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Archaeal community composition affects the function of anaerobic co-digesters in response to organic overload

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ABSTRACT

Microbial community diversity in two thermophilic laboratory-scale and three full-scale anaerobic co-digesters was analysed by genetic profiling based on PCR-amplified partial 16S rRNA genes. In parallel operated laboratory reactors a stepwise increase of the organic loading rate (OLR) resulted in a decrease of methane production and an accumulation of volatile fatty acids (VFA). However, almost three-fold different OLRs were necessary to inhibit the gas production in the reactors. During stable reactor performance, no significant differences in the bacterial community structures were detected, except for in the archaeal communities. Sequencing of archaeal PCR products revealed a dominance of the acetoclastic methanogen *Methanosarcina thermophila*, while hydrogenotrophic methanogens were of minor importance and differed additionally in their abundance between reactors. As a consequence of the perturbation, changes in bacterial and archaeal populations were observed. After organic overload, hydrogenotrophic methanogens (*Methanospirillum hungatei* and *Methanoculleus receptaculi*) became more dominant, especially in the reactor attributed by a higher OLR capacity. In addition, aggregates composed of mineral and organic layers formed during organic overload and indicated tight spatial relationships between minerals and microbial processes that may support de-acidification processes in over-acidified sludge.
Comparative analyses of mesophilic stationary phase full-scale reactors additionally indicated a correlation between the diversity of methanogens and the VFA concentration combined with the methane yield. This study demonstrates that the coexistence of two types of methanogens, i.e. hydrogenotrophic and acetoclastic methanogens is necessary to respond successfully to perturbation and leads to stable process performance.

Keywords methanogens, biogas, VFA, OLR, anaerobic digester

1. Introduction

Anaerobic degradation of organic wastes and by-products from agriculture and the food industry to produce CO_2 and CH_4 and the utilization of the produced biogas as an alternative energy source are common solutions for decentralized waste management. In practice, it became clear that co-digestion of multiple substrates provides an opportunity to improve yields of anaerobic digestion of solid wastes. For this, a major amount of a main basic substrate (e.g. manure or sewage sludge) is mixed and digested together with minor amounts of a single, or a variety of additional substrate such as energy crops or silage (Braun, 2002). This type of waste treatment is still increasingly supported and requested by many European governments, as biogas is regarded as an environmentally friendly energy and an alternative to conventional greenhouse gas emitting fossil fuels (BMU, 2009). Therefore, professional collection of produced biogases and preparation of digested residues are preconditions for an efficient utilization of this energy source.

Comprehension of this complex process is necessary to improve the efficiency and process stability of biogas plants. Knowledge of the microbial aspects of the co-digestion of sewage sludge and various co-substrates is still incomplete despite several studies focused on bacteria and methanogenic archaea (Ahring, 1995a; Fernandez et al., 1999; Karakashev et al., 2005; Leclerc et al., 2004; McHugh et al., 2003). As reasons can be quoted the complex multi-step process of biogas production involves several different physiological groups, the complex and plant-dependent diverse composition of digestion materials, and individual plant parameters like temperature, hydraulic retention time (HRT), mixing, and pH. To avoid an organic overload and a subsequent collapse of gas production that requires long recovery periods or the total exchange of reactor matter, reactors are usually not operated at their maximum organic loading rate (OLR). Thus, biogas plants receiving various types of solid organic wastes have unused capacities, and a better understanding of microbial function could result in increased gas yield.

The paramount importance of methanogens in the anaerobic digestion process was emphasized in several studies focused on nutrient availability, conversion rates, and environmental parameters, such as operating temperature, ammonia levels, volatile fatty acid (VFA) concentrations, and substrates (Ahring et al., 2001; Boone and Xun, 1987; Karakashev et al., 2005; Leclerc et al., 2004). Investigations focused on the impact and activity of methanogens in the degradation of sewage sludge and co-substrates like glucose or organic wastes from abattoirs or food industries were mostly conducted under OLR steady state conditions or single substrate shock loads (Dearman et al., 2006; Fernandez et al., 2000; Karakashev et al., 2005; McHugh et al., 2003). Only a few studies investigated the dynamics of acetogenic and hydrogenotrophic methogens, provoked by organic overload and long-term accumulation of VFA in mesophilic and thermophilic laboratory-scale reactors, respectively (Delbès et al., 2001; Hori et al., 2006; Blume et al., 2010). However, those investigations were conducted for digesters running on mono-type substrates and not for co-digestion.

This study investigates the effects of increased OLR provoked by shock loads of rape oil and resulting in process failure in two laboratory-scale sewage sludge reactors, in reference to microbial activity resulting in the production of biogas and dynamics in microbial community structure. Rape oil was chosen as co-substrate, because of its long chain fatty acids that are frequently found in real wastewaters (Komatsu et al., 1991). According to Galbraith et al. (1971), long chain fatty acids inhibit gram positive bacteria (e.g. *Clostridia*). As these microorganisms are common in anaerobic digesters (e.g. Grady et al., 1999), this inhibition is favourably for the provocation of process instability by shock loads.

Furthermore, three full-scale plants were analysed with regard to operational and chemical characteristics and their microbial community structures within the sludge. The bacterial and methanogenic archaeal diversity was determined by genetic fingerprinting based on partial 16S rRNA genes from the DNA directly extracted from the reactors. PCR-SSCP (Single Strand Conformation Polymorphism) was chosen as profiling technique, allowing direct comparison of the community composition from different samples and the identification of differences by DNA sequencing and phylogenetic analyses (Schwieger and Tebbe, 1998; Dohrmann and Tebbe, 2004). Additionally scanning electron microscopic analyses with energy dispersive spectrometer (SEM-EDS) were done to investigate composition of aggregates formed during acidification in laboratory-scale reactors.

2. Materials and Methods

2.1 Laboratory-scale reactors

Two laboratory-scale reactors were filled with digested sludge from a full-scale plant (R1, Tab. 1) operated with organic waste and sewage sludge at 43°C with an interval of two months. The fermenters possessed a working volume of 23 litres. They were single stage fermenters and supposed to be completely mixed. For the continuous mixing, a pneumatic system was used. A membrane vacuum pump (KNF N86KTE) withdraws a part of the produced biogas of the reactors` gas-phase and pumped it into the liquid-phase at the bottom of the reactor (Fig. 1). The pipelines for the gas circulation were kept free of condensates by different facilities such as a condensate collector and a drying tower.

