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Marker microbiome clusters are determined by operational parameters and specific key taxa combinations in anaerobic digestion

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Abstract

In this study, microbiomes of 36 full-scale anaerobic digesters originated from 22 different biogas plants were compared by terminal restriction fragment length polymorphism (TRFLP) analysis.

Regarding the differences in microbial community composition, a weighting of the environmental parameters could be derived from higher to lower importance as follows: (i) temperature, (ii) TAN and NH_3 concentrations and conductivity, and (iii) the chemical composition of the supplied feedstocks.

Biotic interactions between specific bacterial and archaeal community arrangements were revealed, whereby members of the phyla *Bacteroidetes* and *Cloacimonetes* combined with the archaeal genus *Methanothrix* dominated the conversion of homogeneous feedstocks, such as waste water sludge or industrial waste.

As most of the detected TRFs were only found in a certain number of anaerobic digestion plants, each plant develops its unique microbiome. The putative rare species, the specialists, are potentially hidden drivers of microbiome functioning as they provide necessary traits under, *e.g.*, process-inconvenient conditions.

Keywords

Biogas, operational parameters, TRFLP, microbiome cluster, specific key taxa combination

Highlights

(1) Marker microbiome clusters are determined by operational parameters.

(2) A weighting of operations parameters was derived from higher to lower importance.

(3) Biotic interactions between specific community arrangements were revealed.

(4) Each anaerobic digestion plant develops its own unique microbiome.

(5) Specialists are potentially hidden drivers under process-inconvenient conditions.

1. Introduction

Biogas production has become a common practice worldwide due to several advantages (Appels et al., 2011; Dahiya et al., 2018; Hagman et al., 2018; Hagos et al., 2017; Steigmeier et al., 2015). The production of biogas is independent of daily, seasonal and weather-related fluctuations and can therefore secure basic electricity supply and simultaneously generate heat by a combined heat and power unit. Biogas is suitable as a fuel and as a substitute for natural gas. For the production of biogas, a broad variety of feedstocks can be used, including energy crops, agricultural livestock residues, municipal solid waste, as well as organic commercial and industrial wastes. Biogas plants can be constructed in a wide range of scales, from household to large commercial facilities. Anaerobic digestion can be sustainably integrated into bioeconomic production systems for food, feed, energy and biomaterials.

Current research efforts mainly arise from the requirement of high process stability and efficiency with low susceptibility to instabilities or disturbances to ensure demand-driven gas production, which consequently requires continuous process monitoring (Bensmann et. al., 2016; Carballa et al., 2015; De Vrieze and Verstraete, 2016). Current process control and management strategies are based on process engineering and chemical parameters (Boe et al., 2010; Ward et al., 2008), as well as on the experiences of biogas plant operators. Despite intensive efforts and decisive progress to understand the biogas process microbiology in its taxonomic, functional and ecological diversity, microbial process indicators and hence control and management strategies that consider the microbial demands are still missing (Carballa et al., 2015; De Vrieze and Verstraete, 2016). The main research question remains: who is doing what, when, with whom and why? (De Vrieze and Verstraete, 2016). The challenge is to understand the biological highly sensitive process in its complexity to evolve a knowledge-based microbial diversity management.

Most of today´s knowledge about microorganisms and their physiological capacities has arisen from traditional microbiological methodologies, *e.g.*, isolation, cultivation and characterization of pure and enriched cultures, which were the main tools for generating knowledge about microbial life for many years (Schnürer, 2013). Modern molecular biological tools now enable the generation of detailed information about the genomic structure and gene expression, which reflects the putative and actual microbial metabolism of pure and enriched cultures. These methods also allow the analysis of complex environments and their microbiomes (Alivisatos et al., 2015; Vanwonterghem et al., 2014). The application of next generation sequencing (NGS) tools, including 16S rRNA gene amplicon libraries and metagenome, metatranscriptome, and metaproteome analyses, to unravel the microbial community "black box" led to numerous discoveries of previously unknown microbial life in anaerobic digestion (AD) plants. Although NGS technologies have enabled a new view on microbial diversity, it has mainly yielded snapshots that shed limited light on microbial functions or community dynamics (Alivisatos et al., 2015). Hence, established fingerprinting techniques, such as terminal restriction fragment length polymorphism (TRFLP), are still valuable for swift microbial community screening in full-scale AD, especially regarding the need for microbial monitoring of the AD process (Lim et al., 2018; Prakash et al., 2014).

In this study, anaerobic microbiomes of 36 full-scale anaerobic digesters that originated from 22 different biogas plants located in Belgium and Germany were compared by TRFLP analyses. The objectives of this study were (i) to determine whether prevalent process conditions are related to marker populations or at least to marker microbiome clusters, (ii) to elucidate key abiotic and biotic parameters that determine the bacterial and archaeal community arrangements, and (iii) to derive a weighting of key parameters from higher to lower importance on the microbial community structure development.

2. Methods

2.1 Characteristics of the analysed full-scale anaerobic digestion plants and sampling

Overall, 22 different full-scale anaerobic digestion plants (ADPs) from Belgium (14) and Germany (8) were investigated. Samples with the same number but different small letters either originate from the same ADP at different time points or the ADPs consisted of two main fermenters (only the case for G04). In total, 36 samples were considered for this study. The investigated ADPs differed in their configuration (reactor type, volume), in the supplied feedstocks, in their general process performance (*e.g.*, organic loading rate (OLR), hydraulic retention time (HRT) and process temperature) and consequently in their main chemical characteristics (Table 1).

Information concerning the reactor type and volume, the feedstock supply, the process temperature, as well as the biogas production were provided by the plant operators. Samples of the digester content were taken from the main digester, considering that the obtained samples were representative of the digester content at the current operational conditions. Considering the high fermenter volume and the variance of the supplied feedstocks, the sample properties vary materially, spatially and temporary. Therefore, the requirements of "representativeness" must be adapted to suit the possible variance in the material composition. Sampling was carried out before the next feedstock supply took place (to avoid distortion of values by fresh supplied material) and after the fermenter content was mixed very thoroughly for at least 10 to 15 minutes (homogenization). Samples were taken via a sampling nozzle/port, whereby the pipe section was purged at least twice. The pipe section was intensively flushed, whereby at least 20 L were taken and discarded to ensure that the samples derived from the real fermenter content. Afterwards, several subsamples (overall 5 times 4 L) were collected. Sample bottles (1 L) were filled to 2/3 with the well-mixed composite sample (due to an ongoing gas formation), stored at 4°C to reduce the microbial

activity, and directly transferred to the laboratory. Aliquots were taken for subsequent chemical and molecular biological analyses and stored immediately at -20°C until further analyses.

The following chemical analyses were conducted on all investigated ADPs: total solids (TS), volatile solids (VS), total ammonium nitrogen (TAN), volatile fatty acids (VFA), pH, and conductivity according to standard methods (Greenberg et al., 1992). The NH_3 concentration was calculated as a function of the TAN concentration, the pH and the temperature, according to Hansen et al. (1998).

2.2 Microbial community analyses

To profile the anaerobic microbial communities, one of the most commonly applied and reliable fingerprinting techniques, terminal restriction fragment length polymorphism (TRFLP), was used (Lim et al., 2018; Prakash et al., 2014; Rademacher et al., 2012).

