Originally published as:

Kleyböcker, A., Lienen, T., Liebrich, M., Kasina, M., Kraume, M., Würdemann, H. (2014): Application of an early warning indicator and CaO to maximize the time–space-yield of a completely mixed waste digester using rape seed oil as co-substrate. - *Waste Management*, *34*, 3, pp. 661—668.

DOI: http://doi.org/10.1016/j.wasman.2013.11.011

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Application of an early warning indicator and CaO to maximize the time-space-yield of a completely mixed waste digester using rape seed oil as co-substrate

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Abstract

In order to increase the organic loading rate (OLR) and hereby the performance of biogas plants an early warning indicator (EWI-VFA/Ca) was applied in a laboratory-scale biogas digester to control process stability and to steer additive dosing. As soon as the EWI-VFA/Ca indicated the change from stable to instable process conditions, calcium oxide was charged as a countermeasure to raise the pH and to bind long-chain fatty acids (LCFAs) by formation of aggregates. An interval of eight days between two increases of the OLR, which corresponded to 38% of one hydraulic residence time (HRT), was sufficient for process adaptation. An OLR increase by a factor of three within six weeks was successfully used for biogas production. The OLR was increased to 9.5 kg volatile solids (VS) m^{-3} d^{-1} with up to 87% of fat. The high loading rates affected neither the microbial community negatively nor the biogas production process. Despite the increase of the organic load to high rates, methane production yielded almost its optimum, amounting to 0.9 m^3 $(kg\ VS)^{-1}$. Beneath several uncharacterized members of the phylum *Firmicutes* mostly belonging to the family *Clostridiaceae*, a *Syntrophomonas*-like organism was identified that is known to live in a syntrophic relationship to methanogenic archaea. Within the methanogenic group, microorganisms affiliated to *Methanosarcina*, *Methanoculleus* and *Methanobacterium* dominated the community.

Keywords

Biogas, early warning indicator, high organic loading rates, genetic fingerprinting, microbial community, reactor performance

Abbreviations

CaO, calcium oxide; DGGE, denaturing gradient gel electrophoresis; GC, gas chromatography; HRT, hydraulic residence time; IC, ion chromatography; LCFAs, long chain fatty acids; OLR, organic loading rate; PAOs, phosphorus accumulating organisms; PCR, polymerase chain reaction; SCFAs, short chain fatty acids; SEM, scanning electron microscopy; TS, total solids; VFAs, volatile fatty acids; VS, volatile solids; WWTP, wastewater treatment plant.

1. Introduction

Due to global warming and climate protection, finding a sustainable energy supply is necessary. One flexible and decentralized renewable energy source is biomass, especially organic residue and waste which can be converted into biogas.

Within the biogas formation process complex organic matter is degraded to methane and carbon dioxide (Schink, 1994; Deublein *et al.*, 2008). The process is defined by four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The first three phases are carried out by hydrolytic and fermentative bacteria, while the methanogenesis is performed by archaea.

The biogas production depends on a large number of interactive factors, and many aspects are only incompletely understood, despite decades of research. In consequence, full-scale biogas reactors are still operated as “black boxes”. In order to prevent process failures, waste digesters (continuously stirred tank reactor [CSTR]) are operated at low organic loading rates (OLR) between 1 and 4.5 kg VS m^{-3} d^{-1} (von Felde *et al.*, 2005; DWA, 2009; Hartmann and Ahring, 2005; Janke, 2008; Röske and Uhlmann, 2005; ATV-DVWK, 2002; Jäkel, 2003; FNR-BMELV, 2005). In consequence, full-scale digesters are usually not run at their maximal OLR, taking a loss in capacity utilization to prevent process failures such as over-acidification. Process failures can be caused by different inhibitors such as sulfides, ammonia, heavy metals, and high (> 0.1 mbar) hydrogen partial pressure (Pender *et al.*, 2004; Braun *et al.*, 1981; Karakashev *et al.*, 2005; Harper and Pohland, 1986). Various parameters such as VOA/TIC (volatile organic acids/total inorganic carbonate buffer) value, redox potential, concentration of fatty acids, and hydrogen partial pressure have been investigated in terms of their suitability as an early warning indicator for over-acidifications (Allmann, 2007; Boe, 2006; Chynoweth *et al.*, 1994; Pind *et al.*, 2003; Prechtel *et al.*, 2006; Rieger *et al.*, 2006). However, the indication of a process failure was usually too late to stabilize the process efficiently (Schüsseler, 2008). The profitability decreases dramatically during a process failure due to the lack of biogas production. Furthermore, greenhouse gas emissions increase while the substrate is not digested completely, and methane and ammonia emissions rise if the incomplete fermented sludge is used by farmers as

fertilizer.

