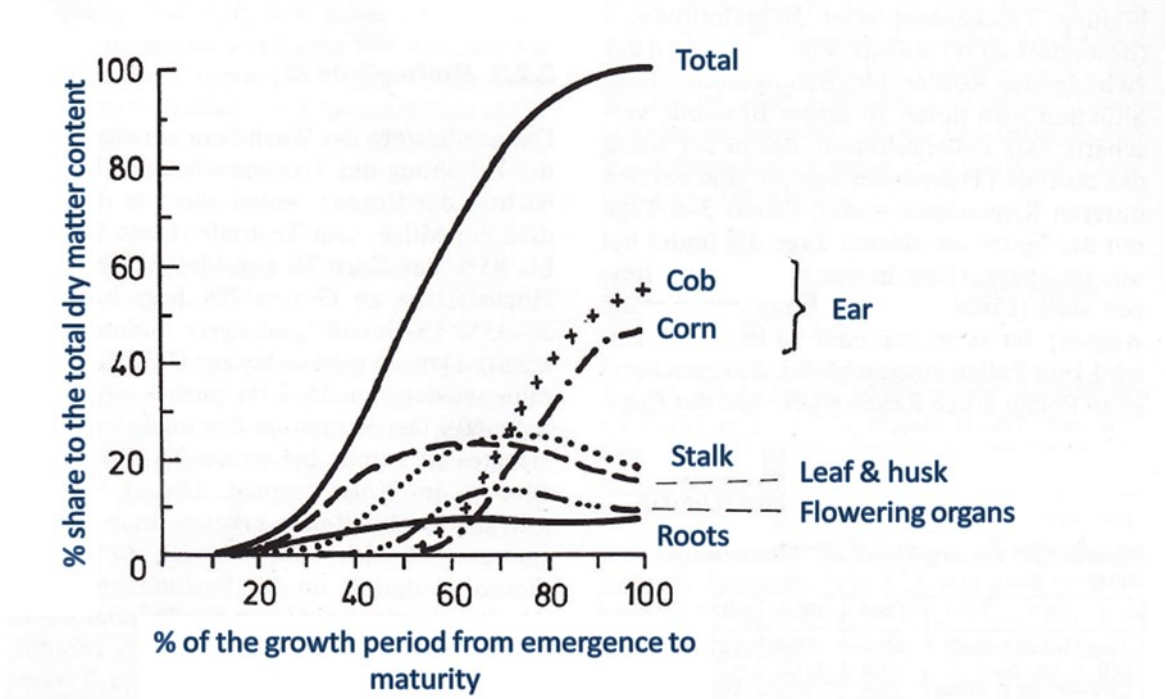

Figure 4: Dry matter accumulation in different organs of maize crop. (adapted after Jeroch et al., 1993).

2.3.2 Maize genotypes and classification

Several traits are targeted in maize breeding. Among the most important traits worldwide are: dry matter yield; maturity; stalk strength; cold tolerance; and drought resistance. More than for any other crop maize production requires appropriate variety choices, meaning that the genotype should be able to reach a physiological maturity for the given location and usage (Zscheischler et al., 1990; Schmidt et al., 2005b).

Based on the length of time needed to reach maturity, namely the thermal time between planting and physiological maturity expressed as growing degree days “GDD” or heat units "HU" (Nielsen, R.L.B., 2012), the FAO has established an international nomenclature according to which the world assortment is classified in values (also called FAO maturity index) ranging from 100 to 900. The rating is done by considering solely the dry matter content of the cob. To identify varieties with

respect to their maturity group, the variety authorities label genotypes with the FAO maturity values.

With the introduction of stay-green varieties, the imperative to consider exclusively the dry matter content of the cob for silo maize was found to be insufficient by the German federal variety authority (Bundessortenamt). Consequently, since 1998 maize varieties are classified in 4 groups in Germany according either to the dry matter content of the cob for grain usage or the total dry matter content of the whole-crop for silage maize. These groups are labeled as early (FAO-index 170-220), mid-early (FAO-index 230-250), mid-late (FAO-index 260-290), and late (FAO-index 300-340) maturity group (Anonym, 2014).

Since Germany is located at the upper boundary of maize cultivation zone, the classification by the German federal variety authority takes into account only the early varieties (FAO-Index < 350). Austria and Switzerland classify assortment of up to FAO value 500. Under central European conditions the difference of 10 FAO maturity values makes approximately 1-2 days difference in maturity or 1-2% dry matter content in corn maize at the time of harvest. Hence, a variety with FAO value of 280 matures under Germany conditions approximately 5-8 days later than one with FAO value of 230. This means that harvested the same day, the dry matter content of the variety with FAO value 280 will be nearly 5-8% lower (Zscheischler et al. 1990). This explains also the reason why the same type of variety might be grouped differently according to countries (or various environmental conditions). Therefore, the classification of maize genotypes of given FAO maturity values into above mentioned groups (early, mid-early, mid-late and late) remains country-specific and thus the boundaries can be shifted for academic sake.

2.3.3 Breeding for high biomass yielding maize - Energy Farming Concept

The process by which solar energy is converted via photosynthesis into chemical energy contained in the biomass components involves complex biochemical and photochemical mechanisms (Gregory, 1989; Salisburi and Ross, 1992). Nevertheless, these can be summarized and simplified in the following chemical reaction and explanation:

$$\mathbf{6\,CO_2 + 6\,H_2O + light \rightarrow C_6H_{12}O_6 + 6O_2 \quad \Delta G^\circ = +2880\,kJ/mol} \qquad \text{(Eq. 3)}$$

During the photosynthesis process, carbon dioxide is reduced and water is oxidized. Thus, the inorganic materials, CO_2 and water, are converted to organic chemicals, and oxygen is released. Depending on species secondary products such as polysaccharides, lipids, proteins may or may not be produced.

Photosynthesis as a conversion process is in technical apprehension an inefficient process. In fact, the upper limit of the capture efficiency of the incident solar radiation in biomass is estimated to range from about 5-15% and in most actual situation generally in the 1% range or less (Gregory 1989; McMahon et al. 2007, Klass 1998). Therefore, to increase the biomass yield per unit of land a strategy that could maximize the capture and conversion of the yearly solar radiation per unit of land into biomass was required. Amon et al. (2006a; 2006b) state that an ideotype energy maize genotype should display both high biomass yielding capacity and high specific methane yield potential.

Based on the work of Haarhof (1990), who found that irrespective of the harvest dates, late maturing varieties showed higher total dry matter per unit of land than the early maturing ones, Schmidt (2005a,b) and Schmidt and Landbeck (2005) showed that the dry matter yield of maize per unit of land could be doubled by delaying the flowering period. The energy maize genotypes produced up to 30 tons DM per hectare, while silage maize varieties commonly grown in the central Europe conditions yield 15-18 tons DM per hectare.

Figure 5 shows exemplarily how the total dry matter yield (TDM/ha) of a maize variety bred for biogas production (also called EM = energy maize) exceeds that of a traditional silage maize variety bred for animal nutrition (SM = silage maize). The black curve shows the trend in the total dry matter yield of a silage maize (SM). The green curve shows the development of the total dry matter yield of an “energy maize” variety (EM). Until flowering of the silage maize variety, the two growth curves show an identical course: The total dry matter yield of both species increases exponentially. After flowering of the silage maize variety, the two growth curves diverge remarkably. The curve of the silage maize continues to rise linearly and then flattens very quickly. Because of the deferred begin of the generative phase, the growth curve of the energy maize variety shows a steady exponential increase. The discrepancy in growth pattern is due to the fact that after flowering of the silage maize, the energy maize variety continued to invest its entire assimilation potential in additional vegetative leaf growth, while the silage maize invests its further assimilation in the cob formation. The additional leaves formed contribute further to the assimilation efficiency of energy maize. On the contrary carbohydrates stored in the cob do not contribute to further assimilation in silage maize variety. The later the harvest the higher the total dry matter yield of the energy maize variety (Schmidt, 2005a).

For the development of hybrids (characterized by a vigorous and quick initial vegetative growth) adapted to the central European climatic conditions where temperatures during the initial vegetative growth might be remarkably low, Schmidt and Landbeck (2005) had recourse to the crossing of Mediterranean high biomass yielding germplasm resources (e.g. Italian) with the German cold-resistant genetic resources.

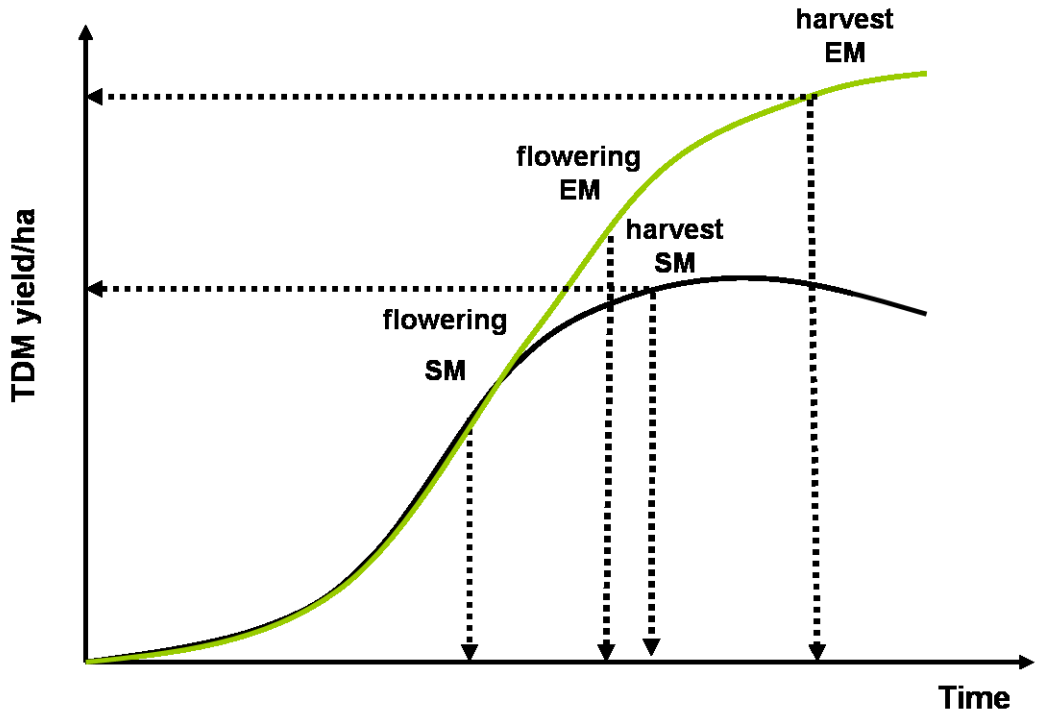

Figure 5: Energy crop breeding strategy showing the increase in total dry matter (TDM) yield following the shift from the conventional silage maize (SM) growth pattern to the novel energy-maize (EM) pattern. (Schmidt 2005a).

Moreover, they integrated tropical short-day gene (e.g. Peruvian) in the central European long-days environment to stimulate biomass yield (Schmidt 2005b). By using the so-called doubled haploids induction method, they could achieve homozygosity in one generation, while five to six generations of self-pollination are normally required (Schmidt and Landbeck, 2005; Messmer et al., 2011). This enabled a fast development of novel "energy crops". These breeding approach together with adjusted agronomical techniques that enable to reach high DM yield per unit of land was named: "energy farming" (Becker, 2007; Kesten, 2007). It is worth to note, however, that an effective increase of the dry matter yield per unit of land requires an adequate water supply and heat summation (Schmidt and Landbeck, 2005, Hahn et al., 2006; Döhler and Schliebner, 2007). The choice for maize was justified by its agronomic and breeding importance as the most thoroughly researched genetic system (Strable and Scanlon, 2009). Furthermore, it was considered that if this strategy works for maize it could be easily transferred to other crops. The conservation techniques for maize are also largely known.

The comparison of different biofuel production lines shows that the use of silage maize as feedstock to produce biogas has a considerable productivity per unit of land (Table 1). The application of an adequate strategy that could maximize the dry matter yield per unit of land has the ability of enhancing sustainably this potential.

Table 1: Comparison of biofuel yields per unit of land. (adapted from FNR 2006 and Meyer et al. 2007)

Biofuel Yield/unit of land					
Typ of crop usage	Crop fraction			Whole crop	
Fuel type	Rape oil	Biodiesel	Bioethanol	Biogas *	Biomass to Liquid (BtL)
Resource	Rapeseed	Rapeseed	Cereal grain	Silo maize	Energy crop
Yield [t FM/ha x yr]	3.4	3.4	6.6	45	15
Biofuel Yield [l/t FM x yr]	435	455	387	79 [kg/t FM x yr]	269
Biofuel Yield [l/ha x yr]	1479	1547	2554	3555 [kg/ha]	4028
Diesel-/Gasoline equivalent [l/ha x yr]	1420***	1408***	1660**	4977**	3907***
Net Energy Yield [GJ/ha]	35	38	30	113	118

* Biomethane produced from traditional silo-maize
** Gasoline equivalent
*** Diesel equivalent

However, because of the prolonged vegetative growth phase in the production of energy maize, the generative phase (i.e. flowering-fruit formation-maturity phase) is shortened. As consequence the huge biomass yield potential of an energy maize variety is achieved to the detriment of starch accumulation. This would be irrevocably negative for animal nutrition, but the authors were encouraged in their strategy by the statement derived from the work of Oechsner et al. (2003). In fact Oechsner et al. (2003) showed that for different crop species, and even within species, only the dry matter yield per unit of land was determinant to increase susceptibly the methane productivity per unit of land. The conclusions that would be drawn from the work of Oechsner et al. (2003) were, however, limited since only few crop genotypes were investigated. To which extend this shift impacts the energy

value of crop as biogas feedstock need to be evaluated. Because of all these issues a work that would consider a larger number of maize varieties was initiated.

2.3.4 Specific methane yield potential of maize

The specific methane yield potentials of maize found in the literature vary greatly. Gronauer and Kaiser (2007) compiled specific methane yields ranging from 195 to 745 l_N CH_4/kg ODM for maize whole-crop. While several laboratories tend to explain this large corridor by the wide variability in the maize biochemical traits, the authors explain rather the extreme discrepancies by the differences in protocols. In fact, before the harmonization of the methane yield determination's protocols by the VDI 4630 Guideline (2006), each laboratory had its own protocol. For instance specific methane yield potentials determined in some laboratories were not expressed in standard conditions of temperature (273 K) and pressure (1013 HPa). In addition, silage samples were treated as non-ensiled crop materials. Therefore, it was obvious that methodological errors led to biases in the results.

Nevertheless even by limiting the literature review to the studies that make reference to the harmonized procedure, as compiled later in the VDI 4630 Guideline (2006), considerable discrepancies are still found. Kaiser and Gronauer (2005) examined three genotypes and determined specific methane yield potentials varying from 250 to 360 l_N CH_4/kg ODM. The variation range represented 30% difference across genotypes and growth stages. In other publications the authors determined methane yield potentials ranging from 319-432 l_N CH_4/kg ODM (Gronauer and Kaiser, 2007). Although the difference across genotypes was limited, the maximum was considerably high. Amon et al. (2003a) investigated 5 genotypes and determined methane yields ranging from 205.8 and 283.7 l_N CH_4/kg ODM. In additional studies, the authors determined specific methane yields varying from 359 to 422 l_N CH_4/kg ODM on a samples set of 7 genotypes. The average specific methane yield for late maturing varieties (FAO 380 to 600) was 398 l_N /kg ODM (Amon et al., 2006a; Amon et al., 2007a). All these studies attributed the large variation ranges to agricultural practices (variation in planting and harvesting dates) and/or genotypes. The yields of the first harvest (at the milk stage) were the highest. The methane yields

decreased with the increasing maturity and were the lowest at the end of the dough stage (Amon et al., 2006a). The validation of these values based on the maize growth pattern, the biochemical composition, the specific lower heating value and the methodological aspects mentioned earlier has not been yet sufficiently clarified.

2.4 Ensiling process

Biogas plants need to be fed with a constant quality of substrate throughout the year while the growing season in almost all countries with temperate climates is restricted. The harvest takes place only at specific time during the year. This makes it necessary to have a standardized feedstock through conservation. In general, energy crops for biogas production are conserved as silages. Silage is the material produced by a controlled fermentation of a high moisture content forage (McDonald 1981). The main aim of this conservation method both for animal nutrition and biogas production is to secure a high quality feedstock with low nutrient and energy losses (Kalzendorf, 2006; Pahlow, 2006) for a longer duration.

The changes that take place when forage crops are ensiled are complex and not fully understood (Woolford, 1984). Nevertheless, it is known that the major metabolic pathway that takes place is a lactic fermentation under anaerobic condition, whereby lactic acid bacteria ferment the naturally occurring sugars to a mixture of organic acids, predominantly lactic acid. The process takes place in three phases. The first phase is called the “aerobic phase” and is due to remaining oxygen in the harvested material. For several hours after ensilage, the crop continues to respire until the oxygen supply has become exhausted and anaerobic conditions are established (Woolford, 1984). During this step the pH is 6.0 - 6.5 and facultative anaerobic microorganisms such as fungi, yeasts, and enterobacteria dominate the microflora (Thylin, 2000). These microorganisms proliferate, oxidizing residual sugars and lactic acid, acetic acid, and ethanol as substrate. When the microbial mass formed is large enough, the heat released from oxidation gives rise to a measurable increase of temperature (Pahlow et al., 2003), which denotes the energy depletion of the crop material. Hence, to avoid this energy depletion, the essential objective

in preserving crops by natural fermentation is the achievement of anaerobic conditions (McDonald 1981).

The removal of oxygen initiates the second phase, also called “the main fermentation phase”. During this phase the groups of microorganisms that proliferate during the aerobic phase are replaced by lacto-bacteria whose fermentation products (mainly lactic acid but also acetic acid) suppress all competing bacteria. The faster the fermentation is completed, the more nutrients will be retained in the silage. Lactic and acetic acids inhibit also the enzymatic depletion of protein compounds of the silage. The success of this phase depends also on the crop properties (e.g. WSC and nitrogen content) and ensiling condition (e.g. compaction grade and yeast population). An ideal crop for ensiling should contain an adequate level of fermentation substrate in the form of water-soluble carbohydrates (WSC) (McDonald, 1981). Additionally, the buffer capacity of the crop to be ensiled should be low in order to maintain a higher WSC/buffer capacity quotient. This quotient expresses the acid-generating capacity of the crop material, and hence the ability of the crop material to undergo a fermentation process. Crop materials with a WSC/buffer capacity quotient below 2.0 are considered to be difficult to ensile (Jänicke, 2006). The minimum WSC content for an optimum fermentation should range between 2.0-3.0% on wet weight basis or 8.0-9.0% on dry weight basis (Nußbaum, 1998). The main fermentation phase is generally accomplished in 7 days (Pahlow, 2006).

With the decrease of the fermentation process, the silage reaches a third phase called “stable conservation phase”. During the stable conservation phase only limited specialized acid-tolerant enzymes continue to degrade polysaccharides. This assures replenishment in simple sugars which are necessary to keep the silage stable over a longer duration of time. During this period the population of lacto-bacteria decreases to 0.1% in comparison to the beginning of the main fermentation phase. However, yeasts can survive in lower pH conditions than lacto-bacteria as spores. When the conditions are favorable (at opening of the silo they will start multiplying with a risk of reducing the nutrition quality of the silage). The German

agriculture society has developed a key for the evaluation of the ensiling success (Kaiser 2006).

Ensiled samples might exhibit different methane yield potential than non-ensiled samples. To compare the specific methane yield potential of silage with that of fresh samples it is of paramount importance to take into account the ensiling losses as shown by Mukengele and Oechsner (2007) and confirmed by Hermann (2011). The procedures for the consideration of the thermolabile compounds available in the silage (fatty acids and alcohols) were provided by Weißbach and Kuhla (1995).

2.5 Biogas technology

Biomasses can be converted into energy via 3 main pathways: thermo-chemical processes (mainly combustion, carbonization and pyrolysis); physico-chemical (e.g. processes based on the use of plant oils) (Kaltschmitt, 2001); and biochemical (alcoholic fermentation and anaerobic digestion). Thermo-chemical and physico-chemical processes are not appropriate options for biomass of high moisture content (e.g. energy crops with moisture content of 65-85%) because of the high energy losses through enthalpy of vaporization (Kesten, 2007). In fact, the water enthalpy of vaporization is more than five times the energy required to heat the same quantity of water from 0°C to the boiling point (100°C) (Anonym, 2010). For this type of biomass the choice of anaerobic digestion as judicious pathway for energy production is justified. In general energy crops based biogas plants operate in semi-continuous mode.

2.5.1 Process

The biogas technology can be defined as the use of anaerobic fermentation for the breakdown of organic matter in order to produce a secondary energy carrier called biogas. Biogas itself is mainly a mixture of methane (50 to 70%) and carbon dioxide (29 to 49%) gases. Other volatile components like hydrogen sulphide (H_2S) and ammonia (NH_3) formed in this process will also end up in the biogas but in small

amounts (Weiland, 2001; Fuchs, 2007). Behind this short definition is hidden a very complex system involving a wide range of microorganisms mainly categorized in 5 groups. The process is generally described as taking place in 4 steps (Fuchs 2007).

The **first step** called "**hydrolysis**" is initiated by hydrolyzing (fermentative) bacteria (lipolytic, proteolytic, and cellulolytic). During this step hydrolyzing bacteria secrete extracellular enzymes (also called exoenzyms) to degrade macromolecules into their component subunits (monomers). Generally, nonstructural carbohydrates are readily degraded. Structural carbohydrates which are predominant in biomass require a complex of cellulolytic enzymes (exo-glucanases, endo-glucanases, cellobiases, etc.) and resist therefore hydrolysis (Fuchs, 2007). For this reason hydrolysis can be a rate-limiting step for methane production from energy crops. The rate of hydrolysis is determined by both microbial constraints (e.g. cellulase production, retention time) and physical and chemical characteristics (e.g. cross-linkages of phenolic units, surface area/particle size ratio). The hydrolysis of fats by extracellular lipase enzymes is generally rapid if fat is soluble. Moreover, fats are more soluble if the pH value is high (pH 8) compared to the pH of acidifying reactors (5.5 to 6.0) where fat is mostly insoluble and the hydrolysis is low (Kortekaas, 2002).

The soluble products of hydrolysis are metabolized intercellularly by a complex consortium of hydrolytic and non-hydrolytic microorganisms (Lubberding, 2002) in a **second step** called "**Acidogenesis**". The products are mainly volatile fatty acids (acetic acid, propionic acid, and butyric acid), hydrogen (H_2), and CO_2. Negligible quantity of alcohol and lactic acid is also formed. The products of the second step are converted to acetic acid, H_2, and CO_2 by the hydrogen-producing acetogenic bacteria. This **third step** is called "**Acetogenesis**" and delivers substrates for methanogenic bacteria.

Most of hydrogen-generating reactions are thermodynamically unfavorable (positive $\Delta G°'$) under standard conditions. Due to the high affinity of the methanogenic bacteria towards H_2, the partial pressure of H_2 is kept as low as 10^{-2} atmosphere in the presence of these microorganisms to make these reactions thermodynamically feasible. Hobson and Wheatley (1993) state that carbohydrate fermentations can

proceed in the absence of hydrogen-utilizing bacteria, while the complete anaerobic metabolism of lipids can only proceed in the presence of a suitable hydrogen-utilizing bacterium.

The fourth step called "**Methanogenesis**" involves the production of methane by methanogenic bacteria. They convert the intermediate products to methane and carbon dioxide via one of two routes. Nearly all known methanogenic species are able to produce methane from H_2/CO_2. Only few species of methanogens isolated up to now are capable of acetoclastic methane formation. The hydrogenotrophic pathway is important to the entire digestion process, since it is responsible for removing H_2 and maintaining the low H_2 partial pressure required for the production of acetate. If H_2 concentrations increase above the threshold level, the fermentative bacteria will change to the production of acids other than acetic acid, and the conversion to acetate by the acetogens will fall (Burton and Turner, 2003). In the whole nutrient chain, only a small portion of energy available is needed for the growth of different bacteria, so that the larger share of the potential energy is kept as methane (Fuchs, 2007). Typically 5-10% of the organic material degraded is utilized to synthesize bacterial mass (Angelidaki and Sanders, 2004).

A stable biogas production process requires that all microorganisms consortia involved remain in a harmonious dynamic equilibrium. In the traditional energy crop based biogas plants this equilibrium is reached by operating at low OLR. In these type of plants (mostly CSTR: "completely stirred tank reactors") the digesters are over-dimensioned to cope with both the resistance of the cell-wall matrix to hydrolysis and the excessive microbial biomass washout (Volume = HRT * daily flow rate).

2.5.1.1 Operation parameters affecting biogas production

There are only few parameters that can be altered within limits to influence the biogas production process (i.e. temperature, organic loading, and retention time). Since the entire complex system can only be controlled using these few levers, the

knowledge of the metabolic pathways and inhibition mechanisms are prerequisites for the adjustment of operation parameters.

Temperature

Three process temperature ranges are recognized: the psychrophilic range (0°C to 20°C); the mesophilic range (20°C to 42°C); and the thermophilic range (42°C to 75°C). The upper limits of these ranges are defined by the temperature at which the decay rate of bacteria in that respective range start to exceed the growth rate, meaning, for example, that the activity of mesophilic bacteria will be low in a psychrophic range (Van Lier 2002). Kelderman (2002) studied a substrate at different temperatures and retention times, and found that methanogenesis shows two optima: one in mesophilic environment (33°C to 42°C) and a second in a thermophilic at 55°C to 60°C. The reaction rate between 45-48°C shows a relative minimum. In Germany, one favors mostly the mesophilic range (40-42°C). In practice, the lower segment of the mesophic range (below 37°C) seems to affect negatively the biogas production rate. The psychrophilic range requires more space because of the low microbial growth rate, while thermophilic processes are prone to consume too much energy, and to be sensitive to slight variations in operating conditions.

In the practice, temperature fluctuations are well resisted by methanogenic bacteria, as long as the upper limits of the process temperature range are not exceeded and the temperature shift is not sudden. For research purpose the operation temperature should be kept constant.

Organic loading rate

The organic loading rate is given by the following formula:

$$OLR = \frac{C_i * Q}{V_R}$$ (Equation 4)

where (*C_i = organic dry matter concentration of the feedstock in %; Q = feedstock quantity in Kg/d; and V_R = digester liquid volume)*

Hydraulic retention time

The hydraulic retention time is the average length of time the substrate remains in the digester for treatment (VDI 4630, 2006) and is given by the following formula:

$$HRT = \frac{V_R}{\dot{V}} \qquad \text{(Equation 5)}$$

where *($\dot{V}$ = daily feeding rate in m^3/d; V_R = digester liquid volume).*

In a CSTR, the HRT expresses the average time of contact between the microorganisms and the feedstock fed to the digester.

pH and buffer capacity

The production of biogas performs in a very narrow pH spectrum between pH 6.8 and 7.5 (Kapp, 1984). Systems operating beyond this range have been reported. However, methanogenic bacteria are impeded below the threshold of pH 6.8. The pH fluctuation in the digester is related with the VFA (volatile fatty acids) accumulation. Since acids show toxicity in their unionized form, the lower the pH, the more toxic the VFAs. In most cases, inhibition increases with the increasing concentration of VFAs and generally the accumulation of VFAs leads to process failure. In some cases, the digester continues to run, but at a suboptimal efficiency level. Such an unstable system can fail whenever a slight change takes place (e.g. change in feeding regime).

To maintain a stable operation, the system should possess an ability to resist a change in pH as VFAs are accumulating (buffer capacity mostly expressed in AD as alkalinity). The bicarbonate ions (HCO_3^-) represent the most important buffer system in a digester. The bicarbonate ions result from the dissolution of (CO_2) in aqueous milieu. In fact, the partial pressure of (CO_2) in the gas phase is, according

to Henry's law, in equilibrium with its dissolved fraction (Bischofsberger et al., 2005; Khanal, 2008). An exceeding increase in VFA causes the bicarbonate alkalinity to decrease.

Trace and oligo-elements

A stable operation of a biogas production process depends largely on an adequate distribution of essential nutrients (macro, oligo, and micro elements). Deficits, excess, or unavailability of these nutrients causes process imbalance or incomplete digestion. Imbalances affect the process performance and in acute cases, they can lead to a total process collapse with tremendous consequences. Trace elements play a major role in different catalytic processes. The lack of trace elements by methanogenic bacteria leads to a reduction of the reaction velocity for the entire process. This means that the conversion/degradation rate of such a process is going to be very low. In general, trace elements have to be provided by the feedstock. If the feedstock does not provide the broad spectrum of essential trace elements, they have to be added. Lemmer et al. (2010) found that beet and grass silage provided considerable amount of both macro and micro-elements in comparison to cereal and maize whole-crops so that such systems require scarcely an additional supply in trace elements. Trace elements are used up and therefore have to be renewed regularly. The most important elements mentioned in the literature include Fe, Ni, Co, Mo, S, P, Cu, Se, W (Zehnder and Wuhrmann, 1977; Schönheit et al., 1979; Oleszkiewicz and Sharma, 1989). The impact of Ni addition on methane generation has been widely studied. Different authors (Oleszkiewicz and Sharma, 1989; Burgess et al., 1999; Haydock et al., 2004) state that methanogenic bacteria have a higher requirement for Ni, which is for all other bacteria in general not necessary.

The trace elements added to the digester should be kept in solution in order to be bio-available. Nevertheless, Snoeyink and Jenkins (1980) state that because of the partial dissociation phenomenon, in a digester at typical pH (7.3-7.6) species responsible for trace element precipitation (S^{-2} and CO_3^{2-}) are present only in small amounts. These small quantities do not cause severe precipitation problem (Lubberding, 2002). Preißler et al. (2007), however, found that the manure free

systems had a high demand in Fe supply than often reported in the literature. The authors found also that manure free digesters had considerably lower concentrations of both macro and micro-elements, especially Mg, Na, B, Co, Cu, Mn, Ni, Se, and Zn, than biogas plants with a high manure content. Oechsner et al. (2011) give the following ranges for a stable energy crop digestion: Ni (3 to 16 mg/kg DM); Co (0.4 to 5 mg/kg DM); Mo (1 to 6 mg/kg DM); Se (0.2 to 2 mg/kg DM); Fe (1500 to 3000 mg/kg DM); Mn (100 to 1500 mg/kg DM); Wo (0.1 to 30 mg/kg DM); Zn (30 to 300 mg/kg DM). Among oligo-elements, Na, K, and Vitamins (B2 and folic acid) are also reported to have a positive impact on methanogens (Scherer and Sahm 1981; Sowers and Ferry 1985).

2.5.2 Specific methane yield of energy crops in semi-continuous flow digesters

As previously stated, the specific methane yield potential of energy crops are determined using batch-tests and these are conducted in a way that the ultimate biochemical potential be determined. In contrast, energy crops based biogas plants generally operate in semi-continuous flow mode. It is known that because of the above mentioned operation parameters (e.g. hydraulic retention time), the specific methane yield in semi-continuous systems are expected to be different from the specific methane yield potential determined in batch systems. Nevertheless, an understanding of the extent to which operational factors affect the specific methane yield generation in semi-continuous systems is important to both crop breeders and bio-process engineers. In fact, comparisons of the theoretical maximum specific methane yield potential, the specific methane yield potential, and the specific methane yield in semi-continuous systems provides important information related to the actual conversion efficiency in different practical conditions and the magnitude of plant breeding effort that can be justified to reach the highest possible bioconversion efficiency.

3 Objectives of this work and approach

This work is one of two PhD theses initiated in the frame of a joint research project entitled, “The development of the biosynthetic potential of local crops as energy crops for biogas production”. Three research partners were involved in this project: the crop breeding company KWS SAAT AG Einbeck; the Bavarian State Research Center for Agriculture – Crops Science and Plant Breeding; and the Institute for Agricultural Engineering and Bioenergy of the University of Hohenheim. The project had four main objectives:

I. The breeding of appropriate high biomass yielding maize varieties for biogas production;
II. The development of a NIRS (near infrared reflectance spectroscopy) calibration to predict the specific methane yield potential of maize whole-crop;
III. The investigation of the influence of ensiling process on the specific methane yield potential of maize;
IV. The scaling-up of the batch fermentation process to a semi-continuous flow digester (simulation of a full-scale plant).

To work out the research topics considered in this project, several field trials were conducted between 2002 and 2006. The breeding issues were covered by KWS SAAT AG Einbeck. The agronomical, NIRS calibration, and sustainability topics of the project were carried out by the Bavarian State Research Center for Agriculture – Crops Science and Breeding, and are reported in the thesis of Eder (2010). Among the research topics assigned to the Institute for Agricultural Engineering and Bioenergy of the University of Hohenheim were: the investigation of the influence of ensiling process on the specific methane yield potential of maize and the scaling-up of the batch results to continuous flow system (simulation of a full-scale plant).

The results of the first assignment are presented in Experiment I. This experiment presents the actual benefit of the ensiling process on maize of different maturity groups. It treats also the methodological aspects related to the use of silage samples for the determination of the specific methane yield potential of energy crop as

already referred to in Mukengele and Oechsner (2007). Although Hermann (2011) validated the results of Mukengele and Oechsner (2007) by investigating various energy crops, the overall impact of the combined effects of mechanical pretreatment and ensiling processes on the determination of the specific methane yield potential of maize (i.e. non-drying-chopping samples versus drying-milling or ensiling-non-drying-chopping samples versus ensiling-drying–milling samples) still needed to be addressed. Furthermore the overall benefit after deducting the inherent conversion losses should be displayed.

The scaling-up of batch results are presented in Experiment II. In this experiment, we performed, additionally, an energy balance to evaluate both the bioconversion/substrate-use efficiency and the reactor-use efficiency. The experiment provided information about the full potential of maize whole-crop and the actual share of the potential that is not tapped in full-scale conditions.

The literature review has shown that the specific methane yield potential of maize varies greatly. This broad variation was putatively explained by differences in the chemical composition and degradability of the biomass used as substrate. We therefore undertook to examine the spectrum of the specific methane yield potential of maize underpinned by both the evolution of the biochemical composition and the absolute lower heating values. Furthermore, the literature review showed that there is a need to point out the biochemical crop traits that characterize “The” biogas maize genotype. In fact, when selecting genotypes for animal nutrition, crop breeders ask of animal scientists for guidance as to which forage characteristics should be modified to achieve the desired improvement in animal performance (Buxton and Casler, 1993). In the same way, to select and breed for high specific methane yield potential, crop breeders challenge bioprocess engineers to point out biochemical crop traits that should be targeted. The values of correlations between specific methane yield potential and the targeted predictors provide the limit in breeding efficiency. The higher the value of the correlations, the better a selection or a breeding program can be carried out.

As shown in the literature review, the selection criteria used as predictors for high energy value in animal production sector have been used as predictors for high specific methane yield potential, and based on this approach different mathematical models haven been developed to predict the specific methane yield potential of energy crops. Because of the inconsistencies mentioned previously (e.g. digestibility versus degradability, drawbacks of the Weender methodology, etc.) it seemed necessary to exam the appropriateness of these predictors for specific methane yield potential selection's purpose. Hence, this work evaluated the appropriateness and validity of different biochemical crop traits and *in-vitro* estimates of digestibility as predictors for high specific methane yield potential. This topic was approached in three steps:

(1) The investigation of the relationships between the biochemical crop traits and the specific methane yield potential in a more artificial way - namely by intentionally blending crop fractions (stover and ear fractions) in specific proportions. In this way, the unpredictable environmental factors that might induce random structural changes in the crop, especially in the cell-wall fractions, and bias the interpretation, were discarded and considered as constants. The main determinants remaining were the absolute values of the targeted traits and the genotypes used. The genotypes used were chosen based on their maturity groups. These were evaluated in Experiment III;
(2) The assessment of the relationships between the biochemical composition traits and the specific methane yield potential of whole-crop materials across genotypes, maturity groups, and growth stages. Here, the environmental effects were considered and the variation ranges of both the biochemical crops traits of maize and the specific methane yield potential were comprehensively examined. The overall objective being to examine whether it is possible or not to point out biochemical traits that characterize "The" biogas maize genotype. These were evaluated in Experiment IV;
(3) The comparison of the *in-vitro* estimates of digestibility and biodegradability (recovery efficiency) as predictors for high specific methane yield potential in AD system. This stage examined the sharpness of both cell-wall content and

in-vitro estimates of digestibility as predictors. These were evaluated in Experiment V.

The questions raised in the discussion above are also of concern for energy crops other than maize. The tendency is to focus on lipid-rich crops. Therefore, we undertook to examine briefly the variation in biochemical traits and specific methane yield potential of both lipid and carbohydrate rich crops (Experiment VI). From a bioprocess engineering standpoint, this experiment helped to formulate general statements about the specific methane yield potential of both carbohydrate and lipid-rich biomass crops, and to evaluate the relationships between the biochemical crop traits and the specific methane yield potential. Agronomical aspects were not considered.

4 Material and Methods

4.1 Batch-test: The Hohenheim biogas yield test (HBT)

In this system, a glass syringe sampler (volume of 100 mL and a graduation of 1/1 mL) rendered gas tight using silicon paste (Baysilone-Paste medium viscosity, BAYER), was used as a mini-digester (Figure 6). The syringe sampler served also as a biogas collector (storage). The mini-digesters were filled with the inoculum and a tiny quantity of the test-substrate (Helffrich and Oechsner, 2003).

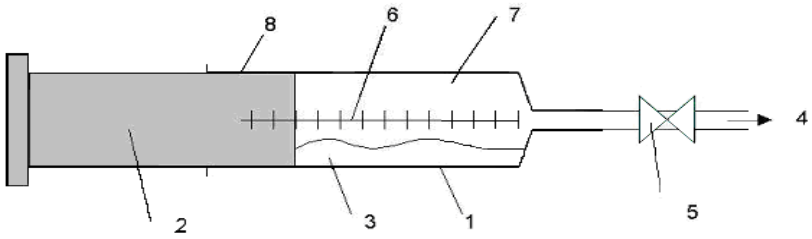

Figure 6: Mini-digester and gas holder with: 1) glass syringe sampler; 2) piston; 3) fermenting substrate (inoculum + test-substrate); 4) opening for gas analysis; 5) hose clamp; 6) graduation 1/1; 7) gas chamber; 8) lubricant and sealant (Helffrich et al. 2005)

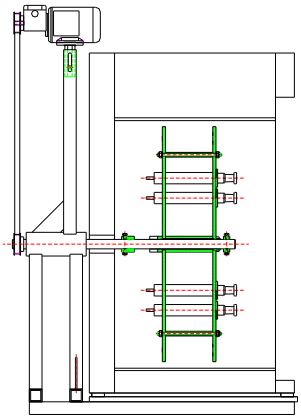

Figure 7: Scheme of the Hohenheim biogas yield test (HBT) comprising syringe sampler, rotating drum placed in the incubator.

The whole apparatus was placed in an incubator chamber equipped with a slow rotating drum (Figure 7) to ensure an intimate contact between the test-substrate and the inoculum, as well as a proper heat distribution at the mini-digester surface. The biogas formed was enclosed in the syringe. The pressure due to gas accumulation in the enclosure caused the piston to be pushed backwards so that the biogas within the enclosure remained at the atmospheric pressure. The biogas volume was read from the graduation of the syringe sampler while the methane content was determined using an infrared methane sensor "Advanced Gasmitter" (Pronova Analysentechnik GmbH & Co. KG, Berlin, Germany). Only CH_4 was analyzed for the batch trials. To assure that methane content is measured in pre-dried biogas, the methane sensor was equipped with two successive gas filters. The first gas filter consisted of a cotton pad and the second was a phosphorus pentoxide drying agent with humidity indicator. Thanks to the humidity indicator, the filters could be changed whenever necessary. The digestion took place at 37°C (±1) for a retention time of 35 days.

Triplicates of a control containing only the inoculum were used as blank for the correction of the specific methane yield potential of the test-substrate. The inoculum was sieved before being fed to the mini-digesters. Two reference standard substrates (standard reference hay and standard cattle concentrate feedstuff) whose methane yield potentials are known were used to assure the repeatability of the test.

The test was carried out in triplicate. Thirty grams (30 g) of inoculum, together with 400 mg of the test-substrate were fed to the mini-digester. The ratio between test-substrate and inoculum was kept constant for all batch-tests; $\left[\frac{ODM_{Substrate}}{ODM_{Inoculum}} \leq 0.5 \right]$.

The results of a batch-test were valorized only if specific conformity values were met, as described by Helffrich et al. (2005) and the VDI 4630 guidelines (2006). In fact, the German version of the guideline VDI-4630 is authoritative. The retention time was 35 days. The specific methane yield potential of a test-substrate was gained by correcting the methane production for the inoculum's own methane production. The results were expressed as the arithmetic mean of the accumulated

methane yields from triplicate experiments, in m^3 $CH_4.kg^{-1}$ ODM at STP (standard condition of temperature and pressure). This Batch-test allows to determine the specific methane yields of energy crops with a coefficient of variation (CV) of 2.8% Mittweg et al. (2012).

4.1.1 Inoculum

The standard inoculum was a mixture of active inocula from different mesophilic biogas plants where the following substrates were digested: cattle manure, different energy crops, and kitchen-food waste. These inocula were brought to the biogas laboratory at the University of Hohenheim, where they were mixed and conditioned in a 400 L digester at 37°C. In order to accommodate a broader spectrum of microorganisms and provide essential trace elements, the blend inoculum was fed daily at an OLR of 0.5 kg VS/m^3*d with mixed feedstocks containing carbohydrates, protein, fat and raw cattle manure. The feeding was conducted in a way that the inoculum's own gas production could be kept at the lowest level possible during batch-tests. The inoculum was sieved before being used in the batch trials.

4.1.2 Standard reference substrates

To secure the results of the batch-tests, standard reference samples were used. At the University of Hohenheim, two substrates of a known composition and methane yield potential were used - namely standard reference hay and cattle concentrate feedstuff. The methane yield potential and digestion behavior of these two substrates are well known, as they were used as reference substrates in experiments described by Helffrich and Oechsner (2003) for HBT and Steingass and Menke (1986) for HFT (Hohenheimer Futtermitteltest).

4.1.3 Samples conditioning

The standard procedure for the conditioning of the test-substrate consisted of drying the sample at low temperature (60°C) followed by milling (using a cutting mill) at a size of 1 mm particles. The samples used were freshly harvested crop material (not silages) and conditioned as mentioned above. The samples so prepared were preserved in hermetically sealed flasks. If not expressly indicated, the above mentioned conditioning method was used. Where other sample conditioning methods (e.g. blending or mixing, conservation in a cool storage) have been applied (Experiment I and Experiment II) additional details related to the methods used are explicitly described in the experimental set-up.

4.2 Laboratory set-up for the semi-continuous flow trial

The laboratory set-up for the semi-continuous flow trial consisted of 6 main units (Figure 8):

- horizontal digester;
- robot feeder (not shown on the scheme);
- mixer frequency and intensity regulation control unit (not shown on the scheme);
- gas storage bag;
- gas analyzer;
- central control unit for the automatic command of the lab and data logging (not shown on the scheme).

The liquid feeding was performed automatically by a robot feeder. The robot was equipped with a calibration system in order to control the accuracy of the daily feeding. The horizontal digester unit was a continuously stirred tank reactor (CSTR) with intermittent overflow through volumetric displacement. The digester was made of a double jacket stainless steel vessel with a liquid volume of 17 L (Figure 9). The mixing was performed by a horizontal paddle stirrer run by an electric motor. The

mixing frequencies and timing were controlled by the central control unit. In order to maintain a constant fermentation temperature, the digesters were heated up to the set temperature by an automatically-regulated heating system. The heating system unit was composed of a thermostat and an external heat exchanger made of a coil heater submerged in a water heating vessel. Water circulation velocity was controlled by a pump. The tubes constituting the water circuit were isolated with glass wool to avoid heat loss through the loop. The inoculum used for the semi-continuous flow trial was not sieved.

The gas generated in the digester was collected in a gas storage bag (Linde). Before entering the gas storage bag, the biogas collected was cooled through a heat exchanging system to condense the water vapor contained in the gas. The intermediate-pressure built by the incoming gas in the gas storage bag was rendered constant with the pressure in the digester by the pressure equalizer device. This mechanism allowed the volume of substrate in the digester to remain constant and avoided substrate overflow due to overpressure. The daily produced biogas was measured using a mass flow measuring device while the biogas quality was analyzed toward its quality (CH_4, CO_2, H_2S) using an electrochemical sensor (Awite Bioenergie GmbH, Langenbach, Germany). After each gas analysis the gas analyzer and the biogas line were purged. The biogas quality analyses were done in triplicate and the results were presented as mean values. The complete biogas laboratory was controlled via a central unit where data generated were also stored. The results were expressed as accumulated methane yield in m^3 $CH_4.kg^{-1}$ ODM at STP (standard condition of temperature and pressure). The specific methane yield of the test-substrate was corrected of the inoculum's own methane production.

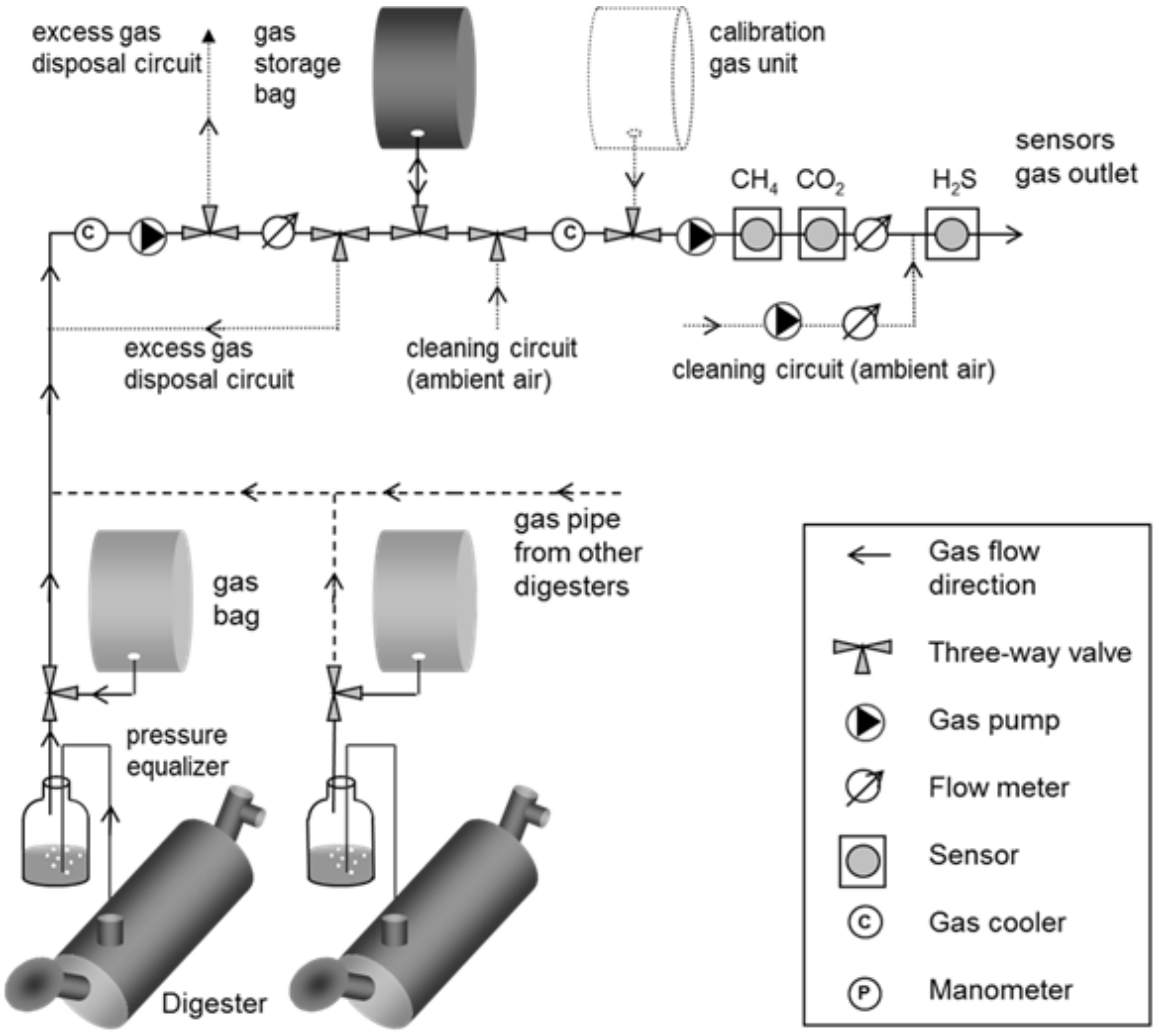

Figure 8: Simplified overall set-up of the Hohenheim laboratory for semi-continuous anaerobic digestion trials. (Brulé, 2014)

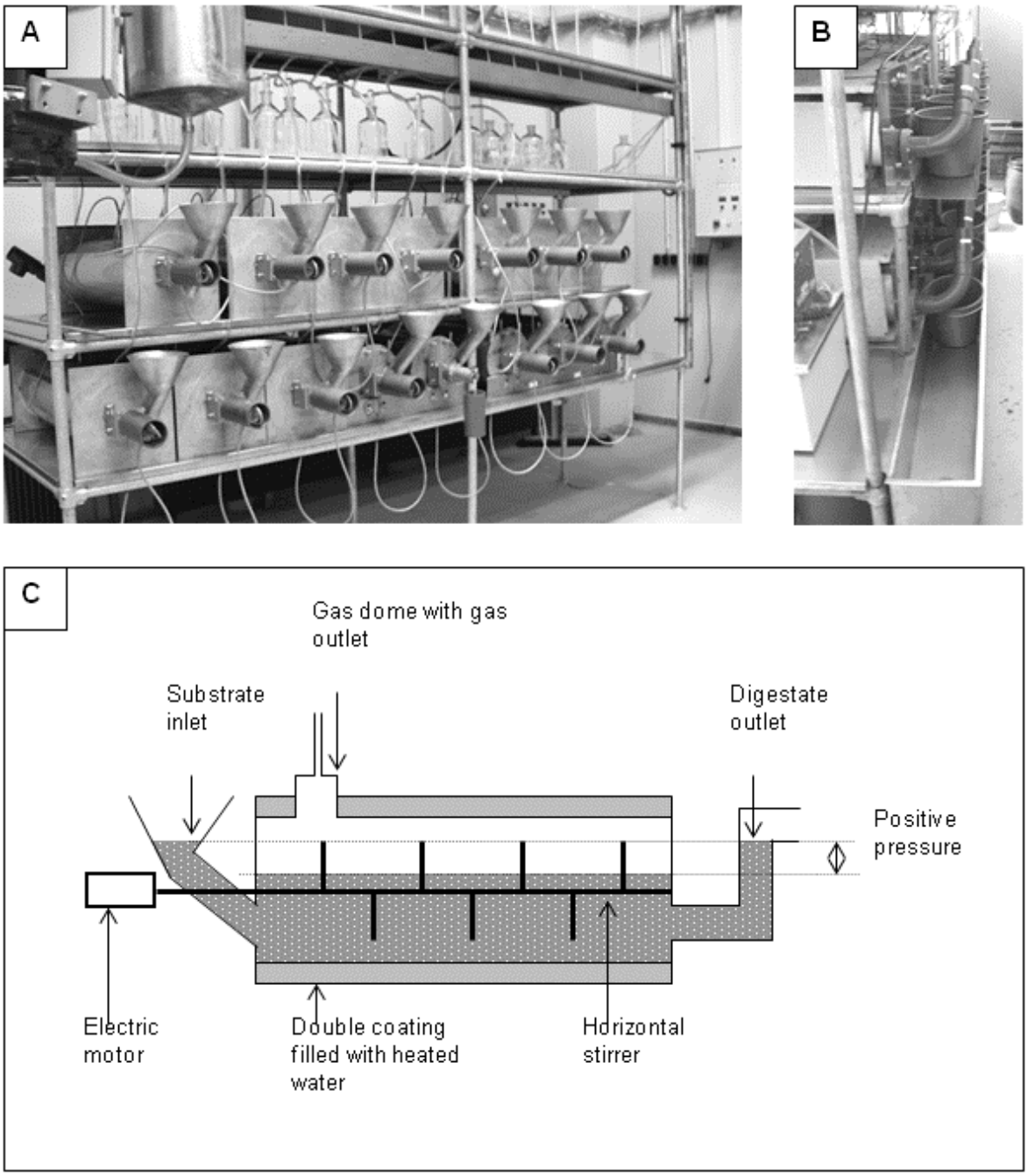

Figure 9: Horizontal biogas digester of the Hohenheim biogas laboratory with a volume of 17 L. (A) front side, (B) back side, (C) scheme.