According to the manufacturer's instructions, genomic DNA was extracted either with the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) in the case of the Belgium samples or with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., USA) in the case of the German samples. As both methods are based on a mechanical (beat beating) and chemical cell disruption, similar results can be expected regarding the microbial community composition (Albertsen et al., 2015). The extracted DNA was used as template for TRFLP analyses, which was performed according to the previously published protocol by Klang et al., (2015). As the TRFLP is based on a restriction digest of fluorescently-labelled PCR products, the bacterial and archaeal 16S rRNA genes were amplified (three replicates per crude DNA extract) with the primer pairs 27F/926MRr (*Bacteria*) and Ar109f/Ar912r (*Archaea*), whereby forward primers were fluorescently labelled with Indodicarbocyanine (Cy5) at the 5´-end. After purification, the PCR products were digested with *Msp*I and *Hin*6I

in the case of the bacterial assay or with *Alu*I for the archaeal assay. The separation of the restriction fragments was carried out using the GenomeLab™ GeXP Genetic Analysis System (AB SCIEX Germany GmbH, Darmstadt, Germany). The obtained raw data were pre-analysed with the GeXP analysis software (version 10.2), whereby only profiles (electropherograms) whose internal standard had a standard deviation of 0.39 nucleotides (nt) or less were considered for further analyses (Rademacher et al., 2012). A detailed analysis was then performed using the software package BioNumerics 7.6 (Applied Maths, Kortrijk, Belgium), according to Klang et al. (2015).

For the phylogenetic identification of the recorded terminal restriction fragments (TRFs), an in-house database was used, currently containing about 3000 16S rRNA gene sequences from various projects carried out at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) during the last ten years. These projects investigated the microbial community structure by applying microbiological (isolation and cultivation of anaerobic microorganisms) and/or molecular biological methods (sequence libraries) in laboratory- and full-scale biogas installations. The obtained sequences were grouped into operational taxonomic units (OTU), which were virtually digested using BioNumerics 7.6 (Applied Maths, Kortrijk, Belgium), as previously described by Klang et al., (2015). The OTUs were phylogenetically identified using the RDP Naïve Bayesian rRNA Classifier Version 2.6 (Wang et al., 2007).

2.3 Statistical analyses

The software package of PC Ord Version 6 (McCune and Mefford, 2011) was used to perform a non-metric multidimensional scaling (NMS) analysis (Clarke, 1993) considering the Bray-Curtis distance (Bray and Curtis, 1957) as it retains sensitivity in more heterogeneous datasets and gives less weight to outliers.

The number of bacterial and archaeal TRFs found in a respective ADP group, as well as the median relative abundance of the bacterial or archaeal TRFs from the respective ADP group, was determined. That means it was counted in how many anaerobic digesters in one TRF was detected. Beyond that, the median relative abundance of each TRF was calculated considering only the ADPs in which this TRF was recorded, and correlated this to the microbial community as a median of all the investigated ADPs. This was done to find TRFs that reflect microorganisms or groups of organisms that either symbolise a type of core-microbiome (generalists) or that represent specialists that potentially occupy specific ecological niches and hence fulfil specific functions in the digester ecosystem.

3. Results and discussion

3.1 Process conditions determine microbiome marker cluster

The investigated ADPs strongly differed in their process engineering and chemical characteristics (Table 1). Considering the wide range of supplied feedstocks, a huge variety in OLR (1.5 - 11.0 kg_{COD} m^{-3} d^{-1} for the Belgian ADP and 1.1 - 5.4 kg_{VS} m^{-3} d^{-1} for the German ADPs) and HRT (16 - 158 days) was recorded. The temperature ranged from mesophilic (33 - 38°C) via intermedium (40 - 45°C) to thermophilic (54°C) conditions. Consequently, the process-chemical parameters also showed a wide range: pH values varied from 6.8 - 8.5, conductivity from 5 - 62 mS cm^{-1}, VFA from below the detection limit to 34.5 $g_{HAc\text{-}Eq}$ L^{-1}, TAN from 0.1 - 6.5 g_N L^{-1}, and NH_3 from 1 - 1177 mg_N L^{-1}. Based on this initial situation, the aim of this study was to determine whether these prevalent process conditions are related to marker populations or at least marker microbiome clusters.

The first main difference between the investigated ADPs was the diversity of the applied feedstocks. While various sources of biodegradable material are used for biogas production in Belgium, the main feedstocks in Germany are energy crops and agricultural animal wastes (Table 1). This is of high importance as the chemical composition of provided feedstocks and the substrate bioavailability further affects the microbial growth and activity and, hence, the formation of specifically adapted microbial communities (De Vrieze et al., 2015; Klang et al., 2015; Luo et al., 2016; Zhang et al., 2014). Three different feedstock groups were identified, which corresponded to the anaerobic conversion of (i) organic biological waste (cluster "biowaste"), (ii) energy crops and agricultural animal waste (clusters "maize, manure" and "40-45°C; maize, rye, cattle") and (iii) waste water sludge and industrial wastes (cluster "lowest values") (Fig. 1). This grouping might be attributed to the chemical composition of the feedstocks. Table 2 gives a compilation of the chemical composition of different feedstocks from both the literature as well as the results of this study. Biowaste, for example, is characterised by high amounts of proteins, lipids and, depending on the origin, different types of carbohydrates; hence, biowaste shows a high complexity of different chemical compounds. However, biowaste is perhaps the most variable feedstock as its chemical composition differs between rural and urbanized areas (including lifestyle and cultural impacts) or undergoes seasonal differences such as higher amounts of garden waste during the summer season (Appels et al., 2011; Ward et al., 2008). In contrast, energy crops are dominated by more recalcitrant compounds, such as cellulose (Table 2), which is often protected from biodegradation as the anaerobically non-degradable lignin forms a matrix that surrounds the (hemi-) cellulose microfibrils (Kirk and Farrell, 1987; Rees et al., 1998). Hence, the chemical complexity here is related to a complex molecule structure. If energy crops are combined (co-digested) with agricultural animal manure, proteins are incorporated into the substrate mixture (Table 2). These chemical characteristics influence biogas production as well as methane yields, with the highest amounts for energy crops and food wastes (Appel et

al., 2011; Ward et al., 2008). However, depending on the supplied feedstocks, the occurring microbial communities are faced with different levels of chemical complexity (either various compounds or complex molecule structures) that require several conversion pathways. Chemically complex and/or heterogeneous feedstocks in function of time (*e.g.*, biowaste as well as energy crops in combination with agricultural animal waste) need a structural more diverse community with members of various phyla (*e.g.*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *Thermotogae*, *Cloacimonetes* as well as *Euryarchaeota*) (*e.g.*, De Vrieze et al., 2015; Klang et al., 2015; Luo et al., 2016; Werner et al., 2011) that ensure a high functional (broad range of metabolic pathways) and ecological (generalists, specialists, redundancy, resilience, concurrence, syntrophy, co-occurrence) diversity to efficiently convert the provided biomass into high-yield, methane-containing biogas. The microbial diversity is symbolised by various detected TRFs (Table 3), whereby the German ADPs showed a higher average number of TRFs (16.8 ± 2.4) compared with the Belgian ADPs (9.6 ± 3.1), indicating that the microbiomes of the German ADPs are exposed to more complex environmental conditions than the microbiomes of the Belgian ADPs. Additionally, the German ADPs clustered closer together, while the mesophilic Belgian ADPs (cluster "biowaste" and "maize, manure") showed a wider distribution within their clusters (Fig. 1). This might be related to the feedstock heterogeneity of the "biowaste" samples (Table 2) or to differences in the release of degradation by-products such as salt, NH_4^+-N or NH_3 (see below). In contrast, more homogeneous feedstocks (meaning chemically stable/similar in function of time), such as waste water sludge and industrial wastes (paper mill or brewery waste water) (Ward et al., 2008), clearly showed a high relative abundance of the phylum *Bacteroidetes* with representatives from the order *Bacteroidales* (symbolised by TRF-88bp, TRF-90bp, TRF-92bp and TRF-95bp) (Fig. 1B). They accounted for 30-40% (relative abundance) of the entire bacterial community (Table 3), similar to previous studies (De Vrieze et al., 2015; Werner et al., 2011). This study further showed that these