In previous studies of Kleyböcker *et al.* (2012a), an early warning indicator, calculated from the relation of the volatile fatty acid (VFA) concentration to the calcium (Ca) concentration (EWI-VFA/Ca), was developed to warn several days before an over-acidification occurs. Additionally, the additive calcium oxide (CaO) was proven to stabilize the biogas production process and the reactor performance after an over-acidification (Kleyböcker *et al.*, 2012b). The formation of aggregates consisting of calcium and long-chain fatty acids (LCFAs) was observed during the de-acidification process and these aggregates contributed to the de-acidification by binding additional acids at their surfaces. The aggregates provided structures for microbial growth with optimal living conditions promoting the process stabilization. Furthermore, due to higher concentrations of VFA in the digester, phosphorus accumulating organisms (PAOs) took up fatty acids and released phosphate.

The aim of the study presented in this paper was to apply the EWI-VFA/Ca in combination with the addition of CaO to run a laboratory-scale biogas digester at high OLRs. Furthermore, the microbial community composition was monitored during the experiment to investigate effects of the OLR increase and the additive addition.

2. Material and methods

The EWI-VFA/Ca was monitored to control the process performance and to steer the additive dosage in order to maximize the time-space-yield. Depending on the trend of the EWI-VFA/Ca, CaO was used as a countermeasure to prevent over-acidification of the biogas formation process.

2.1 Laboratory-scale biogas digester

The laboratory-scale experiment was conducted according to Kleyböcker *et al.* (2012b) and was run for 41 days. The reactor contained 23 L of sludge. It was regulated at 50°C by a thermostat (Thermo Haake B7, Phoenix II) connected to a heating pipe which was placed around the

reactor. The sludge was mixed pneumatically using biogas with a flow of 150 L h^{-1} each day for 15 min before the samples were taken and 15 min after the substrate was introduced. For the biogas recirculation a KNF N86KTE membrane vacuum pump was used. The substrates were sewage sludge (TS ~38 g L^{-1}, VS ~27.5 g L^{-1}, pH 5.8) and rape seed oil, which were charged manually every day. Rape seed oil was chosen as co-substrate due to its degradability to LCFAs which are frequently found in real wastewaters (Komatsu *et al.*, 1991). The OLR was increased from 3.2 kg VS m^{-3} d^{-1} at day 0 stepwise to 9.5 kg VS m^{-3} d^{-1} at day 38 (Tab. 1).

Tab. 1: Feeding and OLR increase during experimental time

Time [d]	Sewage sludge [g VS d^{-1}]	Rape seed oil [g VS d^{-1}]	OLR [kg VS m^{-3} d^{-1}]
0	27.5	45	3.2
1	27.5	65	4.1
4	27.5	85	4.9
6	27.5	105	5.8
12	27.5	125	6.7
20	27.5	145	7.6
31	27.5	165	8.4
36	27.5	185	9.3
38	27.5	190	9.5

While the amount of rape seed oil varied widely (45-190 g d^{-1}), the sewage sludge ranged between 1.0 and 1.2 kg VS m^{-3} d^{-1}. Accordingly, the fat fraction of the daily substrate comprised a range between 44% and 87% VS. With the increasing fat fraction in the OLR, the expected methane yield increased due to a higher potential for methane production. The EWI-VFA/Ca calculated by the relation of VFA to calcium concentration (Kleyböcker *et al.*, 2012a) was applied to control the process performance during the increase of the OLR. Every time the EWI-VFA/Ca indicated an imminent over-acidification, 439 mg CaO L^{-1} d^{-1} was added to stabilize the

process. The hydraulic residence time (HRT) was counted between 20 and 23 days depending on the OLR. The produced biogas was measured with a Ritter gas meter (TG05/5).

For the analyses of the digested sludge, the samples were withdrawn at the outlet of the reactor. The biogas samples were taken at a bypass of the gas pipe between the gas outlet and gas wash bottle, which was located before the gas meter.

2.2 Wet chemical and gas analyses

Chemical and gas analyses were conducted according to Kleyböcker *et al.* (2012a). Temperature and pH of the digested sludge samples were measured by WTW pH 340i using a Sen Tix 41 pH electrode.

For the total solids (TS) and the volatile solids (VS) the samples were dried at 105°C in a Memmert drying chamber for 24 h and later on burned at 550°C (Nabertherm Controller B170). The weight of the samples was determined by a Sartorius CP220S-OCE balance. The TS and VS were analyzed according to the German guideline DIN 38409-1.

Total VFA (LCK 365), phosphate (LCK 350), and calcium (LCK 327) were determined photometrically (Hach-Lange DR2800) after the samples had been centrifuged twice at 10,000 rpm for 10 min (Eppendorf Centrifuge 5804). After the first centrifugation, pellets were transferred to 1.5 mL Eppendorf tubes and stored at -20°C for DNA extraction.

The short chain fatty acids (SCFA) were determined by ion chromatography (DIONEX ICS 3000). The eluent was sodium hydroxide and the IC was equipped with an AS11-HC column. The LCFAs were estimated by the difference of the calculated sum of SCFA (IC) and the sum of VFA (Hach-Lange).

The gas composition was analyzed by gas chromatography (GC) (SRI 8610C; SRI Instruments). The GC was equipped with a silica gel column and a 13X mole sieve column. The carrier gas was argon. The measured gas components were hydrogen, oxygen, nitrogen, methane, and carbon dioxide.

