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Parameters for sustainable and demand-oriented biogas production

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Abstract

Biogas production offers a possibility to generate electricity and heat by renewable sources. It has a great potential in terms of availability. Unlike solar radiation and wind energy, biogas production does not underlie natural fluctuations. Biogas substrates can be stored and are therefore available at all times. In an ideal scenario, biogas production should meet certain criteria. It should be (1) sustainable, (2) provide the highest possible system effectiveness and (3) must be available in times of high electricity demand. This work concentrates on aspects of all three criteria.

Biogas production in a highly sustainable way can be found on organic farms (Manuscript 1). Therefore, we analysed 13 biogas plants on organic farms and looked for improvement potentials. The survey concentrated on biogas plant specifications, fermentation characteristics and feedstock compositions. The biogas plants were tested repeatedly to investigate the impact of fermentation characteristics on volatile solids (VS) degradation, indicating the effectiveness of the anaerobic digestion process. The total feedstock composed mainly of livestock residues (61 %) and grass silage (14 %). Such substrates are considered ecologically beneficial (Danner & Kilian 2012; Insam et al. 2015), when compared to possible negative effects of maize (Svoboda et al. 2015). By comparing the feedstock composition to the biogas plant specifications we found significant correlations between the amount of livestock residues and the dimension of the combined heat and power (CHP) unit. With an increasing share of livestock residues in the feedstock the installed electrical capacity of these biogas plants became smaller. Within the fermentation characteristics, the process temperatures ranged between 17 - 49 °C. Low temperatures were found to be the driving factor for low VS degradation (range 11.67 - 87.70 %) in the residue storage. Significantly better VS degradations were found in biogas plants with two reactor stages compared to one reactor stage. Effluents with high VS concentrations can cause harmful greenhouse gas emissions (Ruile et al. 2015). Backfitting a cover on the residue storage is highly recommended, as most biogas plant residue storages were found to be installed without (> 84 %). To maintain a highly sustainable biogas production on organic farms, the maximisation of VS degradation is required. Therefore, the biogas plants' VS degradation could be raised by increasing the process temperatures and/or the hydraulic retention time.

Most biogas plants in Germany are, however, installed on conventional farms, accounting for > 99 % of the total installed electrical capacity. Here, maize silage is the most important substrate with the highest share on the total feedstock (ca. 40 % fresh weight) (FNR 2014). During the anaerobic digestion in biogas plants, the maximisation of carbon transformation from substrate to biogas is a desired goal of every biogas plant operator. It increases the system effectiveness of the biogas plant and therefore its economical sustainability. The carbon degradation can be quantified by common carbon degradation determination methods (C deg_{mix}) (VDLUFA 1997). With this method, it is not possible to quantify substrate-bound carbon degradation independently of the inoculum. Therefore, we tested an isotope-based method in reactor mixtures of maize silage and inoculum from agricultural biogas plants (Manuscript 2) to identify solely substrate-bound carbon degradation (C deg f_{MS}). As stable isotopes underlie natural variability, the method was tested on six different maize silages of different farming systems (organic or conventional farming) and maize silages of high (MS high) and low (MS low) qualities. A total of 19 lab-scale batch reactors were analysed for digestion parameters, specific biogas (sby) and methane yields (smy), stable isotopes and carbon degradations. Reactors with MS high showed up to 23.8 % higher sby and smy. The carbon degradations in reactors with MS low were significantly lower compared to reactors containing MS high. The C deg f_{MS} values were, however, significantly higher compared to C $deg_{mix,}$ as it excludes the masking effect of the inoculum. At the start of the experiments, the stable carbon isotope values (δ13C) in the reactors were highly variable. Nonetheless, C deg f_{MS} showed reliable values independently of the maize silage qualities. The effect of the farming system of the maize silages was found negligible in both carbon degradation methods. We found that the isotope-based carbon degradation determination method offers a possibility to assess the substrate-bound carbon degradation in more detail and contributes to our understanding of the total system effectiveness.

In a future scenario of 100 % renewable energy in Europe, a large contribution will come from wind and photovoltaic energy generation (Steinke et al. 2013). Such resources underlie, however, strong temporal fluctuations. This creates balancing challenges in the energy systems (Hahn et al. 2014). In times of sun and wind deficits energy shortage could partly be filled in by biogas plants (SRU 2011), having relatively short start-up phases. The biogas production is,

however, dependent on the microbial transformation of the substrate. Therefore, a quickly adaptable microbial community is necessary to cope with flexible substrate inputs. In our survey (Manuscript 3) we focused on structural changes of microbial communities in four lab-scale reactors. Every reactor was continuously fed with different mixtures of maize silage (MS) and sugar beet silage (SBS). The mixing ratio was 1:0 (VS ratios of MS and SBS) in continuous fermenter one (CF1), 6:1 (CF2), 3:1 (CF3) and 1:3 (CF4), respectively. The organic loading rates were equal in all reactors with 1.25 kg VS m^{-3} d^{-1}. Bacterial and archaeal communities' compositions were analysed with 454 amplicon sequencing technique on the basis of 16S rRNA genes. With increasing amounts of SBS, the bacterial and archaeal communities' compositions shifted. Despite the shifts, biogas production rates and methane concentrations were similar in all reactors. This showed that the communities adapted to different environmental conditions induced by different substrate mixtures and ensured biogas production efficiency.

In conclusion, my work contributes to parameters for optimized biogas production scenarios. For sustainable biogas production on organic farms deficits were detected, which could be reduced by increasing the VS degradations during the fermentation process. High VS degradation is a crucial factor for biogas system effectivity. In conventional biogas plants high VS degradation of maize silage is also a desired goal, which is resembled by carbon degradation. The application of a stable isotope-based method for carbon degradation determination of maize silage showed reliable and more detailed results. This increases our understanding of anaerobic degradation effectiveness in biogas reactors. For demand-oriented biogas production, quickly adaptable microbial communities are crucial prerequisites. Different substrate feeding regimes in biogas reactors resulted in compositional changes of microbial communities by simultaneously ensuring biogas production efficiency. This implicates the functional redundancy of microbial communities within biogas plant reactors.

The outcome of this thesis contributes to future-oriented biogas production. Optimised biogas production can improve environmental sustainability and national energy sufficiency. This supports the creditability of biogas production and emphasises its importance among all renewable energies.

INTRODUCTION

This thesis focuses on sustainable biogas production on organic farms and high inner system efficiency. It further depicts the functional adaptability of microorganisms in continuous biogas experiments with focus on demand-oriented biogas production. The introduction gives an overview on the energetic situation of Germany including biogas production and organic farming. It further considers the importance of maize silage on the actual renewable energy production and the future role of biogas as a flexible, on-demand available power source.

Renewable energies in Germany

The worldwide exploitation of natural resources over the last decades has led to apparent affluence but simultaneously to drastic social, ecological and economic conflicts. The primary sources for energy production have been fossil fuels. Their excessive combustion and greenhouse gas emission implicate negative long-term effects like global warming (Meinshausen et al. 2009). In December 2015, the United Nations Framework Convention on Climate Change took place. This international alliance agreed on holding the global average temperature increase to < 2°C compared to pre-industrial times (UNFCCC 2015). They provide obligations and compliance procedures for developed and developing countries to achieve global response to the threat of global warming and climate change. To meet this global target, 33 % of oil, 50 % of gas and > 80 % of coal reserves need to remain unused (McGlade & Ekins 2015). In any case, fossil fuels are expected to exhaust within the next 137 years for coal and the next half century for petroleum and natural gas, or earlier (Brown & Sovacool 2011). An environmentally friendly, conscious and more efficient handling of these finite natural resources is more important than ever for a worldwide sustainable development. Therefore, substitution of fossil fuel based energy production by renewable energies is essential.

The European Union (EU) has been reliant on primary energy imports from other countries for a long time. In 2013 the 28 EU member states' gross inland energy consumption was dependent on 53.2 % of energy imports (Eurostat 2015). To reduce energy import dependency and to reduce greenhouse gas emissions, the European Commission set an ambitious target of a 20.0 %

share of energy from renewable sources in the EU's gross inland energy consumption by 2020 (EU 2009). This 'RES-Directive' sets obligatory targets for each member state. Therefore, the 'EU roadmap' was established to implement and promote the use of energy from renewable sources on national level. According to the RES-Directive, Germany is expected to generate 18.0 % of its gross energy consumption by renewable energies in 2020. Including Germany, 16 countries already forecasted to exceed their national 2020 targets (Zervos et al. 2011).

Before the RES-Directive, German government had already established the Renewable Energies Sources Act (EEG), which entered into force in 2001. This EEG law aimed to establish an expansion of renewable energies by fixed governmental subsidies. A purchase guarantee and the priority feed-in of electricity generated by renewable sources into the grid were included in the EEG to support Germany's energy transition. The EEG guaranteed a continuous fixed feed-in tariff to renewable energy plant operators for electricity fed into the grid over a period of 20 years. In 2014 Germany already had a renewable energy share on gross final energy consumption of 13.7 % and on gross electricity consumption of 27.4 % (BMWi 2015a). In 2014 14.5 billion euro were given out for economic stimuli from the operation of renewable energy plants (BMWi 2015b). Long-term strategies concerning energy security in an economically viable and ecologically sound direction have been proposed in Germany's 'Energy Concept'. The ambitious targets of the Energy Concept aim to increase the renewable energy share on gross final energy consumption to 30 % by 2030 and 60 % by 2050. The gross electricity production from renewable energy sources are planned to be increased to 35 % by 2020, 50 % by 2030, 65 % by 2040 and 80 % by 2050. Simultaneously, greenhouse gas emissions are targeted to be reduced by 40 % in 2020, 55 % in 2030, 70 % in 2050 and >80 % by 2050 compared to base year 1990 (BMWi 2010). The total renewable-based electricity generation in 2015 accounted for 187.3 TWh. Onshore wind energy production and photovoltaics contributed greatly to this with a share of 37.9 and 20.7 %, respectively. The highest contributor in terms of non-intermittent renewable-based electricity production was, however, based on biogas transformation with a share of 16.8 % (AGEE-Stat 2016).

Biogas development in Germany

Biogas combustion is one technique for renewable energy production. During the anaerobic process, biomass gets metabolised to a gas mixture by microbial communities. The gas mixture consists mainly of methane (CH_4), which can be transformed to electrical energy and heat by a combined heat and power (CHP) unit. One CH_4 m³ contains 10 kWh energy, which corresponds to the energy contained in one litre of heating oil (FNR 2014). Biomass is, in contrast to fossil fuels, CO_2 neutral as it releases only the same amount of CO_2 as was previously stored in the biomass. Biomass is regenerative and can contribute as energy source for future generations.

Biogas production already has a long history in Germany. It originates from waste water treatment plants of the early 20th century. The biogas potential of agricultural materials was firstly considered after 1945 (Eder & Schulz 2012). It still took till after the oil crisis in the 1970's to establish biogas plants for energy production from agricultural materials. In the 1980´s ca. 75 to 150 biogas plants were established on farms, mostly in the southern part of Germany. Organic farmers are regarded as the pioneers of energy production through biogas combustion. They often belonged to anti-nuclear or environment protection movements (Anspach 2010) and focused on energy self-sufficiency. A steady increase of newly installed biogas plants appeared with introduction of the EEG 2000. In 2001 a number of 1300 biogas plants were present in Germany which increased to 3500 in 2006 and 8213 in 2012 (German Biogas Association 2016). This rapid development appeared due to a certain bonus system in different EEG amendments, enabling biogas plant operators to increase their profits when fulfilling the according requirements. With the EEG 2004 energy crop and CHP-bonuses were introduced and biogas plant operators gained subsidies for utilizing energy plants and the technical usage of generated heat in the CHP unit (Friedl 2016). The EEG amendment of 2009 introduced bonuses for emission reduction and for installations of new techniques (Balussou et al. 2012). Thus, over the last decades, German industry has become the worldwide technical leader in engineering biogas technique (dena 2013). Together with agriculture they have taken an exemplary role in this segment. From 2009 to 2011 more than 1000 biogas plants were installed annually. However, since the EEG amendment of 2012 this advance obviously decreased. Due to changes in the legal regulations only 163 biogas plant new-installations were recorded in 2014 (German Biogas Association 2016). Today, renewable energy production from biogas plays a decisive role in

Germany´s energy transition. In 2015 a total number of 8856 biogas plants were estimated with an installed electrical capacity of 4.0 GW (German Biogas Association 2016). These combined efforts are able to substitute two nuclear power plants in Germany. In 2014 biogas plants produced 27,580 GWh of electrical energy (FNR 2015). This accounted for ca. 5.0 % of gross electricity consumption and thus avoided ca. 10.7 million tons of CO_2 equivalents (BMWi 2015c). The German Biogas Association even claims that biogas production accounted for a reduction of 20.8 million tons of CO_2 equivalents in 2014 (German Biogas Association 2016).

Agricultural biogas production has proved to be beneficial in several sustainability aspects. Besides environmental aspects, it can contribute to socio-economic benefits, as it positively influences developments in rural areas. Billions of euros have been invested by the government through installations of biogas plants. A high share of these added values reached the local economy, e.g. farms and handicrafts business (Guenther-Lübbers & Theuvsen 2015). This sector simultaneously created several jobs. In 2015 about 44,000 employees were estimated in the biogas sector (German Biogas Association 2016). Beside biogas plant sites, employment is provided to structurally related companies in building industry, mechanical engineering, wood-, metal-, fabric-working industries and others (Guenther-Lübbers 2015). Additionally to these benefits, the existing biogas plants contribute to ensure security of supply and decrease our dependency on fossil fuels. Contributions to environmental benefits are, however, dependent on the specifically utilised biogas substrates.

Biogas substrates

The enormous biogas expansion in the last decades led to an innovative technical engineering process and a great variability in the utilized substrates. While in the 1990s most agricultural biogas plants were based on manure and food remains, the substrate diversity has shifted over the last decades. In 2004 the total feedstock mixture composed mainly of livestock residues and residue materials from industry and agriculture (87 % fresh weight (FW)). Only six years later, the share of energy crops on the total feedstock was 46 % (FW) (DBFZ 2011). A large study on biogas production in Germany considered 413 biogas plants of which 61 were analysed in detail (Gemmeke et al. 2009). It showed that an average biogas plant uses a higher share of renewable energy plants than livestock residues. The livestock residues account in average for 37 % FW of the total substrate input. The highest share of livestock residues is cattle manure with a share of 24 % of the total substrate input. Fewer than 60 % of the analysed biogas plants utilized cattle manure in their feedstock. Renewable energy plants take the other share of 63 % (FW) of the total substrate input. More detailed, the utilized energy plants are mainly maize silage, accounting for 48 % FW of the total substrate input, and grass (including grass silage) with 10 % FW. The survey showed that more than 94 % of the analysed biogas plants have maize silage in their feedstock.

Maize (*Zea mays* L.) is, therefore, the most commonly used energy plant for biogas production in Germany (FNR 2014). In many agricultural aspects maize is preferentially chosen to other energy crops. It has the highest biomass yield potential of agricultural crops grown in Europe (Amon et al. 2007). Maize is a relatively undemanding crop and suitable to be grown in nearly any location in Germany (FNR 2010). A national wide project showed that maize has the highest dry matter production potential on German soil (Herrmann 2013). This is due to its effective photosynthetic C_4 pathway, which leads to a fast growth rate. A yield of up to 16.5 t biomass (for agricultural use also expressed as total solids (TS)) ha^{-1} of energy maize is usually possible at good soil conditions (DMK 2006). Over the last years (2007 – 2014) the annually average maize yield was between 38.97 to 47.61 t (FW) ha^{-1} (DMK 2016). Since the introduction of EEG 2004 an enormous increase of maize acreage took place. In 2015 maize was grown on 2.6 million ha in Germany. Maize for silage production including biogas maize and livestock fodder was cultivated on 2.1 Million ha. This accounts for 12.6 % of the total agricultural area (Federal Statistical Office 2016). The

agricultural area used for energy maize cultivation for biogas production was 0.9 million ha in 2015 (FNR 2016). This shows that 34.6 % of the total maize production area is dedicated to biogas production. As maize mainly composes of starch, it is easily degradable in anaerobic fermentation. Maize is considered as the biogas substrate with the highest methane yield potential per hectare (Herrmann 2013). This shows the importance of maize on the total biogas feedstock in Germany.

The substantial increase of land cultivated under maize has, however, been criticised. In an economically aspect, the total costs for maize silage have been rising extremely over the last years. Including cultivation, transport and ensiling of maize, the total costs can be estimated with up to 40 € t^{-1} (Wagner 2015). In 2010 maize silage was still considered with total costs of 32 € t^{-1} (DBFZ 2011). Thus, profitability of biogas plants has reduced due to higher substrate costs. Also, ecological negative effects were found for soil erosion, soil organic matter degradation and nitrate leaching due to common overfertilisation and continuous maize cropping systems (Svoboda 2015). Therefore, the substrate choice influences sustainability criteria of biogas plants.

Sustainable biogas production

As already described above, biogas production can contribute to socio-economic benefits, crucial for a sustainable development. For environmental sustainability, biogas substrates should not solely meet the criteria for optimal biogas production but should additionally contribute to environmental protection. The use of energy crops in biogas plants increased largely over the last decade due to legislative regulations (DBFZ 2011). Energy plants take the highest share in the total biogas feedstock of Germany. Without energy plants the biogas sector could not contribute to renewable energy production in the present way. The biogas sector significantly contributes to the shift from fossil fuels to renewable energies. Therefore, it is important to maintain energy crops in the total biogas feedstock. However, maize cultivation can have negative effects on the environment (Svoboda 2015) and should, therefore, be realised in the best sustainable way possible. Farmers should focus on a high diversity of crop rotation and on low input of fossil fuel-based mineral fertilisers to increase overall sustainability (Herrmann 2013) during maize cultivation. Additionally, conflicts with food crop production on agricultural farmland should be kept as low as possible, as agricultural farmland is a limited resource. Besides the given criticism on maize cultivation, it is necessary to keep further negative effects of utilising maize in biogas plants to a minimum.

After the maize harvest the ensiling process of maize is important to produce long storable biomass with low energetic losses. For a good silage production, optimal microbial fermentation processes rapidly reduce the pH level to ca. 4.0 within days (Ali et al. 2015). Losses of nutrients should be avoided but can occur during the ensiling process, storage and the feed-out period (Khan et al. 2014). After feeding maize silage to the biogas plant, a high transformation efficiency of biochemically stored energy from the substrate to biogas is important to exploit all available energy from the renewable resource. Therefore, efficient substrate degradation in the biogas plant is necessary to achieve high sustainability. This transformation efficiency underlies, however, various abiotic and biotic factors. For instance, the degradation of organic matter is dependent on substrate quality, substrate input amount, temperature, retention time in the reactor and biochemical inhibiting substances which all interact with the microbial activity (Chen et al. 2008; Mao et al. 2015). Incomplete substrate degradation leads to liquid effluents to the residue storage high in organic compounds and can, therefore, risk significant methane

emissions to the atmosphere (Ruile 2015). In 2011 only 37 % of all biogas plants in Germany were equipped with a residue storage cover (Herrmann 2013). In 2013 this number increased to 68 %, due to legal regulations in the EEG 2012 (DBFZ 2013). To avoid revenue losses and possible greenhouse gas emissions, the maximisation of substrate degradation in the biogas plant has to be a main goal. Inner-system energy cycles in the biogas reactor should be optimised to increase the outcome of energy-rich biogas and reduce energetic losses of unused substrate outflow. Such losses can be evaluated by analysing the volatile solids (VS) content of the reactor effluent. The relative ratio of VS contents of inflowing substrate and outflowing effluent determine the VS degradation of the substrate. The VS degradation is, therefore, an indicator for the transformation process efficiency in the biogas plant. Commonly, VS contents are analysed after VDLUFA (1997) by drying the sample and combusting the organic compounds at 550 °C. The VS content of the utilised substrate can be measured directly with this method. However, a direct analysis of VS content of the substrate after application to the reactor is not possible as it mixes with the reactor content (inoculum) and becomes inseparable. Therefore, a different methodology for analysing the VS degradation independently of the reactor inoculum is highly required. One method for analysing solely the substrate-bound VS degradation of maize silage could be the determination of stable carbon isotopes (^{13}C). During the VS degradation mainly organic compounds are degraded, which are based on carbon bonds. The analysis of stable carbon isotopes could contribute to our understanding of substrate-bound degradation processes and, therefore, general system effectiveness within biogas plants.