communities are also characterized by the presence of members from the phylum *Cloacimonetes* (symbolized by TRF-163bp) (Fig. 1B). This is not surprising in digesters treating waste water sludge, as this phylum was first detected in a municipal waste water plant (Chouari et al., 2005; Pelletier et al., 2008). Most of the so far described members of the phyla *Bacteroidetes* and *Cloacimonetes* are known for their ability to convert easily degradable amino acids, sugars and alcohols into VFA (e.g., Hahnke et al., 2016; Limam et al., 2014; Pelletier et al., 2008), which emphasizes their potential crucial role in acido- and acetogenesis. However, and so far for the first time described, this bacterial community structure (dominance of representatives from the phyla *Bacteroidetes* and *Cloacimonetes*) was correlated with a predominance of members from the methylotrophic or acetoclastic archaeal genera *Methanomethylovorans* (TRF-83bp, Fig. 1C) and *Methanothrix* (TRF-93bp, Fig. 1C), respectively, which accounted for 70 - 90% (relative abundance) of the entire archaeal communities within the respective ADPs (Table 3). This high dominance, especially of the genus *Methanothrix*, could be expected as the prevalent process parameters (Table 1) corresponded to unstressed AD systems (De Vrieze et al., 2012; De Vrieze et al., 2016; Regueiro et al., 2012; Westerholm et al., 2016) with low concentrations of potential process inhibitory factors, such as conductivity (5 - 8 mS cm^{-1}), VFA (not higher than 0.2 $g_{HAc\text{-}Eq}$ L^{-1}) and NH_3 (between 1 and 30 mg_N L^{-1}). These are beneficial conditions for the sensitive genus *Methanothrix* with their rod-shaped cell structure, slow growth rates (μ_{max} = 0.20) and restricted metabolic capacity as obligate acetoclastic methanogens combined with a high affinity for acetate and high sensitivity for potential toxic substances (De Vrieze et al., 2012; Rajagopal et al., 2013). In case that acetic acid cannot be converted directly into methane because, the acetoclastic methanogens are inhibited in their functionality, the microbial community uses the mechanism of syntrophic acetic oxidation (Rajagopal et al., 2013; Westerholm et al., 2016). For example, Alsouleman et al. (2016) showed that a gradual increase in the amount of poultry manure and consequently in the TAN and NH_3

concentrations led to a process disturbance (VFA concentration = 9.6 $g_{HAc\text{-}Eq}$ L^{-1} at 5.9 g NH_4^+-N L^{-1} and 500 mg NH_3 L^{-1}). In this context, the microbial community was restructured from a *Bacteroidetes*-dominated to a *Clostridiales*-dominated bacterial community, accompanied by a shift from the acetoclastic (*Methanothrix*) to the hydrogenotrophic (*Methanobacteriaceae*) pathway of methane formation. Without active counteracting, the microbial community adapted to the new conditions and stabilised the biogas production process. From an ecological point of view, this can be considered as a naturally controlled microbial diversity management. However, the presented results further confirm previous findings by Kirkegaard et al. (2017) who noted a stable microbial community in several waste water treatment plants over a six-year survey period. Therefore, it can be assumed that the recorded microbial communities were highly adapted to their environment, that they are stable over time, and hence that the occurring microbial community composition (*Bacteroidetes-Cloacimonetes-Methanothrix*) is an indicator of a well-running process.

The second main distinction is related to the process performance: the German ADPs were operated at 40°C to 45°C (Table 1), a temperature regime between optimal values for mesophilic (33°C - 38°C) and thermophilic (50°C - 60°C) conditions (Kim and Lee, 2016). This is reflected in the NMS analyses as the German ADPs cluster separately from the mesophilic and thermophilic Belgian ADPs (Fig. 1). This shows that the temperature is one of the most important driving factors for the development of microbial communities, even at intermedium temperature regimes. This is strengthened at thermophilic conditions (cluster "54°C" in Fig. 1), where the temperature, for example, overlaps the impact of the feedstock and thus the nutrient availability effect. Within the thermophilic cluster (Fig. 1), ADPs were identified converting slaughterhouse waste (B02), a mixture of manure, food waste, slaughterhouse waste and energy crops (B04) or maize silage and manure (B05) (Table 1). The bacterial communities of these ADPs were dominated by members of the phyla

Firmicutes (TRF-150bp, TRF-171bp, TRF-178bp and TRF-181bp) and *Thermotogae* with the genus *Defluviitoga* (TRF-68bp) in combination with a predominance of hydrogenotrophic methanogens represented by the archaeal family *Methanobacteriaceae* (TRF-337bp) (Fig. 1B and 1C, Table 3), which confirmed previously published studies (De Vrieze et al., 2015; Niu et al., 2013; Rademacher et al., 2012; Pap et al., 2015). Under thermophilic conditions, other environmental effects derived from potential process-inhibiting factors (*e.g.*, salt or NH_3 content) also have no impact or only a minor impact on the microbiome structure. All thermophilic ADPs exceeded the frequently described and generally accepted threshold values of 30 mS cm^{-1} for the salt content (Chen et al., 2008; De Vrieze et al., 2012; De Vrieze et al., 2017) and of 300 mg_N L^{-1} for the NH_3 content (Westerholm et al., 2016). In contrast, these parameters are of crucial importance under mesophilic conditions, which is shown by the presented study (see below).

The NMS analysis revealed that the salt content (conductivity) as well as the TAN and NH_3 concentrations are key abiotic parameters that affect the microbiome composition under mesophilic process conditions. Both factors are by-products of the biochemical process chain, meaning that they are either released by the breakdown of organic compounds (salt and NH_4^+-N) or that they are the result of specific physicochemical process conditions (NH_3). In the case of potential salt stress, it has been frequently described that conductivity values over 30 mS cm^{-1} can cause microbial cell dehydration due to osmotic pressure (Chen et al., 2008; De Vrieze et al., 2012; De Vrieze et al., 2017). In this study, five mesophilic operating ADPs (B03, B06, B08, B09 and B10) exceeded this threshold value (32 - 62 mS cm^{-1}). With exception of B03, those ADPs were additionally characterized by TAN and NH_3 concentrations exceeding 4.0 g_N L^{-1} and 300 mg_N L^{-1}, respectively (Table 1); values frequently described and generally accepted as process inhibition thresholds (Westerholm et al., 2016). The microbial communities of B01 and B10, as well as G07 (Fig. 1A) were only

affected by their elevated levels of TAN and NH3 concentrations (Table 1). However, no clear effect on the overall biogas production was evaluated. B01, B10 and G07, for example, showed comparable high concentrations of the mentioned nitrogenous compounds (TAN: 4.6 to 6.5 g_N L^{-1}, NH_3: 700 to 1500 mg_N L^{-1}) with putative high biogas amounts for B01 (3.73 m^3 kg_{COD}^{-1}), while B10 and G07 showed with 0.53 m^3 kg_{COD}^{-1} and 0.32 m^3 kg_{VS}^{-1}, respectively, low biogas amounts. A potential overestimation of the calculated NH_3 concentrations by 10 - 40%, due to disregarding the ionic strength effect or the NH_4^+-N activity coefficient, could be assumed (Hafner and Bisogni, 2009; Nielsen et al., 2008; Rajagopal et al., 2013), but even this does not explain the absence of a correlation with the produced biogas. However, it can be assumed that the occurring microbiomes or specific community members are well-adapted to these putative process-inconvenient conditions. The NMS analysis revealed three bacterial TRFs (TRF-64bp, TRF-162bp and TRF-228bp) that are characteristic for these clusters (Fig. 1B), but they could not be assigned to any known bacteria. Nevertheless, it can be suggested that the TRF-related, yet unknown, species are highly adapted and resistant to these prevalent environmental conditions and occupy a specific ecological niche that makes them important for their microbiomes and their functionality. Regarding the archaeal community, the hydrogenotrophic pathway of methane formation could be considered, as there was a high pre-dominance of members of the genus *Methanoculleus* (symbolised by TRF-428bp, Fig. 1C). This is in general accordance with previously reported studies (*e.g.*, Westerholm et al., 2016).