4.3 Characterization of the feedstocks

Dry matter (DM) and organic dry matter (ODM)

Crop materials were characterized according to standard methods:

- The determination of dry matter (DM) was performed according to the DIN EN 12880 (2001);
- The determination of the organic dry matter (ODM) was performed according to the DIN EN 12879 (2001);

Biochemical composition

The biochemical composition of the feedstocks was determined using either the wet chemical analyses according to “Methodenbuch VDLUFA Bd.III: Untersuchung von Futtermittel” (VDLUFA, 1988) at the Institute for Chemistry, University of Hohenheim or the NIRS (near infrared reflectance spectroscopy). The NIRS spectra were recorded using a spectroscope developed by the company Foss Instruments, Modell NIR System 5000 (NIRSystems, Silver Spring, MD, USA) by the Bavarian State Research Center for Agriculture – Crops Science and Plant Breeding. Data processing was performed by the same institution using the statistic program (WIN ISI II), Infrasoft International Inc. (Port Matilda, PA, USA) and Table 2 describes parameters that have been measured (Eder 2010):

Table 2: Description of NIR measured parameters. (adapted from Eder 2010)

Parameter	Description
	Protoplasm
Starch	Starch content of whole-crop [in %] according to EWERS (1908)
WSC	Water soluble carbohydrates [in %] according to LUFF & SCHOORL (1928)
WSC-R	Water soluble carbohydrates in the stove
XP	Crude protein content [%] according to KJELDAHL (1883)
	Cell-Wall
NDF	neutral detergent fiber content according to VAN SOEST (1963)
NDF-R	neutral detergent fiber content in the stove
ADF	acid detergent fiber content according to VAN SOEST (1963)
	Digestibility
CDOMD (ELOS)	cellulase digestible organic matter of the dry matter (CDOMD) [%] according to DE BOEVER et al. (1986)
IVDOM	*In-vitro* digestibility of ODM of the stove [%] according to TILLEY and TERRY (1963)
IVDOM-R	*In-vitro* digestibility of ODM [%] according to TILLEY and TERRY (1963)
DNDF-R	digestibility of NDF in the stover [%] according to VAN SOEST (1963) and TILLEY and TERRY (1963)

The volatile compounds (fatty acids, alcohols, etc.) of the silage were analysed using the HPLC (high performance liquid chromatography) at the "Landwirtschaftliches Zentrum für Rinderhaltung, Grünlandwirtschaft, Michwirtschaft, Wild und Fischerei Baden-Württemberg" in Aulendorf. The correction of the dry matter and organic dry matter content was achieved using the exact methodology proposed by Weißbach and Kuhla (Weißbach, 1994; Weißbach and Kuhla, 1995).

4.4 Methodologies

4.4.1 Correction of the organic dry matter losses

The organic dry matter losses of the silage samples were taken into account by correcting the dry matter content as proposed by Weißbach and Kuhla (Weißbach, 1994; Weißbach and Kuhla,1995):

$$\boldsymbol{DM_{sc}} = \boldsymbol{DM_{su}} + (\boldsymbol{VFA_s} - \boldsymbol{VFA_d}) + (\boldsymbol{A_s} - \boldsymbol{A_d}) + (\boldsymbol{NH_{3S}} - \boldsymbol{NH_{3S}}) + \mathbf{0.08}\,\boldsymbol{LA} \quad \text{(Equation 6)}$$

where:

DM_{sc}= the dry matter of the silage corrected
DM_{su}= the dry matter of the silage uncorrected
VFA_s= the sum of volatile fatty acids in the silage (formic acid not included)
VFA_d= the sum of volatile fatty acids in the dried sample (formic acid not included)
A_s= the sum of monohydric alcohols in the silage
A_d= the sum of monohydric alcohols in the dried sample
NH_{3S}= Ammoniac content of the silage
NH_{3d}= Ammoniac content of the dried sample
LA= lactic acid content of the silage

4.4.2 Procedure for the determination of the hectare-methane yield

The specific methane yield per unit of land also referred to as hectare-methane yield [m_N^3 CH_4/ha] was calculated as following:

$$\boldsymbol{Y_{Mha} = SMY * DMY_{ha} * ODM\% * 1000} \quad \text{(Equation 7)}$$

Where:

Y_{Mha} Specific methane yield per unit of land (the hectare-methane yield) [m_N^3 $CH_4.ha^{-1}$]

SMY Specific methane yield of the crop per unit of ODM [m_N^3 $CH_4.kg^{-1}$ ODM]

$ODM\%$ ODM content [in % of DM]

4.4.3 Procedure for the determination of the theoretical maximum methane yield and the biodegradability

The theoretical maximum methane yield potential was derived from the lower heating value (net calorific value). The higher heating value was determined using a bomb calorimeter and the lower heating value was calculated as described by AFNOR (2004). The theoretical lower heating value so computed was converted to methane yield potential using the methane energy density factor of 35,802 MJ/m^3 CH_4 (Anonym, 2014) and deduced of 10% to account for the typical anaerobic

conversion's energy losses (biomass growth and heat production) according to Angelidaki (2002) and Spanjers (2011). The result was expressed in m^3 $CH_4 * kg^{-1}$ ODM.

$$\boldsymbol{Y_{CH_4\,max} = \frac{[LHV]}{35.802} * 0.90} \qquad \text{(Equation 8)}$$

Where:

$Y_{CH_4\,\max}$ Theoretical maximal methane yield (m_N^3/kg ODM)

LHV Lower heating value of the sample (expressed in MJ/kg ODM).

The biodegradability (bioconversion efficiency) was expressed as percentage of the theoretical maximum specific methane yield potential removed from the system (digester) or converted into methane using the equation below. It was also compared to the *in-vitro* digestibility (CDOMD) in absolute term as justified in Section 2.2.3.

$$\boldsymbol{\eta = \frac{SMY}{Y_{CH_4 max}} * 100} \qquad \text{(Equation 9)}$$

Where:

η Biodegradability (or bioconversion efficiency) in AD

SMY Specific methane yield potential in batch or CSTR (m_N^3/kg ODM)

$Y_{CH_4\,max}$ Theoretical maximal methane yield potential (m_N^3/kg ODM)

4.5 Statistical Methods

The statistics shown in this section were performed using the software SPSS-Statistics package 23.

4.6 Overview of the experimental design

Six different experiments were conducted in this work. The experimental set-up and the specific objectives for each experiment are given in corresponding sections. Table 3 shows the overview of the experiments carried out.

Table 3: Overview of the experiments carried out.

Experiment		Trials/Methods	Parameters	Information
I	Quantification of the effect of ensiling and drying process on the determination of the specific methane yield potential of maize whole-crop	Ensiling Batch tests HPLC Mechanical pretreatments	Volatile solids losses Chemical composition Specific methane yield	Quantification of the drying losses Quantification of the impact of the ODM correction and drying pretreatment on the determination of the sp. methane yield potential Overall quantification of the influence of the ensiling process on the specific methane yield potential
II	Upscaling batch results - Assessment of the bioconversion efficiency in semi-continuous flow system	Batch tests Semi-continuous process Bomb calorimeter	Specific methane yield Chemical composition Energy balance	Process behaviour and Influence of OLR on methane yield Quantification of the residual methane yield Quantification of the Conversion efficiency in different systems
III	Influence of the biochemical crop traits on the specific methane yield potential of intentionally blended maize fractions (ear and stover)	Batch tests	Chemical composition Specific methane yield	Influence of biochemical traits on specific methane yield potential when environmental effects are intentionally discarded Methane yields of crop fractions Thresholds and variation ranges
IV	Influence of the biochemical crop traits on the specific methane yield potential of maize whole-crop	Batch tests	Chemical composition Specific methane yield	Tresholds and variation ranges of the key biochemical traits Influence of biochemical traits on specific methane yield potential
V	Assessment of the *in-vitro* estimate of digestibility for whole-crop (CDOMD) and the biochemical traits as predictors of the biodegrability in AD batch system	Batch tests Bomb calorimeter	Theoretical methane yield Specific methane yield Chemical composition	Theoretical methane yield potential Variation ranges and conversion efficiency Assessment of the applicability of the ruminal digestibility in AD
VI	Evaluation of the specific methane yield potential of various crops alternative to maize	Batch tests	Specific methane yield Chemical composition	Methane yield potential of whole crops and crop fractions Tresholds, variation ranges of the key crop features

4.7 Experiments

4.7.1 Experiment I: Quantification of the effect of ensiling and drying process on the determination of the specific methane yield potential of maize whole-crop

The investigation was conducted using three maize genotypes of three different maturity groups, namely the mid-early (FAO-index 250), mid-late (FAO-index 280) and late (FAO-index 600). The crop materials were grown in Freising, Bavaria and harvested at three different occasions using a forage harvester. After harvest, samples were taken for the determination of the DM and ODM. A portion of the sample was dried at 60°C (24h) and milled using a cutting mill (sieve diameter 1 mm), the second portion was ensiled in preserving glass jars (from the company J. Weck GmbH u. Co. KG, Wehr, Germany) as lab silos, and the third portion was kept deep frozen (so that all samples could be tested in the same batch). The latter is referred to as the fresh variant.

The crop material to be ensiled were compacted in the jars using a pestle. The glass jars used were equipped with a rubber seal and lid to allow for the release of gases while the silage juice was retained in the jars. The samples were not treated with silage additives. For each maize genotype, three glass jars of 2 L were filled. After 6 weeks, the ensiling process was deemed to be complete so that the preserving glass jars could be opened. At the same time, the fresh samples were taken out of the deep freezer and chopped using a laboratory blender (Büchi lab mixer) to have homogenous particles. The silage samples were divided into three portions. The first portion was chopped using the same laboratory blender (Büchi lab mixer), while the second portion was dried at 60°C (24h). The third portion was dried at 105°C. Silage samples were analysed for their volatile compound content (fatty acids, alcohols, etc.). The analyses were performed using the HPLC (high performance liquid chromatography) at the “Landwirtschaftliches Zentrum für Rinderhaltung, Grünlandwirtschaft, Michwirtschaft, Wild und Fischerei Baden-Württemberg” in Aulendorf. The correction of the dry matter and organic dry matter content was

achieved using the exact methodology proposed by Weißbach and Kuhla (Weißbach, 1994; Weißbach and Kuhla, 1995).

The fresh milled-dried (60°C) variant was compared to the fresh-chopped (deep frozen) variant to study the effect of the mechanical pretreatment/conditioning processes on the determination of the specific methane yield potential. The fresh-chopped (deep frozen) variant was compared to the silage variant to quantify the influence of ensiling process on the specific methane yield potential. The silage samples dried at 60°C and 105°C were used to study the volatile solids profile of silage samples and the ODM loss at different temperatures.

The specific methane yield potentials were determined using the Hohenheim biogas yield test (HBT). Table 4 shows the maturity groups of the genotypes used, the growing durations and different pretreatments applied.

Table 4: Maize genotypes, growing durations and mechanical processes investigated.

Genotype	Maturity FAO - Index	Vegetation duration [d]	Variants			
			Fresh and chopped	Fresh dried & milled	Silage chopped	Silage dried & milled
B	250	127	x	x	x	x
		148	x	x	x	x
		168	x	x	x	x
J	280	148	x	x	x	x
		168	x	x	x	x
G	600	127	x	x	x	x
		148	x	x	x	x
		168	x	x	x	x

The overall goal was to quantify the impact of the ensiling process on the specific methane yield potential of maize.

4.7.2 Experiment II: Up-scaling the batch results - Assessment of the bioconversion efficiency in semi-continuous flow system

For this experiment maize silage (Variety: Eurostar; FAO-Index 240) was collected from the research station Meiereihof at the University of Hohenheim and used without additional treatment. During the entire experiment period, the sample was kept in a cool room at 4°C in tight containers. In addition, winter wheat grain was collected from the research station Ihinger Hof of the University of Hohenheim. Prior to the use of wheat-grain for the semi-continuous experiment, a batch-test was performed in order to determine the appropriate way in which wheat-grain had to be used. The influence of different mechanical pretreatments, namely crushing and milling were investigated. The appropriate variant was retained based on the digestion kinetics and the specific methane yield potential.

For the semi-continuous flow digestion, the substrate (maize silage and wheat-grain) were used either single or mixed together in a mixture ratio of (1:1) on ODM basis. The Substrates were fed to the digester together with pre-digested cattle manure collected from the outlet of a full-scale biogas plant. The Manure was added to the energy crop to reach a manure/energy crop ratio of [1:6] on ODM basis. For all the variants, pre-digested cattle manure was used as inoculum (Table 5). The manure for the daily feeding was refrigerated at 4°C over the experiment period.

Table 5: Experimental design of the semi-continuous experiment.

OLR		Total ODM	Shares		crop/manure Ratio	Digester Liq. Vol.
			crop	manure		
(g ODM/l*d)		g ODM/d	g ODM/d	g ODM/d	(ODM)	(L)
Low	2.5	42.5	36.4	6.1	6:1	17
High	4.0	68.0	58.3	9.7	6:1	

Digesters of 17 L capacity were fed at two different organic loading rates (OLR); namely at 2.5 g ODM/l*d (referred to as low OLR) and 4 g ODM/l*d (referred to as high OLR). The digestion proceeded for a hydraulic retention time of 35 days at the temperature of 37°C (± 1°C). The experiment was started with digesters being fully filled with pre-digested cattle manure. The digesters were fed subsequently with the corresponding substrates (i.e. maize silage, wheat-grain or the mixture of both substrates) together with pre-digested manure at the low OLR during seven days. After the seven days' starting phase, the feeding load of 2.5 g ODM/l*d was maintained for the digesters fed at the low OLR, while the OLR was increased to 4 g ODM/L*d for the digesters fed at the high OLR. To keep the hydraulic retention time equal for all variants, a calculated amount of water was added to crop material (Table 6). The experiment was further conducted for more than 3 successive retention times, over a period of 123 days. Two control digesters fed only with the pre-digested cattle manure were run concurrently. Each variant was run in duplicates, except for maize silage at the high OLR which was run in triplicate. Samples were regularly taken from the digesters over the whole experiment. The Volatile Fatty Acids (VFA), the Total Anorganic Carbon (TAC), ammonium, DM and ODM contents were frequently monitored. Both biogas production and biogas quality were analyzed daily. Figure 8 and Figure 9 show the overall set-up of the Hohenheim biogas laboratory.

Table 6: Detailed description of the experimental set-up.

Substrate	N	OLR [g ODM/l*d]	Fresh weight [g/d]			
			Maize silage	Wheat-grain	Water	Manure
Manure (control)	2	1.1				486
Maize silage	2	2.5	129		200	156
Wheat-grain	2	2.5		42	287	156
Mixture (maize + wheat)	2	2.5	64	21	244	156
Maize silage	3	4	206		29	250
Wheat-grain	2	4		67	168	250
Mixture (maize + wheat)	2	4	103	33	99	250

After the experiment the conversion efficiency of the semi-continuous process at different organic loading rates was determined by performing an energy balance. The semi-continuous specific methane yields were compared to two reference values. The first reference value was the theoretical maximum methane yield potential of the substrates calculated from the lower heating values of the samples as described in Section 4.4.3. The second reference value was the "specific methane yield potential" determined in HBT Batch-test. The HBT Batch-test proceeded for a retention time of 35 days at 37°C (± 1°C).

During the semi-continuous digestion, samples were collected three times from the outlet of the digesters (at the 105th, 108th and 115th day) and mixed to determine the residual methane yields. The residual methane yields were determined according to the protocol described by Oechsner (2013). These residual methane yields were measured in order to analyse the performance of the system at different organic loading rates. Figure 10 shows schematically the approach that has been adopted for the performance analysis.

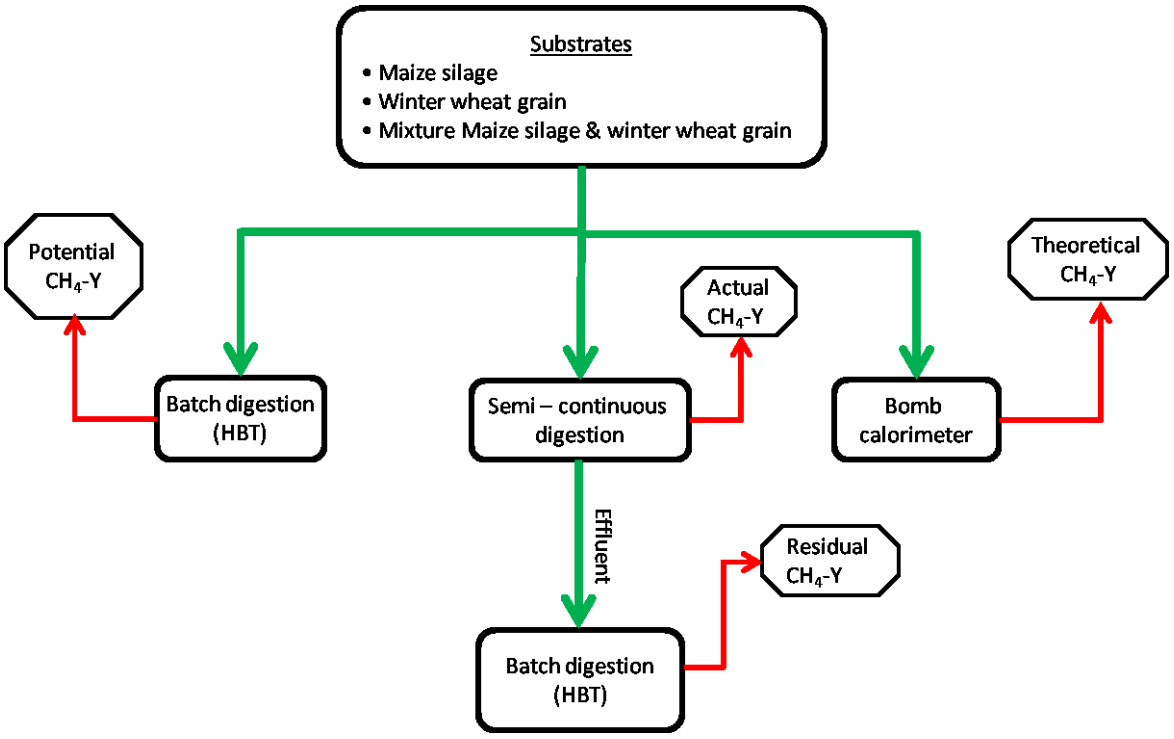

Figure 10: Schematic description of the methodology used for the performance analysis.

The main goal was to scale-up the batch results and simulate hence the actual bioconversion efficiency at different OLR in full-scale CSTR plant. Since the crop mixtures could affect the conversion efficiency, a mixture with less fibrous crop material (wheat-grain) was included in the test and an overall energy balance performed.

4.7.3 Experiment III: Influence of the biochemical crop traits on the specific methane yield potential of intentionally blended maize fractions (ear and stover)

The experiment was carried out as following: three maize genotypes of different maturity groups (mid-early, mid-late and late) were harvested at the same harvesting date in the same locality (Freising, Bavaria). The climatic data of the site are shown in Table 8. For each genotype, two crop fractions, namely the ear fraction (corn-cob) and the stover (stalk + leaf) were separated from one another and dried at 60°C. The two fractions were afterward blended in various proportions on a weight

basis. By doing so, the potential environmental factors that contribute randomly to crop traits (especially cell-wall) can be controlled. The absolute content of the biochemical traits was increased while the inner physiological status remained constant. Hence, the influence of the absolute values of cell-wall fractions on the specific methane yield potential could be investigated. The samples so prepared were analyzed toward their biochemical composition using the NIRS as descripted in the Section 4.3. Each mixture proportion was afterward digested in sixplicate using the HBT. Table 7 gives the details for the crop materials and the mixtures.

Table 7: Genotypes and mixture proportions of corn-cob and stalk-leaf fractions.

		Mixture proportions [Corn-cob : Stalk-Leaf] in %					
genotype	FAO-Index	[0:100]	[20:80]	[40:60]	[60:40]	[80:20]	[100:0]
B	250	x	x	x	x	x	x
C	280	x	x	x	x	x	x
G	600	x	x	x	x	x	x

The main goal of this experiment was to study the influence of the absolute contents of the biochemical traits on the specific methane yield potential of maize whole-crop.

4.7.4 Experiment IV: Influence of the biochemical crop traits on the specific methane yield potential of maize whole-crop

A set of 304 maize samples from different agricultural field trials between 2002 and 2006 was investigated. The agricultural field trials were conducted in 9 different locations (6 in Germany and 3 in Luxemburg) by both the breeding company KWS SAAT AG Einbeck and the Bavarian State Research Center for Agriculture – Crops Science and Plant Breeding. Table 8 shows the description of the locations and Table 9 indicates the main agronomic and/or breeding research issues treated in each field experiment from which the samples were collected and the number of samples that have been analyzed toward their specific methane yield potential. The genotypes investigated covered a broad spectrum of maturity groups spreading from FAO-index 220 to 700 and growth stages (beginning of the milk stage to the

end of the dough stage). The set included 8 brown-midrib mutants. The plants containing a brown midrib mutation (bm3) exhibit a reddish brown pigmentation of the leaf midrib starting when there are four to six leaves. These mutations are known to be associated with a low lignin content (Riboulet et al., 2008) and altered lignin composition (Vignols et al., 1995). They exhibit higher digestibility than their counterparts. Because of their high ruminal digestibility, it was expected that their inclusion in the set would widen the range of the specific methane yield potential of maize.

Table 8: Description of the locations.

Location	Country	Altitude over sea level (m)	Temperature (°C)*	Precipitation (mm)*
Bernburg	Germany	80	9.7	511
Freising	Germany	454	7.5	750
Ingolstadt	Germany	365	7.6	700
Ismaning	Germany	485	9.8	800
Kehlen	Luxemburg	330	9.8	862
Marnach	Luxemburg	498	8.8	755
Pleschetterhof	Luxemburg	344	9.8	862
Tittenkofen	Germany	-	-	-
Weser Ems	Germany	9	9	750

adapted from Eder, B (2010) and Agrimeteo Luxemburg
* annual long term average

Table 9: Number of observations for each year and the agricultural/breeding research questions investigated during the field trials.

			Observations [n]
Experiment year	2002		71
	2003		51
	2004		66
	2005		77
	2006		39
Agronomical/-breeding research questions	Exp.1	Comparison of different sowing densities	37
	Exp. 2	Comparison of brow midrib (bm3) and not brown midrib (bm3) Genotypes	8
	Exp. 3	Comparison of different harvest dates	126
	Exp. 4	Comparison between extremely low and high DM content crop materials	21
	Exp. 5	Variation of both sowing and harvesting times	21
	Exp. 6	Comparison of different Genotypes and sowing densities	71
	Exp. 7	Comparison of different Genotypes	20

Four genotypes (FAO 250, 280, 600, and 700) extracted from the set of 304 samples were considered to analyze the evolution of the biochemical crop traits at different physiological growth stages. These genotypes were sown on April 28th 2004 in Ismaning, Bavaria, Germany and harvested after 121, 139, 161 and 196 days growth periods. The FAO 250 genotype (Gavott) was a variety adapted to the German climatic conditions and generally used as silage maize. The FAO 280 genotype (KXA 4171) was an experimental hybrid. The FAO 600 (Mikado) and FAO

700 (Doge) were late maturing genotypes of warmer Mediterranean regions used in the selection for higher biomass yield.

The objectives of this experiment were:

- The assessment of the evolution of the main biochemical traits through the relevant growth period where maize is generally harvested for biogas production;
- The determination of the absolute upper and lower boundaries within which the biochemical main crop traits vary, irrespective of genotypes and growth stages (including both conventional silage maize with high DM content and non-conventional crop materials with low DM content);
- The assessment of the relationships between the biochemical crop traits
- The determination of the absolute upper and lower boundaries within which the specific methane yield potential of maize whole-crop vary (effects due to ensiling process excluded);
- The evaluation of the relationships between the crop biochemical traits and the specific methane yield potential.

The experiment should examine whether it is possible, or not, to point out biochemical traits that characterize “The” biogas genotype at the specific methane yield potential’s regard.

4.7.5 Experiment V: Assessment of *in-vitro* estimate of digestibility for whole-crop (CDOMD) and the biochemical traits as predictors of the biodegradability in AD batch system

To assess the appropriateness of *in-vitro* estimate of digestibility (CDOMD) and the absolute values of the biochemical crop traits as predictors of the biodegradability in AD batch system, eight (8) maize genotypes (FAO-index 240 to 700) collected from a field trail conducted in Weihenstephan, Bavaria (Germany) were investigated (Table 10). The genotypes were harvested at 5 different dates so that not only the genotype effect was taken into account, but also the physiological status. The field trial and the determination of the chemical composition was performed by the

Bavarian State Research Center for Agriculture – Crops Science and Plant Breeding, using the near infrared reflectance spectroscopy (NIRS) as described in Section 4.3. The specific methane yield potentials and the calorimetric measurements were performed by the University of Hohenheim (Institute for Agricultural Engineering and Bioenergy). The HBT was used for batch analysis.

Table 10: Maturity index and growing durations of different maize Genotypes investigated.

genotype	FAO-Index	Growing duration [d]				
		121	**139**	**161**	**177**	**196**
A	240	x	x	X	X	X
B	250	x	x	X	X	X
C	280	x	x	X	X	X
D	280	x	x	X	X	X
E	400	x	x	X	X	X
F	400	x	x	X	X	X
G	600	x	x	X	X	X
H	700	x	x	X	X	X

Hence both very young crop materials (rich in WSC) and very ripe crop materials (with high starch content) were examined. The field trial took place in Weihenstephan, Bavaria. The samples were conditioned as described in Section 4.1.4. A portion of the sample was used for the determination of the gross energy content using a bomb calorimeter at the Institute of Animal Husbandry and Animal Breeding (University of Hohenheim). The values gained were corrected to get the net calorific values (lower heating values). The bomb calorimeter analysis method and the procedure for the determination of the net calorific values are described in (AFNOR, 2004). The net calorific values were afterward used to calculate the theoretical maximum methane yield potential and the recovery efficiency as

described in Section 4.4.3. The second portion of the sample was used for the determination of the specific methane yield potential in batch-test (HBT).

The main goal was to assess the sharpness of both *in-vitro* estimate of ruminal digestibility for whole-crop (especially the CDOMD) and cell-wall content as predictors for biodegradability (bioconversion efficiency in AD system). The biodegradability was expressed as percentage of the theoretical maximum specific methane yield potential converted actually into methane or the share of the gross energy removed from the system (digester) as described in Section 4.4.3. The results were compared to the CDOMD (in absolute terms) as the *in-vitro* estimate of ruminal digestibility (CDOMD) expresses the fraction (in percentage) of the total ODM that disappears (or is removed) from the system (rumen).

4.7.6 Experiment VI: Evaluation of the specific methane yield potential of various crops alternative to maize

The major objectives of this experiment were to examine the variation ranges of the specific methane yield potential of both lipid and carbohydrate rich crops and to evaluate the influence of the biochemical composition on the specific methane yield potential.

4.7.6.1 Sunflower (*Helianthus annuus* L.)

The crop materials were provided by the State Plant Breeding Institute, University of Hohenheim (research group legumes and sunflower - Eckartsweier). This investigation was conducted in two steps. In the first step, seven (7) whole-crop samples (including cultivars and experimental hybrids) were investigated in order to determine the range of variability in specific methane yield potential.

In the second step, crop fractions of both established oil sunflower cultivar and experimental high biomass-yielding hybrid were investigated at different planting and harvesting dates. At harvest, the crop materials were dissected into three

fractions: stem, leaf, and crown, and conditioned according to the standard HBT procedures (see Section 4.1.3). The biochemical composition was determined using the wet chemical analysis methods as described by VDLUFA (1988). Cell-wall fractions were determined according to Van Soest procedure (Van Soest, 1967). The specific methane yield potential was determined using the Hohenheim biogas yield test (HBT).

Both field trials took place in Eckartsweier, Baden-Wuerttemberg (long-term averages: 726 mm annual mean precipitation and 9.9°C annual mean temperature). The sowing and harvesting dates of the crop material used in the first step of the experiment were not known. Table 11 shows the planting and harvesting dates as well as the corresponding growth periods for each of the crop material used in the second step of the investigation.

Table 11: Planting and harvesting dates and the growth duration of sunflower.

Planting dates		Harvesting dates		Growth duration [d]
Description	Date	Description	Date	
Early Planting	11-May	1st harvest	17-Aug	98
		2nd harvest	14-Sep	126
		3rd harvest	12-Oct	154
Late Planting	2-Jun	1st harvest	31-Aug	90
		2nd harvest	27-Sep	117

4.7.6.2 Rape (*Brassica napus* L.)

For this investigation two different sets of materials were considered. The first set of crop materials (here referred to as Set I) was harvested at three different physiological stages at the research station Ihinger Hof of the University of Hohenheim (long-term averages: 693 mm annual mean precipitation and 8.1 °C

annual mean temperature) using a forage maize chopper. The second set of materials (here referred to as Set II) was harvested manually from the federal varieties comparison trial at the research station Ihinger Hof of the University of Hohenheim:

Set-I: an unknown cultivar was harvested at three different physiological growth stages (full flowering, pods elongation and full maturity) as whole-crop. To avoid possible seed losses the harvest at the full maturity stage took place one week before the proper harvest date for rape seeds.

Set-II: Set II was made of 5 different genotypes (Inbred Lines and hybrids) harvested at the full maturity. One of the genotypes was classified as a high erucic acid content genotype. After the harvest a share of the whole-crop material was split into crop fractions, namely the green fraction (stalk and empty pods) and the seed fraction. Both whole-crop and crop fractions were analyzed. The harvest took place manually. Table 12 shows the main characteristics of rape genotypes investigated.

Table 12: Main characteristics of rape Genotypes investigated. [according to the federal variety authority - Bundessortenamt 2011]

Cultivar	Typ	Seeds yield	Oil yield	Oil-content	Glucosinolates content	Erucic acid content
Aurum	line	high	medium to high	medium to high	low	very low
Oase	line	medium to high	high	high to very high	low	very low
Elektra	hybrid	high	high	medium to high	low	very low
Trabant	hybrid	high	medium to high	medium to high	low	very low
Maplus	line	-	-	-	low	very low to low

4.7.6.3 Rye (*Secale cereal* L.)

Two sets of samples both made of two rye hybrid genotypes (Visello and Picasso), one population rye genotype (Recrut) and one forage rye genotype (Vitallo) were investigated. The crop materials were harvested by the State Plant Breeding Institute, University of Hohenheim (Research Group Rye). The field trials were conducted in two different locations, namely in Hohenheim (731mm annual mean

precipitation and 10.5°C annual mean temperature) and Wohlde (1035 mm annual mean precipitation and 10.9°C annual mean temperature). The first set was composed of whole-crop materials harvested at the beginning of heading (BBCH-Scale EC51) using a forage maize chopper. The second set was made of crop fractions (ear, stalk-leaf and stubble) split after that the whole-crop was harvested manually. The crop materials constituting the second set were harvested at two different physiological growth stages (as described by Meier, 2001), namely at the early milk stage (BBCH-Scale EC73) and at the late milk-early dough stage (BBCH-Scale EC77/83). The biochemical composition was determined using the wet chemical analysis methods as described by VDLUFA (1988). The analyses were conducted at the State Institute of Agricultural Chemistry (University of Hohenheim). The biogas yield potential was determined using the Hohenheim biogas yield test (HBT).

4.7.6.4 Sorghum

A set of samples made of 4 sorghum varieties (*Sorghum bicolor*) and 4 sorghum hybrids (*S. bicolor* x *S. sudanense*) harvested at two different occasions were investigated. The growth durations were of 117 and 133 days, respectively. The crop materials were provided by a project partner (Agrisem GmbH, Dr. Friedrich Jäger). The biochemical composition was determined using the wet chemical analysis methods as described by VDLUFA (1988). The biogas yield potential was determined using the Hohenheim biogas yield test (HBT). The planting and harvesting dates are given in Table 13.

Table 13: Planting and harvesting dates of various sorghum cultivars.

Cultivar	Species	Planting date	Harvest dates [Growth duration]	
Susu	Sudan grass hybrid (S. bicolor x S. sudanense)	24.5.06	1st Harvest 18.09.06 [117 days]	2nd Harvest 04.10.06 [133 days]
Bovital	- ditto -			
Lussi	- ditto -			
Gradavan	-			
Ronal 1	(Sorghum bicolor)			
Super Sile 18	- ditto -			
Super Sile 20	- ditto -			
Celu SC	-			

5 Results

5.1 Experiment I: Quantification of the effect of ensiling and drying process on the determination of the specific methane yield potential of maize whole-crop

As mentioned in Section 4.7.1, the main goal of this experiment was to quantify the actual benefit of the ensiling process on the specific methane yield potential of maize whole-crop. However, because of differences in the methodologies and the use of both different sample materials and mechanical preconditioning techniques, further aspects were investigated. The results for this experiment are presented in the following structure:

- Influence of the physiological maturity on the profile of volatile solids and loss potential at different drying temperatures;
- Influence of mechanical pretreatment/conditioning processes on the specific methane yield potential;
- Effect of volatile solids compensation on the specific methane yield potential of silages;
- Influence of ensiling process on the specific methane yield potential of maize.

Influence of the physiological maturity on the volatile solids profile and loss potential at different drying temperatures

Genotypes of maize plants with different maturity indexes accumulate dry matter (DM) differently over the growing period. A difference of 10 FAO-index points corresponds almost to a maturity difference of 1 to 2 days, or 1 to 2% in DM content of grains at the time of harvest (Zscheischler et al., 1990). Therefore, the DM content of the crop materials at harvest were considered to reflect the maturity index of the genotypes. Table 14 describes the crop material used and the DM content at harvest. Two maize genotypes (B and G) were harvested at three different harvest dates, and genotype J was harvested only at two harvest dates.

While genotype B (FAO-index 250) reached higher dry matter content (27%, 31% and 37%) at almost all harvest dates, genotype G (FAO-index 600) reached scarcely 24%, even after a growing period of 168 days. Genotype J (FAO-index 280) reached 32% at the third harvest (168 days growth period). The dry matter content of genotype G was too low for a stable ensiling process in a traditional bunker silo where organic dry matter losses due to excessive liquid losses might be considerable.

At the opening of the preserving glass jars, no butyric acid was found in the silage and the pH values were ≤ 4. This indicated an optimal ensiling process. The presence of butyric acid in silage indicates that the silage has undergone high dry matter loss, since butyric acid is a metabolite of saccharolytic bacteria (*Clostridium ssp.*). The development of saccharolytes destabilizes the silage by consuming lactic acid and proteins (Jeroch et al., 1999).

Table 14: Maturity index, growing durations and the dry matter content at harvest.

genotype	Maturity	Sowing	Harvesting	Growing duration	DM_h
	FAO – Index	Date		[d]	[%]
B	250	05.12.05	09.16.05	127	27.3
			10.07.05	148	31.0
			10.27.05	168	37.3
J	280		10.07.05	148	24.7
			10.27.05	168	31.9
G	600		09.16.05	127	19.6
			10.07.05	148	20.9
			10.27.05	168	23.7

Note: (DM_h: Dry matter content at harvest)

The analysis of the silage materials showed that the dry matter contents of the silages were, for all variants, lower than in the fresh crop material before ensiling (Table 15).

The high discrepancy between the DM content of the maize crop at harvest and silage was an apparent loss of dry matter content, since the contribution of volatile compounds in the silage was not yet included in the balance. The apparent dry matter loss ranged from 3.6% and 10.5%. The highest apparent dry matter losses were measured in the crop material, with the lowest dry matter content at harvest.

Table 15: Comparison of the dry matter content at harvest and after silo opening.

genotype	Maturity	Sowing	Harvesting	DM_h	DM_{su}	pH
	FAO - Index	Date		[%]	[%]	
B	250	05.12.05	09.16.05	27.3	24.8	3.93
			10.07.05	31.0	29.3	3.97
			10.27.05	37.3	34.9	4.01
J	280		10.07.05	24.7	23.2	4.08
			10.27.05	31.9	30.8	4.13
G	600		09.16.05	19.6	18.1	3.92
			10.07.05	20.9	18.7	4.08
			10.27.05	23.7	21.7	3.90

Note: (DM_h: Dry matter content at harvest; DM_{su}: Dry matter content of silage uncorrected)

Table 16 shows the results after correction. By taking into account the organic dry matter fraction contained in the solution, the actual dry matter losses attributed to ensiling process were barely lower, and ranged between 0.3-5.2%.

Table 16: Comparison of apparent and actual dry matter losses during the ensiling process of various maize genotypes.

genotype	Maturity	DM_h	DM_{su}	DM_{sc}	DM-$loss_{ap}$	DM-$loss_{ac}$
	FAO - Index	[%]	[%]	[%]	[%]	[%]
B	250	27.3	24.8	25.9	9.1	5.2
		31.0	29.3	30.6	5.4	1.3
		37.3	34.9	35.6	6.5	4.6
J	280	24.7	23.2	24.6	5.8	0.3
		31.9	30.8	31.3	3.6	2.1
G	600	19.6	18.1	19.3	7.8	1.6
		20.9	18.7	20.6	10.5	1.6
		23.7	21.7	22.8	8.7	3.8

Note: (DM_h: Dry matter content at harvest; DM_{su}: Dry matter content of silage uncorrected; DM_{sc}: Dry matter content of silage corrected; DM-$loss_{ap}$: Apparent loss of dry matter; DM-$loss_{ac}$: Actual loss of dry matter)

The portion of soluble organic compounds (volatile fatty acids, alcohols, etc.) was found to be very high in young crop materials. In fact, Figure 11 shows that the percentage of soluble organic matter in the silage was highly correlated with the dry matter content of the crop at harvest. The lower the dry matter content of the crop at harvest, the higher the content of volatile compounds in the solution.

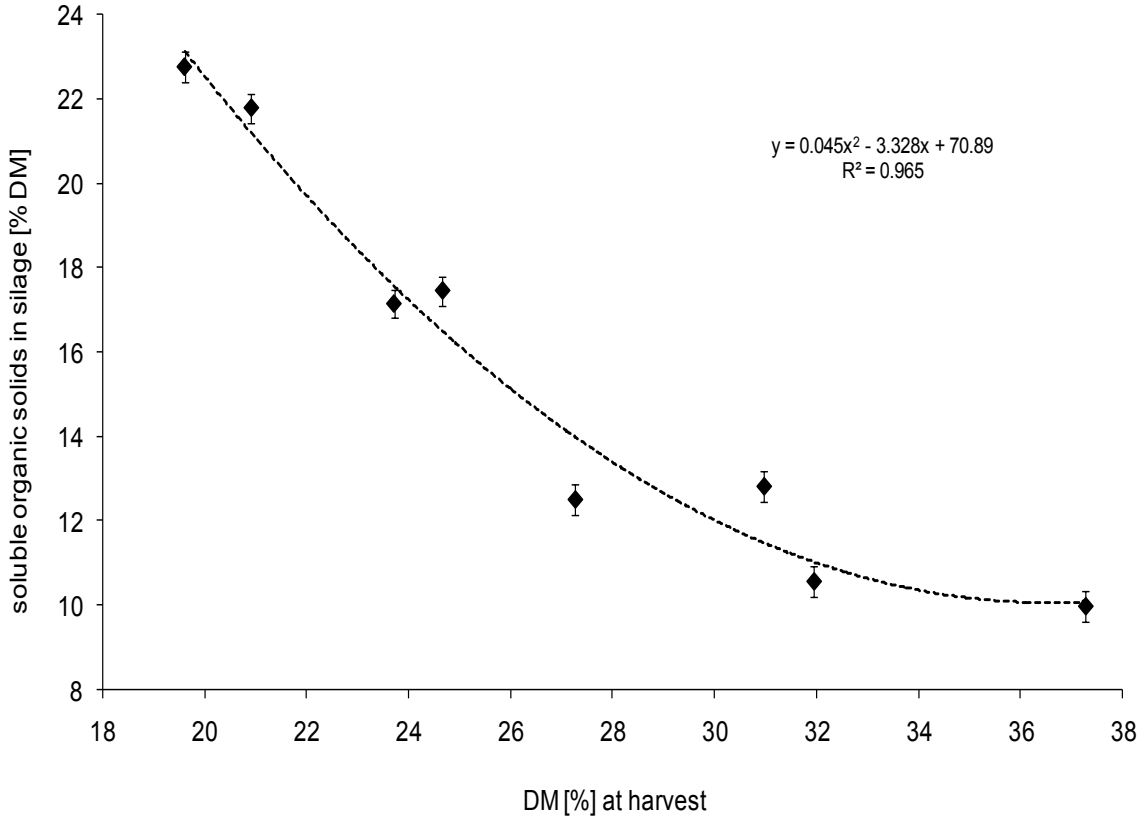

Figure 11: Relationship between the DM at harvest and the soluble organic solids in silage.

The profiles of organic acids in the silage revealed both the microbiota and the biochemical pathways that were active during the ensiling process. Figure 12 and Figure 13 show the profiles of organic acids and alcohols in the silages of crop materials with different physiological maturities. The figures show also the variation in the profiles of the soluble organic compounds after the samples were exposed to the drying process at different temperatures. These results reveal that lactic acid was dominant in the silage material. This is an indication of a successful ensiling process. In fact, by the time of the shift to the anaerobic phase during the ensiling process, the most active microbes were enterobacteria and lactic acid bacteria (LAB). In general, these microorganisms proliferate and produce neutral and acidic end-products. The acidic end-products reduce silage pH and favor growth of the more acid-tolerant LAB. When substrate is not limiting, LAB dominate the fermentation, producing lactic acid and acidify the silage until a pH is attained which suppresses LAB growth, resulting in a stable silage (Rooke and Hatfield 2003).

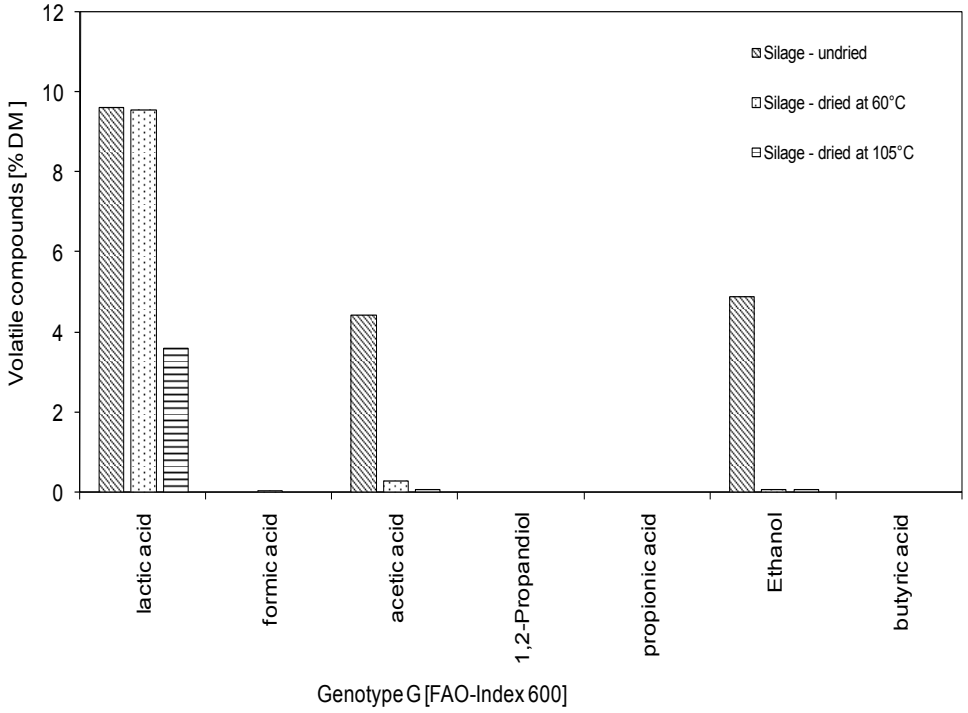

Figure 12: Profile of organic acids and alcohols in the silage of a late-maturing maize genotypes (FAO-Index 600) after a growing period of 148 days.

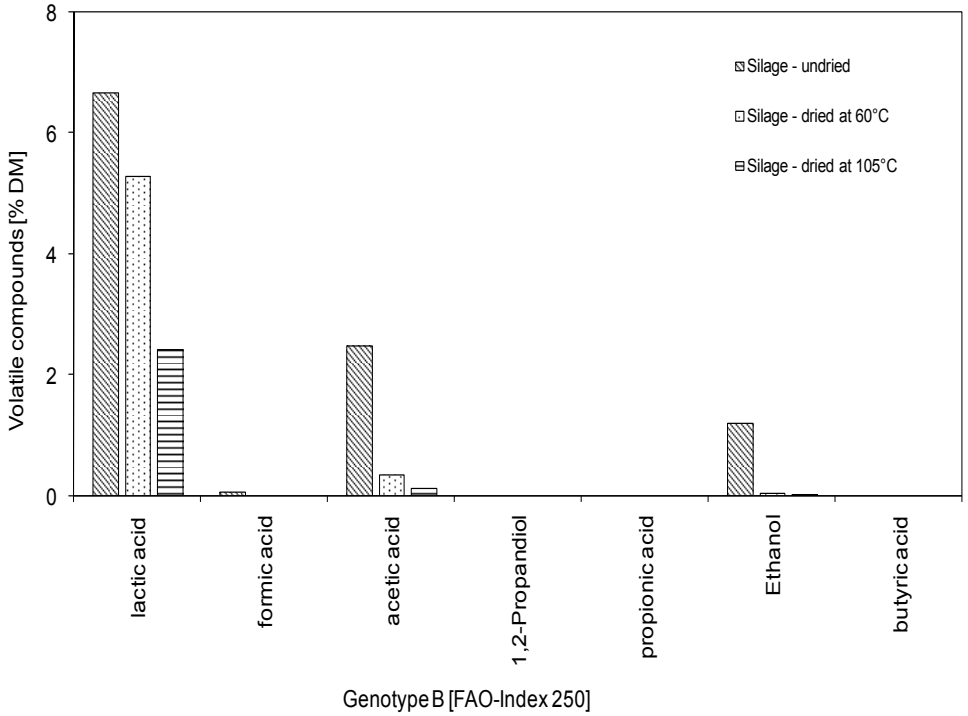

Figure 13: Profile of organic acids and alcohols in the silage of a medium-early maturing maize genotype (FAO-Index 250) after a growing duration of 148 days.

In easily fermentable substrates, such as maize, the production of lactic acid starts shortly after the crop material is covered, so that after 7 days the pH reaches the preservation zone below pH 4 (Kalzendorf 2006). Exposing silage to heat modifies its volatile solid composition and the extent of evaporation of the volatile solids depends on both the temperature and the pH (Weißbach 1994; Weißbach and Kuhla 1995). At 60°C, 98.4% of the ethanol and 93.2% of the acetic acid was lost in genotype G (FAO-Index 600), while in genotype B (FAO-index 250), ± 97.0% of the ethanol and 85% of the acetic acid was lost. At 105°C the concentration of ethanol in genotype G was almost the same as at 60°C, but in genotype B no alcohol could be found. The losses of acetic acid were close to 98.4% in genotype G and 95.2% in genotype B.

The level of lactic acid in the dried crop materials was found to be lower than in fresh silage. Because of its high boiling point (118°C) lactic acid is not lost to the same extend as other acids. At temperatures applied here, lactic acid undergoes only condensation reactions through which it is converted into lactids, which are not measurable with the usual analytical procedures. Because of this reason, volatile compounds losses in the dry matter content were adjusted to 8% to account for the losses attributed to lactic acid (Weißbach and Kuhla 1995). Hence the lower levels of lactic acid measured in crop material dried at 60°C and 105°C were apparent losses.

Influence of the mechanical pretreatment/conditioning processes on the determination of the specific methane yield

The samples used to determine the specific methane yields of crops are generally chopped directly after harvest, or dried and milled. It may be argued that these preconditioning processes affect the samples so that the specific methane yield potentials determined are substantially different. Hence, there is a need to quantify the effect of these mechanical conditioning processes on the measurement of the specific methane yield potential. Figures 14, 15, 16 show the effect of the two mechanical conditioning processes (chopping versus drying-milling) on the specific methane yield potential of maize of genotypes B, J, and G, respectively, at different

harvest dates. The specific methane yield potentials of the non-dried samples varied between 0.342 and 0.354 m_N^3 CH_4/kg ODM. The specific methane yield potential of the dried-milled variants ranged from 0.339 and 0.350 m_N^3 CH_4/kg ODM. The differences between the dried-milled and the non-dried variants ranged from 0% and 3%.

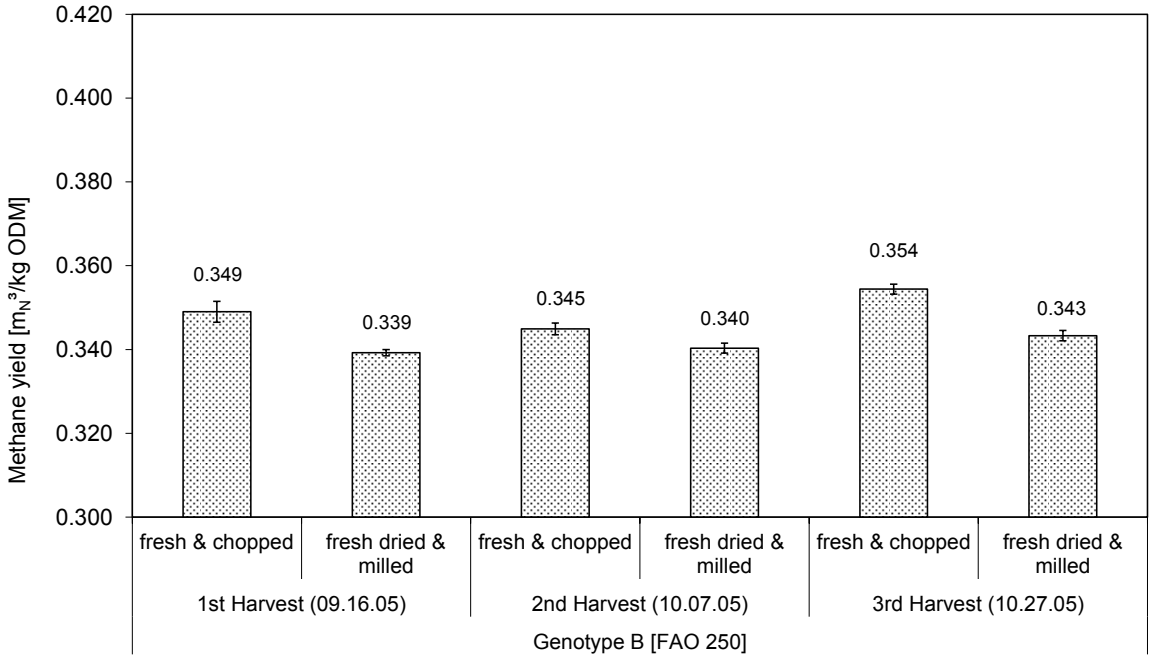

Figure 14: Effect of mechanical conditioning processes on the specific methane yield potential of maize at different harvesting dates (genotype B).

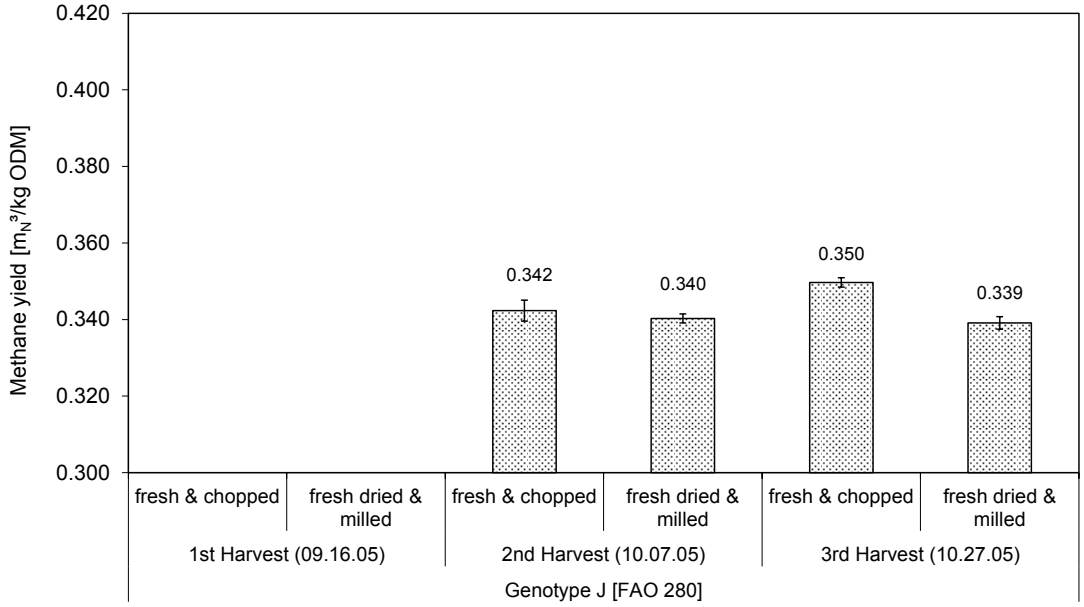

Figure 15: Effect of mechanical conditioning processes on the specific methane yield potential of maize at different harvesting dates (genotype J).

