Microbiota in anaerobic digestion of sewage sludge with and without co-substrates

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Abstract
In this study, seven full-scale anaerobic digesters, with or without co-substrate regime, were analysed by physicochemical and molecular biological methods. A combination of robust community fingerprinting and Illumina MiSeq sequencing revealed a core bacterial community dominated by Chloroflexi, Firmicutes, Bacteroidetes and Proteobacteria, with variations in the profiles because of differences in the co-substrate feeding regime. Despite these differences, physicochemical properties revealed a stable performance of all reactors, indicating a resilient bacterial microbiota in all full-scale reactors. A rich bacterial core community ensured reactor functionality, whilst feeding regime and reactor type impacted the overall and the core bacterial diversity. Within the Archaea, Methanoseta dominated in all reactors. Results indicated no relationship between the archaeal community structure and the type of co-substrate digested. Methanogens rely on the metabolic end products of bacterial activity and are thus less dependent on differences in the initial co-substrate regime.

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Introduction
At wastewater treatments plants (WWTPs) costs for both the pre and postprocessing and the deposition of sewage sludge (SS) often exceed the costs for the treatment of the wastewater (Yang et al. 2014; Guo et al. 2015). Thus, treating excess SS in an ecologically and economically suitable way is one of the major challenges in WWTP operation. Amongst the treatment options, anaerobic digestion (AD) is the most commonly applied technique, serving hygienisation requirements and reducing the volume of sludge (Loustarinen et al. 2009; Yang et al. 2014). AD is cost-effective because no aeration is required and the maintenance effort needed is comparatively low. Additionally, the gaseous methane (CH₄) generated may be used for generating electricity and/or serves as a source of heat energy. Complementing SS with co-substrates from various waste streams is an interesting option to improve the efficiency of the process through the priming effect (Aichinger et al. 2016; Insam & Markt 2016): The nutrient-rich organic waste not only increases the yield of CH₄ as a result of its inherent biomethane potential, but also facilitates the degradation of SS, because of synergistic effects attributed to an improved nutrient balance (Loustarinen et al. 2009). In other words, sweets for microorganisms render the staple palatable (Insam & Markt 2016). The complex process of AD is based on close interactions between numerous microorganisms, which degrade organic polymers in a sequence of steps involving hydrolysis, acidogenesis, acetogenesis and methanogenesis, resulting in the production of CH₄, carbon dioxide (CO₂), and water (Ahring 2003). In recent years, considerable efforts have been made to reveal the microbial
communities involved and to understand the interactions between the microbiota and their environment. Because the vast majority of microorganisms are yet to be cultured, culture-independent molecular biological techniques were developed, particularly targeting the 16S rRNA gene. Genetic fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), are able to provide a profile representing the genetic diversity of a microbial community from environmental samples in a rapid and reliable way (Ercolini 2004). However, since 2005, advances in next-generation sequencing (NGS) technologies have revolutionized environmental microbiology. In contrast to traditional Sanger-sequencing, allowing to sequence isolates, NGS platforms have made it possible to recover DNA sequence data directly from environmental samples, leading to a better representation of sample diversity at a reasonable cost (Shokralla et al. 2012).

To date, few NGS studies have been conducted on the microbial community structure of anaerobic digesters at WWTPs, especially regarding the addition of co-substrate: In a study of Ye & Zhang (2013), bacterial communities in different sections of a municipal WWTP were revealed by 16S rDNA 454 pyrosequencing. Yang et al. (2014) investigated the microbial communities and functions in AD sludge from two WWTPs, the Shatin and Shek Wu Hui Sewage Treatment Works. Guo et al. (2015) used an Illumina HiSeq 2000 platform to characterize the microbial community structure of the AD sludge from a full-scale municipal WWTP in Beijing, China.

The main objective of this study was to reveal both the bacterial and archaeal communities present in seven full-scale anaerobic digesters operated under different reactor configurations and with different co-substrate types. A combination of the robust fingerprinting technique DGGE and Illumina MiSeq sequencing was applied to characterize the taxonomic composition of reactors. The results and conclusions of this study should increase the knowledge on the ecology and function of the involved microbial consortia, and thus allow a better control and optimization of AD in digestion towers at WWTPs.