In contrast to reactor R1, from which the inoculum originated, the reactors were operated thermophilic at 50°C, because a thermophilic process is more sensitive to failures. The reactors were run with a start-up organic loading rate (OLR) of 2.5 kg total volatile solids (TVS) m^{-3} d^{-1} (data not shown). After 110 days, the OLRs of both reactors were increased to 4.5 kg TVS m^{-3} d^{-1} (normal level) within 32 days. The outlet for the digested sludge was located in the middle at the bottom of the reactor. At normal level, once a day, one litre of digested sludge was withdrawn at the outlet. This matter was used for chemical and microbiological analyses. The substrates were manually put into the reactor at its top. Each day, one litre of sewage sludge (TVS: ~30 g l^{-1}, pH 6.1) and 72.5 ml of rape oil were fed after the digested sludge had been withdrawn. The hydraulic retention time (HRT) in the reactors was 21 days.

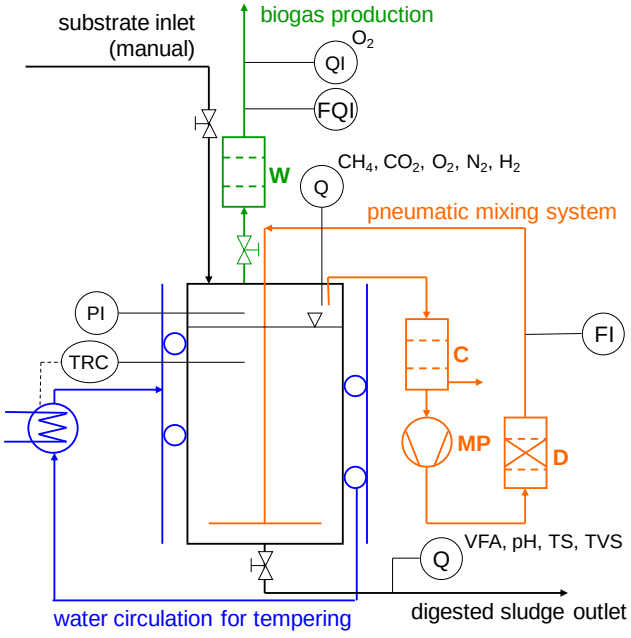

Fig. 1 Scheme of laboratory-scale reactor. bold: Wash bottle (W), condensate collector (C), membrane pump (MP), drying tower (D); normal: quality (Q, if first letter), quantity (Q, if following letter), indicating (I), flow (F), controlling (C), pressure (P), temperature (T), recording (R)

Table 1 Operation parameters of full-scale reactors

Reactor	Operating temperature [°C]	Digestion materials	OLR [kg TVS m^{-3} d^{-1}]	HRT [d]	VFA [mg l^{-1}]	CH_4-yield [m^3(kg TVS)$^{-1}$]
Semi-continuously stirred reactor (R1)	43	organic waste (68% TVS), sewage sludge (22% TVS), grease (10% TVS)	3.2	19	500	0.56
Plug flow reactor (R2)	37	leftovers (41% TVS), liquid manure and crop (59% TVS)	5.6	12	3.000	0.34
Plug flow reactor (R3)	37	leftovers (41% TVS), liquid manure and crop (59% TVS)	5.6	12	11.000	0.28

TVS total volatile solids, *OLR* organic loading rate, *HRT* hydraulic retention time, *VFA* volatile fatty acids

After several months of stable reactor operation at normal level, the OLR of the reactors was increased per diem by applying multiples of the daily oil charge until gas production decreased significantly, indicating process failure (Tab. 2). Even though the OLR of sewage sludge was also slightly increased during that time by a factor

of 1.38, however, the organic loading rate of rape oil was increased by a factor of almost 12 from the "normal" to the maximal level. Thus, the organic shock load consisted mainly of rape oil.

Table 2 Organic loading rates and hydraulic retention time of laboratory-scale reactors

Sewage sludge + rape oil	Sewage sludge		Rape oil		
OLR [kg TVS $(m^3\ d)^{-1}$]	OLR [kg TVS $m^{-3}\ d^{-1}$]	V [ml]	OLR [kg TVS $m^{-3}\ d^{-1}$]	V [ml]	HRT [d]
2.5 (start-up)	0.3	250	2.2	50	76
4.5 (normal level)	1.3	1,000	3.2	72.5	21
11.0 (S1)	1.5	1,000	9.5	218	19
14.3 (S2)	1.6	1,200	12.7	290	18
17.5 (S3)	1.7	1,200	15.8	362	17
27.3 (S4)	1.9	1,200	25.4	580	14
40.3 (S5)	2.1	1,500	38.2	870	12

OLR organic loading rate, *TVS* total volatile solids, *V* volume, *HRT* hydraulic retention time

Subsequent nomination of days bases on the conducted experiment. The previous phase of stable reactor operation lasting several months was not considered in the figure. The OLRs were increased as follows: reactor 1: day 13 (OLR 11.0 kg TVS $m^{-3}\ d^{-1}$) and at day 15 (OLR 14.3 kg TVS $m^{-3}\ d^{-1}$) and reactor 2: day 15 (OLR 14.3 kg TVS $m^{-3}\ d^{-1}$), day 17 (OLR 11.0 kg TVS $m^{-3}\ d^{-1}$), day 19 (OLR 14.3 kg TVS $m^{-3}\ d^{-1}$), day 21 (OLR 17.5 kg TVS $m^{-3}\ d^{-1}$), day 26 (OLR 27.3 kg TVS $m^{-3}\ d^{-1}$), day 28 (OLR 40.3 kg TVS $m^{-3}\ d^{-1}$). Between subsequent shock loads, the OLR was again 4.5 kg TVS $m^{-3}\ d^{-1}$. Table 2 presents the composition of sewage sludge and rape oil at different values of the OLR. After the provoked process failure, organic substrates were further supplied for three days at normal level (OLR 4.5 kg TVS $m^{-3}\ d^{-1}$). Afterwards, no substrates were charged in reactor 2, indicating the end of the experiment. To analyse the effects of restarting the substrate charge, the substrate input was restarted with an OLR of 4.5 kg TVS $m^{-3}\ d^{-1}$ in reactor 1, 13 days after the provoked process instability.

Gas production and composition, pH, and the total and specific concentrations of VFAs were analysed once or twice a day, depending on reactor performance. The gas production was measured with a gas meter (Ritter, Bochum, Germany). The gas composition was determined by gas chromatography (SRI 8610C; SRI Instruments, Torrance, USA), and the concentrations of the single VFAs were determined by ion chromatography (DIONEX ICS 3000, CA, USA). The total concentration of VFAs was quantified photometrical (Dr. Lange LCK 365, Hach Lange GmbH, Düsseldorf, Germany).

For microbial community analyses, sludge samples were taken in intervals of two or ten days over a period of 45 days (reactor 1: day 1, 3, 7, 9, 16, 25, 35, 42; reactor 2: day 7, 8, 9, 11, 20, 29, 36, 45). Sludge samples (70 g wet weight) were centrifuged twice at 10,000 x g (Eppendorf 5804, Hamburg, Germany), and pellets were aliquoted and stored at -75°C until further analysis.