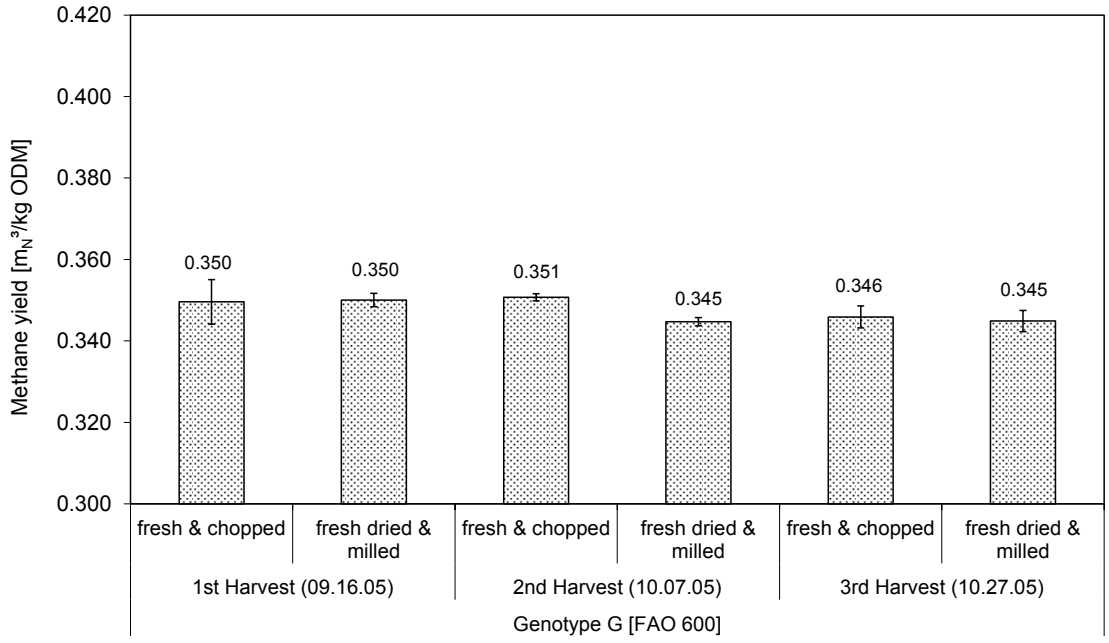

Figure 16: Effect of mechanical conditioning processes on the specific methane yield potential of maize at different harvesting dates (genotype G).

Table 17 summarizes the effect of different pretreatment/conditioning methods on the determination of specific methane yield potential. The numbers in brackets show the relative standard deviation in percentage. The table shows also the absolute difference between the drying-milling in fresh chopped processes.

Table 17: Specific methane yield potential of maize genotypes depending on the mechanical conditioning process applied. Mean values of three independently replicated experiment (n = 3). Values ± relative SD in %. Significant difference (*) to a tolerance degree of 5% ($p < 0.05$) relative to the fresh dried-milled variant.

	Genotype B [FAO 250]			Genotype J [FAO 280]			Genotype G [FAO 600]		
Vegetation duration (d)	fresh-chopped	fresh-dried milled	Difference	fresh-chopped	fresh-dried milled	Difference	fresh-chopped	fresh-dried milled	Difference
127	0.349 [±4.0]	0.339 [±0.9]	2.9%				0.350 [±3.1]	0.350 [±0.9]	0.0%
148	0.345 [±1.6]	0.340 [±1.4]	1.4%	0.342 [±1.6]	0.340 [±0.7]	0.5%	0.351 [±0.5]	0.345 [±0.6]	1.7%
168	0.354 [±1.3]	0.343 [±1.4]	3.1%*	0.350 [±0.7]	0.339 [±1.0]	3.1%*	0.346 [±1.6]	0.345 [±1.5]	0.2%

The dried-milled variants showed slightly lower methane yield potential in comparison to the fresh-chopped materials. Nevertheless, the differences were in general not significant ($p < 0.05$). The dried-milled process showed an additional advantage as dried-milled samples were easy to handle.

Effect of volatile solids compensation on the determination of the specific methane yield potential of maize silages (whole-crop)

Figures 17, 18, 19 show the specific methane yield potential of fresh silage variants, with and without correction for loss of volatile solids. Without correction, the specific methane yield potentials were found to vary between 0.376 and 0.381 m_N^3 CH_4/kg ODM for genotype B (FAO-Index 250), 0.363 and 0.383 m_N^3 CH_4/kg ODM for genotype J (FAO-Index 280), and 0.355 and 0.407 m_N^3 CH_4/kg ODM for genotype G (FAO-Index 600). These values were excessively high in comparison to the yields observed on fresh dried and fresh non-dried variants (Figures 14, 15 and 16). After correction, the specific methane yield potentials were between 0.364 and 0.369 m_N^3

CH_4/kg ODM for genotype B (FAO-Index 250), 0.357 and 0.360 m_N^3 CH_4/kg ODM for genotype J (FAO-Index 280), and 0.336 and 0.380 m_N^3 CH_4/kg ODM for genotype G (FAO-Index 600). The overestimation of specific methane yield potentials due to lack of correction for volatile solid losses in silages was estimated to vary between 2.1% and 4.4% for genotype B (FAO-Index 250), 1.7% and 5.9% for genotype J (FAO-Index 280), and between 5.3 and 9.6% for genotype G (FAO-Index 600). In general, the overestimation of the specific methane yield potential decreased with the increasing crop maturity. In fact, the increasing crop maturity was accompanied by the decrease in volatile compounds' content.

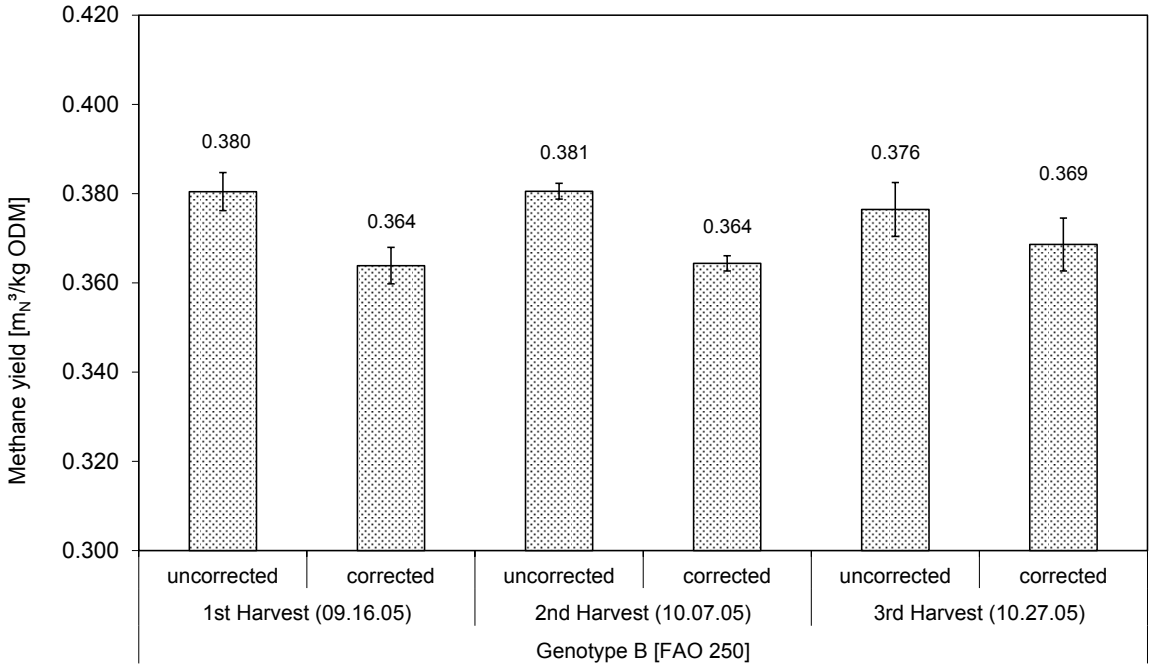

Figure 17: Impact of correcting for the DM content on the specific methane yield potential of ensiled maize crop (FAO-Index 250) at different growth stages.

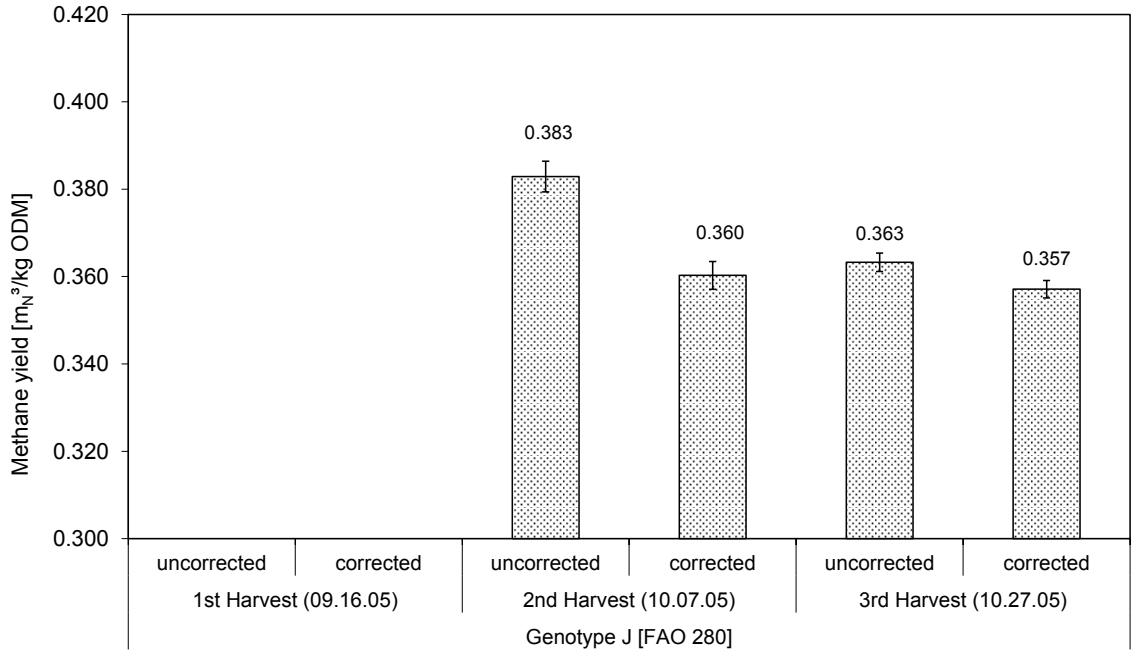

Figure 18: Impact of correcting for the DM content on the specific methane yield potential of ensiled maize crop (FAO-Index 280) at different growth stages.

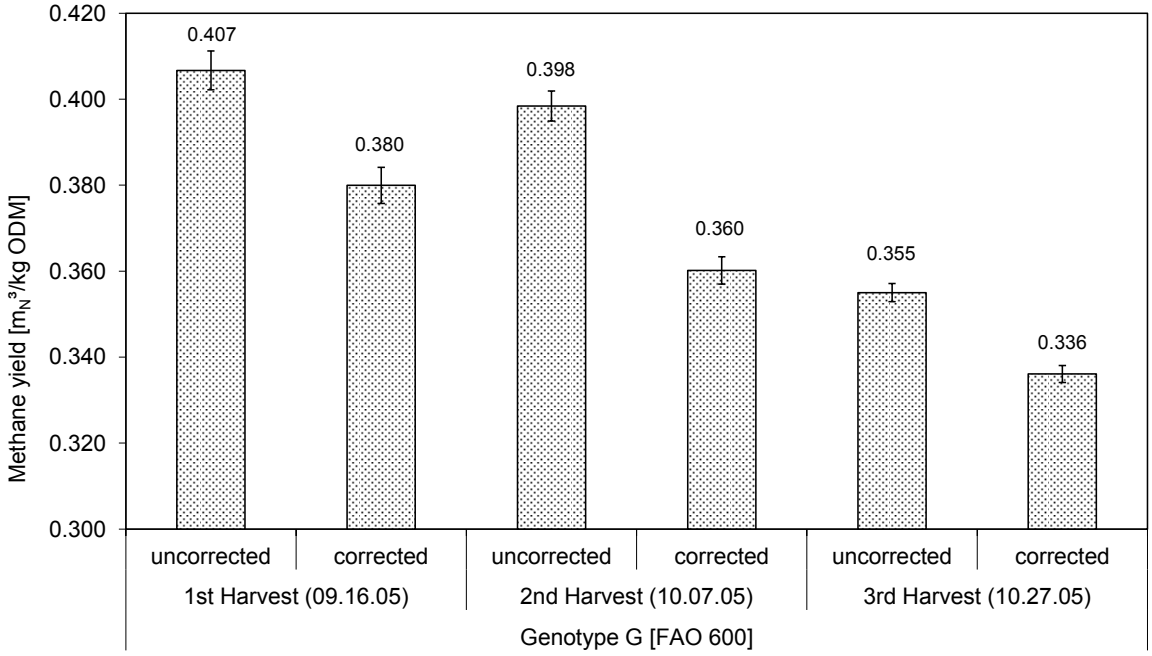

Figure 19: Impact of correcting for the DM content on specific methane yield potential of ensiled maize crop (FAO-Index 600) at different growth stages.

Influence of ensiling process on the specific methane yield potential of maize

The influence of ensiling process on the specific methane yield potential of maize is presented in two steps (Figure 20 and Figure 21). In both figures, the fresh (chopped) variant is compared to the silage (chopped) variant. In Figure 20, the specific methane yield potential of the fresh (chopped) variant is compared to that of the ensiled variant (silage chopped) after the compensation for volatile solids losses (drying losses). The top dark section represents the yield increase that is attributed to ensiling process (the figures give the exact values of the yield increase/decrease in comparison to the fresh-chopped variant). The results show that ensiling process enhances the specific methane yield potential of maize for almost all the variants analyzed. The highest effect was found on very young crop material (+8.6%), namely the late-maturing genotype G (FAO-Index 600) early harvested (after 127 days vegetation period).

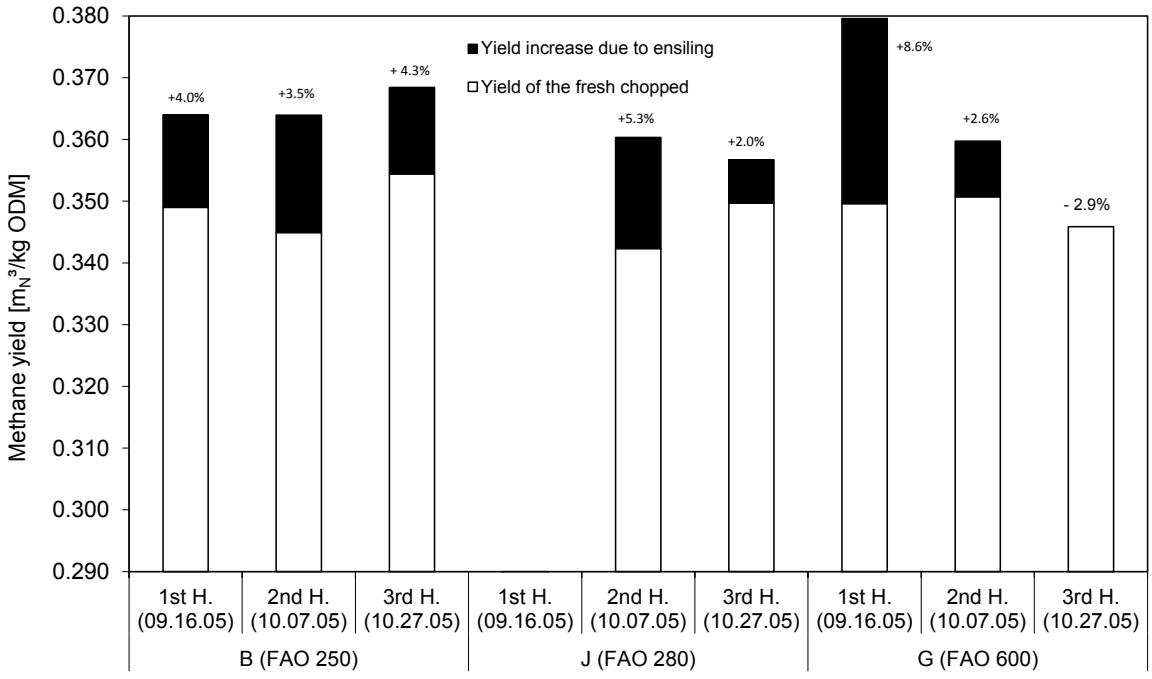

Figure 20: Effect of ensiling on the specific methane yields of crop materials (yields of the fresh chopped variant plus the additional increase due to ensiling).

In Figure 21 the specific methane yield potential of the fresh (chopped) variant is compared to that of the silage (chopped) variant after compensation for both the

volatile solids losses and the inherent ensiling losses. The inherent ensiling loss is the actual ODM depletion that takes place during the ensiling process. After including these losses in the balance the results show that the overall effect of ensiling process on the specific methane yield potential is ambivalent and varied between -0.8% and +4.2% for genotype B (FAO-Index 250), 0.0% and 4.9% for genotype J (FAO-Index 280) and -6.5% to 7.0% for genotype G (FAO-Index 600).

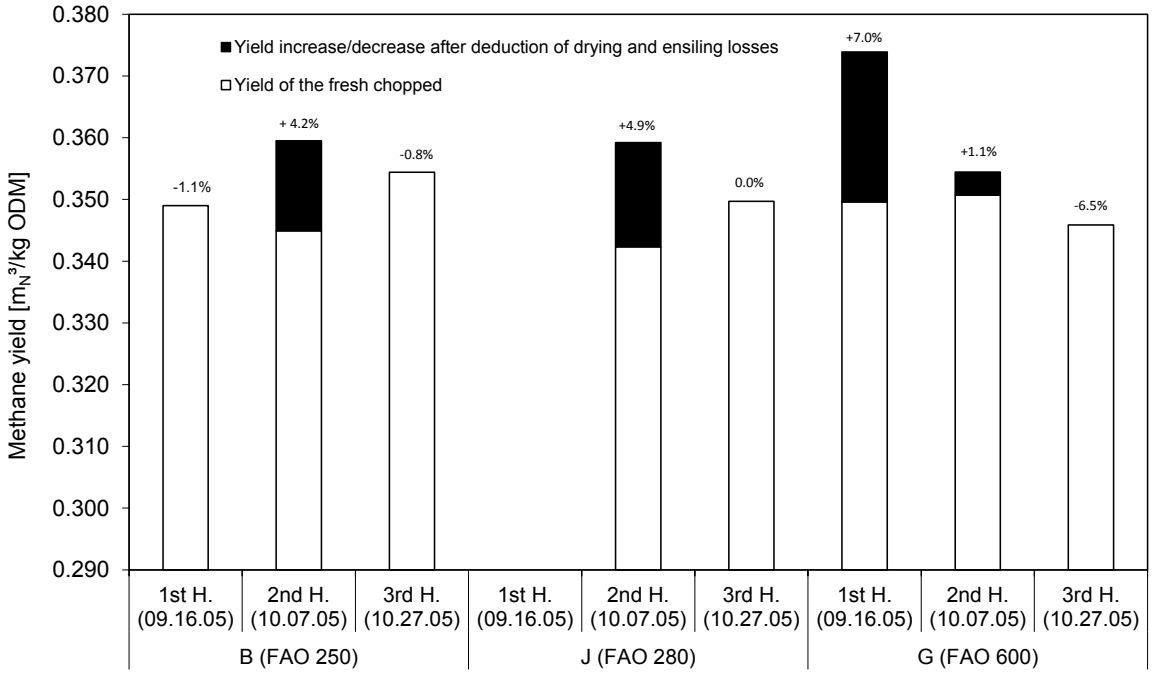

Figure 21: Specific methane yields of fresh crop materials and that of silages after correction of both drying and ensiling DM losses.

5.2 Experiment II: Up-scaling the batch results - Assessment of the bioconversion efficiency in semi-continuous flow system

As mentioned in Section 4.7.2, the main goal of this experiment was to scale-up the batch results, in order to simulate the specific methane yield of maize (whole-crop silage) to be expected in a full-scale digester. Two reference values were set for the evaluation, namely the theoretical maximum methane yield potential (derived from the lower heating values) and the specific methane yield potential (batch). Based on these, reference values for the bioconversion efficiencies in batch and semi-

continuous flow systems were calculated. Variants, including wheat-grain, were tested in order to evaluate the influence of co-digestion of crops on bioconversion efficiency. Prior to the use of wheat-grain in the semi-continuous trial, different wheat-grain pretreatment variants (whole-grain, milled, crushed) were examined in batch-tests in order to determine the most appropriate way of using wheat-grain.

The results of this experiment are presented in the following structure:

- Influence of different mechanical pretreatments on both the digestion kinetics and the specific methane yield potential of wheat-grain;
- Scaling-up of the batch results – bioconversion/substrate-use efficiency in different systems;
- Influence of the OLR and wheat-grain's addition on the reactor use-efficiency.

Table 18 shows the DM content and the main biochemical traits of the crop materials used for this experiment. Maize silage had a moderate NDF value of 43.6%. Wheat-grain had lower NDF value of 11.0%. The lignocellulosic fraction (ADF) was 2.3% and 24.3% for wheat-grain and maize silage respectively. As expected the crude protein level of wheat-grain was higher (11.8%) than that of maize silage (8.0%). The lipid content in the two crop materials was slightly different. Both crop materials had low lignin content of 0.7 and 2.7% for wheat-grain and maize silage respectively.

Table 18: The DM and the crop biochemical traits of different substrates investigated.

Substrate	DM content	XP	XL	XF	NDF	ADF	ADL	XA
	[% FM]	[% DM]						
Maize silage	29.4	8.0	3.5	20.5	43.6	24.3	2.7	4.0
Wheat-grain	88.6	11.8	2.2	2.0	11.0	2.3	0.7	1.6

Influence of different mechanical pretreatments on both the digestion kinetics and the specific methane yield potential of wheat-grain

The results of the batch-tests showed the degree to which the mechanical pretreatment needed to be applied to wheat-grain prior to its use in a semi-continuous operating digester. Figure 22 shows, both the specific methane yield potential for all variants, and the influence of particle size reduction on the digestion kinetics of wheat-grain. After a retention time of 35 days, all variants tested showed the same ultimate methane yield of 0.384 m_N^3 CH_4/kg ODM. This reveals that the mechanical pretreatments used had neither a positive, nor a negative impact on the specific methane yield potential of wheat-grain.

Despite the similarities in ultimate specific methane yield potentials, the kinetics of methane production were found to be affected considerably by the pretreatment. For untreated whole-grain, a retention time of 20 days was needed to reach the amount of methane that was collected after 8 days with both crushed and milled variants. In light of these results, the crushed variant was chosen for the subsequent step, namely the semi-continuous digestion trial.

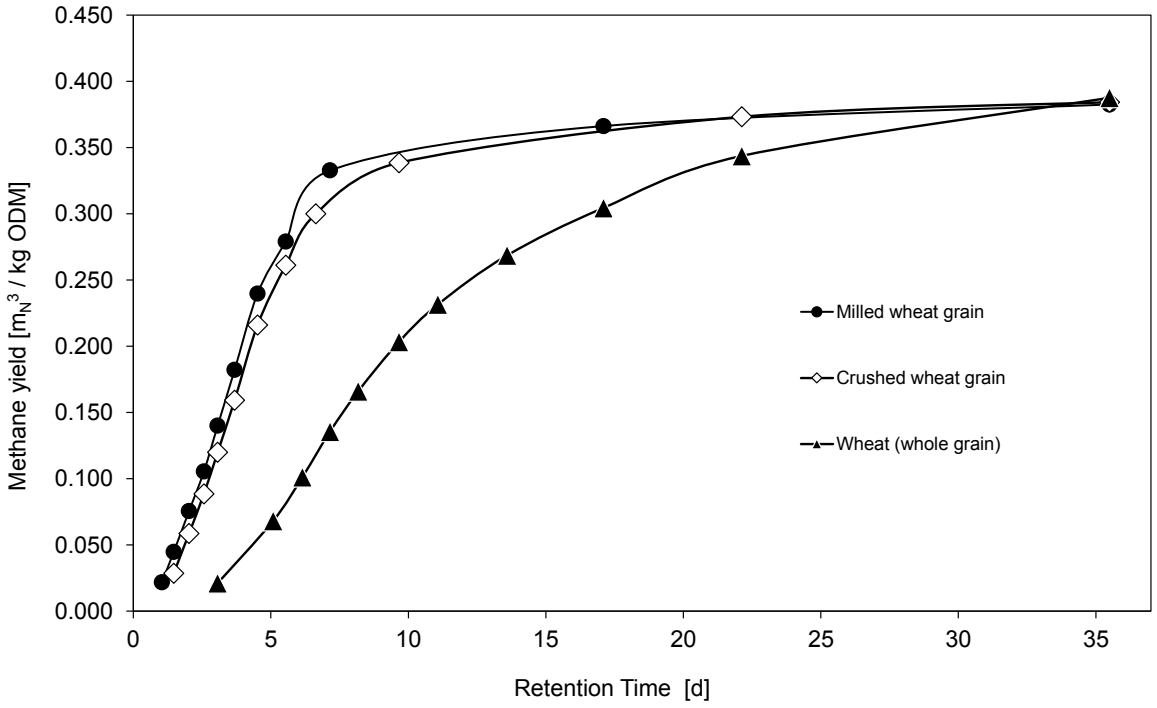

Figure 22: Influence of mechanical treatments on the digestion kinetics of wheat-grain.

Scaling-up of the batch results – bioconversion/substrate-use efficiency in different systems

Figure 23 shows the theoretical maximum methane yield potential, the specific methane yield potential (batch), the specific methane yields generated in semi-continuous flow system, as well as the residual methane yield potential in the effluent of the semi-continuous digesters.

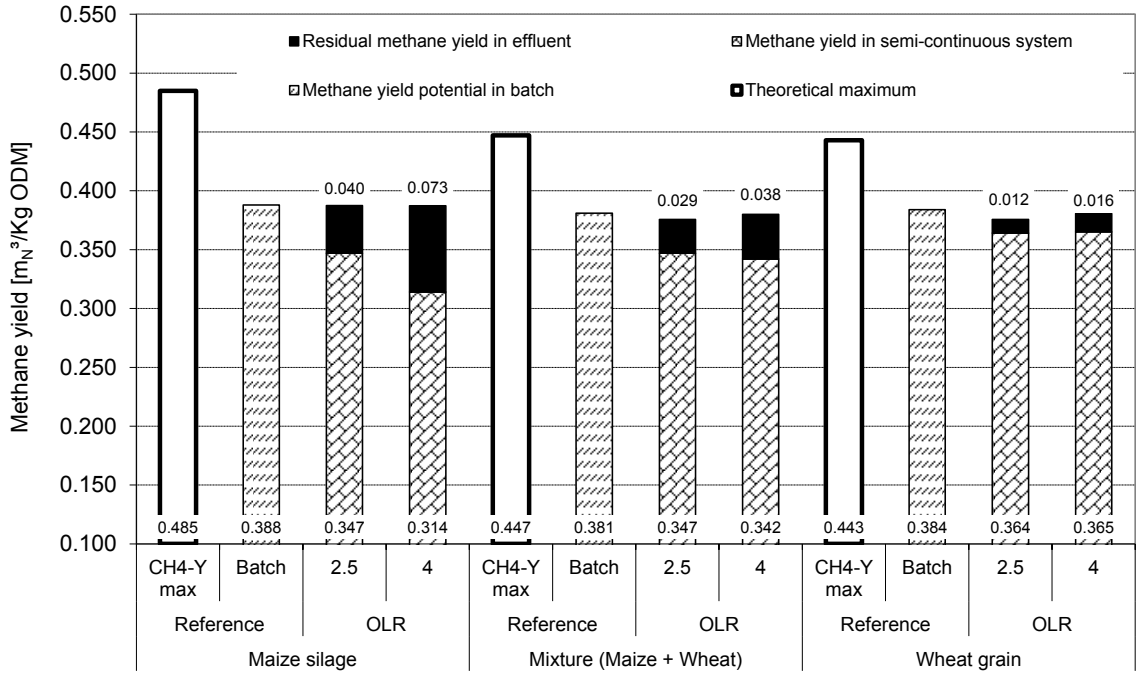

Figure 23: Theoretical maximum methane yield potential (bomb calorimeter), specific methane yield potential (batch), specific methane yields generated in semi-continuous system and residual methane yield potential in the effluent of the semi-continuous digesters.

The specific methane yield potential recovered in batch showed average of 0.388, 0.381 and 0.384 m_N^3 CH_4/kg ODM for maize silage, the mixture (maize-wheat grain) and wheat-grain respectively. All three substrates showed more or less the same level for the specific methane yield potential. By scaling-up to semi-continuous mode the specific methane yields decreased of 10.6% to 19.1% for maize, 8.9% to 10.2% for maize-wheat grain mixture, and 4.9% to 5.2% for wheat-grain, depending on the OLR. The higher the OLR, the lower the recovery efficiency. The specific methane yield varied from 0.347 m_N^3 CH_4/kg ODM to 0.314 m_N^3 CH_4/kg ODM for maize, and from 0.347 m_N^3 CH_4/kg ODM to 0.342 m_N^3 CH_4/kg ODM for the maize-wheat grain mixture. The OLR had very little effect on the specific methane yield for wheat-grain (0.364 m_N^3 CH_4/kg ODM and 0.365 m_N^3 CH_4/kg ODM).

Based on the specific methane yield generated in the semi-continuous trial, the residual methane yield (energy loss in the effluent) varied from 11.6% to 23.3% for maize, 8.3% to 11.1% for the maize-wheat grain mixture, and 3.2% to 4.3% for

wheat-grain only (Figure 24). By increasing the OLR from 2.5 to 4, the residual methane yield in the effluent was nearly doubled for maize silage.

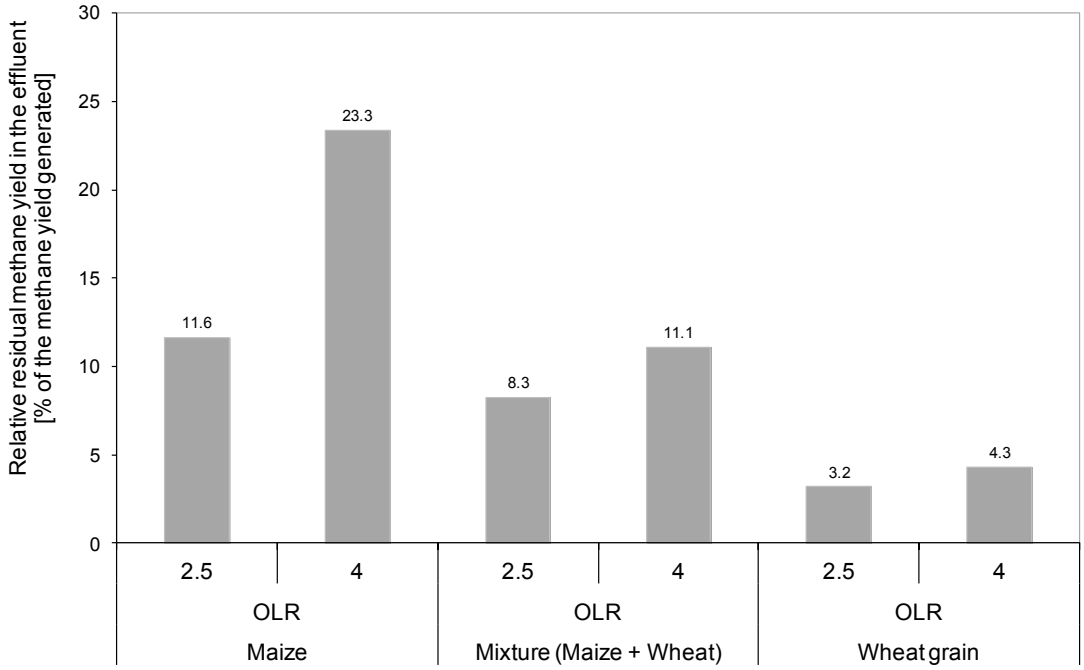

Figure 24: The relative residual methane yields in the effluent of digesters fed with various substrates at different OLR (percentage of the methane yield generated in the semi-continuous flow system).

Both specific methane yield potential in the batch trial and specific methane yield in the semi-continuous trial were relative values and, thus, provide less information with respect to the absolute energy losses. Therefore, the conversion efficiency based on the theoretical maximum specific methane yield potential was conducted. These experiments provided information about the magnitude of the untapped potential in the digestion of energy crops and assessed the substrate-use efficiency.

The mean values (followed by the standard deviations) of the calculated theoretical maximum methane yield potentials were: 0.485 [±1.3%], 0.477 [±2.9%] and 0.443 [±0.02%] m_N^3 CH_4/kg ODM for maize silage, the maize-wheat grain mixture, and wheat-grain only, respectively. Based on these values, conversion rates of 80%, 85%, and 87% were achieved in the batch system for maize silage, the maize-wheat grain mixture, and wheat-grain, respectively (Figure 25).

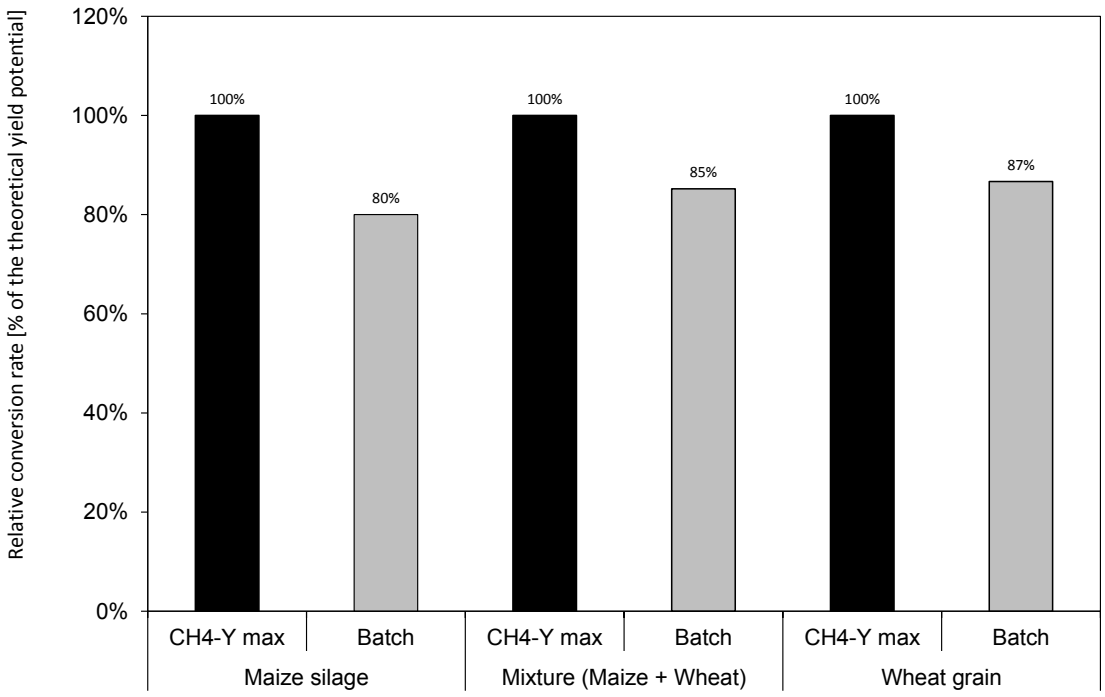

Figure 25: Relative conversion rate of various energy crops digested in batch system (based on the calculated theoretical specific methane yield potential).

Figure 26 shows the comparison of the theoretical maximum specific methane yield potentials to the specific methane yields in the semi-continuous process. These data revealed low recovery efficiencies in the semi-continuous process. Furthermore, the conversion efficiency decreased generally with increasing OLR. For maize silage, the decrease was more noticeable - from 71.6% at the low OLR down to 64.7% at the high OLR. For the of maize-wheat grain mixture, the conversion efficiency dropped slightly from 77.6% at the low OLR to 76.5% at the high OLR. The OLR minimally affected the conversion efficiency for wheat-grain. Its conversion rate was higher than that of the two other substrates (82.2% to 82.4%).

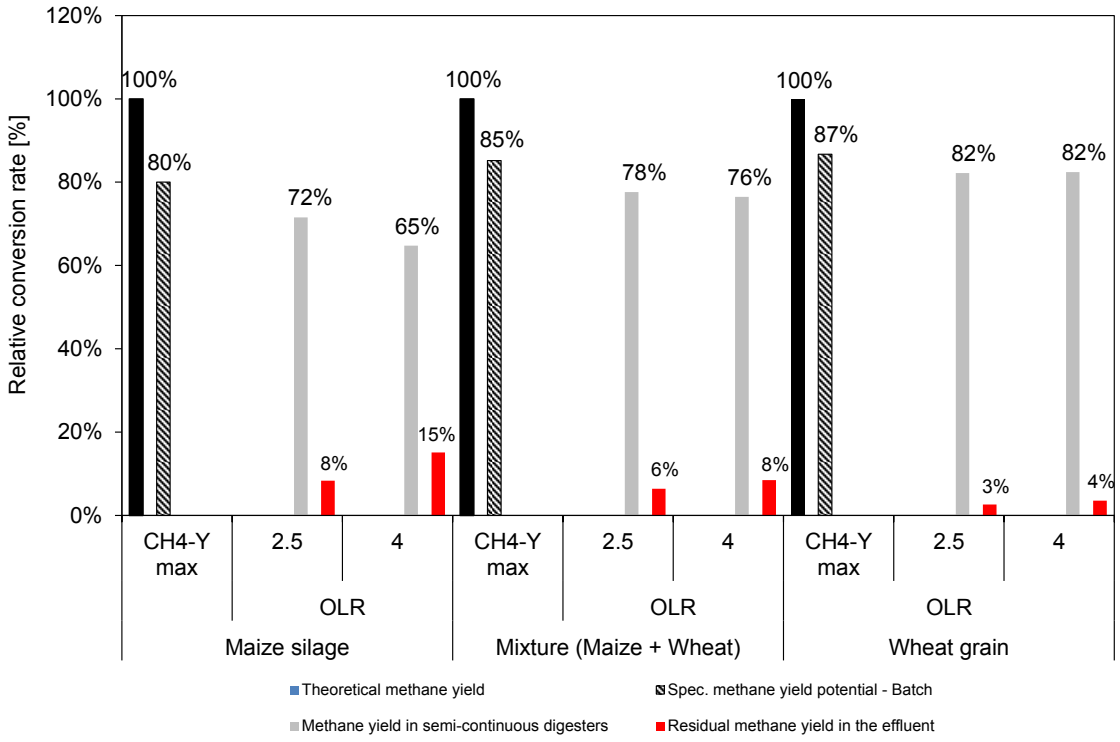

Figure 26: Relative conversion efficiencies of the theoretical specific methane yield potential (bomb calorimeter) of various energy crops digested at different OLR.

The residual specific methane yields, based on the theoretical maximum specific methane yield potential, were less than 20%, and were affected by both the crop characteristics and the OLR (Figure 26). The absolute losses varied between 8% - 15% for maize silage. The digestion of wheat-grain revealed the lowest absolute energy losses in the effluent (2.6% to 3.5%). The maize-wheat grain mixture displayed moderate energy losses in the semi-continuous digesters (6.4% - 8.4%).

The positive impact of the feedstock mixture on the conversion efficiency was revealed also by the analysis of DM accumulation in the digesters. The analysis of the DM and ODM accumulation (Figure 27) showed that the DM content in the digesters fed with wheat-grain remained low at both low and high OLRs. However, the DM content increased with the increasing OLR for digesters fed with maize silage. The DM content of digesters fed with the maize-wheat grain mixture was moderate.

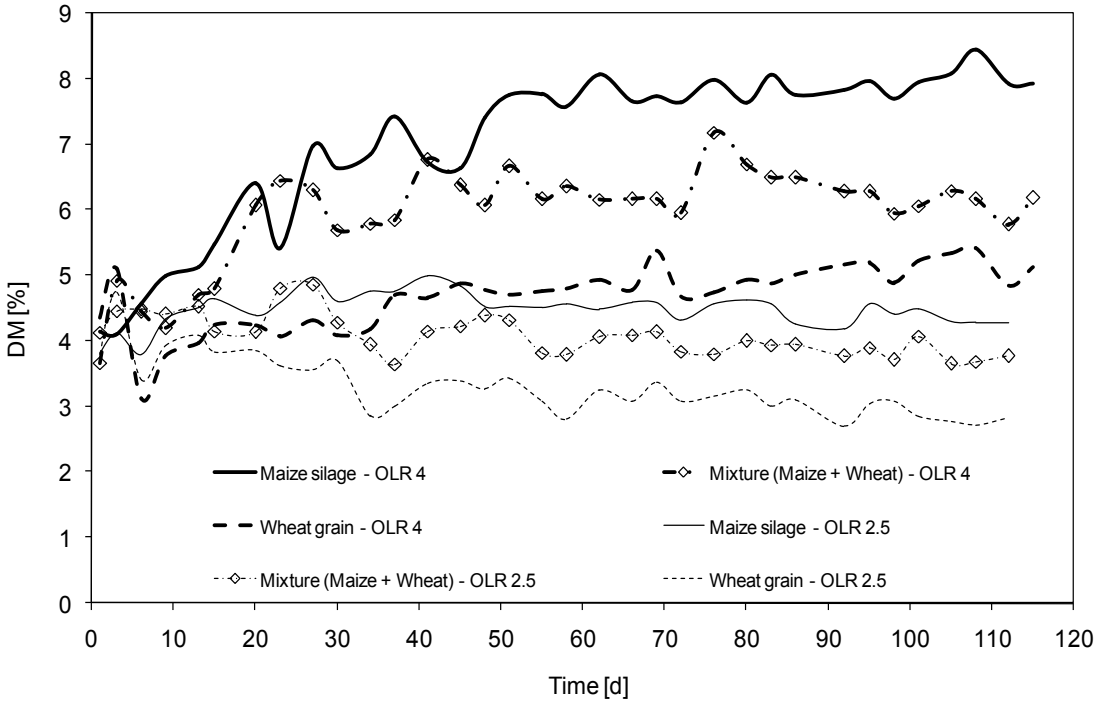

Figure 27: Evolution of the DM content in the continuously driven digesters fed at low and high organic loading rates.

Influence of the OLR and wheat-grain's addition on the reactor use-efficiency

In contrary to the results on the impact of the OLR on the bioconversion/substrate-use efficiency, the influence of the OLR's increase on the reactor-use efficiency was unanimously positive for all variants. As shown in Figure 28, at the low OLR the reactor-specific methane yields were more or less similar for all variants and lied by 0.8 m_N^3/m^3*d. By increasing the organic loading rate, the reactor-use efficiency increased for all variants from 0.8 to 1.3 m_N^3 CH_4/m^3*d. The increase were of 46%, 57% and 61% for maize silage, mixture maize-wheat and wheat-grain respectively.

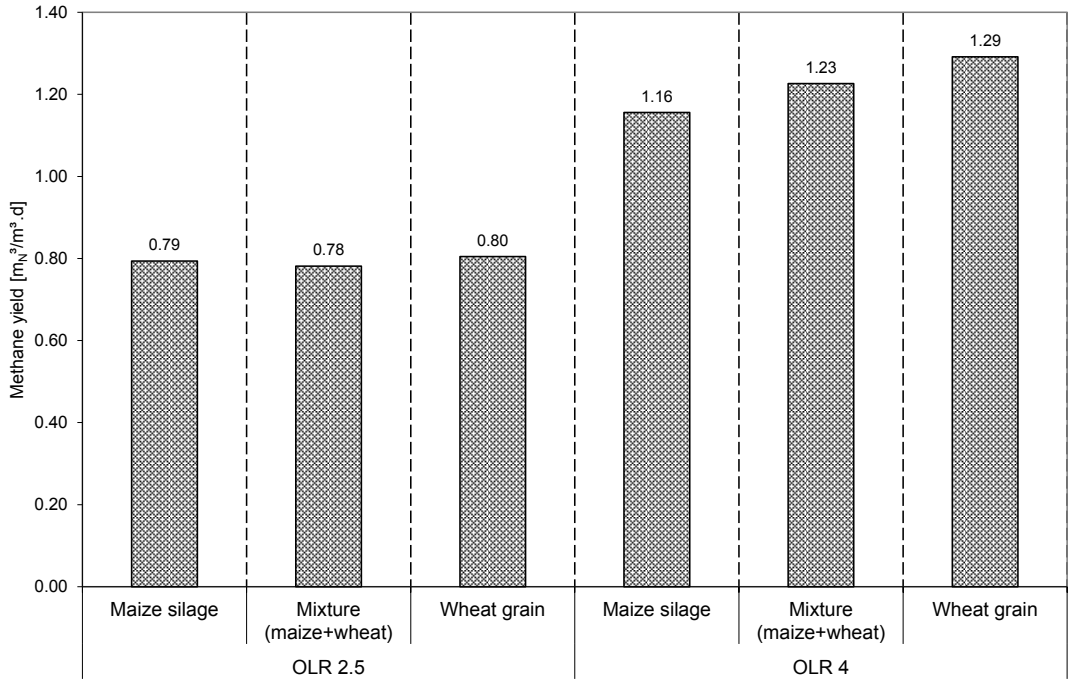

Figure 28: Reactor-specific methane yield of different variants at low and high OLR.

5.3 Experiment III: Influence of the biochemical crop traits on the specific methane yield potential of intentionally blended maize fractions (ear and stover)

The first step toward elucidating the influence of biochemical traits on the specific methane yield potential of maize whole-crop was to study the relationships between the biochemical composition and the specific methane yield potential by blending the crop fractions (ear and stover fractions) in different proportions. By doing so, the potential environmental factors that contribute randomly to crop traits (especially cell-wall) can be controlled. The absolute content of the biochemical traits was increased while the inner physiological status remained constant. Hence, the influence of the absolute values of cell-wall fractions on the specific methane yield potential could be analyzed. Two commonly used genotypes in central Europe (FAO 250 and 280) and a high biomass yielding genotype of warmer Mediterranean region (FAO 600) were investigated.

Table 19 shows the biochemical composition of the mixtures. The original crop materials (not the mixtures) revealed some differences in their biochemical composition. No starch was found in the stalk-leaf (stover) fraction of all three genotypes. As expected, the corn-cob fraction of the mid-early genotype [FAO-Index 250] had higher starch content (65%), while the late maturing genotype [FAO-Index 600] had lower starch content (56.4%). The corn-cob fraction of the mid-early genotype showed also an enzymatic digestibility (CDOMD) of 91.2%, which was greater than that of the late-maturing genotype (86.8%). The cell-wall content (NDF) was 5 units higher in mid-early genotype than in the late-maturing genotype.

Table 19: Main features of the corn-cob and stalk-leaf fractions and their blends.

Genotype	Corn-cob share [%]	Starch	XP	XF	NDF	DNDF	ADF	Enzymatic digestibility of ODM	Specific CH_4 - Yield [m_N^3/kg ODM]
		[%]							
B [250]	0	0.0	6.5	33.7	78.5	50.6	39.8	44.7	0.302 [±0.1]
	20	10.2	7.3	28.8	65.5	55.7	33.2	53.5	0.322 [±0.7]
	40	25.9	8.0	22.0	51.9	60.1	24.4	63.9	0.335 [±1.8]
	60	36.9	8.2	17.3	40.8	66.5	18.9	71.4	0.346 [±0.6]
	80	49.0	8.5	11.9	29.9	71.2	12.7	79.9	0.348 [±1.7]
	100	65.1	9.2	4.7	17.6	77.8	6.7	91.2	0.368 [±1.8]
C [280]	0	0.0	4.8	35.5	78.9	54.7	41.5	44.2	0.293 [±1.0]
	20	12.8	6.0	28.2	63.4	58.4	32.7	55.4	0.311 [±1.0]
	40	25.7	6.9	22.4	52.3	63.1	25.1	64.7	0.335 [±0.6]
	60	37.2	7.3	17.3	40.9	66.9	19.0	72.3	0.341 [±1.1]
	80	48.9	8.0	11.9	30.6	71.1	13.1	80.3	0.347 [±1.3]
	100	63.3	8.7	5.7	19.9	78.0	8.1	90.6	0.363 [±0.9]
G [600]	0	0.0	7.2	32.2	73.0	59.6	38.3	48.1	0.307 [±0.1]
	20	12.4	7.9	26.8	60.9	64.0	31.7	56.8	0.310 [±2.9]
	40	24.6	8.1	21.8	50.3	66.9	25.0	65.2	0.326 [±1.7]
	60	33.8	8.1	18.1	42.6	69.2	20.2	71.3	0.346 [±2.3]
	80	43.0	8.2	14.4	34.4	71.7	16.1	77.2	0.348 [±2.5]
	100	56.4	9.0	8.6	25.5	75.5	10.6	86.8	0.355 [±1.9]

[ ] = % relative standard deviation

The cell-wall content (NDF) in the stalk-leaf fraction of the mid-early genotype was of 78.9% compared to 73% for the late-maturing genotype. These results ascertain that at the harvest date the mid-early genotype had reached its full maturity with a higher NDF value in its vegetative parts and more mature grains in the ear (i.e. high CDOMD value exceeding 90%). At the same time the late-maturing genotype was in an earlier stage of development, with a low NDF value in the vegetative fraction and less mature grains in the cob (i.e. relatively low value for the enzymatic digestibility). The mid-late genotypes showed values in between.

By blending the crop fractions, the starch content in the mixtures went increasing with the increased share of the corn-cob fraction as expected. The mid-late genotype showed values far much closer to that of the mid-early genotype.

The stalk-leaf fraction had the lowest specific methane yield potential (0.293 to 0.307 m_N ³ CH_4/kg ODM), but the specific methane yield potential differed from one genotype to another. These yields were in the range of those of hay (± 0.300 m_N ³ CH_4/kg ODM), a standard reference substrate used commonly in HBT trials. The stalk-leaf fraction of the late-maturing genotype (FAO-index 600) had a higher methane yield potential in comparison to that of the other stalk-leaf fractions. This might be due to the age of its tissues at the harvest (younger tissues in comparison to that of the mid-early maturing genotype as the values of NDF and CDOMD show).

The specific methane yield potential of the corn-cob fraction was higher for the mid-early maturing genotype than that for the late maturing genotype. The specific methane yield potentials of the crop materials varied from 0.293 m_N³ CH_4/kg ODM in stalk-leaf fraction to 0.368 m_N³ CH_4/kg ODM in corn-cob fraction. The specific methane yield potential went increasing with the increased corn-cob share in the mixture (Figure 29).

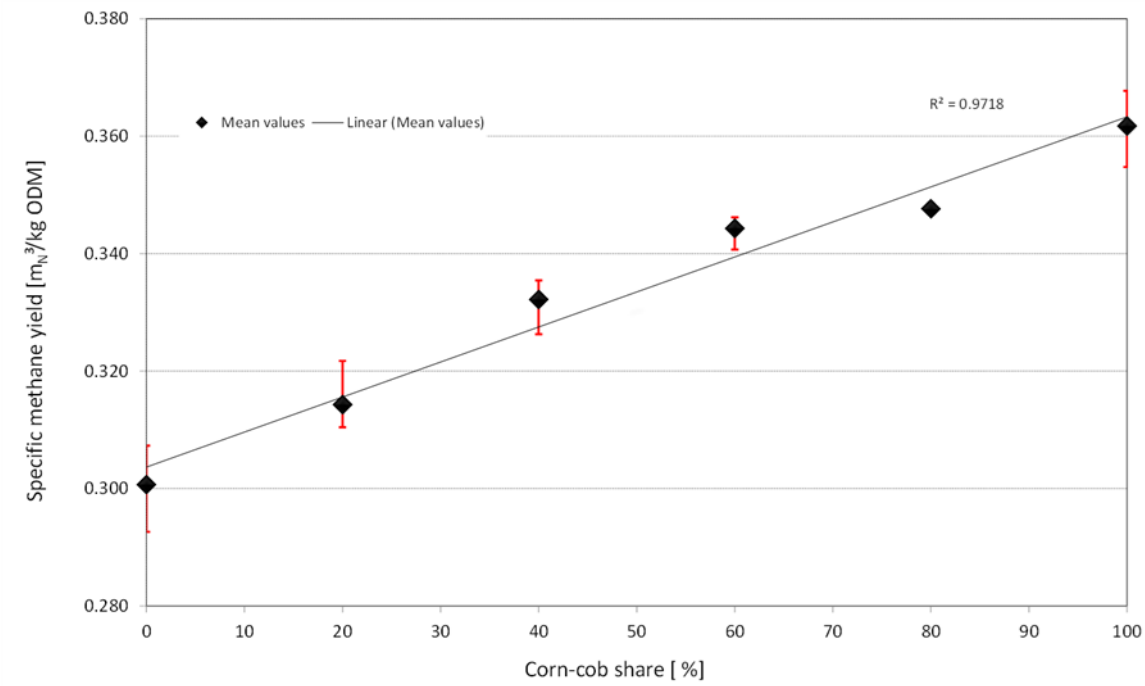

Figure 29: Relationship between the corn-cob share and the specific methane yield of intentionally blended crop fractions of three maize genotypes.