**Methods**

**Reactor configuration and sampling**

Digester sludges from seven full-scale anaerobic digesters of five different WWTPs in Tyrol (Austria) and South Tyrol (Italy) were collected in October 2013. Details about reactor configurations and performance, as well as schematic figures of reactors are presented in Table 1 and in the supplementary material, respectively. All reactors treated SS, in a mixture of primary and secondary sludge. The reactor R1 (Unteres Pustertal, Italy) co-digested residues from dairy production (flotation sludge; dry matter (DM) 74.6%; loss on ignition 88.8%) at low concentrations. The reactor R2 (Wasserfeld, Italy) treated SS solely, without any co-substrate. The twin reactors R3a and R3b (Tobl, Italy) were switched between serial or parallel mode in 2013, according to requirement, and co-digested residues from dairy production (whey; DM 40%; loss on ignition 70%). In October, reactors were operated in parallel mode. Biowaste from source-separate collection of the organic fraction of household wastes (mush, DM 13.4%; loss on ignition 78.3%) was used as a co-substrate in the twin reactors R4a and R4b, which were operated in parallel mode (Strass, Austria) and in the reactor R5 (Zirl, Austria), respectively. A high volume load of co-substrates resulted in a comparatively increased biogas and methane production in R5.

From all sampling sites, 5 L of homogeneous sludge samples were taken.

All reactors, except reactors R3a and R3b, were sampled during a steady-state period of operation with constant biogas production. Samples were frozen and stored at −20°C until needed for further processing.

**Physicochemical analysis**

The parameters pH, temperature and electric conductivity (EC) were measured in freshly digested sludge directly at the respective facility using a portable Multi 340i (WTW, Weilheim, Germany). After solids had been removed by dialysis, volatile fatty acid (VFA) concentrations were determined by High Performance Pressure Liquid Chromatography (HPLC) (Wagner et al. 2016). To measure DM, approximately 50 g of digested sludge (fresh matter, FM) was dried at 105°C for 24 h and weighed after cooling in a desiccator. Volatile solids (VS) were calculated as the loss of weight after igniting 2 g of the oven-dried residue at 550°C in a muffle furnace for 5 h. Sludge sample characteristics are listed in Table 2.

**DGGE and Illumina MiSeq sample preparation, sequencing and analysis**

DNA extraction was conducted in triplicate for each of the sludges. Sample volumes of 12 mL were centrifuged at 3000 g for 3 min. Approximately 500 mg pellet material was used for extraction, according to the manufacturer’s instructions (NucleoSpin® Soil DNA isolation Kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany). Qualities and concentrations of extracted DNA were determined by agarose gel electrophoresis and NanoDrop2000c microspectrophotometry (Thermo Fisher Scientific, USA). Extracts were stored at −20°C until use.

DNA for DGGE analysis was amplified by the polymerase chain reaction (PCR). For total Bacteria, the primers F984-4C clamp (AAC GGC AAC AAC CTT AC) and R1378 (CGG TGT GTA CAA GCC CCG GGA ACG) were used, targeting the bacterial 16S rRNA gene. PCR cycling comprised an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min. An elongation step at 72°C for 15 min completed DNA amplification. For Archaea, the primers 0357F-GC (TAC GGG GCG CAG CAG) and 0691R (TTA CAR GAT TTC AC) also targeting the 16S rRNA gene were used. Thermal cycling comprised an initial denaturation at 94°C for 10 min, which was followed by 35 cycles.
at 94°C for 1 min, 49°C for 2 min and 72°C for 2 min. An elongation step at 72°C for 15 min completed DNA amplification. Each PCR reaction contained 0.2 µM of each primer, 0.5 U/µL MyTaq DNA polymerase (Bioline, UK), 1 X MyTaq reaction buffer, 0.4 mg/mL bovine serum albumin (BSA) and 1 X enhancer (Peqlab, Germany). Finally, 1 µL extracted DNA was added to the 24 µL of the PCR mastermix. PCR products were loaded into a 7–8% (w/v) polyacrylamide gel with a denaturing gradient of 40–65% (100% denaturant consists of 7 M urea plus 40% formamide in TRIS based buffer) and gels were run for 16 h at 100 V in 1 X TAE buffer (pH 7.4) at a constant temperature of 60°C. Gels were stained with silver nitrate in an automated gel stainer (Amersham Pharmacia Biotech, Germany). Resulting digitalized band patterns were analysed with the GelCompar II software package (version 4.0, Applied Maths, Ghent, Belgium). Calculation of the pairwise similarities was based on the Dice correlation coefficient. Dendrograms were created using the algorithm of Ward (Legendre & Legendre 1998).