To assess a stable and efficient biogas process, multiple parameters have to be considered. This is obvious for the samples B06b and B11, which showed very high VFA concentrations (B06b: VFA = 21.2 $g_{HAc\text{-}Eq}$ L^{-1}, B11: VFA= 34.5 $g_{HAc\text{-}Eq}$ L^{-1}). An "inhibited steady-state" could be possible under certain conditions, but the biogas production values are quite good compared to the other investigated ADPs (B06: 0.93 m^3 kg_{COD}^{-1}, B11: 1.14 m^3 kg_{COD}^{-1}). A

stable biogas production process is further confirmed by the detected pH values (B06: 8.0, B11: 7.8) as well as the NH_3 concentration (B06b: 268 mg_N L^{-1}, B11: 287 mg_N L^{-1}), which are not at toxic levels. Interestingly, B11 was dominated by TRF-428bp (*Methanoculleus*) with 42.8%, while B06b showed a high relative abundance of TRF-106bp (*Methanotrix*) with 72.6%. For the latter case, Chen et al. (2015) reported that *Methanothrix* (synonym *Methanosaeta*) can dominate the archaeal community at high acetate levels.

To summarize, the comparison of 36 anaerobic digestion microbiomes revealed that prevalent process conditions are related to different marker microbiome clusters, whereby it is difficult to distinctly interlink one specific parameter to the development of a specific microbiome. Most often, various environmental parameters affect the microbial community composition and hence the functional and ecological diversity within anaerobic digestion plants. Based on the presented results, a weighting of affecting environmental parameters can be derived from higher to lower importance as follows: (i) thermophilic conditions (temperature); (ii) TAN and NH_3 concentrations, as well as conductivity; and (iii) the chemical composition (complexity) of the supplied feedstocks and hence the nutrient availability.

3.2 Each biogas fermenter has its own microbiome

This study showed that it was possible to define marker microbiome clusters reflecting the effects of, *e.g.*, temperature, TAN and NH_3 concentrations, conductivity, and feedstock composition (nutrient availability/accessibility), on the microbiome structure. Additionally, biotic interactions between specific bacterial and archaeal community arrangements were revealed. Nevertheless, one current important research challenge is the reversed approach in terms of exploring the influence of the microbial community on the digester functioning and stability (Venkiteshwaran et al., 2016).

From an ecological point of view, the members of a microbial community can be separated into generalists and specialist. Generalists exist (meaning that they are present and functionally active) under various conditions and hence are found in most of the biogas plants in high relative abundances. The Pareto principle states that roughly 20% of the occurring species are responsible for 80% of the energy flux (De Vrieze and Verstraete, 2016). Consequently, the remaining 80% are suggested to be specialists that fulfil specific functions in the digester's ecosystem, occupying specific ecological niches, and are less frequent and often digester specific. The 20% of the TRFs that were most often and most frequently detected in this study accounted for 65% and 60% (relative abundance) of the bacterial and archaeal community, respectively (Fig. 2C and 2D).

In the presented dataset, no TRF was recorded in all the investigated ADPs. Only two bacterial TRFs (TRF-84bp and TRF-150bp) were found in 25 of the 36 investigated ADPs, whereby these TRFs had a median relative abundance of 23% (Fig. 2A and 2C). At the archaeal level, one TRF was detected in 32 ADPs, with a median relative abundance of 36% (Fig. 2B and 2D). Most of the detected TRFs were only found in a certain number of ADPs, which reflects that each ADP develops its own unique microbiome, specifically adapted to their local environmental conditions. The putative rare species, the specialists, are potentially hidden drivers of microbiome functioning (Jousset et al., 2017), as they provide necessary traits under process-inconvenient conditions, resulting in an overall stable biogas production process. For example, TRF-162bp was found in seven ADPs with a median relative abundance of 1.7%, whereby TRF-228bp was recorded in 12 ADPs with a median relative abundance of 1.5%. The TRF-related (yet unknown) members of the microbial community are suggested to be resistant against elevated levels of salt and/or TAN and NH_3 (see above); hence, they can be used as potential markers for their microbiome cluster. The question at this point is: what specific ecological function do these microorganisms have? Do they

compensate the function of inhibited community members and hence rescue the entire community from lethal stress by *e.g.* VFA removal? This shows that further research is required (i) to identify and describe unknown microorganisms and classify their system ecological function; (ii) to explore the influence of single microorganisms (populations), groups of microorganisms or entire communities on the digester function and stability by elucidation of mechanisms that regulate the spatiotemporal, biotic and abiotic interactions; and (iii) to identify microbial indicators to develop new diagnostic and assessment procedures.

Conclusions

Each ADP develops its individual microbiome. The NMS analysis (β-diversity) revealed marker microbiome clusters corresponding to prevalent environmental factors. A weighting of key parameters can be derived from higher to lower importance as follows: (i) thermophilic conditions, (ii) TAN and NH_3 concentrations, as well as conductivity, and (iii) nutrient availability. Thermophilic digesters showed similar microbial communities, independent of the nutrient availability or potential process-inhibiting factors, which were the driving factors at mesophilic conditions. Biotic interactions between specific bacterial and archaeal community arrangements were recorded, as well as rare species that can be hidden drivers of microbiome functioning at process-inconvenient conditions.

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Table legends

Table 1: Characteristics of the investigated full-scale anaerobic digestion plants (ADPs). For each ADP, the reactor type and volume, the supplied feedstock, the organic loading rate (OLR) and hydraulic retention time (HRT), the applied process temperature, as well as the main chemical parameters conductivity, pH, total volatile acids (VFA), total ammonium nitrogen (TAN), ammonium nitrogen (NH_4^+-N), ammonia nitrogen (NH_3) and biogas production are given. The main process parameters are highlighted in colours, from green for the lowest values to red for the highest values. CSTR = continuous stirred tank reactor, UASB = upflow anaerobic sludge blanket reactor, PF = plug flow reactor, FW = food waste, and ns = not specified.

Table 2: Comparative overview of the biochemical composition of feedstocks used in this study. TS = total solids, FM = fresh mass, VS = volatile solids, na = not available, * = given as total carbohydrates.

Table 3: Median relative abundance ($n \geq 3$) of the detected bacterial and archaeal TRFs of the investigated anaerobic digestion plants (ADPs) highlighted in colours, from green for the lowest values to red for the highest values of the detected TRFs. Only TRFs with a relative abundance higher than 2% are given. The ADPs are sorted according to NMS clustering (Fig. 1). TRFs given in bold were most often and most frequently found, accounting for 65% and 60% (relative abundance) of the bacterial and archaeal community and respresenting 20% of the occurring species that putatively are responsible for 80% of the energy flux. Additionally, the phyolgenetic assignment is given for each TRF based on an an in-house database, currently containing about 3000 16S rRNA gene sequences. TRF = terminal restriction fragment, B = Belgian ADPs, G = German ADPs.

Figure legends

Fig. 1: Non-metric distance scaling (NMS) analysis using the Bray-Curtis dissimilarity distance to elucidate key abiotic parameters (A), as well as the main biotic parameters, in this case, selected terminal restriction fragments (given numbers) of the bacterial (B) and archaeal communities (C), which characterize the recorded microbiomes within the investigated full-scale anaerobic digestion plants. The final stress and instability in all three cases was 11.3 and 0.00000, respectively. NMS analysis was performed using the software package of PC Ord Version 6 (McCune and Mefford, 2011).