2.2 Full-scale reactors

Three mesophilic full-scale plants, of two different construction types were chosen for the analyses: one semi-continuously stirred tank reactor (R1, Tab. 1) and two plug flow reactors (R2, R3, Tab. 1). For the semi-continuously stirred tank reactor, the substrates were organic waste such as expired foodstuff (TVS: 61 g l^{-1}, pH 4.2; OLR: 2.2 kg TVS $m^{-3}\ d^{-1}$), sewage sludge (TVS: 29 g l^{-1}, pH 6.1; OLR: 0.7 kg TVS $m^{-3}\ d^{-1}$), and grease (TVS: 58 g l^{-1}, pH 6.2; OLR: 0.3 kg TVS $m^{-3}\ d^{-1}$), while crops (wheat) mixed in liquid cow manure (TVS: 60 g l^{-1}, pH 6.9; OLR: 3.3 kg TVS $m^{-3}\ d^{-1}$), and leftovers originating from restaurants (TVS: 185 g l^{-1}, pH 4.6; OLR: 2.3 kg TVS $m^{-3}\ d^{-1}$) were charged into the plug flow reactors.

R1 was chosen for sampling, because the digested sludge of R1 was used as inoculum for the laboratory experiments. Compared to R1, Reactor R2 and R3 differed in type and substrates, whereby R2 performed well and R3 produced not as much biogas as expected.

A volume of 1 litre sludge of each full-scale plant was withdrawn at the outlet of the fermenter for the digested sludge and was taken for the chemical analyses and the determination of the microbial composition. Sludge samples (70 g wet weight) were centrifuged twice at 10,000 x g (Eppendorf 5804, Hamburg, Germany), and pellets were aliquoted and stored at -20°C until further analysis.

2.3 DNA extraction and PCR amplification of partial 16S rRNA genes

Genomic DNA was extracted from centrifuged sludge samples with the FastDNA® SPIN Kit for Soil (Q-BIOgene, Carlsbad, USA). For genetic profiling, partial sequences of the 16S rRNA genes were amplified by PCR. The primer sets used in this study are listed in Table 3. The primer set com1/com2-ph was used to amplify the bacterial 16S rRNA gene. The archaeal 16S rRNA was amplified by the primer set w039-ph/w036. The PCR reactions were performed using the universal or archaea-specific primers with PCR cycling conditions as follows: initial denaturation at 95°C for 15 min; 30 cycles, each consisting of denaturation for 1 min at 94°C, primer annealing at 50°C for 1 min, and extension for 70 sec at 72°C, with a final extension at 72°C for 5 min. All PCR reactions were carried out in a total volume of 100 µl containing 4 µl of extracted DNA (1:100 dilution of original DNA solution), 0.2 mM each deoxynucleoside triphosphate (Qbiogene, Heidelberg, Germany), 0.5 µM of each primer (MWG Biotech, Ebersbach, Germany), and 1.25 U Taq polymerase (Hotstar Taq, Qiagen) with the corresponding 1X PCR buffer containing 1.5 mM $MgCl_2$. For each sample, three independent 100 µl PCR reactions were pooled to minimize PCR variability. All PCR amplifications were conducted in a Primus 96 thermocycler (MWG Biotech, Ebersbach, Germany). The size and yield of PCR products were analysed by electrophoresis in a 1% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}).

Table 3 Primers used for amplification of 16S rRNA gene fragments

Primer	Sequence	Specificity	Position[1]	Reference
com1	CAG CAG CCG CGG TAA TAC	SSU rDNA *Bacteria*	F519	Schwieger and Tebbe 1998
com2-ph	CCG TCA ATT CCT TTG AGT TT	SSU rDNA *Bacteria*	R926	Schwieger and Tebbe 1998
w036	TCC AGG CCC TAC GGG G	SSU rDNA *Archaea*	F333	Leclerc et al. 2001
w039-ph	CTC CCC CGC CAA TTC CT	SSU rDNA *Archaea*	R915	Leclerc et al. 2001

[1] position according the 16S rDNA of *E.coli*, *ph* phosphorylated, *F* forward primer, *R* reverse primer

2.4 Genetic profiling by single strand conformation polymorphism (SSCP) and DNA-sequence analyses

The SSCP analysis was carried out according to the guidelines of Schwieger and Tebbe (1998) and Dohrmann and Tebbe (2004). Briefly, partial 16S rRNA genes of the microbial communities were directly amplified from total DNA by PCR using universal and archaea-specific primers as described above. After subsequent single strand removal, the single strand PCR products were separated electrophoretically in non-denaturing polyacrylamide gels (MDE, FMS Bioproducts) in a Macrophor electrophoresis unit (Amersham Bioscience, Freiburg, Germany) at a temperature of 20°C for 17 h at 400 V (Bacteria) and 600 V (Archaea). The SSCP profiles were visualized by silver staining (Bassam et al., 1991).
DNA sequencing from bands of SSCP gels was performed as described by Dohrmann and Tebbe (2004) and included cutting out the bands of interest, elution by the crush and soak procedure, PCR of single strand DNA, ligation of the PCR products into the pGEM vector, and transformationto *E.coli* competent cells. Additionally, DNA of clones with vector inserts was amplified by PCR and the single strand DNA was separated again by electrophoresis and compared with the original SSCP community profile. In case of identical profiles, the plasmid DNA of the corresponding sample was further analysed by DNA sequencing with the SequiTherm EXEL™//DNA Sequencing KIT-LC (Epicenter Technologies, USA) and a DNA-Analyser Gene Readir 4200 (MWG Biotech, Ebersbach, Germany). The sequencing results were examined with the program IMAGE IR "Consed". Sequences were aligned using the software package ARB (http://www.arb-home.de), and the nucleotide sequences were compared with the Basic Local Alignment Search Tool (BLAST) function of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences analysed in this study have been deposited in the EMBL database of the European Bioinformatics Institute (www.ebi.ac.uk/embl) under the accession numbers GQ483472 to GQ483502.

2.5 Mineralogical analyses

Four weeks after organic overload laboratory scale reactors were opened and aggregates of different size, ranging between 0.5 and 2 cm in diameter, were found in the sludge. Scanning Electron Microscope (SEM) Ultra 55 Plus (Carl Zeiss SMT) with energy dispersive spectrometer EDS (with analytical system NSS) was used to determine the chemical composition and the morphology of aggregates that formed in laboratory scale reactors

due to organic overload. Aggregates and their fragments used for analysis were carbon coated and the Back Scattered Electron (BSE) technique was used in order to determine spatial relationship between sample components and microorganisms, and to identify elements and detect changes in composition within the aggregates.