Figure 30 and Figure 31 show the relationships between the main biochemical crop traits (ADF, NDF, and starch) and the specific methane yield. The crop biochemical traits were found to account, to a very high degree, for the variability in specific methane yield potential. For instance with the increasing starch or corn-cob content in the mixture the specific methane yield potential increased almost linearly (R^2 = 0.93 and 0.97). Similarly, the increase in stalk-leaf fraction (i.e. increase in cell-wall content) caused the specific methane yield potential to decrease.

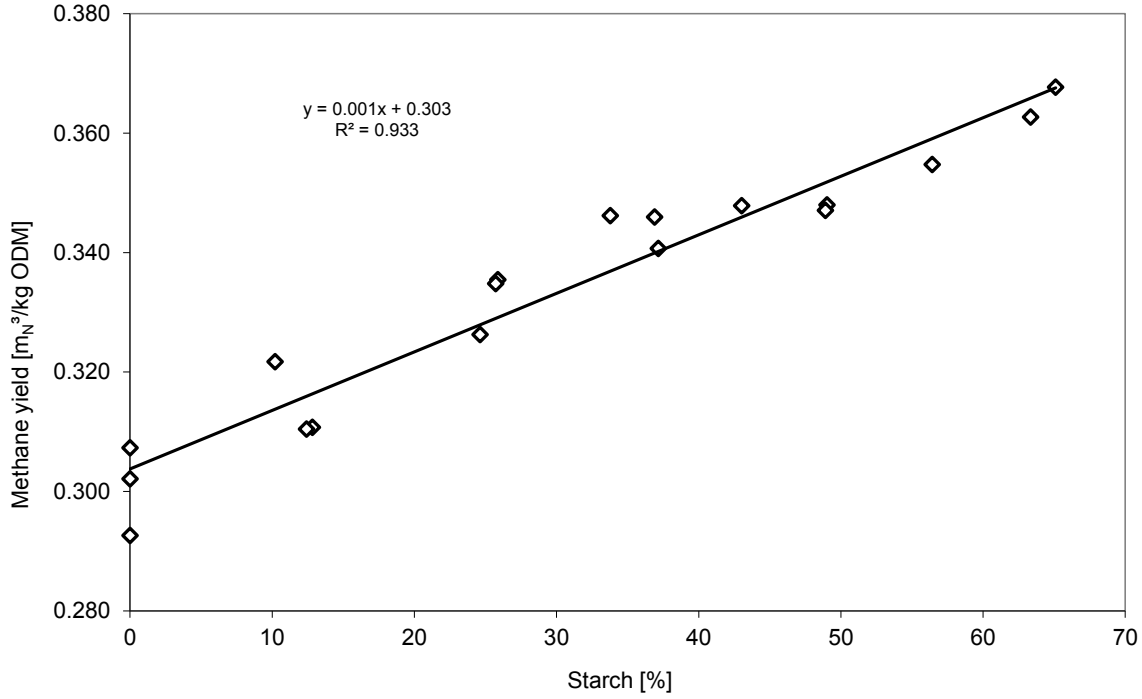

Figure 30: Relationship between the starch content and the specific methane yield potential of intentionally blended crop fractions of three maize genotypes.

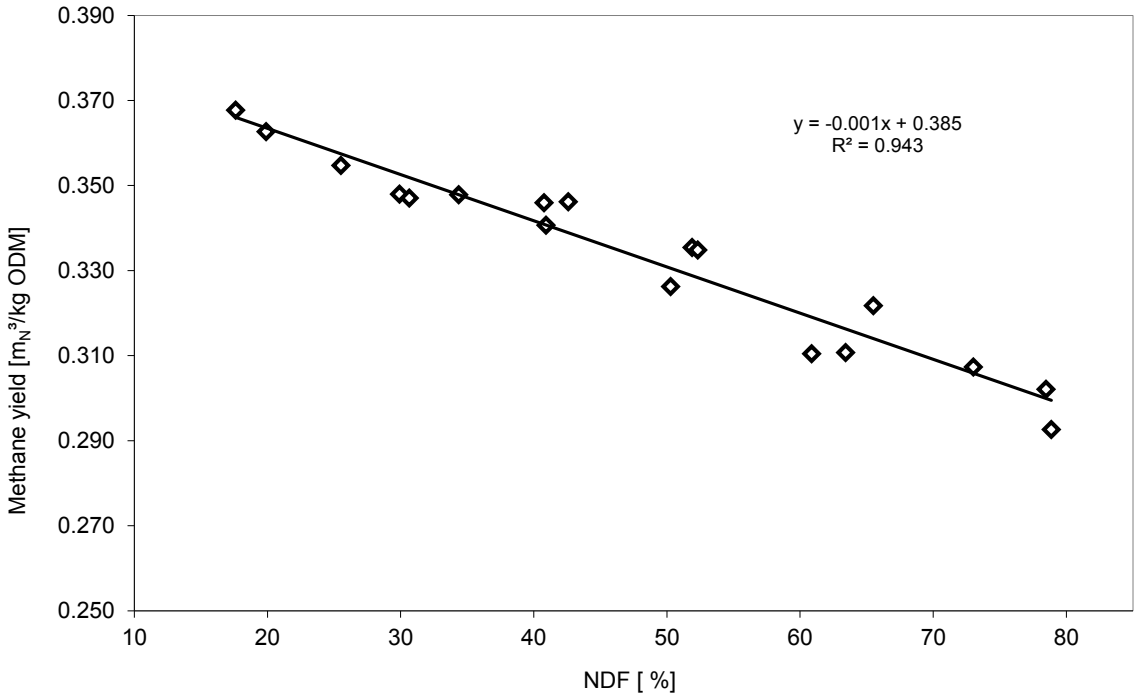

Figure 31: Relationship between the absolute NDF content and the specific methane yield potential of intentionally blended crop fractions of three maize genotypes.

Apart from the ultimate specific methane yield potential determined in this experiment, it was observed that samples of high starch or low cell-wall contents showed a benefit of a high biogas yield rate. Figure 32 shows the positive effect of starch on the biogas production kinetics. The higher the corn-cob proportion in the blend the quicker the biogas production rate. After 8 days retention time, almost 90% of the ultimate methane yield was reached for the corn-cob fraction, while only 70% was reached for the stalk-leave fraction. At the eighth day, the difference between the specific methane yield of the corn-cob fraction and that of the stalk-leaf fraction was larger for genotype B (FAO-Index 250) and smaller for genotype G (FAO-Index 600).

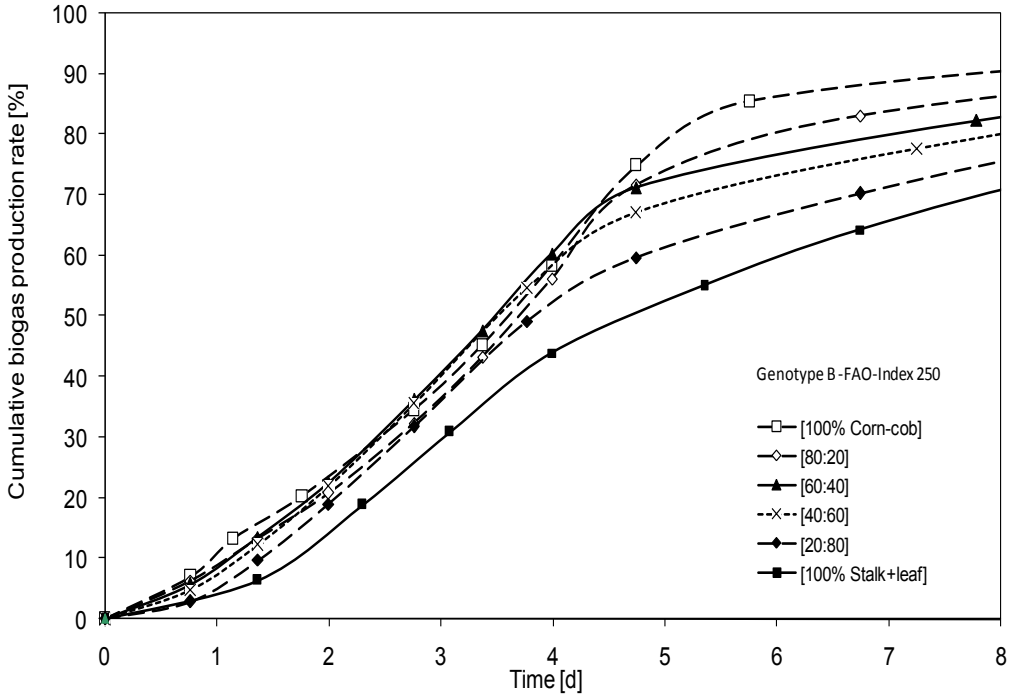

Figure 32: Cumulative biogas production for maize genotype B (exemplarily for FAO-Index 250).

5.4 Experiment IV: Influence of the biochemical crop traits on the specific methane yield potential of maize whole-crop

The difference in the specific methane yield potential of maize whole-crop and the absolute upper and lower boundaries are dictated by the crop's biochemical composition. Therefore, we examined the biochemical composition patterns of maize across different physiological stages. The evolution of the biochemical crop traits of four genotypes (FAO 250, 280, 600, and 700) extracted from the set of 304 samples (see Section 4.7.3) is presented in Figure 33. The growth periods used exceeded those commonly observed for silage maize or biogas production under the central European climatic conditions.

Evolution of maize's main biochemical traits

Figure 33 shows the evolution of the main biochemical crop traits in maize varieties of different maturity grades from the 121st to the 196th growing day. The biochemical composition patterns vary with the maturity grade and physiological growth stages.

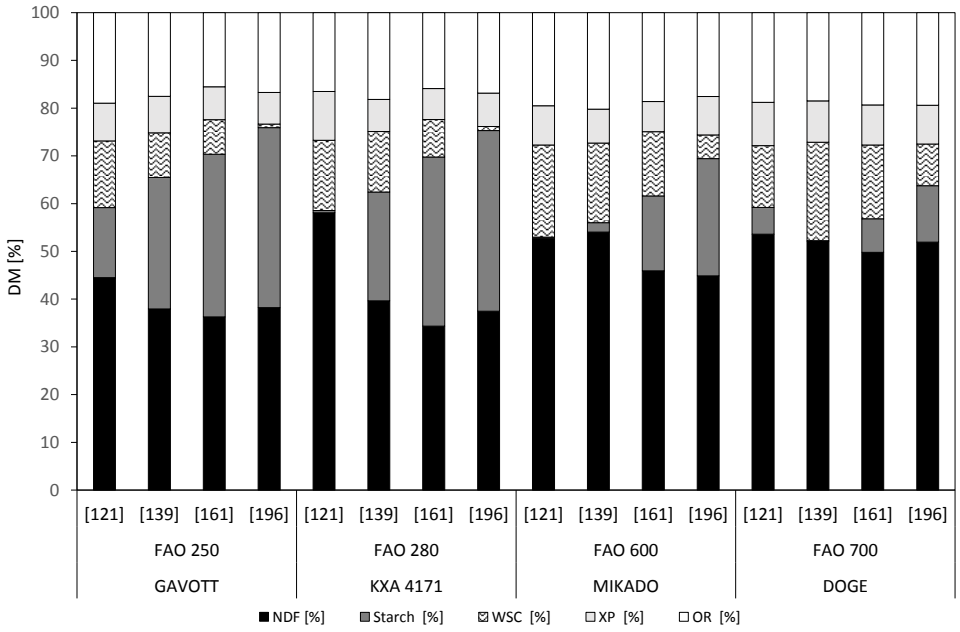

Figure 33: Change in biochemical composition for maize Genotypes after different growing duration.

The main biochemical traits of maize are: NDF (neutral detergent fibers), WSC (water soluble carbohydrates), protein and starch. The NDF content expresses the cell-wall content while WSC, protein and starch represent more or less the cell content fraction. Other components, here expressed as OR or “organic rest” represent lipids and other compounds whose estimation is not performed with the routine NIRS method.

The results show that cell-wall make the predominant share of the biochemical traits of maize. Across the genotypes and harvest dates the NDF content showed a minimum value of 34.4% and a maximum value of 58.1%. The average was 44.7%. The mid-early and the mid-late genotypes showed slight differences of NDF values. Apart from the first harvest date (at the 121st growing day) where the mid-late genotype showed a higher NDF value of 58.10%, both genotypes showed low NDF values (below 40%) at the following harvest dates. The NDF values of the late maturing genotypes (FAO-Index 600 and 700) were higher than that of the mid-early and that of the mid-late genotypes at each harvest date. The NDF values of the late maturing genotype DOGE (FAO 700) did not drop below 49% throughout the growing period.

The starch content in the mid-early and the mid-late genotypes went increased drastically with the stage of physiological growth and reached 37.86% at the late harvest occasion. The starch contents in the late maturing genotypes were low and reached maximum values of 11.82% and 24.54% for the FAO 700 and FAO 600 genotypes respectively. This shows that despite the long growing period beyond the normal growing season, the late-maturing genotypes had not reached their full physiological maturity. This is worth to be noted since central European climatic conditions and on good soils, silage maize is generally harvested after 130 to 160 growing days.

The WSC content decreased with the increasing starch accumulation to reach the lowest values at the late harvest date. Following values were measured: 0.75%, 0.83%, 4.96% and 8.75% for the mid-early, mi-late genotypes and for the late genotypes (FAO 600 and 700) respectively. The *in-vitro* estimate of digestibility

(CDOMD value) showed 22.55 units' difference with values ranging from 51.85 to 74.40% (Figure 34).

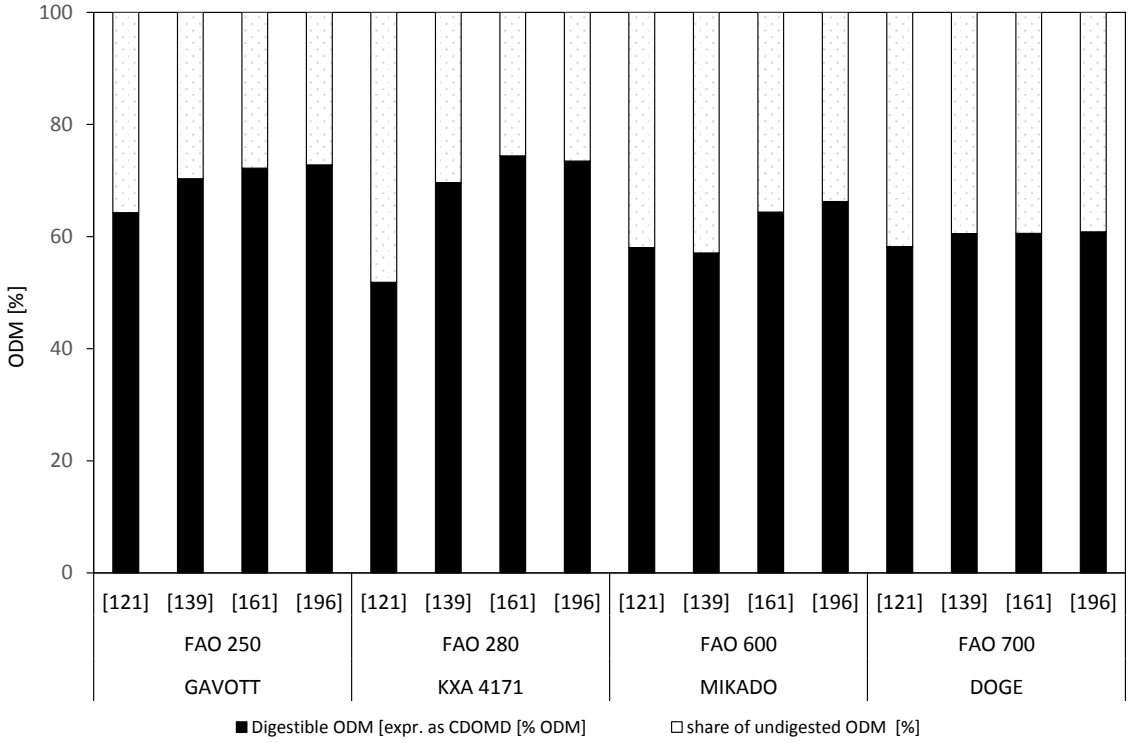

Figure 34: The share of the estimated digestible ODM (expressed by the CDOMD values) in comparison to the estimated undigestible fraction of ODM for maize whole-crop throughout the growing duration.

Variation ranges and relationships of the main crop biochemical traits of maize whole-crop

The entire samples set covered whole-crop materials of even a wider physiological range. Table 20 shows the upper and lower limits and variation ranges of the biochemical traits for the samples analysed.

Table 20: Descriptive statistics of the main crop features of maize whole-crop.

	N	Range	Min.	Max.	Mean	Std. Deviation
DM Yield [t/ha]	291	19.0	10.0	29.0	20.0	4.1
Whole-crop DM [%]	296	41.3	14.6	55.9	31.4	8.4
Starch [%]	303	44.1	0.0	44.1	24.8	10.9
WSC [%]	283	20.6	0.4	21.0	8.7	4.3
Crude protein [%]	304	6.6	5.4	12.0	7.7	1.0
NDF [%]	296	39.9	30.7	70.6	43.9	6.0
ADF [%]	283	29.0	15.4	44.4	23.7	4.3

The total dry matter (DM) content ranged from 15% to 56%. The cell-wall content (Cellulose, hemicellulose and lignin) expressed as NDF and the ligno-cellulose fraction (ADF) varied also in a very broad-range. NDF values varies from 30.7% to 70.6% (40 unit difference) while ADF varies from 15.4% to 44.4% (29 unit difference). The starch accumulation showed also a high variation range (44 unit difference). Hence, the set covers almost all variability in biochemical composition that would represent genotypes and physiological maturity grades needed for the assessment of the influence of the biochemical crop traits on the specific methane yield potential of maize whole-crop.

The key relationships between main crop traits, CDOMD and the specific methane yield potential for the five years are presented in Table 21 and Figures 35 and 36. As expected, the increase in starch accumulation was significantly negatively correlated with NDF and ADF ($p < 0.01$). The *in-vitro* estimate of digestibility for whole-crop (CDOMD) was significantly positively correlated with the starch content ($p < 0.01$). All the main biochemical traits and the *in-vitro* estimate of digestibility (CDOMD) showed significant correlation to each other (R^2= 0.68-0.74).

Table 21: Relationships between key crop traits, CDOMD and the specific methane yield potential of maize whole-crop.

Correlations

		Spec. Methane Yield (m_N^3/kg ODM)	Whole crop DM (%)	CDOMD (%)	Starch (%)	NDF (%)	ADF (%)	WCS (%)	Crude protein (%)
Spec. Methane Yield (m_N^3/kg ODM)	Pearson Correlation	1	.291**	.439**	.477**	-.463**	-.415**	-.233**	-.008
	Sig. (2-tailed)		.000	.000	.000	.000	.000	.000	.883
	N	304	296	283	303	283	283	283	304
Whole crop DM (%)	Pearson Correlation	.291**	1	.676**	.783**	-.567**	-.690**	-.686**	-.471**
	Sig. (2-tailed)	.000		.000	.000	.000	.000	.000	.000
	N	296	296	275	295	275	275	275	296
CDOMD (%)	Pearson Correlation	.439**	.676**	1	.859**	-.948**	-.969**	-.465**	-.320**
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.000	.000
	N	283	275	283	283	283	283	283	283
Starch (%)	Pearson Correlation	.477**	.783**	.859**	1	-.840**	-.822**	-.801**	-.294**
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.000	.000
	N	303	295	283	303	283	283	283	303
NDF (%)	Pearson Correlation	-.463**	-.567**	-.948**	-.840**	1	.890**	.428**	.344**
	Sig. (2-tailed)	.000	.000	.000	.000		.000	.000	.000
	N	296	275	283	283	283	283	283	283
ADF (%)	Pearson Correlation	-.415**	-.690**	-.969**	-.822**	.890**	1	.423**	.218**
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.000	.000
	N	283	275	283	283	283	283	283	283
WCS (%)	Pearson Correlation	-.233**	-.686**	-.465**	-.801**	.428**	.423**	1	.311**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000		.000
	N	283	275	283	283	283	283	283	283
Crude protein (%)	Pearson Correlation	-.008	-.471**	-.320**	-.294**	.344**	.218**	.311**	1
	Sig. (2-tailed)	.883	.000	.000	.000	.000	.000	.000	
	N	304	296	283	303	283	283	283	304

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 35 shows that starch continued to accumulate in maize with the increasing DM content until the dry matter content reached 40%. From this point onward starch content reaches a plateau. The maximum starch content was around 44%. Figure 37 shows the same trend for the relationship between *in-vitro* estimate of digestibility for whole-crop (CDOMD) and whole-crop DM. In both figures, the area considered to be the optimum harvest-window for silage maize (28% to 40% DM content) showed great variability in CDOMD and starch content.

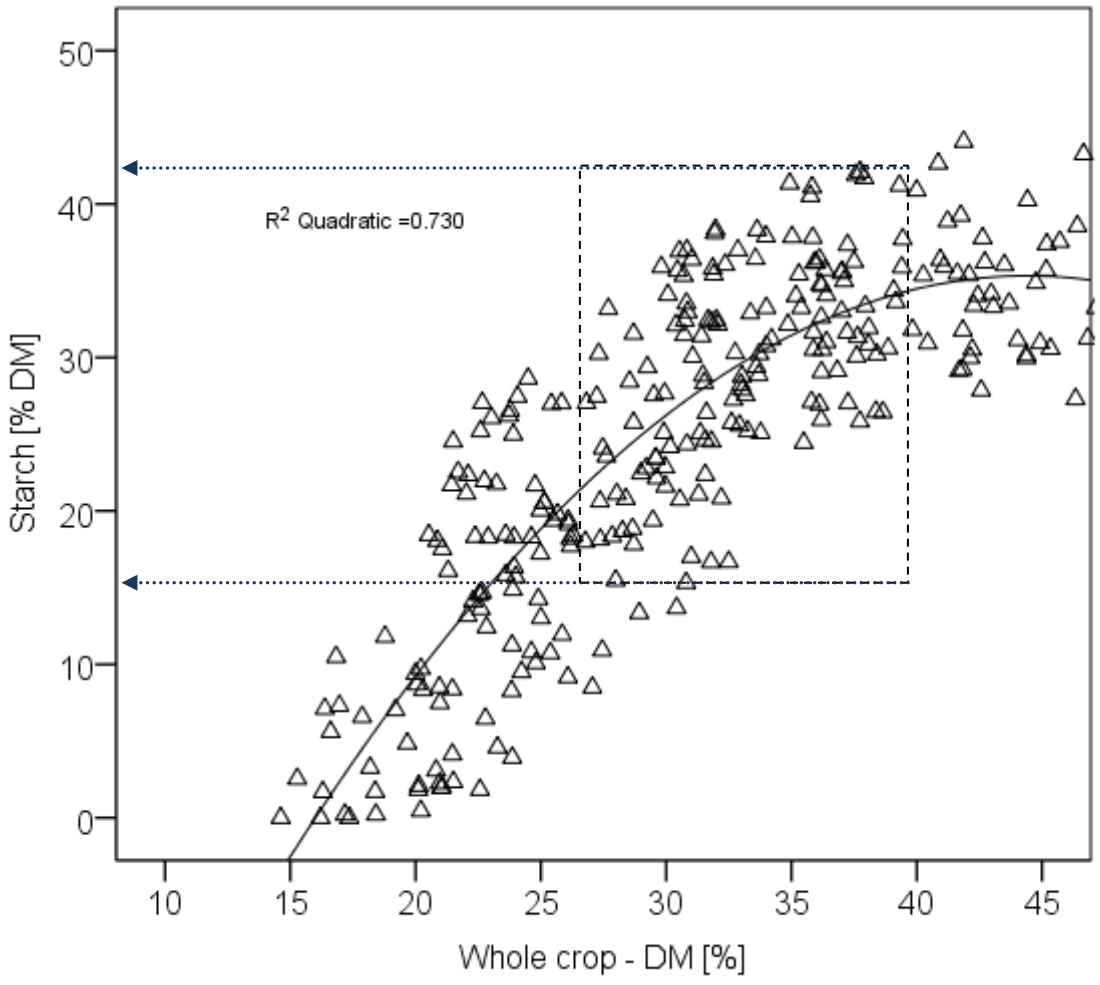

Figure 35: Relationship between the total DM of the whole-crop and the starch content (the crosshatched area shows the variation in starch content in the zone commonly considered as optimum for silage maize harvest).

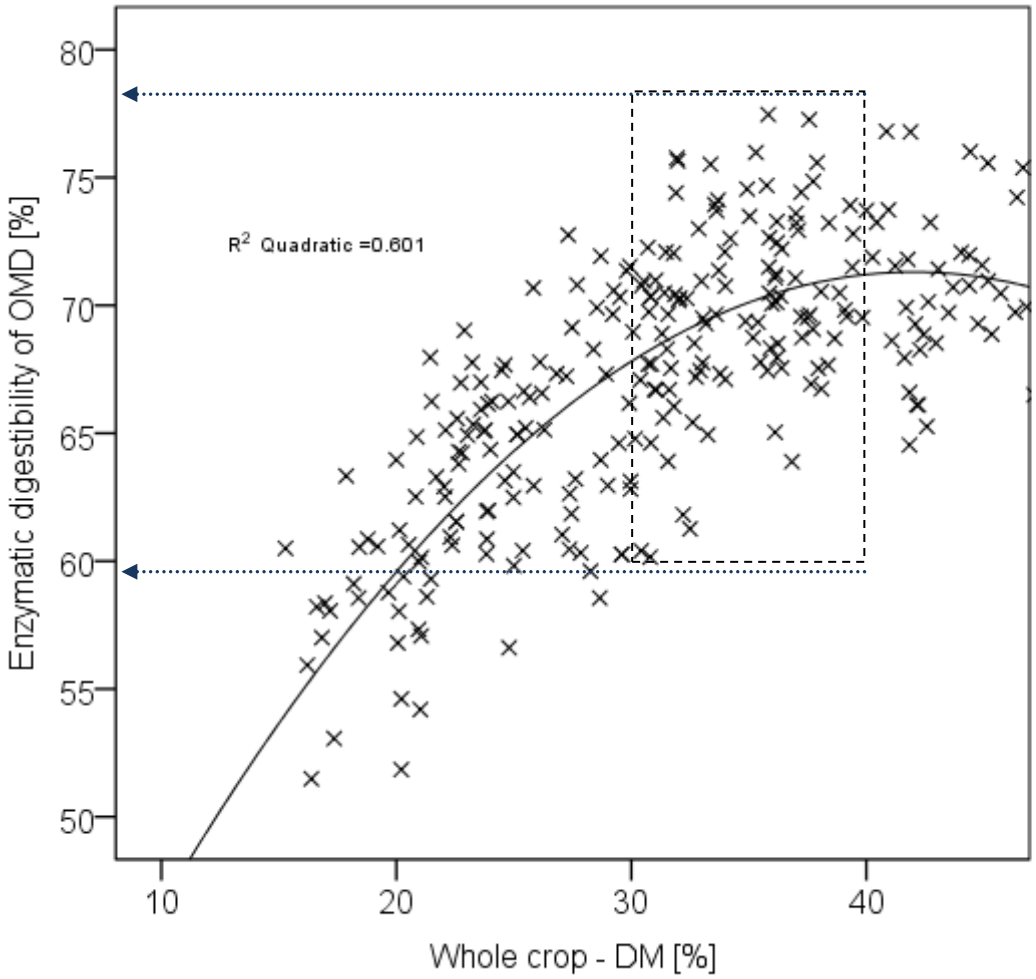

Figure 36: Relationship between CDOMD (also called enzymatic digestibility of ODM) and total DM of the whole-crop (the crosshatched area shows the digestibility variations in the optimum silage maize harvest zone).

Influence of the crop traits on the specific methane yield potential of maize whole-crop

Table 21 above shows that the correlation between the total DM content of maize whole-crop and the specific methane yield potential for all the five years and nine locations was quasi inexistent. The results suggest hence that it is not possible to characterize whole-crop maize toward its specific methane yield potential based on the total DM content. In fact, in the zone that is considered to be the optimum for silage maize (DM 28% to 40%), the specific methane yield potential vary in a 12%-range, but without clear trend. The relationships between the starch content, NDF,

CDOMD and the specific methane yield potential revealed minor effect (R^2 = 21% to 24%) at $p < 0.01$.

The year 2003 was marked by extreme heat and dryness. The crop materials in 2003 experienced severe environmental stresses, so that the relationships between the crop traits and the specific methane yield potential were assumed to be distorted. Hence, the samples from 2003 were discarded and the influence of the crop features on the specific methane yield potential reevaluated for the other years. The correlations increased for almost all parameters. The results are shown in Figure 37 and 38. More than 30% of the increase in specific methane yield was found to be influenced by the decrease in ADF content or the increase in *in-vitro* enzymatic digestibility of ODM.

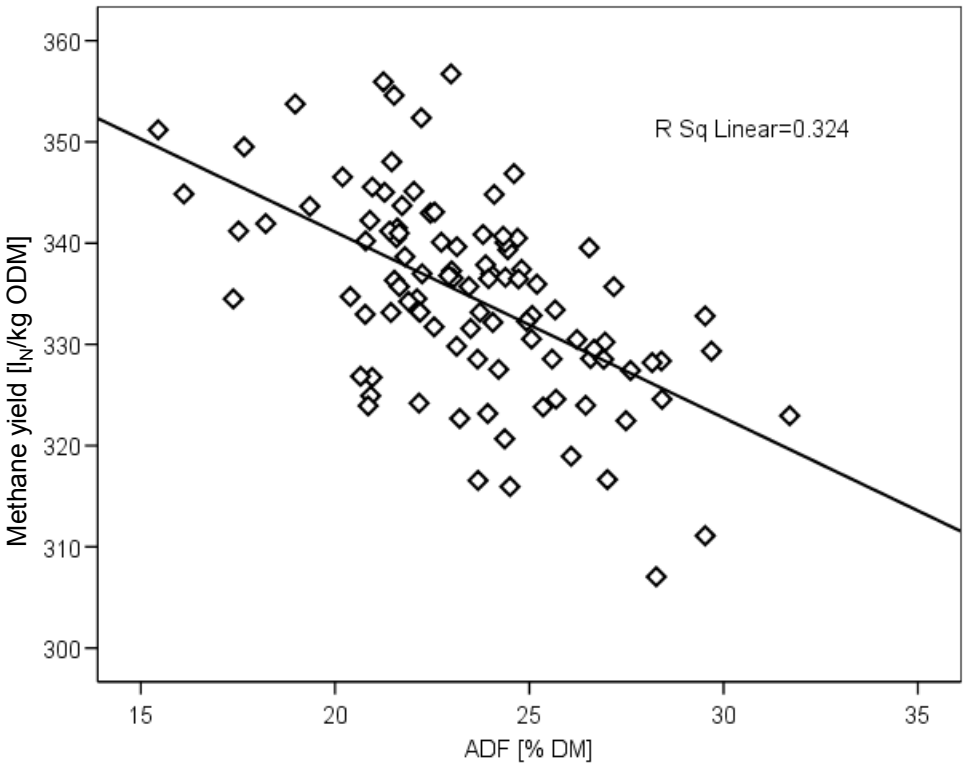

Figure 37: Relationship between acid detergent fiber content (ADF) and specific methane yield of various maize genotypes (samples of the year 2003 discarded).

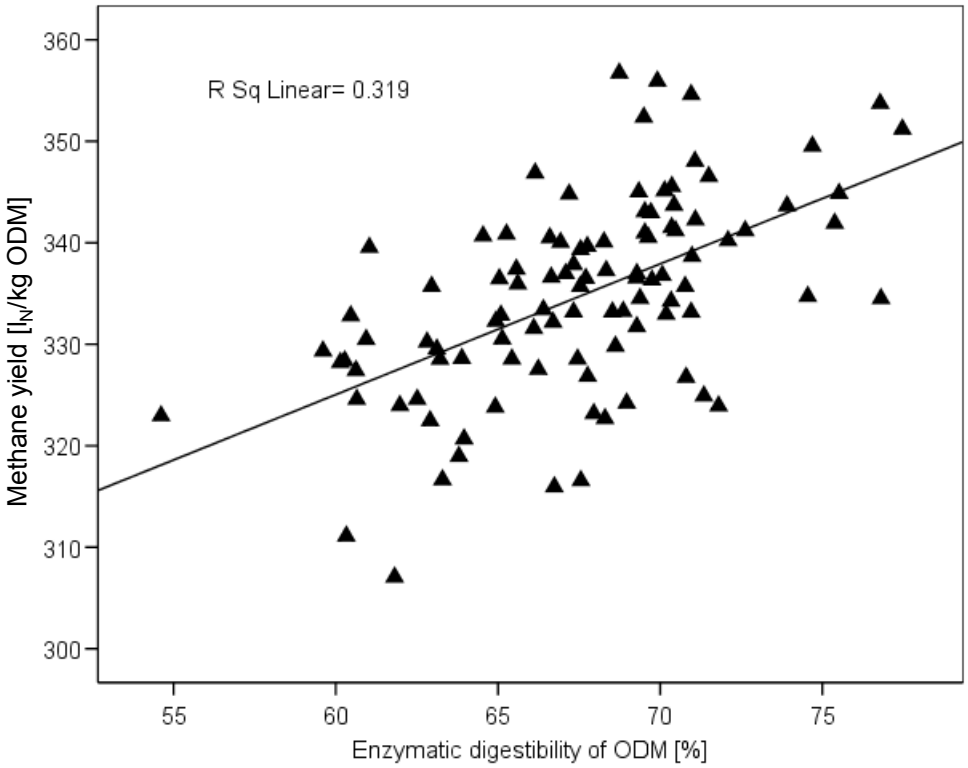

Figure 38: Relationship between the enzymatic digestibility of ODM and the specific methane yield of various maize genotypes (samples of the year 2003 discarded).

Specific methane yield potential of maize whole-crop

The specific methane yield potential and the spread for the 304 samples of maize whole-crop analyzed over the five years are shown by the box plot (Figure 39). The variation widths of the specific methane yield potential of maize genotypes were slightly different from year to year, as represented by the medians.

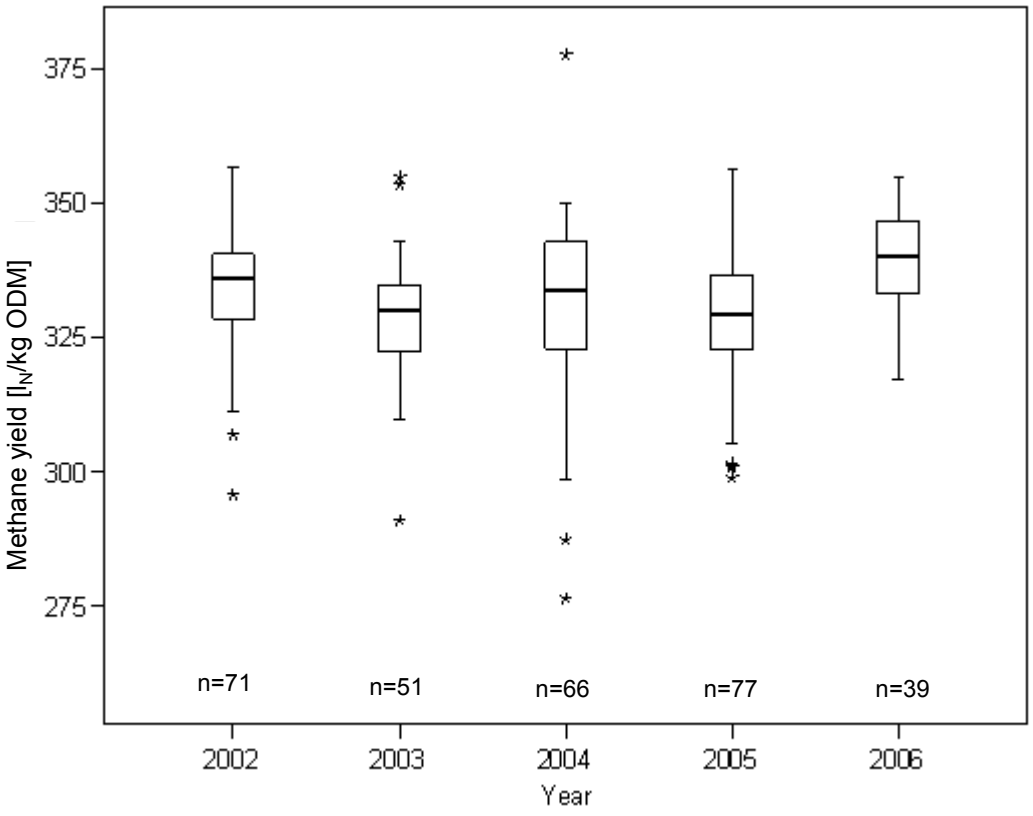

Figure 39: Box plot of the specific methane yields of various maize genotypes with different maturity.

The analysis (Table 22) showed that only the mean of the year 2006 was significantly different to the means of preceding years ($p < 0.05$). The slight differences over the years might be due to the fact that the research questions addressed by the agricultural and breeding studies were different from year to year, so that the populations were not homogeneous.

Table 22: Post Hoc test (Games-Howell) - analysis of variance in specific methane yield over five years.

Multiple Comparisons

Dependent variable: Spec. methane yield [m_N^3/kg ODM] Games-Howell

(I) Year	(J) Year	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2002	2003	.004975	.001940	.084	-.00041	.01036
	2004	.002790	.002351	.759	-.00373	.00931
	2005	.004587	.001867	.106	-.00057	.00974
	2006	-.005591*	.001926	.037	-.01096	-.00023
2003	2002	-.004975	.001940	.084	-.01036	.00041
	2004	-.002186	.002479	.903	-.00906	.00469
	2005	-.000388	.002025	1.000	-.00600	.00522
	2006	-.010566*	.002080	.000	-.01636	-.00477
2004	2002	-.002790	.002351	.759	-.00931	.00373
	2003	.002186	.002479	.903	-.00469	.00906
	2005	.001798	.002422	.946	-.00491	.00851
	2006	-.008380*	.002469	.008	-.01523	-.00153
2005	2002	-.004587	.001867	.106	-.00974	.00057
	2003	.000388	.002025	1.000	-.00522	.00600
	2004	-.001798	.002422	.946	-.00851	.00491
	2006	-.010178*	.002012	.000	-.01577	-.00458
2006	2002	.005591*	.001926	.037	.00023	.01096
	2003	.010566*	.002080	.000	.00477	.01636
	2004	.008380*	.002469	.008	.00153	.01523
	2005	.010178*	.002012	.000	.00458	.01577

*. The mean difference is significant at the 0.05 level.

The means of the 6 locations in Germany were not significantly different. They were however significantly different to means of the 3 locations in Luxemburg at $p < 0.001$ as shown by the Welch one-way ANOVA (Table A-2) and the *post-hoc* analyses in Table A-3 and Table A-4 (Appendix).

Despite the large variation in populations, locations (9), and years (5), it is however interesting to note that the yearly medians varied only between 0.325 and 0.345 m_N^3 CH_4/kg ODM. Although *in-vitro* enzymatic digestibility of ODM for whole-crop revealed an extreme variation of 40 units (from 40.6% to 77.5%), the specific

methane yield potential varied only from 0.300 and 0.356 m_N^3 CH_4/kg ODM. This is equivalent to 57 l_N CH_4/kg ODM, which corresponds to 15% difference from the lowest to the highest yield. The 5-years median lies by 0.332 m_N^3 CH_4/kg ODM.
Table 23 shows descriptive statistics for *in-vitro* estimates of digestibility for whole-crop, the specific methane yield potential and the hectare-specific methane yield. Contrary to the small variation in specific methane yield potential (15%), the variation in hectare-specific methane yield (m_N^3 CH_4/ha) reached 68.6%. The average hectare-specific methane yield was of 6443 m_N^3 CH_4/ha. The minimum and the maximum values were 2916 and 9277 m_N^3 CH_4/ha respectively. This represents a range of 6443 m_N^3/ha. The hectare-specific methane yield was found to be highly correlated with the total DM yield per ha ($R^2 = 0.96$) at $p<0.01$. Figure 40 shows the relationship between the total DM yield per ha and the hectare-specific methane yield.

Table 23: Descriptive statistics of maize genotypes analysed.

	N	Range	Minimum	Maximum	Mean	Std. Deviation
IVDOM [%]	304	31.30	51.15	82.45	72.62	3.97
CDOMD [%]	283	36.86	40.60	77.46	67.05	5.56
Methane yield [m_N^3/kg ODM]	304	0.057	0.298	0.356	0.332	0.010
Methane yield [m_N^3/ha]	291	6,361	2,916	9,277	6,443	1,365

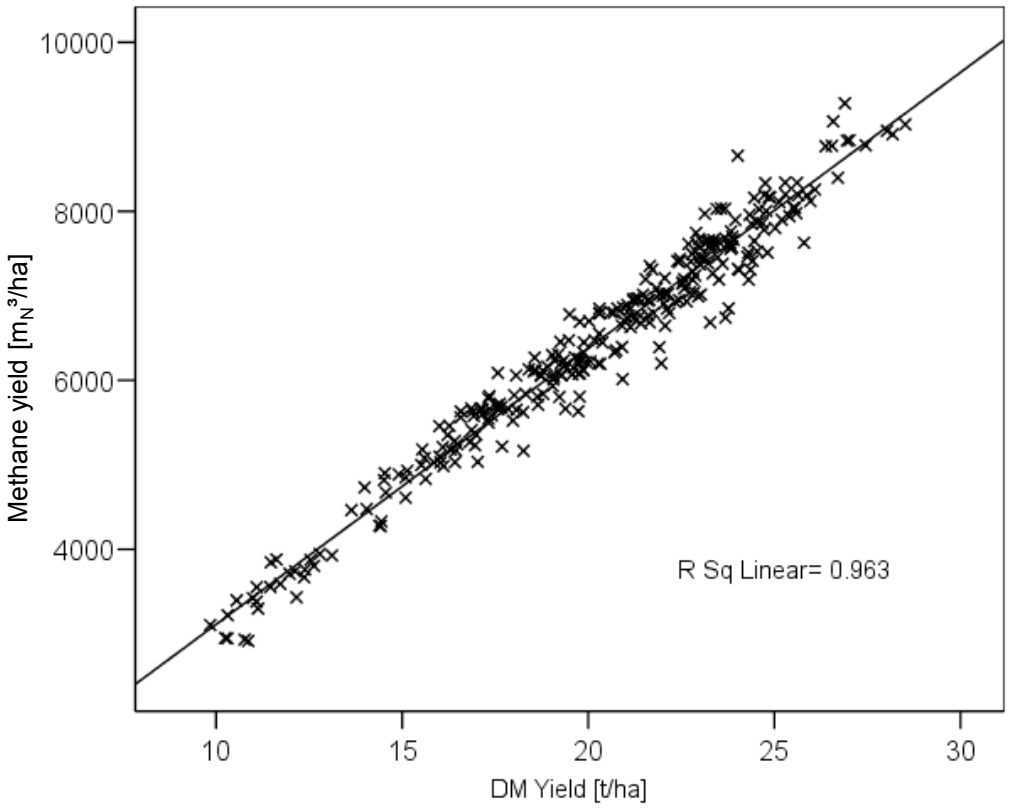

Figure 40: Influence of dry matter yield on the methane yield potential per unit of land (ha).

5.5 Experiment V: Assessment of *in-vitro* estimate of digestibility for whole-crop (CDOMD) and biochemical traits as predictors of biodegradability in an AD batch system

Among the most targeted parameters in the maize selection for high energy value are: starch, NDF, ADF, ADL, and digestibility of ODM. As shown in Experiment IV, a very high correlation exists between the absolute values of different cell-wall fractions and *in-vitro* digestibility, which in turn reflects the crop energy value. As far as the specific methane yield potential of maize whole-crop is concerned, the results of Experiment IV showed that these causalities were not as high as expected, especially when considering samples across years. We suggested, *inter alia*, that both absolute values of crop traits and *in-vitro* estimates of digestibility, as known from ruminal digestion, were not sharp enough to assess the ability of a crop to be biodegraded in AD systems. Consequently in this experiment we undertook to evaluate this by comparing *in-vitro* enzymatic digestibility of ODM (CDOMD) to

biodegradability (bioconversion efficiency in AD system). Furthermore we examined, the theoretical maximum methane yield potential across genotypes and through the growing season (at 5 different harvest dates) and reevaluated the relationships between the crop biochemical composition and the specific methane yield potential.

Table 24a and Table 24b show the theoretical maximum methane yield potential, the biodegradability (or the bioconversion efficiency), and the biochemical traits of different genotypes harvested at different physiological growth stages. The results show that the theoretical maximum methane yield potentials were more or less constant across genotypes and physiological growth stages. The variations were in a very small range between 0.447 and 0.469 m_N^3 CH_4/kg ODM. The absolute difference in energy content was of 4.7%. These results suggest that irrespective of genotype and physiological maturity, the absolute difference in energy content of maize whole-crop is very limited (4.7 units difference). Moreover, the energy content did not increase consistently with the increasing maturity as expected. Trends were different according to genotypes, but no clear patterns could be recognized. The slight differences in energy content may be attributed to moderate differences in lipid and protein contents across genotypes and physiological maturity. As carbohydrate compounds (WSC, starch, and complex carbohydrates) have the same specific energy values, it is obvious that maize genotypes generate more or less the same levels of specific methane yield potential.

Table 24a: Theoretical methane yields, recovery efficiency and crop features of maize crop genotypes after different growing periods.

Genotype	Growth duration	Theoretic CH_4-Yield	Recovered energy	Specific CH_4-Yield	NDF	ADF	Starch	Enzymatic digestibility of ODM
	[d]	[m_N^3/kg ODM]	[%]	[m_N^3/kg ODM]	[% DM]	[% DM]	[% DM]	[%]
A [240]	121	0.457	68.9	0.315	43	25	16	66
	139	0.458	73.9	0.338	37	21	29	72
	161	0.456	75.2	0.343	35	21	35	72
	177	0.454	76.9	0.349	34	19	41	74
	196	0.456	69.7	0.318	34	18	42	76
B [250]	121	0.457	69.7	0.319	45	26	15	64
	139	0.458	69.6	0.319	38	23	28	70
	161	0.460	75.0	0.345	36	21	34	72
	177	0.463	71.5	0.331	37	21	36	71
	196	0.467	73.6	0.343	38	20	38	73
C [280]	121	0.469	64.0	0.300	58	37	0	52
	139	0.449	76.9	0.345	40	23	23	70
	161	0.453	74.1	0.336	34	19	35	74
	177	0.457	76.5	0.350	37	22	33	71
	196	0.454	75.8	0.344	37	20	38	73
D [280]	121	0.462	69.3	0.320	53	30	5	59
	139	0.457	72.2	0.330	41	24	21	68
	161	0.454	74.0	0.336	36	22	32	72
	177	0.456	73.6	0.335	42	24	29	68
	196	0.460	71.8	0.330	40	21	35	71

Table 24b: Theoretical methane yields, recovery efficiency and crop features of maize crop genotypes after different growing periods.

Genotype	Growth duration	Theoretic CH_4-Yield	Recovered energy	Specific CH_4-Yield	NDF	ADF	Starch	Enzymatic digestibility of ODM
	[d]	[m_N^3/kg ODM]	[%]	[m_N^3/kg ODM]	[% DM]	[% DM]	[% DM]	[%]
E [400]	121	0.463	69.8	0.323	55	33	2	54
	139	0.457	75.5	0.345	43	26	18	67
	161	0.461	75.5	0.348	40	24	29	70
	177	0.467	74.6	0.348	41	26	31	67
	196	0.469	69.1	0.324	41	23	36	70
F [400]	121	0.457	65.4	0.299	53	30	2	58
	139	0.452	77.3	0.349	41	24	19	68
	161	0.453	77.2	0.350	37	21	32	72
	177	0.451	76.1	0.344	36	22	35	72
	196	0.447	76.1	0.341	37	20	38	74
G [600]	121	0.454	68.8	0.312	53	32	0	58
	139	0.459	68.1	0.313	54	33	2	57
	161	0.459	69.4	0.318	46	27	16	64
	177	0.468	74.2	0.347	43	26	19	65
	196	0.456	72.2	0.329	45	25	25	66
H [700]	121	0.458	71.4	0.327	54	30	6	58
	139	0.455	66.8	0.304	52	30	0	61
	161	0.455	75.4	0.344	50	29	7	61
	177	0.458	74.1	0.339	50	30	8	59
	196	0.469	70.8	0.332	52	28	12	61

Figure 41 shows the relationship between the theoretical maximum specific methane yield potential and the specific methane yield potential generated in batch. The results show that for a given theoretical maximum methane yield potential, the specific methane yield recovered in batch fermentation reactor can vary up to 14.6 percentage units, with specific methane yields ranging from 0.299 to 0.350 m_N^3 CH_4/kg ODM. Since the theoretical methane yield potentials were more or less similar and that the cell contents (starch, WSC, protein, etc.) are mobilized with ease in AD, the high variation (14.6%) in specific methane yield potential seemed to be ruled strongly by the biodegradability, most probably that of the cell-wall.

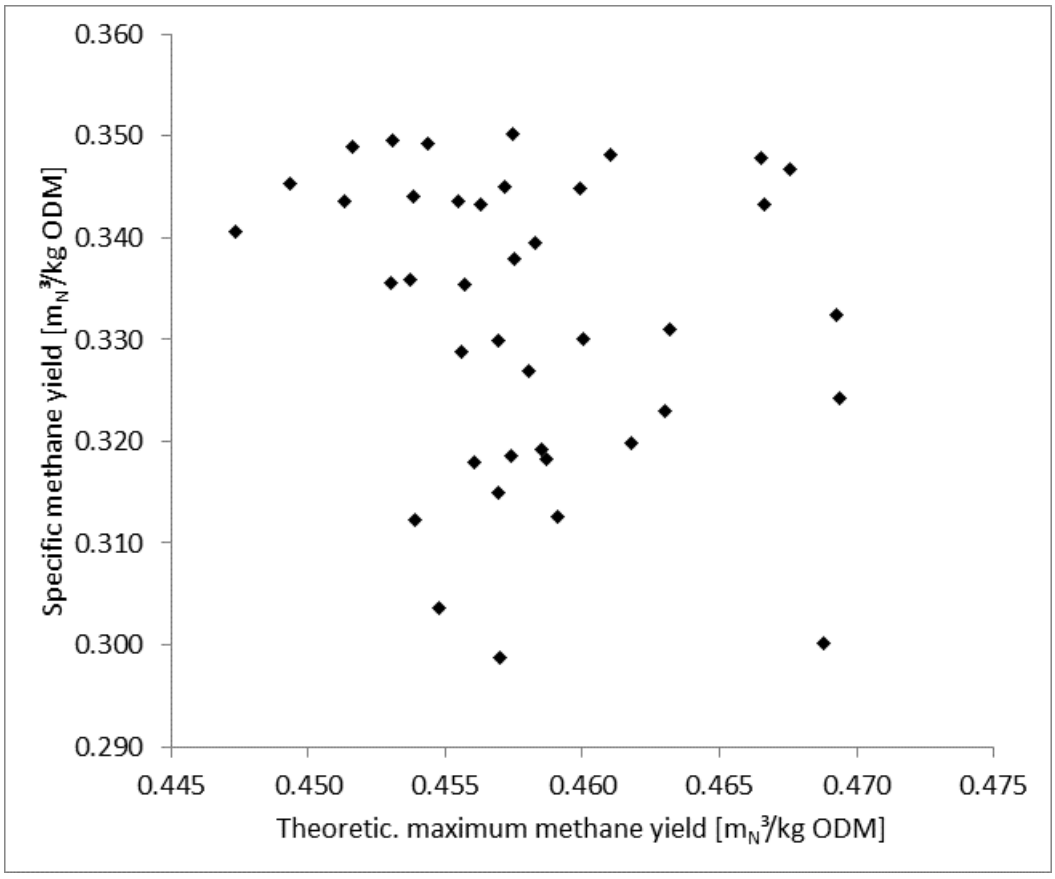

Figure 41: Relationship between the theoretical maximum methane yield and the specific methane yield recovered in batch-test.