16S rRNA gene amplicon sequencing was performed by Microsynth (Switzerland) on a MiSeq Illumina sequencer, using a bp paired end approach and the primers 515F (GTG CCA GCM GCC GCG GTA A) and 806R (GGA CTA CHV GGG TWT CTA AT) targeting the bacterial V4 region of the 16S rDNA (Caporaso et al. 2011). DNA from replicate extracts was mixed prior to sequencing after confirming similar clustering in DGGE analysis.

To process raw sequences, the CoMA analysis pipeline (Hupfauf et al. 2017) was applied to assemble paired-end reads using the default settings. After quality checking, merged reads, barcodes and primers were trimmed. Low-quality reads with an average quality score below 30, with >1 ambiguous base, or a length <240 bp or >270 bp were removed. To perform alignment and taxonomic assignment of sequences, a blast algorithm using SILVA SSU (release 123) and Greengenes (release 13_5) database was conducted, by using a similarity level of 97%. Rare OTUs with a sum of reads below two within all samples were removed from onward analysis. Finally, after checking rarefaction curves, samples were subsampled to the depth of the smallest sample.
Results and discussion

Physicochemical parameters reveal stable reactor performances

Physicochemical characteristics of large-scale WWTP anaerobic digesters operating with different co-substrates were determined to indicate unfavourable AD conditions at the time point of sampling. Despite differences in the input materials and operational mode of the AD processes from which the sludges were collected, the similarity of physicochemical characteristics amongst sludges from the different plants was high, with pH values ranging between 7.20 and 7.53, DM between 2.85 ± 0.03% (FS) and 3.48 ± 0.03% (FS) and VS between 1.71 ± 0.02 and 2.22 ± 0.04% (DM). EC ranged between 9.88 mS/cm and 15.4 mS/cm in R1 and R2, respectively. Low VFA concentrations, ranging from 13.6 to 45.9 mg/L for acetate, <2.22 to 10.7 mg/L for propionate, 3.79 to 4.93 mg/L for butyrate and <1.26 to 1.80 mg/L for lactate were measured (Table 2). All physicochemical parameters indicated favourable conditions for biomethanisation (BMVIT: Bundesministeriums für Verkehr, Innovation und Technologie, 2007).

DGGE band profiling results indicate a rich bacterial community diversity

DGGE was conducted to compare the composition of microbial communities present in anaerobic digesters and their replicability. For Bacteria, a higher number of bands (20.2 ± 3.88) was observed in DGGE analyses, indicating a higher community richness in comparison to the Archaea (11.1 ± 0.64 bands). Cluster analysis of banding patterns showed that co-substrate addition influenced the bacterial community and increased the heterogeneity within the communities of AD plants (Fig. 1a). Obviously, the digestion of SS supported a core bacterial community that can be found in all reactors, whereas the addition of different co-substrates changed the abundance of Bacteria present. Likely, these differences in banding patterns reflect substrate responses of hydrolysing bacteria involved in the first step of AD: Reactor R1, treating low loadings of dairy products revealed a similarity of >76% in banding pattern to R2, treating SS solely. Reactors treating biowaste were found in a separate cluster, revealing similarities of 71% (R4a, R4b) and 69% (R5), indicating related communities. DNA from sludges of the reactors R3a and R3b, which co-fermented residues from dairy production, grouped distinct from all other samples (62% similarity). Furthermore, differences between sludges from R3a and R3b were higher than these found in the reactors R4a and R4b.