Alternatively to maize, residue materials are ideal substrates for biogas production. Such substrates do not impede with other land uses like food crop production (Johansson & Azar 2007). Grassland, for instance, offers a large potential for biogas production. Traditional grassland management can, however, stand in conflict with modern economic decisions. The possible abandonment of grassland management could lead to reduction of biodiversity (Isselstein et al. 2005). Alternatively, this grassland could be used for biogas production by simultaneously contributing to nature conservation. Farms with husbandry can utilize livestock residues in biogas plants and contribute to sustainable goals. During anaerobic digestion of livestock residues, biogas is produced and important nutrients for fertilisation (nitrogen, potassium and phosphor) are not exhausted. By using the digestate for fertilisation, it

contributes to closed nutrient cycles and replaces fossil fuel based mineral fertilisers. In an agricultural scenario without biogas, livestock residues get conventionally stored in residue storages. In most agricultural farms such residue storages are not covered and climate noxious gases emit uncontrolled. By treating livestock residues in biogas plants before being stored in residue storages, such emissions get significantly reduced (Ruile 2015).

The most sustainable biogas production can be reached by combining biogas production with an already sustainable-oriented farming systems (e.g. organic farming) (Danner 2012). The substrate feedstocks of biogas plants on organic farms have generally positive ecologically effects. Livestock residues, along with grass and clover, are mostly available as organic residues on these farms. Grass and clover are important intercrops in organic farming, which makes them ideal biogas co-substrates. The substrates contribute to closed nutrient cycles, biodiversity, resource and environment protection (Danner 2012; Insam 2015). These substrates meet the overall goals of organic farming.

Biogas production from residue substrates on organic farms has unused potentials. With the still available residue materials it would be possible to run 1,975 organic biogas plants and thus supply one million households with electricity and more than 35,000 houses with heat (Grieb & Zerger 2015). To support this, the valid EEG 2014 offers possibilities for new-installations of small-scaled biogas plants (≤ 75 kW) with high shares of livestock residues (≥ 80 %), which are possible on both, organic and conventional farms. The governmental compensation for such biogas plants is set to 23.73 ct kWh^{-1} (Federal Government 2014), which offers economically interesting options. The combination of biogas production with organic agriculture is an ideal scenario for creating sustainable renewable energies.

Organic agriculture in Germany

At present, Germany has a total utilised agricultural area of 16.7 Million ha. This area is managed by 280,800 farms. By average, every farm has to manage an agricultural area of ca. 60 ha (Federal Statistical Office 2016). When focusing on organic farming this share reduces drastically. 6.3 % of all farms are working according to organic standards. These 18,000 farms manage 6.0 % of the total agricultural land of Germany. Therefore, every organic farm manages an average of 5.8 ha of the total 1,047,000 ha agricultural farmland cultivated according to organic standards (Federal Statistical Office 2014).

Farms which produce organic plant and livestock products are bound to the Council Regulation (EC) No 834/2007 of organic agriculture in Europe (EU 2007). This regulation lays down precisely the legal production and manufacture requirements for organic products. It arose from a compromise of European Union member states and describes therefore minimum standards for the production of organic products. Within Germany, most organic farms are members of various organic farming associations which support on a regional level (BMEL 2016). These farms are subordinated to the standards of the individual associations. All associations apply ethical principles for organic-biological (e.g. Bioland, Naturland) or bio-dynamic farming (e.g. Demeter) which further includes anthroposophical humanities (Koepf et al. 1996). These legal regulations make organic farming beneficial in many aspects.

Compared to conventional farming, organic farming can have greater benefits to consumers and to the environment (Eurostat 2016). It aims to achieve closure of nutrient cycles, preservation and improvement of soil fertility and animal welfare in livestock management. Therefore, it has a range of positive effects on biodiversity, protection and conservation of the cultivated landscape and rural area employment (Federal Government 2012). Organic farms are bound to restrict the use of external inputs, exclude the use of genetically modified organisms and strictly limit the use of chemically synthesised materials. They are also bound to minimise the use of non-renewable resources (EU 2007). Generally, organic farming contributes to sustainability as it protects natural resources. These sustainable effects have also been acknowledged by the Federal Government of Germany. Therefore, organic farming plays an important role in Germany's National Sustainable Development Strategy. In this strategy it was included to

increase the share of environmentally sound farm production. The share of organically managed farmland shall be increased to 20 % in the upcoming years. However, no precise time frame has been given for achieving this goal (Federal Government 2012).

It was shown in several publications that organic farming not only contributes to environmental protection but offers potentials for sustainable worldwide food security considering the increasing world population (e.g. Badgley et al. 2007). By further focusing on the energetic sector the question arises whether organic farms can also contribute to sustainable energy production. By looking retrospectively on this sector, it becomes clear that organic farmers already took a pioneering role in renewable energy production over 30 years ago (Anspach et al. 2010). They were the establishers of biogas producing plants in Germany. But can organic farms still contribute to sustainable biogas production nowadays?

Biogas production on organic farms

On the base of both sustainability goals, sustainable energy production and sustainable agriculture, it has widely been discussed how renewable energy production can be implemented in organic agriculture. The focus lies here on biogas production on organic farms. Although organic farmers were the pioneers of biogas production they have become relatively small among modern biogas farms. After the EEG amendment of 2004 an enhanced increase of biogas plants was also visible in organic farming. About 40 % of all organic biogas plants and ca. 78 % of their total installed electrical capacities were facilitated after 2004. Today, most modern biogas plants are installed on conventional farms. In 2010, 160 to 180 biogas plants were situated on organic farms with an average installed electrical capacity of 34 to 38 MW_{el}. This accounts for < 2.5 % of the total installed biogas plants and < 1.0 % of the total installed electrical capacity of biogas (Anspach 2010).

Through the rapid increase of the biogas sector and the huge demand of farmland for energy production, biogas production has triggered controversial discussions. Concerning organic agriculture, it has widely been argued whether biogas production contradicts the core ideas of organic agriculture (Blumenstein et al. 2014). Organic biogas production should meet the goals of sustainability by focusing on accordance of social demands, organic agriculture principles and economic viability. Arguments concern the increase of external co-substrate inputs. A monitoring of biogas plants on organic farms conducted in 2011 showed that among 86 biogas plants more than 50 % were utilizing conventionally produced substrates, mostly maize. These biogas plants provide 80 % of the installed electrical capacity with the help of conventional co-substrates. Reasons for the purchase of conventional substrates are mainly economic aspects (Siegmeier et al. 2013). However, the utilization of conventionally produced substrates can be seen critical as it contradicts the idea of a closed farm nutrient cycle. It further threatens the credibility of organic agriculture. This shows that the purchase of conventional substrates for organic biogas production with exclusive focus on economic advantages is not a viable future option (Blumenstein 2014).

A further argument is the discussion about food vs. bioenergy production. Organic farms are facing difficult competitions with conventional farms and their interest in land for bioenergy

substrate production. The intense subsidising of substrates for biogas production led to an increase of rents of agricultural land. The rents are estimated to be higher than affordable for most organic farms and form, thus, conflicts between organic agriculture and bioenergy synthesis (Meyer & Priefer 2012). In order to promote biogas production and organic farming, it is advisable to increase the share of residual substrates for biogas generation. The most excellent requirements for sustainable organic biogas production seem to be preferentially residual material input on confined biogas scales (Blumenstein 2014).

Under given excellent conditions, biogas production on organic farms not only contributes positively to renewable energy production but also to food production security and nutrient supply. Recent surveys also stated that the integration of biogas production can have positive impacts on the organic farming systems. E.g. the economic situation of an organic farm can be improved with a biogas system (Serdjuk & Hülsbergen 2015). It can also be beneficial to intra-farm nutrient cycles and increased yields (Grieb 2015). Further, it holds the possibility for a positive energy balance on the farm, to be self-sufficient with organic nitrogen fertilizers and to reduce greenhouse-gas emissions (Pugesgaard et al. 2014). The application of digested manure as fertilizer can have benefits as it has a positive effect on humus, soil aggregate stability and earthworm (*Lumbricus*) populations (Hülsbergen 2016). The integration of biogas production in organic agriculture can, therefore, contribute to nature protection and to sustainable resource handling (SRU 2007). In such circumstances, biogas production on organic farms can be seen differently to biogas production on conventional farms. Actions have been taken to implement terms like ‘BioBiogas’ or ‘Sustaingas’ to refer to organic biogas production with focus on manure and residue substrate digestion (e.g. grass, straw) (FiBL 2013). By putting high priority on intra-farm nutrient cycling, biogas production on organic farms can be seen as a highly sustainable way to achieve renewable energy production and enhance, simultaneously, the productivity of organic farming systems (Grieb & Gerlach 2013).

Future role of biogas production

During the twentieth century industrial refineries concentrated on the production of multiple goods on basis of coal, petroleum and natural gas. These fossil resources are, however, not sustainable in aspects of economy, ecology and environment. The demand for sustainable and environmentally friendly energy sources has become urgent over the last years. The use of fuels from plants and organic wastes is of large interest. Its utilisation supports the reduction of oil dependencies and, simultaneously, the reduction of greenhouse gas emissions. In recent years, initial steps towards replacing fossil fuels were taken by introducing 'first generation' biofuels, amongst others biogas. Benefits of first generation biofuels can be seen in reduction of CO_2 emissions and energy security improvement. However, such technologies need feedstock sources. These feedstocks can also have negative effects on biodiversity and land use. One main disadvantage of first generation feedstocks is the competition with food crops. The role of agriculture as an energy provider, additionally to food provider, has peaked in the food-versus-fuel debate. Therefore, the idea of utilising 'second generation' biofuels has generated great interest. Such feedstocks are plant materials which could significantly reduce CO_2 emissions and do not interfere with agricultural food production. This includes especially substrates with high contents of lignocelluloses (e.g. wheat straw, forestry materials) (Naik et al. 2010). Up to now, the utilisation of such substrates in biogas plants is still rare, due to limited biotransformation by microorganisms. Second generation biofuels further include residue materials from agriculture (e.g. livestock residues, grass). Such materials have already been used for biogas production and proofed sustainably positive. An increase of residue materials in biogas feedstocks in the future would contribute to the idea of second generation biofuels.

With the increasing contribution of renewable energy to the total energy production in Germany, technical difficulties arose. Germany's energy infrastructure was mainly based on relatively steady electrical supply by large power plants. A rather dynamic and quickly adjustable energetic system has to be realised with an increasing amount of many small, decentralised renewable energy power plants. In a future scenario of 100 % renewable energy, a large contribution will come from wind and photovoltaic energy generation (Steinke 2013). Such resources are, however, known for strong temporal fluctuations and thus lead to an unsteady power generation (Hahn 2014). These intermittent renewable energy sources will therefore

cause problems in the balancing of the energetic system. To provide energy sufficiency at all times by renewable energy sources, large energy backups will be necessary (Steinke 2013). One important renewable energy backup could be biogas. Beneficially, energy rich biomass is storable and can be utilised in biogas plants continuously or in a regulated regime. In times of low energy generation by wind or photovoltaics, the power supply will have to be replaced by a different renewable energetic source (Friedl 2016). Therefore, the demand for flexible power generation is high. By considering biogas production as a flexible process, we could compensate energetic shortage by adequately activating biogas combusting generators. However, the realisation of demand-oriented biogas plants meets new challenges.

The main goal of most installed biogas plants is to operate at full capacity and to produce maximal electrical energy. Such biogas plants run with a relatively constant feeding regime. As mentioned above, most biogas plant feedstocks comprise of maize and grass silage. The required retention time for such substrates in the biogas plant is about 100 days, whereas fibre-rich substrates are not fully degraded in this time. The substrate addition to such biogas reactor systems occurs on average 20 times per day (Gemmeke 2009). However, with the introduction of the EEG 2014 and the EEG amendment of 2017, demand-driven biogas production gains increasing importance. With the demand-oriented biogas production, energy should be generated in times of high electrical needs. This is attended by new challenges for the established biogas systems.

In a first scenario, a flexible biogas plant could still produce biogas in the common constant way and collect the produced biogas in a biogas reservoir. The biogas reservoirs could be used as backup energy while biogas combusting CHP units would produce energy in times of demand. However, such biogas reservoirs are hardly realisable because of space demand and cost efficiency. Additionally, the on-farm biogas storage capacity is legally limited to a maximum of 50 tons (ca. 39000 m^3 biogas) by the German Federal Pollutant Control Act (BImSchG) (Hahn 2014). Therefore, backup energy provision by on-farm biogas storages is not advisable.

In a second scenario, the biogas production of a biogas plant could be regulated by altering the substrate feeding regime. Ideally the substrate addition to the biogas plant would result in an immediate biogas production. As energetic deficits are not always predictable long beforehand,

biogas plants with short start-up phases would greatly contribute in such times. Flexible biogas production in a relatively common biogas plant with 500 kW installed electrical energy and a feedstock composing of manure, maize silage and wheat corn was analysed by Hartmann et al. (2010). It showed that biogas production can be regulated within a period of several days by a variable feedstock. However, faster biogas production response after substrate addition would be beneficial. Implementing such a fast adaptable biogas system would require substrates that can be transformed to biogas more rapidly. Such substrate can be found in sugar beet. It contains easily degradable sugars and alcohols (after ensiling), which can be metabolised to biogas rapidly and offers similar biogas yield potentials compared to maize silage. However, the monofermentation of sugar beet silage can cause process problems in forms of acidification and foam formation. The utilisation of sugar beet silage as a co-substrate with, for instance, grass or maize silage is highly recommended (Ahmed et al. 2016). Therefore, the regulated input of substrate mixtures with sugar beet silage could reduce the start-up time for biogas production. Such periodical substrate inputs are likely to result in high organic loading rates (OLR) in the biogas reactor. High organic loading rates are challenging for the microbial communities (Burgstaler et al. 2010). An optimal biogas production process requires an efficient microbial community in order to deal with changing environmental conditions in form of altering substrate compositions. Therefore, it is essential to test the adaptability of microbial communities to different substrate mixtures with sugar beet.

Motivation of doctoral thesis

This doctoral thesis was motivated in order to support optimised biogas production scenarios presented in the introduction. To contributing to parameters of sustainable biogas production this thesis hypothesised that the feedstocks of biogas plants on organic farms support sustainable biogas production and that improvement strategies can be identified by analysing biogas process parameters (Manuscript 1).

This thesis further contributes to sustainable biogas production by considering parameters for highest possible system effectiveness. Therefore, it concentrated on the subarea of organic compound degradation of maize silage in biogas plants. It hypothesised that analysing the carbon degradation of maize silages independently of the inoculum is possible by applying a stable-isotope approach (Manuscript 2). This provides a more detailed insight in maize silage degradation processes.

Future prospects of biogas production are demand-oriented biogas production. Such biogas plants should compensate electrical deficits in the national grid. Assuming sugar beet as a suitable substrate for timely predictable biogas production, this thesis hypothesised that microbial communities in biogas plants are adaptable to different substrate mixtures with sugar beet (Manuscript 3).

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Manuscript 1

Characterisation of biogas plants on organic farms and potentials for improvement

Daniel Einfalt, Marian Kazda

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Personal contribution

Planning: 90 %

Experimental procedures: 100 %

Writing the manuscript: 90 %

Anaerobic digestion process characteristics have been only rarely considered in recent studies on biogas production on organic farms. In a survey of 13 biogas plants fermentation characteristics, biogas plant specifications and feedstock compositions were investigated and analysed for inherent problems.

Repeated sampling was examined on biogas plants of organic farms in order to investigate the impact of fermentation characteristics on volatile solids (VS) degradation, indicating the effectiveness of the digestion process. The main fermentation characteristics factor for low VS degradation was process temperature, ranging from 17 to 49 °C. Among biogas plant specifications, two reactor stages significantly improved the VS degradation.

Livestock residues and grass silage had the highest shares on the feedstock composition (61 % and 14 %, respectively, of the total fresh weight). The calculated specific methane yield of the individual feedstocks (median 256.34 m^3 kg-1 VS) were considered low, compared to Germany's average biogas plants.

For improving the VS degradation, higher temperatures and longer hydraulic retention times are required. Coverage of the residue storage would lead to lower greenhouse gas emissions. Such improvements would positively contribute to Germany's National Sustainability Development Strategy.

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Characterisation of biogas plants on organic farms and potentials for improvement

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Abstract Biogas plants on organic farms contribute little to the total installed electrical capacity of biogas in Germany (<1.0 %). To increase the energy efficiency, parameters of biogas plant specifications, fermentation characteristics and feedstock composition were examined for inherent problems in 13 biogas plants. Biogas plants with two reactor stages were found to have significantly higher substrate degradation efficiency and better overall process performance than single-stage biogas plants. The main factor for low volatile solid (VS) degradation was process temperature (range 17 to 49 °C) before hydraulic retention time (HRT) and organic loading rate (OLR). Low VS degradation was found in biogas plants with only one reactor stage, low fermentation temperature and a high share of livestock manure. The total feedstock of the biogas plants comprised mainly of livestock residues (61 %) and grass silage (14 %). The individual biogas plant feedstocks showed strong intake variability and, in general, low specific methane yields (median 256.34 m^3 kg^{-1} VS) when compared to German average. The presented study specified several optimisation pathways for biogas production within the organic farming. Residue storage covers would lessen ecological and economic disadvantages of high VS contents in the effluents considerably. Technical changes in combined heat and power (CHP) units and reactor content recirculation show a way to increase process temperatures and HRT which, in turn, would improve VS degradation efficiency. Moreover, the German Renewable Energies Sources Act 2014 offers possibilities for new small-scaled (≤75 kW_{el}) manure processing (≥80 % fresh weight (FW)) biogas plant installations, an opportunity to increase the total installed electrical capacity of biogas plants on organic farms.

Keywords Organic farming · Livestock residues · Process temperature · Degradation · Specific methane yield

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Introduction

Biogas plays a decisive role in the composition of renewable energies in Germany. In 2014, 27.8 % of electricity production originated from renewable energy sources (BMWi 2015). With the revised Renewable Energies Sources Act (EEG) 2014, the German government set a strict threshold for subsidising the use of energy crops for biogas fermentation. Small-scale (≤75 kW_{el}) manure processing (≥80 % fresh weight (FW)) biogas plants are in favour (Bundesregierung 2014). The German government aims to further increase sustainable agriculture in its National Sustainability Development Strategy, as organically farmed

agricultural land will be raised by up to 20 % (Bundesregierung 2012).

The combination of organic farming and biogas production was already established as early as 1981 (Möller and Anspach 2007) with the organic farms being regarded as the pioneers of renewable energy production within the biogas branch. In 2010, about 160 to 180 biogas plants were present on organic farms with a total of 34 to 38 MW_{el} (Anspach et al. 2010). They contribute to the 7944 total biogas plants with <2.5 % and with only <1.0 % to the total installed electrical capacity of biogas (3895 MW_{el}) in 2014 (German Biogas Association 2014). When compared to the amount of agricultural land being organically farmed (6.3 %; 1,044,953 ha) (BÖLW 2014), the share of biogas on organic farms is low. The integration of biogas in organic farming systems is a beneficial addition, as it contributes to the objectives of organic farming. It can help to close nutrient cycles, maintain soil fertility, contribute to nature conservation and handle resources and energy responsibly (Anspach and Möller 2009; Reents et al. 2011; SRU 2007).

Previous studies concentrated on structures and economic potentials of biogas plants on organic farms. Fermentation process characteristics have, however, not been investigated (Anspach 2010; Anspach et al. 2010; Danner and Kilian 2012; Siegmeier et al. 2013). The aim of this paper was to find solutions for increasing the biogas production efficiency on organic farms, as it contributes to overall sustainability goals. The hypothesis was that the small share in electricity produced from biogas generation results from (1) substrates of relatively low specific yields and (2) a low effectiveness of the fermentation process itself. As the latter offers chances for improvement, biogas plant specifications and fermentation characteristics including feedstock compositions were analysed. The improvement strategies defined from this analysis meet the aims of organic farming.