Figures 42, 43 and 44 show the comparison between *in-vitro* enzymatic digestibility of ODM (CDOMD) and the biodegradability for maize genotypes of different maturity grades at different physiological stages. For all variants, the biodegradability (bioconversion efficiency) was higher than could be predicted by *in-vitro* estimate of

digestibility for whole-crop (CDOMD). The values were, in most cases, several units higher than those of *in-vitro* enzymatic digestibility. The *in-vitro* estimate of digestibility for whole-crop (CDOMD) seems to underestimate the bioconversion efficiency in AD systems. The greatest underestimation was observed for late maturing genotypes. Moderate to low underestimation was observed for the mid-early maturing genotypes. If the process inherent losses are considered in the balance, the share of the biochemical energy removed in AD system would exceed significantly the presumed *in-vitro* digestibility of ODM. Therefore the information this parameter deliver is, to a certain extent, misleading for an accurate appraisal of genotypes' effects. This is more probably the major hindrance for further selection.

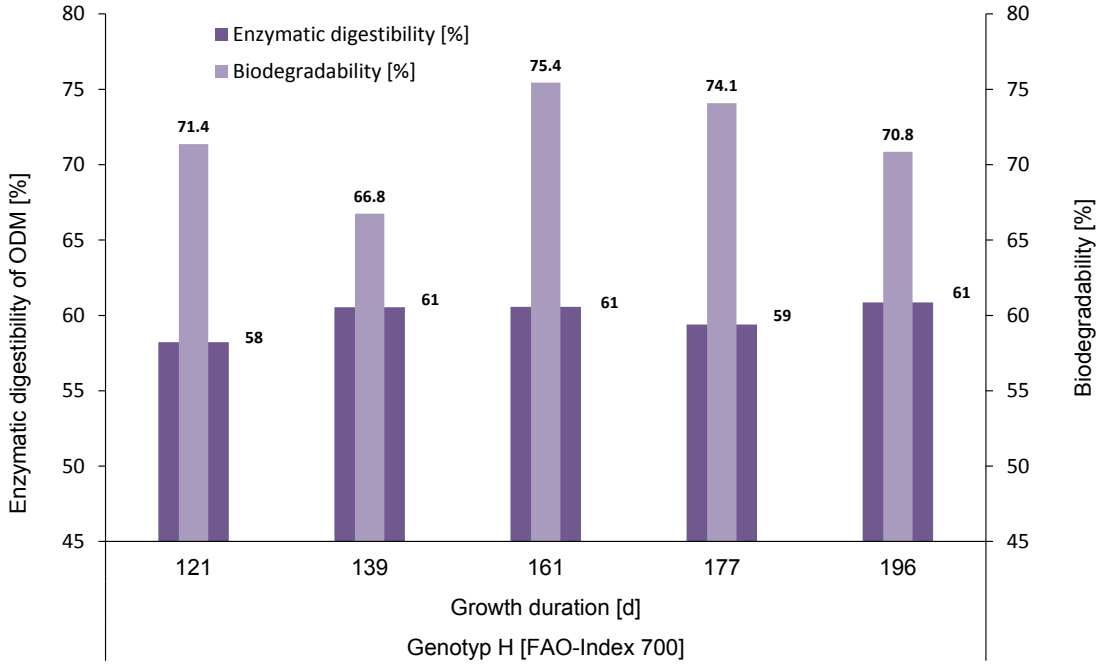

Figure 42: Comparison between *in-vitro* estimates of digestibility for whole-crop and the biodegradability in batch for the late-maturing maize genotype (FAO-Index 700).

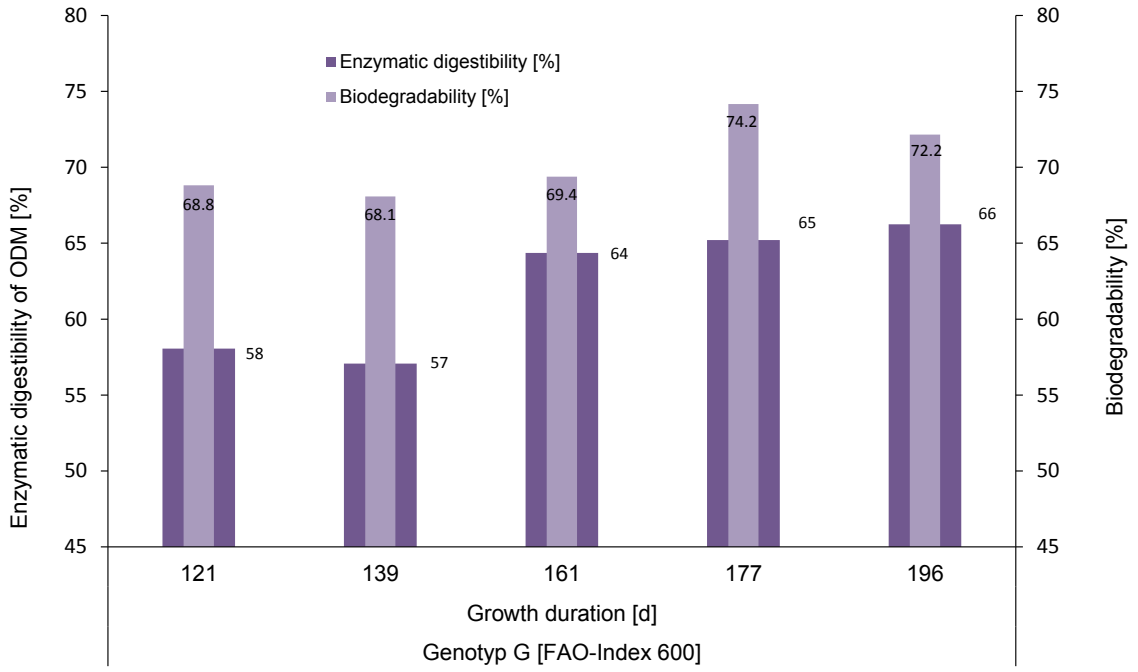

Figure 43: Comparison between *in-vitro* estimates of digestibility for whole-crop and the biodegradability in batch for the late-maturing maize genotype (FAO-Index 600).

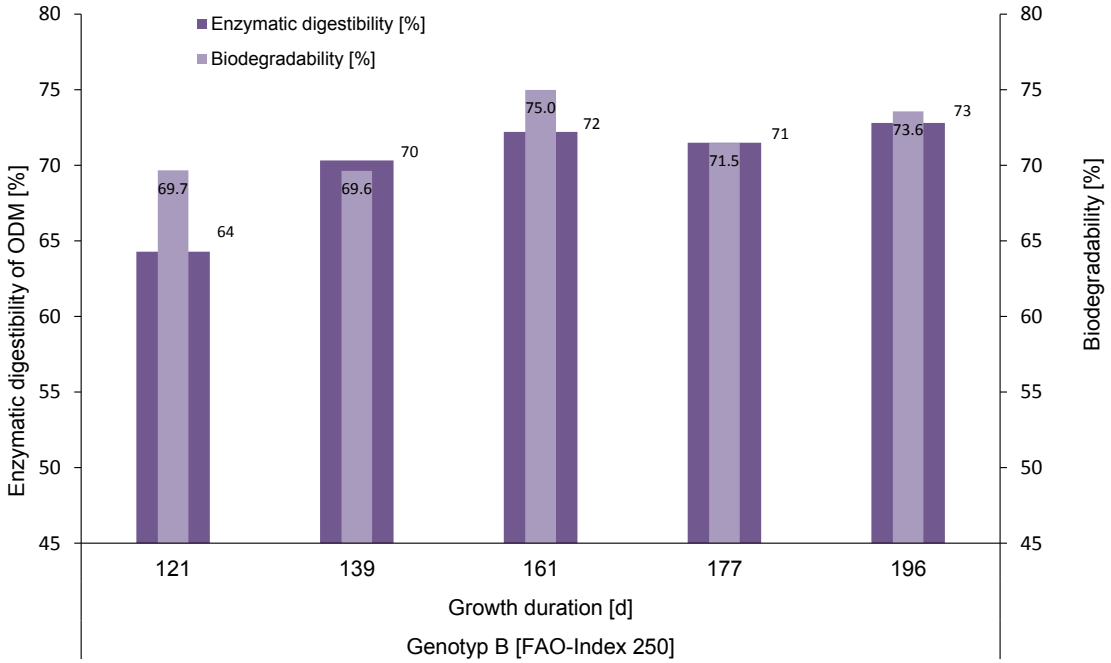

Figure 44: Comparison between in-vitro estimates of digestibility for whole-crop and the biodegradability in batch for the mid-early maturing maize genotype (FAO-Index 250).

The biodegradability increases with the increasing enzymatic digestibility of ODM (Figure 45). Nevertheless, *in-vitro* enzymatic digestibility of ODM for whole-crop (CDOMD) explained only 40% the ability of a crop material to be digested in AD system.

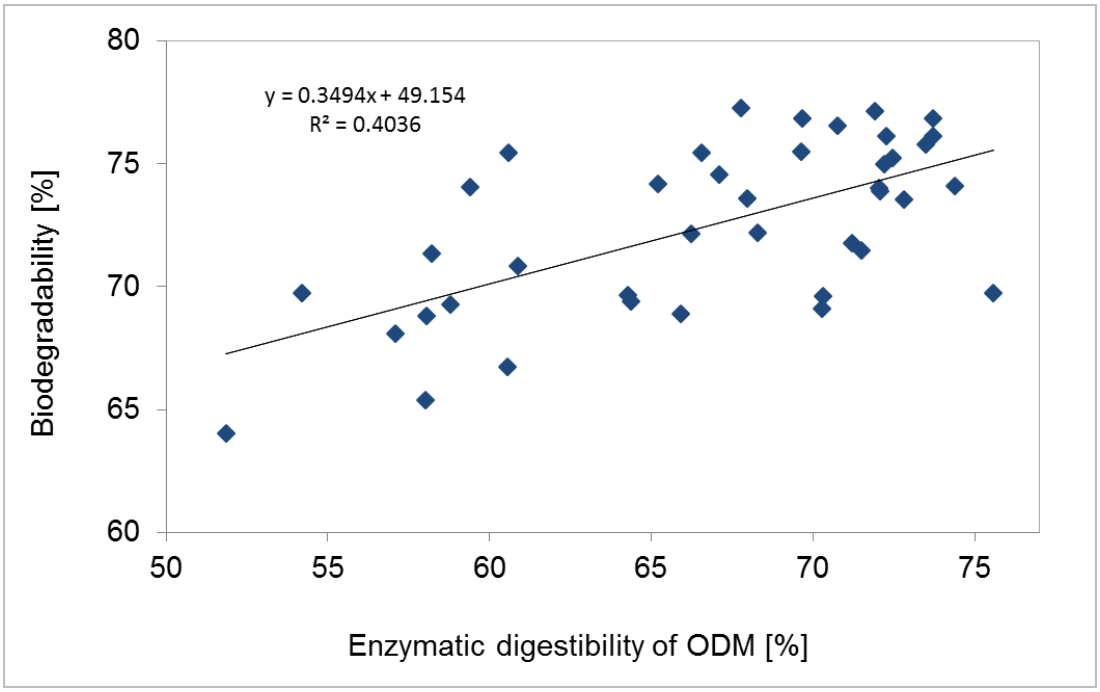

Figure 45: Relationship between enzymatic digestibility of ODM and the energy recovery efficiency in anaerobic bath system (HBT) for maize whole-crops.

The analysis of the cell-wall fractions shows that the mid-early and mid-late maturing genotypes showed lower NDF values. Genotype B [FAO 250] for instance showed NDF values varying from 36 to 45%. The NDF content went on decreasing with the increasing physiological growth. Its biodegradability increased only slightly throughout the physiological growth. The late-maturing genotypes had extremely high concentration of cell-wall content than the mid-early maturing genotypes. The late-maturing genotypes (FAO Index 600-700) showed, for instance, high NDF values throughout the growing period (43 to 54%). Despite the low starch content and high cell-wall contents (i.e. NDF, ADF values), the biodegradability was found to be high. Figures 46, 47 and 48 show the results for genotypes with FAO-Index 700, 600 and 250.

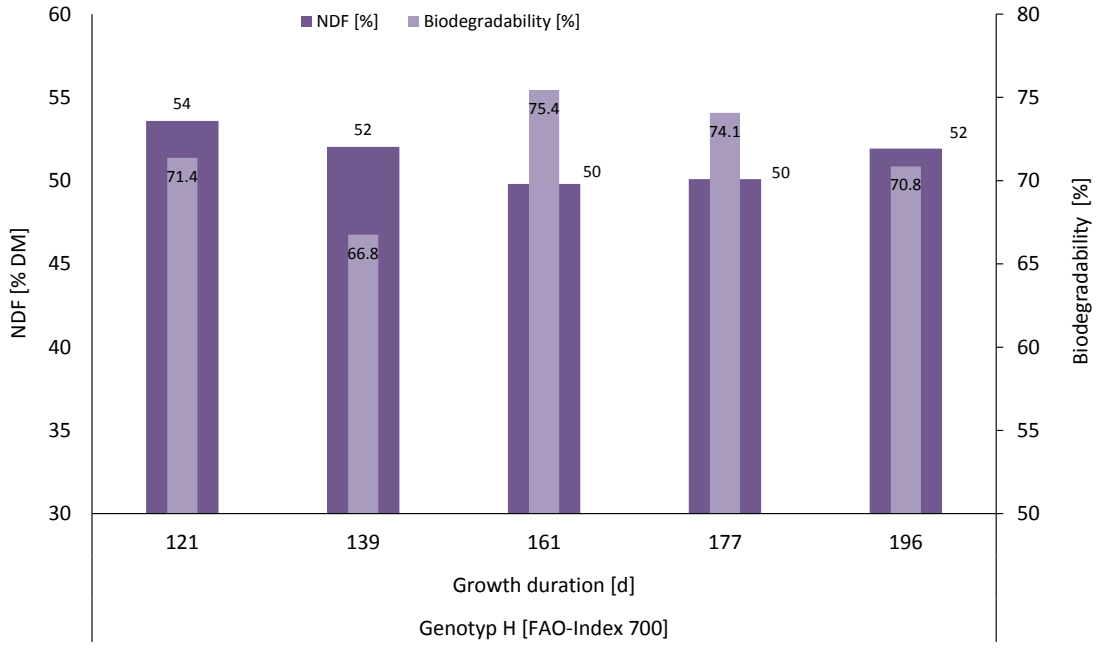

Figure 46: Comparison of absolute cell-wall content and the biodegradability for the late-maturing maize genotype (FAO-Index 700).

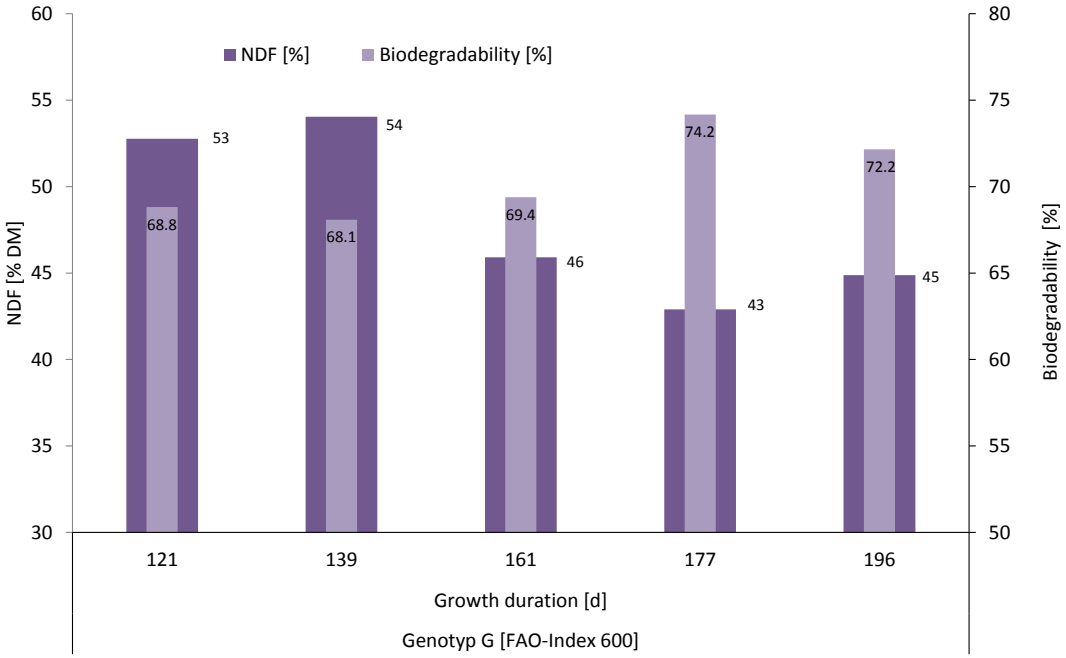

Figure 47: Comparison of absolute cell-wall content and the biodegradability for a late-maturing maize genotype (FAO-Index 600).

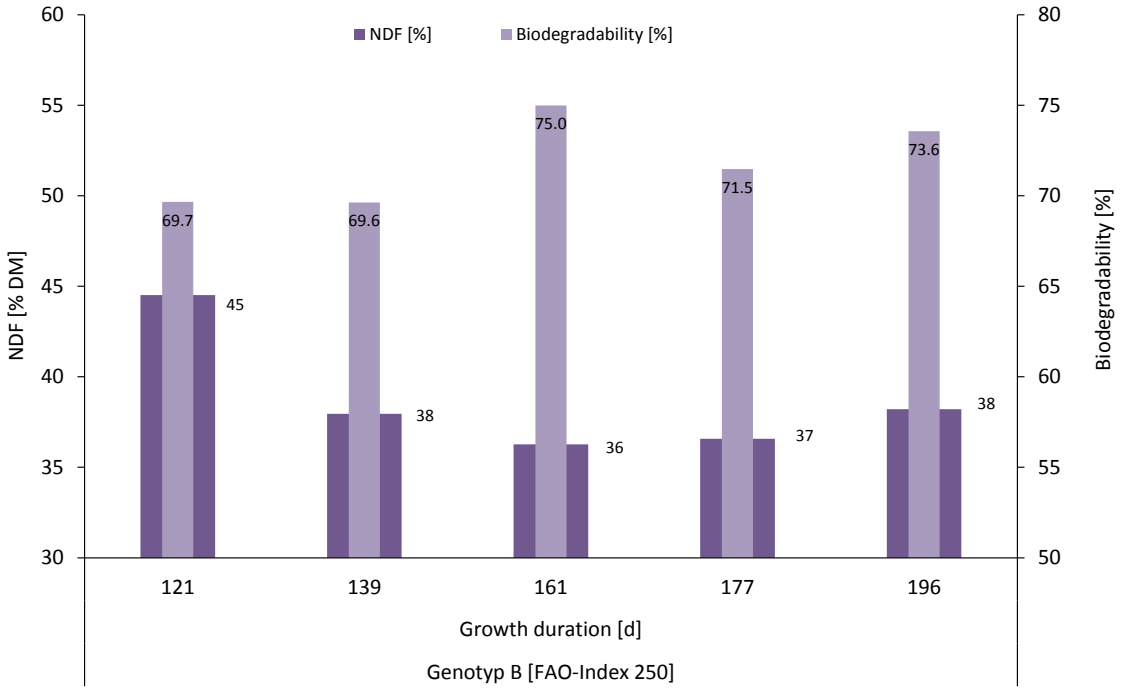

Figure 48: Comparison of absolute cell-wall content and the biodegradability for the mid-early maturing maize genotype (FAO-Index 250).

According to Daniel (1984) a 1% increase in crude fiber content (almost the same expression for ADF) results in 2% decrease of the ODM digestibility. This suggests a strong linear correlation between cell-wall fractions and digestibility as that found between cell-wall fractions and *in-vitro* enzymatic digestibility (Figures 49 and 50).

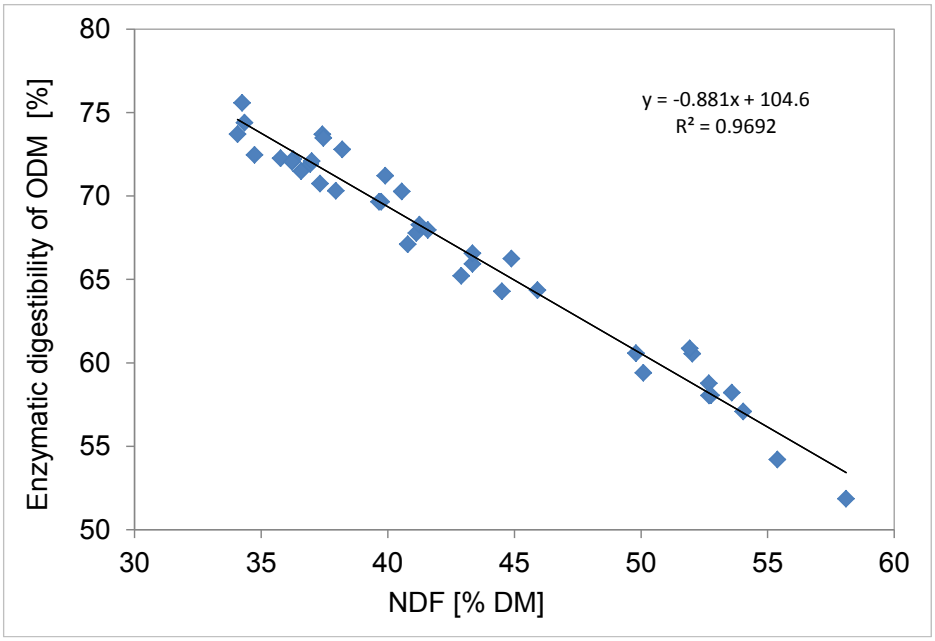

Figure 49: Relationship between the neutral detergent fiber (NDF) content and the enzymatic digestibility of ODM for various maize whole-crops.

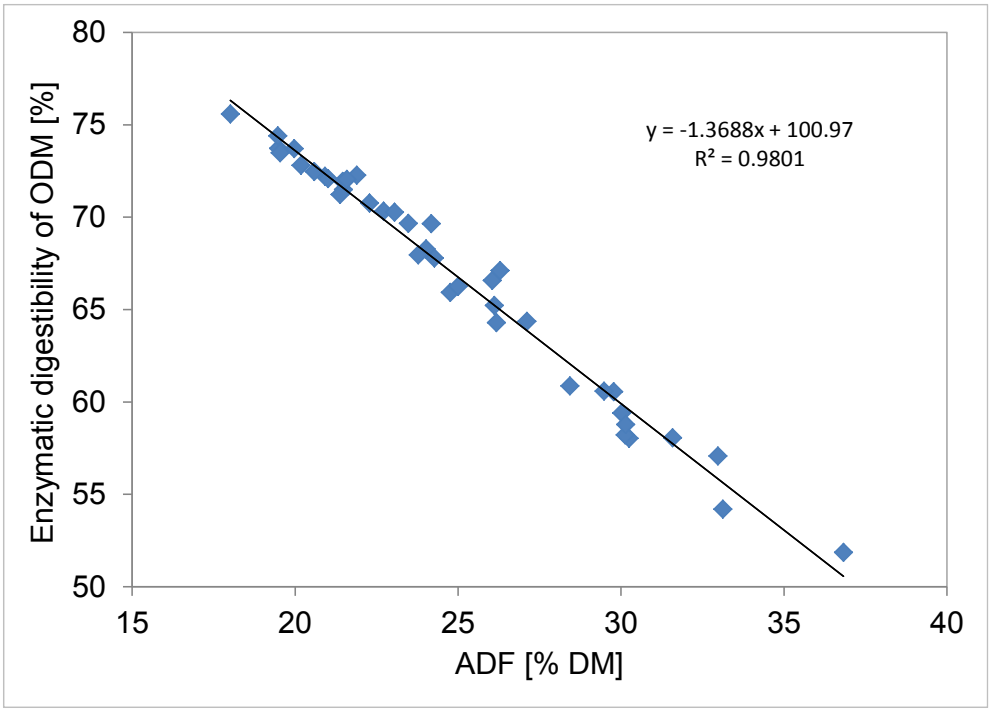

Figure 50: Relationship between the acid detergent fiber (ADF) content and the enzymatic digestibility of ODM for various maize whole-crops.

However, the biodegradability (or the bioconversion efficiency in AD) of maize whole-crop showed only moderate correlations. In fact, the reevaluation of the relationships showed that only 37% to 43% of the variations in the biodegradability could be explained by the absolute values of different cell-wall fractions of maize whole-crop (Figures 51, 52, 53).

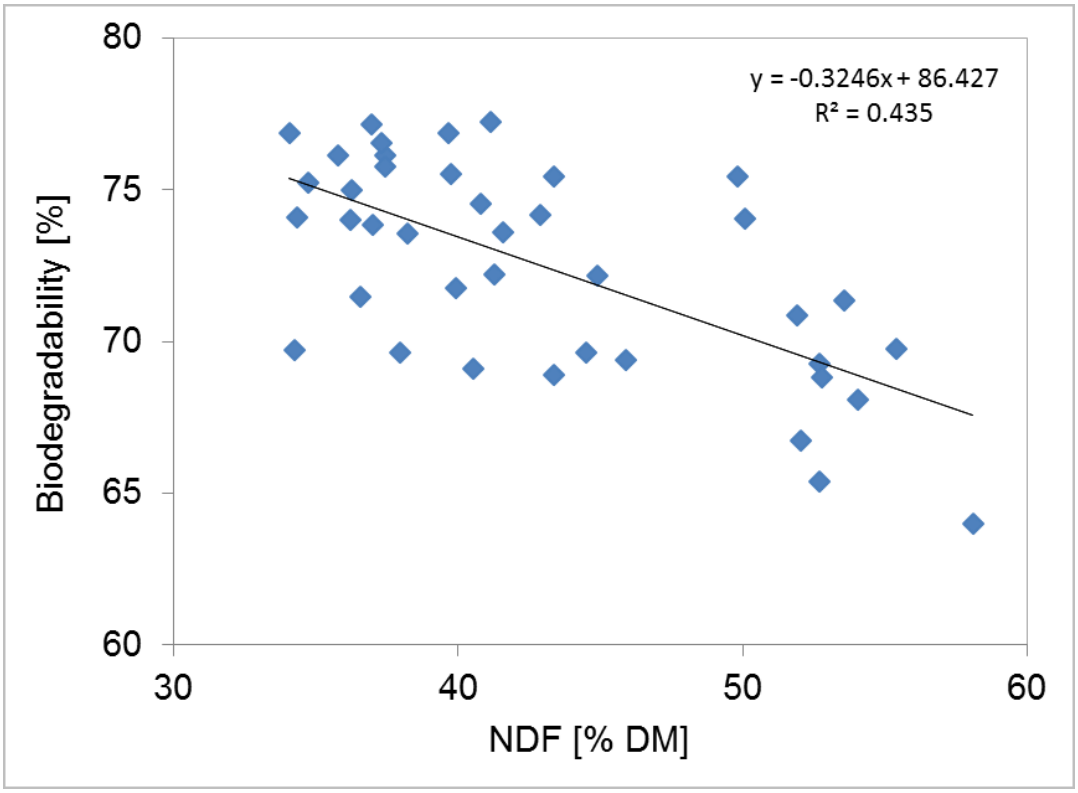

Figure 51: Relationship between the neutral detergent fiber (NDF) content and the energy recovery efficiency in anaerobic batch system (HBT) for various maize whole-crops.

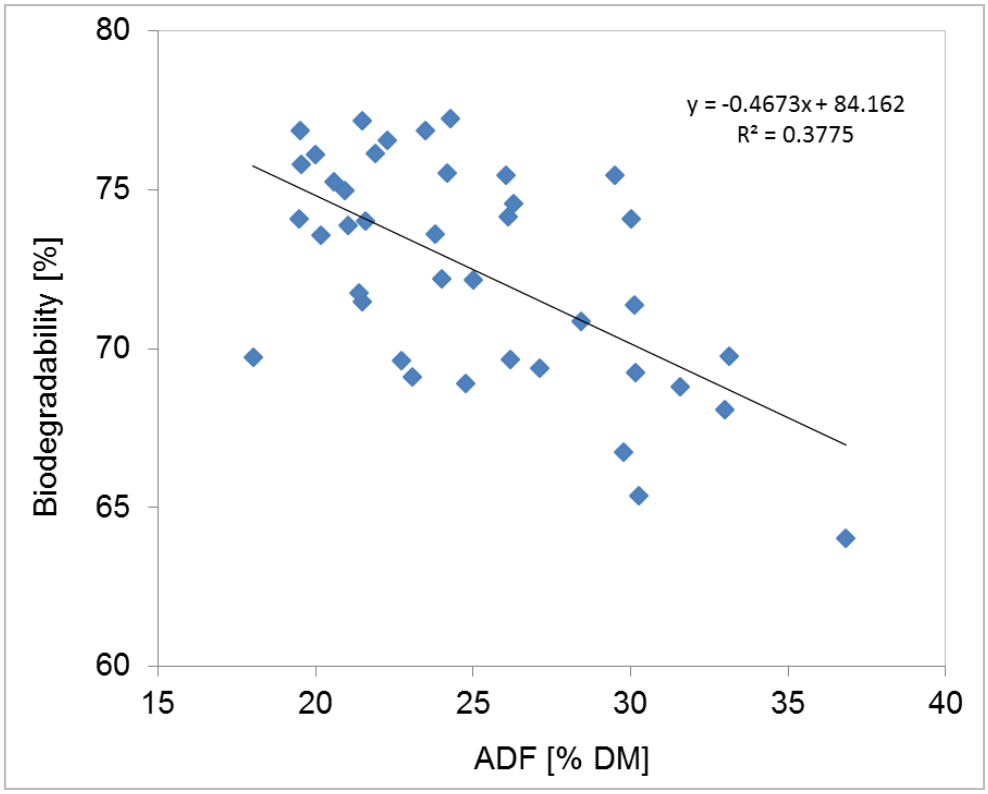

Figure 52: Relationship between acid detergent fiber (ADF) content and the energy recovery efficiency in anaerobic batch system (HBT) for various maize whole-crops.

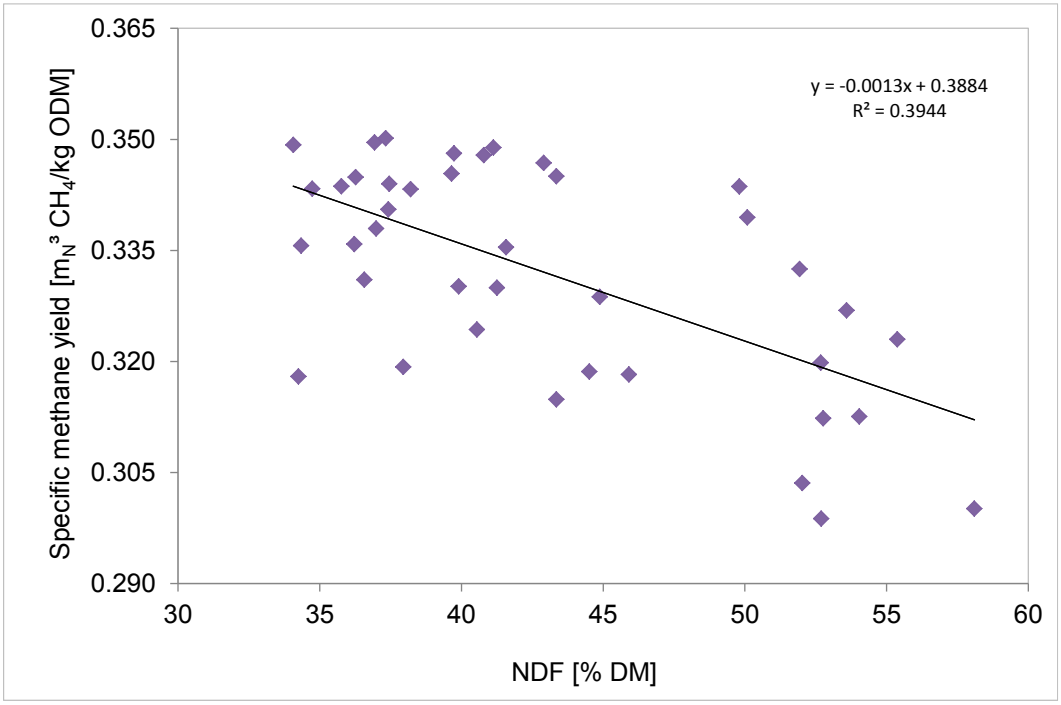

Figure 53: Relationship between neutral detergent fiber (NDF) content and the absolute specific methane yields generated in anaerobic bath system (HBT) for various maize whole-crops.

5.6 Experiment VI: Evaluation of the specific methane yield potential of various crops alternative to maize

As stated in the Section 4.7.6., the major objectives of this experiment were to examine the variation ranges of the specific methane yield potential of lipid and carbohydrates rich crops, and to evaluate the influence of the biochemical composition on the specific methane yield potential. In this section, therefore, these parameters were investigated using sunflower (*Helianthus annuus L.*), rape (*Brassica napus L.*), rye (*Secale cereal L.*), and sorghum (*Sorghum bicolor L.*).

5.6.1 Sunflower (*Helianthus annuus L.*)

In contrary to the results on maize whole-crop (Experiment IV) sunflower whole-crop showed a wider range of variation in specific methane yield potential. Figure 54 shows that the specific methane yield potential of sunflower whole-crop varied from 0.249 to 0.343 m_N^3 CH_4/kg ODM, with a median of 0.274 m_N^3 CH_4/kg ODM. The range of 94 l_N CH_4/kg ODM represents 27.3% difference.

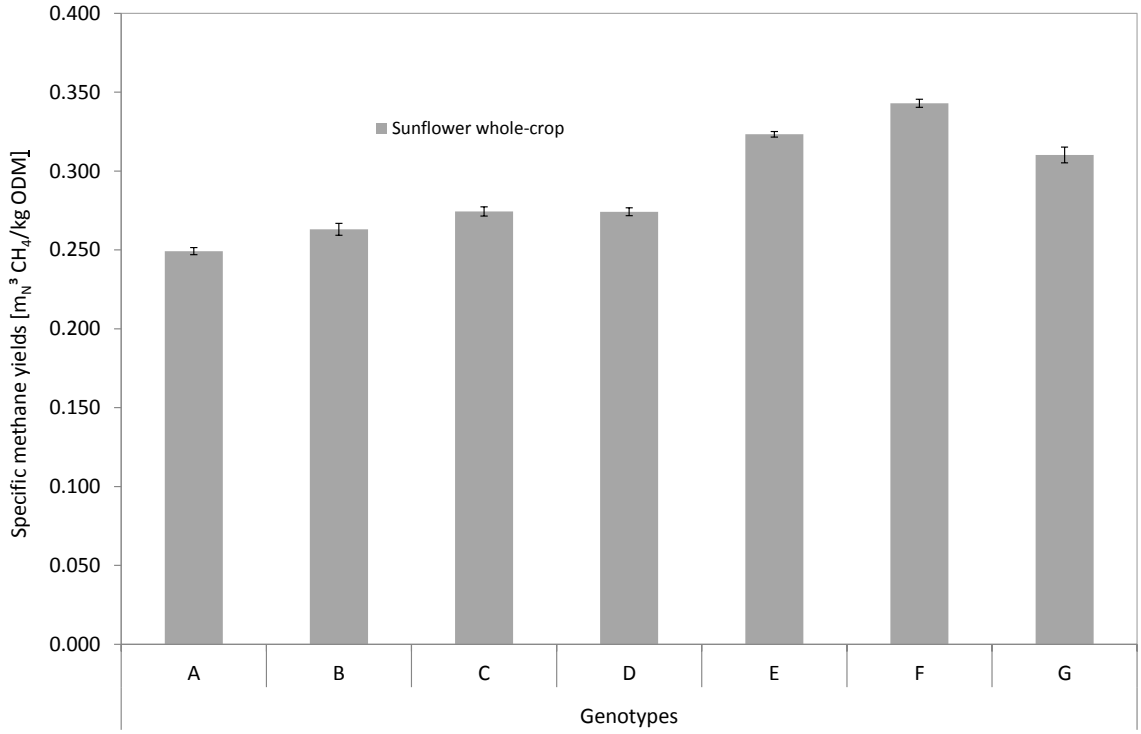

Figure 54: Specific methane yield potential of different sunflower genotypes (whole-crop).

Figure 55 shows the specific methane yield potential of both leaf and stem fractions. The specific methane yield potential of the stem fraction varied between 0.241 and 0.284 m_N^3 CH_4/kg ODM for the oil cultivar and between 0.201 and 0.244 m_N^3 CH_4/kg ODM for the experimental biomass hybrid. They were generally lower than that of the leaf fraction. The specific methane yield potential of the leaf fraction varied between 0.268 and 0.321 m_N^3 CH_4/kg ODM for the oil cultivar and between 0.235 and 0.287 m_N^3 CH_4/kg ODM for the experimental biomass hybrid.

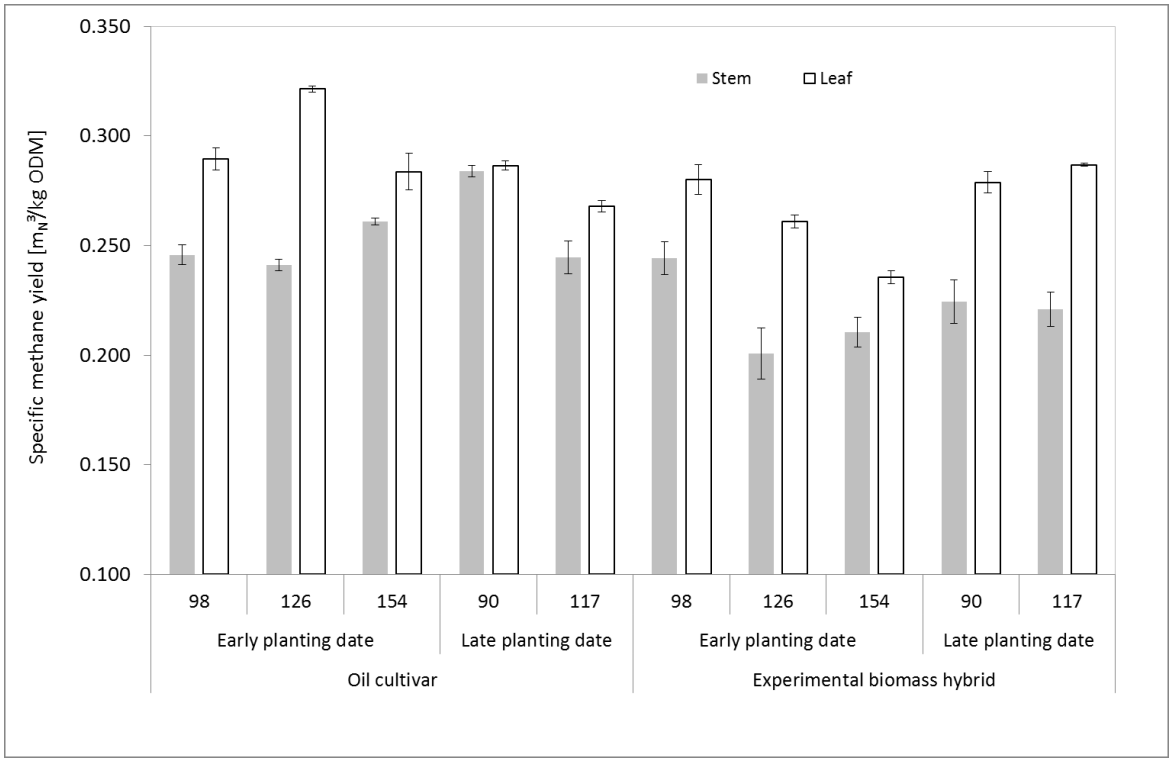

Figure 55: Specific methane yield potential of sunflower stem and leaf fractions after various growing periods.

The specific methane yield potential of the crown fraction varied between 0.367 and 0.455 m_N^3 CH_4/kg ODM for the oil cultivar and between 0.294 and 0.388 m_N^3 CH_4/kg ODM for the experimental biomass hybrid. The specific methane yield potential of the crown fraction was in general higher than that of the other plant fractions.

The cell-wall content of the stem fraction was extremely high varying from 56.7% to 74.1% across the cultivars and planting/harvesting occasions. The lignocellulosic fraction (ADF) represents the preponderant share of the cell-wall content (47.6 to 60.3%). Figures 56 and 57 show the NDF and ADF values. The share of both NDF and ADF were also relatively high in the crown fraction as shown in Figure 58 and Figure 59.

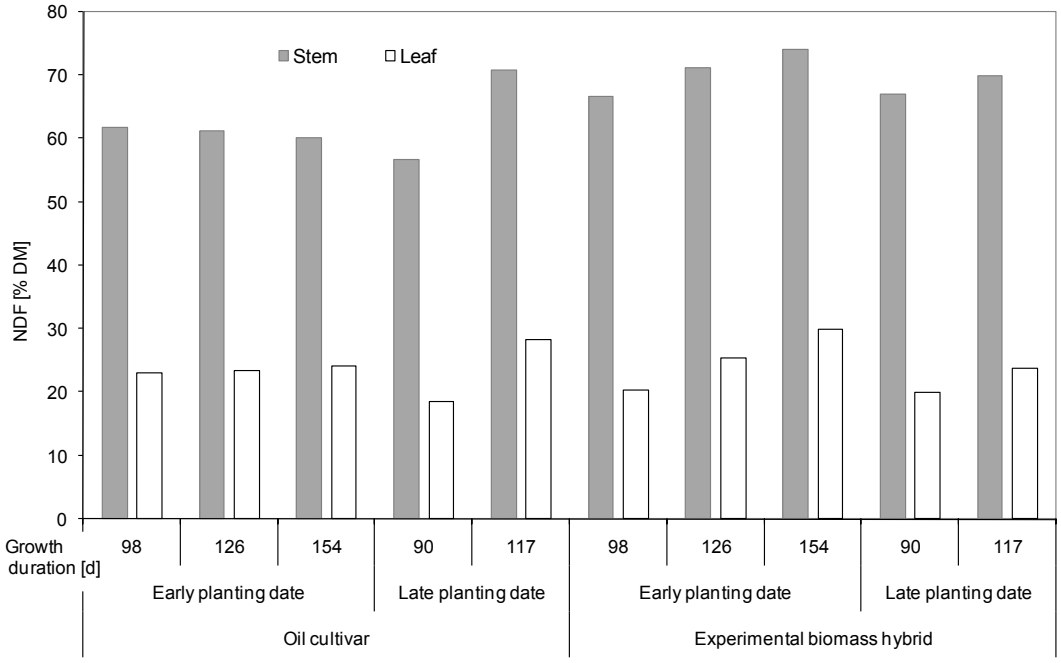

Figure 56: Cell-wall neutral detergent fiber (NDF) content of sunflower stem and leaf after various growing periods.

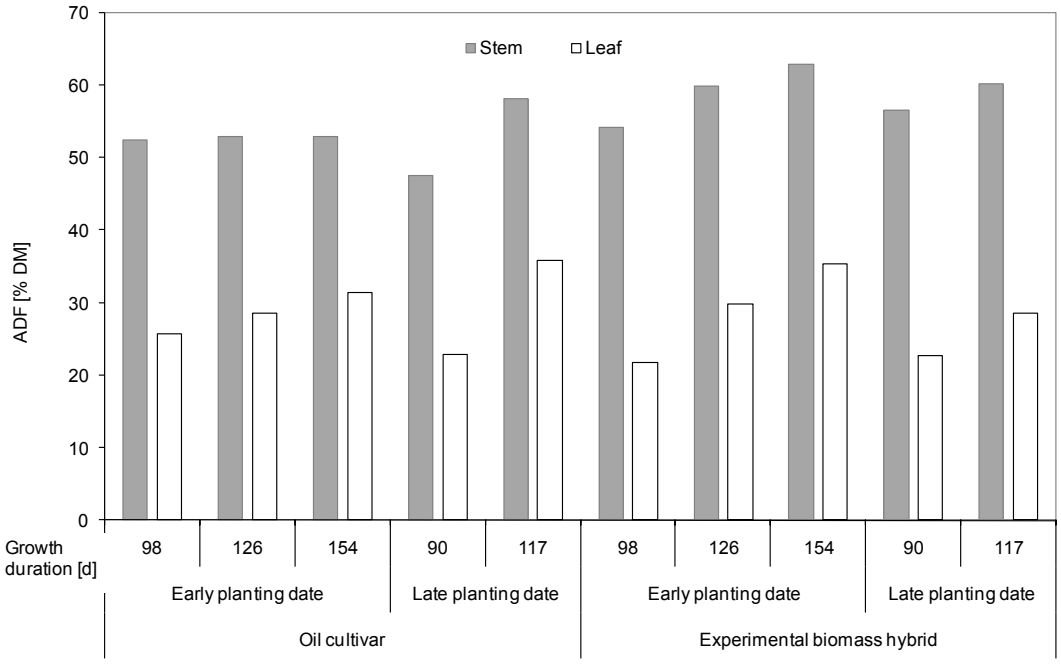

Figure 57: Lignocellulosic (ADF) content of sunflower stem and leaf after various growing periods.

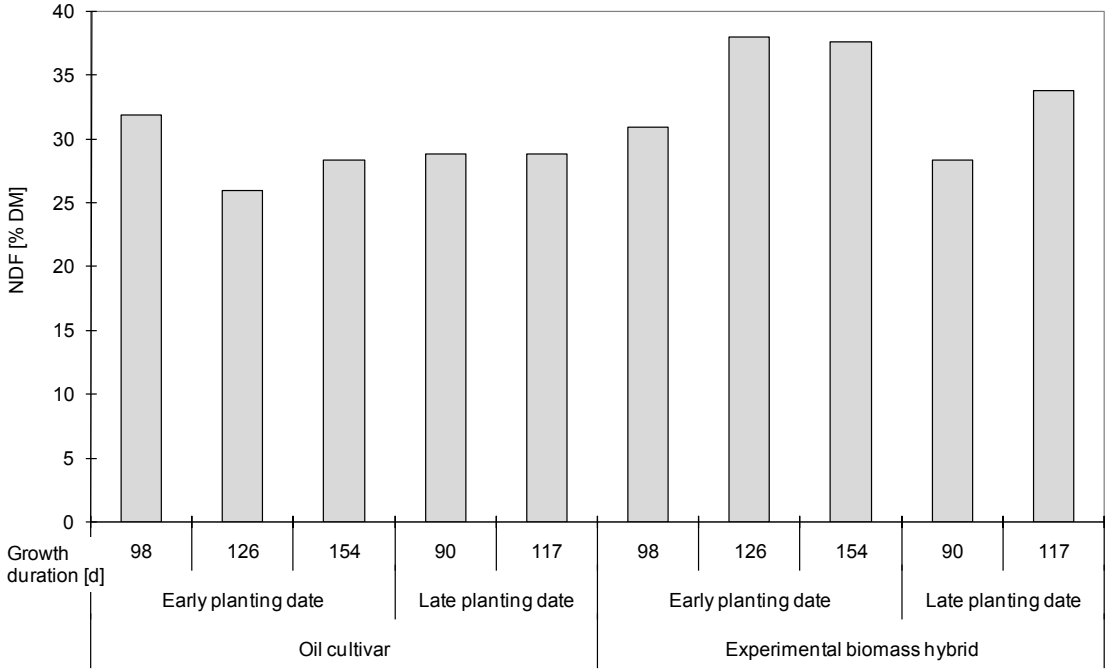

Figure 58: Neutral detergent fiber (NDF) content of sunflower crown after various growing periods.

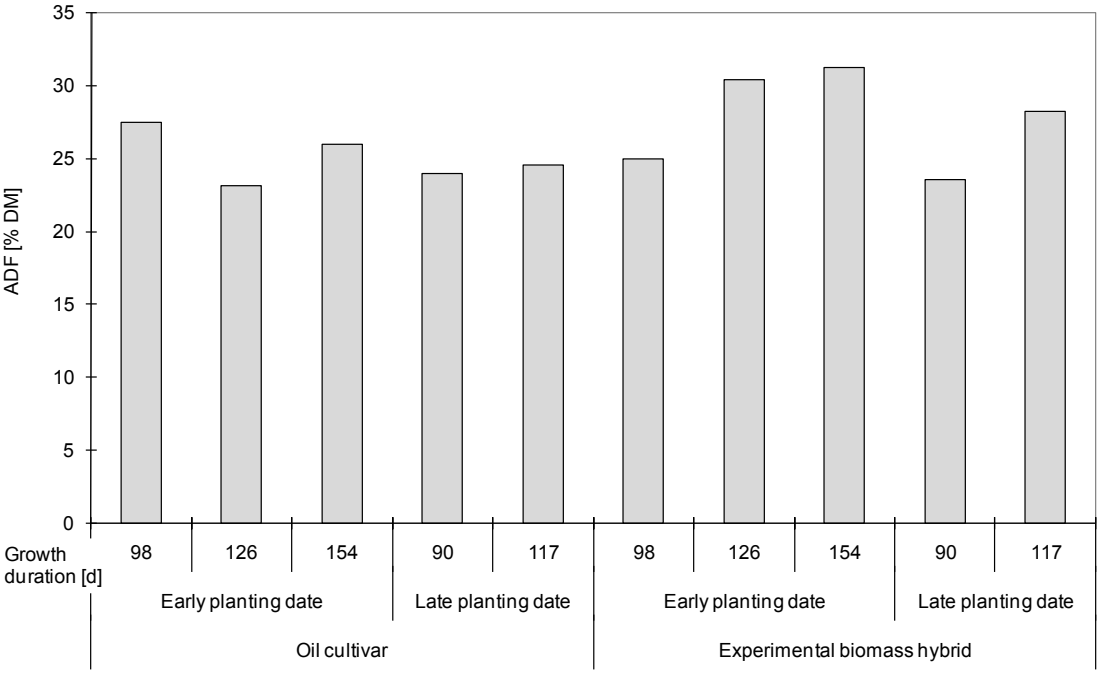

Figure 59: Acid detergent fiber (ADF) content of sunflower crown after various growing periods.

The chemical analysis showed also that both the stem and leaf fractions contained a considerable amount of lipid (Figure 60 and Figure 61). The lipid content in the stem fraction varied between 0.9% and 3.6% across cultivars. In the leaf fraction, the lipid content varied between 4.2% and 10.6% at the first planting date and from 4.9% to 6.2% at the second planting date for the oil cultivar. The leaf fraction of the experimental biomass hybrid showed a lower lipid content, varying between 3.4% to 6.7% at the first planting date and from 3.5% to 6.3% at the second planting date.

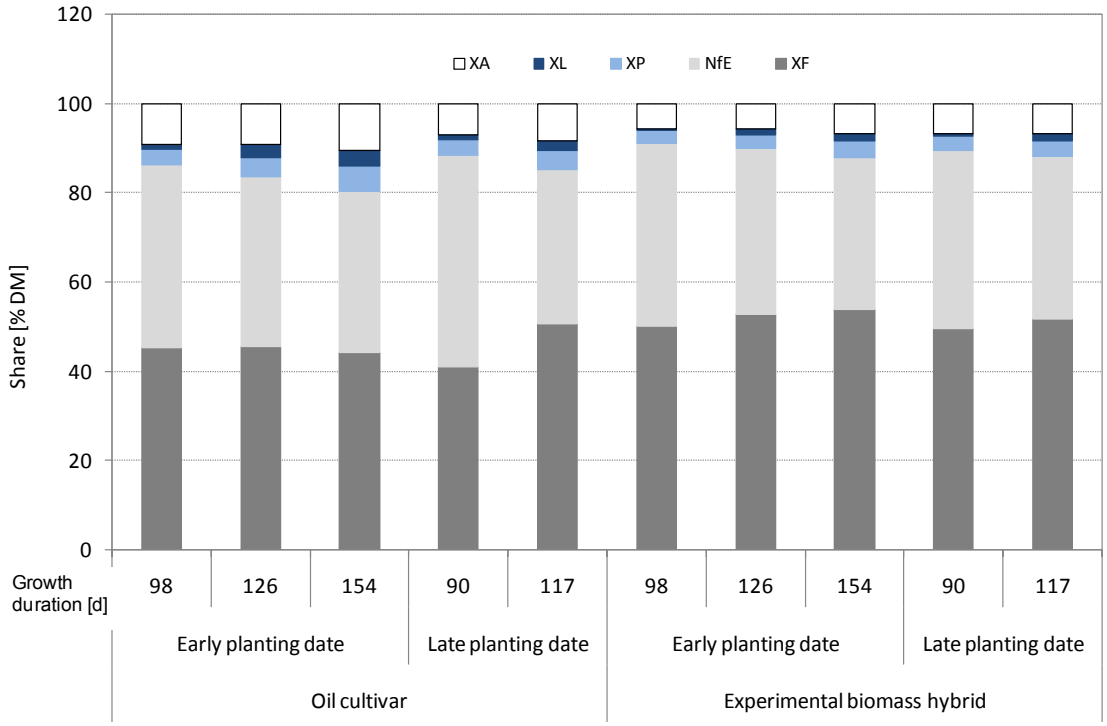

Figure 60: Biochemical composition of sunflower stems after various growth periods.