Archaeal communities (Fig. 1b) showed more homogenous banding patterns (>86% similarity), indicating less differences between reactor samples. This may be attributed to the fact that methanogens rely on the metabolic end products of acetogens and acidogens and are thus less dependent on the original substrate composition. Additionally, methanogenic communities employed in AD reactors in WWTPs may be mainly recruited from SS, and co-substrates may have a minor impact on the archaeal diversity, when compared to bacteria. These findings are in line with the results of Rincón et al. (2008) and Carballa et al. (2011), where the authors also observed a richer and more diverse bacterial community compared to the archaeal community through DGGE and T-RFLP.

Next generation sequencing reveals core microbiota with bacterial adaptations to co-substrate

Overall, 1032 MB sequencing data were generated, revealing 9.34 x 10^6 reads. Using quality filtering, 17.4 ± 2.81% of total reads were removed, resulting in an average of 39,682 ± 17,441 reads per sample. After BLAST, singleton removal and subsampling, 38,452 reads were obtained per sample, resulting in 1817 OTUs based on 97% similarity.

In all samples, more than 96.8 ± 1.39% of the sequenced reads taxonomically annotated were affiliated to Bacteria (1782 OTUs), whilst 3.11 ± 1.34% were assigned to Archaea (19 OTUs). This distribution of Bacteria and Archaea is in line with studies of other authors (Sundberg et al. 2013; Yang et al. 2014; Guo et al. 2015), who published archaeal abundances of 2.7–4.1%, 5.6% and 2.0–20.0% in AD reactor sludges, respectively.

A consortium of Chloroflexi, Firmicutes, Bacteroidetes and Proteobacteria were found to be dominant in digester samples, accounting for 69.9% of all sequences. In this study, Chloroflexi were found to be the most abundant phylum (24.6 ± 10.3%), followed by Firmicutes (22.9 ± 9.37%) and Bacteroidetes (7.79 ± 2.92%). Other phyla, detected at percentages >2% were affiliated to Actinobacteria (4.6%), Atribacteria (4%), Synergistetes (3.4%) and Saccharibacteria (2.7%).

The overall dominance of these phyla has been described previously. In a study by Riviére et al. (2009), in which seven full-scale anaerobic digesters in France, Germany and Chile were monitored, a consortium of Proteobacteria, Bacteroidetes, Firmicutes and Chloroflexi was found to dominate. Similarly, Yang et al. (2014) identified a predominant community of Proteobacteria, Bacteroidetes and Firmicutes in two digestion towers over time. Sundberg et al. (2013) found Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Chloroflexi and Spirochaetes to be the dominant populations in AD reactors digesting SS, whilst Firmicutes were the most prevalent phylum when co-digesting various combinations of wastes from restaurants, households and slaughterhouses.

The metabolic potential of the genus Chloroflexi is still unclear, but several studies suggest a role in carbohydrate degradation (Riviére et al. 2009).

The most abundant group of Chloroflexi at the class level found in sludges belonged to Anaerolineae...
including Anaerolineales (order) and Anaerolineaceae (family) – with minor deviation amongst digester samples (Fig. 2). This class represented 95 ± 1.19% of all Chloroflexi reads. According to Yamada et al. (2007), members of this group are saccharolytic bacteria that can metabolize polysaccharides, such as pectin or xylan.