Material and methods

Biogas plant assessment

A total of 13 full-scale biogas plants situated on organic farms were studied in Bayern and Baden-Württemberg, Germany. The biogas plant specifications, individual feedstock compositions and intakes were assessed by questionnaire. Substrates and biogas reactor contents were sampled several times. Biogas plant specifications were analysed for year of installation, number of reactor stages, installed electrical capacity, digester volume, digester technique (vertical/horizontal), combined heat and power (CHP) technique (spark ignition/dual fuel) and residue storage cover. Fermentation characteristics were assessed for feedstock intake, livestock residue share of the feedstock, process temperature, organic loading rate (OLR), hydraulic retention time (HRT), specific methane yield, volatile solids (VS) degradation, pH, volatile fatty acids (VFA)/total alkaline capacity (TAC) buffer stability (VFA/TAC) and carbon/nitrogen ratio (C/N). Reactor contents and slurry samples were homogeneously extracted and transported in sealed plastic boxes (VDLUFA 1983). Temperatures of reactor contents were measured immediately after sample extraction on site. The survey was performed between April 2012 and Mai 2014. As fermentation characteristics differed largely during biogas plant visits, each of the 25 samplings was considered a separate fermentation process.

Sample analysis and evaluation

Reactor contents were analysed for pH and VFA/TAC buffer stability immediately after returning to the laboratory (max 6 h). VFA/TAC was measured with two-point titrations to pH 5.0 and pH 4.3 (Voß et al. 2009). All substrate and reactor samples were examined for total solids (TS), VS, C and N (TruSpec CN, LECO, St. Joseph, USA). The measurement of TS and VS was carried out as described in VDLUFA (1983). Data of samples taken directly on site were used for calculations. For unavailable feedstock samples, either data were used from earlier sampling on site or data from the literature (Online Resource 1). Cattle manure is a mixture of cattle slurry and cattle dung and was sampled separately as far as possible. The calculated VS degradation was based on the difference of daily VS input of feedstock and the VS in the reactor content of the according reactor stage. OLR calculations comprised the volume capacity of the first reactor (R1) stage, and HRT calculations were based on the total reactor volume capacity. The specific methane yields were calculated with substrate standard data (KTBL 2013; KTBL 2015; LFL 2015) and the daily VS feedstock intake. For tables and diagrams of feedstock composition, fresh grass and clover grass silage were included in category grass

silage. Rye silage and grain husk were included in category grain silage. Straw was integrated in livestock residues.

Statistical analysis

Spearman test from the statistical analysis software SPSS 20 (SPSS Inc., Chicago, USA) was used to find correlations in biogas plant specifications (year of installation, reactor stages, digester volumes, installed electrical capacity) and fermentation characteristics (feedstock intake, livestock residue share, process temperatures, OLR, HRT, specific methane yield, VS degradation, pH, VFA/TAC, C/N). The parametric free Kruskal-Wallis test was applied to find significant differences ($p < 0.05$) in such categories. A principal component analysis (PCA) was performed to find integral relationships. The PCA results were rotated using the varimax method. Diagrams were generated with Microsoft Excel 2010.

Results

Biogas plant specifications

All biogas plants used completely stirred tank reactors (CSTR) and CHP units (Table 1). The oldest biogas plant was installed in the year 1988 (40 kW_{el}) and the newest in 2011 (250 kW_{el}) with a median in 1994. Independently of the age of the biogas plant, all used two types of CHP technique. Fifty-four percent of the biogas plants used spark ignition CHP units and 38 % used dual fuel CHP units. Biogas plant No. 13 used two units with both CHP techniques.

Biogas plants No. 1 to 6 used one reactor stage (single-stage digestion system) and No. 7 to 13 operated with two CSTR reactors in series (two reactor stages). Most plants used vertical CSTR systems whereas biogas plants No. 7 and 9 used also horizontal plug flow digesters. The total reactor volume showed a median of 700 m^3 (range 240–3200 m^3). The installed electrical capacity of the CHP units ranged from 13 to 280 kW_{el} (median 40 kW_{el}). Biogas plants built before 2004

Table 1 Description and technical data of the biogas plant specifications

No.	Reactor stages	Electrical capacity (kW)	Installation year	Digester volume (m^3)	Digester technique	CHP technique	Residue storage cover
1	1	13	1994	500	Vertical	Spark ignition	No
2	1	22	1997	270	Vertical	Dual fuel	No
3	1	25	1992	300	Vertical	Spark ignition	No
4	1	28	1994	350	Vertical	Spark ignition	No
5	1	30	1992	1000	Vertical	Dual fuel	No
6	1	40	1994	600	Vertical	Spark ignition	No
7	2	22	2000	120	Horizontal	Spark ignition	Yes
				120	Horizontal		
8	2	40	1988	350	Vertical	Spark ignition	No
				350	Vertical		
9	2	50	2005	100	Horizontal	Spark ignition	No
				950	Vertical		
10	2	60	2004	350	Vertical	Dual fuel	No
				1000	Vertical		
11	2	80	1989	880	Vertical	Dual fuel	No
				780	Vertical		
12	2	250	2011	1500	Vertical	Dual fuel	No
				1500	Vertical		
13	2	280	2008	1600	Vertical	Spark Ignition	Yes
				1600	Vertical	Dual fuel	

(69 %) showed a significantly smaller installed electrical capacity (median 28 kW_{el}) than later built biogas plants (median 155 kW_{el}) ($p < 0.05$, $n = 13$).

All biogas plants, independently of plant age, used residue storage tanks without a heating system. The survey showed that only two of the 13 biogas plants were equipped with residue storage cover. The two largest biogas plants (total volume 3000 and 3200 m^3, respectively) cooperated with conventional farms to ensure substrate sufficiency. Single-stage digestion systems showed significantly lower total reactor volumes and installed electrical capacities than biogas plants with two reactor stages (Table 3).

Fermentation characteristics

Feedstock

The results of this investigation showed a big variety of fermentation characteristics that are shown in Table 2. Within the 25 samplings from the 13 biogas plants, mostly grass silage, grain silage and maize silage were co-substrates that were used alongside livestock residues. Biogas plants No. 2 and 5 were found to utilise cattle manure exclusively, having a low installed electrical capacity of up to 30 kW_{el}. The feedstock intake ranged from 2.20 to 17.00 t day^{-1} FW and 0.18 to 3.28 t day^{-1} VS. As expected, the two largest biogas plants, No. 12 and 13, showed the highest feedstock intake between 15.50 and 17.00 t day^{-1} FW and 2.35 to 3.28 t day^{-1} VS. A correlation between the feedstock intake and biogas plant specifications or fermentation characteristics could not be found.

According to the VS of the feedstock and the dimensions of the reactor volume, a wide range of OLR from 0.27 to 4.83 kg VS m^{-3} day^{-1} was observed (median 1.22 kg VS m^{-3} day^{-1}). Significant differences of OLR were found between biogas plants with one reactor stage and two reactor stages (Table 3). The HRT differed between only 26 and 160 days with a median of 110 days.

The percentage of livestock residues in the total feedstock (FW) in this study was 61 % (Fig. 1a). Cattle manure had the biggest share with 33 %, followed by 11 % for cattle dung. Only biogas plants No. 3 and 10 used swine residues for biogas production. Biogas plant No. 10 utilised exclusively livestock residues from swine. The share of swine dung and swine manure accounted for 10 and 7 % of the total feedstock.

In this survey, it was found that an increasing livestock residue share of the feedstock correlated with a decreasing installed electrical capacity of the biogas plants ($r = -0.79$, $n = 25$, $p < 0.001$). For instance, biogas plants No. 1 to 9 utilised >73 % livestock residues in their individual feedstocks and showed a maximum of only 50 kW_{el} of installed electrical capacity. Also, a significantly higher share of livestock residues were utilised in biogas plants with one reactor stage (median 92.94 %) compared to biogas plants with two reactor stages (median 64.52 %) (Table 3).

Grass silage was the most utilised co-substrate, holding a share of 14 % of the total feedstock. Maize silage was only used in biogas plants No. 3, 10 and 13. Biogas plants No. 10 and 13 cooperated with conventional farms and utilised non-organic maize silages.

The average biogas feedstock in Germany (Gemmeke et al. 2009) was highly different to the feedstock in this survey (Fig. 1). The countries' total feedstock comprised mainly of maize silage. Livestock residues held a share of 35 % while only 4 % for grass silage.

Temperature

The temperature, an essential parameter in anaerobic digestions, ranged between 17 and 49 °C (median 38 °C). As expected, many significant correlations with the fermentation temperature R1 could be found (Fig. 2). For instance, it significantly correlated with the VS degradation in R1 ($r = 0.69$, $n = 18$, $p < 0.05$) and the residue storage (RS; $r = 0.66$, $n = 15$, $p < 0.05$). A negative correlation of the temperature in R1 was found for the livestock residue share ($r = -0.42$, $n = 19$, $p < 0.05$). Moreover, it correlated with the C/N ratio in R1 ($r = -0.56$, $n = 19$, $p < 0.05$) and the pH in R1 ($r = 0.70$, $n = 19$, $p < 0.05$). Single-stage reactor systems showed a median temperature of 31 °C which differed significantly from biogas plants with two reactor stages (median 42 °C) (Table 3).

Degradation

In this survey, the VS degradation varied largely between the 25 biogas plant samplings. The VS degradation in R1 showed a median of 62.66 % (range 19.46 to 75.50 %, $n = 19$). Two-stage reactor systems showed a VS degradation between 59.96 and 79.24 % in reactor 2 (R2) with a median of 72.68 % ($n = 11$). The highest variability of VS degradation was found in the RS

Table 2 Description of fermentation characteristics and feedstock compositions

No.	Sample #	Daily feedstock$_{FW}$ (t day^{-1}) (VS)	Feedstock$_{FW}$ (%)	Temp (°C)	OLR (kg VS m^{-3} day^{-1})	HRT (day)	Specific methane yield (m^3 kg^{-1} VS)	VS degradation (%)
1	1	7.43 (0.48)	Cattle manure (91.88)	33	0.95	60	253.92	20.69 (R1)
			Grass silage (8.12)					
	2	7.43 (0.42)	Cattle manure (91.88)	33	0.83		258.81	59.80 (RS)
			Grass silage (8.12)					
2	1	3.70 (0.18)	Cattle manure (100)	26	0.68	80	209.00	19.46 (R1)
	2	3.74 (0.26)	Cattle manure (100)	17	0.95		208.88	22.17 (R1)
3	1	2.26 (0.37)	Swine solids (66.37)	49	1.22	130	262.02	62.66 (R1)
			Cattle manure (22.12)					84.25 (RS)
			Grass silage (6.42)					
			Maize silage (2.88)					
			Food residues (2.21)					
	2	2.20 (0.33)	Swine solids (68.34)	49	1.11		256.34	75.50 (R1)
			Cattle manure (22.78)					
			Grass silage (6.61)					
			Grain silage (2.28)					
4	1	10.45 (1.05)	Cattle manure (88.44)	25	3.01	26	239.47	45.20 (R1)
			Grass silage (11.56)					51.99 (RS)
	2	10.45 (1.04)	Cattle manure (88.44)	33	2.96		235.72	31.26 (R1)
			Grass silage (11.56)					54.75 (RS)
5	1	4.83 (0.27)	Cattle manure (100)		0.27	110	224.14	29.25 (RS)
	2	4.83 (0.27)	Cattle manure (100)		0.27		224.14	14.57 (RS)
6	1	5.33 (0.30)	Cattle manure (95.75)	29	0.49	120	239.91	27.25 (R1)
			Grass silage (2.83)					34.66 (RS)
			Grain silage (1.42)					
	2	5.43 (0.54)	Cattle manure (94.00)	25	0.90		236.03	26.42 (R1)
			Grass silage (4.60)					11.67 (RS)
			Grain silage (1.39)					
7	1	6.38 (0.62)	Cattle manure (95.27)	45	4.83	40	232.04	
			Grass silage (4.73)	30				63.23 (R2)
								63.11 (RS)
	2	6.38 (0.55)	Cattle manure (95.27)	45	4.58		230.55	
			Grass silage (4.73)	31				59.96 (R2)
								61.36 (RS)
8	1	3.80 (0.69)	Cattle manure (73.68)	32	1.98	130	251.50	63.68 (R1)
			Grass silage (26.32)	37				77.10 (R2)
	2	3.80 (0.81)	Cattle manure (73.68)	41	2.33		268.84	70.80 (R1)
			Grass silage (26.32)	43				71.79 (R2)
								87.70 (RS)
9	1	6.81 (0.65)	Cattle manure (73.40)	42	0.62	150	272.80	
			Grass silage (26.60)	40				61.55 (R2)
	2	7.50 (0.70)	Cattle manure (86.67)	42	0.66		262.71	
			Grass silage (6.67)	36				64.38 (R2)
			Grain silage (6.67)					
10	1	5.70 (0.87)	Swine manure (38.60)	46	2.48	37	307.41	64.81 (R1)
			Grass silage (56.14)	34				79.24 (R2)
			Maize silage (5.26)					85.11 (RS)
11	1	7.58 (1.86)	Cattle manure (47.22)	41	2.12	90	300.47	70.04 (R1)
			Grass silage (52.78)	38				71.60 (RS)

Table 2 (continued)

No.	Sample #	Daily feedstock$_{FW}$ (t day^{-1}) (VS)	Feedstock$_{FW}$ (%)	Temp (°C)	OLR (kg VS m^{-3} day^{-1})	HRT (day)	Specific methane yield (m^3 kg^{-1} VS)	VS degradation (%)
	2	7.58 (1.07)	Cattle manure (38.24)	44	1.21		286.69	64.87 (R1)
			Grass silage (32.35)	40				64.77 (RS)
			Grain silage (29.41)					
12	1	15.50 (2.35)	Cattle manure (64.52)	42	1.57	160	289.39	61.87 (R1)
			Grass silage (35.48)	30				74.53 (R2)
								72.64 (RS)
	2	16.30 (2.88)	Cattle manure (57.06)	43	1.92		293.55	71.98 (R1)
			Grass silage (42.94)	35				73.37 (R2)
								71.91 (RS)
13	1	17.00 (3.18)	Cattle manure (38.24)	40.5	1.99	120	307.39	69.54 (R1)
			Grass silage (20.59)	38				75.76 (R2)
			Grain silage (20.59)					76.38 (RS)
			Maize silage (20.59)					
	2	17.00 (3.28)	Cattle manure (38.24)	46	2.05		303.86	63.85 (R1)
			Grass silage (32.35)	40				72.68 (R2)
			Grain silage (29.41)					

ranging between 11.67 and 87.70 % (median 63.11 %, $n = 17$).

Biogas plants No. 2, 5 and 6 showed a low VS degradation in R1 (max 27.25 %) and in RS (max 34.66 %) with low process temperatures of 17 to 29 °C. Here, the HRT ranged between 80 and 120 days. The highest VS degradations in RS were found in biogas plants No. 3, 8 and 10 (range 84.25 to 87.70 %) with a HRT between only 37 and 130 days and a process temperature of 43 to 49 °C.

Significantly higher VS degradation in R1 ($n = 15$) and RS ($n = 17$) were found in fermentation processes with two reactor stages compared to biogas plants with single-stage fermentation (Table 3). Also, VS degradation correlated with the livestock residue share in R1 ($r = -0.70$, $n = 19$, $p < 0.05$) and RS ($r = -0.73$, $n = 17$, $p < 0.05$).

Further fermentation process parameters indicated stable process performances and no VS degradation inhibitions were induced. The VFA/TAC in R1 showed a median of

Table 3 Differences in biogas plant specifications and fermentation characteristics of biogas plants with one reactor stage and two reactor stages

Biogas parameters	Number (n_1)	BGPs 1 reactor stage	Number (n_2)	BGPs 2 reactor stages	p
Volume total (m$^{3)}$	6	425 (312.50/575)	7	1350 (875/2330)	<0.05
Electrical capacity (kW$_{el}$)	6	26.50 (22.75/29.50)	7	60 (45/165)	<0.05
Livestock res (%)	12	92.94 (90.46/100)	13	64.52 (47.22/73.68)	<0.001
OLR (kg VS m^{-3} day^{-1})	12	0.93 (0.63/1.14)	13	1.99 (1.57/2.33)	<0.05
VS deg. (R1 %)	10	29.26 (23.23/42.22)	9	64.87 (63.85/70.04)	<0.01
Temp R1 (°C)	10	31.00 (25.25/33.00)	13	42.00 (41.00/45.00)	<0.05
pH R1	11	7.89 (7.63/8.03)	9	8.19 (8.12/8.39)	<0.05
VS deg. RS (%)	8	43.32 (25.58/56.01)	9	71.91 (64.77/76.38)	<0.01
Specific methane yield (m^3 kg^{-}1 VS)	12	237.75 (224.14/254.52)	13	286.69 (262.71/300.47)	<0.01

Deg. degradation, Results in median (25 % quartile/75 % quartile)

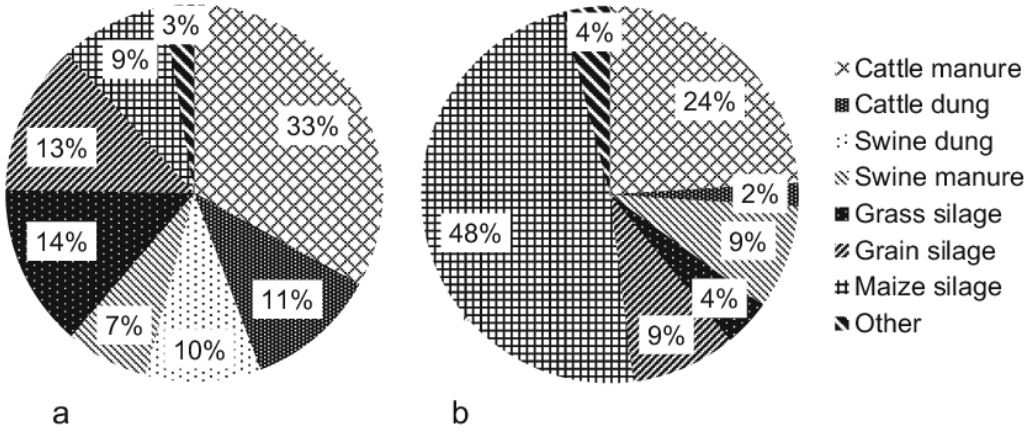

Fig. 1 Mean substrate inputs of the total feedstock in % FW of **a** this survey and **b** Germany (Gemmeke et al. 2009)

0.06 with its maximum in biogas plant No. 3 with 0.14. The pH in R1 ranged from 7.55 to 8.55 with a median of 8.04. The C/N ratio (R1) varied between 10.61 and 17.91 with the highest value found in biogas plant No. 11.

Specific methane yield

The specific methane yield varied between 208.88 and 307.41 m^3 kg^{-1} VS and showed a median of 256.34 m^3 kg^{-1} VS. As expected, it increased significantly with a decreasing share of livestock residues ($r = -0.93$, $n = 25$, $p < 0.001$) and an increase of co-substrates. Biogas plants No. 2 and 5, which utilised exclusively livestock residues, showed the lowest specific methane yields between 208.88 and 224.14 m^3 kg^{-1} VS. The highest specific methane yields (307.41 and 307.39 m^3 kg^{-1} VS) were found in biogas plants No. 10 and 13 with high shares of co-substrates in the individual feedstocks of 61.40 and 61.76 %. A significantly higher specific methane yield was found for fermentations in two-stage reactor systems than in single-stage systems (Table 3). Further significant correlations of the specific methane yield were found for VS degradation in R1 ($r = 0.73$, $n = 19$, $p < 0.001$), the VS degradation in RS ($r = 0.76$, $n = 17$, $p < 0.001$) and the process temperature in R1 (Fig. 2a).

PCA

A PCA of all factors of the biogas plant specifications and fermentation characteristics gave four major components (PC1–PC4) capturing 90.24 % of the variance (Table 4). Seven factors in PC1 accounted for 48.52 % of the variance and were all found to be positively correlated for the Spearman test. The results showed that the VS degradation in R1 and RS was strongly related with, i.a., the process temperature in R1, the number of reactor stages and the livestock residue share. The second component (PC2) included three biogas plant specification factors, which accounted for 19.97 % of the variance. The installed electrical capacity was, however, not correlated to the installation year of the biogas plant. The fermentation characteristic factors in PC3 and PC4 explained 13.68 and 8.08 % of the variance. These four factors showed no internal correlations.