3. Results

In this study, the effects of organic shock loads of rape oil in two thermophilic laboratory-scale co-digesters were analysed. Gas production, gas composition, and the concentration of volatile fatty acids (VFAs) were used to indirectly quantify the activity of the microbial community in producing biogas and indicating process failure, respectively. Changes in bacterial and archaeal community structures were investigated by genetic fingerprinting (PCR-SSCP). Aggregates that formed during organic overload were investigated using SEM and BSE analyses in order to determine the aggregate structure with respect to the spatial relationship between sample components and microorganisms. Additionally, three mesophilic full-scale reactors were studied to correlate the microbial community structure with plant parameters such as methane yield and concentration of volatile fatty acids.

3.1 Laboratory-scale reactors

Two laboratory-scale reactors inoculated with the sludge from a full-scale plant were operated for two months at 50°C with a daily organic loading rate (OLR) of 4.5 kg TVS m^{-3} d^{-1} (normal level) after start-up using rape oil and sewage sludge as substrates. During stable reactor performance at normal OLR level (data subset shown for reactor 1: day 1-12 and reactor 2: day 1-14, Fig. 2), the gas production rate (GPR), pH, and the total and single concentrations of VFAs coincided in both reactors with average values of GPR 4.5 l h^{-1}, pH 7.2 and 370 mg l^{-1} VFA. Low concentrations of acetate were quantified with an average value of 30 mg l^{-1}. The gas composition was equal for both reactors (CH_4 ~73 Vol % / CO_2 ~20 Vol %, data not shown).

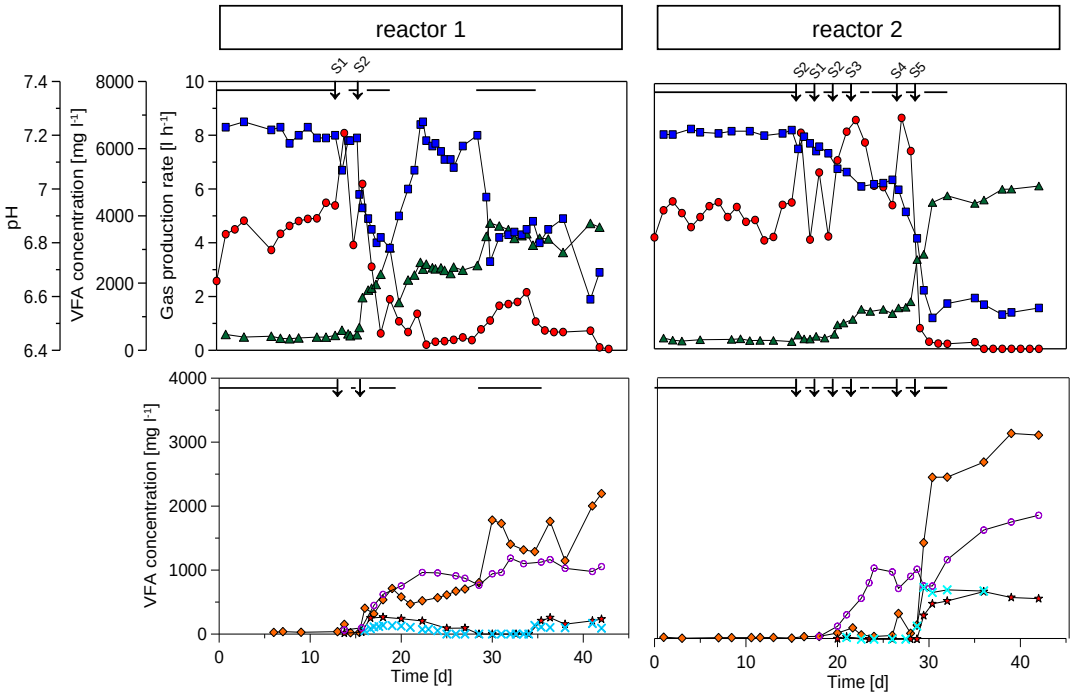

Fig. 2 Monitored gas production, pH, concentration of VFA (sum parameter) and concentration of single VFAs before and after organic overload in two thermophilic laboratory-scale reactors. *OLR* normal level (—) 4.5 kg TVS m^{-3} day^{-1}, shock loads (↓) *S1* 11.0 kg TVS m^{-3} day^{-1}, *S2* 14.3 kg TVS m^{-3} day^{-1}, *S3* 17.5 kg TVS m^{-3} day^{-1}, *S4* 27.3 kg TVS m^{-3} day^{-1}, *S5* 40.3 kg TVS m^{-3} day^{-1}. Gas production rate (red filled circle), pH (blue square), total concentration of VFA (green triangle), concentration of specific volatile fatty acids: acetate (orange diamond), propionate (purple circle), butyrate (red asterix), valerate (light blue cross)

As a consequence of a three-fold increased OLR at day 15 subsequent to an initial GPR increase, the production rate decreased drastically in reactor 1. However, an almost three-fold higher OLR was necessary for the collapse in gas production to less than 1.0 l h^{-1} in reactor 2 (Fig. 2). Along with the collapse in gas production in both reactors, the methane content in the gas in reactor 1 and 2 decreased until day 18 and day 30, respectively, from 73 Vol % to 68 Vol % and the carbon dioxide content increased from 20 Vol % to 28 Vol % on average. Hydrogen became detectable and increased to 0.3 Vol % and 0.05 Vol % in reactor 1 and reactor 2, respectively (data not shown). After the overload, the concentration of VFA increased rapidly to approximately 2,000 mg l^{-1} in reactor 1 and 5,800 mg l^{-1} in reactor 2, and resulted in a decrease in pH from 7.2 to 6.8 and 6.5, respectively. The short chain fatty acids consisted mainly of acetate and propionate (Fig. 2). In reactor 1, acetate and propionate increased in parallel to the same concentration level (720 mg l^{-1}) until day 19. When the GPR reached its minimum at 0.2 l h^{-1} on day 22, the propionate concentration was almost double as high as the acetate concentration at 520 mg l^{-1}. In reactor 2, however, the acetate concentration remained low at 40 mg l^{-1}, while propionate amounted to 1,090 mg l^{-1}. On day 30, when the substrate input was restarted at reactor 1 and another shock load was charged to reactor 2, higher concentrations of acetate than of propionate were detected in both reactors. Butyrate and valeriate became detectable, but remained at low concentrations for reactor 1. In reactor 2, butyrate and valeriate increased in parallel on day 30 to 730 mg l^{-1} and 840 mg l^{-1}, respectively.
The restarted oil and sludge supply in reactor 1 at day 28, 13 days after process failure, induced a further increase in VFA concentration up to 3,800 mg l^{-1}, a decrease in pH to 6.7, and a slightly increased gas production rate of up to 2.0 l h^{-1}.