Firmicutes are well described fermenting bacteria often acting as syntrophs to methanogens, by degrading VFA, such as butyrate and its analogues (Garcia-Peña et al. 2011). The released H₂ is scavenged by methanogens, making the reaction thermodynamically possible for both partners. Within the phylum Firmicutes, Clostridia was found to be the major class, accounting for 95.5 ± 2.32% of Firmicutes reads. The most abundant taxons within Clostridiales were affiliated to Ruminococcaceae (e.g. genus Fastidiosipila), and ‘Family XI’ (e.g. genus Sedimentibacter), comprising between 3.56% in R3a and 71.4% in R5, and 16.6% in R5 and 60.9% in R3b of all Clostridia reads, respectively (Fig. 2). Species of the class were reported as highly versatile organotrophs capable of degrading proteins, lipids, and polymeric carbohydrates to produce VFAs and ammonia (Garcia-Peña et al. 2011; Sundberg et al. 2013).

Bacteroidetes are often proteolytic bacteria, and most classes are capable of metabolizing carbohydrates to produce VFAs (Garcia-Peña et al. 2011).

In the phylum Bacteroidetes, the classes ‘Bacteroidetes vadinHA17’ and Bacteroidia (including the order

Fig. 1. Cluster analysis of reactor samples R1 to R5 by DGGE fingerprints of bacterial (a) and archaeal community (b). The scale bars represent similarities between samples. [Colour figure can be viewed at wileyonlinelibrary.com]
**Bacteroidia Incertae Sedis with Draconibacteriaceae** dominated, with 36.2% (R4b)– 81.1% (R3b) and 9.82% (R1)–60.8% (R4b) annotated *Bacteroidetes* sequences in all digested samples, respectively. Members of the family *Draconibacteriaceae* were primarily found in reactors where kitchen waste was used as a co-substrate, accounting for 3.50, 3.40 and 2.02% of all reads in R4a, R4b and R5, respectively (Fig. 2).

The phylum *Proteobacteria* comprised approximately 6% of sequenced reads in reactors R1, R2, R3b, R4a, R4b, R5; however, numbers of this phylum were significantly higher in R3a (64.1%). Taxa of *Proteobacteria* are well-known glucose-, propionate-, butyrate- and acetate-utilizing microorganisms (Guo et al. 2015). Considering the composition of the less abundant phylum *Proteobacteria* in the sludges, the subphylum *Gammaproteobacteria* with *Pseudomonadales*, *Pseudomonadaceae* and genus *Pseudomonas* were predominant in R3a, representing 74.7% of all *Proteobacteria* sequences in this reactor. The dominance of *Pseudomonas*, a well-known long-chain fatty acid degrader (Duarte et al. 2016), may be attributed to high loadings of dairy waste, which is known to contain high concentrations of fats and oils (Perle et al. 1995).

Lower percentages were found in all other reactors, comprising between 8.91% in R1 and 15.7% in R2. In these reactors, mainly Alpha- (<65% of *Proteobacteria* reads in R4b) and Deltaproteobacteria (<40.0 of *Proteobacteria* reads in R3b) were detected (Fig. 2). Results underline the operational changeover between different modi in plant 3.

Mixed results have been published by other authors on the impact of co-substrate addition to microbial communities (Mata-Alvarez et al. 2014). According to literature, the introduction of co-substrates tends to increase the microbial diversity (Supaphol et al. 2011; Jensen et al. 2014), however Sundberg et al. (2013), who evaluated 14 full-scale co-digestion plants, reported that some co-digestion reactors revealed a lower bacterial diversity in comparison to SS digesters. Supporting the findings of Jensen et al. (2014) and Supaphol et al. (2011), sludge from the AD R2 using no co-substrate revealed slightly lower numbers of OTUs (717) and a lower Shannon Index (3.66) in comparison to R1 (871 and 4.3), R3b (845 and 4.02), R4a (912 and 4.3), R4b (838 and 4.2) and R5 (756 and 3.62). However, sludge R3a revealed even lower values of 670 and 2.82, respectively, probably as a result of operational changeover.

However, microbial diversity was shown not to be a critical indicator for a functionally successful anaerobic microbial community (Dearman et al. 2006; Carballa et al. 2011).