Discussion

Biogas plant specifications

In general, the contribution of biogas plants on organic farms to the total renewable energies is small. All biogas plants were equipped with similar CSTR and CHP technique. This led to comparable fermentation processes independent of the age of the biogas plants. The median installed electrical capacity of 40 kW_{el} was much smaller than the German average of 495 kW_{el} (Gemmeke et al. 2009). In this survey, it was found that >69 % of the biogas plants were constructed before the second EEG 2004. These biogas plants showed significantly smaller installed electrical capacities (median 28 kW_{el}) than biogas plants built since (median 155 kW_{el}). Siegmeier et al. (2013) found similar results with an average of ca 50 kW_{el} (<2004). The EEG 2004 had a positive effect on new installations of biogas

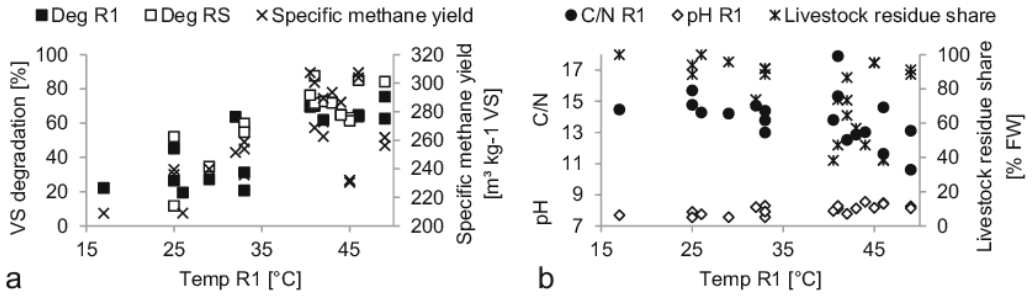

Fig. 2 **a** Correlation of the fermentation temperature in reactor 1 (R1) with the VS degradation in R1 ($r = 0.69$, $n = 18$, $p < 0.05$) and residue storage (RS) ($r = 0.66$, $n = 15$, $p < 0.05$) and the specific methane yield of the feedstock ($r = 0.49$, $n = 23$, $p < 0.05$). **b** Correlation of the fermentation temperature in R1 with the C/N ratio in R1 ($r = -0.56$, $n = 19$, $p < 0.05$), the pH in R1 ($r = 0.70$, $n = 19$, $p < 0.01$) and the livestock residue share ($r = -0.42$, $n = 23$, $p < 0.05$). *Deg* degradation

plants on organic farms with higher installed electrical capacities (Anspach 2010; Anspach and Möller 2008a; Möller and Anspach 2007; Siegmeier et al. 2013). However, a correlation of the installation year and the installed electrical capacity was not found in this survey. This shows that small-scaled biogas plants are currently still important in organic farming.

The major factor of the biogas plant specifications affecting the fermentation characteristics was found in the number of reactor stages within the PCA (PC1). Biogas plants with two reactor stages showed in general a better process performance than single-stage biogas plants. The installation year, total reactor volume and the installed electrical capacity were also important factors (PC2) as they explained ca 20 % of the data variance in the PCA.

Independent of the installation year, >45 % of the biogas plants were equipped with biogas/diesel dual fuel CHP technique. This CHP technique can be chosen for fermentation process criteria. It can be used for increasing the fermentation temperature in biogas reactors in periods of low biogas production (Van Haren and Fleming 2005). From a legal point of view, the diesel fuel has to originate from vegetable oil (Bundesregierung 2014), which derives from conventional farming. Problematically, this can also be seen as utilising conventional substrates for the biogas process and a reason why many organic farmers might use spark ignition engines.

Most biogas plants (>84 %) had no residue storage cover and greenhouse gas emissions were not inhibited. Gemmeke et al. (2009) found similar results with an average of 84 % of single-stage and 71 % of multi-stage biogas plants in Germany having no residue storage cover.

Fermentation characteristics

Feedstock

The total feedstock consisted mainly of livestock residues, grass silage and grain silage. The survey demonstrated that biogas plants that utilised higher shares of livestock residues had lower installed electrical capacities. A decrease of the livestock residue share linked with an increase of installed electrical capacity was also found by Siegmeier et al. (2013). The feedstock intake varied largely and no correlations with other parameters were found. Also OLR and HRT had a wide range. This shows that every biogas plant worked with an independent feeding plan, resulting from substrate availability and its energetic differences.

The total feedstock in this survey contrasted widely to the average feedstock used in Germany's biogas plants, which was mostly situated on conventional farms. There the feedstock mainly comprised of energy crops such as maize silage (48 %), while less so for livestock residues (35 %) (Gemmeke et al. 2009). The expansion of maize has been criticised as it can lead to negative ecological effects (Svoboda et al. 2015). In contrast, the utilised feedstock substrates on organic farms have been found to be ecologically beneficial. They have positive effects on closed-cycle farm management, environment, resource protection and biodiversity (Danner and Kilian 2012; Insam et al. 2015). These substrates meet the overall goals of organic farming. Livestock residues, along with grass and clover, are mostly available as organic residues on these farms. Grass and clover are important intercrops in organic farming, which makes them ideal biogas co-substrates.

They can be used energetically via biogas production and afterwards also substantially as fertilisers, without competing with food production (Stinner 2005). On farms without biogas plants, they are often mulched and nitrogen losses can result in negative environmental effects (Graß 2008). Hölker (2009) even showed positive effects of cattle manure on feedstock degradation in anaerobic digestion. The use of biogas slurry as a fertiliser can have further positive outcomes for organic farming systems. It has been researched to increase the abundancy of many soil organisms (e.g. earthworms) and enhance soil attributes (Koblenz et al. 2015; Petz 2000; Wolfrum et al. 2011).

Temperature

The survey showed that many biogas plants had fermentation temperatures far below the German average of 41.8 °C (Gemmeke et al. 2009), which negatively affects the anaerobic degradation. The significance of a high process temperature could clearly be seen on the VS degradation. Also, a correlation between the temperature and the livestock residue share was found. This shows that biogas plants with a high share of livestock residues should increase their fermentation process temperature to achieve a better VS degradation and a higher biogas yield. A decrease of the C/N ratio and an increase of the pH were found in R1 for increasing process temperatures, as more carbon was metabolised to biogas. Biogas plants with two reactor stages had a median process temperature according to the German average. However, biogas plants with only one reactor have been found to run with significantly lower fermentation temperatures. They are also advised to increase their process temperature.

Table 4 PCA with factors of biogas plant specifications and fermentation characteristics

Factors	PC1	PC2	PC3	PC4
pH R1	0.910			
VS deg. R1	0.903			
Temp R1	0.902			
VS deg. RS	0.892			
Specific methane yield	0.777			
Reactor stages	0.733			
Livestock res	−0.732			
Electrical capacity		0.942		
Volume total		0.926		
Install year		0.853		
HRT			0.802	
OLR			−0.886	
VFA/TAC R1			0.745	
C/N R1				−0.873

Deg. degradation, *res* residues, *temp* temperature

Degradation

The degradation of VS during the anaerobic digestion process indicates the degradation efficiency of the biogas plant. The VS degradation depends mainly on OLR, HRT and the fermentation temperature (Mähnert 2007; Naik et al. 2014; Sakar et al. 2009). The national average of VS degradation in RS is 76 % (Gemmeke et al. 2009). In this survey, only four fermentation processes were found to have VS degradations (RS) of >76 %. High OLR can lead to inhibitions and a decrease in VS degradation. This might have been the case in biogas plant No. 7 with an OLR of 4.58 to 4.83 kg VS m^{-3} day^{-1}. However, it still showed a reasonable VS degradation of 61.36 to 63.11 % (RS). General consensus implies that the HRT has a large impact on the degradation efficiency. However, in this survey, it was found that, despite a HRT of only 37 days in biogas plant No. 10, a high VS degradation of 85.11 % in RS was possible. Similar results were found by Koch et al. (2009). Low VS degradation efficiencies were mainly found in fermentation processes with low process temperatures, one reactor stage and high shares of livestock residues. Moreover, no correlation of the OLR or HRT was found for VS degradation (RS). In contrast, the process temperature was correlated to all fermentation characteristic factors in group PC1 of the PCA (Table 4). It shows that the fermentation temperature in R1 had the biggest effect on the VS degradation in this survey. This seems contradictory to the results of Ruile et al. (2015) who found the HRT to be the driving factor for degradation efficiency in biogas plants using conventional substrates, while not the process temperature or the reactor stages. Their results are, however, only valid for higher process temperatures (investigated range 40 to 53 °C).

Specific methane yield

Generally, the feedstock composition in this study showed a relatively low specific methane yield (median

256.34 m^3 kg^{-1} VS) when compared to the national average biogas feedstock with 371 m^3 kg^{-1} VS (Gemmeke et al. 2009). This can be explained by the relatively high share of livestock residues in the organic biogas plants' feedstock and its low energetic output (specific methane yield 209 to 252 m^3 kg^{-1} VS (KTBL 2013; LFL 2015)). Energy crops like maize silage can be prone to high methane outputs with 340 m^3 kg^{-1} VS (KTBL 2013) and are utilised to a high share in the national average biogas plants (Fig. 1b). To increase the biogas output of livestock manure, many parameters have to be considered. The biogas potential of livestock manure is largely influenced by the VS content, the moisture content, storage conditions, animal feeding interval and the livestock performance level (Amon et al. 2007; Møller et al. 2014). Kaparaju et al. (2009) found that the biogas production of manure processing biogas plants can be optimised by using two reactor stages with equal process temperatures. This was confirmed by the results in this survey (biogas plants No. 3, 8 and 9). Grass silage can vary in its fibre content which, in-turn, influences the specific methane yield during the biogas process. Fibre-rich substrates show generally lower methane yields (Mukengele et al. 2006), which could be increased by elongating the HRT.

The real methane yield can also differ to the theoretical specific methane yield (Ruile et al. 2015). But KTBL (2013) showed that calculated theoretical specific methane yields were mostly correct, with a deviation of ±20 % in 82 % of 1854 investigated biogas plants. However, it has to be considered that the biogas plants in this survey, showing low degradation efficiencies, did not meet the calculated theoretical specific methane yields.

Residual methane potential

High amounts of VS in the fermenter effluents result in a high residual methane potential. The maximisation of the VS degradation in the biogas process has to be the main goal in organic farming to achieve ecological and economic benefits. Financial losses occur when high residual methane potentials impair the possibly higher energy production of the CHP unit. Furthermore, high residual methane potentials are a danger to the environment, as harmful greenhouse gas emissions can occur. In this investigation, it was found that only two biogas plants were equipped with residue storage cover. Coverage could prevent such emissions. Many single-staged biogas plants showed a high share of livestock residues and a low VS degradation in RS. Such biogas plants are advised to increase their VS degradation and reduce the residual methane potentials. The installation of a residue storage cover and integration of its methane emissions into the biogas concept would increase the economic and ecological benefits. Without coverage, a low residue storage temperature (<20 °C) is recommended to reduce methane emissions (Angelidaki et al. 2005; Ruile et al. 2015).

Improvement strategies

To gain a better biogas output in biogas plants on organic farms, the utilisation of a higher share of energy crops would seem to be sensible by contributing to an increase of the specific methane yield and the OLR. However, studies showed that growing energy crops for biogas production in organic farming is not economical (Anspach and Möller 2008b; Johansson et al. 2011). The shift to energy crops from conventional farming should not be the goal, as it contradicts the aims of organic farming. The main aspect of improving anaerobic digestion in organic farming is by increasing the VS degradation. This can be done by raising the process temperature to an acceptable mesophilic level. Such is possible by installing biogas/diesel dual fuel CHP engines. They have the benefit of being able to run with (organically produced) biodiesel and/or biogas. Moreover, a biogas plant operator can decide when the use of such supportive fuels is necessary.

After improving the process temperatures, the HRT is also a factor for optimising substrate degradation. The survey showed that biogas plants with two reactor stages favoured the VS degradation. The installation of a second reactor stage is advisable, as it also leads to a longer HRT; however, it is highly costly. A possibility to gain a higher HRT without a new installation of reactors is by recirculating reactor content from R2 or RS to R1. For that, the reactor volume in R1 has to be sufficient. For environmental criteria, residue storage cover is highly advised. Considering these improvement strategies, an increase of sustainable renewable energy production from biogas plants on organic farms can be met.

EEG 2014

The EEG 2014 deals with these problems by obligating all new biogas plant installations to have a HRT of

≥150 days and a residue storage cover (Bundesregierung 2014). Further, it offers possibilities for small-scaled biogas plants (≤75 kW) with high livestock residue shares (≥80 % FW) in the feedstock by subsidising its generated electrical energy with 23.73 ct kW h^{-1} (Bundesregierung 2014). As such biogas plant concepts are relevant in organic farming, this is a chance for increasing new installations of small-scaled biogas plants and its contribution to the total installed electrical capacity. An increase of such sustainable energetic concepts would also contribute to the National Sustainability Development Strategy.

Conclusion

The survey of 25 fermentation processes on organic farms showed large varieties in biogas plant specifications, fermentation characteristics and feedstock. Biogas plants with two reactor stages indicated, in general, better degradation performances than single-stage biogas plants. It was found that the process temperature had the largest impact on the VS degradation, not HRT and OLR. Poor degradation efficiency could be increased by raising the process temperature. Residual storages should have coverage to avoid greenhouse gas emissions. The utilised feedstock consisted mainly of livestock manure with low specific methane yield and grass silage. These substrates result in a lower energy production compared to average biogas plants in Germany. However, they are far more valuable in terms of sustainability when compared to maize silage. The EEG 2014 offers important chances for small-scale manure processing biogas plants on organic farms. An increase of biogas plants on organic farms would raise the sustainable renewable energy production and simultaneously support the National Sustainability Development Strategy.

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Manuscript 2

Stable carbon isotopes reveal that silage quality – not farming system – drives carbon degradation in anaerobic batch fermentations

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Personal contribution

Planning: 80 %

Experimental procedures: 100 %

Writing the manuscript: 80 %

Biogas plant operators aim to maximise the transformation of substrate-bound carbon (C) to biogas during the fermentation process. Common carbon degradation determination methods (C deg_{mix}) can be applied to identify the effectiveness of this process. These methods, however, depict the carbon degradation of the substrate and the inoculum inseparably. In our study we tested an alternative carbon isotope-based approach to identify solely substrate bound carbon degradation of maize silage (C deg f_{MS}). As stable isotopes vary in their natural abundance, the applicability of the alternative method was tested on maize silages of different farming systems (organic farming, conventional farming) and on maize silages with high (MS high) and low (MS low) qualities.

We digested six different maize silages in 19 lab-scale batch fermentations and analysed digestion parameters, specific biogas (sby) and methane yields (smy), stable isotopes and carbon degradations. Reactors with MS high showed up to 23.8% higher sby and smy compared to MS low. Significantly lower carbon degradations were found in reactors containing MS low compared to reactors with MS high within C deg_{mix} and C deg f_{MS}. Despite a high variability of the

stable carbon isotope values ($\delta^{13}C$) in the reactors at the start of the experiment, the isotope-based method showed reliable C deg f_{MS} values irrespective of maize silage qualities. Maize silages of different farming systems showed no prominent differences within reactors regarding digestion parameters and carbon degradation methods. The isotope-based approach offers a way to determine maize silage-bound carbon degradation more detailed by focusing on substrate degradation and excluding masking effects of the inoculum.

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Stable carbon isotopes reveal that silage quality – not farming system – drives carbon degradation in anaerobic batch fermentations

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Abstract

In anaerobic digestions carbon degradation indicates the effectiveness of the process. However, common carbon degradation determination methods (C deg_{mix}) handle substrate and inoculum inseparably. The aim of this study was to test an isotope-based method that allows solely maize silage bound carbon degradation (C deg f_{MS}) determination, digested in agricultural biogas plant inoculum. As the natural abundance of stable isotopes can differ, the method's applicability was tested on maize silage with high (MS high) and low (MS low) qualities and on maize silages of different farming systems.

Six maize silages were digested in a total of 19 lab-scale batch fermentations and analysed for digestion parameters, specific biogas (sby) and methane yields (smy), stable isotopes and carbon degradations. Reactors with MS low showed up to 23.8% lower sby and smy compared to MS high. After artificially reducing the maize silage qualities, significantly lower carbon degradations were detected in reactors with MS low compared to reactors containing MS high within C deg_{mix} and C deg f_{MS}. Despite highly variable stable carbon isotope values ($\delta^{13}C$) in the reactors at experimental start, the isotope-based method showed reliable C deg f_{MS} values independently of the maize silage qualities. Reactors with maize silages of different farming systems showed no prominent differences within digestion parameters and between all carbon degradation methods. The isotope-based method offers the possibility to assess maize silage-bound carbon degradation more detailed by excluding the masking effect of the inoculum.

Keywords: $\delta^{13}C$, carbon degradation, maize silage, organic farming, specific methane yield, stable isotopes

Abbreviations:

C deg_{mix} = Common carbon degradation determination method of reactor mixture

C deg_{MS} = Common carbon degradation determination method of maize silage

C deg f_{MS} = Carbon degradation determination method of maize silage using stable isotopes

MS conv = conventionally produced maize silage

MS high = maize silage of high quality

MS low = maize silage of low quality

MS org = organically produced maize silage

1. Introduction

Among the most important substrates in biogas production in Germany are maize silages [1]. A most effective transformation of substrate-bound carbon (C) to biogas during the anaerobic digestion process is the key aim for every biogas plant operator. Hence, many lab-scale biogas reactor investigations focused on maize silage degradations in order to analyse the effectiveness of the substrate's carbon transformation. The common degradation determination methods are mostly based on volatile solids (VS)[2] and on this basis specific yields are calculated. More detailed information on carbon degradation is gained by analysing the samples carbon concentrations by elemental analysers. Carbon mass quantification at experimental start and end is a common method for determining carbon degradation [3] in anaerobic digestions (C deg_{mix}). Many lab-scale anaerobic fermentations are set up in batch processes which, in the simplest case, are run with one inoculum and one substrate (e.g. maize silage). The common carbon degradation determination method C deg_{mix}, however, offers no possibility to identify maize silage carbon degradation (C deg_{MS}) during the anaerobic digestion individually, without taking the applied inoculum into account. Only an indirect approximation of available maize silage carbon in a reactor mixture is possible by taking separately digested inoculum into account. Therefore, it is highly required to establish a more accurate method to identify the degradation of maize silage carbon independently of the inoculum contribution.

Environmental sciences and geosciences have intensively used the natural abundance of stable isotopes ($^{13}C/^{12}C$, $^{15}N/^{14}N$) to investigate diverse physical, chemical and biological processes in oxic [4–6] and anoxic [7] environments. They are expressed as $\delta^{13}C$ or $\delta^{15}N$ values. Such studies often depended on substrate labelling or the natural variation of $\delta^{13}C$ in C_3 and C_4 vegetation. In anaerobic milieus, stable

isotopes have been successfully used to identify microbiological reaction pathways (e.g. [7–12]). Most studies were conducted using either in-vitro methods of specific microorganisms, municipal wastewater treatment plants, or by concentrating exclusively on the exhausted gasses. Investigations into agricultural biogas reactor contents and substrates are rare.

The aim of this survey was to implement a simple method to identify solely maize silage-bound carbon degradation (C deg f_{MS}) by stable isotopes, digested with agricultural biogas plant inoculum. Maize silage offers an outstanding possibility as its carbon is highly enriched in the heavier ^{13}C isotope as a consequence of the C_4 metabolic pathway in maize photosynthesis compared to C_3 plants [13]. The natural abundance of stable isotopes of farm products can, however, be influenced by different farming systems [14,15]. Therefore we included maize silages from different conventional farms (MS conv) and maize silages produced on organic farms (MS org) in the experimental design. Maize silages cultivated under organic farming standards (MS org) occur regularly as biogas substrates [16]. So far MS org have not been qualitatively investigated regarding anaerobic digestion performances. Thus, this investigation further aimed to identify differences in anaerobic digestion performances between MS conv and MS org.

Our main hypothesis was that identifying C deg f_{MS} by stable isotopes is possible. Therefore we artificially lowered the maize silage quality to test the applicability of the stable isotope method. By comparisons between maize silages of high quality (MS high) with maize silages of low quality (MS low), we expected to see significant carbon degradation differences with both, common carbon degradation method and the stable isotope method. Additional analyses of biogas and methane yields of qualitatively different maize silages offers the possibility to identify losses due to ensilage and storage failures.