2.3 DNA extraction and PCR-DGGE analysis

From 350 mg of the pellets full genomic deoxyribonucleic acid (DNA) was extracted using a commercial DNA isolation kit according to the manufacturer's instructions (MP Fast DNA Spin Kit for Soil). Additionally, DNA was isolated using a modified protocol according to Rohland and Hofreiter (2007).

To compare the diversity in the microbial community, the partial 16S rRNA genes of bacteria, PAOs and methanogenic archaea were amplified by polymerase chain reaction (PCR) using the primer pair 341F-GC/907R (Muyzer *et al.*, 1993; Amann *et al.*, 1992) for the bacteria (94 °C 2:45 min, 94 °C 0:45 min, 56 °C 0:45 min, 72 °C 0:50 min, 72 °C 30 min, 40 cycles), the primer pair 462F-GC/846R (Crocetti *et al.*, 2000) for the PAO (94 °C 2:45 min, 94 °C 0:45 min, 56 °C 0:45 min, 72 °C 0:50 min, 72 °C 30 min, 40 cycles), and the primer pair 348F-GC/786R for the methanogens (94 °C 2:45 min, 94 °C 0:45 min, 56 °C 0:45 min, 72 °C 0:50 min, 72 °C 10 min, 40 cycles). The PAO primer pair was specific for the order *Rhodocyclales*. 50 µl reactions were mixed containing 5 µl 10x buffer (Genecraft), 6 µl dNTPs (10 mM, Fermentas), 3 µl $MgCl_2$ (50 mM, Genecraft), 3 µl forward primer (10 mM), 3 µl reverse primer (10 mM), 0.4 µl BSA (20 mg/ml, Fermentas), 0.3 µl Taq polymerase (5 u/µl, Genecraft), 28.3 µl RNA/DNA-free water (Fermentas), and 1 µl template. Amplicons were purified subsequently (Fermentas GeneJET PCR Purification Kit). Denaturing gradient gel electrophoresis (DGGE) was performed afterwards with equal DNA concentrations of amplicons and a gradient of 35% to 65% urea and 6% to 9% acrylamid for bacteria and PAO and 40% to 60% urea and 6% to 9% acrylamid for the methanogenic archaea using the Biorad DCode System. The DGGE gel was run for 17 h at 110 V and 60°C. The gels were silver stained and the gel bands were excised and reamplified using the primer pair 341F/907R (94 °C 1:30 min, 94 °C 0:30 min, 56 °C 0:30 min, 72 °C 0:30 min, 72 °C 10 min, 30 cycles), 462F/846R and 348F/786R (94 °C 1:30 min, 94 °C 0:30 min, 56 °C 0:30 min, 72 °C 0:30 min, 72 °C 10 min, 30 cycles). PCR products were cleaned up using the Fermentas GeneJET PCR Purification Kit. Amplicon DNA concentrations were measured fluorimetrically (BMG Labtech FLUOstar OPTIMA). PCR Products were sent in and sequenced by GATC Biotech AG. Sequences homologies were checked by BLAST (Basic

Local Alignment Search Tool).

3. Results

3.1 Increase and optimization of the space-time yield of a biogas reactor using the EWI-VFA/Ca

While the OLR was increased from 3.2 kg VS m^{-3} d^{-1} at day 0 stepwise to 9.5 kg VS m^{-3} d^{-1} at day 38, every increase of OLR was accompanied by an increase of EWI-VFA/Ca (Fig. 1).