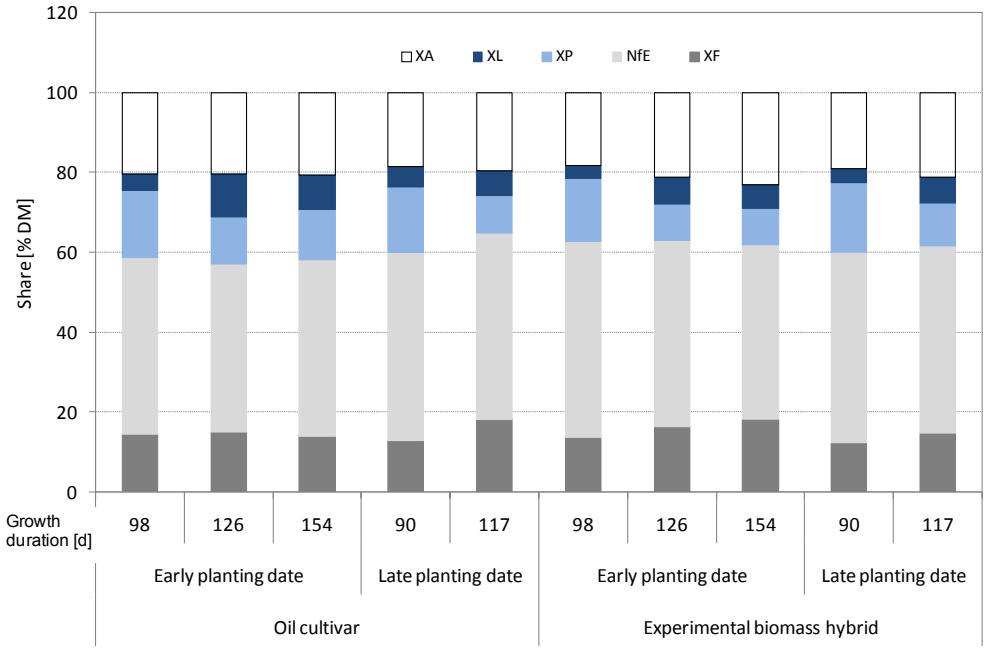

Figure 61: Biochemical composition of sunflower leaf after various growth periods.

The lipid content of the crown fraction varied between 15.3% and 28.8% in the oil cultivar at the first planting date and between 13.4% and 26.3% at the second planting date. In the experimental biomass hybrid, it varied to a greater extent, from 3.5% to 23.4% at the first planting and from 3.0% to 20.3% at the second (Figure 62).

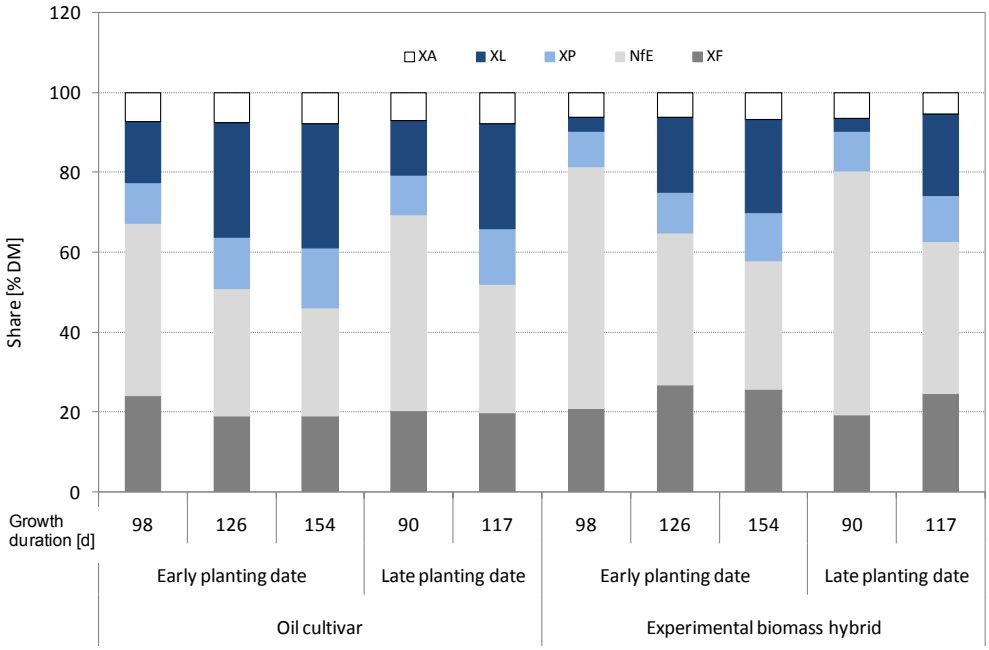

Figure 62: Biochemical composition of sunflower crown after various growing durations.

Neither the lipid nor the protein, content of the stem and leaf fractions showed any correlation with the specific methane yield potential. In contrast, both the cell-wall content (NDF) and the lignocellulosic fraction (ADF) were found to be negatively correlated with the specific methane yield potential (Figure 63 and Figure 64). The cell-wall content explained to over 70% the variability in specific methane yield potential in these crop fractions.

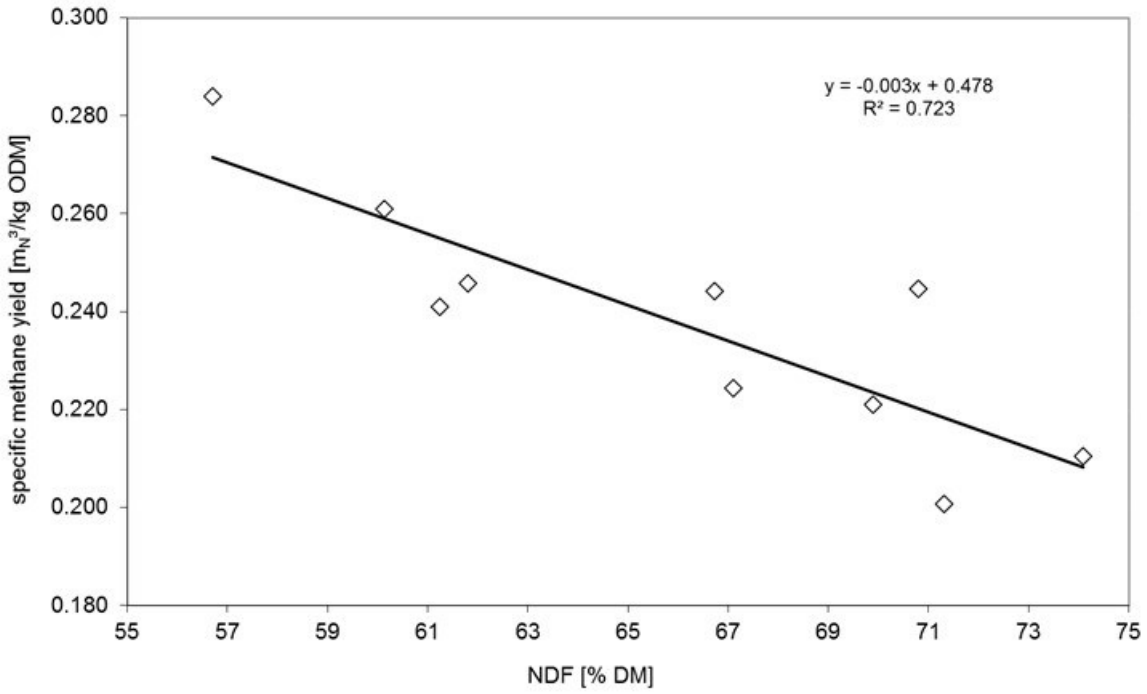

Figure 63: Relationship between neutral detergent fiber (NDF) content and specific methane yield of sunflower stem.

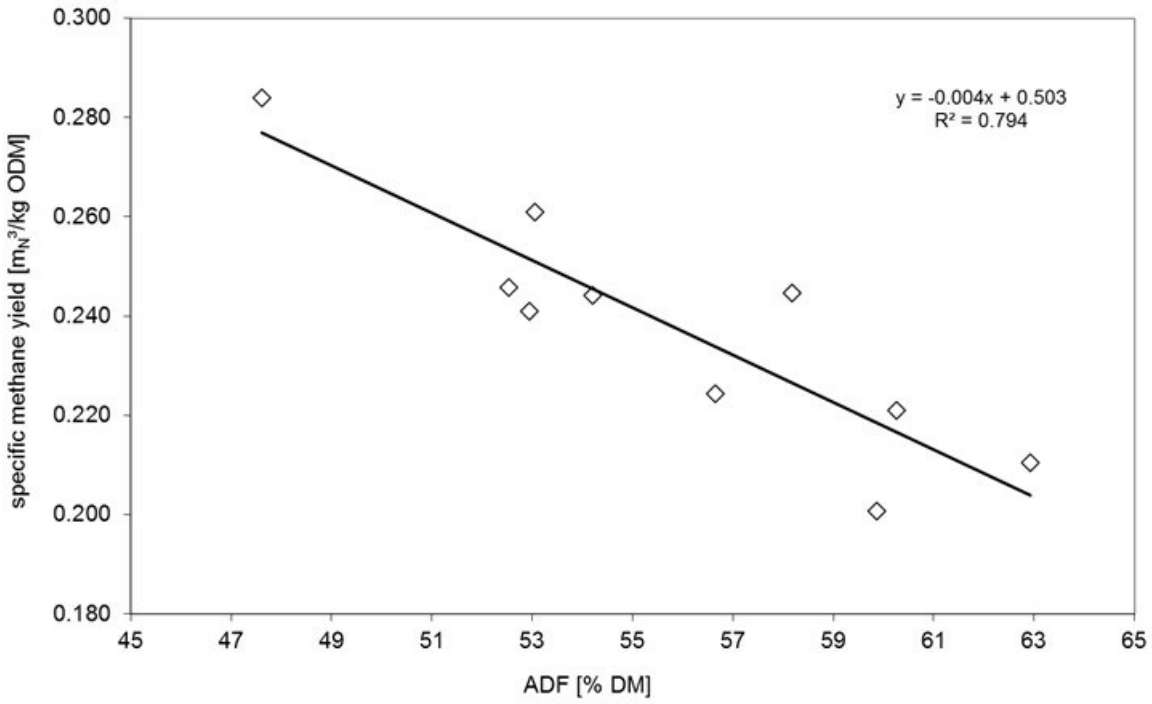

Figure 64: Relationship between acid detergent fiber (ADF) content and specific methane yield of sunflower stem.

The lipid content explained over 90% the variability in the specific methane yield potential of the crown fraction (Figure 65). At a lipid concentration of 3.0% to 3.5% in the crown, the specific methane yield potentials were found to be very low, varying between 0.294 and 0.299 m_N^3 CH_4/kg ODM (Figure 66). This level of methane yield potential corresponds more or less to that of hay or maize stover.

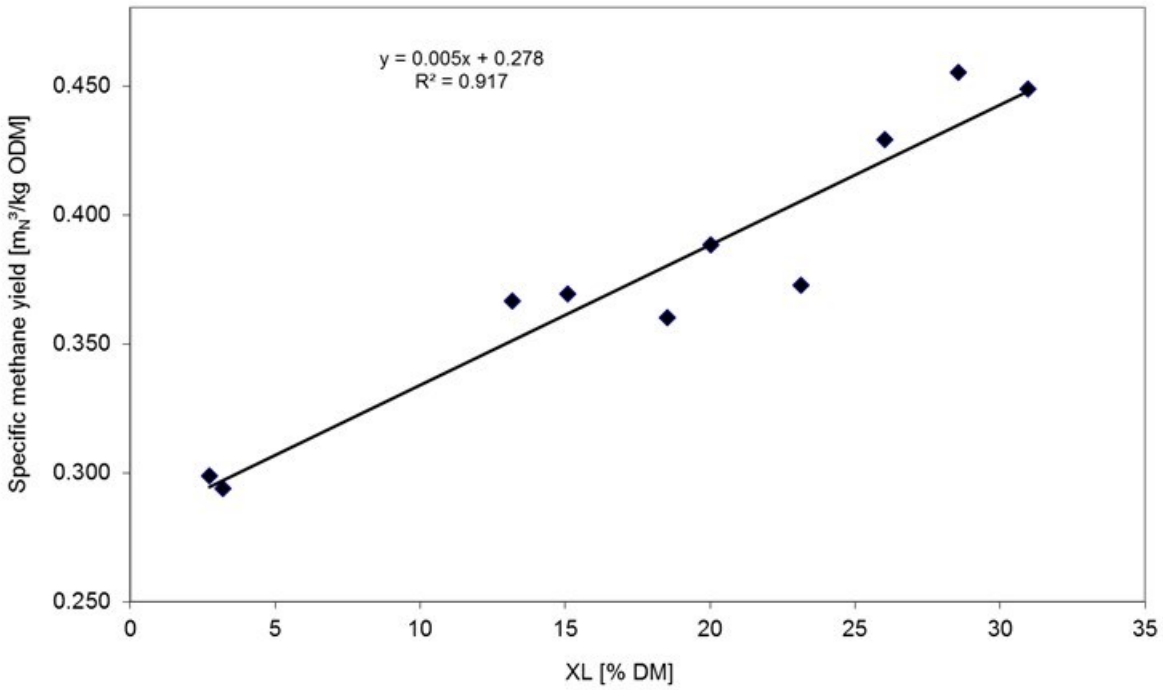

Figure 65: Relationship between lipid content and specific methane yield of sunflower crown.

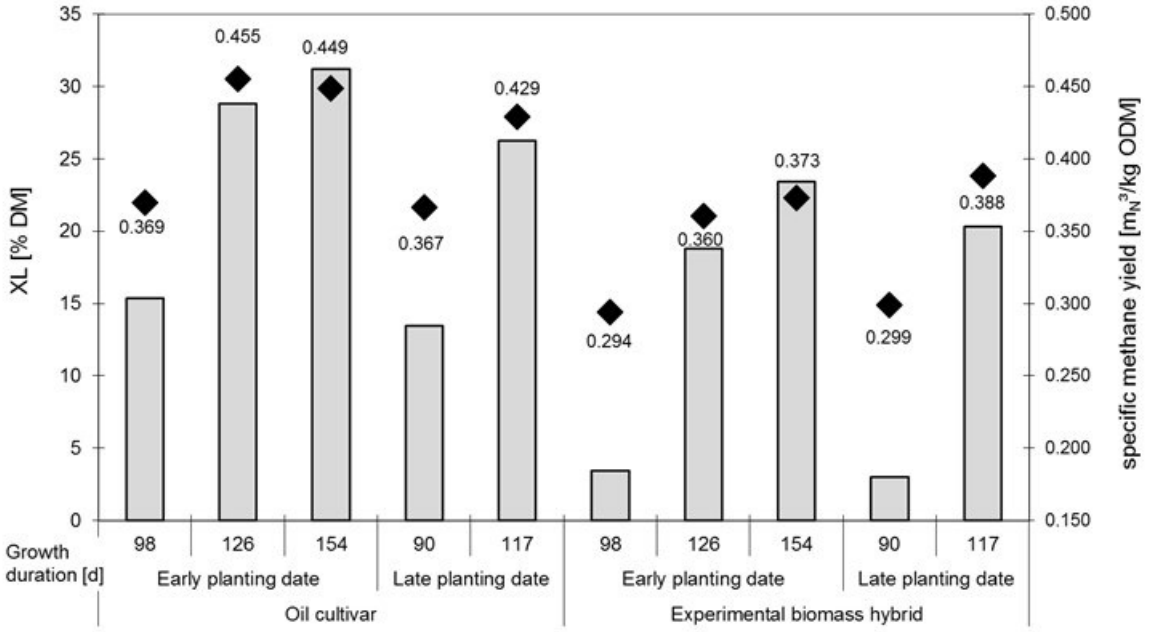

Figure 66: Lipid content of the crown and the corresponding specific methane yields after various growing durations.

The specific hectare-methane yield was strongly affected by the portion of the crown fraction in the total DM of the crop. The contribution of the crown fraction to the total dry matter yield continued to increase with increased growth duration, and thus contributed to the increase in hectare-methane yield (Figure 67 and Figure 68). For the oil cultivar, the contribution of the crown to the hectare-methane yield increased from 47.3% to 59.9% at the early planting date and from 43.3% to 60.1% at the late planting date. For the experimental biomass hybrid, it increased from 28.9% to 65% at the early planting date and from 24.6% to 54.4% at the late planting date. The specific hectare-methane yield of the oil cultivar varied between 3642 and 5593 m_N^3 CH_4/ha at the early planting date and between 3375 and 3020 m_N^3 CH_4/ha at the second date. The experimental biomass hybrid showed specific hectare-methane yield varying between 4237 and 3859 m_N^3 CH_4/ha at the first planting date, and from 3720 and 7021 m_N^3 CH_4/ha at the second.

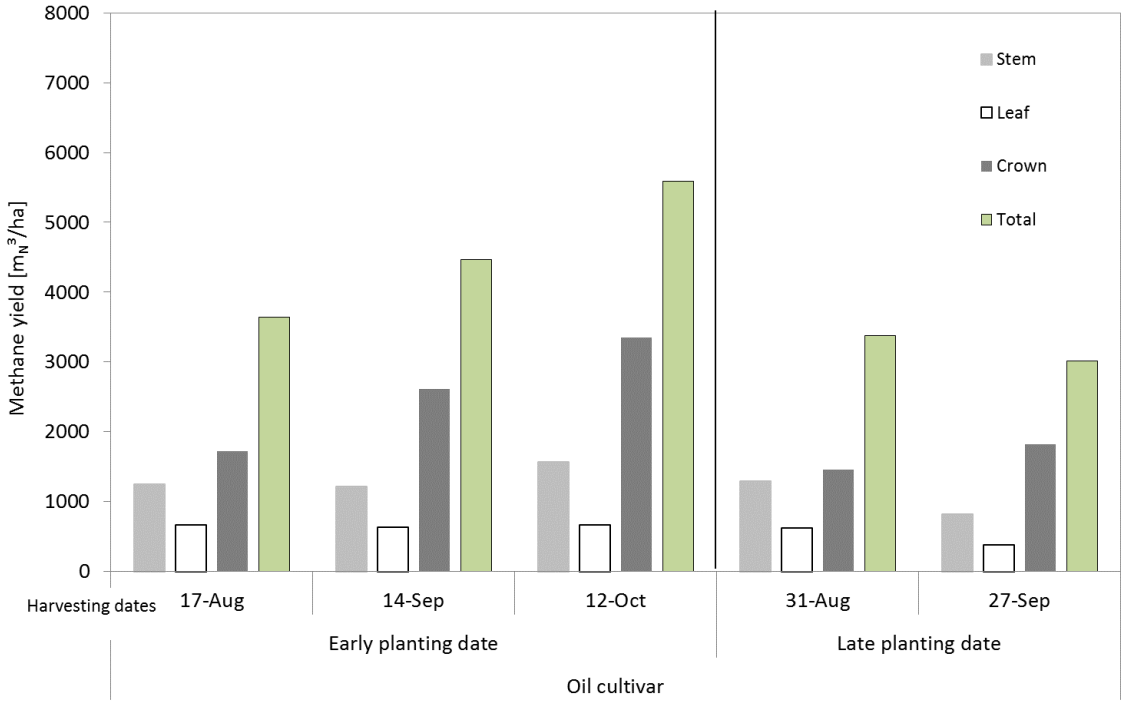

Figure 67: Hectare-methane yield of sunflower crop fractions at different harvesting dates (oil cultivar).

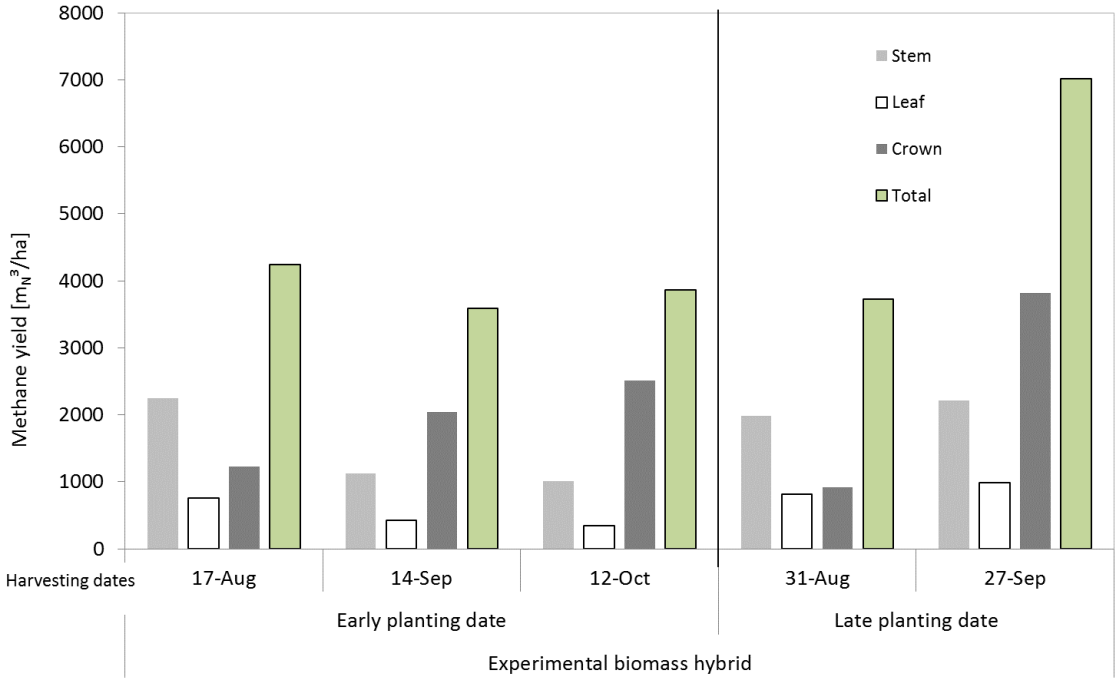

Figure 68: Hectare-methane yield of sunflower crop fractions at different harvesting dates (experimental biomass hybrid).

5.6.2 Rape (*Brassica napus L.*)

The results of the first set of crop materials (**Set I**) are presented in Tables 25, 26 and 27. Table 25 shows that the specific methane yield potential varied between 0.275 and 0.307 m_N^3 CH_4/kg ODM and did not increase with the proceeding maturity. At the pod elongation stage, the specific methane yield was the lowest. This was more probably due to both the decrease in stalk biodegradability and the high proportion of the green fraction. In general, the older the stalk the lower its biodegradability. Although the crop fractions were not weighted, the ratio seeds to straw (stalk - empty pod fraction) is more or less of 1 to 3 (own observation). This high share of the straw fraction might have impacted negatively the specific methane yield potential. Furthermore a possible seed loss due to mechanical harvest using a forage maize chopper cannot be totally excluded although the harvest took place one week before the presumed full maturity.

Table 25: Specific methane yield potential of rape at different growth stages.

Growth stage	Harvest date	Methane yield	CH_4-conc.	ha-methane yield
		[m_N^3/kg ODM]	[%]	[m_N^3/kg ODM]
Full flowering	9-May	0.307	52	1759
Pods elongation	1-Jun	0.275	52	2410
Full maturity	5-Jul	0.302	60	3166

The DM content was also low and increased from 10.5% at the full flowering stage to 29.4% at the full maturity stage (Table 26). At both the full flowering and pods elongations stages the crop had not yet reached the dry matter content suitable for a bulky ensiling process.

Table 26: Dry matter and organic dry matter yields of rape at different growth stages.

Growth stage	Harvesting date	DM [%]	ODM [%]	FM Yield [t/ha]	DM Yield [t/ha]
Full flowering	9-May	10.5	86.8	62.6 [± 4.3]	6.6 [± 4.5]
Pods elongation	1-Jun	14.6	90.4	66.5 [± 4.3]	9.7 [± 4.5]
Full maturity	5-Jul	29.4	92.0	38.8 [± 9.1]	11.4 [± 9.0]

The protein and water soluble carbohydrates contents decreased substantially with the proceeding maturity (Table 27). At the same time, the lipid content increased. At the full maturity the crop showed a high lipid content of 12.4%. Despite this increase in lipid content the specific methane yield potential remained low as shown above in Table 25.

Table 27: Chemical composition of rape at different growth stages.

Growth stage	Harvest date	XP	XL	Starch	WSC
		[% DM]			
Full flowering	9-May	19.3	3.4	1.9	11.5
Pods elongation	1-Jun	12.3	2.4	3.1	13.8
Full maturity	5-Jul	9.4	12.4	2.2	2.5

The specific hectare-methane yield was dictated by the dry matter yield per hectare. From the full flowering to the full maturity stage, the specific hectare-methane yield

grew of about 45%. At the full maturity the total methane yield reached 3166 m_N^3 CH_4/ha.

The results of the second set of crop materials (**Set II**) are presented in Table 28 and in Figures 69 to 71. Table 28 shows that all the genotypes used in Set II reached a higher dry matter content at the harvest (DM > 30%) than the genotypes used in Set I (Table 26). Figure 69 shows that the specific methane yield potential of rape whole-crop varied between 0.339 m_N^3 CH_4/kg ODM and 0.405 m_N^3 CH_4/kg ODM (16.2 percentage units' difference).

Table 28: Dry matter content of different rape cultivars.

Cultivar	Oil-content *	Glucosinolates content *	Erucic acid content *	DM		ODM	
				[%]	STD [%]	[%]	STD [%]
Aurum	medium to high	low	very low	34.2	4.7	92.5	0.2
Oase	high to very high	low	very low	30.8	3.6	92.5	0.2
Elektra	medium to high	low	very low	32.5	5.4	92.4	0.4
Trabant	-	low	very low to low	31.4	1.1	92.0	0.5
Maplus	medium to high	low	very low	31.9	3.3	92.7	0.1

*classification according to the federal variety authority (Bundessortenamt 2011)

According to the classification of the Federal variety authority (Bundessortenamt), the cultivar Oase has a high oil content and hence was expected to yield the highest methane yield potential. The results show, however, that Aurum and Maplus had the highest specific methane yield potential, followed by Oase and Trabant. Elektra showed the lowest specific methane yield potential among the genotypes. These results are corroborated by Schumacher et al. (2007).

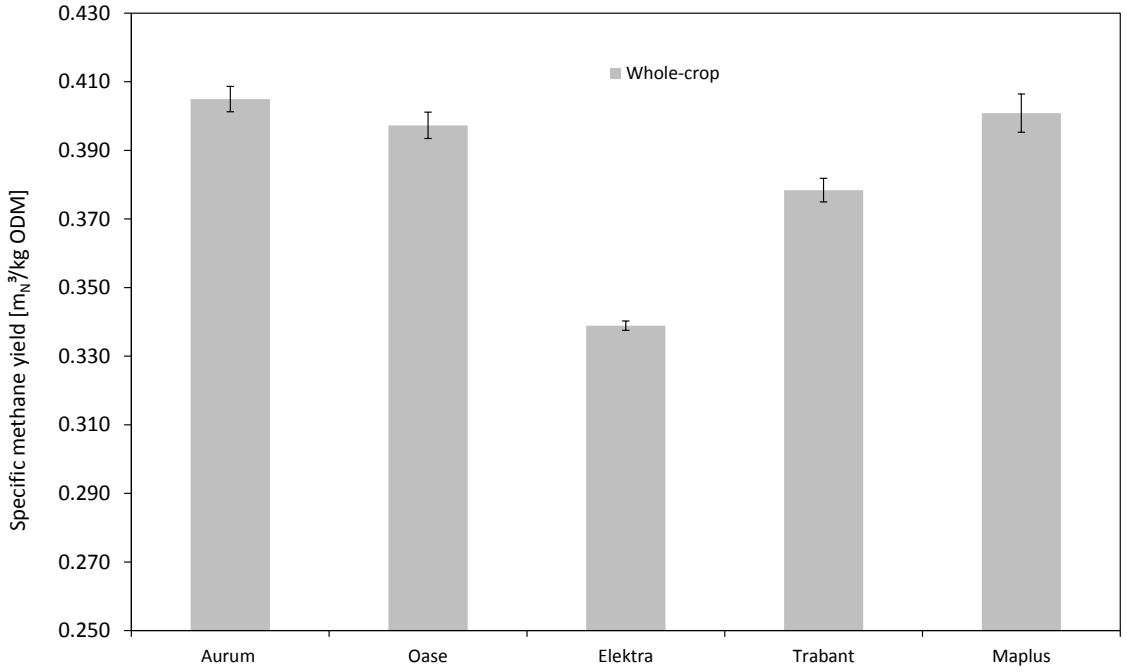

Figure 69: Specific methane yield potential of various rape cultivars (whole-crop).

The crop fractions showed also differences in specific methane yield potential. The specific methane yield potential of the green fraction (stalk-empty pods) were in the range of that of sunflower stalks varying between 0.246 and 0.288 m_N^3 CH_4/kg ODM (Figure 70). The specific methane yield potential of the seed fraction varied between 0.547 and 0.598 m_N^3 CH_4/kg ODM (Figure 71). This represents an 8.5% difference. The relative high erucic acid content in the hybrid Maplus (as classified by the Federal variety authority “Bundessortenamt”) was not found to affect negatively the specific methane yield potential. On the contrary the seeds of Maplus showed the highest specific methane yield potential (Figure 71).

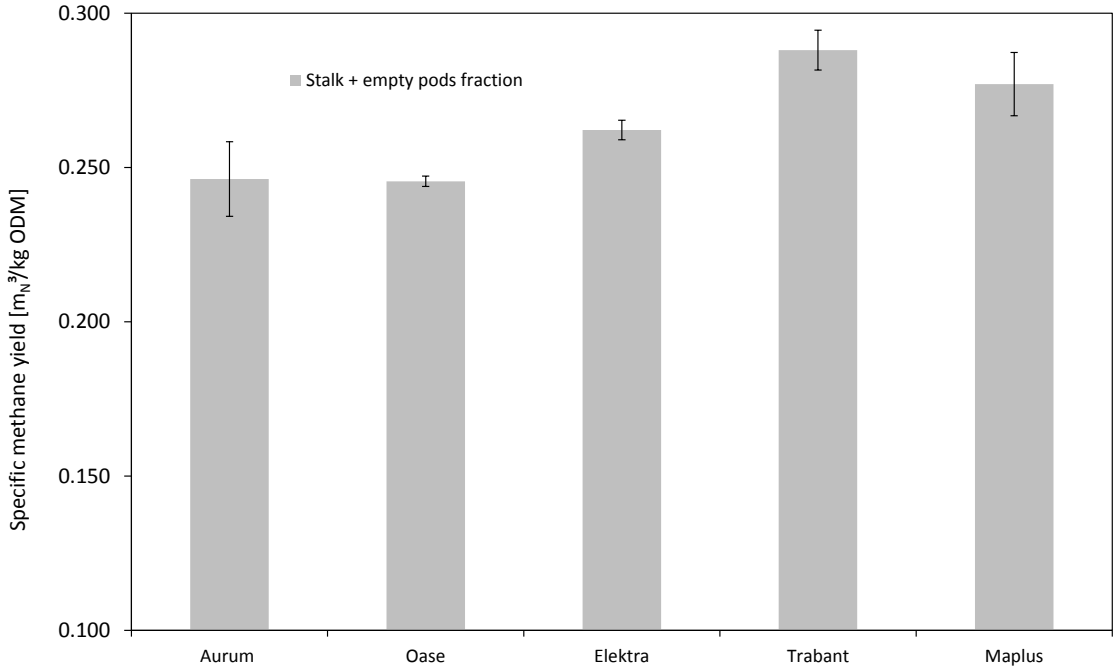

Figure 70: Specific methane yield potential of the green fraction (stalk-empty pods) of various rape cultivars.

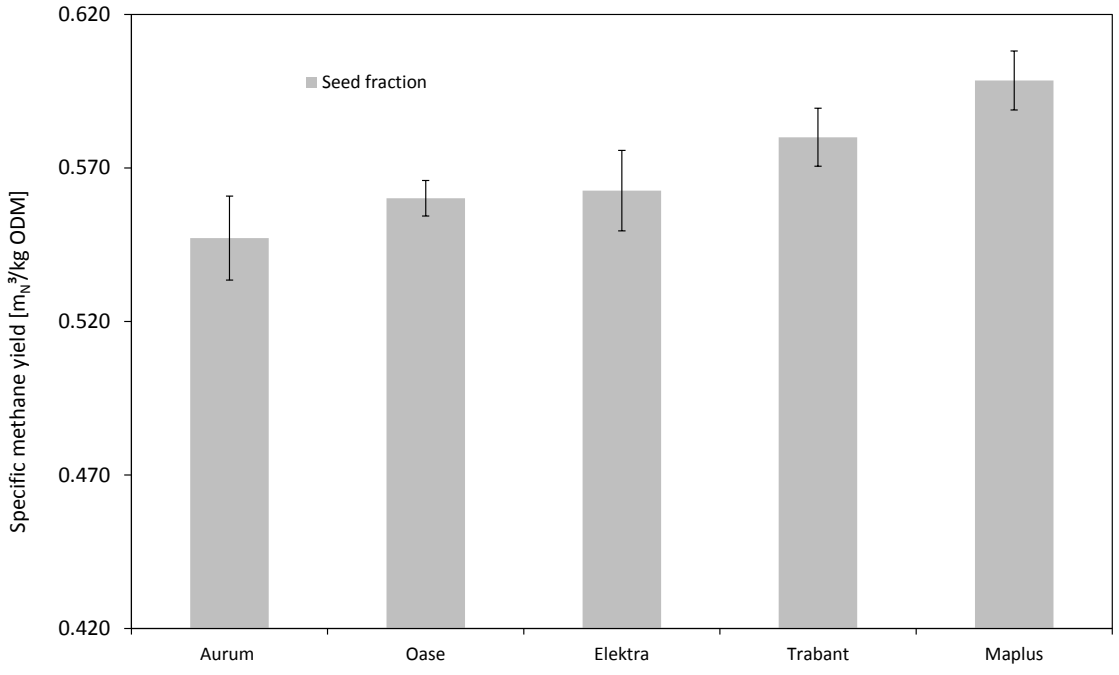

Figure 71: Specific methane yield potential of the rape seed fraction for various cultivars.

5.6.3 Rye (*Secale cereal L.*)

At the beginning of heading (BBCH-Scale EC 51), the total DM content of the whole-crop samples analyzed showed the following means and standard deviations: 24.12% (± 1.35) in Hohenheim and 20.69% (± 1.23) in Wohlde. Following mean values were measured in Hohenheim for NDF, ADF, and ADL respectively: 57.55% (± 1.4), 34.11% (± 1.7), and 3.40% (± 0.3). These values were higher than those measured in Wohlde: 48.55% (± 1.6), 27.57% (± 1.5), and 2.28% (± 0.4). The grain hybrids did not exceed the other genotypes in specific methane yield potential (Figure 72). In fact, the grain hybrids (Picasso and Visello), the population genotype (Recrut) and the forage rye genotype (Vitallo) showed following mean specific methane yield potentials across locations: 0.333 (± 1.6%), 0.332 (± 0.9%), and 0.323 (± 1.7%) m_N^3 CH_4/kg ODM, respectively. Neither the cell-wall fractions, nor the lipid contents were correlated to the specific methane yield potential of rye whole-crop.

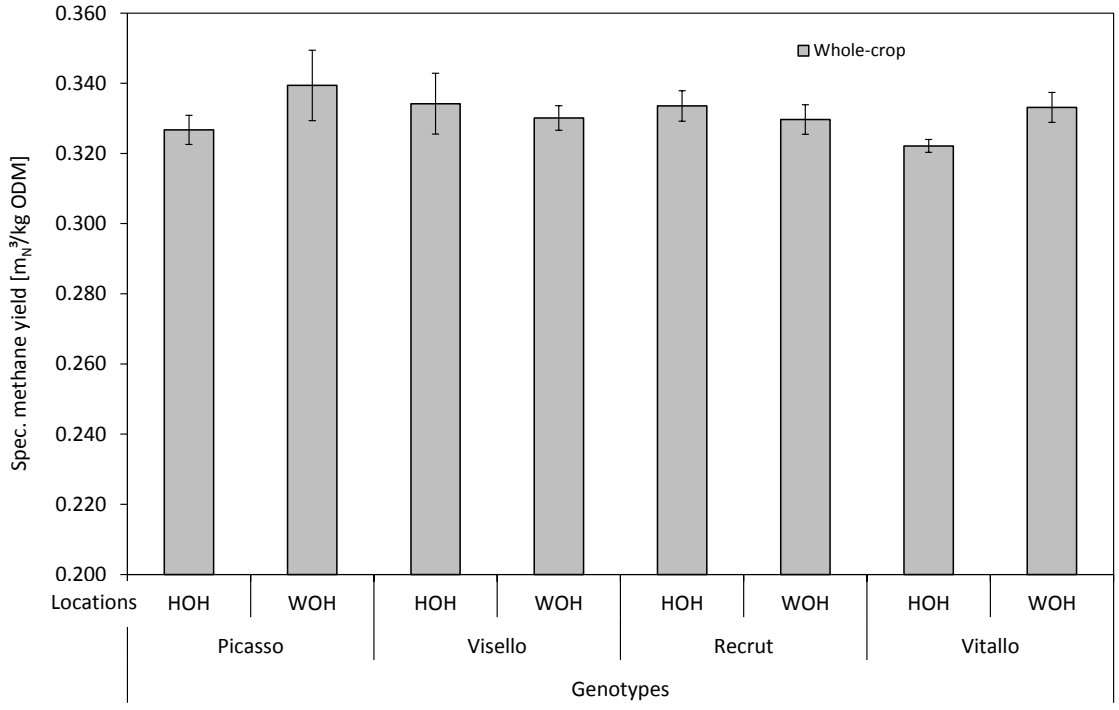

Figure 72: Specific methane yield potential of various Rye genotypes (whole-crop) at the beginning of heading (EC 51) in two different locations

At the EC51 the average specific hectare-methane yield for all variants lied by 2668 m_N^3 CH_4/ha (Figure 73). At this growth stage, the forage genotype (Vitallo) showed higher hectare-methane yield (3129 m_N^3 CH_4/ha) than its counterparts. Its specific methane yield potential was also consistent across growth stages.

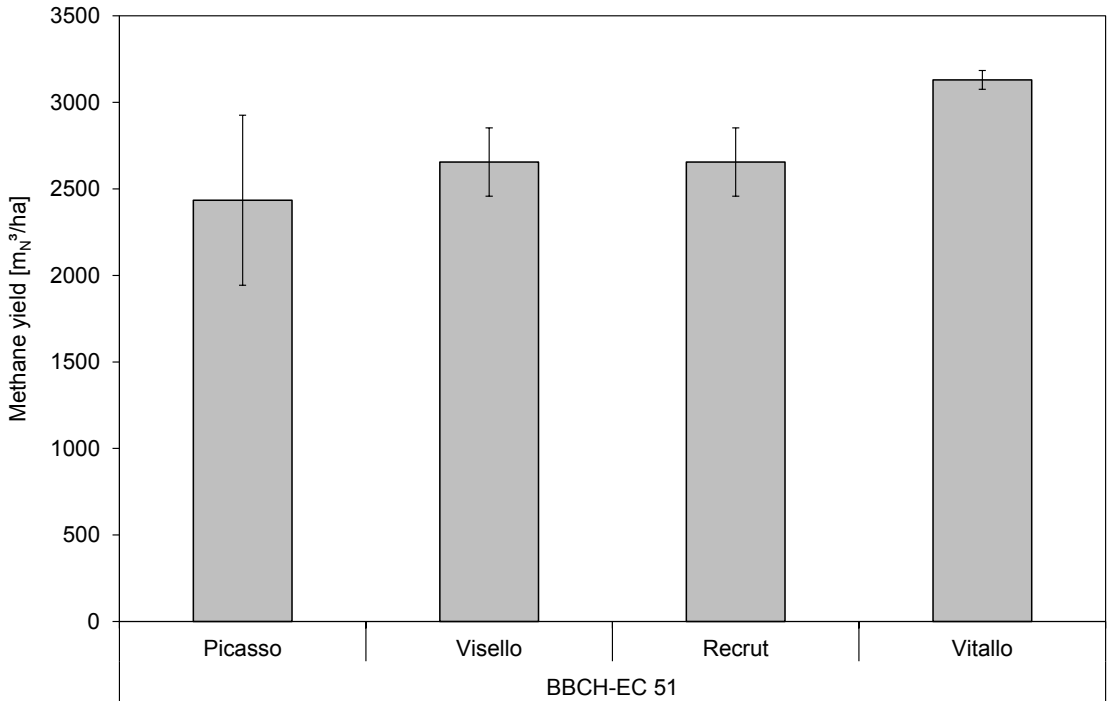

Figure 73: Mean hectare-methane yields of different rye genotypes at the early milk growth stage BBCH-EC51 (two locations).

At both the early milk (EC 73) and the late milk-early dough (EC 77/78) stages, the specific methane yield potential of the crop fractions varied in a very narrow range despite the broad variation range for the biochemical traits (Table 29), and the biochemical composition showed mitigated influence on the specific methane yield potential. Neither the cell-wall content (NDF, ADF and ADL), nor the lipid content were correlated with the specific methane yield potential of the ear fraction. NDF, ADF, and ADL were moderately negatively correlated to the specific methane yield potential of the stalk-leaf fraction with coefficients of determination (R^2) of 0.28, 0.29, and 0.43, respectively. In contrast, ADL was highly negatively correlated with the specific methane yield potential of the stubble fraction (R^2 = 0.66), as shown in (Figure 74). Table A-5 and Table A-6 (see in appendix) give details for the

biochemical composition and the specific methane yield potential of each crop fractions.

Table 29: Biochemical composition, methane yields, DM content and hectare yields of rye whole-crop and crop fractions (mean values).

Growth stage/ Crop fraction	Biochemical composition							Specific CH_4-Yield	DM content and ha-Yields		
	NDF	ADF	ADL	XF	XL	XP	XA	CH_4-Yield	DM	DM-Yield	CH_4-Yield
	(%)							(m_N^3/kg ODM)	(%)	(t/ha)	(m_N^3 CH_4/ha)
EC51 Whole crop	53.1 [10]	30.8 [13]	2.9 [26]	27.7 [13]	2.3 [15]	10.7 [27]	5.9 [4]	0.331 [2]	22.4 [11]	8.6 [15]	2,668 [14]
EC73											
Ear	41.8 [13]	19.7 [20]	3.0 [5]	16.6 [23]	1.9 [7]	9.4 [4]	3.7 [14]	0.314 [3]	39.3 [6]	4.7 [30]	1,392 [28]
Stalk-leaf	58.7 [3]	37.5 [4]	4.2 [14]	33.6 [5]	1.7 [9]	5.6 [13]	4.4 [10]	0.319 [3]	36.6 [10]	8.5 [12]	2,586 [10]
Stubble	59.5 [5]	38.9 [6]	4.8 [13]	34.5 [6]	0.9 [13]	2.4 [27]	4.2 [7]	0.312 [3]	34.8 [9]	1.9 [7]	578 [9]
EC 77/83											
Ear	34.3 [10]	14.0 [13]	2.4 [9]	10.9 [15]	1.9 [6]	8.3 [6]	3.2 [11]	0.321 [2]	42.5 [9]	6.7 [29]	2,060 [28]
Stalk-leaf	63.8 [3]	41.3 [3]	4.9 [14]	36.3 [5]	1.6 [10]	5.1 [19]	4.6 [10]	0.310 [3]	32.9 [11]	7.6 [14]	2,222 [14]
Stubble	64.7 [2]	43.2 [4]	5.7 [14]	37.3 [5]	0.9 [16]	2.3 [26]	4.9 [9]	0.305 [5]	32.1 [16]	1.8 [29]	517 [29]

[ ] = % relative standard deviation

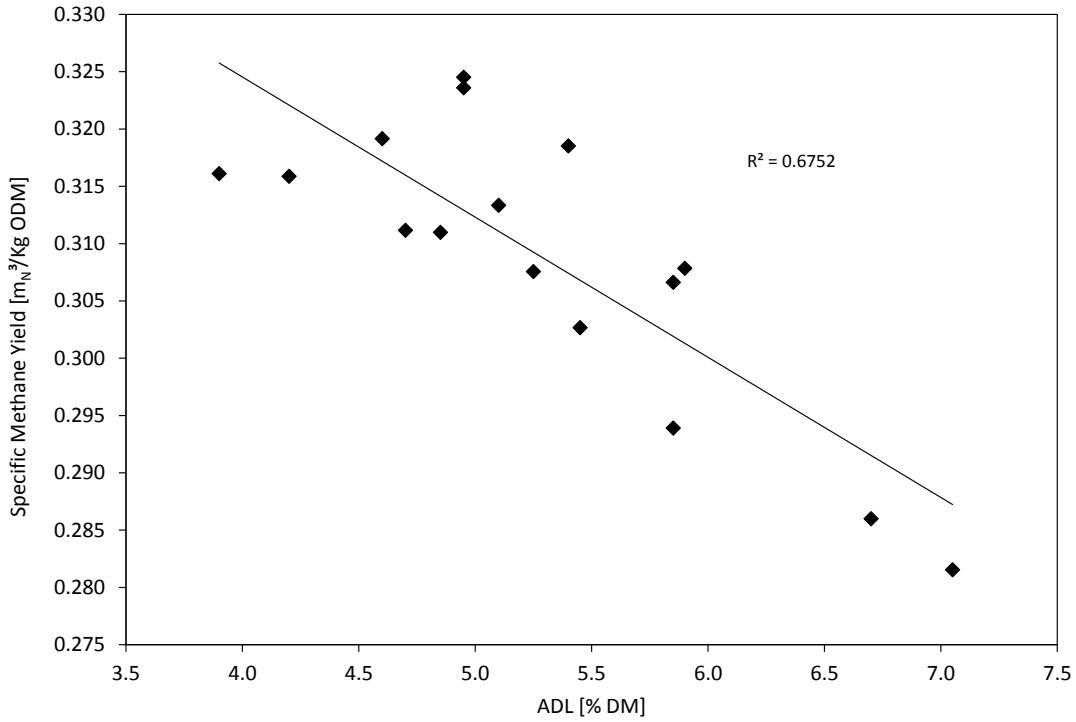

Figure 74: Relationship between the acid detergent lignin and the specific methane yield potential of rye stubbles (mean values of 4 genotypes and 2 locations).

5.6.4 Sorghum (*Sorghum bicolor L.*).

The results of the biochemical composition and specific methane yield potential of sorghum genotypes are clustered per growing period (117 and 133 days) and are presented in Tables 30 and 31. The mean specific methane yield potentials at the first harvest date were of 0.325 and 0.323 m_N^3 CH_4/kg ODM for *S. bicolor* and for the hybrid *S. bicolor* x *S. sudanense* (Sorghum) hybrid genotypes, respectively. At the second harvest, *S. bicolor* maintained its yield level (0.323 m_N^3 CH_4/kg ODM), while the specific methane yield potential of the Sorghum hybrid decreased to 0.302 m_N^3 CH_4/kg ODM.

At the first harvest date, the two groups of crop materials (*S. Bicolor* and Sorghum hybrid) showed substantial differences in sugar and starch contents. The mean sugar contents of 20.3% and 13.4% were measured for *S. bicolor* and the Sorghum hybrid genotypes, respectively. The high sugar content in *S. bicolor* was accompanied by a lower starch content. In fact, the mean starch content of the *S.*

bicolor genotypes (0.1%) was far much lower than that of the Sorghum hybrid genotypes (6%). This suggests that after a growing duration of 117 days the nutrient translocation process had progressed further for the hybrid genotype than it had for the *S. bicolor* genotype. The two groups showed also a 0.6% difference for lignin content. The mean values for protein and crude fiber contents were similar for both crop groups.

Table 30: Biochemical composition of different sorghum genotypes after 117 days growing duration and the corresponding specific methane yield potentials.

Genotype		Lipid	Sugar	Starch	Protein	Crude fiber	Lignin	Ash	CH_4-Yield
		[% DM]							[m_N^3/kg ODM]
Ronal1	S. bicolor	1.33	24.56	0.25	9.52	25.95	3.6	5.27	0.321
Supersile 18		1.37	19.51	0	9.48	30.33	4.2	6.31	0.322
Supersile 20		1.88	18.47	0	10.8	29.26	3.8	6.14	0.332
Cellu SC		1.66	18.51	0.24	9.84	28.64	4.4	6.21	0.326
Susu	S. bicolor x S. sudanense	1.21	17.78	6.23	9.41	26.53	4.5	6.14	0.319
Bovital		1.31	15.84	4.96	8.36	26.72	4.4	5.9	0.334
Gardavan		1.73	11.06	5.12	10.55	28.84	5.1	6.2	0.331
Lussi		1.74	8.84	7.5	10.47	29.73	4.8	5.94	0.309

The translocation process seemed to take place at a different pace in the different genotype groups, as growing period progressed (from 117 to 133 days growth period). For the *S. bicolor* genotypes, the average WSC content increased slightly from the first to the second harvest date (20% to 22%). The maximum value was however 27.3% (Table 31). The fact that *S. bicolor* genotypes still contained high sugar contents, even after 133 days of growth, suggests that they had not yet completed their vegetative growth and had not reached full maturity. Only two of the four genotypes showed starch accumulation, but the level remained lower than that of the Sorghum hybrid genotypes. Their specific methane yield potential remained high at both harvest occasions.

Table 31: Biochemical composition of different sorghum genotypes after 133 days growing duration.

Genotype		Lipid	Sugar	Starch	Protein	Crude fiber	Lignin	Ash	CH4-Yield
		[% DM]							[m_N^3/kg ODM]
Ronal1	S. bicolor	1.16	22.26	7.49	7.85	22.24	3.4	5.4	0.318
Supersile 18		0.98	24.64	0	7.81	25.86	4.8	6.23	0.333
Supersile 20		1	27.33	0	7.95	24.8	3.9	6.96	0.330
Cellu SC		1.73	16.52	8.91	9.1	22.71	3.8	5.73	0.307
Susu	S. bicolor x S. sudanense	2.66	10.39	13.5	10.11	21.39	4.4	5.75	0.307
Bovital		2.52	7.72	15.16	10.37	25.12	4.4	5.43	0.307
Gardavan		2.23	8.51	15.37	10.45	23.75	3.8	5.69	0.317
Lussi		2.46	6.85	13.48	9.55	24.91	4.9	5.16	0.279

Conversely, the sugar content in the hybrid genotypes decreased with the increasing growing period (from 13.4% to 8.4% in average). At the same time the starch content increased of several units, from 6% to 14.4% (on average), while the lignin content increased only slightly. However, their specific methane yield potential decreased drastically to 21 l_N CH_4/kg ODM. More probably, the translocation phenomenon is accompanied by an abrupt decrease in the degradability of the stalk. As Figure 75 shows, irrespective of the genotype group, the median was higher at the first harvest date (0.324 m_N^3 CH_4/kg ODM) than at the second (0.312 m_N^3 CH_4/kg ODM).