2. Material and Methods

2.1. Experimental setup

Three anaerobic batch digestion experiments were conducted to test maize silages originating from conventional and organic farming systems in high and low qualities. The first experiment (E1) was performed with nine one-litre glass flask reactors which were situated in a closed PVC box and heated continuously to 38.0 ± 0.5 °C. Every flask had a gastight sealing including a biogas outlet. The biogas was led through flexible tubes into a water-filled glass to prevent reverse aeration before being diverted to

discharged air. All reactors were continuously stirred on a platform shaker (110 rpm). They contained 700 ml inoculum (Inoc1) and MS high from four different farms (MS1 to MS4). MS1 and MS2 were conventionally produced maize silages, while maize silages MS3 and MS4 originated from organic farming (Tab. 1). All maize silages were digested in two reactors as duplicates. One reactor contained only inoculum Inoc1 and run as control.

The second experiment (E2) had a similar setup with a different inoculum (Inoc2, 700 ml) and the same maize silages MS1 to MS4 digested in duplicates. Prior to the start of experiment E2, maize silages MS1 to MS4 were kept for 47 days at 21 ± 1 °C while stored in gastight plastic bags to simulate poor storage conditions and achieve maize silages of low quality. The maize silages of experiment E2 are therefore referred to as incubated maize silages. Here, reactor R3 (MS3) could not be evaluated, owing to technical problems. One control reactor contained only inoculum Inoc2.

After addition of maize silage to the inocula, each reactor in experiments E1 and E2 was filled with up to 150 ml of water to achieve a consistent total volume of 900 ml. In this survey, reactor contents, after addition of maize silages to the inocula, are referred to as mixtures (mix). The organic loadings (OL's) of the reactors at start of experiments E1 and E2 were, respectively, 13.32 ± 1.14 and 13.92 ± 0.00 kgVS/m³. The duration of both experiments was 29 days.

A third experiment (E3) was carried out with four 12 litre lab-scale batch reactors. A water bath was used to maintain the temperature of the digesters at 42 ± 0.5 °C. The reactors were repeatedly stirred (40 rpm) with installed paddle mixers for three minutes with a pausing interval of 15 minutes. Two different maize silages (MS5 and MS6) were digested in duplicates with inoculum (Inoc3, 9000 ml) (Tab. 1). Maize silage MS5 originated from conventional farming and was categorised as MS high while maize silage MS6 was organically produced maize silage and categorised as MS low due to the level of pH. Each reactor was then filled with water to a volume of 9600 ml. Produced biogas was analysed for volumes and methane concentrations. A fifth reactor (1 l glass flask) was added to the water bath and operated as a control containing solely inoculum Inoc3. The OL was set to 15.00 kgVS/m³ and the experimental duration to 64 days.

2.2. Substrates

Inocula Inoc1 and Inoc2 originated from an organic farm where a biogas plant was operated at 38 °C, digesting cattle manure, cattle dung and clover silage (52.78% fresh weight (FW) of total feedstock). The inocula were extracted separately and stored under anaerobic conditions at 38 °C for 4 days. Inoculum Inoc3 was extracted from a mesophilic (40 °C) biogas plant using conventional substrates: cattle manure, cattle dung, maize silage (50.00% FW of total feedstock) and grass silage (16.67% FW of total feedstock). The inoculum Inoc3 was stored in the reactors of experiment E3 for 11 days at 42 °C for microbial adaptation and reduction of background biogas production by the inoculum. It showed an average of 13.67 l_N/(kgVS d) residual biogas production prior to the start of the experiment. Maize (*Zea mays* L.) silages were collected at different conventional and organic farms with installed biogas plants. Organic maize silage MS6 was ensiled with sunflower plants (ca. 5% FW).

2.3. Sample analysis and evaluation

Generally, all inocula, maize silages and reactor mixtures (start/end) were analysed for total solids (TS), VS and pH (excluding MS1 – MS4) according to VDLUFA (1997) [2]. Total carbon (C) and nitrogen (N) concentrations were analysed in dried (60 °C) and ground (particle size < 0.5 mm) samples with an elemental analyser (LECO TrueSpec CN, LECO, St. Joseph, USA). Inocula were tested for VFA/total alkaline capacity (TAC) buffer stability (VFA/TAC) [17] (Tab. 1).

Tab. 1 Substrate characteristics before experimental start

	Inoc1	Inoc2	Inoc3	MS1	MS2	MS3	MS4	MS5	MS6
Farming	org	org	conv	conv	conv	org	org	conv	org
TS [%]	4.93	4.53	4.77	39.02	33.67	34.47	27.58	30.55	31.03
VS [%]	56.29	64.27	57.11	96.40	80.49	95.51	96.69	84.45	80.98
C [%]	38.87	39.76	39.40	45.91	46.56	45.82	47.03	46.05	46.90
N [%]	2.20	2.49	3.33	1.26	1.33	1.27	1.20	1.20	1.63
pH	8.02	7.94	7.99	———	———	n.d.	———	3.78	5.71
VFA/TAC	0.067	0.056	0.063	———	———	n.a.	———	———	———

C = carbon concentration, conv = conventional, Inoc = inoculum, MS = maize silage, N = nitrogen concentration, n.a. = not applicable, n.d. = not determined, org = organic, TS = total solids, VS = volatile solids, VFA/TAC = volatile fatty acids/total alkaline capacity

In experiment E1, three major factor groups were analysed consisting of digestion parameters, stable isotopes and carbon degradations. Experiment E2 focussed mainly on stable isotopes and carbon degradations. Data from reactors containing MS conv (MS1, MS2) and MS org (MS3, MS4) were compared

to elucidate differences between maize silages produced by conventional and organic agricultures, respectively, using both experiments E1 and E2. Experiments E1 and E2 were additionally compared to identify prominent differences between maize silages of both high and low quality. Experiment E3 was analysed for differences in digestion parameters, specific biogas (sby) and methane yields (smy), respectively, stable isotopes and carbon degradations.

The digestion parameters consisted of pH, VFA/TAC, acetic, propionic and butyric acids, which were analysed consecutively in experiment E1 (ten times) and experiment E3 (twelve times). Samples were pre-treated to 50% (v/v) with formic acid (≥ 99%) and stored immediately at -21 °C. Organic acids were analysed after centrifugation by gas chromatography (GC) (CP 9001, FID, SGE HT5, 300 °C, Chrompack Inc., USA). Samples of experiment E3 were analysed with a Clarus 580 GC (FID, Elite Wax, 280 °C, PerkinElmer, USA).

Sby and smy were determined by recording volumes (MGC-1, Ritter GmbH & Co. KG, Germany) and methane concentrations (BCP-CH4, BlueSens gas sensor GmbH, Germany) of the produced biogas in experiment E3. Data were logged hourly with the software BACVis (Version 7.6.2.1, BlueSens gas sensor GmbH, Germany) and corrected to standard conditions (273.15 K and 101.325 kPa). The sby and smy were measured in norm litres (l_N) produced per kg volatile solids (kgVS) of the according substrate.

Stable carbon isotopes were evaluated in all maize silages, inocula and reactor mixtures at beginning and at the end of the experiments. In addition, nitrogen isotope signatures ($\delta^{15}N$) were assessed for all maize silages. Analyses were conducted with 0.7 mg of dried and ground samples at the Institute of Food Chemistry, University of Hohenheim. Acetanilide was used as the laboratory standard (isotopic and elemental drift control). Samples were measured with an elemental analyser (Euro EA C/N analyser, EuroVector, Italy), which was coupled to an isotope ratio mass spectrometer (Thermo Finnigan Delta plus Advantage, Thermo Fisher Scientific Inc., USA). The applied laboratory reference gases were CO_2 and N_2 (USGS40 calibrated) [18].

Stable isotope signatures are expressed by convention as

$$\delta X\ (‰) = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000 \qquad (1)$$

where X is ^{13}C or ^{15}N and R is the corresponding isotope ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The isotope signatures are referred to $R_{standard}$, being the international PDB (Pee Dee Belemnite) standard of 0.0112372 [19] for $\delta^{13}C$ and atmospheric N_2 of 0.3663 for $\delta^{15}N$ [20].

The carbon degradations of the reactor mixtures (C deg_{mix}) were determined by carbon mass quantification at experimental start and end, with the common carbon degradation determination method

$$\mathrm{C\,deg_{mix}}(\%) = \frac{\mathrm{C\,mass_{mix}}\,start - \mathrm{C\,mass_{mix}}\,end}{\mathrm{C\,mass_{mix}}\,start} \times 100 \quad (2)$$

Generally, the carbon mass of mixtures (C $mass_{mix}$), maize silage (C $mass_{MS}$) and inoculum (C $mass_{Inoc}$) were analysed by

$$\mathrm{C\,mass_{A\,B}\ (g)} = \mathrm{FW_{A\,B}} \times \mathrm{TS_{A\,B}} \times \mathrm{C_{A\,B}} \quad (3)$$

with *A* = exclusively mix, MS or Inoc and *B* = exclusively start or end of the experiment. This formula included the fresh weight (FW [g]), the percentage of total solids (TS [%]) and carbon concentrations (C [%]) of the according sample.

For elucidating the maize-bound carbon degradation on the basis of the common carbon degradation (C deg_{MS}) we used the following equation:

$$\mathrm{C\,deg_{MS}}(\%) = \frac{\mathrm{C\,mass_{MS}}\,start - (\mathrm{C\,mass_{mix}}\,end - \mathrm{C\,mass_{Inoc}}\,end)}{\mathrm{C\,mass_{MS}}\,start} \times 100 \quad (4)$$

As the carbon mass of the maize silage is not detectable at the end of the experiment, it was approximated by an indirect approach. Therefore, the carbon mass of the inoculum (C $mass_{Inoc\ end}$), digested in the control reactor, was subtracted from the carbon mass of the reactor mixture at experimental end (C $mass_{mix\ end}$).

For calculating the carbon degradations of the maize silages on basis of the stable isotopes (C deg f_{MS}) the following equation was applied:

$$\mathrm{C\,deg\,f_{MS}}\ (\%) = \frac{\mathrm{C\,mass_{MS\ start}} - \mathrm{C\,mass_{MS\ end}}}{\mathrm{C\,mass_{MS\ start}}} \times 100 \quad (5)$$

Here, the carbon mass of the maize silages were analysed at experimental start (C mass $f_{MS\ start}$) and end (C mass $f_{MS\ end}$) by

$$\text{C mass}\, f_{\text{MS A}}\,(\text{g}) = \text{C mass}_{\text{mix A}} \times f_{\text{MS }A} \quad (6)$$

with *A* = exclusively start or end of the experiment. For this formula the stable carbon isotope fractions (f_{MS}) of the maize silages were analysed by applying $\delta^{13}C$ values of the respective mixture ($\delta^{13}C_{mix}$), inoculum ($\delta^{13}C_{inoc}$) and maize silage ($\delta^{13}C_{MS}$) on the following stable isotope mixing model:

$$f_{\text{MS }A} = \frac{\delta^{13}C_{\text{mix }A} - \delta^{13}C_{\text{Inoc }A}}{\delta^{13}C_{\text{MS }start} - \delta^{13}C_{\text{Inoc }A}} \quad (7)$$

with *A* = exclusively start or end of the experiment. The values for inocula were taken from the according control reactors. This stable isotope mixing model was modified after Werth & Kuzyakov [5].

During anaerobic fermentations $\delta^{13}C$ values of reactor mixtures shift towards a specific direction. This shift is described as

$$\Delta\delta^{13}C\,(‰) = \delta^{13}C_{\text{mix end}} - \delta^{13}C_{\text{mix start}} \quad (8)$$

2.4. Statistical analysis

Different statistical tests were applied to find significant ($p < 0.05$) differences between reactors containing MS high and MS low and between reactors with MS conv and MS org. Online-measured digestion parameters were analysed by repeated measures analysis of variance (ANOVA). Here, experiment E1 and experiment E3 were analysed separately, including the first 29 days for analysis. Nonparametric Mann-Whitney-U tests with the factors 'maize silage quality (high/low)' and 'farming system (conv/org)' were applied to find significant differences in $\delta^{13}C$ values of the reactor mixtures and the three carbon degradation determination approaches C deg_{mix}, C deg_{MS} and C deg f_{MS}. Pearson tests were applied to find correlations within the analysed parameters. The software SPSS 20 (SPSS Inc., USA) was used for statistical analysis. Diagrams were generated with Microsoft Excel 2010. Mean values are given with standard deviations.

3. Results

3.1. Digestion parameters

In experiment E1, reactors with MS conv (MS1, MS2) showed comparable pH courses to reactors containing MS org (MS3, MS4) (Fig. 1). The maximum VFA/TAC value was found with 0.35 in reactor R2 (MS1) on day

four. As expected, the pH courses developed according to the VFA/TAC values. The highest acetic acid concentrations in experiment E1 were found in reactors with MS conv with 5.26 g l^{-1} for maize silage MS1 and 4.67 g l^{-1} for maize silage MS2. Taking the whole experimental phase of experiment E1 into account, significantly higher acetic acid ($F = 11.69$, $n = 10$, $p < 0.05$) and propionic acid concentrations ($F = 10.82$, $n = 10$, $p < 0.05$) were found in reactors containing MS conv compared to MS org.

In experiment E3, reactors with maize silage MS6 showed higher pH values on day one compared to reactors with maize silage MS5. The VFA/TAC showed values of 0.18 (R3) and 0.15 (R4) on day seven in reactors with maize silage MS6, while both reactors with maize silage MS5 showed values of 0.04. Acetic and propionic acid concentrations were found in reactors with maize silage MS6 on day seven, but were already depleted in reactors with maize silage MS5. In experiment E3, significantly higher VFA/TAC values ($F = 50.525$, $n = 8$, $p < 0.05$) and acetic acid concentrations ($F = 26.05$, $n = 8$, $p < 0.05$) were found in reactors with maize silage MS6 compared to reactors containing MS5.

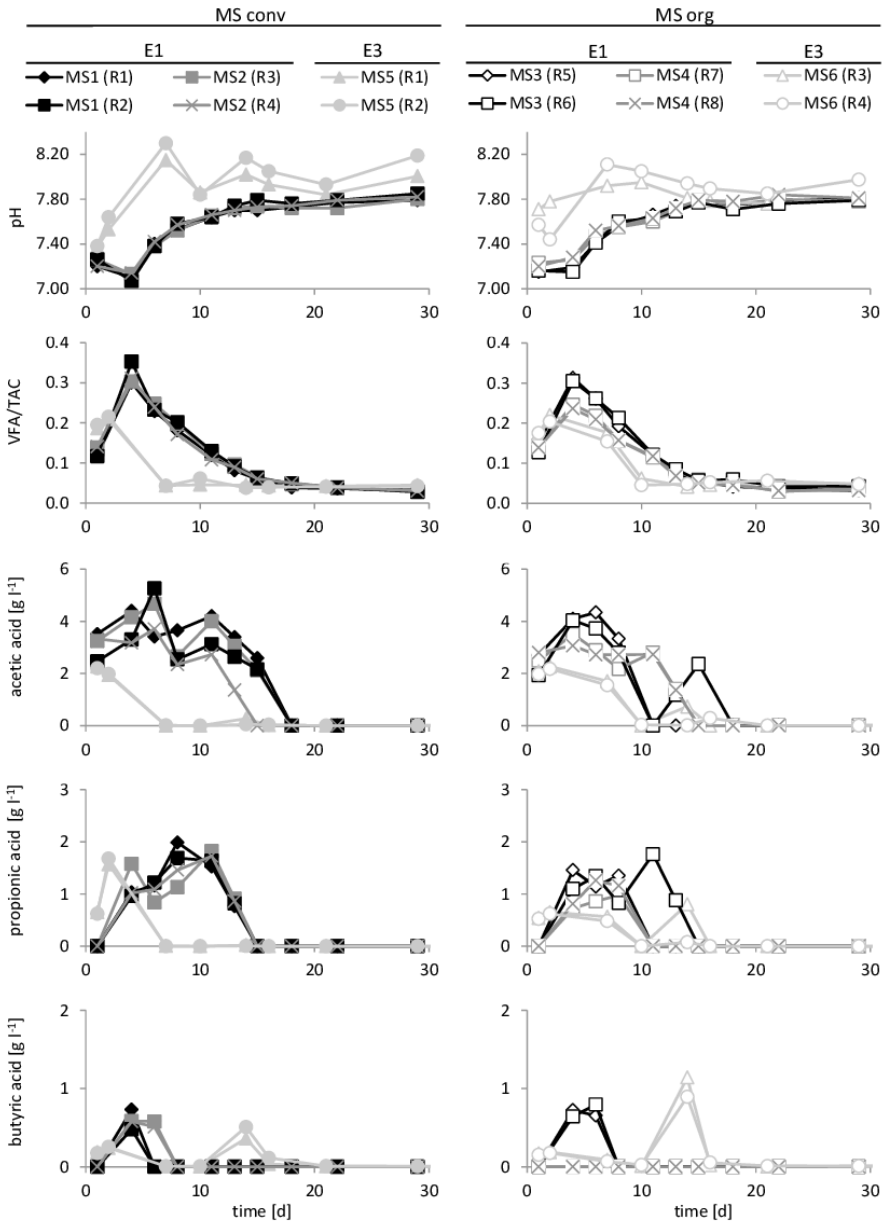

Fig. 1 Digestion parameters of different maize silages (MS) originating from conventional (conv) and organic (org) farming in experiments E1 and E3 (first 29 days depicted). R = reactor, VFA/TAC = volatile fatty acids/total alkaline capacity buffer. Note different y-axis scaling

3.2. Specific biogas and methane yields

Standard protocols for assignment of specific yields [21] require that the inoculum has a low residue biogas production at experimental start, which was given in experiment E3. Maize silage MS5 showed higher

cumulated sby of 708 and 702 l_N/(kgVS), respectively, than MS6 with 648 and 621 l_N/(kgVS) (Fig. 2). The cumulated smy was also higher in reactors containing maize silage MS5 with 461 and 392 l_N/(kgVS), respectively, compared to reactors containing maize silage MS6 with 360 and 352 l_N/(kgVS). Reactors with maize silage MS6 produced 7.6 - 12.2% lower sby and 8.3 - 23.8% lower smy, respectively, than reactors with maize silage MS5. A shift in the steepness of sby and smy courses was visible. For reactors with maize silage MS5 the courses' steepness changed from day six to seven and for reactors with maize silage MS6 on day eight to nine.

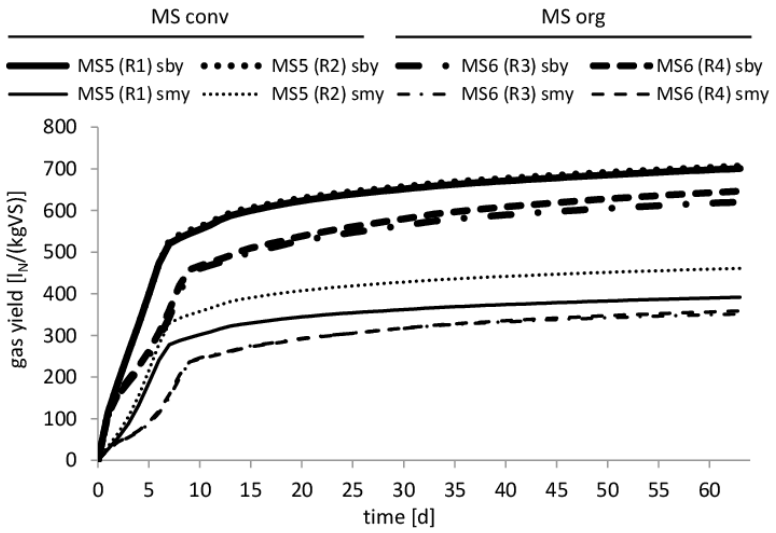

Fig. 2 Specific biogas (sby) and methane yields (smy), respectively, of different maize silages (MS) originating from conventional (conv) and organic (org) farming in experiment E3. R = reactor, VS = volatile solids

3.3. Stable isotopes

The $\delta^{13}C$ values of maize silages MS1 to MS4 ranged from -12.160 - -13.068 ‰ (Tab. 2). The $\delta^{15}N$ values of maize silages MS1 to MS4 ranged from 6.165 - 7.486 ‰. In experiment E3, maize silage MS5 and MS6 showed $\delta^{13}C$ values of -12.371 and -18.626 ‰, respectively, and inoculum Inoc3 a $\delta^{13}C$ value of -17.581 ‰. In average, MS conv (MS1, MS2) showed slightly more negative $\delta^{13}C$ values compared to MS org (MS3, MS4). MS conv also showed slightly higher average $\delta^{15}N$ values compared to MS org.