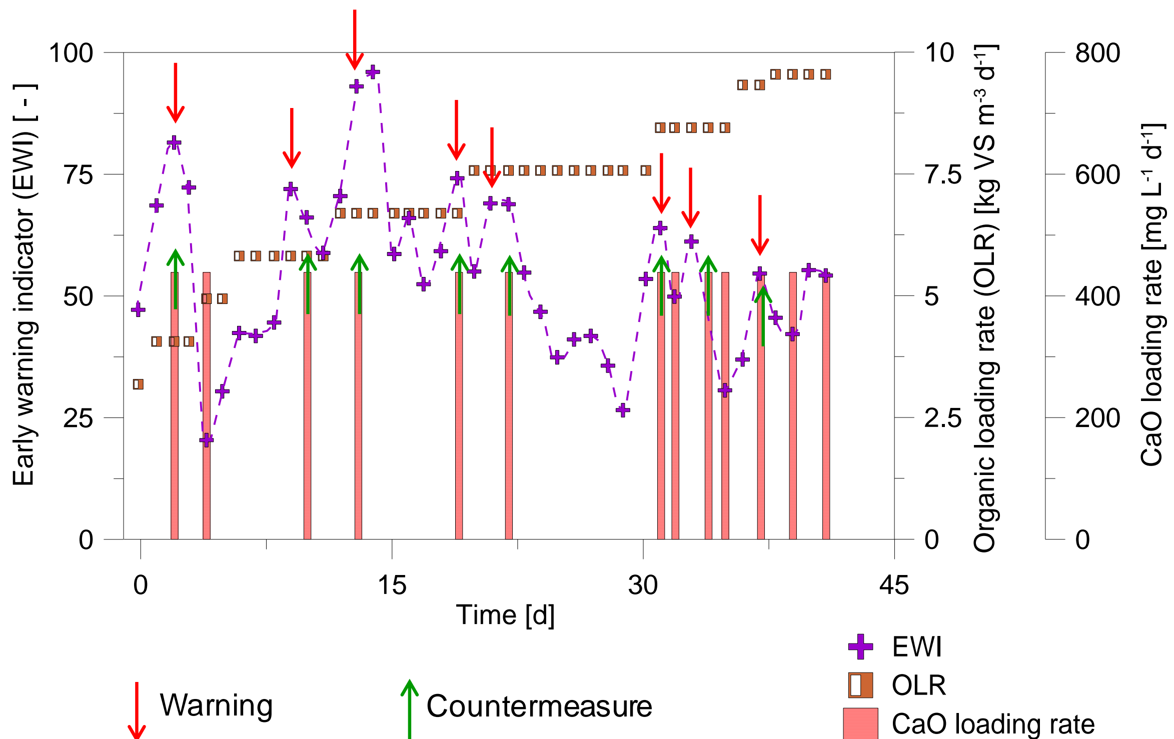

Fig. 1: Trend of the EWI-VFA/CA due to the increased OLR and the addition of CaO depending on the trend of the EWI-VFA/CA.

As soon as the EWI-VFA/Ca amounted to the two to three fold value referring to normal operation conditions at low OLRs, CaO was dosed as a countermeasure. The methane yield ranged between 0.58 and 0.88 m^3 (kg $VS)^{-1}$ depending on the interval between two OLRs increments and the added amount of CaO (Fig. 2). On average, the methane yield was 17% less than its expected value for high OLRs such as 6.7 kg VS m^{-3} d^{-1} and above. At an OLR of 9.5 kg VS m^{-3} d^{-1} and after the addition of CaO, the methane yield increased to 0.88 m^3 (kg $VS)^{-1}$ which corresponded to 94% of its expected value. The interval between two OLR rises amounted on average to 7.6 d which corresponded to 38% of the HRT. At low OLRs, such as

less than 5 kg VS m^{-3} d^{-1}, the averaged interval between two rises was only 2.5 d.

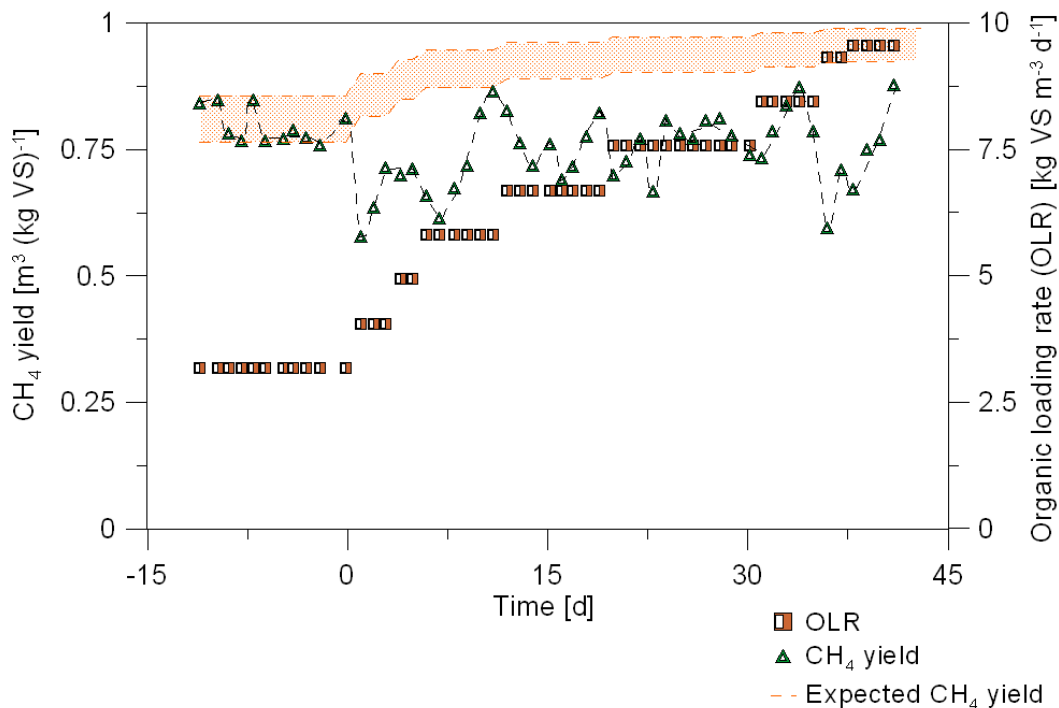

Fig. 2: Trend of the methane yield compared to the expected methane yield.