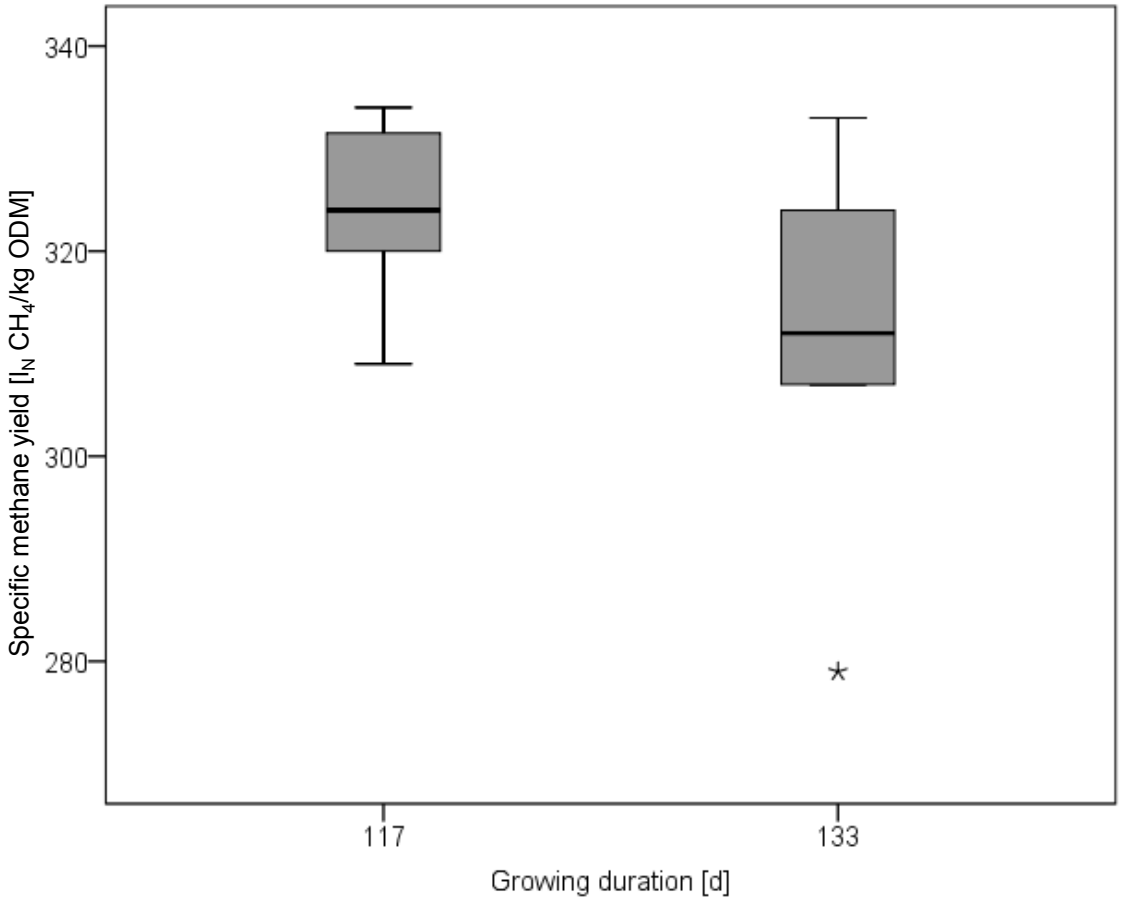

Figure 75: Specific methane yields of sorghum genotypes after different growing periods.

The analysis of the relationships between different crop biochemical traits showed that the crude fiber content was highly correlated to the sum of WSC and starch content (Figure 76). Furthermore the lignin content was negatively correlated with the sum of WSC and starch content (R^2=0.40) as shown in the appendix (Table A-7). Nevertheless, neither the crude fiber content nor the sum of WSC and starch content showed correlation to specific methane yield potential. The WSC fraction considered alone explained however to 38% the variability in the specific methane yield potential (Figure 77).

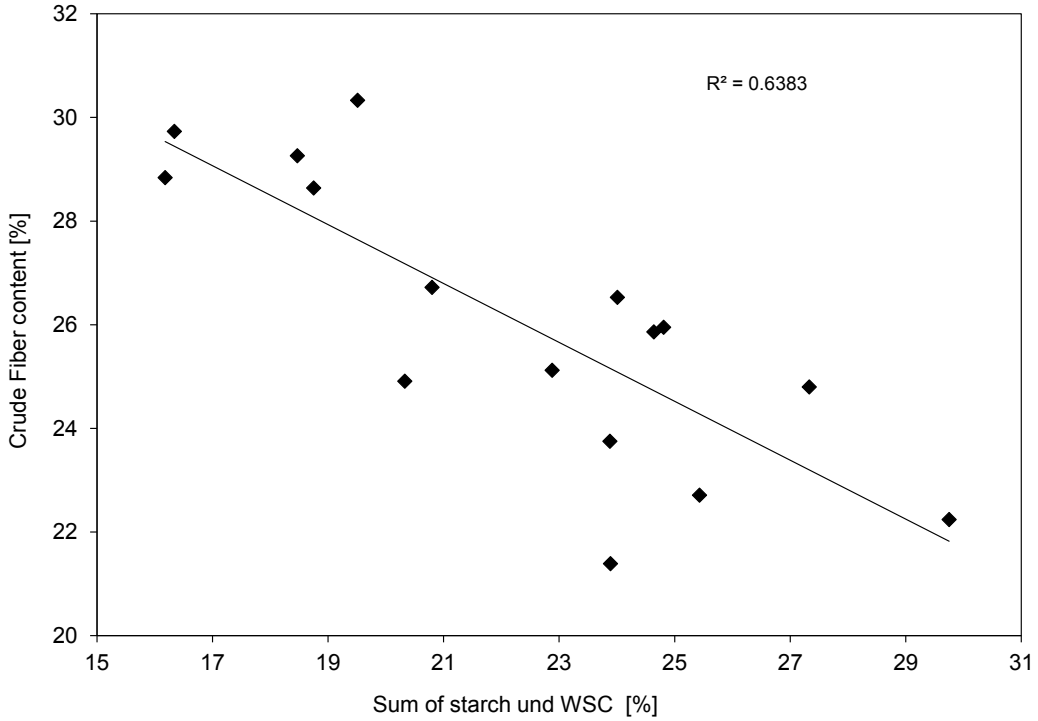

Figure 76: Relationship between crude fiber content and the sum of WSC and starch content of sorghum.

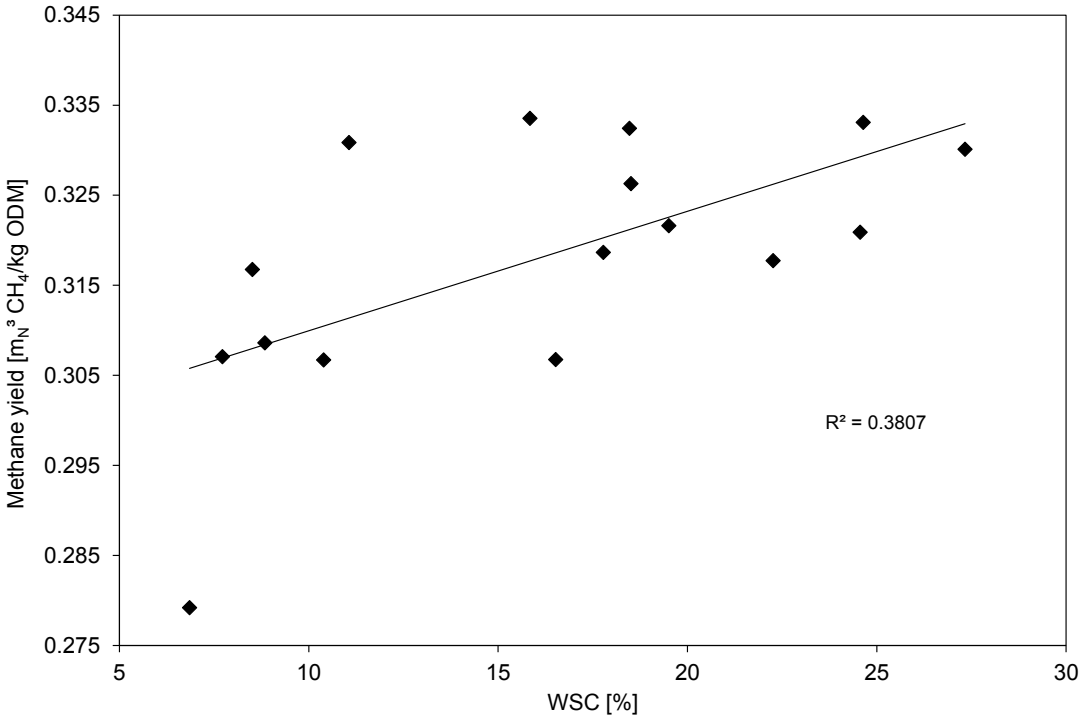

Figure 77: Relationship between specific methane yield and the WSC content of sorghum.

5.7 General comparison

A cross-section through the totality of samples investigated (carbohydrate and lipid rich energy crops) shows that the specific methane yield potential of energy crops and crop fractions can vary over a very wide range (0.207 to 0.621 m_N^3 CH_4/kg ODM). This wide variation range was also reflected by the variability in the biochemical crop traits as shown in Table 32.

Table 32: Descriptive statistics for the main crop traits of both carbohydrate and lipid rich energy crops.

	N	Range	Minimum	Maximum
Crude protein [% DM]	342	16.2	3.1	19.3
Starch [% DM]	301	65.1	.00	65.1
WSC [% DM]	251	26.9	.4	27.3
Crude fiber [% DM]	339	49.1	4.7	53.8
NDF [% DM]	302	61.3	17.6	78.9
ADF [% DM]	302	55.7	6.7	62.4
Lipids [% DM]	80	31.8	.5	32.3
Methane yield [m_N^3/kg ODM]	477	.414	.207	.621

The analysis of crop fractions showed that the highest specific methane yield potential and the largest yield discrepancies between vegetative and reproductive fractions were observed with rape (Figure 78). The sunflower stem fraction showed remarkably the lowest specific methane yield as already stated. The high range in the biochemical composition of the vegetative and reproductive fractions of lipid rich crop fractions explained the spread in specific methane yield potential of this fractions.

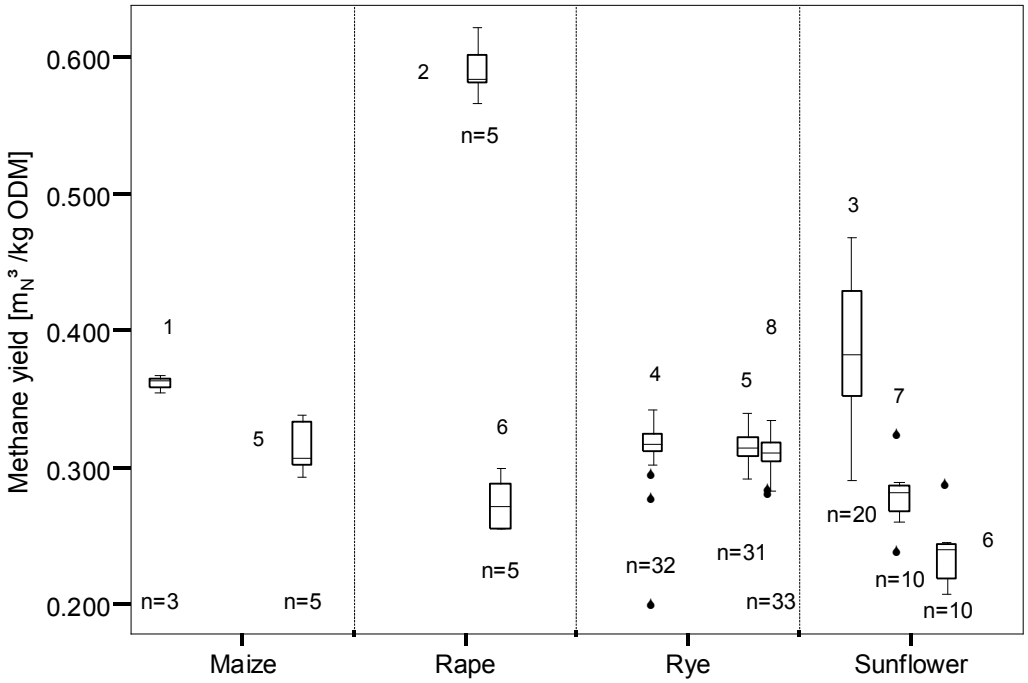

Figure 78: Specific methane yield potential of different crop fractions. 1) corn-cob; 2) seed; 3) crown; 4) ear; 5) stalk-leaf; 6) stalk for sunflower or stalk-empty pods for rape; 7) leaf; 8) stubble.

6 Discussion

This work had four main objectives: 1) investigation of the influence of ensiling process on the specific methane yield of maize whole-crop; 2) scaling-up of the batch results to a semi-continuous flow system; 3) analysis of the biomass biochemical composition and its influence on the specific methane yield potential of maize whole-crop; and 4) analysis of the biomass biochemical composition and its influence on the specific methane yield potential of other crops alternative to maize. The work was performed in a series of different experiments.

6.1 Quantification of the effect of ensiling and drying process on the determination of the specific methane yield potential of maize whole-crop

Experiment I dealt with the quantification of the effect of ensiling process on the methane yield potential of maize whole-crop. Furthermore, the impact of the mechanical conditioning processes (drying–milling and non-dried–chopping) on the determination of the specific methane yield potential was assessed.

The results showed that silage juices have a considerable share of volatile organic solids. The organic solids were mainly made of WSC and volatile organic acids. The level of the volatile organic solids in the silage juice was negatively correlated with the DM content of the crop material. This is due to the fact that physiologically young crop materials are prone to release a higher share of water soluble carbohydrates (WSC) in the silage juice than do mature crop materials. WSC are partially converted into organic acids. The sum of WSC and volatile organic acids remains higher in the silage juices of younger crop material than in the mature ones.

The profile of the volatile organic acids in the silage juices differed also with the physiological maturity level. Hetero-fermentative bacteria seemed to be more active in younger maize crop materials. In fact, material with low dry matter content (21%) showed a predominant share of acetic acid and alcohols. According to several

authors, products other than lactic acid should not be fostered in the silage if the silage is to be kept stable for a longer storage period (Thylin, 2000; Rooke and Hatfield, 2003).

These results have also implications on both the data computation procedure and the interpretation of specific methane yield potential gained using silages samples. When silages are used to determine the specific methane yield potential of a crop, it is mandatory to correct for the volatile compounds losses due to the drying process (Weißbach, 1994; Weißbach and Kuhla, 1995). Otherwise the specific methane yield potential determined is overestimated. Secondly, by comparing the results of different trials it is necessary to make sure that the crop materials used have undergone the same preconditioning processes and that adequate procedures have been chosen to account for losses of volatile solids during both the ensiling and the drying processes. The younger the crop material, the higher the risk of overestimating the specific methane yield potential. Therefore, the risk of overestimating the specific methane yields of late-maturing maize genotypes is high (Mukengele and Oechsner, 2007). This might be the reason why some earlier studies (Herrmann et al., 2006), where the correction for volatile loss was not indicated, report extremely high methane yield potential for silages, especially for late-maturing maize early harvested.

Nevertheless despite the discrepancies due to non-compensation for volatile solids' loss, some authors report higher specific methane potential for silages than measured in this work. Neureiter et al. (2005) report a 20% specific methane yield potential's increase due to ensiling process. They report also higher values for maize than those determined in this work, ranging from 383 to 480 l_N CH_4/kg ODM. This study found however that ensiling does not always improve the specific methane yield of energy crops. In fact, after correcting for inherent ensiling process losses, the results were ambivalent according to genotypes. Additionally, the authors noted that the specific methane yield increased with the increasing storage duration. This might be due to the fact that after a long period of storage, specialized acid-tolerant microorganisms can start degrading polysaccharides. According to the theory this phenomenon assures replenishment in simple sugars which are

necessary to keep the silage stable over a longer duration of time (Nußbaum, 1998). Nevertheless, the magnitude of this phenomenon is known to be limited. In a more recent work by Herrmann et al. (2011), the authors report specific methane yield's increase of 3% to 6% after correcting for ODM losses. These later results were consistent with those determined in this work and are sustained by the literature. In fact, the literature reports similar, limited, positive effects of ensiling on the energy values of silages (Ferris et al., 2005). Furthermore, according to the maize ensiling theory, at the time of ensiling, WSC will provide the primary source of energy to the LAB because of their availability. Microorganisms stop almost completely their activity as soon as the pH in the silage is lower than four (4.0). After reaching the stable conservation phase the level of lacto-bacteria decreases considerable and the silage remains stable so that further degradation of complex carbohydrates is negligible. In addition, it is reported that LAB lack hydrolytic activity towards complex carbohydrates and can only metabolize simple sugars and a few disaccharides (Rooke and Hatfield, 2003). Since both the activity and the effect of LAB on complex carbohydrates are limited it is explainable that the methane yield potential increase due to ensiling be also limited.

The comparison of the two samples preconditioning processes (drying-milling versus non-drying chopping) showed, in general, no significant differences. The minor differences might be explained by the proceeding respiration during the drying process. This phenomenon might have caused additional depletion. Therefore a quick drying after harvest can be recommended to diminish the risk for bias due to further respiration losses. The fresh-chopped variant might have also benefited from the cellular freeze-cracking caused by the freezing process. In fact, it is known that freezing can cause change in the texture and structure of vegetables leading to cellular freeze-cracking (Van Buggenhout et al., 2008). To perform the experiment in the same batch, fresh samples had to be frozen while the other variant was being dried. Because of the advantages of the drying-milling process (better homogeneity, matching standard samples pretreatment's require for other chemical analyses), it can be generally considered as an acceptable compromise.

6.2 Up-scaling the batch results - Assessment of the bioconversion efficiency in semi-continuous flow system

As stated in the objectives of Experiment II, the main goal of this experiment was to scale-up the batch results, to investigate to what extent the results gained in the batch experiment would be reproduced in the semi-continuous process and to determine hence their validity for practical use in full scale operation. Additionally an energy balance was performed in order to evaluate both the bioconversion/substrate-use efficiency and the reactor-use efficiency. Prior to the use of wheat-grain for the semi-continuous flow trial, a batch-test was conducted in order to determine the appropriate mechanical pretreatment to be applied.

The results of the batch-test showed that the simple and low-energy consuming crushing was sufficient and suitable as pretreatment for wheat-grain. This suggests that the increase of the substrate's specific area was not responsible of the higher kinetics but rather the breaking of the cuticle barrier. Therefore the intensive wheat-grain's pretreatment through milling, as it is commonly done on biogas plants, is superfluous. Furthermore, the results suggest that none of the mechanical pretreatments applied was able to alter substantially the recalcitrance of the grain fibers. In fact, the ultimate specific methane yield potential remained the same for all the variants.

The comparison of the theoretical specific methane yield and the actual specific methane yield potential in batch-test showed that 80% to 87% of the theoretical potential could be converted in batch-test depending on the substrate. In fact, the conversion rates were of 80%, 85%, and 87% for maize silage, the maize -wheat grain mixture, and wheat grain only, respectively. For a bulky substrate such as the maize whole-crop, a conversion efficiency of 80% can be considered to be high. This high conversion efficiency might be due to the ensiling process and storage duration. We had shown previously that the ensiling process can have a certain positive effect on maize digestion. Furthermore, maize silage used for this experiment was collected at the research station where the storage duration was unknown.The samples were kept further for an longer period at 4°C. Neureiter et al.

(2005) and Hermman et al. (2011) have observed that the specific methane yield of ensiled crop materials increases with the increasing storage duration.

By scaling-up the batch-test to the semi-continuous flow mode, the specific methane yield decreased depending on OLR. The higher the OLR, the lower the recovery efficiency. The decrease was 10.6% and 19.1% for maize, 8.9% and 10.2% for the maize-wheat grain mixture, and 4.9% and 5.2% for wheat-grain. Batch-tests are conducted under optimal conditions (e.g. the ratio between the test-substrate and the inoculum), and thus it is expected that the conversion efficiency in batch-tests may be higher than in a semi-continuous flow mode. However, to extrapolate the batch-test values to a semi-continuous flow system, both the biochemical composition of the crop and the OLR have to be taken into account. For an appropriate dimensioning of a full-scale biogas plant, it is necessary to consider these types of losses.

The relative residual methane yield (% of the methane yield generated in semi-continuous mode) measure in this work vaired between 11.6% to 23.3%, 8.3% to 11.1%, and 3.2% to 4.3% for maize, the maize-wheat grain mixture, and wheat grain, at low and high OLR, respectively. These values were comparable to those found in the literature. In fact, between 2001 and 2003 Oechsner et al. (2006) measured average relative residual methane yield of 15% in one-stage full scale biogas plants. The hydraulic retention times of these biogas plants were between 40 and 60 days. Vogtherr et al. (2008) measured losses of 15 to 30% for biogas plants with hydraulic retention times of 30 to 50 days. The residual methane yield in the influent increased with the decreasing retention time.

Mixing substrates of different characteristics was found to have a positive effect on the overall conversion efficiency. In fact, by mixing wheat-grain with maize silage (1:1 ratio on ODM basis), the overall conversion efficiency in the semi-continuous mode was shifted from 65% to 76% at the high OLR. This positive effect of the mixture was observed in both batch and semi-continuous systems, and was correlated with the DM accumulation in digesters. Digesters with maize-wheat grain mixture did not show a steadily increase in DM accumulation. The DM accumulation

in a digester is an indication of an inefficient digestion system since where the organic feeding regime is coupled with all the conversion steps, DM/ODM do not accumulate. This phenomenon is often observed in full-scale energy crop based CSTR digesters. To deal with it and its mechanical consequences, CSTR are either run at a low OLR (over-dimensioned) or run at a high OLR with an option whereby a certain amount of the sludge is regularly removed from the system to be pressed while the liquid fraction is returned to the digester to keep the DM content of the sludge in the digester at a constant level. Increasing the OLR of an inefficient system reduces not only the hydraulic retention time, but the latter causes in turn the sludge retention time (SRT) to be reduced so that the SRT necessary to control a viable population of microbial biomass for a given degradation is not existent as the theory requires (Khanal 2008). Therefore the DM/ODM accumulation in these CSTR systems is inevitable. Because of all these reasons this simple process management's strategy, namely the mixing of crops of different characteristics, presents a practical advantages for the energy crop based biogas plants to guarantee a better conversion efficiency at high OLR. The results showed also that wheat-grain's addition allowed to increase the reactor-use efficiency (57%) without having to jeopardize considerably the conversion efficiency.

6.3 Influence of the crop biochemical traits on the specific methane yield potential of intentionally blended maize fractions (ear and stover)

The main objective of this experiment was to evaluate the influence of the biochemical crop traits on the specific methane yield potential when the absolute contents of the crop biochemical traits were intentionally modified. This intentional modification was achieved by blending the crop fractions (ear and stover) in different proportions.

The results showed that by modifying intentionally the composition of maize genotypes of different maturity groups, the biochemical crop traits (e.g. cell-wall and starch contents) were highly correlated to the specific methane yield potential (R^2 = 0.93-0.94). Weißbach (2009; 2010) suggested that the reason why it is challenging

to use the biochemical traits to predict the specific methane yield potential of maize is the high standard error inherent to anaerobic batch-tests. This has been a limiting factor to further breeding for high specific methane yield. The results of this work show, however, that the influence of the crop biochemical traits on the specific methane yield potential may be assessed with high accuracy using a batch-test. Therefore, we suggest that the limiting factor for further breeding is not the high standard error due to batch-tests, but rather the inappropriateness of biochemical crop traits chosen to characterize genotypes toward their specific methane yield potential as explained below in Section 6.4. Furthermore, this experiment demonstrates the accuracy of the Hohenheim biogas yield test (HBT) as the curves depicting the cumulative methane yield potential increase in parallel with constant increasing of the corn-cob share.

The results reveal also the averages specific methane yield potential to be expected for maize (effects of preconditioning and ensiling process not considered). In fact, it is worth to note that even in these intentionally blended crop materials, where the starch content varied from 0 to 65%, the difference in ultimate methane yield potential of the variant with the lowest starch content and that with the highest starch content did not exceed 20%. This makes once more clear the fact that the biochemical composition of the crop dictates to a larger degree the variability in specific methane yield potential. If the biochemical profile of a crop shows components of similar nature (e.g. different shares of carbohydrates and negligible difference in lipid content), it will be also more likely that the variability in specific methane yield potential of genotypes be limited and confined in a tight spectrum. In other words, as far as maize genotypes are not bred for high lipid content, it cannot be expected that one genotype will be 20% better than another as sometime found in the literature (see Section 2.2.5). Commonly used silage maize genotypes have in general starch content varying between 20% and 35%. Figure 30 shows that intentionally blended crop materials with starch content between 20% and 35% reach more or less the same level of specific methane yield potential of maize whole-crop as given by KTBL (2010).

6.4 Influence of the biochemical crop traits on the specific methane yield potential of maize whole-crop

The overall objective of this experiment was to point out the biochemical crop traits that characterize “The” biogas maize genotype with regard to the specific methane yield potential. In fact, both breeders and agronomists look to bioprocess engineers for guidance as to which biochemical crop traits it should be targeted in order to increase the specific methane yield of energy crops. From the bioprocess engineering standpoint there are two prerequisites: 1) the genotypes or physiological stages considered should present clear biochemical features or characteristics; and 2) the biochemical crop traits used for genotypes characterization or maturity classification should reflect accurately the defined or presumed quality difference. In the field of forage breeding for animal nutrition, the values of correlations between the crop traits and energy value have provided the limits in breeding efficiency. In the same manner, for biogas production the correlations between the biochemical crop traits and the specific methane yield potential provide the edges in breeding efficiency. The higher the value of the correlations the better a breeding program will be carried out.

The biochemical composition pattern of maize is in accordance with the literature. The mid-early and the mid-late maturing genotypes represent classical silage maize varieties adapted to the climate and grown in central Europe. If they had the chance, to come into the dough ripe level, their starch content is generally high and their share of the cell-wall relatively low. The late maturing genotypes are high biomass yielding varieties originating from warmer regions. In central European climatic conditions they often have the problem that they do not reach maturity so that their high biomass yield potential is not fully exploited.

The results showed, in accordance with the literature (KWS 2007), that the most important change that takes place in maize crop throughout the growing period is the shift in carbohydrate fractions. Cell-wall complex carbohydrates (NDF) decrease as the starch content increases. In fact, through this shift, the share of the corn-cob fraction in the total organic matter content increases, while the share of cell-wall rich

organs (i.e. stalk and leaf) decreases. The protein content remains more or less constant. Nevertheless, the broad variation ranges for NDF, ADF, WSC, starch and DM contents confirm the fact that maize crop traits vary greatly across genotypes and growth stages. It also attests the fact that the set of samples considered was constituted of genotypes of different maturity indexes. In fact genotypes differ considerably in terms of vigor, partitioning of assimilates and quality (Dolstra et al. 1993; Argillier 1995). In this regard genotypes of different maturity indexes and/or crop materials harvested at different growth stages are totally different individuals. The variations in the set of samples investigated in this experiment are much larger than that observed by Andrieu et al. (1999) although the authors studied as well a large population of 150 samples including 12 bm3 samples. In their set, the NDF content varied from 36% to 57%, while the ADF varied from 17% to 32%. This might be explained by the fact that the authors focused on mature silage maize for ruminant nutrition with DM content ranging from 28% to 40% and this study does not give indication toward the number of years considered.

The relationships between different key crop biochemical traits revealed, as expected, that starch and water-soluble carbohydrates were negatively correlated with NDF and ADF (R^2 = 0.67 to 0.70). The enzymatic digestibility of ODM (CDOMD) was positively correlated to starch content. For both breeder and animal nutritionist, these relationships are of paramount importance. For instance the relationship between the cell-wall and starch contents determines digestibility which in turn reflects the energy value of the forage. In this perspective, the higher the cell-wall content the lower should be the energy value. In fact, cell-wall ripening is known to be accompanied by an increase in the proportion of lignin and cellulose in the cell-wall, and by a decrease in the proportion of hemicelluloses (Argillier 1995). The increase in lignin content affects negatively the digestibility of ODM. Furthermore the starch accumulation is known to be accompanied by the increase in total DM content of a crop. This justifies the use of both digestibility (Argillier et al., 2000) and total dry matter content as major energy value criteria in breeding for animal nutrition. Based on these parameters crop materials can be classified toward their energy content. Therefore, it could be expected, on the one hand, that the specific methane yield potential vary greatly and, on the other hand, that the biochemical

traits explain to a larger degree the variability in specific methane yield potential as found in the literature.

In contrast to the large range of specific methane yield potential values for maize whole-crop found in the literature (see Section 2.5.2), the results presented in this work reveal that the specific methane yield potential of maize whole-crop vary in a very narrow range (0.300 to 0.356 m_N^3 CH_4/kg ODM), despite a broad variation range in crop traits (NDF, starch content and digestibility). Both the level and the variability in the specific methane yields of maize whole-crop presented in this work are supported by the theory (i.e. sugar translocation or shift in carbohydrates forms without substantial change in lipid or protein concentration susceptible of increasing considerably the level of the specific methane yield potential per se). Irrespective of genotypes, maize remains exclusively a carbohydrate rich crop. Hence it is more obvious that the specific methane yield potentials of various genotypes were confined in a narrow range of variation, namely that of other carbohydrate rich substrates (e.g. molasses, sugars and starch). The limited variation in specific methane yield potential can therefore be attributed to both the moderate difference in energy content (slight differences in lipid and protein contents) and variability in degradability, especially that of the stalk fraction.

These results were corroborated by other authors. Lemmer (2005) investigated silages of 9 maize genotypes at different growth stages and found that the biochemical composition of different maize genotypes were quiet homogenous despite the difference in growth stages. The author determined methane yield potential ranging from 0.310 and 0.380 m_N^3 CH_4/kg ODM. The arithmetic mean was 0.322 m_N^3 CH_4/kg ODM. The minimum value of 0.310 m_N^3 CH_4/kg ODM was that of a very late maturing genotype (FAO 700) harvested in the very early growth stage (with a total DM content of 19.8 % at the harvest). Böhmel (2007) investigated 8 different varieties (FAO 250 to 700) in two different crop production strategies and determined that the methane yield potential varied between 0.333 and 0.358 m_N^3 CH_4/kg ODM. Schumacher (2008) investigated 9 Maize varieties and determined specific methane yield potential ranging from 0.307 and 0.357 m_N^3 CH_4/kg ODM. Hermann (2010) determined specific methane yield potential of maize in the range

of 0.327 and 0.388 m_N^3 CH_4/kg ODM. Ohl (2011) determined specific methane yield potential ranging from 0.358 and 0.378 m_N^3 CH_4/kg ODM, with part of the samples being silages. KTBL (the German association for technology and structures in agriculture) gives values varying from 0.332 and 0.347 m_N^3 CH_4/kg ODM for maize of different maturity level (KTBL 2010).

In general maize growth is always linked with the increase in the total DM content of the crop (as discussed in the literature). In this regard DM content of the whole-crop can be considered as quality parameter. Still, by zeroing in the area considered to be the optimum harvest-window for silage maize (28% to 40% DM content), one notices that for a given total DM content, the starch content or the *in-vitro* enzymatic digestibility of ODM vary so greatly that the relationship between the total DM content of maize whole-crop and the intrinsic crops characteristics (NDF, starch, etc.) can be considered to be somehow loose. This can be explained by the fact that the total DM content of whole-crop, being a result of the physiological growth and environmental factors, will not correspond necessarily to a given specific quality (especially when severe environmental conditions and stresses impede the normal physiological growth). Therefore at the same DM content, the quality can be substantially different. Amler (2003) confirmed this statement. This might be the reason why the analysis of the relationships between total DM and the specific methane yield potential showed that total DM content of the whole-crop had no influence on the specific methane yield potential (R^2=0.13). Still Kaiser and Gronauer (2007) found that the DM of whole-crop justified to 48% the variability in specific methane yield potential of maize silage. Struik (1983) states that the quality of maize whole-crop is highly related to the composition/physiological status of plants at harvest, which in turn depends on the growing conditions, i.e. weather, soil and cultural practices. Though the effect of crop material (hybrid or line) can be fairly large on the nutritive value, seasonal, cultural and environmental variation influence highly the digestibility of maize whole-crop (Dolstra et al., 1993). This negates the use of this parameter as main quality parameter and makes it an inappropriate criterion for the breeding of genotypes with high specific methane yield potential. Its consideration as energy value criteria is hence overestimated. The statement of Kaiser (2007) toward the influence of the total DM on the specific methane yield may

be due to the limited number of genotypes and locations considered in his study. Herrmann (2010) found that the increase in dry matter was accompanied by a slight decrease in specific methane yield but could not generalize the trend with reliability for all genotypes.

The evaluation of the influence of the biochemical traits on the specific methane yield potential showed that both starch and cell-wall content explained only to a very limited degree the variability in specific methane yield potential (R^2 = 0.22 to 0.24). These weak correlations suggest that the absolute values of crop traits per se (NDF, ADF, WSC and starch) do not deliver enough information with respect to the crop quality and methane yield potential. The locations and year effects seem to weaken considerably the prediction power of the commonly used crop biochemical traits for whole-crop, so that their use as predictors of specific methane yield potential is inappropriate. Although there is scarcely literature on the appropriateness of the biochemical crops traits on the specific methane yield potential per se, some authors have already evoked similar observations in the breeding of maize for cattle nutrition. Fontaine et al. (2003) found, for instance, very low correlation between absolute cell-wall content (NDF value) and digestibility of the cell-wall. In general, maize lines of low lignin content showed also high digestibility. But some lines of even lower lignin content revealed however a lower digestibility. At the same time specific lines of significantly high lignin content had a high cell-wall digestibility. Struik (1983) observed considerable differences in cell-wall digestibility of populations. He could ascribe the variation to none of the known plant or site characteristics. He stated that the variation was partly associated with the cell-wall content (absolute values) and partly with the cell-wall digestibility or both. However, the degree to which this takes place in different genotypes is not known. Barrière et al. (2005) stated that variation in cell-wall digestibility that was not explained by lignin content (often higher than 50%) could be attributed to variation in lignin structure. This study suggested that cell-wall structure and organization, and lignified tissue patterning, are undoubtedly involved in maize digestibility. Therefore, it is not possible to make for further progress in plant cell-wall digestibility without understanding the biochemical and molecular basis of cell-wall biogenesis,

organization, and lignification. This shows the limit of using digestibility as predictor for specific methane yield potential.

Furthermore, the inappropriateness of the use of absolute values of crop traits as predictor for specific methane yield potential can be exposed by the drawbacks inherent to the Van Soest methodology. In an investigation where soil particles were added to the sample, it was found that almost all ash derived from soil contamination was determined as NDF and ADF. The NDF value can contain as much as 47% ash and the ADF 61% (Aerts et al., 1978). The authors state furthermore that the over- or underestimation of NDF and ADF results in a faulty figure for the quantity of hemicellulose, as the latter is measured as the difference between both. This means that climatic conditions at the harvest and soil type and cutting length can cause biases to the actual quality of the cell-wall absolute values.

These results bring to light a phenomenon worth consideration and explain actually the reason why in Experiment III high correlations between biochemical crop traits for whole-crop and specific methane yield potential could be observed, while in Experiment IV they were less likely to be observed. In fact, in Experiment III the composition of the crop material was intentionally modified (i.e. ear and stover fractions were blended in specific proportions). The increase in cell-wall fractions or starch content was not a product of a physiological change but rather that of an intentional mechanical mixing of crop fractions. In this way, the structural changes (e.g. that of the cell-wall matrix) were eliminated and could be therefore considered as constants. The equation was hence simplified. It becomes as if one could move a cursor along a variable. The “only” factor influencing the specific methane yield potential was then restricted to the linear increase or decrease of the absolute content of a biochemical trait. Therefore, the absolute values of the biochemical crop traits accounted for variability in specific methane yield with high coefficient of determination. Conversely in experiment (IV) the changes took place in nature, environmental factors affecting the structure of maize genotypes randomly. In this way, the absolute contents of the biochemical crop traits, especially the cell-wall fractions (NDF, ADF, ADL) alone delivered insufficient indications toward the tissue structure and the degree to which the crop would be degraded.

Because of the inappropriateness of the *in-vitro* estimates of digestibility and biochemical crop traits to characterize sufficiently genotypes toward their methane yield potential, it was not possible either to point a specific genotype as “The” energy maize genotype or to point out with accuracy the whole-crop traits that need to be targeted as predictor for high specific methane yield potential. As far as the hectare-methane yield is concerned, the results showed that “The” energy maize genotype remains that with the highest dry matter yield per hectare.

6.5 Assessment of the *in-vitro* estimate of digestibility for whole-crop (CDOMD) and the biochemical traits as predictors of the biodegradability in AD batch system

The results of Experiment V showed that the *in-vitro* estimate of digestibility for whole-crop (CDOMD) although highly correlated to commonly used biochemical whole-crop traits (NDF, ADF, WSC and starch), underestimate the genotypes’ net energy recovery efficiency (biodegradability) in AD system. Neither low CDOMD values for whole-crop nor high cell-wall contents were necessarily in conjunction with low specific methane yield potential. Young crop materials or late-maturing genotypes were severely misrepresented based on their high cell-wall contents and low values of the *in-vitro* estimates of digestibility while their actual degradability in AD was found to be high.

The results of this experiment are corroborated by other works in field of ruminants’ nutrition. Andrieu et al. (1999), Kruse et al. (2006) observed similar inconsistencies between *in-vitro* estimates of digestibility for whole-crop and the gas yielding potentials. Deinum and Struik (1989) showed that cell contents (starch, WSC, etc) and cell-wall contents are very much affected by environmental and developmental variations. Several other authors ascertain that cell-wall contents and their digestibility were independent (Dolstra and Medema 1990; Dolstra et al., 1993; Andrieu et al., 1993; Argillier et al., 1995). These observations suggests that two genotypes of the same NDF value can still reveal different degradability in AD system and therefore display different specific methane yield potential. In a review of different studies, Barrière et al. (2005) state that the *in-vitro* estimates of

digestibility (IVDOM) for whole-crop explained partly (50-60%) the variation in animal performance. The authors note furthermore that when two genotypes of similar cell-wall digestibility but different starch contents were compared, the parameter enzymatic digestibility of whole-crop (CDOMD) appeared to be overestimated for whole-crop with high starch content than for the crop with lower starch content. Hence the *in-vitro* estimates could not, notably, distinguish hybrids with a high grain content and a low stover digestibility, from hybrids with a lower grain content, but a higher stover digestibility (Argillier et al., 1995).

The incubation time and milieu are additional factors that might explain further the inappropriateness of the use of both *in-vitro* estimates for digestibility whole-crop and absolute values of biochemical traits as predictors for specific methane yield potential. By the way the *in-vitro* estimates of digestibility for whole-crop express the percentage of ODM digested after a 48-h incubation period (Hansey et al., 2010). This incubation duration might be accurate for ruminants' nutrition but the literature shows that the digestion *in-situ* increases with the increasing incubation time. Steingaß (2007) in an incubation trial observed that the DM of a maize silage continued to be degraded up to 96 h-incubation period. Traxler et al. (1998) observed continued digestion after 96 h in some forages and recommended 144h-fermentation as necessary. By increasing the incubation time from 48-h to 96-h Raffrenato et al. (2009) found that the *in-vitro* digestibility of NDF in maize increased of 13 percentage units.

Furthermore, it might be that some tissues that are not digested in rumen are degraded in AD systems. Despite all the limitations, especially the fact that the effects of the environment on the cell-wall degradation in maturing plant tissue are yet not fully understood (Buxton and Russell, 1988; Buxton and Redfearn, 1996; Grabber, 2005; Jung and Casler, 2006), the effluence of the milieu is referred to in the literature. A review by Hobson and Wheatley (1993) notes that cellulolytic bacteria predominant members of the microorganisms flora of the feedstock (slurry) fed to the digester were not found to be predominant in the anaerobic digesters. The group of cellulolytic bacteria, which were afterward predominant seemed to have been selected by growth in the digester from bacteria which made up a small

proportion of the bacteria in the digester feedstocks. Additionally, the authors state that the anaerobic digesters seemed to contain a much more diverse population of cellulollytic bacteria than those of the rumen, where three or four genera and species comprise the main cellulolytic population. After a certain retention time they could not find rumen cellulolytic rumen bacteria in digesters fed with pig- or cattle-manure. Many of the cellulolytic digester bacteria were found to be spore formers. Therefore the authors suggested that these would survive better in a digester with a long retention time and substrates of poorly-degradable fiber than would the non-sporulating rumen bacteria adapted to system of short retention and substrates of much higher degradability. Moreover, they noticed hemicellulotytic activity in cellulotytic bacteria isolated in these experiment. The cellulolytic bacteria grew and hydrolyzed cellulose optimally at about 35°C and pH 6.5 to 7; i.e., under the normal mesophilic digester conditions. This might explain why the crop cell-wall are, hydrolyzed to a great extent in a digester than expressed by the rumen as depicted by *in-vitro* estimates of digestibility. More recently Kumaravelayutham (2015) studied the impact of simple and complex substrates on the composition and diversity of microbial communities and the end-product and found that both the microbial community and the end-products were different depending on the source of carbon. The author observed a much higher diversity of the microbial population when the microorganisms were grown on complex substrate such as wheat straw than when they were grown on pure substrates such as D-glucose or α-cellulose. It was also observed in this study that certain microorganisms could only grow on specific substrates at 37 °C and pH 7.2, irrespective of the diversity of microbial population present in the seed inoculum. The microbial communities affected also the biogas composition and quantity.

Therefore, it seems necessary to define additional predictors for high specific methane yield than absolute cell-wall contents and in-vitro estimates of digestibility for whole-crop. Conversely Grieder et al. (2011) established a NIRS (near infrared reflectance spectroscopy) model whereby the specific methane yield potential of maize genotypes could be predicted using both the estimated chemical composition and Van Soest cell-wall fractions. Darnhofer et al. (2009) did not succeed to

elaborate a NIRS based model for maize and suggested, *inter alia*, that additional sources of variations (year and environmental effects) weakens the model.

6.6 Evaluation of the specific methane yield potential of various crops alternative to maize

Maize is widely used as biogas feedstock. For biomass supply security and environment management's purposes other crops come into consideration. In Experiment VI we have investigated both lipid and carbohydrate rich crops alternative to maize.

The comparison between maize and sunflower revealed that the specific methane yield potentials of sunflower whole-crop were spread over a wider range. While maize whole-crop showed only a 15% difference in specific methane yield potential, sunflower reached a 27.4% difference. The specific methane yield potential varied from 0.249 to 0.343 m_N^3 CH_4/kg ODM. The median value across genotypes was however far much lower (0.274 m_N^3 CH_4/kg ODM) than that of maize whole-crop (0.332 m_N^3 CH_4/kg ODM). Since the specific methane yield potential of the stalk and leaf fractions were confined in a narrow range, the large variability in specific methane yield potential of sunflower whole-crop could be explained mainly by the crown's lipid content. The crop materials used comprised both oil and high biomass yielding genotypes. These crop materials had different lipid content as shown by the analyses of the crop fractions (Figures 60, 61 and 62), especially for the crown fraction. The low median value was attributed to both high cell-wall contents and the biodegradability of the stem cell-wall. The digestion recalcitrant's cell-wall fractions in both crown and stem might have thwarted the benefit of high lipid content in a way that the specific methane yield potential of sunflower whole-crop could not exceed that of maize.

A closer look on the crop fractions showed that the specific methane yield potential of the sunflower's stem were lower than that of other crops (rape, maize and rye). This suggests a poor degradability of the sunflower stem cell-wall. Although sunflower's stem revealed as high NDF values as maize stalk, its lignocellulosic

fraction was higher (56% against 40% for maize stalk). Furthermore, sunflower's crown showed also high NDF and ADF contents (26% to 38% and 23% to 31.2%, respectively). These values are considerably higher than those generally measured on maize ear (17% to 25% NDF and 6% to 11% ADF). It is well documented that because of the cross-linkages in lignocellulosic fibers, ADF represents the share of cell-wall which is the most recalcitrant to digestion.

In contrast to maize, where the relationships between the crop biochemical traits and the specific methane yield potential were loose, the cell-wall content (absolute values) seems to control the methane yield potential of sunflower stem, while the lipid content controls the methane yield potential of the crown. In fact, the specific methane yield potentials of the sunflower stem were negatively correlated with NDF (R^2 = 0.72) and ADF (R^2 = 0.79). The crown fraction was positively correlated with the lipid content (R^2 = 0.91). These robust correlations lay down a foundation for an efficient selection and breeding program (to this regard).

Because of its strong tap-root system, rape contributes to maintain a better soil structure. It also contributes to increase the yields of cereals (10%) when sown afterward (Bundessortenamt, 2011). Nevertheless, rape plants grown for oil production might show considerable yield fluctuations due to seed losses when precipitation hinders timely harvest after full maturity. The use of the whole-crop for biogas production might be an alternative.

Rape whole-crop harvested at different growth stages showed lower specific methane yield potential (0.275 to 0.307 m_N^3 CH_4/kg ODM) than maize. This low specific methane yield potential was attributed to the biochemical composition of the genotype used. In fact, at the two first harvest occasions (at the full flowering and pods elongation stages) the lipid content showed a minor increase from 2.4% to 3.4%. This level of lipid content is equal or slightly higher than that of maize whole-crop. Accordingly higher specific methane yield potential than that measured on maize would not be expected. At the third harvest occasion (full maturity) the lipid content reached 12.4%, but apparently the lignification of the stalk fraction was so advanced that the positive effect of the high lipid content might have been thwarted

by the decreasing degradability of the stalk fraction. Since the cell-wall fractions were not analyzed, this hypothesis could not be directly confirmed. Nevertheless, in the second step of this experiment, where rape crop fractions were investigated, the rape green fraction at full maturity revealed a lower specific methane yield potential far below that of maize stover (0.265 m_N^3 CH_4/kg ODM). Furthermore, Lancaster et al. (1990) reported a higher share of lignocellulosic fiber content for rape whole-crop (36 to 40%) than those of maize whole-crop (17 to 23%). All these factors appear to explain the low specific methane yield potential of the rape genotype used in samples set I. In opposition to this picture, when rape crops of higher total DM contents (advanced maturity) than that previously used were considered, higher specific methane yield potentials ranging from 0.339 to 0.405 m_N^3 CH_4/kg ODM were observed. The median lied by 0.375 m_N^3 CH_4/kg ODM. The lipid rich seed fraction appeared to explain the high specific methane yield potential. In fact, rape seed showed higher specific methane yield potential (0.547 to 0.598 m_N^3 CH_4/kg ODM) than sunflower crown (0.367 to 0.455 m_N^3 CH_4/kg ODM). The high specific methane yield potential of rape seed were explained by both low cell-wall contents and high lipid content.

Although the cell-wall and lipid contents of rape seed were not analyzed, Jeroch et al. (2008) give values of 16% and 14% for NDF and ADF, respectively. These values are lower than those measured on sunflower crown in this work. In fact, the sunflower crowns investigated in this work showed values of 26% to 38% and 23% to 31.2% for NDF and ADF, respectively. Furthermore, Velasco et al. (1999) measured higher lipid content in rapeseed (28.5 to 54.9%) than those measured in sunflower in this work (13.4% to 28.8%). Even though the lipid content, in general, boosts the specific methane yield potential of energy crops, stalk cell-wall degradability remains the limiting factor that hinders a successful use of rape and sunflower for biogas production. Nevertheless, compared to maize, cell-wall and lipid contents of lipid rich crops can be considered to be acceptable predictors for specific methane yield potential.

Rye is a carbohydrate rich crop, like maize. Nevertheless, it is seen as an alternative substrate to maize because of its ability to grow successfully and produce high dry matter yields on sandy soils (Hübner, 2011). Rye whole-crop harvested at the beginning of heading showed in average similar median specific methane yield potential as maize whole-crop (0.332 m_N^3 CH_4/kg ODM), but the range of 17 l_N CH_4/kg ODM was lower than that measured on maize genotypes (56 l_N CH_4/kg ODM). The comparison of the crop fractions of these two crops showed clearly that the specific methane yield potential of rye fractions were close together, while maize fractions showed considerable differences (Figure 78). The rye ear fraction yielded in average 0.317 m_N^3 CH_4/kg ODM while maize ear revealed higher specific methane yields of 0.368 m_N^3 CH_4/kg ODM. Its stalk-leaf fraction yielded 0.314 m_N^3 CH_4/kg ODM in average. This difference was partially explained by the cell-wall contents. The comparison of genotypes toward the specific methane yield potential revealed that the grain hybrids did not surpass the forage genotypes. For this crop the dry matter yield remains the determinant factor for high specific hectare-methane yield.

Like maize sorghum belongs to the Family of Poaceae and the principal species cultivated for grain is *Sorghum bicolor*. Both the grain species (*S. bicolor*) and the forage hybrid (Sudan grass) are used for biogas production. Several cultivars and hybrids have been developed in the last years. Sorghum is an important alternative to maize because of its growth flexibility. In contrary to maize, sorghum has the ability to bridge drought periods, meaning that the crop stops temporarily its vegetative growth during drought period and resume growth when precipitations are again available (Jäkel, 2012). Sorghum shows also low requirements on both soil and water supply (Klostermann and Oechsner, 2008).

The comparison of different sorghum genotypes (*S. bicolor* and Sorghum hybrids) revealed that the *S. bicolor* genotypes are rich in WSC than their counterparts (Sorghum hybrids). Their WSC content was high throughout the growing period so were also their specific methane yield potential. They showed, however, low starch content. The high share in WSC at both harvest occasions reveals that the plant has longer vegetative phase and more probably a higher biomass yielding potential. In

general, in this growth phase the cell-wall are more degradable so that their mobilization for methane generation should be easier. This appears to explain the high specific methane yield potential of the *S. bicolor* genotypes at both harvest dates. The Sorghum hybrids (*S. bicolor* x *S. sudanense*) could not maintain the high specific methane yield when the growth duration was prolonged. At the second harvest date (133 days growing period), they all showed lower specific methane yield potential. Their high starch content showed no positive impact on the specific methane yield potential. Therefore, it seems to be more appropriate to favor rather the high WSC content than starch for this particular crop.

These observations are corroborated by a comprehensive study where various sorghum genotypes were investigated for six consecutive years in different locations in Germany. According to this study Sorghum hybrids (*S. bicolor* x *S. sudanense*) require a shorter growing period to reach maturity (Zander, 2012). A longer growing period would produce more lignified crops. This might explain why at the second harvesting date the Sorghum hybrids showed low specific methane yield potential. The crop might have exceeded the optimal growing period. Due to the fact that they enter quickly the reproductive phase, they show also low to moderate biomass yielding potential than the *S. bicolor* genotypes. However, the author found that the *S. bicolor* genotypes were heterogeneous with respect to DM yield per hectare and that the high biomass yielding genotypes had a poor standability because of the height. This reflects the rich WSC crop materials displayed in this work.

7 Outlook and further research need

This work dealt with energy crops for biogas production, as a pioneer work born right after the amendment of the Renewable Energy Act in 2004, in the turmoil search for the best energy crop. Its character is broad and it encompasses both pure academic and practical issues. Meanwhile some of the issues treated in this work were clarified by other studies. The search for lipid-rich maize genotypes is proceeding at a high pace. Despite these breakthroughs, bioprocess engineers have not been yet able to provide breeders with accurate information about to which crop biochemical traits should be targeted for the selection and breeding of maize genotypes with high specific methane yield potential although both NIRS (Near Infrared Reflectance Spectroscopy) and HBT (Hohenheim Biogas Yield Test) are economically viable tools that help time and cost for laboratory analyses to be kept in acceptable limits.

The results on maize showed, however, that because of the inappropriateness of both *in-vitro* estimates for digestibility of whole-crop and absolute values of biochemical crop traits as predictors for specific methane yield potential, it was not possible to point out with accuracy the crop traits that characterize an ideotype energy maize (with respect to specific methane yield potential). The results suggested that the environment had a high influence, *inter alia*, on cell-wall structure and organization, so that the use of whole-crop biochemical traits and *in-vitro* estimates of digestibility for whole-crop, parameters on which all current selection efforts are based, are misleading. Furthermore the drawbacks of the Van Soest system increase the risks for misinterpretation. For instance, in the narrow harvest window within which maize is harvested for ensiling (e.g. 28% to 40% DM content), impurities due to rain during harvest can become a limiting factor and cause considerable biases in the determination of the crop biochemical traits. Additionally both the milieu and incubation period used to determine the *in-vitro* estimates for degradability seem to be inadequate.

For future research on this subject it seems of paramount importance to make adjustments at three levels: 1) choice of predictors; 2) determination of cell-wall fractions; and 3) incubation milieu and duration.