In experiments E1 and E2 all reactor mixtures had less negative $\delta^{13}C$ values compared to inocula Inoc1 and Inoc2 with -27.111 and -26.840 ‰, respectively. In experiment E1 (MS high) the mixtures showed mean $\delta^{13}C$

values of -21.98 ± 0.11 ‰ at experimental start ($\delta^{13}C_{mix\ start}$) and -25.68 ± 0.20 ‰ at experimental end ($\delta^{13}C_{mix\ end}$) (Fig. 3). The mixtures showed a mean $\Delta\delta^{13}C$ shift of -3.70 ± 0.13 ‰ in experiment E1. When low quality silages were digested in experiment E2, the mixtures showed mean $\delta^{13}C_{mix\ start}$ values of -23.60 ± 0.78 ‰ and mean $\delta^{13}C_{mix\ end}$ values of -25.84 ± 0.37 ‰. The $\delta^{13}C_{mix}$ values shifted in average by $\Delta\delta^{13}C$ -2.24 ± 0.74 ‰. The $\delta^{13}C_{mix\ start}$ values were significantly more negative ($z = -3.252$, $n = 15$, $p < 0.01$) in experiment E2 compared to experiment E1. The $\delta^{13}C_{mix\ start}$ values correlated with the $\delta^{13}C_{mix\ end}$ values ($r = 0.858$, $n = 8$, $p < 0.01$) in experiment E1. Further, a correlation was found between the $\delta^{13}C_{mix\ start}$ values and the stable isotope shift $\Delta\delta^{13}C$ ($r = 0.885$, $n = 7$, $p < 0.01$) in experiment E2. Generally, all $\delta^{13}C_{mix\ end}$ values were more negative compared to the $\delta^{13}C_{mix\ start}$ values. Also the $\delta^{13}C_{Inoc\ end}$ values of the inocula Inoc1, Inoc2 and Inoc3 were more negative compared to their $\delta^{13}C_{Inoc\ start}$ values.

Tab. 2 $\delta^{13}C$ and $\delta^{15}N$ stable isotope values of maize silages MS1 to MS6 (n =3).

Maize silage	Farming	$\delta^{13}C$ [‰]	$\delta^{15}N$ [‰]
MS1	conv	-12.558 ± 0.14	7.486 ± 0.09
MS2	conv	-13.068 ± 0.21	6.798 ± 0.26
MS3	org	-12.577 ± 0.15	6.615 ± 0.22
MS4	org	-12.160 ± 0.07	6.165 ± 0.08
MS5	conv	-12.371 ± 0.09	6.536 ± 0.70
MS6	org	-18.576 ± 0.67	6.223 ± 0.32

conv = conventional, org = organic

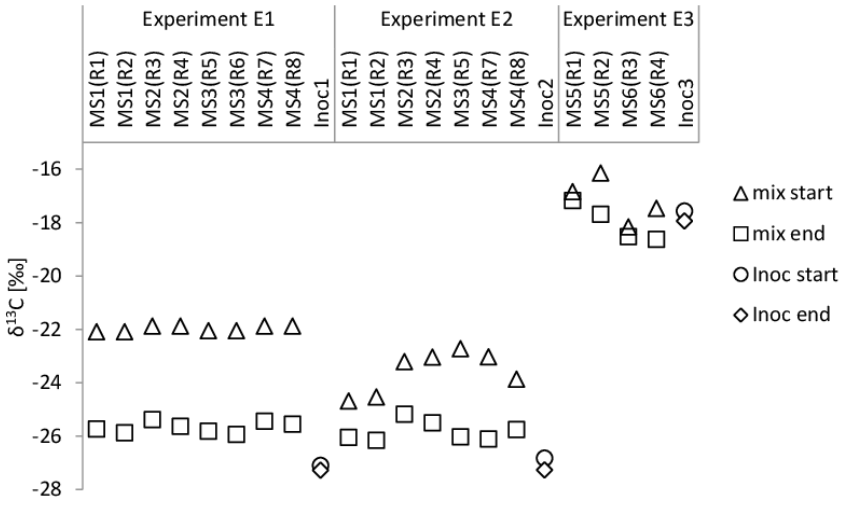

Fig. 3 $\delta^{13}C$ values of reactor mixtures (mix) and inocula (Inoc) at start and end of experiments E1, E2 and E3. MS = maize silage, R = reactor

3.4. Carbon degradations

As expected, the carbon mass in each reactor was higher at experimental start (C $mass_{mix\ start}$) compared to the end of the experiments (C $mass_{mix\ end}$) (Tab. 3). This was also found for the carbon mass of the maize silages. The carbon degradations showed C deg_{mix} values ranging from 31.64 - 48.67% for the whole reactor mixtures in experiment E1. Significantly higher C deg_{mix} values were found in experiment E1 compared to experiment E2 ($z = -2.893$, $p < 0.01$, $n = 15$) with low quality maize silages, ranging from 23.10 - 36.98%. The carbon degradations of experiment E3 showed C deg_{mix} values ranging between 61.23 and 62.93%.

When applying the common carbon degradation method, C deg_{MS} ranged from 49.54 - 85.62% in experiment E1 and from 46.66 - 81.12% in experiment E2. No significant differences were found for C deg_{MS} between both experiments with this method. However, slightly higher average C deg_{MS} values were found in experiment E2 with 71.48 ± 11.9% compared to experiment E1 with 65.56 ± 14.5%. Experiment E3 showed C deg_{MS} values ranging from 79.50 - 95.32%.

The application of the stable isotope mixing model allowed the determination of maize-silage specific carbon degradation C deg f_{MS} (cf. equation 7). The C deg f_{MS} values in experiment E1 ranged from 75.35 - 86.48% and were significantly higher ($z = -2.777$; $n = 15$; $p < 0.01$) compared to the values found in

experiment E2 with C deg f_{MS} ranging from 58.38 - 79.24%. The carbon degradation determination method using stable isotopes C deg f_{MS} was found to be not applicable in experiment E3. Different farming systems of the maize silages showed no significant differences between MS conv and MS org for C deg_{mix}, C deg_{MS} and C deg f_{MS}.

Tab. 3 Carbon mass of the mixtures (C $mass_{mix}$) and maize silages (C $mass_{MS}$, C mass f_{MS}) for each reactor at the start and the end of experiments E1 and E2. Common carbon degradation calculations of mixtures (C deg_{mix}) and maize silages (C deg_{MS}) originating from conventional (MS conv) and organic (MS org) farming. Maize silage bound carbon degradation with applied isotope mixing model (C deg f_{MS}). Experiment E1 represents maize silages of high quality (MS high) while experiment E2 represents maize silages of low quality (MS low). Letters refer to differences between different carbon degradation determination methods of experiments E1 and E2. Variants with the same letter are not significantly different at the 0.05 level.

			MS conv				MS org				total average	
			MS1		MS2		MS3		MS4			
			R1	R2	R3	R4	R5	R6	R7	R8		
E1 (MS high)	C $mass_{mix\ start}$	[g]	24.35	24.35	23.58	23.58	27.12	27.12	25.05	25.05		
	C $mass_{mix\ end}$	[g]	14.72	14.54	16.12	15.60	15.92	13.92	16.07	14.25		
	C deg_{mix}	[%]	39.57	40.28	31.64	33.83	41.32	48.67	35.86	43.10		
	Ø C deg_{mix}	[%]	36.33 ± 4.3				42.24 ± 5.3				39.28 ± 5.5	a
	C $mass_{MS\ start}$	[g]	5.97	5.97	6.05	6.05	5.95	5.95	6.11	6.11		
	C $mass_{MS\ end}$	[g]	1.65	1.48	3.05	2.54	2.85	0.86	3.00	1.19		
	C deg_{MS}	[%]	72.34	75.25	49.54	58.06	52.16	85.62	50.91	80.57		
	Ø C deg_{MS}	[%]	63.80 ± 12.1				67.32 ± 18.4				65.56 ± 14.5	b
	C mass $f_{MS\ start}$	[g]	8.39	8.39	8.77	8.77	9.17	9.17	9.01	9.01		
	C mass $f_{MS\ end}$	[g]	1.54	1.39	2.16	1.81	1.54	1.24	2.01	1.68		
	C deg f_{MS}	[%]	81.58	83.40	75.35	79.33	83.17	86.48	77.66	81.40		
	Ø C deg f_{MS}	[%]	79.92 ± 3.5				82.18 ± 3.7				81.05 ± 3.5	c
E2 (MS low)	C $mass_{mix\ start}$	[g]	23.33	20.74	22.93	26.17	21.19	n.d.	25.87	21.70		
	C $mass_{mix\ end}$	[g]	16.70	15.95	17.01	16.49	16.12	n.d.	18.07	16.10		
	C deg_{mix}	[%]	28.43	23.10	25.84	36.98	23.93	n.d.	30.14	25.78		
	Ø C deg_{mix}	[%]	28.59 ± 6.0				26.62 ± 3.2				27.74 ± 4.8	d
	C $mass_{MS\ start}$	[g]	5.97	5.97	7.25	7.25	6.01	n.d.	6.09	6.09		
	C $mass_{MS\ end}$	[g]	1.88	1.13	2.19	1.67	1.30	n.d.	3.25	1.28		
	C deg_{MS}	[%]	68.50	81.12	69.80	76.93	78.41	n.d.	46.66	78.94		
	Ø C deg_{MS}	[%]	74.09 ± 6.0				68.00 ± 18.5				71.48 ± 11.9	b
	C mass $f_{MS\ start}$	[g]	3.50	3.33	6.03	7.19	5.92	n.d.	6.90	4.50		
	C mass $f_{MS\ end}$	[g]	1.39	1.21	2.51	2.05	1.32	n.d.	1.43	1.66		
	C deg f_{MS}	[%]	60.34	63.67	58.38	71.49	77.66	n.d.	79.24	63.10		
	Ø C deg f_{MS}	[%]	63.47 ± 5.8				73.34 ± 8.9				67.70 ± 8.42	b

MS = maize silage, n.d. = not determined, R = reactor

4. Discussion

4.1. Digestion parameters

Metabolic inhibitions due to high VFA/TAC or low pH values [17] were not detected in the experiments. Acid production is the key intermediate during anaerobic digestion. Acetic acid showed the highest concentrations of all analysed acids. This was also found by Mösche & Jördening [22]. Significant different acetic acid concentrations were found between reactors with MS conv and reactors with MS org in both experiment E1 and experiment E3. In experiment E1 reactors with MS conv (MS1, MS2) showed significantly higher acetic and propionic acid concentrations than reactors with MS org (MS3, MS4). In experiment E3, however, the acetic acid concentrations were significantly higher in reactors with MS org compared to MS conv. These contradictory results indicated that organic acid production cannot be linked to a specific farming system producing maize silages.

McDonald et al. [23] stated that pH values less than 4.0 indicate a good silage quality, which was present in maize silages MS5 (pH = 3.78) of experiment E3. The higher acetic acid concentrations we found in this maize silage, compared to maize silage MS6, was possibly due to beneficial substrate break-down by lactic acid fermentation during ensilage [23]. This break-down did not take place in maize silage MS6, as indicated by higher pH values, and slowed the anaerobic digestion metabolism. As a result it led to a prolonged presence of acetic acid in reactors with maize silage MS6. These results indicated that the digestion parameters were more influenced by the quality of the maize silages than by the farming system itself.

4.2. Specific biogas and methane yield

Of the analysed acids, acetic acid has the highest conversion rates to methane [24]. Herrmann et al. [25] and Vervaeren et al. [26] showed a close relationship of acetic acid concentrations to the smy. The effect of acetic acid on the biogas and methane production was also visible in our survey. With depletion of acetic acid the steepness of the sby and smy courses changed visibly. Higher sby and smy were found for reactors with maize silage MS5 compared to reactors with MS6. This showed that good silage quality, as evidenced in maize silage MS5, is beneficial for biogas and methane production. Low quality maize silages appear frequently throughout farming [27] which are often preferably utilised in biogas plants instead of being used as livestock fodder. In agreement with our survey, Amon et al. [28] also found smy to be increased by up to 25%, while maize showed good ensilage properties compared to that of freshly harvested maize. The higher

sby and smy of reactors with maize silage MS5 compared to reactors with maize silage MS6 are therefore likely to be related to beneficial ensilage processes and good storage conditions of the maize silage [29].

4.3. Stable isotopes

The investigated maize silages showed $\delta^{13}C$ values corresponding to literature values for maize [30,31] and maize silages [32]. An exception was found in maize silage MS6, which showed more negative $\delta^{13}C$ values. Sunflowers have, as C_3-plants, a $\delta^{13}C$ of about -28 ‰ [33], which explains the more negative values found in maize silage MS6 due to mixed ensilage. Therefore, reactor mixtures of experiment E3 were not considered for further stable isotope analyses. Our survey showed slightly more negative $\delta^{13}C$ values of MS conv (MS1, MS2) compared to MS org (MS3, MS4) (Tab. 2). However, Haubert et al. [30] found contradictory results with marginally significant lower $\delta^{13}C$ values in organically cultivated maize leaves (-13.4 ± 0.37 ‰) and roots (-13.3 ± 0.06 ‰) compared to conventional maize. This indicated that the farming system has no specific impact on the $\delta^{13}C$ ratios in maize. The slightly higher $\delta^{15}N$ values of MS conv, compared to MS org, might be a result of different agricultural fertilization systems. Combinations of mineral and organic fertilisers are often applied in conventional farming, whereas mineral nitrogen fertilisers are not allowed in organic farming practice [34]. Shearer et al. [35] showed that manure is ^{15}N enriched with $\delta^{15}N$ values of > 5.0 ‰ and mineral fertilisers had $\delta^{15}N$ values of < 4.0 ‰. Higher $\delta^{15}N$ values of MS conv might result from an increased ^{15}N uptake due to a quantitatively higher manure fertiliser application in conventional farming systems.

The inocula Inoc1 and Inoc2 showed relatively similar $\delta^{13}C$ values at the start of each experiment. The $\delta^{13}C$ differences between these inocula (Inoc1 and Inoc2) compared to inoculum Inoc3 originated from different substrates utilised in the according biogas plants. The biogas plant of inocula Inoc1 and Inoc2 was situated on an organic farm. Such biogas plants are generally prone to utilise high amounts of C_3 plants and low amounts of maize silages (C_4 plants) [16]. This resulted in $\delta^{13}C$ values more depleted in the ^{13}C isotope than inoculum Inoc3, which originated from a conventional farm's biogas plant that utilised maize silages. The incubation of maize silages MS1 to MS4 prior to experiment E2 (MS low) led to significantly more negative $\delta^{13}C_{mix\ start}$ values compared to the same maize silages in experiment E1 (MS high) (see Fig. 3). This shows that maize silages with storage failures can be identified by negatively-shifted $\delta^{13}C$ values in the reactor mixture. The correlation of $\delta^{13}C_{mix\ start}$ in experiment E1 demonstrated that the reactor mixtures with high

quality maize silages tended to shift similarly towards a more negative $\delta^{13}C_{mix\,end}$ value. This could also be seen by the relatively low standard deviation of the average $\Delta\delta^{13}C$ values. Therefore, the heavy ^{13}C isotope was depleted equally in maize silages of high quality throughout all reactors.

The relatively high variation in $\delta^{13}C_{mix\ start}$ values of the maize silages in experiment E2 showed that the maize silages incubated differently. The incubation led to different ^{13}C-depleted maize silages and reactor mixtures at the start of experiment E2. Despite the different average $\delta^{13}C_{mix\,start}$ values between experiments E1 and E2, relatively similar $\delta^{13}C_{mix\,end}$ values were found in both experiments (Fig. 3). The $\delta^{13}C_{mix\,end}$ values of experiments E1 and E2 were clearly shifted towards the $\delta^{13}C_{Inoc}$ values. This was also shown by the correlation of the $\delta^{13}C_{\ mix\,start}$ values with the $\Delta\delta^{13}C$ shifts in experiment E2. Despite highly variable $\delta^{13}C_{mix\,start}$ values the $\Delta\delta^{13}C$ shift was limited to a certain threshold. This shows that the ^{13}C depletion of the maize silage is limited by the quantitatively higher amount of inocula.

Most biochemical processes discriminate against the heavier carbon isotope creating products depleted in ^{13}C and leaving reactants enriched in ^{13}C [5,7,36]. Such results were, however, mainly observed in experiments of either pure C_3 or pure C_4 samples. In our survey we worked with mixed C_3 and C_4 samples and found a negative shift of all $\delta^{13}C_{mix}$ and $\delta^{13}C_{Inoc}$ values when comparing the experimental start with the experimental end. This indicated a ^{13}C depletion of the reactant. Similar results were found by Laukenmann et al. [12] who digested C_3 and C_4 mixtures in batch biogas systems and analysed $\delta^{13}C$ values of produced methane and CO_2. The authors found a shift of the gases towards more negative $\delta^{13}C$ values for the first five days, leaving the mixture enriched in ^{13}C isotopes. This was also found by Francioso et al. [8] who reported a positive shift of $\delta^{13}C$ values in the mixtures during the first six days. However, in the proceeding experimental time of Laukenmann et al. [12] the gases shifted towards less positive $\delta^{13}C$ values, demonstrating a depletion of the ^{13}C isotopes in the mixture. This occurred despite methane formation, which is a strongly ^{13}C discriminating biodegradation process (methane $\delta^{13}C$ values from -47 - -89 ‰) [37]. An explanation can be found in the limitation of the carbon pool. As Laukenmann et al. [12] showed, the gases were isotopically depleted at the beginning of the experiment. During this time the mixtures became enriched in ^{13}C. However, as the carbon pool is limited in a batch digestion experiment, the ^{13}C enriched mixture becomes eventually metabolised to methane and CO_2 which, in turn, shows increasingly less

positive $\delta^{13}C$ values. These results indicated a depletion of ^{13}C in the reactor mixtures of anaerobic batch digestion experiments when the carbon pool becomes increasingly limited.

The negative shift of all $\delta^{13}C_{mix}$ and $\delta^{13}C_{Inoc}$ values at the experimental end was also the result of variable degradability of different biochemical fractions. Sugars, starch, proteins and organic acids are considered easily degradable in anaerobic digestions, while having less negative $\delta^{13}C$ values [4,38]. Lignin and lipids are difficult substrates concerning degradability [39] and have more ^{13}C-depleted $\delta^{13}C$ values [4,38]. The relative amount of biochemical fractions of low degradability increased during the experimental retention time, shifting $\delta^{13}C$ towards more negative values, and, in addition, to the continuous depletion of maize silage in the mixtures.

4.4. Application of stable isotopes for carbon degradation assessment

The significantly lower C deg_{mix} in both experiments, E1 and E2, compared to C deg_{MS} and C deg f_{MS} can be explained as the carbon mass of the reactor mixtures includes the inoculum (cf. equation 2). The carbon degradation, however, occurs mainly in the added substrate while the inoculum, still rich in carbon, provides the necessary microbial communities and enzymes. Therefore, the carbon degradation evaluation of the substrate in batch experiments is masked by the quantitatively high amount of inoculum, resulting in an underestimation of the actual substrate-bound carbon degradation. The expectedly low C deg_{mix} of experiment E2 compared to experiment E1 was as a result of lost organic compounds during the incubation of the maize silages. The maize silage degradability can be influenced negatively by temperature and long storage conditions [40]. During the incubation of the maize silages, previous degradation processes of available carbohydrates appeared. Losses of easily degradable substances (e.g. non-structural carbohydrates) increased the relative amount of poorly degradable substances (e.g. fibres). Their degradation is a relatively slow process and needs a long retention time [41] which was not the case in experiment E2 with a retention time of 29 days.

The highest C deg_{mix} and C deg_{MS} values were found in experiment E3 with a retention time of 64 days. This can be explained by the results of Ruile et al. [42], who found the retention time to be the driving factor for degradation efficiency, besides temperature. Additionally, temperature is a key factor in biogas production [43–45] influencing the metabolism of the microorganisms and thus the substrate degradation efficiency

[46]. As a longer retention time and higher digestion temperatures were present in experiment E3, compared to experiments E1 and E2, beneficial conditions increased the carbon degradation.