3.2 Microbial community structures in laboratory-scale reactors

To understand which organisms contribute to the microbial communities in the reactors, genetic SSCP profiles based on PCR-amplified partial 16S rRNA genes were generated from DNA extracted from sludge samples of the reactors. During the stable performance period (data subset shown for reactor 1: day 1-12 and reactor 2: day 1-14), the analysis of the bacterial and archaeal community structures revealed a high level of consistency of the dominant bands in both reactors with slight differences in band intensities (Fig. 3).

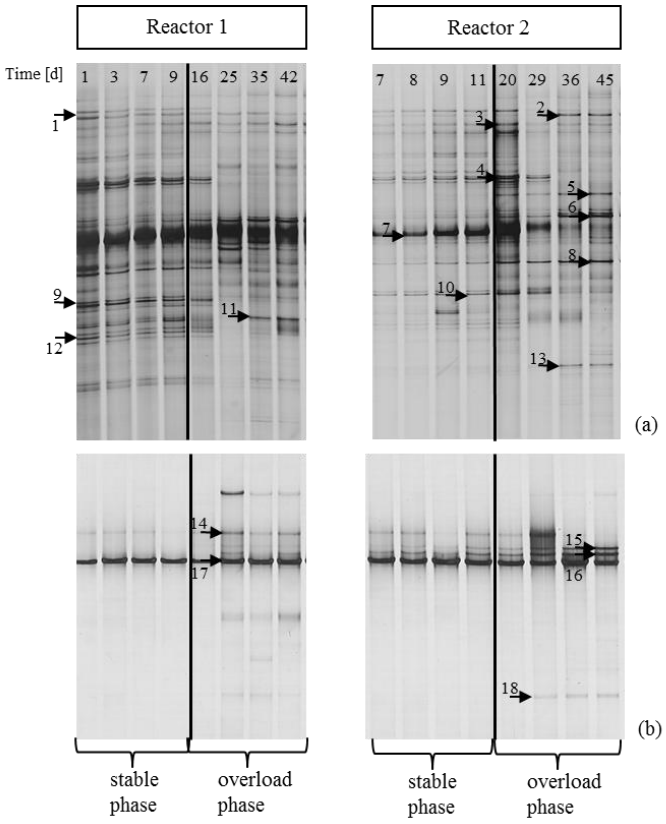

Fig. 3 SSCP profiles of bacterial (a) and archaeal (b) communities in two laboratory-scale reactors during stable phases (left lanes) and after organic overloads (right lanes). The timeline indicates sludge samples taken for SSCP analysis during sample periods. Arrows indicate the positions of bands that were sequenced.

The bacterial profiles were comprised of about 25 bands, while the archaeal profiles showed less diversity with up to 6 dominant bands and a higher variability between reactor 1 and reactor 2. DNA sequencing of the dominant bands of the bacterial SSCP profiles revealed the presence of *Clostridia*, *Bacteroidetes*, and *Actinobacteria* with 94 to 100% identity to sequences in the GenBank database (Table 4). The predominant DNA sequence in the archaeal profiles of both reactors before the organic overload was attributed to *Methanosarcina thermophila* (band 17, 99 % sequence similarity) and to a moderate extent to *Methanobacterium* clone KT27 (band 14, 99 % sequence similarity) (Table 4). In reactor 2, two additional bands were detected with 98 and 99 % identities to sequences of the genus *Methanospirillum* (bands 15 and 16). The increase of the daily oil rate resulted in changes in the bacterial community structures in both reactors. In the course of the overload, some bands, corresponded to *Clostridia*, *Bacteroidetes*, and *Actinobacteria*, gradually disappeared (bands 1, 4, 7, 9, 10, and 12), while others intensified (band 6) or appeared (band 5). Additional changes were reactor specific (band 2, 3, 8, 11, and 13) and characterized by non-uniform band disappearance, intensification, or consistency.
In the archaeal profiles, one band appeared in both reactors due to the organic overload (band 18). The sequence was affiliated to a sequence related to *Methanoculleus receptaculi*. In contrast to reactor 1, the two bands related to members of *Methanospirillum* were clearly intensified in the SSCP profile of reactor 2 (bands 15 and 16).

Table 4 Phylogenetic affiliation of partial *Bacteria* and *Archaea* 16S rRNA gene sequences recovered from SSCP profiles with DNA extracted from sludge of laboratory-scale reactors

Band	Domain	Class	Family	Closest relative (GenBank accession number)	Similarity [%]	GenBank accession number
1	*Bacteria*	*Clostridia*	*Family XVIII. Incertae Sedis*	uncultured bacterium, clone QEDP1CE01 (CU924683)	97	GQ483472
2			*Family XVIII. Incertae Sedis*	uncultured bacterium, clone QEDP1CE01 (CU924683)	100	GQ483473
3			*Thermoanaerobacteraceae*	uncultured bacterium, clone AP-B-13 (AB260006)	94	GQ483474
4			*Syntrophomonadaceae*	uncultured bacterium, clone FB13 (HQ403024)	98	GQ483475
9			*Clostridiaceae*	uncultured bacterium, clone MOL5 (AB232571)	97	GQ483480
13			*Family XI. Incertae Sedis*	*Sporanaerobacter acetigenes* , strain DSM 13106 (GQ461827)	99	GQ483484
7		*Bacteroidetes*	*Marinilabiaceae*	uncultured bacterium, clone PPf50E2 (AY548787)	100	GQ483478
10			*Cytophagaceae*	uncultured bacterium, clone AHU 21 (AB092906)	100	GQ483481
11			*Cryomorphaceae*	uncultured bacterium, clone R1-4 (EU009350)	97	GQ483482
6		*Actinobacteria*	*Intrasporangiaceae*	uncultured bacterium, clone Ebpr20 (AF255628)	99	GQ483477
5		*Bacteria*		uncultured OP8 bacterium, clone QEEB2CE02 (CU918277)	100	GQ483476
8				uncultured bacterium, clone B_98 (EF029339)	99	GQ483479
12				uncultured WWE1 bacterium, clone QEDQ2AE08 (CU923265)	98	GQ483483
14	*Archaea*	*Methanobacteria*	*Methanobacteriaceae*	*Methanobacterium formicicum* strain Mb10 (JN205061)	99	GQ483485
15		*Methanomicrobia*	*Methanospirillaceae*	*Methanospirillum hungatei* -like archaeon (AB517987)	98	GQ483486
16			*Methanospirillaceae*	uncultured *Methanospirillum* sp., clone DI_G07 (AY454788)	99	GQ483487
17			*Methanosarcinaceae*	*Methanosarcina thermophila* -like archaeon (NR_044725)	99	GQ483488
18			*Methanomicrobiaceae*	*Methanoculleus receptaculi* -like archaeon (NR_043961)	99	GQ483489

3.3 Formation of multi-layered aggregates

During the organic overload aggregates of different size, ranging between 0.5 and 2 cm in diameter, were formed in both laboratory-scale reactors (Fig. 4).