Due to the addition of CaO, the pH was kept neutral. The sum of VFAs usually ranged between 700 mg L^{-1} and 900 mg L^{-1} during the experiment, but on day 13 the VFA concentration reached 1,180 mg L^{-1}. After the further addition of CaO, the sum of VFAs decreased immediately. The acetic acid concentration ranged between 130 mg L^{-1} and 450 mg L^{-1}, while the propionic acid did not exceed 190 mg L^{-1}. Butyric acid, iso-butyric acid, and valeric acid remained below 40 mg L^{-1}, 180 mg L^{-1} and 17 mg L^{-1}, respectively. The hydrogen partial pressure was between 0.09 and 0.51 mbar which is in the range to allow for the degradation of butyric acid, but it was actually too high to allow for the degradation of propionic acid referring to standard conditions (Fig. 3). Even though the CaO charge was increased from day 31 on, the calcium concentration in the liquid phase did not increase and varied between 15 and 30 mg L^{-1} during the course of the experiment. The phosphate concentration was around 300 mg L^{-1} until day 31 and then decreased to a level around 200 mg L^{-1} within 3 days where it remained until the end of the experiment (Fig. 4).

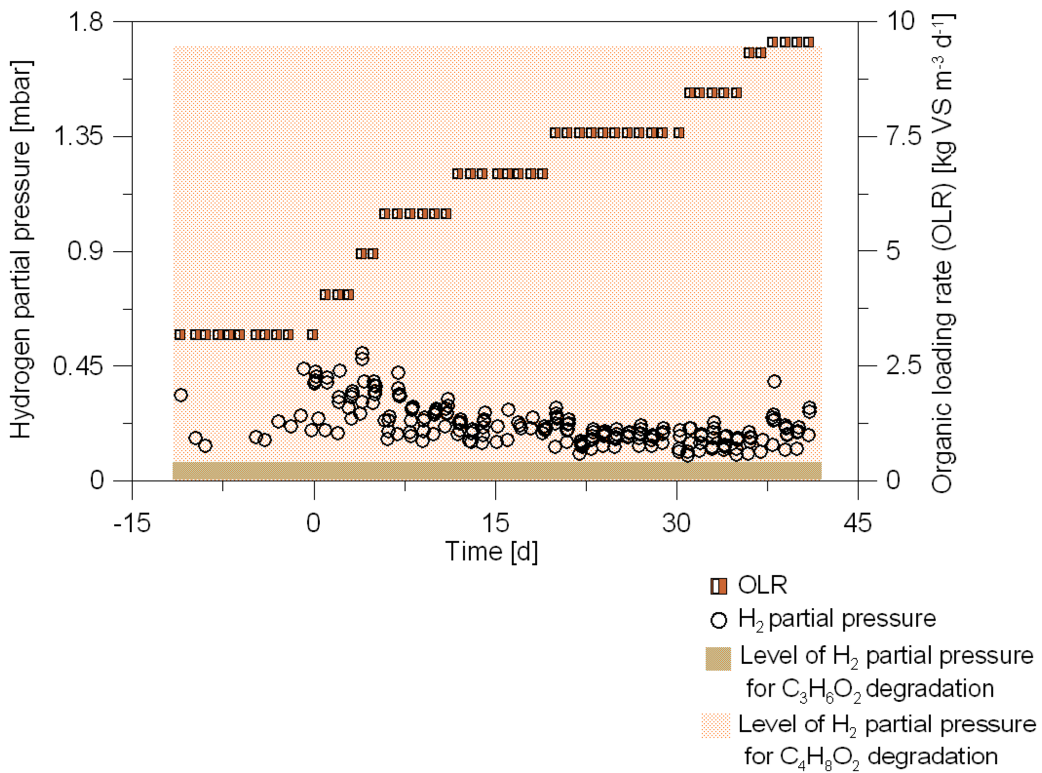

Fig. 3: Hydrogen partial pressure and organic loading rate in the course of the experiment.