Fig. 2: Number of bacterial (A) and archaeal (B) TRFs found in a certain anaerobic digestion plant (ADP) group, as well as the relative abundance of all bacterial (C) or archaeal (D) TRFs from the certain ADP group to elucidate generalists and specialists within the investigated ADP. For example, only two bacterial TRFs were found in 25 of the 36 investigated ADPs, (A) whereby these TRFs had a median relative abundance of 23% (C).

Table 1: Characteristics of the investigated full-scale anaerobic digestion plants (ADPs). For each ADP, the reactor type and volume, the supplied feedstock, the organic loading rate (OLR) and hydraulic retention time (HRT), the applied process temperature, as well as the main chemical parameters conductivity, pH, total volatile acids (VFA), total ammonium nitrogen (TAN), ammonium nitrogen (NH4+-N), ammonia nitrogen (NH3) and biogas production are given. The main process parameters are highlighted in colours, from green for the lowest values to red for the highest values. CSTR = continuous stirred tank reactor, UASB = upflow anaerobic sludge blanket reactor, PF = plug flow reactor, FW = food waste, and ns = not specified.

Plant name	Reactor type	Reactor volume [m^3]	Composition of the supplied feedstock	OLR [$kg_{COD}\ m^{-3}\ d^{-1}$]	HRT [d]	Temperature [°C]	Conductivity [$mS\ cm^{-1}$]	pH [-]	VFA [$g_{HAc\text{-}Eq}\ L^{-1}$]	TAN [$g\ L^{-1}$]	NH_3 [$mg\ L^{-1}$]	Biogas [$m^3\ kg_{COD}^{-1}$]
B01	CSTR	1500	Maize, Manure	1.5	100	38	29	8.5	5.4	4.6	1460	3.73
B02a	CSTR	1000	Slaughterhouse waste	11.0	20	54	31	8.0	10.4	2.4	696	0.68
B02b	CSTR		Slaughterhouse waste			54	25	8.0	10.4	2.4	696	
B03a	CSTR	1200	Maize, Manure	4.0	40	34	40	8.0	4.4	3.5	338	0.28
B03b	CSTR		Maize, Manure			34	40	8.0	4.2	3.5	380	
B04a	CSTR	3600	Manure, FW, Slaugtherhouse waste, Energy crops	5.1	40	54	32	8.0	6.2	3.3	363	0.29
B04b	CSTR		Manure, FW, Slaugtherhouse waste, Energy crops			54	33	8.0	7.1	3.2	353	
B05a	CSTR	2000	Maize, Manure	4.0	30	54	39	8.0	7.6	3.1	743	1.85
B05b	CSTR		Maize, Manure			54	38	8.0	7.4	3.3	701	
B06a	CSTR	1500	Organic biological waste	3.0	80	34	32	8.0	5.2	4.0	402	0.93
B06b	CSTR		Organic biological waste			34	32	8.0	21.2	2.7	268	
B06c	CSTR		Organic biological waste			34	32	8.0	0.8	4.2	417	
B07a	CSTR	4000	Wastewater sludge	3.0	18	34	8	7.4	0.0	1.1	25	0.67
B07b	CSTR		Wastewater sludge			34	7	7.5	0.0	1.0	30	
B07c	CSTR		Wastewater sludge			34	6	7.4	0.0	1.0	26	
B08	CSTR	1500	Organic biological waste	2.5	80	34	32	8.1	0.2	3.9	459	2.56
B09	CSTR	1250	Maize, Manure	2.5	80	34	42	8.1	0.7	5.0	530	1.64
B10	CSTR	3200	Maize, Manure	4.0	40	34	62	8.3	0.4	6.4	1020	0.53
B11	CSTR	3000	Maize, Manure	5.0	40	34	25	7.8	34.5	5.0	287	1.14
B12	UASB	1210	Paper mill wastewater	5.6	ns	35	7	7.2	0.2	0.2	4	0.21
B13	CSTR	3255	Sludge, Manure	3.0	20	33	8	7.4	0.0	0.5	11	0.90
B14a	UASB	274	Brewery wastewater	3.3	ns	34	6	7.1	0.0	0.3	4	0.45
B14b	UASB		Brewery wastewater			34	5	6.8	0.0	0.1	1	

Table 1: continued.

Plant name	Reactor type	Reactor volume [m^3]	Composition of the supplied feedstock	OLR [kg_{VS} m^{-3} d^{-1}]	HRT [d]	Temperature [°C]	Conductivity [mS cm^{-1}]	pH [-]	VFA [g_{HAc-Eq} L^{-1}]	TAN [g L^{-1}]	NH_3 [mg L^{-1}]	Biogas [m^3 kg_{VS}^{-1}]
G01	CSTR	1130	Maize silage, Tricale, Cattly slurry	4.1	54	44	14	7.7	1.1	2.3	166	0.66
G02	PF	270	Maize silage, Rye silage, Grass silage, Pig manure, Cattly slurry	5.4	16	42	16	7.8	1.4	2.1	169	0.63
G03a	CSTR	1500	Maize silage, Rye silage, Cattle manure, Cattle slurry	2.5	86	45	20	7.9	0.3	3.2	296	0.52
G03b	CSTR		Maize silage, Rye silage, Cattle manure, Cattle slurry			45	19	7.7	0.1	2.8	440	
G04a	PF	270	Maize silage	3.5	158	44	14	7.3	7.8	2.5	93	0.46
G04b	CSTR	1065	Maize silage			42	19	8.1	0.2	3.3	490	
G05	CSTR	1750	Maize silage, Rye silage, Cattle manure, Slurry mixture	1.1	68	40	11	7.7	0.9	2.2	135	1.09
G06a	CSTR	1750	Maize silage, Rye silage, Grass silage, Cattle manure, Cattle slurry	4.0	45	43	19	7.9	1.2	3.2	331	0.38
G06b	CSTR		Maize silage, Rye silage, Oat silage, Cattle manure, Cattle slurry			41	14	7.6	0.0	2.1	126	
G07a	CSTR	2280	Maize silage, Rye silage, Chicken manure	3.4	70	41	27	8.3	0.6	6.5	1177	0.32
G07b	CSTR		Maize silage, Chicken manure			41	29	8.0	0.3	5.9	712	
G08a	CSTR	1400	Maize silage, Grass silage, Slurry mixture	1.7	125	40	15	7.8	0.5	3.5	280	1.12
G08b	CSTR		Maize silage, Grass silage, Slurry mixture			40	19	7.8	0.1	3.6	243	

Table 2: Comparative overview of the biochemical composition of feedstocks used in this study. TS = total solids, FM = fresh mass, VS = volatile solids, na = not available, * = given as total carbohydrates.