The determination of C deg_{MS} is based on an estimation of the available maize silage carbon mass in the reactor mixtures by considering the carbon mass of the separately digested inoculum (cf. equation 4). C deg_{MS} showed no significant difference between experiments E1 and E2. The average C deg_{MS} was found slightly higher in experiment E2 compared to experiment E1. This can be seen critical, as the incubation of the maize silages cannot have improved the available total carbon mass. Also, higher standard deviations of the average C deg_{MS} were found compared to average C deg_{mix} and average C deg f_{MS}, indicating a high variability in the method. As the determination of C deg_{MS} is bound to elucidate C $mass_{MS\ end}$ by subtracting the inoculum's carbon mass from the mixture's carbon mass, small inhomogeneities in the sample extraction of the reactor mixtures cause a large deviation of the C deg_{MS} determination. The expected lower carbon degradation of the maize silage in experiment E2, compared to experiment E1, was not detectable with this method.

The carbon degradation determination C deg f_{MS} showed a significant difference between experiments E1 and E2. By applying the stable isotope mixing model, the $\delta^{13}C$ values of the according inoculum, the maize silage and the mixture were taken into account (see equation 7). Compared to the common carbon degradation determination methods (C deg_{mix} and C deg_{MS}), more parameters are taken into account and the carbon degradation of the investigated maize silage are validated in more detail. This gives an immediate result of the substrate's carbon degradation, independently of the inoculum.

As significant differences between experiments E1 and E2 were found with the common carbon degradation determination method C deg_{mix} and the carbon degradation on basis of the stable isotope mixing model C deg $f_{MS,}$ it was shown that the stable isotope mixing model can be applied on maize silages of different qualities.

The determination of C deg f_{MS} in experiment E3 was not possible. The stable isotope mixing model must be applied to substrates of pure C_3 sources in combination with substrates of pure C_4 sources. As maize silage MS6 and inoculum Inoc3 already contained mixtures of C_3 and C_4 sources, no precise ^{13}C fractionation was determinable. Therefore, the experimental setup design should consist of inocula of biogas reactors utilizing solely C_3 plants (e.g. organic farming) and pure C_4 plant substrates (e.g. maize silages) or vice versa.

The lack of significant differences found between reactors containing MS conv and MS org within all applied carbon degradation determination methods indicated that the stable isotope carbon degradation determination method can be applied on maize silages independently of the farming system.

5. Conclusion

The experiments revealed that stable isotopes offer the possibility to determine C deg f_{MS}. Artificially lowering the maize silage quality by unfavourable storage conditions (47 days, 21 ± 1 °C) significantly influenced stable isotope compositions and carbon degradations. MS low produced lower sby and smy. Maize silages of different farming systems showed no prominent differences in the digestion performances.

The method was found applicable for maize silages of different qualities and farming systems, demonstrating that the stable isotope mixing model has the advantage to test maize silage carbon degradations by excluding the masking effect of the inoculum. This offers the possibility to implement a standardised stable isotope method for solely maize-silage bound carbon degradation in anaerobic batch fermentations.

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Manuscript 3

Functionally redundant but dissimilar microbial communities within biogas reactors treating maize silage in co-fermentation with sugar beet silage.

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Personal contribution

Planning: 40 %

Experimental procedures: 50 %

Writing the manuscript: 10 %

Recent publications focused on the flexibility of microbial communities in anaerobic digestions. In our survey we focused on process parameters in four differently fed lab-scale reactors and possible functional and structural changes of the microbial communities. The 12 l reactors were fed in a continuous mode, continuously stirred and operated at 39 °C. The reactors were fed with different substrate ratios of maize silage (MS) and sugar beet silage (SBS) of 1:0 (volatile solids (VS)/VS) in continuous fermenter 1 (CF1), 6:1 in CF2, 3:1 in CF3 and 1:3 in CF4. All reactors operated with the same organic loading rate (OLR) of 1.25 kg VS m^{-3} d^{-1}.

The process parameters indicated stable process performances, similar average methane concentrations and similar biogas production rates in all reactors, despite different substrate ratios. The bacterial and archaeal community compositions were analysed with a 454 amplicon sequencing approach based on 16S rRNA genes. The community compositions altered with an increasing share of SBS in the substrate ratio. The archaeal community changed towards more microorganisms of the genus *Methanosarcina* while *Methanosaeta* declined simultaneously.

Similar biogas production rates were found despite changes of the microbial communities. This indicated that bacterial and archaeal communities adapted to their individual, substrate-dependent environment with easily degradable SBS. Despite dissimilar microbial communities, they fulfilled functional processes in anaerobic digestion similarly.

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Functionally redundant but dissimilar microbial communities within biogas reactors treating maize silage in co-fermentation with sugar beet silage

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Summary

Numerous observations indicate a high flexibility of microbial communities in different biogas reactors during anaerobic digestion. Here, we describe the functional redundancy and structural changes of involved microbial communities in four lab-scale continuously stirred tank reactors (CSTRs, 39°C, 12 L volume) supplied with different mixtures of maize silage (MS) and sugar beet silage (SBS) over 80 days. Continuously stirred tank reactors were fed with mixtures of MS and SBS in volatile solid ratios of 1:0 (Continuous Fermenter (CF) 1), 6:1 (CF2), 3:1 (CF3), 1:3 (CF4) with equal organic loading rates (OLR 1.25 kgVS m^{-3} d^{-1}) and showed similar biogas production rates in all reactors. The compositions of bacterial and archaeal communities were analysed by 454 amplicon sequencing approach based on 16S rRNA genes. Both bacterial and archaeal communities shifted with increasing amounts of SBS. Especially pronounced were changes in the archaeal composition towards *Methanosarcina* with increasing proportion of SBS, while *Methanosaeta* declined simultaneously. Compositional shifts within the microbial communities did not influence the respective biogas production rates indicating that these communities adapted to environmental conditions induced by different feedstock mixtures. The diverse microbial communities optimized their metabolism in a way that ensured efficient biogas production.

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Introduction

In 2013, 7.6% of the end energy consumption in Germany was gained from biogas. 48% of the 7850 biogas plants in Germany use renewable raw materials as feedstocks, such as maize silage (MS, 73%) and grass silage (12%) (FNR, 2014).

Utilization in biogas reactors of these plant materials requires retention times of about 100 days even though fibre-rich feedstocks may not get fully degraded (Boe and Angelidaki, 2009; Procházka *et al*., 2012). In contrast, feedstocks containing easy-degradable sugars and alcohols, such as sugar beet silage (SBS) can be rapidly degraded leading to an acidification of the biogas sludge and consequently to an inhibition of methane production (Kryvoruchko *et al*., 2009). Furthermore, SBS is a difficult feedstock in terms of the availability of nutrients and buffering capacity (Demirel and Scherer, 2008). However, it is an interesting co-feedstock for biogas production (Weissbach and Strubelt, 2008) with a similar biogas yield compared with maize silage (Klang *et al*., 2015). Co-fermentation of SBS with other feedstocks can lead to a stable process and has been shown to enhance biogas production resulting in a more efficient biogas process (El-Mashad and Zhang, 2010). However, currently just 2% of the renewable raw materials used in biogas plants in Germany account for SBS (FNR, 2014).

Within the biogas process, methane is mainly produced from hydrogen and carbon dioxide and/or acetate by hydrogenotrophic or acetoclastic methanogenic Archaea (Liu and Whitman, 2008). In general, hydrogenotrophic methanogens are dominant in most agricultural biogas plants especially at high ammonia concentrations (Weiland, 2010). Two members of genera within the methanogenic Archaea, *Methanosarcina* and *Methanosaeta*, can use acetate as substrate for methane formation (Liu and Whitman, 2008). Since these two genera can degrade the same substrate using analogous enzymes, they are defined as functionally redundant in this study. Abundances of different acetoclastic methanogenic species are generally depended on their tolerance towards acetate (Ros *et al*., 2013).

However, an optimal biogas production process relies on a complex and efficient microbial community in order to deal with changing environmental conditions as well as

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Table 1. Biogas production characteristics of the studied CSTRs. Data recorded for 80 days at OLR of 1.25 kgVS m^{-3} d^{-1}.

CSTR	CF1	CF2	CF3	CF4	
Feedstock mixture (MS : SBS)	1:0	6:1	3:1	1:3	
Average methane concentration (%)	59 ± 4.3	59 ± 6.2	61 ± 3.9	60 ± 8.2	(n = 80)
Cumulative specific biogas yield (l_N kg^{-1}VS)	755	726	746	799	
Expected specific biogas yield (l_N kg^{-1}VS)	650	657	662	687	
Avg. biogas production rate (l_N h^{-1})	0.39 ± 0.04	0.38 ± 0.06	0.36 ± 0.05	0.41 ± 0.09	(n = 80)
pH	7.8 ± 0.15	7.9 ± 0.10	7.9 ± 0.15	7.8 ± 0.10	(n = 20)
VFA/TIC ratio	0.06 ± 0.04	0.07 ± 0.04	0.06 ± 0.03	0.07 ± 0.04	(n = 20)
C/N ratio	11.1 ± 0.38	11.1 ± 0.24	11.3 ± 0.39	11.4 ± 0.77	(n = 5)
NH_4^+–N [g l^{-1}]	2.7 ± 0.09	2.6 ± 0.010	2.5 ± 0.09	2.1 ± 0.19	(n = 4)

l_N kg^{-1}VS, norm litre per kilogram volatile solids; VFA/TIC ratio, volatile fatty acids/total inorganic carbon ratio; C/N ratio, carbon/nitrogen ratio.

altering substrate compositions. Although, similar archaeal and bacterial groups are present in different biogas reactors, compositions of each of the microbial communities are unique (Sundberg *et al.*, 2013). Their identical end-products (CO_2 and CH_4) indicate that these microbial communities carry out similar functional processes, regardless of differences in their compositions (functional similarity) (Allison and Martiny, 2008).

In this study, the effect of different feedstock mixtures composed of MS and SBS (CF1, 1:0; CF2, 6:1; CF3, 3:1; CF4, 1:3) on the biogas process was assessed in four continuously fed 12 L lab-scale CSTRs. Therefore, we hypothesized that the biogas production from MS could be improved by co-fermentation of SBS in terms of biogas yield and process stability. Furthermore, in order to correlate possible changes in terms of an improvement of the biogas production, the archaeal and bacterial communities were investigated in relation to different feedstock mixtures by a 454-pyrosequencing approach based on 16S rRNA gene analysis and analysed by RDP (RIBOSOMAL DATABASE PROJECT, Release 11.1; Cole *et al.*, 2013).

Results

Process performance

All reactors performed well at high yields during the main experimental phase of 80 days at feedstock supply at 1.25 kgVS m^{-3} d^{-1}. The cumulative specific biogas yields of all CSTRs (Table 1; Fig. S1) were higher compared with expected values calculated from KTBL data (KTBL 2013, Faustzahlen Biogas).

Cumulative specific biogas yields of all reactors differed slightly (Table 1; Fig. S1), whereas the highest average methane concentration (61%) was determined for the 3:1 (MS : SBS) mixture (CF3). Regarding the process dynamics, no substantial differences between the individual mixtures were obvious, while a comparatively higher specific biogas yield (799 l_N kg^{-1}VS) has been found for CF4 containing the highest rate of SBS, which goes back to slightly higher biogas potential of SBS compared with MS.

Moreover, all reactors showed a stable process performance in terms of pH (7.5–7.9), VFA/TIC (0.04–0.18), C/N ratio (10.6–12.6) and ammonia concentrations (2.1–2.8 g l^{-1}) (Table 1).

Overall phylogenetic analysis

454-amplicon sequencing of the five analysed biogas-producing microbial communities (inoculum and four CSTRs) resulted in a total of 399 258 archaeal and bacterial 16S rRNA gene sequences. The average sequence read length was 713 bp for bacterial and 559 bp for archaeal 16S rRNA gene sequences. The total numbers of sequences per sample obtained by the RDP workflow were given in Table S2 and used for further downstream analysis.

Taxonomic assignment of 16S rRNA gene sequences by the RDP classifier showed a high identification due to the read length of bacterial (760 bp) and archaeal sequences (550 bp). On average, 93% of the bacterial sequences were assigned at phylum level, 61% sequences on family level and 36% sequences at genus level. Furthermore, 99.7% of the archaeal sequences were assigned on phylum level, 89.8% sequences on family level and 87.8% sequences on genus level.

Species richness and biodiversity

At a sequence homology of 97%, the number of operational taxonomic units (OTUs) based on bacterial 16S rRNA gene sequences was on average 3474 and the Shannon index ranged from 4.0 to 7.4. Data analysis of archaeal 16S rRNA gene sequences resulted in average in 861 OTUs. The respective archaeal Shannon index ranged from 3.1 to 5.1. In general, the estimated numbers of archaeal OTUs as well as the diversity indices were lower compared with the corresponding values for Bacteria (Table S2).

Beta-diversity measurements (weighted UniFrac, Table S3) showed that there were high dissimilarities between bacterial communities (Fig. 1A). Compared with

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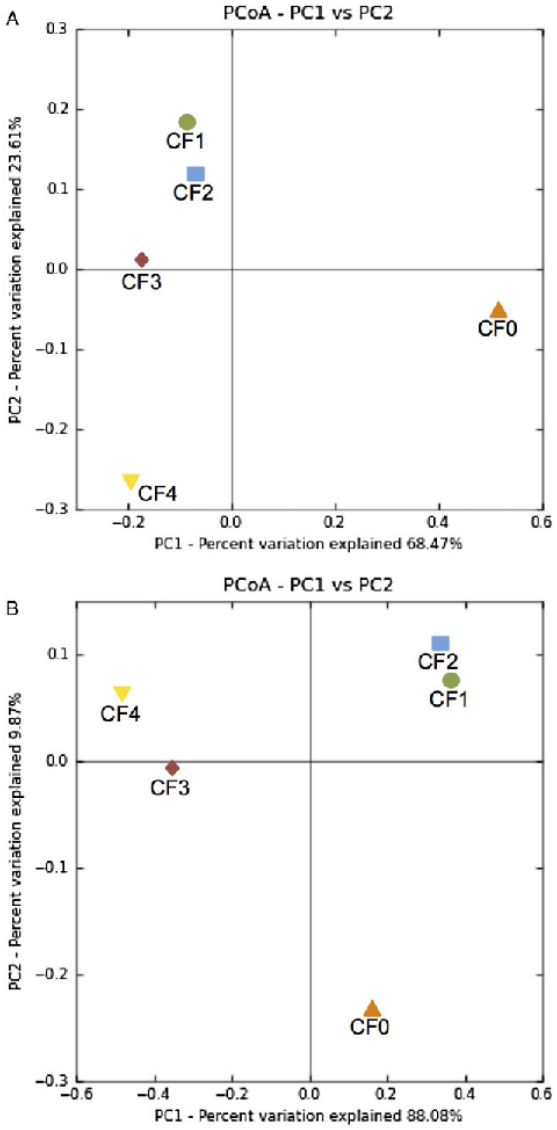

Fig. 1. Two-dimensional PCoA of dissimilarities between (A) bacterial and (B) archaeal communities in biogas reactors based on weighted UniFrac analysis. Similar microbial communities are depicted near from each other. PC1 and PC2 explain (A) 92% and (B) 98% of the variation.

the inoculum (CF0), bacterial communities have shifted in similar way in respect to PCoA axis PC1. In comparison with reactor CF1, bacterial communities lined up along PCoA axis PC2 (Fig. 1A).

The archaeal community has also changed compared with the inoculum but at a relatively low extent as the PCoA axis PC2 explains only 9.9% of the total variance (Fig. 1B). The archaeal community in reactor CF1 with MS as mono-substrate was very similar to reactor CF2 with MS and only 14% SBS. Whereas, archaeal communities in reactors CF3 and CF4 with high amounts of SBS were highly dissimilar in comparison to reactors CF1 and CF2.

Bacterial communities

The bacterial population in the inoculum was dominated by the phyla *Proteobacteria* (63%) and *Firmicutes* (14%). In all CSTRs, the phylum *Firmicutes* (CF1, 77%; CF2, 70%; CF3, 80%; CF4, 78%) was prevalent. Furthermore, *Bacteroidetes* and *Actinobacteria* were present in the inoculum and all four CSTRs. Relative abundances of members of bacterial families comprising at least 1% of the communities were shown in Fig. 2A. At the start (CF0) of the experiment, the bacterial population was dominated by members of the families *Comamonadaceae* (30%), *Pseudomonadaceae* (15%), *Enterobacteriaceae* (5.8%) (*Proteobacteria*), *Propionibacteriaceae* (12%) (*Actinobacteria*) and *Clostridiaceae* 1 (3.4%) (*Firmicutes*). Only 6.9% of the sequences obtained from sample CF0 remained unassigned. With the addition of feedstock mixtures to the reactors CF1–CF4 over a period of 80 days, bacterial abundances were more unevenly distributed compared with the start (CF0), and the proportion of unassigned sequences was on average 43% (±6). Furthermore, the bacterial community structure changed completely. Besides *Clostridiaceae* 1, the abundances of the mentioned families found in the inoculum (CF0) were smaller than 1% in all CSTRs (CF1-CF4).

The most dominant families of the domain Bacteria during monofermentation of MS (CF1) were classified as *Ruminococcaceae* (22.8%), *Lachnospiraceae* (7.2%), *Clostridiaceae* 1 (2.8%) within the phyla *Firmicutes* and *Microbacteriaceae* (3.8%) within the phylum *Actinobacteria* (Fig. 2A, marked in yellow-orange tones). With increasing amounts of SBS, the bacterial community shifted gradually, except for *Ruminococcaceae*. Bacteria belonging to the family *Ruminococcaceae* were present in high abundances in all reactors (CF2, 14%; CF3, 16%; CF4, 18%). Most notably, the abundances of members of *Clostridiales Insertae Sedis* XI increased considerably from 3% in reactor CF1 up to 30% in reactor CF4 (CF0, 0.6%; CF2, 5%; CF3, 18%) with the addition of SBS. Furthermore, relative abundances of the families *Streptococcaceae* (CF1, 0.9%; CF2, 1.6%; CF3, 1.1%; CF4, 3.4%), *Enterococcaceae* (CF1, 0.7%; CF2, 0.7%; CF3, 0.8%; CF4, 1.4%) and *Porphyromonadaceae* (CF1, 1%; CF2, 1.1%; CF3, 2.1%; CF4, 4.8%), (Fig. 2A, marked in blue tones) increased slightly with increasing amounts of SBS.

Archaeal communities

The Archaea detected in all samples belonged to the phyla *Euryarchaeota* (93.3%) and *Crenarchaeota* (6.4%).

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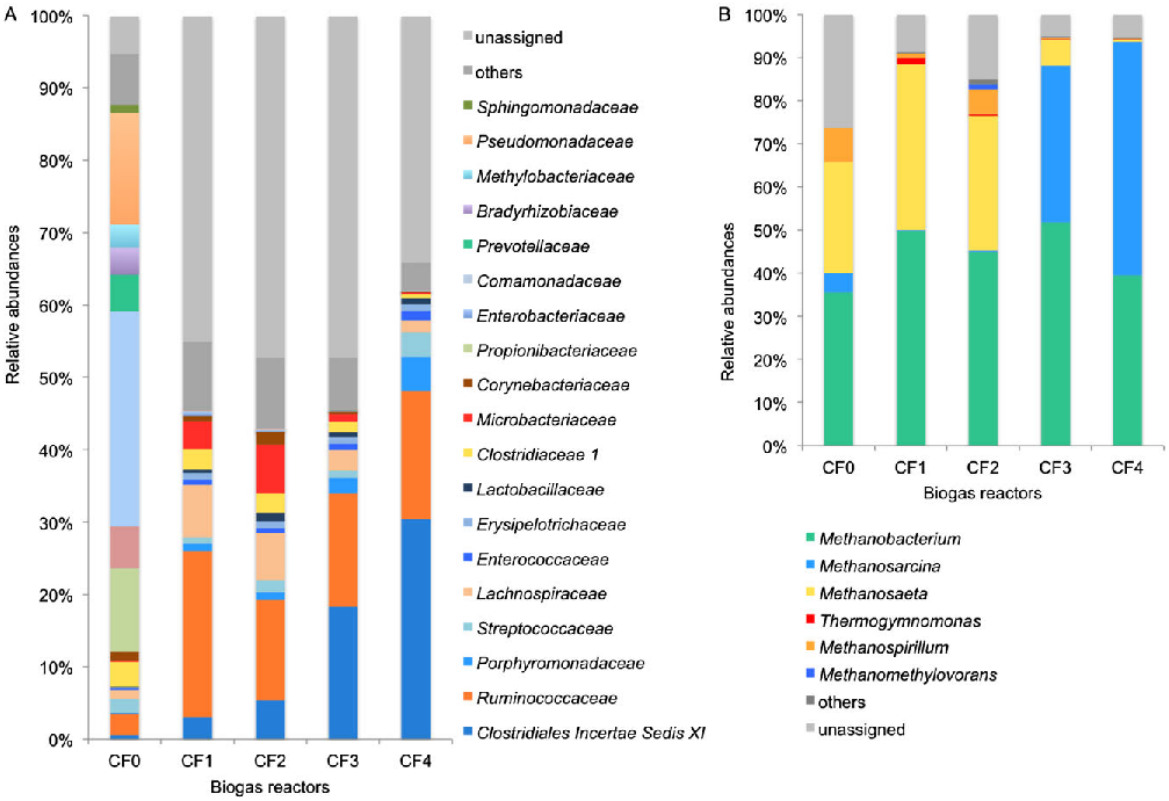

Fig. 2. A. Bacterial community composition on family level and (B) archaeal community composition on genus level at the beginning of the experiment in the inoculum (CF0) and after 121 d AD in four lab-scale biogas reactors CF1–CF4 resulting from the 454 amplicon data analysis (confidence cut-off 80%).