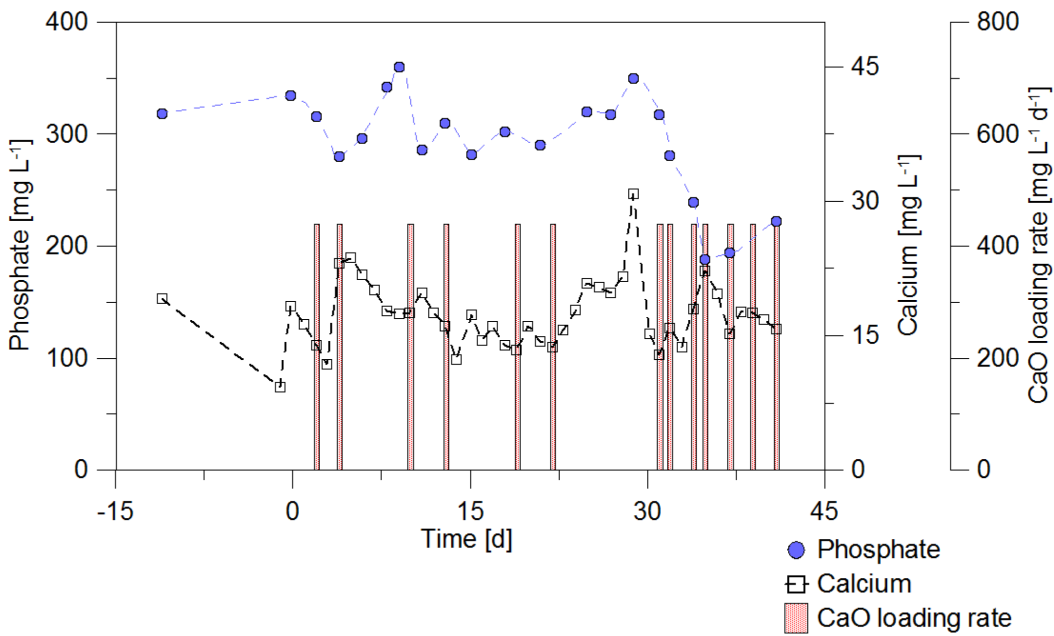

Fig. 4: Phosphate and calcium concentrations in comparison to CaO additions

3.2 Aggregate formation during the increase in OLR

After the addition of CaO, irregular shaped aggregates in the size of up to 1 mm were formed. Scanning electron microscopy (SEM-EDS) observations revealed layered structures. The layers

differed in the compactness from very dense in the inner part to less compact in the outer part. The surface of the aggregates was homogenous, composed of carbon and calcium, with attached rod-shaped microorganisms. In the inner part rolled, prismatic “crystals” composed of carbon and calcium were noted (Fig. 5a) as well as biofilms forming aggregate walls (Fig. 5b).

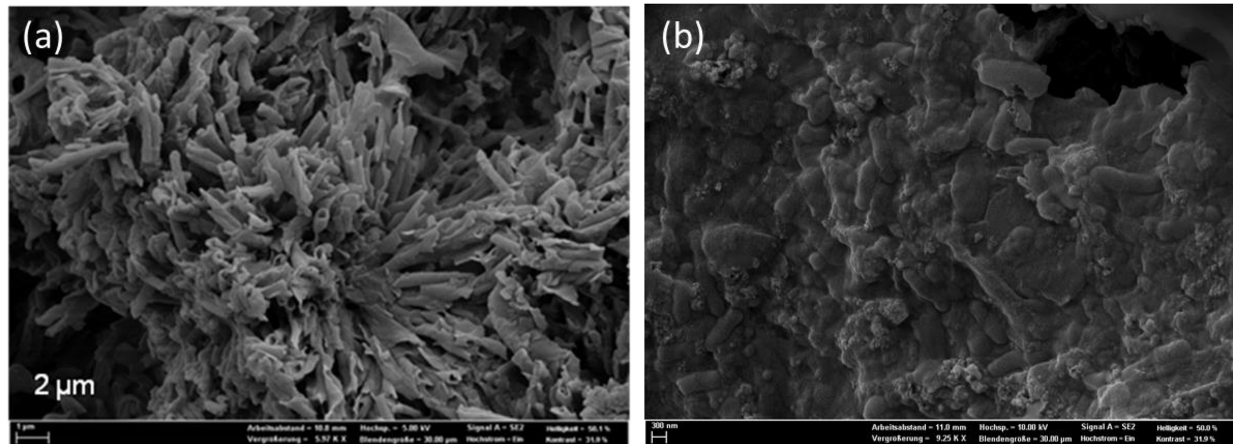

Fig. 5: (a) SEM-EDS analyses of the inner part of the aggregates. Detection of rosette-like crystals with rolled plates consisting of calcium and organic carbon probably originating of LCFA; (b) aggregate wall composed of biofilms: rod shaped microorganisms attached to each other and/or covered with organic material, probably EPS.

Inorganic components such as silicates, carbonates, and phosphates were also detected. However, their content was quantitatively much lower in comparison to the organic material. Carbonates were represented by calcite or aragonite. Iron, aluminium, magnesium, and calcium phosphate minerals with prismatic or rounded crystals or plates were present as well (Fig. 6). SEM analysis showed elongated, rod-shaped and rounded microorganisms covered by extracellular polymeric substances (EPS) in the inner part of the aggregates as well as large filamentous structures.

3.3 Microbial community composition during the increase in OLR

The bacterial community composition was dominated by few organisms indicated by strong band intensities in the genetic fingerprinting (Fig. 7).

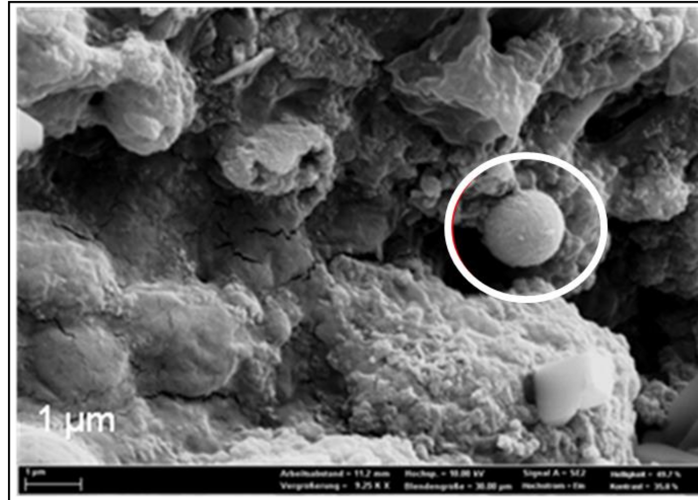

Fig. 6: **SEM-EDS analyses of the aggregates. Detection of rounded crystal of Ca-phosphate present in the circled area.**