Feedstocks	TS (% FM)	VS (% TS)	Protein (% TS)	Lipid (% TS)	Hemicellulose (% TS)	Cellulose (% TS)	Lignin (% TS)	References
Energy crop - Maize	30.2 ± 13.5	95.8 ± 1.6	8.1 ± 3.7	2.5 ± 1.5	15.3 ± 2.8	**21.9 ± 8.7**	3.3 ± 2.6	Herrmann et al. 2016
Energy crop - Maize	33.7 ± 1.9	96.2 ± 0.3	na	na	18.3 ± 2.8	19.1 ± 2.6	3.1 ± 1.5	this study
Energy crop - Tricale	35.2 ± 13.0	95.1 ± 1.2	9.2 ± 3.1	2.3 ± 0.8	**32.4 ± 20.7**	**27.8 ± 6.1**	4.7 ± 1.7	Herrmann et al. 2016
Energy crop - Rye	33.1 ± 2.7	94.8 ± 0.8	8.8 ± 1.4	1.9 ± 0.3	19.2 ± 1.6	**30.4 ± 3.9**	5.8 ± 1.8	Herrmann et al. 2016
Energy crop - Rye	29.9 ± 12.9	89.7 ± 2.9	na	na	**23.6 ± 1.7**	**34.2 ± 5.6**	4.3 ± 1.3	this study
Energy crop - Grass	28.7 ± 13.1	90.1 ± 2.8	**13.3 ± 5.5**	3.3 ± 1.2	15.4 ± 3.1	**25.8 ± 4.5**	4.9 ± 3.5	Herrmann et al. 2016
Energy crop - Grass	39.1 ± 5.6	87.6 ± 4.8	na	na	19.0 ± 1.4	**25.3 ± 2.4**	4.6 ± 1.2	this study
Energy crop - Oat	37.9 ± 13.0	92.4 ± 2.1	9.5 ± 3.5	3.1 ± 0.6	21.4± 1.4	**28.1 ± 3.1**	5.9 ± 1.9	Herrmann et al. 2016
Pig (Sow) manure	37.2 ± 1.1	75.8 ± 0.2	**14.9 ± 0.6**	2.1 ± 0.1	15.5 ± 0.5	12.9 ± 0.7	16.2 ± 0.3	Cu et al. 2015
Dairy cow manure	16.0 ± 0.2	79.0 ± 0.1	**12.1 ± 0.2**	2.3 ± 0.1	**27.6 ± 0.4**	19.6 ± 0.7	11.0 ± 0.4	Cu et al. 2015
Cattle manure	25.4 ± 3.4	78.2 ± 9.8	na	na	13.0 ± 6.5	**23.5 ± 6.5**	15.4 ± 4.7	this study
Cattle slurry	7.0 ± 2.7	77.8 ± 5.0	na	na	13.9 ± 2.5	14.0 ± 4.7	11.1 ± 5.6	this study
Chicken manure	37.9 ± 0.3	66.7 ± 1.0	**18.4 ± 0.4**	2.4 ± 0.1	19.9 ± 0.6	11.1 ± 0.3	5.2 ± 0.2	Cu et al. 2015
Chicken manure	57.9 ± 1.8	83.9 ± 3.2	na	na	14.0 ± 2.6	14.7 ± 0.1	6.9 ± 0.8	this study
Slautherhouse waste (Cow)	17.0 ± 1.2	82.6 ± 0.4	**12.5 ± 0.7**	5.0 ± 0.1	**31.1 ± 1.0**	**20.4 ± 0.6**	9.6 ± 0.5	Cu et al. 2015
Slautherhouse waste (Pig)	16.2 ± 0.9	91.7 ± 0.1	**17.5 ± 0.5**	**14.4 ± 0.4**	14.2 ± 0.7	9.0 ± 0.3	8.0 ± 0.1	Cu et al. 2015
Residual municiple solid waste	39.5 ± 6.2	69.7 ± 1.2	8.1 ± 0.7	**13.3 ± 0.8**	17.4 ± 2.9	**33.0 ± 19.4**	na	Bayard et al. 2015
Biowaste (not further defined)	47.4 ± 3.4	61.9 ± 6.5	**13.6 ± 5.6**	**13.0 ± 2.3**	17.8 ± 3.3	**31.7 ± 5.6**	na	Bayard et al. 2015
Biowaste (household)	21.8 ± 6.4	98.6 ± 0.1	**11.2 ± 2.0**	2.4 ± 1.3	**54.2 ± 6.0**	**26.1 ± 2.7**	5.0 ± 1.9	Cu et al. 2015
Biowaste (catering)	31.0 ± 2.3	94.0 ± 2.0	**13.0 ± 0.5**	**17.3 ± 0.3**		69.7 ± 0.8 *		Fisgativa et al. 2017
Biowaste (vegetarian restaurant)	20.1 ± 1.1	84.3 ± 9.5	**12.7 ± 3.4**	**18.8 ± 1.4**		68.5 ± 2.0 *		Fisgativa et al. 2017
Biowaste (municipality)	33.7 ± 0.5	85.6 ± 3.6	**15.9 ± 2.0**	**19.8 ± 1.2**		64.3 ± 0.8 *		Fisgativa et al. 2017

Table 3: Median relative abundance (n ≥ 3) of the detected bacterial and archaeal TRFs of the investigated anaerobic digestion plants (ADPs) highlighted in colours, from green for the lowest values to red for the highest values of the detected TRFs. Only TRFs with a relative abundance higher than 2% are given. The ADPs are sorted according to NMS clustering (Fig. 1). TRFs given in bold were most often and most frequently found, accounting for 65% and 60% (relative abundance) of the bacterial and archaeal community and respresenting 20% of the occurring species that putatively are responsible for 80% of the energy flux. Additionally, the phyolgenetic assignment is given for each TRF based on an an in-house database, currently containing about 3000 16S rRNA gene sequences. TRF = terminal restriction fragment, B = Belgian ADPs, G = German ADPs..