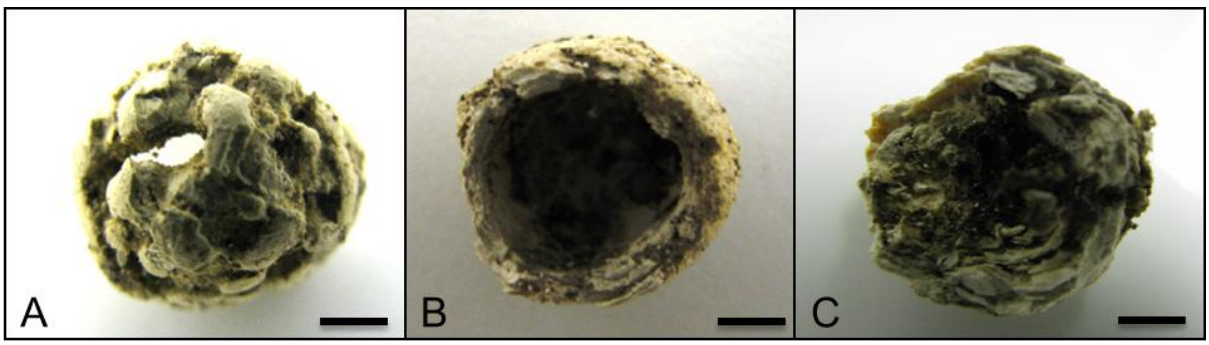

Fig. 4 Aggregates that formed during the organic overload in laboratory-scale reactors. (A) Exterior view of aggregate surface, (B) Opened aggregate with a hollow core, (C) Opened aggregate with a filled core. (scale: 0.5 cm)

Aggregates were still detected four weeks after organic overload, while during that time pH had increased to neutral level (pH 7.38) and the VFA concentration had decreased significant from > 6,000 mg l^{-1} to 138 mg l^{-1}. The aggregates shaped as irregular spheres had a central core that was made of organic matter mixed with crystalline phases like vivianite ($Fe_3(PO_4)_2 \cdot 8H_2O$), K-feldspar ($KAlSi_3O_8$), plagioclases ($NaAlSi_3O_8$ - $CaAl_2Si_2O_8$), and quartz (SiO_2). These phases were surrounded by layers (characteristic lamination), composed of long chain fatty acids which bond Ca (Al), Ca (Mg, Fe) or aluminium phosphate ($AlPO_4$). Figures 4 und 5 present images produced with digital camera and scanning electron microscope (SEM) showing the aggregates surface and the materials detected in the aggregates, like silicified seeds, vivianite crystals, as well as filamentous and curved rod bacteria.

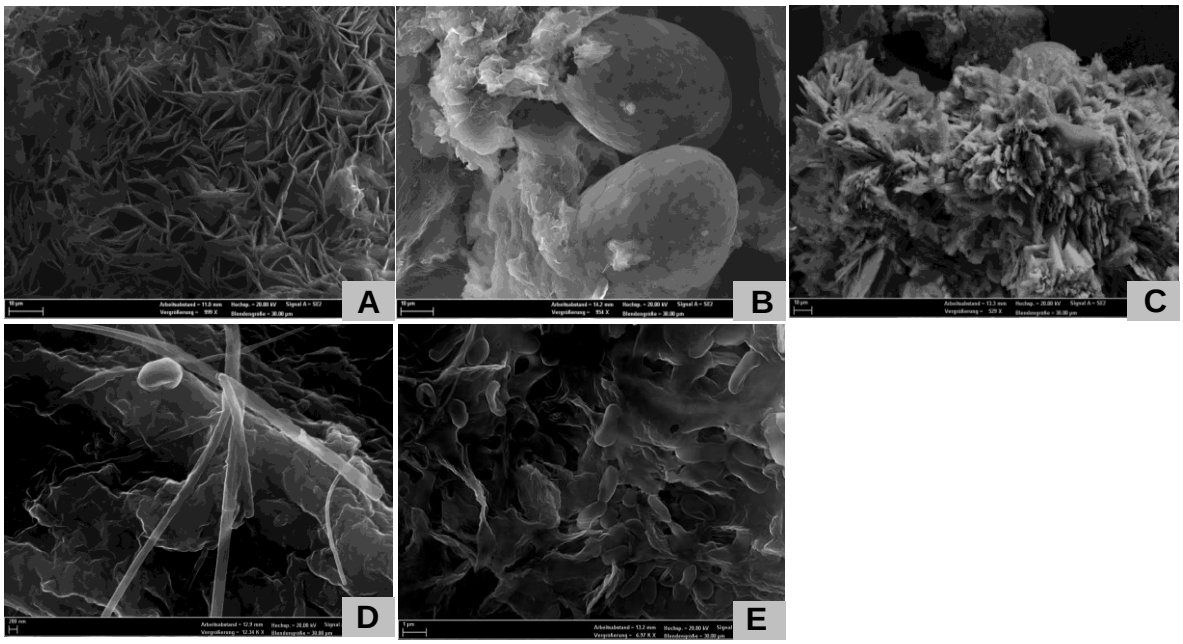

Fig. 5 SEM images of aggregate surface (A) and materials detected in the aggregates. (B) silicified seeds, (C) vivianite crystals ($Fe_3(PO_4)_2 \cdot 8H_2O$) present in the central part of the sample (small aggregates), (D) filamentous structures and curved rods (E) found in the samples.

3.4 Full-scale reactors

For the comparison of the laboratory-scale reactor performance with full-scale biogas plants, representative samples of one semi-continuously stirred tank reactor (R1) and two plug flow reactors (R2, R3) were analysed. Despite being operated by the same OLR of 5.6 kg TVS m^{-3} d^{-1} and calculated HRT, the two plug flow reactors contained different VFA concentrations of 3,000 and 11,000 mg l^{-1}, respectively (Tab. 1). The concentration of VFA in the semi-continuously stirred tank reactor (R1) was significantly lower (500 mg l^{-1}) and the methane yield was 0.56 m^3 $(kg\ TVS)^{-1}$, almost two-fold higher than in the plug-flow reactors.