At the beginning of the experiment (CF0), the archaeal community showed a rather balanced composition between the four genera *Methanobacterium* (35.6%), *Methanosaeta* (25.8%), *Methanospirillum* (7.8%) and *Methanosarcina* (4.5%), while about 25% remained unclassified (Fig. 2B). After anaerobic digestion (AD) for 80 days, the abundances of the hydrogenotrophic *Methanobacteria* (CF1, 50%; CF2, 45%; CF3, 52% and CF4, 40%) in all samples (CF1–CF4) were similar to the start (CF0) independent from the amount of SBS fed to the four CSTRs (Fig. 2B). Furthermore, different members of acetoclastic Archaea were observed in relation to SBS. In reactors fed with MS mainly, *Methanosaeta* species (CF1, 38% and CF2, 32%) dominated. However, with increasing amounts of SBS the archaeal community shifted gradually. In reactors with high amounts of SBS, *Methanosaeta* proportion was as low *as* 6% in CF3 and almost absent in CF4 (0.4%), whereas members of the genus *Methanosarcina* were prevalent in reactors CF3 (36%) and CF4 (54%).

Discussion

Process performance

All four lab-scale CSTRs reached high-specific biogas yields (Table 1; Fig. S1). Biogas yields are significantly affected by system technology as well as feedstock quality (KTBL 2013; Mähnert and Linke, 2009). Moreover, high biogas yields are typical for reactors at low organic loading rates (OLRs; Mähnert and Linke, 2009). Biogas production rates and methane concentrations were similar in all CSTRs (Table 1). pH values between 7 and 8, ammonia concentrations below 5 g l^{-1} and low volatile fatty acid/total inorganic carbon (VFA/TIC) ratios ($< 0.07 \pm 0.04$) indicate process stability (Drosg, 2013). Furthermore, C/N ratios were within the optimum range of 10–15 (Weiland, 2010). Consequently, there was no limitation in any of the reactors in terms of nutrients and process instability.

Feedstock characterization (Table S1) showed that SBS contains mainly ethanol, short chain fatty acids as well as

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sugar, which promote faster feedstock degradation (Weissbach, 2009; Böttcher *et al*., 2011). The comparatively higher specific cumulative biogas yield of reactor CF4 containing the highest rate of SBS (MS : SBS-1:3) goes back to slightly higher biogas potential of SBS (Table 1; Fig. S1). Consequently, the effect of SBS on AD of MS was not quite obvious in terms of biogas yields, methane concentrations and especially in terms of biogas production rates. Thus, the hypothesis of improving biogas production from MS with addition of SBS was rejected.

Species richness and biodiversity

Results showed a great species richness (OTUs) in all reactors (Table S2). Nelson and colleagues (2011) found similar numbers of archaeal (296 OTUs) and bacterial OTUs (5926) investigating multiple anaerobic digesters by a meta-analysis based on 16S rRNA gene sequencing. However, a high number of OTUs is not only caused by species richness but also by the number of sequence reads obtained by high-throughput sequencing (Roesch *et al*., 2007).

Regarding the total number of species/OTUs, the calculation of the Shannon Index showed that the biodiversity (H′) of the archaeal community was lower compared with bacterial biodiversity in biogas reactors (Table S2). This result is in line with other studies (Fernandez *et al*., 1999; Bengelsdorf *et al*., 2013; Gagliano *et al*., 2015). Differences in feedstock mixtures did not affect the biodiversity notably as indicated by similar values for H′.

Bacterial communities

CSTRs mainly fed with fibre-rich MS (i.e. CF1 and CF2; Fig. 2A) were dominated by cellulolytic Bacteria such as members of the families *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae* 1 and *Microbacteriaceae*. For example, members of the families *Ruminococcaceae* and *Lachnospiraceae* persist in fibrolytic communities (Brulc *et al*., 2009). They contain a wide range of glycoside hydrolases and carbohydrate-binding modules, enabling them to colonize complex plant material and to degrade recalcitrant polymers, such as cellulose and hemicellulose. Furthermore, they are able to cleave bonds of starch, cellobiose, cellodextrin and chitobiose (Biddle *et al*., 2013). Therefore, members of these families play a common role as active plant degraders (Biddle *et al*., 2013).

In contrast, CSTRs fed with high amounts of SBS were dominated by members of the families *Clostridiales Insertae Sedis* XI, *Porphyromonadaceae*, *Streptococcaceae* and *Enterococcaceae* (Fig. 2A). The family *Clostridiales Insertae Sedis* XI is a heterogeneous group that includes a wide range of different Bacteria (Pagnier *et al*., 2014), and any functional prediction for clostridia only by using phylogenetic information would be highly speculative due to their broad metabolic potential (Fernandez *et al*., 2000). Bacteria of the family *Porphyromonadaceae* are commonly found in mesophilic full-scale biogas plants (Liu *et al*., 2009; Bengelsdorf *et al*., 2013; Li *et al*., 2013; Pope *et al*., 2013). These Bacteria play a central role in glucose fermentation (Li *et al*., 2009). Members of the families *Streptococcaceae* and *Enterococcaceae* are facultative anaerobic, use homolactic acid fermentation (Fisher and Phillips, 2009; Whiley and Hardie, 2009) and were found in other mesophilic biogas reactors (Li *et al*., 2013). Moreover, *Streptococcaceae*-related microorganisms were dominant in biogas reactors fed with high amounts of glucose (Fernandez *et al*., 2000). An increased availability of monosaccharides in CSTRs with high amounts of SBS could have led to the increase of their relative abundances.

Altogether, the composition of each bacterial community was adapted to the supplied feedstock mixture. Thus, functional similarity of highly diverse bacterial communities led to similar biogas production rates in all reactors.

Archaeal communities

Methane production from acetate is a redundant function performed either by *Methanosaeta* and/or by *Methanosarcina* species (Liu and Whitman, 2008). Due to high affinity to acetate, *Methanosaeta* probably outcompeted *Methanosarcina* in reactors with low amounts of SBS (Fig. 2B). As indicated in Fig. 1B, this pronounced shift took place at the increasing share of SBS addition from 14% (CF2) to 25% (CF3). In reactors with high amounts of SBS, *Methanosarcina* became dominant most likely due to its tolerance to high acetate levels (Ros *et al*., 2013). Consequently, the diverse and functionally redundant members of archaeal communities are able to adapt to changing environmental conditions, and the most competent species consortium is prevailing in the metabolic functions.

Process performance linked to microbial community structures

Dynamics of microbial communities in biogas reactors depend on several factors for instance on hydraulic retention time, temperature, pH, OLR and ammonia concentration (Dollhopf *et al*., 2001; Ye *et al*., 2007; Rademacher *et al*., 2012; Hai *et al*., 2014; Werner *et al*., 2014). In this study, shifts in microbial communities (Fig. 2) were most likely induced by feedstock compositions varying in the proportions of MS and SBS. With respect to the hypothesis, we showed that these shifts clearly not affect the biogas production rates of CSTRs digesting complex feedstock mixtures (Table 1). A similar result was found by

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Fernandez and colleagues (2000). They indicated that the functional stability of AD was linked to a flexible microbial community. Moreover, Briones and Raskin (2003) pointed out the importance of diverse and flexible communities for a stable process performance.

In this study, microbial communities carried out functional processes at similar rates, regardless of differences in composition (Fig. S1; Fig. 2A). The individually composed bacterial communities in each CSTR had fulfilled similar functions (functional similarity), which finally resulted in similar biogas production rates in each reactor.

Even more important is the shift in the Archaea composition towards *Methanosarcina* with increasing proportion of SBS. Archaeal communities (Fig. 2B) newly established after SBS co-fermentation are functionally redundant, and thus biogas production rates and methane concentrations (Table 1) were not altered by compositional shifts (Allison and Martiny, 2008).

Functional redundancy is considered as insurance to maintain ecosystem functions under changing environmental conditions (McMahon *et al.*, 2007). Moreover, stable ecosystem functions could be sustained by highly dynamic communities (Friedrich *et al.*, 2003; Stamper *et al.*, 2003; Wittebolle *et al.*, 2008; Ayarza *et al.*, 2010; Cabrol *et al.*, 2012).

Conclusions

In this study, microbial communities stabilized the digestion process and led to an efficient biogas production from different feedstock compositions. Both bacterial and archaeal communities are responding simultaneously to the supply of easy-to-degrade feedstocks. This finding is unique for the AD research and explains the common observation from biogas practitioners that each biogas plant is an own specific ecosystem, hardly comparable to a second plant.

Experimental procedures

Experimental set-up

Anaerobic digestion was performed continuously in four mixed 12 L lab-scale CSTRs (operating volume 10 L, temperature 39°C).

The cylindrical, double-layered biogas reactors made of stainless steel were built in the mechanical workshop of the Ulm University under the supervision of the authors' institute. Reactor temperature was maintained by passing water from additional hot water bath through copper pipes surrounding the reactors. The water inside the water bath was heated up to 42°C by a heating circulator (JULABO GmbH, Seelbach, Germany).

Feedstocks for biogas production were introduced into the reactors by screw conveyors placed on the bases of each feedstock storage tubes. The corresponding operating unit allowed the subdivision of each rotation into 12 impulses (1 impulse = 30°). Digital card under LabVIEW sent fixed number of impulses to the feeding system at given time interval. In the present experiment, the number of screw conveyor impulses was set individually for each mixture, and the feedstock input was realized automatically on hourly basis. Mixing of SBS with MS might produce high viscosity on the surface of the carrier. Thus, all surfaces were regularly cleaned for good performance as suggested by Scherer and colleagues (2009). The automatic feeding system was controlled daily by the operators of CSTRs. Homogeneous mixing inside CSTRs was achieved by stirring every 10 min for 5 min at 80 r.p.m.

The inoculum originated from a mesophilic (40°C) full-scale biogas plant (Ulm-Gögglingen, Germany) fed with MS (56%), pig manure (30%) and grass silage (14%). Inoculum was used after filtration and kept in the reactors at 40°C for 19 days in order to minimize its background methane production (Li *et al.*, 2013; VDLUFA, 2011). Maize silage and SBS were obtained from the above-mentioned biogas plant (Gögglingen, Germany) and the Raiffeisenwarengenossenschaft Emsland-Süd (Lünne, Germany) respectively. Feedstock characteristics (Table S1) make obvious that SBS contains easily degradable components like alcohol, carboxylic acid and sugar, which leads to intensive formation of organic acid that causes low pH values (Böttcher *et al.*, 2011). Furthermore, mono-fermentation of SBS requires larger volume of reactors or reduction of OLR and causes foam evolution (Kaiser *et al.*, 2008; Böttcher *et al.*, 2011).

The underlying aim of the study was to test the influence of different amounts of SBS on the reactor performance. Therefore, the lab-scale CSTRs were fed with different feedstock mixtures of MS and SBS in a ratio (based on volatile solids, VS) of 1:0 in CF1, 6:1 in CF2, 3:1 in CF3 and 1:3 in CF4 respectively. Anaerobic digestion in all CSTRS was started with a low OLR at 0.69 kgVS m^{-3} d^{-1} for a period of 41 days. After the process was stable in terms of biogas yield as well as VFA/TIC values (0.05 ± 0.01), OLR was increased to 1.25 kgVS m^{-3} d^{-1}, and the feeding continued for further 80 days.

The experiment was designed for online monitoring of the biogas volume and methane concentrations in each reactor. The volume was assessed by Miligascounter (Ritter GmbH, Bochum, Germany) and given at standard condition (1.013 bar, 0°C and 0% RH). Methane concentrations were measured by infrared sensors (Bluesens GmbH, Herten, Germany, relative accuracy: ± 2% of the measured value). The sensors were calibrated with a 60% CH_4 certified calibration gas.

Other process parameters like pH, the ratio of VFA/TIC, carbon/nitrogen (C/N) ratio and the amount of total solids (TS) and volatile solids (VS) were determined weekly. Total solids and VS were determined according to APHA methods 2540B and 2540E (APHA, 1999). The pH of the sludge was determined using Metrohm 605 (Filderstadt, Germany). The VFA/TIC ratio was measured by automatic titration (Dosimat 665, Metrohm, Hersau, Switzerland) with 1 M HCl to endpoints of pH 5 and 4.3 (Voß *et al.*, 2009). The analysis of C and N in the feedstocks and in the sludge was made by TrueSpec C/N analyser (LECO Instrumente GmbH, Mönchengladbach, Germany). Ammonia-nitrogen (NH_4^+–N) concentrations were determined with a gas-sensitive ammonia electrode (Type NH 500/2, WTW GmbH, Weilheim, Germany).

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Ash-free neutral detergent fibre (NDFom), ash-free acid detergent fibre (ADFom), acid detergent lignin (ADL) and relevant characteristics (crude protein, crude fat, starch, sugar, crude fibre) were determined in the accredited Institute for Oil and Environment, LUFA Nord-West (Oldenburg, Germany). Cellulose, hemicellulose and lignin amounts were obtained from NDFom, ADFom and ADL.

Biogas yields, average methane concentrations, biogas and methane production rates were calculated on hourly basis respectively. Cumulative specific biogas yields were calculated based on cumulative biogas yields for total volatile solid inputs to each individual reactor. Methane concentrations as well as biogas production rates were calculated from average values per day (Table 1).

Sampling and deoxyribonucleic acid (DNA) extraction

For microbial community analysis, one sample was drawn from the inoculum (CF0) at the beginning of the experiment. Further samples were taken from each reactor (CF1, CF2, CF3 and CF4) after 80 days of AD. Samples were stored at −20°C before DNA was extracted by a modified protocol of Klocke and colleagues (2008). To remove polymerase chain reaction (PCR)-inhibiting substances, 4 ml of each sample were washed with 20 ml sodium phosphate buffer (Hugenholtz *et al.*, 2002), 1 ml polyvinylpyrrolidon solution (35%) and 2 ml cetyl trimethylammonium bromide solution (10%) and centrifuged at 5000 r.p.m. for 20 min. The supernatants were removed, and the washing step was repeated 3–4x until the supernatants became clear. After washing, the respective pellets were suspended in 5 ml of a saline EDTA buffer (0.1 mol l^{-1} EDTA, 0.15 mol l^{-1} NaCl), and cells were lysed by Rybolyser (Hybaid, Middlesex, UK) for 3x 45 s at a speed of 6 ms^{-1}. Total DNA of the samples was extracted based on phenol/chloroform DNA extraction as described in the protocol of Klocke and colleagues (2008). Deoxyribonucleic acid concentration of the extracted DNA was measured with the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmingtion, USA) at 260 nm.

PCR amplification and sequencing

Barcode-tagged primers (Table S4) were used to amplify the V4-V6 region of archaeal 16S rRNA genes (555 bp) and the V3-V6 region of bacterial 16S rRNA genes (760 bp) from the extracted DNA of samples CF0, CF1, CF2, CF3 and CF4 by PCR. Approximately 100 ng of genomic DNA isolated from biogas reactors was used as template for each PCR reaction.

Archaeal 16S rRNA genes were amplified by *ReproFast*-DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany) under the following thermal cycling conditions: a prior denaturation step of 95°C for 5 min, a first loop of 15 cycles of 95°C for 45 s, 55°C for 60 s and 72°C for 60 s, and a second loop of 15 cycles of 95°C for 45 s, 57°C for 60 s and 72°C for 60 s, followed by a terminal elongation step of 72°C for 10 min. Amplification of bacterial 16S rRNA genes for the pyrosequencing approach was done with a DNA-free *Taq DNA Polymerase* (AppliChem GmbH, Darmstadt, Germany) under appropriate buffer conditions. Temperature steps of the PCR were: a prior denaturation step of 94°C for 5 min, a first loop of 10 cycles of 94°C for 60 s, 55°C for 30 s and 72°C for 60 s and a second loop of 20 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, followed by a terminal elongation step of 72°C for 10 min. 16S rRNA gene amplification and amplicon length were checked on a 2% agarose gel stained with ethidium bromide. For each sample, three independent PCR reactions were accomplished for either, archaeal and bacterial 16S rRNA genes. Merged PCR amplicons were purified by the purification kit DNA clean & concentrator-5 (ZYMO Research Europe GmbH, Freiburg, Germany). Deoxyribonucleic acid concentration of the purified PCR amplicons was measured with the NanoDrop 2000 Spectrophotometer at 260 nm. Finally, bar code-tagged DNA fragments of bacterial and archaeal 16S rRNA genes were equimolarly merged, and sequencing was performed by Roche GS FLX++ chemistry (MWG eurofins, Berlin, Germany) on 0.5 plate.

Data analysis

The analysis of 454 amplicon sequencing data was performed by Ribosomal Database Project (RDP). Following the RDP supervised workflow, archaeal and bacterial 16S rRNA gene sequences were sorted according to their bar codes and trimmed. Sequences with a deviation greater than ± 10% from the expected fragment lengths were discarded. Low-quality sequences (minimal quality score 25) were filtered out, and unspecific sequences were excluded by RDP's sequence selection tool. Remaining sequences were checked for chimeras in the *de novo* mode by USEARCH 6.0 (Edgar, 2010). After filtering processes, 55% of the sequences were discarded. Thus, 180 752 sequences remained for downstream analysis. These sequences were aligned by the INFERNAL aligner (Nawrocki and Eddy, 2013). Based on the aligned sequences, complete linkage clustering (farthest neighbour method) was performed with a distance cut-off of 0.03.

The resulting cluster file was used to calculate rarefaction and alpha diversity (Shannon and Chao1 index). Furthermore, differences between microbial communities (ß-diversity) were measured by a weighted UniFrac analysis using QIIME (Lozupone *et al.*, 2007; Caporaso *et al.*, 2010). Therefore, the depth of coverage was adapted to the lowest number of sequences within bacterial or archaeal samples for even sampling depth. A three-dimensional Principal Coordinates Analysis (PCoA) plot based on weighted UniFrac analysis was generated by EMPEROR for visualization of dissimilarities between microbial communities (Vazquez-Baeza *et al.*, 2013).

Representative sequences were picked from the created clusters. Taxonomy was assigned to sequences by the RDP classifier with a confidence cut-off of 80 (Wang *et al.*, 2007). Analysis was performed on RDP server infrastructure while data were uploaded. Bacterial and archaeal 16S rRNA gene sequences were deposed in the EMBL database under the study accession number PRJEB7938.

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Conflict of interest

None declared.

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ADDITIONAL PUBLICATIONS

Ahmed S, Einfalt D, Kazda M (2016)

Co-digestion of sugar beet silage increases biogas yield from fibrous substrates

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Langer S G, Gabris C, Einfalt D, Wemheuer B, Kazda M, Bengelsdorf F R

Different response of bacteria, archaea and fungi to process parameters in nine full-scale anaerobic digesters

submitted November 2016 to The ISME Journal

ADDITIONAL INFORMATION

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Declaration of independent scientific work

I declare that I prepared this doctoral thesis independently and did not use any sources or means other than those indicated by me and, furthermore, I cited the passages where I quote or referred to works by other people and their contents and I comply with the Statutes of Ulm University on Safeguarding Good Scientific Practice in the applicable version.

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Parts of this dissertation have already been published in the following journlas:

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Characterisation of biogas plants on organic farms and potentials for improvement

Organic Agriculture 6:243–254

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