	TRF	cluster "lowest values"							cluster "biowaste"				cluster "maize, manure"				cluster "40-45°C; maize, rye, cattle" and cluster "chicken"												cluster "54°C"						Phyogenetic assignment of the detected TRFs
	[bp]	B07a	B07b	B07c	B14a	B14b	B13	B12	B06a	B06b	B06c	B08	B01	B09	B10	B11	G08a	G08b	G07a	G07b	G06a	G06b	G05	G04a	G04b	G03a	G03b	G01	G02	B03a	B03b	B02a	B02b	B04a	B04b	B05a	B05b	[sorted by phylum, class, order, family, genus]
Bacteria	**64**		6,3	5,3	4,2		4,4		5,8		9,0	6,6	6,7	5,3	13,9																						*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	68			7,4			7,2	3,3																3,2	3,3					20,0	14,9	22,5	14,1	34,7			*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	77						5,4																														*Firmicutes*
	84		5,3	4,7	4,7	7,4			5,1	7,5	3,6			8,7	5,6	7,7	5,2		16,2		8,7	5,3		12,7	13,3	9,4	6,4	5,5	2,9	6,0	5,3							unknow bacterium
	88				6,4	5,9	6,4													5,0		3,5															*Bacteroidete, Flavobacteriia, Flavobacteriales, Flavobacteriaceae*
	90	8,3	8,1	9,1	11,3	3,7	8,6	6,7	5,7			9,2	10,0					5,9		11,4			14,4														*Bacteroidete, Flavobacteriia, Flavobacteriales, Flavobacteriaceae*
	92	14,3	8,3	7,7		7,8	8,6	15,3								6,7			3,8		3,4	3,5		6,6	5,7		3,5										*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	94	5,5	5,7	5,6	5,6	8,5	5,4	17,8									3,9	4,2			3,1	5,7	2,4	10,0	12,5	15,0	8,6	13,6		7,7	7,6	8,3	8,3					*Bacteroidetes, Bacteroidia, Bacteroidales, Porphyromonadaceae*
	95		6,5		7,5	7,8																3,7						4,7									*Bacteroidetes*
	99				5,6	7,2		4,8												3,3			4,0			3,7		3,4				9,9		7,8		6,7	7,8	*Bacteroidetes, Bacteroidia, Bacteroidales*
	127		5,8			3,4									12,6																						*Proteobacteria*
	132						18,2																			5,4												*Firmicutes, Clostridia, Bacillales, Bacillaceae*
	143						5,3																														*Firmicutes, Clostridia, Clostridiales*
	148		5,3					4,0	9,8	7,7										3,7	4,9		4,2	4,2			4,6	9,2	5,0								*Firmicutes, Clostridia*
	150						7,9						6,2	14,9	7,8			10,0	11,4	4,8	26,4	14,7	5,6	17,7	24,8	19,3	28,8	28,3	1,1	23,8	22,8	22,5	23,7	15,2	51,5	4,9	39,3	*Firmicutes, Clostridia*
	159																																				*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	161	7,3	9,3	7,9		4,1	8,7			8,1														14,8				3,3								*Cloacimonetes, Cloacimonadia, Cloacimonadales, Cloacimonadaceae*
	162									7,3	11,3			5,3	6,2			6,6																			unknow bacterium
	163				5,2	3,2			14,4	5,2		12,5										12,2	3,2														*Cloacimonetes, Cloacimonadia, Cloacimonadales, Cloacimonadaceae*
	166																		3,4	3,0			3,3									5,2					*Cloacimonetes, Cloacimonadia, Cloacimonadales, Cloacimonadaceae*
	167				4,2				6,6	5,4				18,7												3,2				7,6	7,9							*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	171																																		9,9	9,3	*Firmicutes*
	174	9,8	6,3	7,5			4,9																														*Firmicutes*
	178	5,8			5,6																7,5			4,3	7,9	8,4	11,6	3,8			9,9	9,6			6,2	6,2	*Firmicutes*
	180																6,0	13,2	4,8	7,6					9,4	5,9	9,0		6,8				5,7					*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	181				4,8								5,3		7,4		3,3	4,7	6,5	5,2			7,0		3,7				3,5	8,7	9,7					13,4	11,9	*Firmicutes, Clostridia*
	183																																				*Firmicutes*
	186											5,7					4,9	9,8		11,2		9,3	13,9		3,4			4,5										*Bacteroidetes, Bacteroidia, Bacteroidales*
	188																	3,4		5,2			5,6														*Firmicutes, Clostridia, Clostridiales*
	198		5,7	5,8	4,8		4,5																														*Firmicutes, Clostridia, Clostridiales, Lachnospiraceae*
	204		5,3	8,2		17,1																															*Firmicutes, Clostridia, Clostridiales, Lachnospiraceae*
	210					5,5				8,7																											*Firmicutes, Clostridia, Clostridiales*
	218																3,3		3,7	7,7	4,6	3,5	5,1	3,8		4,6		6,4	3,2									*Firmicutes, Clostridia, Clostridiales, Ruminococcaceae*
	222								5,3			6,7					3,6	3,5	3,7		4,6					4,6												*Firmicutes, Clostridia, Clostridiales*
	224						3,9			5,8							3,0	3,2	5,4	4,9			4,8															*Firmicutes, Clostridia, Clostridiales*
	225																					3,9																*Firmicutes, Clostridia, Clostridiales*
	228									5,2	5,5				5,2	9,3					3,1		2,5															unknow bacterium
	230					5,2							14,5																										*Firmicutes, Clostridia, Clostridiales, Clostridiaceae*
	233																											5,3										*Firmicutes, Clostridia, Clostridiales, Clostridiaceae*
	240									5,9																												*Bacteroidetes, Bacteroidia, Bacteroidales*
	243							7,4																														*Firmicutes, Clostridia, Bacillales*
	260																															9,9	5,3					*Firmicutes*
	289									5,8																												*Firmicutes, Clostridia, Thermoanaerobacterales, Thermodesulfobiaceae*
	291															9,7																						*Firmicutes*
	294																				3,7																*Firmicutes, Clostridia, Clostridiales*
	296										19,1								3,3																			*Firmicutes, Clostridia, Clostridiales*
	298										6,3				5,1	19,5																						*Firmicutes, Clostridia, Clostridiales*
	300														6,8																							*Firmicutes, Clostridia, Clostridiales*
	304											5,5														3,2												*Firmicutes, Clostridia , Clostridiales, Defluviitaleaceae*
	352							6,6																														*Proteobacteria*
	363						6,0																															*Proteobacteria*
	502																								3,6	4,6	3,3	4,3	4,5									unknow bacterium
	519				7,5	5,6																																*Chloroflexi, Anaerolineae, Anaerolineales, Anaerolineaceae*
	542																			3,8			4,5															*Firmicutes, Clostridia, Lactobacillales, Streptococcaceae*
	554								5,7													3,4																*Firmicutes, Clostridia, Lactobacillales*
	558																3,2																					*Firmicutes, Clostridia, Lactobacillales*
	564								7,1									3,7					3,6					4,2										*Firmicutes*

Table 3: continued.

	TRF [bp]	cluster "lowest values" B07a	B07b	B07c	B14a	B14b	B13	B12	cluster "biowaste" B06a	B06b	B06c	B08	cluster "maize, manure" B01	B09	B10	B11	cluster "40-45°C; maize, rye, cattle" and cluster "chicken" G08a	G08b	G07a	G07b	G06a	G06b	G05	G04a	G04b	G03a	G03b	G01	G02	B03a	B03b	cluster "54°C" B02a	B02b	B04a	B04b	B05a	B05b	Phyogenetic assignment of the detected TRFs [sorted by phylum, class, order, family, genus]
Archaea	83	4,4	6,2	4,7			7,7	11,6				7,1					5,1				8,2		4,4			3,5	3,6			3,8							2,7	2,8	*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanomethylovorans*
	93		13,4	13,5			7,3					5,8		7,2																								*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanotrichaceae, Methanotrix*
	108-m	61,8	59,0	63,5	9,0	64,1	64,2	8,6	59,6	72,1	7,5		7,7	56,8	39,7	12,5	4,7	19,1		8,9	9,7	6,5	6,8	12,7	27,3	3,5	4,8	8,6		9,7	92,3							*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanotrichaceae, Methanotrix*
	108-t																															36,0	26,9	5,0	10,7	91,2	93,3	*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanobacterium*
	174																6,9		14,9	7,4	3,5		3,7			3,8	3,3		4,6									unknown archaeon
	318																7,0		4,5	7,8			4,8			5,4	5,3		5,8									unknown archaeon
	337	5,2			4,8			4,8																								25,8	59,9	95,3	89,6	2,7		*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae*
	339				5,5				3,2	2,5						17,1	13,5	58,8					5,6	22,5	4,3	2,3	4,3	57,2	13,5				12,7						*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae*
	341			4,6		8,5	6,1		5,9	4,7	23,1		11,3		13,1	16,2			8,2	6,7	68,4	69,8										7,9						*Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, Methanobrevibacter*
	428	**7,6**	**9,3**	**8,6**	**6,9**	**8,6**	**9,2**		**26,7**	**14,7**	**51,3**	**31,7**	**35,8**	**27,5**	**32,6**	**42,8**		**8,6**			**4,4**	**7,5**		**4,8**	**52,4**	**2,2**		**7,7**										*Euryarchaeota, Methanomicrobia, Methanomicrobiales, Methanomicrobiaceae, Methanoculleus*
	469	**4,5**	**5,9**							**2,1**	**4,3**	**48,9**	**34,7**				**6,4**		**70,0**	**29,2**		**2,5**	**29,5**	**14,8**	**8,7**	**49,5**	**3,4**	**6,6**	**5,6**		**3,4**			**4,0**				*Euryarchaeota, Thermoplasmata, Methanomassiliicoccales, Methanomassiliicoccaceae, Methanomassiliicoccus*
	626					8,6		3,1				6,6					6,1			4,4			45,4	4,5		33,2	7,5	7,1	72,6			18,6	4,5					*Euryarchaeota, Methanomicrobia, Methanosarcinales, Methanosarcinaceae, Methanosarcina*