3.5 Microbial community structures in full-scale reactors

The analysis of the bacterial and archaeal community structure in sludge samples from the semi-continuously stirred tank reactor (R1) and the plug flow reactors (R2, R3) showed distinctive profiles (Fig. 6). The bacterial community profiles were comprised of up to 25 dominant bands that differed among the three reactors in abundance and intensity. Results of sequencing revealed a dominance of members of *Clostridia* and *Bacteroidetes* with 85 to 99 % identity to sequences in the GenBank database (Tab. 5). In accordance with the bacterial profiles, the archaeal community patterns differed among the three reactors, but showed a minor diversity comprising up to 15 bands. For archaea, the most dominant band in all reactor profiles was affiliated to *Methanosaeta concilii* (bands 10 and 13, 99 % sequence similarity). In the semi-continuously stirred reactor, *Methanobacterium* and *Methanobrevibacter smithii*-like sequences represented dominant bands that were not seen in the plug flow reactors (bands 11 and 12).

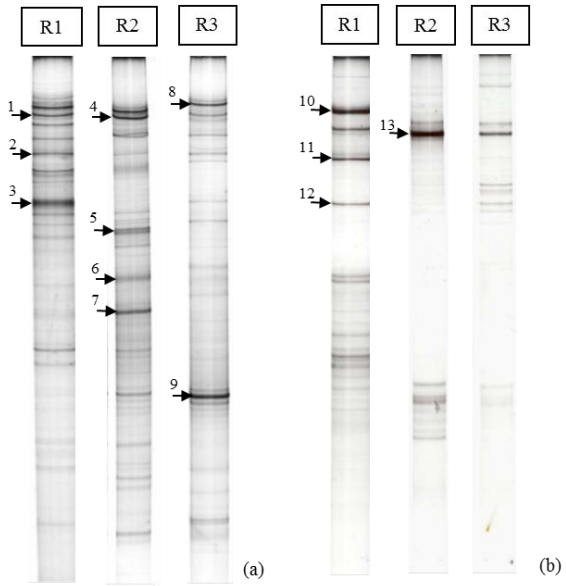

Fig. 6 SSCP profiles of microbial communities in full-scale reactors. *R1* (semi-continuously stirred reactor, VFA 500 mg l^{-1}), *R2* (plug flow reactor, VFA 3,000 mg l^{-1}), *R3* (plug flow reactor, 11,000 mg l^{-1}). (a) *Bacteria* and (b) *Archaea*. Arrows indicate positions of bands that were sequenced.

Table 5 Phylogenetic affiliation of partial *Bacteria* and *Archaea* 16S rRNA gene sequences recovered from SSCP profiles with DNA extracted from sludge of full-scale reactors

Reactor	Band	Domain	Class	Family	Closest relative (GenBank accession number)	Similarity [%]	GenBank accession number
R1	1	*Bacteria*	*Clostridia*	*Syntrophomonadaceae*	uncultured bacterium, clone OTU-9 (AB530690)	96	GQ483490
	2			*Clostridiaceae*	uncultured bacterium, clone OTU-B8 (AB428532)	98	GQ483491
	3			*Thermodesulfobiaceae*	uncultured *Coprothermobacter* sp., clone SHBZ422 (EU638952)	85	GQ483492
	10	*Archaea*	*Methanomicrobia*	*Methanosaetaceae*	*Methanosaeta concilii* GP-6 (CP002565)	99	GQ483499
	11		*Methanobacteria*	*Methanobacteriaceae*	*Methanobacterium formicicum* strain Mb10 (JN205061)	99	GQ483500
	12			*Methanobacteriaceae*	*Methanobrevibacter smithii* ATCC 35061 (CP000678)	99	GQ483501
R2	4	*Bacteria*	*Bacteroidetes*	*Bacteroidaceae*	uncultured bacterium, clone QEEB1CB09 (CU918125)	99	GQ483493
	5			*Bacteroidaceae*	uncultured bacterium, clone QEEB1CH09 (CU917556)	94	GQ483494
	6			*Porphyromonadaceae*	uncultured bacterium, clone CFB-C-3-5 (JN255091)	94	GQ483495
	7			*Porphyromonadaceae*	uncultured bacterium, clone CFB-C-3-5 (JN255091)	94	GQ483496
	13	*Archaea*	*Methanomicrobia*	*Methanosaetaceae*	*Methanosaeta concilii* GP-6 (CP002565)	98	GQ483502
R3	8	*Bacteria*	*Clostridia*	*Family XVIII. Incertae Sedis*	uncultured bacterium, clone 151_BE1_9 (FJ825471)	98	GQ483497
	9			*Family XI. Incertae Sedis*	uncultured bacterium, clone 190_BE1_11 (FJ825495)	99	GQ483498

4. Discussion

This study gives an overview of the composition and the dynamics of the bacterial and archaeal communities present in two laboratory-scale reactors that failed in operation after rape oil shock loads, and three continuously operated full-scale biogas plants, as references. The composition of the microbial communities within the laboratory and full-scale reactors was investigated by PCR-SSCP analysis using representative samples taken over the course of 45 days and once only, respectively. In addition, spatial relationship between mineral formation and microbial processes was indicated by multi-layered aggregates that formed during organic overload in laboratory reactors.

During stable reactor performance with an organic loading rate (OLR) of 4.5 kg TVS m^{-3} d^{-1} the bacterial community composition, indicated by the banding pattern of the SSCP profiles, was widely similar in both reactors, although the intensity of the bands was different (Fig. 3). Partial 16S rRNA sequences were affiliated to fermentative bacteria classically identified within anaerobic digesters such as *Clostridia*, *Bacteroidetes*, and polyphosphate-accumulating *Actinobacteria* (Archer and Kirsop, 1999; Crocetti et al., 2000; Klocke et al., 2007; Leven et al., 2007). The bacterial communities in the full-scale plants used as reference were comparable in phylogenetic structure and diversity with the laboratory-scale reactors, but differed among themselves in a manner dependent on the operation mode. Phylogenetic affiliation of dominant sequences also revealed associations with typical digester anaerobic fermentative *Clostridia* and *Bacteroidetes*.

Archaeal methanogens in laboratory- and full-scale reactors were assigned to already known and cultivated genera and were less diverse than the bacterial communities. This lower diversity corresponds with results of several other studies (Fernandez et al., 1999; Zumstein et al., 2000; Leclerc et al., 2001; Hori et al., 2006). In

both laboratory-scale reactors, the archaeal community profile was clearly dominated by one band whose DNA was affiliated to *Methanosarcina thermophila,* a common representative in anaerobic digesters, indicating that the methanogenesis took place preferentially via acetoclastic metabolism (Visser et al., 1991; Chachkhiani et al., 2004; Hori et al., 2006; Thummes et al., 2007). Several studies revealed a predominance of *Methanosarcina* at high acetate concentrations, while the second acetoclastic genus *Methanosaeta* dominates at lower acetate concentrations because of a higher substrate affinity (e.g. McMahon et al., 2001; Schmidt and Ahring, 1999). In contrast, despite of low acetate concentrations (av. 30 mg l^{-1}) in both reactors, representatives of *Methanosaeta* were not detected in laboratory-scale reactors, but in the full-scale reactors. In addition, beside substrate affinity, parameters like the feeding interval, the mixing intensity and the dilution rate also affect the abundance of acetoclastic methanogens (Conklin et al., 2006; Tang et al., 2007; Hoffmann et al., 2008). Moreover, under stable reactor performance of laboratory-scale reactors, sequences of hydrogenotrophic methanogens, namely *Methanobacterium* and *Methanospirillum hungatei,* were detected to a minor extend, with *Methanospirillum hungatei* exclusively in reactor 2.