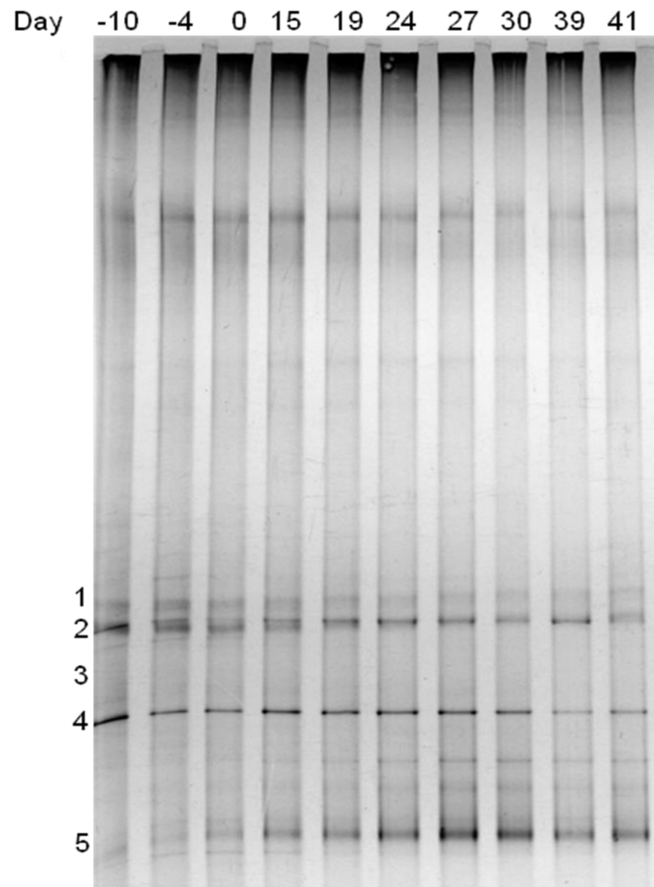

Fig. 7: **Comparative DGGE-analysis of bacterial 16S rRNA gene fragments DNA from sludge samples taken on several days during the increase in OLR. Numbered bands were sequenced. The band intensity of a *Syntrophomonas*-assigned organism (band 5) increased with higher loading rates.**

The identified sequences were all affiliated to bacteria of the phylum *Firmicutes* (Tab. 2).

Tab. 2: Partial 16S rRNA gene sequences retrieved from DGGE genetic fingerprints and sequencing of excised bands. Closest relatives are shown according to Genbank accession number reference.

Band ID	Closest relatives	Phylum	Genbank accession number	Similarity
1	*Clostridiales* bacterium	*Firmicutes*	JF808025.1	93%
2	*Clostridiaceae* bacterium	*Firmicutes*	KC594798.1	90%
3	*Clostridium* sp.	*Firmicutes*	AB093546.1	97%
4	*Clostridiaceae* bacterium	*Firmicutes*	FJ805840.2	91%
5	*Syntrophomonas wolfei subsp. wolfei*	*Firmicutes*	NR_074750.1	96%
Not shown	Candidatus *Accumulibacter phosphatis*	*Betaproteobacteria*	CP001715.1	96%
Not shown	*Dechloromonas* sp.	*Betaproteobacteria*	DQ413167.1	98%
Not shown	*Rhodocyclales* bacterium	*Betaproteobacteria*	EF636146.1	96%
6	*Methanosarcina thermophila*	*Euryarchaeota*	JQ346758.1	100%
7	*Methanobacterium formicicum*	*Euryarchaeota*	JN205061.1	100%
8	*Methanoculleus* sp.	*Euryarchaeota*	GQ872113.1	99%

A *Syntrophomonas*-like organism was present throughout the complete experiment. The associated band intensified with increasing the OLR to 7.6 kg VS m^{-3} d^{-1} and afterward the band intensity remained consistent when the OLR was further increased to 9.5 kg VS m^{-3} d^{-1}. Additionally, three sequences were detected with *Rhodocyclales*-specific primers (Tab. 2). While closest relatives of *Dechloromonas* and a *Rhodocyclales* bacterium dominated the PAO community indicated by strong band intensities, the abundance of an *Accumulibacter*-like organism was minor (data not shown). Among the methanogenic archaea, acetoclastic as well as hydrogenotrophic organisms were identified (Tab. 2). A *Methanosarcina*-affiliated facultative acetoclastic organism was shown to dominate the community during the whole experiment which was indicated by strong band intensities (Fig. 8).