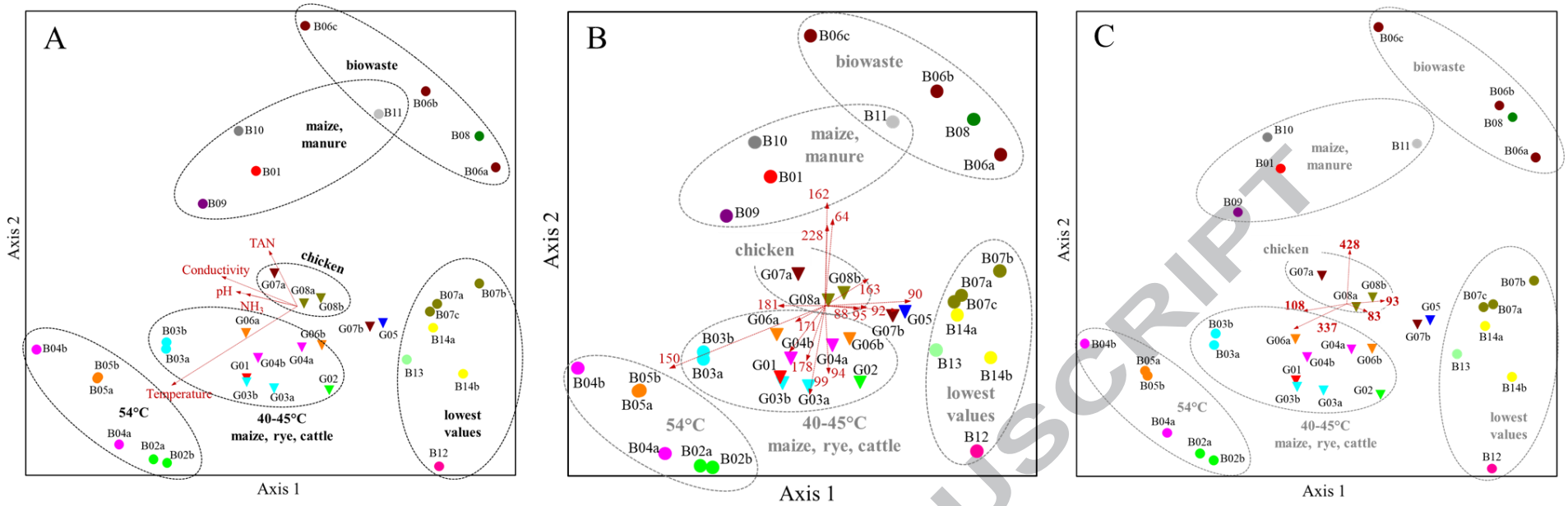

Fig. 1: Non-metric distance scaling (NMS) analysis using the Bray-Curtis dissimilarity distance to elucidate key abiotic parameters (A), as well as the main biotic parameters, in this case, selected terminal restriction fragments (given numbers) of the bacterial (B) and archaeal communities (C), which characterize the recorded microbiomes within the investigated full-scale anaerobic digestion plants. The final stress and instability in all three cases was 11.3 and 0.00000, respectively. NMS analysis was performed using the software package of PC Ord Version 6 (McCune and Mefford, 2011).

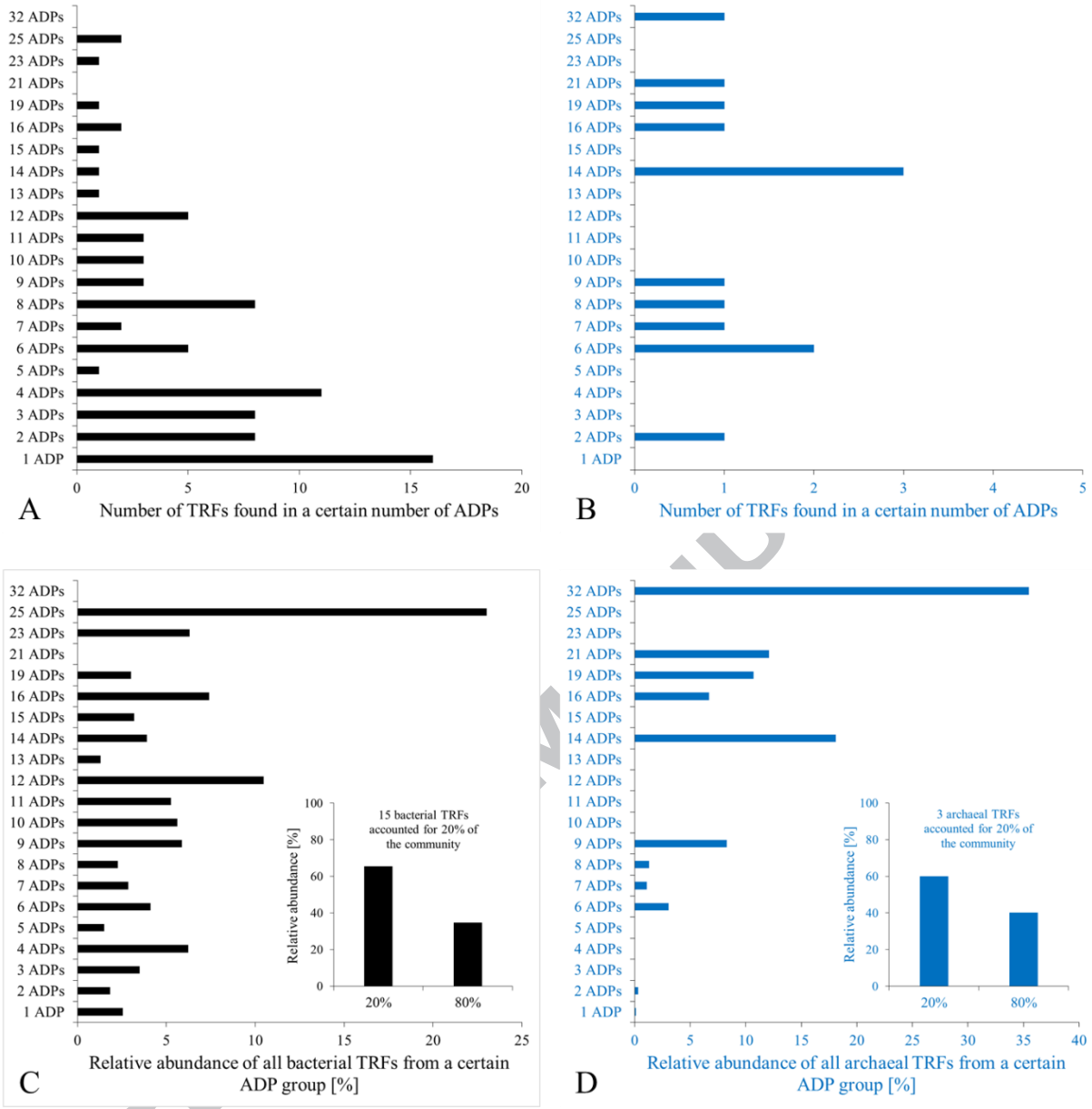

Fig. 2: Number of bacterial (A) and archaeal (B) TRFs found in a certain anaerobic digestion plant (ADP) group, as well as the relative abundance of all bacterial (C) or archaeal (D) TRFs from the certain ADP group to elucidate generalists and specialists within the investigated ADP. For example, only two bacterial TRFs were found in 25 of the 36 investigated ADPs, (A) whereby these TRFs had a median relative abundance of 23% (C).

Highlights

- Marker microbiome clusters are determined by operational parameters.
- A weighting of operations parameters was derived from higher to lower importance.
- Biotic interactions between specific community arrangements were revealed.
- Each anaerobic digestion plant develops its own unique microbiome.
- Specialists are potentially hidden drivers under process-inconvenient conditions.