Despite the same operation conditions in the laboratory-scale reactors lasting for two months, the maximum OLR, which led to operation failure, differed by a factor of almost three between the reactors. The organic overload caused an accumulation of volatile fatty acids and a decrease in pH and gas production rate within one day after feeding the maximal OLR (Fig 2). Similar effects were observed in several studies (Björnsson et al., 2000; Stroot et al., 2001; McMahon et al., 2004; Nielsen et al., 2007). The main components of the short chain fatty acids were acetate and propionate. Propionate accumulated before acetate until day 29 for both reactors. Scherer (2007) and Weiland (2008) found process instabilities correlating with the increase of propionate, which was higher in its concentration than the acetate one. Thomé-Kozmiensky (1995) describes propionic acid at high concentrations as inhibitory to the metabolism of microorganisms. The accumulation of butyrate was observed by Ahring et al. (1995b) as an early indicator for process failures. However in the conducted experiments butyrate was detected only in very small amounts for reactor 1 and in slightly higher amounts for reactor 2 after the process had already failed. Restarting oil and sludge supply in reactor 1, 13 days after process failure, induced a further increase of VFAs and decrease in pH, indicating that the perturbation of the microbial community was still enduring.

The organic overload resulted in a shift in the bacterial and archaeal community. In total, the successions of the bacterial banding patterns was highly complicated, as shifts were not uniformly definable for certain classes or genera and significant changes in pH and volatile fatty acids did not evoke a major shift in the phylogenetic classes of *Clostridia* and *Bacteroidetes*. Similar observations in bacterial community successions due to increased VFA concentrations and dependent from pH in digesters were detected for *Clostridia* by Delbes et al. (2001) and Hori et al. (2006). Polyphosphate accumulating *Actinobacteria* probably were involved in the formation of multi-layered aggregates that formed due to organic overload. The formation of aggregates indicates mineral creation processes during the biogas production process and probably supports de-acidification processes of the sludge since pH was at neutral level and the VFA concentration has declined substantially, a few weeks after organic overload. Aggregates were probably formed due to binding of VFA to iron phosphate minerals such as vivianite ($Fe_3(PO_4)_2 \bullet 8(H_2O)$), while phosphate was released from phosphate-accumulating *Actinobacteria*. However, this study gives not a comprehensive description of these processes; however, aggregate formation and subsequent sludge de-acidification under similar operation conditions will be discussed in detail by Kleyböcker et al. (subm.). Similar observations concerning anaerobic granulation were done for up flow anaerobic sludge blanket (UASB) reactors, treating industrial and municipal wastewater (Liu et al. 2003). However, there are significant differences in aggregate composition as the granules in our study had cores filled with organic matter and minerals. Further investigations focusing this aggregate formation process are required.

With regard to the methanogen archaea, the organic shock loading initiated an enrichment of hydrogenotrophic methanogens as consequence of an increased availability of hydrogen from VFA degradation. In reactor 2, showing a higher organic loading capacity the abundance of hydrogenotrophic *Methanospirillum hungatei* increased, linking to the lower hydrogen content in reactor 2 (H_2 maximum Vol 0.05 %) compared to reactor 1 (H_2 maximum Vol 0.3 %), despite the two-fold higher VFA concentration. Consistently, *M. hungatei*-like sequences were not found to be dominant in reactor 1. Within further increasing VFA concentrations in both reactors, other hydrogenotrophic methanogens related to *Methanoculleus receptaculi* became detectable while *Methanobacterium* relatives disappeared in the sludge of reactor 2. Hori et al. (2006) who investigated the methanogenic population dynamics in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester detected also a shifting of hydrogenotrophic methanogens due to increased VFA

concentrations. The correlation between the hydrogen concentration and methanogenesis from CO_2 and H_2 was also discussed by Demirel and Scherer (2008). According to their results, hydrogenotrophic methanogenesis is especially important at high VFA concentrations.
In contrast to the laboratory-scale reactors, acetoclastic methanogens related to the genus *Methanosaeta* were detected in all full-scale reactors which is in agreement with the mesophilic operation mode (Visser et al., 1991). Remarkably, the presence of this acetoclastic genus seems to be less sensitive to VFAs with concentrations of up to 11.000 mg l^{-1}. In addition, hydrogenotrophic methanogens of the genera *Methanobacterium* and *Methanobrevibacter* characterized the archaeal community in the reactor R1, whereas they were not detected in the reactors R2 and R3, showing higher VFA concentrations.
The comparison of the two plug-flow full-scale reactors demonstrated that despite parallel performance, the concentration of VFA can differ significantly. This result supports the findings of the laboratory-scale reactors that in spite of same inoculum and parallel reactor performance, different individual microbial community structures may establish leading subsequently to specific reactor characteristics, like GPR and VFA concentration for the plug-flow reactors, or, in case of the laboratory reactors to individual maximal organic loading capacities. As methanogenesis is the key process within complete anaerobic digestion especially the abundance, diversity, and metabolic activity of methanogens influence the current state and the operational stability of reactors, whereas the operational and environmental conditions select different methanogens in a biogas reactor (Demirel and Scherer, 2008). The presence of a complex food web, including several organisms of one physiological group, may especially ensure stable reactor status that is adapted to variable reactor operation, e.g., OLR. These results are in agreement with the conclusions of Fernández et al. (1999, 2000). The ability of shifting the electron and carbon flow through various metabolic pathways and interacting species is, thus, necessary to resist external disturbances. In addition, studies of Fernández et al. (1999), Tang et al. (2007, 2011), and Zumstein et al. (2000) revealed that the complexity of the anaerobic microbial communities in wet digestion systems depend on the complexity of substrates.
In summary, our study demonstrates the importance of the composition of the microbial community and its ability to generate a flexible response to operational perturbation. Thereby, the co-existence of hydrogenotrophic and acetoclastic methanogens seems to be necessary to respond to perturbation and leads to stable process performance.
A further understanding of the impact of the methanogenic diversity and quantities on the process stability could be gained by the construction of clone libraries and real-time qRT-PCR analyses and should provide better knowledge for optimizing management practises in the future.

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