Many bacterial groups are capable to degrade a wide range of macromolecules, such as cellulose, pectin, chitin, proteins and various xenobiotic compounds, and thus can quickly react to substrate changes (Chouari et al. 2003). These generalists can potentially perform similar ecological functions and this functional redundancy ensures that even with changing environmental conditions, the ecosystem will stay functional, because its functional niches are occupied. Thus, regardless of competition for...
resources and space between species, ecosystem functioning is not directly dependent on the overall community structure in terms of diversity (Briones & Raskin 2003). In fact, community evenness (Wittebolle et al. 2009) and adequate dynamics of a bacterial community or flexibility to adapt to changing environmental conditions (Miura et al. 2007) are more important for ensuring stable reactor performance. All 

Archaea detected by next generation sequencing belonged to the phylum Euryarchaeota (Fig. 3). Every reactor analysed in this study contained predominantly acetoclastic as well as a number of hydrogenotrophic Archaea. This configuration was described earlier by Leclerc et al. (2004) and could resemble the ‘minimum Archaea microflora needed for stable functioning of the anaerobic digester’.

Presented in relative abundances, sequences were assigned to genera in the classes Methanomicrobia (77.4 ± 18.8%), Thermoplasmata (6.9 ± 4%) and Methanobacteria (ranging from 0.23% in R4a to 41.8% in R3b). Amongst the hydrogenotrophic Euryarchaeota, abundances > 2% were found for two genera: Methanobacterium, which was detected at levels >10 times higher in reactors R1 (21%), R3a (38%) and R3b (41.1%) compared to other reactors, especially those co-fed with biowaste, and Methanospirillum, present in all digested samples, and accounting for 0.41% in R4b to 11.9% in R1.

The strictly acetoclastic genus Methanosaeta was found to be predominant in all reactor samples, accounting for 47.7% in R3a to 95.4% in R4b. Moreover, Methanosarcina, an acetoclastic that can also grow on C1 compounds like CO₂ as well as H₂, did not reach an abundance > 1% in any of the sludge samples. The authors propose acetate utilization as the main pathway in all reactors, performed by Methanosaeta species. A high-affinity adenosine monophosphate (AMP)-forming acetyl-CoA synthetase enables Methanosaeta to outcompete Methanosarcina in environments with low acetate concentrations, such as AD sludge in digestion towers.

These findings are in line with results of Sundberg et al. (2013), Rivière et al. (2009), Walter et al. (2012), Franke-Whittle et al. (2014) and others. Methylo trophic genera showed an abundance < 1%. The results are also in line with DGGE banding patterns, revealing low richness and a high homogeneity in the archaeal communities in the different sludges, in contrast to the bacterial communities. Moreover, results indicate no relationship between archael community structure and the type of substrate digested, a finding also observed by Leclerc et al. (2004), after mapping the archaeal populations of 44 anaerobic digesters in 8 different countries.

**Conclusion**

Although AD has received much attention in the past few decades, the process is still far from being fully understood. Next generation sequencing holds enormous potential to uncover composition and dynamics of complex microbial communities. In this study, a combination of robust DGGE and Illumina MiSeq sequencing revealed a complex bacterial community structure responsive to the type of co-substrate. Regarding Archaea, despite different co-substrates and an overall dominance of Methanosaeta, a high similarity between reactors was detected. Methanogens rely on the metabolic end products of bacterial activity and are thus less dependent on differences in the initial co-substrate regime. Bacterial community composition was dependent on the co-substrate used in AD, whilst archael communities were uniform across all reactors. Even reactors R3a and R3b, sampled in an operational changeover of serial to parallel mode, revealed stable physicochemical properties. According to the DGGE and 16S rRNA gene amplicon sequencing results of this

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**Fig. 3.** Archaeal community composition (reads) at the genus level in reactors R1 to R5. [Colour figure can be viewed at wileyonlinelibrary.com]
study, richness within a bacterial core community ensured reactor functionality, whilst feeding regime and reactor type had an impact on overall and core bacterial diversity.

To which extent microbial community composition could be used to predict reactor performance remains to be investigated.

**Competing interests**
The authors declare that they have no competing interests.

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**Conflict of interest**
We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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