More accurate predictors for high specific methane yield potential are needed. Only a clear and accurate expression of the crop quality is able to reveal the actual genetic variation across crop materials and provide a possibility of assessing afresh the effects of the crop traits on the variability in specific methane yield potential. As far as cell-wall fractions are concerned, some literature (Dolstra and Medema, 1990; Dolstra et al., 1993; Argilier et al., 1995; Barière et al., 2005) in the animal nutrition field suggest, *inter alia*, the use of the digestibility of the stalk cell-wall or the *in-vitro* digestibility of the non-starch and non-soluble carbohydrate plant part (IVDNSC). According to the authors, this selection criterion is stable and highly heritable from about one month and is quite independent of the development stage of the crop. This makes sense for energy crops based AD systems where the stalk, generally rich in cell-wall content, is the limiting factor for methane generation. Nevertheless the authors suggest that in a breeding program the starch content be also controlled in order to avoid the risk of drift toward genotypes of low starch content and generally of less energy content. This requires also that the *in-vitro* estimates be analyzed in an adequate milieu. Another approach suggests the targeting of specific tissues (e.g. rind of stem, tassel, leaf sheath, and mid-rib). Nevertheless because of the importance of lignin to provide mechanical support for stems, to impart strength and rigidity to plant walls, to provide resistance to diseases, insects, cold temperatures, and other biotic and abiotic stresses, practical limits exist as to how much lignin and other cell-wall constituents can be reduced through breeding without adversely affecting the ability of crops to grow and survive in field environments (Buxton and Redfearn, 1996). Because of this limiting factor extensive degradability should be achieved through pretreatment methods and bioprocess management, for instance by the uncoupling of the sludge retention time from the hydraulic retention time. This means however moving from CSTR to other novel AD systems.

Furthermore in order to get the true genotypic variability with respect to *in-vitro* estimates of degradability for whole-crop or stalk fraction, additional incubation time

seems to be necessary. It can be that genotypes or crop fractions presumed to be different after 48-h or 72-h incubation, exhibit after a longer retention time, no difference in degradability. The *in-vitro* estimates need also to be measured in AD environment as the literature notes difference with respect to microbiota and carbon source. It might be that some tissues which are not digested in rumen are digested in AD systems. This would be the true anaerobic biodegradability which needs to be known to differentiate genotypes.

For lipid rich crops alternative to maize, further breeding efforts to increase the stalk degradability is necessary and apparently achievable since reliable predictors are yet available. Nevertheless, the harvest of mature lipid rich energy crop (with high share of seeds) for biogas production presents a technical challenge as mature pods and crown are prone to lose seeds at the harvest. This requires advanced harvest techniques.

Carbohydrate rich crops present a good alternative to maize, but their stalk's quality seems to depreciate quickly as the crop enters the reproductive phase. For crops exhibiting high specific methane yield potential and low DM contents for conventional bunker storage (e.g. *S. bicolor*), the drawbacks can be dealt with by ensiling the low DM forage on the top of existing high DM content maize silage, especially when the harvests of the two crops coincide. This strategy is rational in sandy soil's regions where maize yields and quality are threatened by short drought or heat waves (e.g. Brandenburg region).

8 Summary

This thesis had an overall objective of analyzing the biomass biochemical composition and its influence on the specific methane yield potential of energy crops. This was meant to provider breeders, molecular geneticists and agronomists with information as to which biochemical crop traits it should be targeted in order to increase the specific methane yield potential of energy crops. The main crop evaluated comprehensively was maize and in addition to it: sunflower, rape, rye and sorghum. The analysis on maize covered: the evaluation of the biochemical crop traits, the evaluation of the variation range in specific methane yield potential, the influence of the biochemical traits on the specific methane yield potential, and the viability of the biochemical composition and *in-vitro* enzymatic digestibility of whole-crop as predictors of the specific methane yield potential. Prior to this in-depth analysis the influence of the ensiling technique on the methane yield potential, and the specific methane yield potential gained using a batch-test scaled up to semi-continuous flow system were assessed. The scaling-up involved also the evaluation of the bioconversion efficiency of both batch and semi-continuous flow digester.

The experiment on the influence of ensiling process on the specific methane yield potential showed that by exposing silage samples to the drying process, the quasi-totality of ethanol and acetic acid were already lost at 60°C. Silage of low DM content were more prone to drying losses than were the high DM content samples. Hence the risk of over-estimating the specific methane yield potential of this samples is particularly high. Nevertheless, the investigation showed that through ensiling up to 8.6% higher methane yield potential could be achieved. The impact was different depending on the maturity index of the crop material. By considering the ODM losses inherent to ensiling, the benefit of ensiling process on specific methane yield potential was ambivalent ranging from minus 6.5% and plus 7%.

The evaluation of the bioconversion efficiency in batch and semi-continuous flow digester showed that 80% to 87% of the theoretical methane yield potential could be recovered in a batch-test. By scaling up batch results to semi-continuous flow digester the bioconversion efficiency decreased of up to 19%. The bioconversion

efficiency in semi-continuous flow system depends on both the biochemical composition and the OLR. The mixture of two substrates of different characteristics at high OLR was found to have a positive impact on the reactor-use efficiency without jeopardizing the bioconversion efficiency.

The investigation on maize showed that despite the wide variation range in crop biochemical traits and *in-vitro* estimates of digestibility for whole-crop across genotypes and maturity stages, specific methane yield potential varied in a very narrow range (15% difference). The evaluation of the influence of the biochemical composition on the specific methane yield potential showed that by mixing intentionally the ear fraction to the stover, high correlations between the biochemical crop traits and the specific methane yield potential were obtained. The biochemical crop traits of whole-crop showed however moderate to poor correlations to specific methane yield potential. Hence absolute values of the biochemical crop traits and *in-vitro* estimates of digestibility for whole-crop, commonly used as selection criteria for high energy values, were found to be poor predictors for high specific methane yield potential (R^2 = 0.31 to 0.32). Consequently it was not possible to point out with accuracy the biochemical crop traits that could characterize “The” biogas genotype (with respect to specific methane yield potential). Furthermore, the results suggest that breeding progress on maize is not limiting by the error inherent to the batch-tests, but rather by the choice of inappropriate traits to characterize genotypes and crop materials toward their specific methane yield potential.

Other crops alternative to maize showed a wider variation range in specific methane yield potential. In this case the specific methane yield potential was very much affected by the absolute values of the biochemical crop traits. Reproductive crop fractions of lipid rich crops revealed higher specific methane yields reaching 0.455 m_N^3 CH_4/ kg ODM in sunflower crown and 0.598 m_N^3 CH_4/ kg ODM in rape seed. The stalk/stem fraction of these crops seemed to be the most limiting factor for degradability. For instance, despite the high share of lipid and protein in the sunflower stem, its methane yield was far much lower (0.201 to 0.284 m_N^3 CH_4/kg ODM) than that of maize stover (0.300 m_N^3 CH_4/kg ODM in average). Conversely, carbohydrates rich crops (rye and sorghum) showed methane yields slightly lower

or equal to those of maize. They offer hence an option as substrate where the requirements for maize production are not optimum.

9 Zusammenfassung

Zur Steigerung des spezifischen Methanertrages von Energiepflanzen orientieren sich Pflanzenzüchter, Mikrobiologen und Agrarwissenschaftler an der biochemischen Zusammensetzung des Substrates. Dies setzt zwei Dinge voraus: (1) die betrachteten Genotypen oder Entwicklungsstadien sollten eindeutige biochemische Eigenschaften und Charakteristiken aufweisen; (2) die biochemischen Pflanzenmerkmale, welche zur Typisierung der Genotypen und der Bonitur herangezogen werden, sollten die geforderte Qualität zutreffend wiedergeben.

Diese Arbeit soll dazu beitragen diese Vorbedingungen zu klären. Die vier Hauptziele der Arbeit, durchgeführt in sechs Experimenten, waren: 1) die Quantifizierung des Einflusses der Silierung auf den Methanertrag von Mais; 2) die Übertragung der Batchergebnisse auf das Durchflussbetrieb sowie das Erstellen einer Energiebilanz; 3) die Ermittlung des spezifischen Methanertragspotentials von Mais und anderen Ganzpflanzen als Alternativen und 4) die Einschätzung des Einflusses der biochemischen Zusammensetzung und die *in-vitro* Abschätzungen der Verdaulichkeit von Ganzpflanzen zur Vorhersage der spezifischen Methanausbeute;

Die Quantifizierung des Einflusses der Silierung auf die spezifischen Methanerträge zeigte, dass der beim Trocknungsprozess auftretende Verlust an organischer Substanz (flüchtige Fettsäuren, Alkohol) korrigiert werden muss, um die spezifischen Methanerträge von Silagen genau angeben zu können. Die Untersuchung ergab, dass ohne Korrektur durch das Silieren um bis zu 9.6% höhere Methanerträge suggeriert werden. Der Einfluss variierte je nach Reife-Index des Ernteguts. Unter Berücksichtigung der genannten Trockenmasseverluste wurde der positive Effekt des Silierungsprozesses auf den spezifischen Methanertrag auf 7.0% reduziert.

Die Untersuchung zur Übertragung der Batchergebnisse auf den Semikontinuierlichen Betrieb zeigt, dass die Effizienz der Vergärung sowohl von

Substratmerkmalen als auch von verfahrenstechnischen Parametern wie z.B. der Raumbelastung abhängt. Im Allgemeinen gilt, je niedriger die Raumbelastung, desto höher die Substratumsetzungseffizienz und desto stabiler ist der anaerobe mikrobielle Prozess. Die Effizienz des Reaktorvolumens lässt aber bei niedriger Raumbelastung nach. Es wurde herausgefunden, dass die Mischung von zwei Substraten mit unterschiedlichen Eigenschaften einen positiven Einfluss auf die Reaktor- und Substratumsetzungseffizienz hat. Die Zugabe von gequetschten Weizenkörnern erhöht die Reaktoreffizienz um 57%, ohne die Substratumsetzungseffizienz zu gefährden. Die Verluste in den Fermentern mit Weizenkornmischung waren moderat (8,3 - 11,1%). Am höchsten waren sie in den Fermentern, die mit Maissilage (11,6 - 23,3%) beschickt wurden. Je höher die Raumbelastung und je sperriger das Pflanzenmaterial, desto höher die Restmethanwerte. Somit kann das Abdecken des Gärrestlagers zur Minimierung unkontrollierter Methanverluste sehr sinnvoll sein.

Bei Einsatz von Weizenkorn brachte dessen Quetschen denselben Methanertrag und zeigte die gleiche Kinetik wie die intensive Zerkleinerung durch das Mahlen des Korns.

Die Untersuchung von Mais ergab, dass trotz der großen Variationsbreite der biochemischen Pflanzeneigenschaften und *in-vitro* Schätzungen der Verdaulichkeit für Ganzpflanzen in Genotypen und Reifestadien, die spezifischen Methanerträge in einem sehr engen Bereich (300 - 356 l_N CH_4/kg oTS) lagen. Der Unterschied zwischen den schwächsten und besten Varianten betrug nicht mehr als 15%. Die Ergebnisse zeigen auch, dass die Umweltfaktoren die Struktur der biochemischen Zusammensetzung enorm beeinflussen, sodass sowohl die biochemischen Pflanzeneigenschaften (z.B. NDF, ADF) als auch die *in-vitro* Abschätzungen der Verdaulichkeit nur in begrenztem Maße (R^2=0,31 - 0,32) für die Variabilität der spezifischen Methanerträge verantwortlich sind. Der Umwelteinfluss auf die biochemische Struktur der Pflanzenmerkmale scheint in diesem Zusammenhang die größte Rolle zu spielen. Diese Ergebnisse deuten darauf hin, dass weitere Zuchtfortschritte bei Mais nicht von den Abweichungen innerhalb des anaeroben Batch-Vergärungs-Systems eingeschränkt werden, sondern vielmehr durch die

Wahl ungeeigneter Pflanzenmerkmale zur Charakterisierung von Genotypen und Pflanzenmaterialien unterschiedlicher Reifegrade.

Alternative Energiepflanzen zu Mais zeigten eine größere Variationsbreite in ihren spezifischen Methanerträgen. Die spezifischen Methanerträge wurden dabei sehr stark von der chemischen Zusammensetzung beeinflusst. Reproduktive Pflanzenteile von fettreichen Pflanzen (Sonnenblume und Raps) wiesen höhere spezifische Methanerträge mit einem Durchschnitt von 0.598 m_N^3 CH_4 / kg oTS auf. Der Stängel dieser Pflanzen schien der am meisten limitierende Faktor für die Zersetzung zu sein. Trotz des hohen Anteils an Fett und Protein im Sonnenblumenstängel lag der Methanertrag (0,201 - 0,284 m_N^3 CH_4/kg oTS) deutlich unter dem von grünem Maisstroh (0.300 m_N^3 CH_4/kg oTS). Umgekehrt dazu zeigten kohlenhydratreiche Pflanzen den gleichen oder einen geringfügig niedrigeren Methanertrag als Mais. Diese Pflanzen kommen als Alternative infrage, wenn die klimatischen Bedingungen für den Maisanbau ungünstig sind.

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11 Appendix

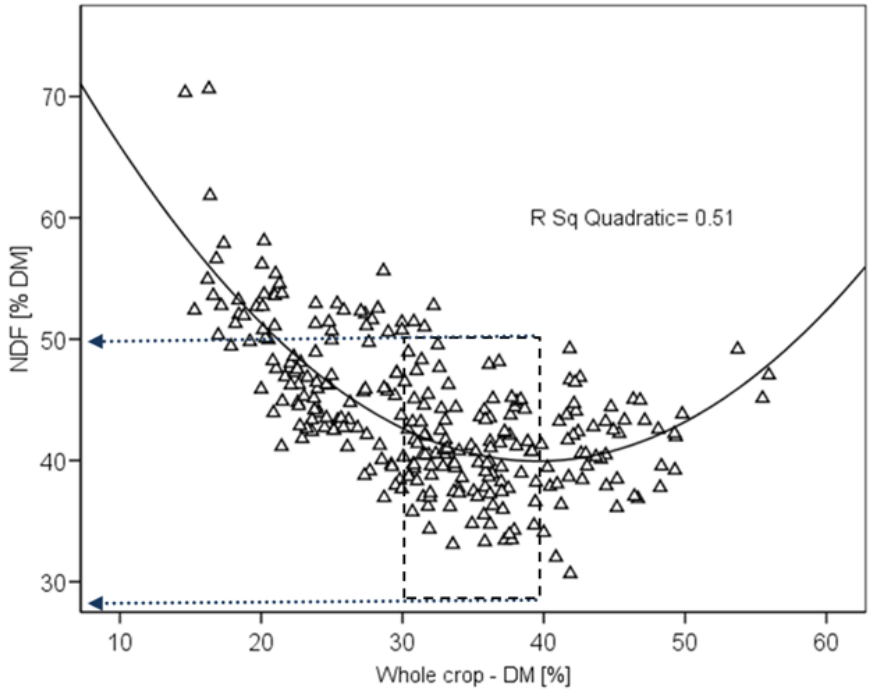

Figure A-1: Relationship between cell-wall (NDF) and total DM contents for various maize genotypes (the crosshatched area shows the NDF content variation in the zone commonly considered as optimum for silage maize harvest) [n=304].

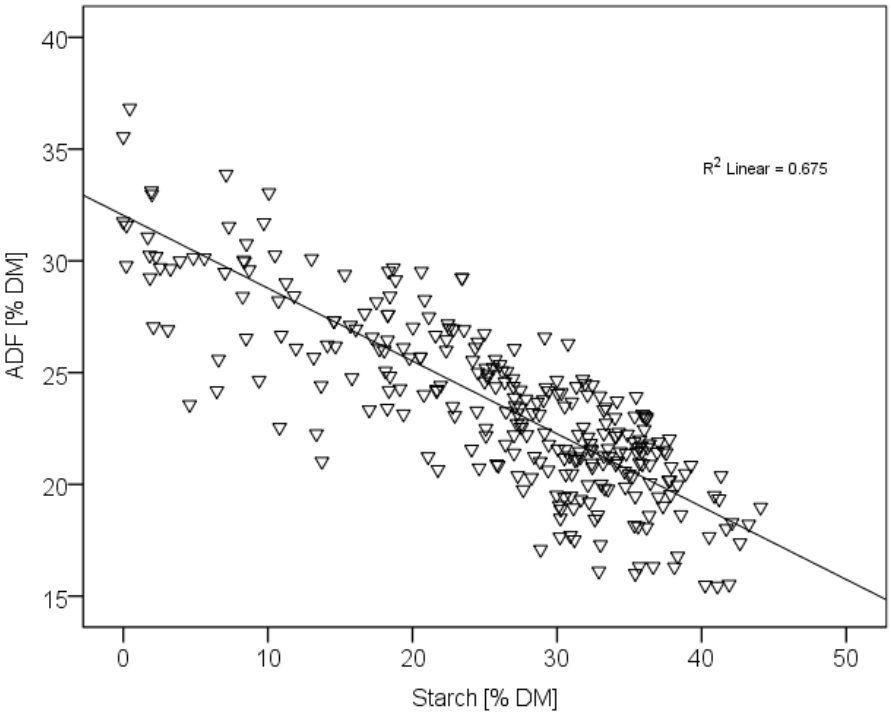

Figure A-2: Relationship between acid detergent fiber (ADF) and starch content of maize.

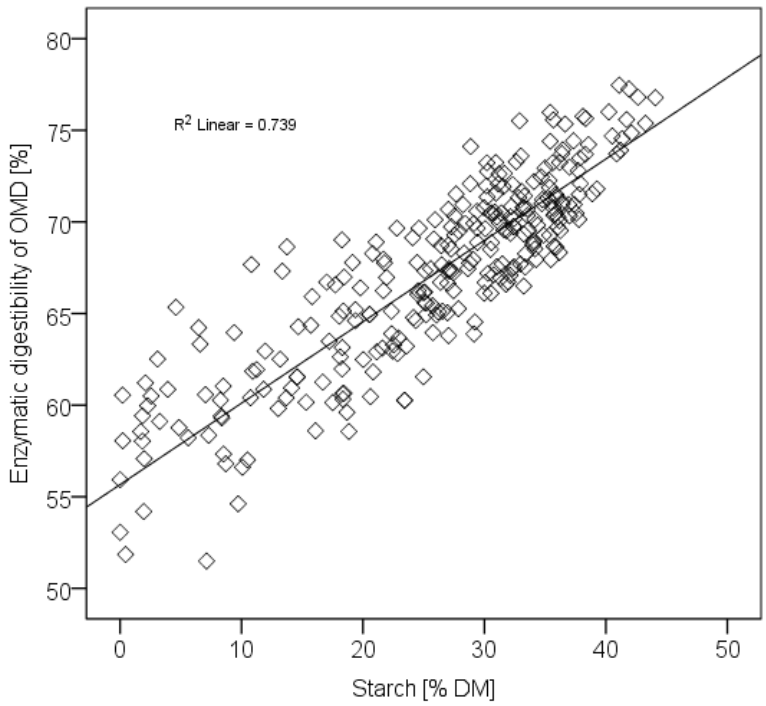

Figure A-3: Relationship between CDOMD (also called enzymatic digestibility of ODM) and the starch content of maize.

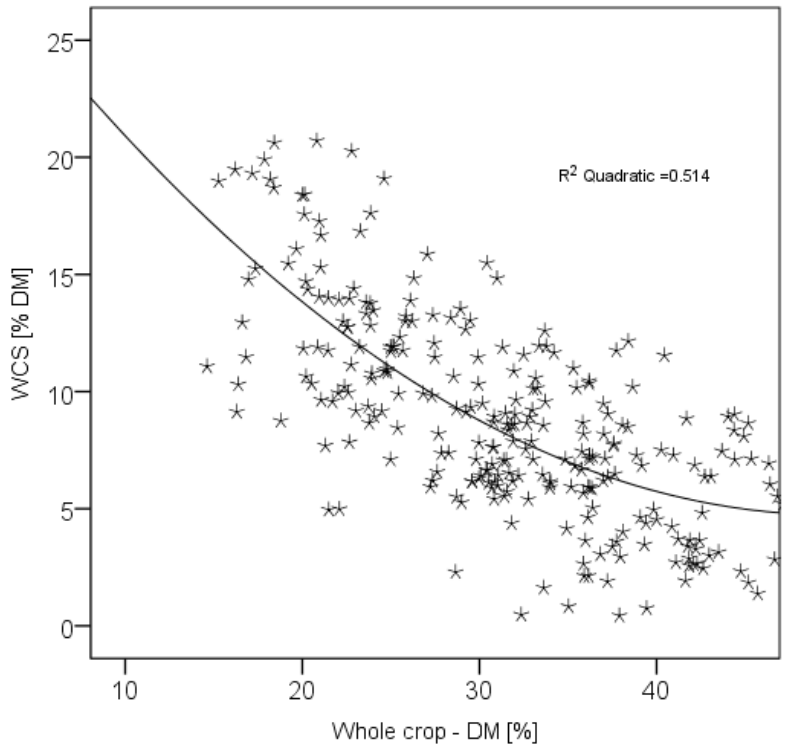

Figure A-4: Relationship between water soluble carbohydrates (WSC) and total dry matter (DM) contents for various maize genotypes.

Table A-1: Welch one-way analysis of variance for specific methane yield between years.

Robust Tests of Equality of Means

Spec. methane yield [m_N^3/kg ODM]

	Statistic[a]	df1	df2	Sig.
Welch	8.783	4	139.118	.000

a. Asymptotically F distributed.

Table A-2: Welch one-way analysis of variance for specific methane yield potentials between locations

Robust Tests of Equality of Means

Spec. methane yield [m_N^3/kg ODM]

	Statistic[a]	df1	df2	Sig.
Welch	20.955	8	38.616	.000

a. Asymptotically F distributed.

Table A-3: Post Hoc test (Games-Howell) for the analysis of variance between locations.

Multiple Comparisons

Dependent variable: Spec. methane yield [m_N^3/kg ODM] Games-Howell

(I) Location		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Weser Ems (D)	Freising (D)	.000290	.001502	1.000	-.00443	.00501
	Karlshof (D)	.002819	.002593	.974	-.00549	.01113
	Ingolstadt (D)	-.003302	.002810	.956	-.01264	.00604
	Bernburg (D)	-.004745	.002704	.707	-.01529	.00580
	Kehlen (Lu)	.017765*	.004067	.035	.00118	.03435
	Marnach (Lu)	.027367*	.002906	.002	.01367	.04107
	Pletschetterhof (Lu)	.009643*	.001441	.000	.00480	.01449
	Tittenkofen (D)	-.007305	.002510	.128	-.01571	.00110
Freising (D)	Weser Ems (D)	-.000290	.001502	1.000	-.00501	.00443
	Karlshof (D)	.002529	.002616	.988	-.00584	.01090
	Ingolstadt (D)	-.003592	.002832	.933	-.01298	.00580
	Bernburg (D)	-.005035	.002726	.657	-.01559	.00552
	Kehlen (Lu)	.017475*	.004082	.038	.00089	.03406
	Marnach (Lu)	.027077*	.002927	.002	.01344	.04071
	Pletschetterhof (Lu)	.009353*	.001483	.000	.00440	.01430
	Tittenkofen (D)	-.007595	.002534	.106	-.01606	.00087
Karlshof (D)	Weser Ems (D)	-.002819	.002593	.974	-.01113	.00549
	Freising (D)	-.002529	.002616	.988	-.01090	.00584
	Ingolstadt (D)	-.006121	.003533	.724	-.01749	.00525
	Bernburg (D)	-.007564	.003449	.442	-.01935	.00422
	Kehlen (Lu)	.014946	.004596	.101	-.00197	.03186
	Marnach (Lu)	.024548*	.003610	.000	.01109	.03801
	Pletschetterhof (Lu)	.006824	.002581	.193	-.00151	.01515
	Tittenkofen (D)	-.010124	.003299	.073	-.02075	.00050
Ingolstadt (D)	Weser Ems (D)	.003302	.002810	.956	-.00604	.01264
	Freising (D)	.003592	.002832	.933	-.00580	.01298
	Karlshof (D)	.006121	.003533	.724	-.00525	.01749
	Bernburg (D)	-.001443	.003616	1.000	-.01379	.01091
	Kehlen (Lu)	.021067*	.004722	.012	.00389	.03824
	Marnach (Lu)	.030668*	.003769	.000	.01683	.04451
	Pletschetterhof (Lu)	.012945*	.002800	.002	.00359	.02230
	Tittenkofen (D)	-.004003	.003473	.962	-.01533	.00732
Bernburg (D)	Weser Ems (D)	.004745	.002704	.707	-.00580	.01529
	Freising (D)	.005035	.002726	.657	-.00552	.01559
	Karlshof (D)	.007564	.003449	.442	-.00422	.01935
	Ingolstadt (D)	.001443	.003616	1.000	-.01091	.01379
	Kehlen (Lu)	.022510*	.004660	.009	.00512	.03990
	Marnach (Lu)	.032112*	.003691	.000	.01776	.04647
	Pletschetterhof (Lu)	.014388*	.002693	.008	.00379	.02498
	Tittenkofen (D)	-.002560	.003388	.997	-.01435	.00924

*. The mean difference is significant at the 0.05 level.

D: Germany Lu: Luxemburg

Table A-4: Post Hoc test (Games-Howell) for the analysis of variance between locations.

Multiple Comparisons

Dependent variable: Spec. methane yield [m_N^3/kg ODM] Games-Howell

(I) Location		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Kehlen (Lu)	Weser Ems (D)	-.017765*	.004067	.035	-.03435	-.00118
	Freising (D)	-.017475*	.004082	.038	-.03406	-.00089
	Karlshof (D)	-.014946	.004596	.101	-.03186	.00197
	Ingolstadt (D)	-.021067*	.004722	.012	-.03824	-.00389
	Bernburg (D)	-.022510*	.004660	.009	-.03990	-.00512
	Marnach (Lu)	.009602	.004780	.567	-.00852	.02772
	Pletschetterhof (Lu)	-.008122	.004060	.579	-.02473	.00849
	Tittenkofen (D)	-.025070*	.004550	.003	-.04200	-.00814
Marnach (Lu)	Weser Ems (D)	-.027367*	.002906	.002	-.04107	-.01367
	Freising (D)	-.027077*	.002927	.002	-.04071	-.01344
	Karlshof (D)	-.024548*	.003610	.000	-.03801	-.01109
	Ingolstadt (D)	-.030668*	.003769	.000	-.04451	-.01683
	Bernburg (D)	-.032112*	.003691	.000	-.04647	-.01776
	Kehlen (Lu)	-.009602	.004780	.567	-.02772	.00852
	Pletschetterhof (Lu)	-.017724*	.002896	.018	-.03152	-.00393
	Tittenkofen (D)	-.034672*	.003551	.000	-.04821	-.02113
Pletschetterhof (Lu)	Weser Ems (D)	-.009643*	.001441	.000	-.01449	-.00480
	Freising (D)	-.009353*	.001483	.000	-.01430	-.00440
	Karlshof (D)	-.006824	.002581	.193	-.01515	.00151
	Ingolstadt (D)	-.012945*	.002800	.002	-.02230	-.00359
	Bernburg (D)	-.014388*	.002693	.008	-.02498	-.00379
	Kehlen (Lu)	.008122	.004060	.579	-.00849	.02473
	Marnach (Lu)	.017724*	.002896	.018	.00393	.03152
	Tittenkofen (D)	-.016948*	.002499	.000	-.02539	-.00851
Tittenkofen (D)	Weser Ems (D)	.007305	.002510	.128	-.00110	.01571
	Freising (D)	.007595	.002534	.106	-.00087	.01606
	Karlshof (D)	.010124	.003299	.073	-.00050	.02075
	Ingolstadt (D)	.004003	.003473	.962	-.00732	.01533
	Bernburg (D)	.002560	.003388	.997	-.00924	.01435
	Kehlen (Lu)	.025070*	.004550	.003	.00814	.04200
	Marnach (Lu)	.034672*	.003551	.000	.02113	.04821
	Pletschetterhof (Lu)	.016948*	.002499	.000	.00851	.02539

*. The mean difference is significant at the 0.05 level.

D: Germany Lu: Luxemburg

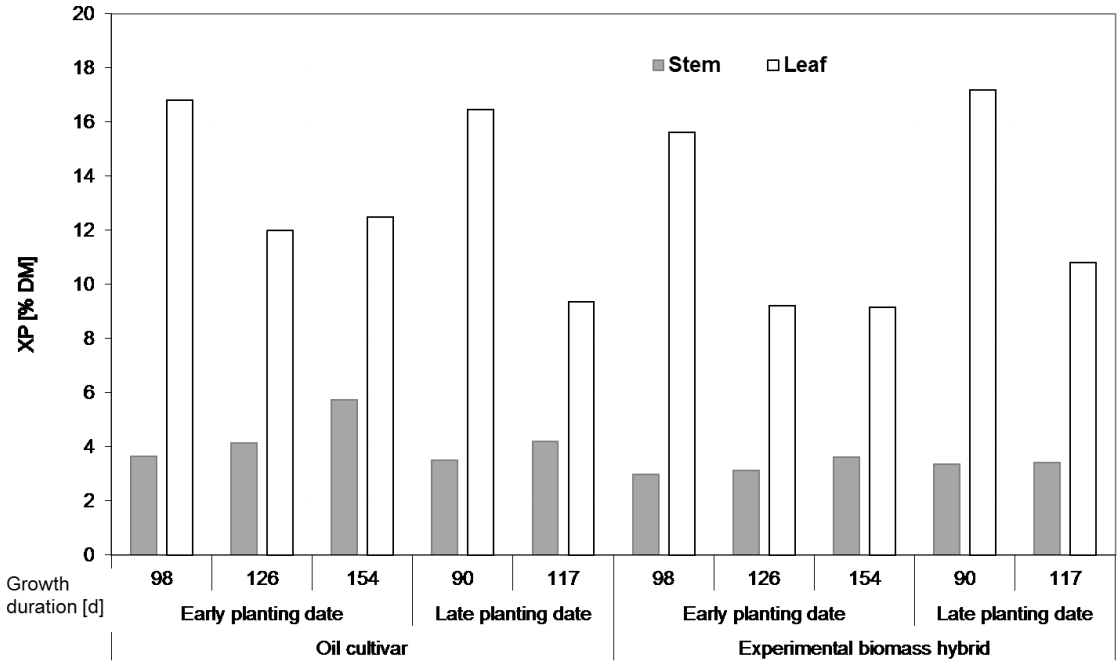

Figure A-5: Protein content of the sunflower leaf and stem at different growth stages.

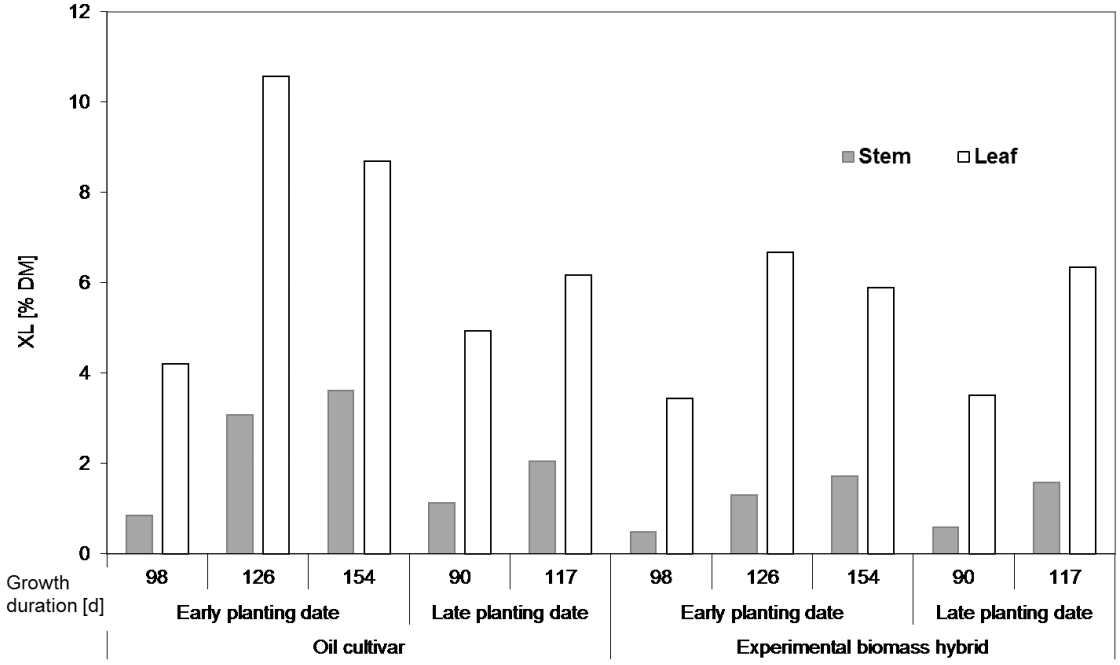

Figure A-6: Lipids content of the sunflower leaf and stem at different growth stages.

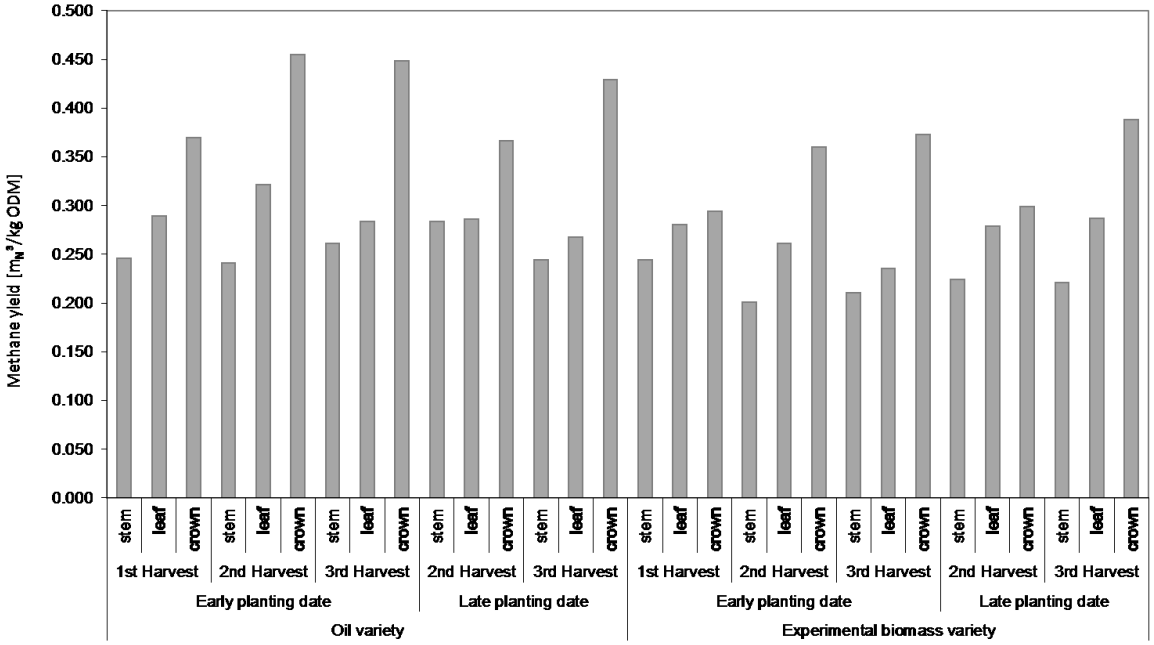

Figure A-7: Specific methane yields of different fractions of sunflower.

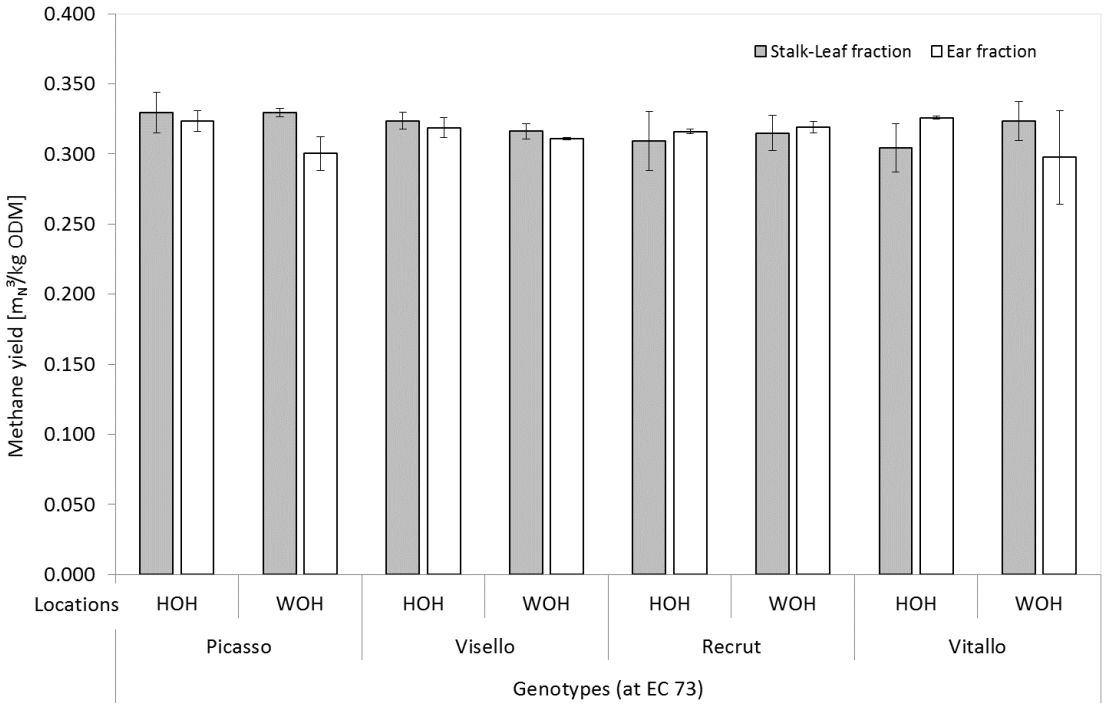

Figure A-8: Specific methane yield potential of Rye crop fractions at the early milk stage (EC 73) in two different locations.

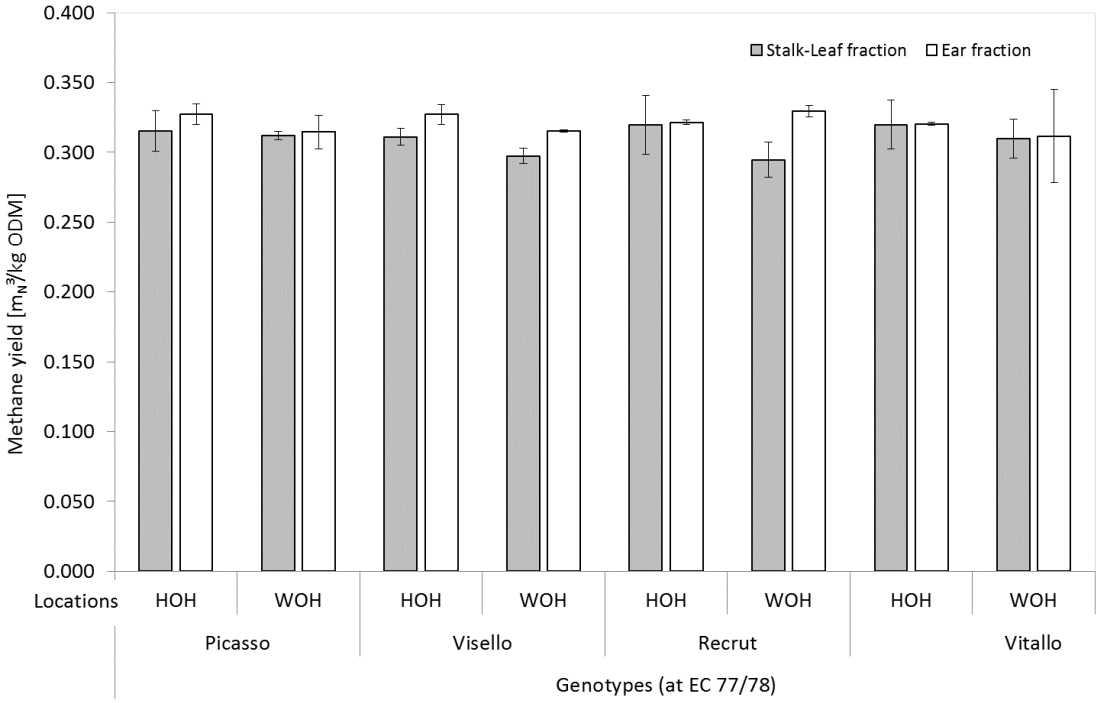

Figure A-9: Specific methane yield potential of Rye crop fractions at the late milk-early dough stage (EC 77/78) in two different locations.

Table A-5: Biochemical traits and spec. methane yield of rye fractions at EC73.

Location	BBCH-Scale	Genotype	Fraction	Spec. CH_4 - Yield	XP	XL	XF	ADF	ADL	NDF
				[m_N^3 CH_4/kg ODM]	[% DM]					
HOH	EC73	Picasso	Ear	0.324 [±1.6%]	9.60 [±1.0%]	1.90 [±10.5%]	21.35 [±1.2%]	24.20 [±1.2%]	3.00 [±3.3%]	45.70 [±0.2%]
		Picasso	Stalk-Leaf	0.330 [±3.1%]	5.30 [±3.8]	1.70 [±17.6%]	31.70 [±1.6%]	35.10 [±1.1%]	3.35 [±4.5%]	56.00 [±1.4%]
		Picasso	Stubble	0.316 [±1.6%]	1.90 [±5.3%]	0.83 [±9.1%]	30.85 [±4.1%]	34.75 [±3.9%]	3.90 [±2.6]	53.80 [±4.1%]
		Visello	Ear	0.319 [±1.5%]	9.25 [±1.6%]	1.85 [±13.5%]	20.80 [±0.5%]	23.80 [±0.4%]	2.90 [±0.0%]	44.10 [±0.5%]
		Visello	Stalk-Leaf	0.324 [±1.3%]	5.55 [±6.3%]	1.75 [±2.9%]	32.55 [±1.4%]	36.00 [±0.8%]	3.50 [±2.9%]	57.65 [±0.8%]
		Visello	Stubble	0.316 [±2.7%]	1.95 [±7.7%]	1.04 [±5.8%]	32.60 [±4.0%]	36.45 [±4.5%]	4.20 [±9.5%]	56.85 [±3.3%]
		Recrut	Ear	0.316 [±0.4%]	8.90 [±1.1%]	1.70 [±11.7%]	20.35 [±6.1%]	24.10 [±5.0%]	3.25 [±1.5%]	43.70 [±4.6%]
		Recrut	Stalk-Leaf	0.309 [±4.8%]	4.90 [±2.0%]	1.85 [±2.7%]	32.60 [±6.4%]	37.20 [±4.3%]	4.10 [±2.4%]	57.65 [±4.2%]
		Recrut	Stubble	0.313 [±2.2%]	1.85 [±8.1%]	1.05 [±4.8%]	34.55 [±4.2%]	39.30 [±3.8%]	5.10 [±0.0%]	59.90 [±4.3%]
		Vitallo	Ear	0.326 [±0.2%]	8.95 [±0.6%]	1.95 [±17.9%]	17.60 [±0.0%]	21.10 [±1.4%]	2.95 [±5.1%]	40.15 [±1.4%]
		Vitallo	Stalk-Leaf	0.304 [±4.0%]	4.65 [±5.4%]	1.50 [±0.0%]	32.05 [±1.4%]	36.55 [±1.0%]	4.10 [±4.9%]	56.95 [±0.1%]
		Vitallo	Stubble	0.303 [±1.8%]	1.70 [±0.0%]	0.85 [±18.3%]	33.70 [±1.2%]	38.90 [±0.3%]	5.45 [±0.9%]	58.15 [±0.8%]
HOH	EC77/83	Picasso	Ear	0.327 [±3.5%]	8.15 [±0.6%]	2.00 [±0.0%]	13.24 [±2.7%]	16.15 [±3.4%]	2.60 [±7.7%]	37.95 [±0.1%]
		Picasso	Stalk-Leaf	0.315 [±1.2%]	5.00 [±2.0%]	1.60 [±12.5%]	34.45 [±2.2%]	39.80 [±2.0%]	4.20 [±2.4%]	62.35 [±1.5%]
		Picasso	Stubble	0.311 [±0.1%]	2.00 [±5.0%]	0.72 [±11.1%]	34.70 [±2.9%]	41.25 [±4.5%]	4.85 [±11.3%]	63.00 [±2.1%]
		Visello	Ear	0.327 [±2.7%]	8.10 [±1.2%]	1.75 [±8.6%]	12.05 [±0.4%]	15.65 [±3.5%]	2.65 [±5.7%]	35.45 [±3.8%]
		Visello	Stalk-Leaf	0.311 [±0.3%]	5.20 [±2.0%]	1.70 [±5.9%]	35.70 [±1.7%]	40.80 [±0.7%]	4.35 [±3.4%]	64.00 [±0.3%]
		Visello	Stubble	0.324 [±2.1%]	2.05 [±7.3%]	1.15 [±4.3%]	35.30 [±1.9%]	41.60 [±1.2%]	4.95 [±3.0%]	63.55 [±2.1%]
		Recrut	Ear	0.321 [±2.9%]	7.50 [±1.3%]	1.75 [±2.9%]	12.15 [±1.2%]	15.50 [±1.9%]	2.35 [±2.1%]	34.75 [±1.9%]
		Recrut	Stalk-Leaf	0.320 [±1.4%]	4.20 [±4.8%]	1.55 [±3.2%]	35.05 [±3.9%]	40.75 [±2.1%]	4.45 [±3.4%]	61.90 [±1.5%]
		Recrut	Stubble	0.308 [±1.3%]	1.85 [±8.1%]	1.01 [±9.5%]	36.35 [±1.8%]	43.20 [±1.9%]	5.25 [±4.8%]	64.70 [±2.5%]
		Vitallo	Ear	0.320 [±1.2%]	8.17 [±0.3%]	1.95 [±2.6%]	11.95 [±2.9%]	15.30 [±0.7%]	2.15 [±2.3%]	35.75 [±3.8%]
		Vitallo	Stalk-Leaf	0.320 [±0.24%]	3.80 [±0.0%]	1.30 [±0.0%]	34.45 [±0.7%]	40.30 [±0.2%]	4.25 [±3.5%]	61.55 [±0.6%]
		Vitallo	Stubble	0.308 [±0.4%]	1.55 [±3.2%]	0.77 [±2.6%]	35.80 [±1.1%]	42.45 [±0.6%]	5.90 [±1.7%]	62.50 [±0.3%]

Table A-6: Biochemical traits and spec. methane yield of rye fractions at EC77/78.

Location	BBCH-Scale	Genotype	Fraction	Spec. CH_4 - Yield	XP	XL	XF	ADF	ADL	NDF
				[m_N^3 CH_4/kg ODM]			[% DM]			
WOH	EC73	Picasso	Ear	0.300 [±2.8%]	9.85 [±0.5%]	1.95 [±2.6%]	14.05 [±3.2%]	16.90 [±3.6%]	3.00 [±0.0%]	38.10 [±2.6%]
		Picasso	Stalk-Leaf	0.330 [±0.7%]	6.50 [±0.0%]	1.50 [±6.7%]	34.80 [±0.9%]	38.55 [±0.4%]	4.50 [±2.2%]	60.30 [±0.0%]
		Picasso	Stubble	0.311 [±0.1%]	3.30 [±6.1%]	1.00 [±0.0%]	35.55 [±0.4%]	39.55 [±0.1%]	4.70 [±2.1%]	61.05 [±2.0%]
		Visello	Ear	0.311 [±0.2%]	9.55 [±0.5%]	2.10 [±0.0%]	12.40 [±2.4%]	16.10 [±3.7%]	3.10 [±3.2%]	36.10 [±2.5%]
		Visello	Stalk-Leaf	0.316 [±1.2%]	6.60 [±3.0%]	1.80 [±0.0%]	34.35 [±0.1%]	38.20 [±0.3%]	4.25 [±3.5%]	60.00 [±0.7%]
		Visello	Stubble	0.319 [±2.4%]	3.10 [±3.2%]	0.99 [±1.5%]	35.35 [±0.4%]	39.45 [±0.1%]	4.60 [±4.3%]	61.05 [±0.1%]
		Recrut	Ear	0.319 [±0.9%]	9.40 [±3.2%]	2.10 [±4.8%]	13.05 [±0.4%]	16.05 [±2.2%]	2.80 [±3.6%]	35.40 [±3.1%]
		Recrut	Stalk-Leaf	0.315 [±2.8%]	5.25 [±2.9%]	1.65 [±9.1%]	36.30 [±1.9%]	40.05 [±0.6%]	5.25 [±1.0%]	61.35 [±1.2%]
		Recrut	Stubble	0.294 [±3.1%]	2.70 [±3.7%]	1.00 [±0.0%]	37.50 [±2.7%]	42.40 [±1.7%]	5.85 [±0.9%]	63.70 [±2.2%]
		Vitallo	Ear	0.298 [±7.9%]	9.80 [±3.1%]	1.85 [±2.7%]	12.90 [±13.9%]	15.45 [±4.2%]	2.85 [±8.8%]	51.15 [±35.7%]
		Vitallo	Stalk-Leaf	0.324 [±3.0%]	5.70 [±22.8%]	1.48 [±11.9%]	34.70 [±2.3%]	38.60 [±2.1%]	4.50 [±0.0%]	59.85 [±1.8%]
		Vitallo	Stubble	0.325 [±3.3%]	2.95 [±25.4%]	0.72 [±8.3%]	36.00 [±2.5]	40.05 [±1.1%]	4.95 [±3.0%]	61.20 [±1.3%]
WOH	EC77/83	Picasso	Ear	0.315 [±4.0%]	8.75 [±1.7%]	1.95 [±2.6%]	10.40 [±1.9%]	13.35 [±3.4%]	2.55 [±2.0%]	32.90 [±0.3%]
		Picasso	Stalk-Leaf	0.312 [±0.6%]	6.60 [±6.1%]	1.60 [±6.3%]	36.57 [±1.3%]	41.45 [±1.1%]	5.05 [±8.9%]	64.45 [±0.1%]
		Picasso	Stubble	0.307 [±3.8%]	3.35 [±10.4%]	0.90 [±10.6%]	37.90 [±1.1%]	43.35 [±0.3%]	5.85 [±0.9%]	65.30 [±0.9%]
		Visello	Ear	0.316 [±1.2%]	8.20 [±0.0%]	1.70 [±0.0%]	9.45 [±2.6%]	12.10 [±3.3%]	2.36 [±1.9%]	38.45 [±19.9%]
		Visello	Stalk-Leaf	0.297 [±0.8%]	6.30 [±3.2%]	1.82 [±1.1%]	36.85 [±0.7%]	40.95 [±1.1%]	5.15 [±1.0%]	64.75 [±0.1%]
		Visello	Stubble	0.319 [±0.2%]	2.85 [±8.8%]	1.07 [±22.1%]	38.26 [±1.5%]	42.90 [±2.3%]	5.40 [±0.0%]	65.15 [±1.2%]
		Recrut	Ear	0.329 [±3.9%]	8.50 [±1.2%]	1.90 [±5.3%]	9.25 [±3.8%]	12.35 [±3.6%]	2.30 [±0.0%]	29.80 [±3.4%]
		Recrut	Stalk-Leaf	0.295 [±0.2%]	5.25 [±2.9%]	1.60 [±6.3%]	38.70 [±0.3%]	43.45 [±0.1%]	6.10 [±3.3%]	65.40 [±0.3%]
		Recrut	Stubble	0.282 [±0.7%]	2.70 [±3.7%]	1.01 [±9.5%]	39.85 [±0.1%]	46.10 [±0.9%]	7.05 [±2.1%]	67.35 [±1.1%]
		Vitallo	Ear	0.312 [±0.0%]	9.30 [±3.2%]	1.85 [±8.1%]	8.60 [±3.5%]	11.55 [±4.8%]	2.10 [±4.8%]	29.50 [±0.3%]
		Vitallo	Stalk-Leaf	0.310 [±1.4%]	4.30 [±7.0%]	1.40 [±7.1%]	38.30 [±0.8%]	43.10 [±0.9%]	5.30 [±9.4%]	66.05 [±0.8%]
		Vitallo	Stubble	0.286 [±2.9%]	2.05 [±7.3%]	0.84 [±14.2%]	39.85 [±0.6%]	44.55 [±0.3%]	6.70 [±3.0%]	65.90 [±0.2%]

Table A-7: Descriptive statistics of eight sorghum genotypes harvested after 117 and 133 days growing periods.

	N	Minimum	Maximum	Mean	Std. Deviation
Starch [% DM]	16	0.0	15.4	6.1	5.8
WSC [% DM]	16	6.9	27.3	16.2	6.6
XF [% DM]	16	21.4	30.3	26.0	2.8
Crude protein [% DM]	16	7.8	10.8	9.5	1.0
Lignin [% DM]	16	3.4	5.1	4.3	0.5
Methane yield [m_N^3/kg ODM]	16	0.279	0.334	0.318	0.014
Valid N (listwise)	16				