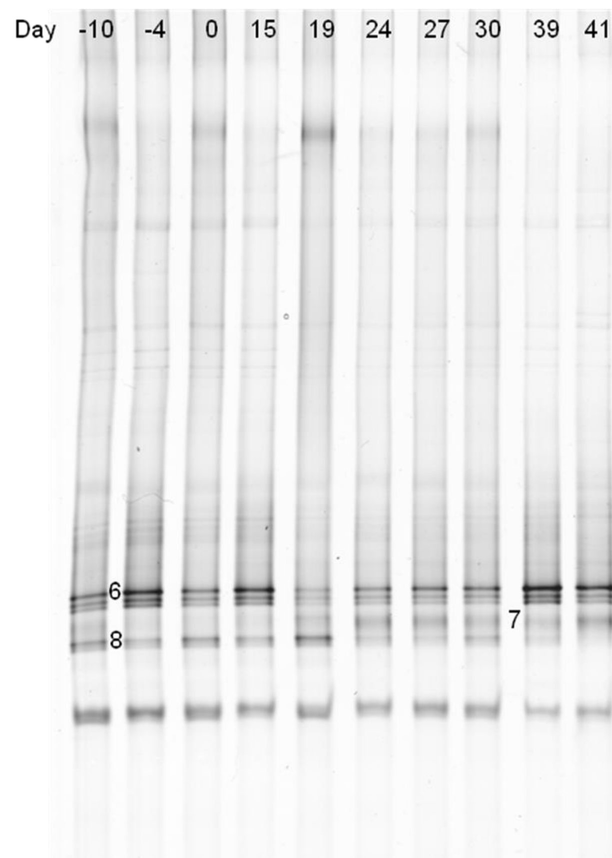

Fig. 8: Comparative DGGE-analysis of methanogenic archaeal 16S rRNA gene fragments DNA from sludge samples taken on several days during the increase in OLR. Numbered bands were sequenced and identified as relatives of the genera *Methanosarcina* (band 6), *Methanobacterium* (band 7) and *Methanoculleus* (band 8).

Additionally, a closest relative of the hydrogenotrophic methanogen *Methanoculleus* was consistently detected by genetic fingerprinting. Furthermore, a *Methanobacterium*-like hydrogenotrophic organism became more dominant at OLRs higher than 7.6 kg VS m^{-3} d^{-1} and the strongest intensity for the *Methanobacterium*-assigned band was detected at an OLR of 9.5 VS kg m^{-3} d^{-1}.

4. Discussion

Although the OLR was increased by a factor of three within a short period of 38 days, the process of biogas formation was stable with VFA concentrations below 1000 mg L^{-1} and a high methane yield of 0.88 m^3 (kg VS)$^{-1}$. However, the hydrogen partial pressure in the gaseous phase was actually too high to allow for the degradation of propionic acid to methane. According to Harper and Pohland (1986), the hydrogen partial pressure should remain below 0.1 mbar to allow for propionic acid degradation. Nevertheless, in our experiment, the concentration of propionic acid remained at a low level despite H_2 concentrations of 0.09 to 0.51 mbar. As already proposed in a previous study (Kleyböcker *et al.* 2012b), we assume that aggregation played an important role in terms of process stability and regulated the availability of LCFAs which precipitated and/or adsorbed on the precipitates and subsequently formed the aggregates which served as a buffer for LCFAs. LCFAs are a degradation product of rape seed oil, which was used as substrate. In low concentrations, they are already inhibitory to anaerobic microorganisms as observed by Angelidaki and Ahring, 1992 and Hwu *et al.*, 1998.

Because the concentration of propionic acid remained at a low level, we assume that within these aggregates different microhabitats existed which favored conditions for propionic acid degradation. The positive effect of aggregation on the process stability due to formation of interfaces and microhabitats was also discussed in other studies (Kasina *et al.*, in prep.; Kleyböcker *et al.*, 2012b; Rajeshwari *et al.*, 2000; Mac Leod *et al.*, 1990; Hwu *et al.*, 1998). In the present study, the surface of the observed aggregates was characterized by SEM analysis as calcium and carbon rich. Probably, LCFAs and calcium precipitated and stimulated the aggregation. However, the methane yield was only 10% to 20% lower than expected because

the aggregates were probably degraded by syntrophic consortia located on their interfaces. Among other compounds, the aggregates also contained phosphate. During high CaO loads, the phosphate concentration decreased, while the calcium concentration did not increase as expected giving a hint to precipitation and/or adsorption processes. At high acid concentrations, PAOs are known to take up acids and simultaneously release phosphate under anaerobic conditions (Seviour *et al.*, 2003; Röske and Uhlmann, 2005). As already discussed in our previous study (Kleyböcker *et al.*, 2012b), the released phosphate also might contribute to the formation of the aggregates. This assumption is corroborated by the detection of three different sequences by the *Rhodocyclales*-specific genetic fingerprinting analysis, of which especially *Accumulibacter phosphatis* is known to be present in wastewater treatment plants (WWTP) with enhanced biological phosphorus removal (EBPR) and to take part in the acid uptake and phosphate release mechanism (Hesselmann *et al.*, 1999; Fukushima *et al.*, 2007). In contrast, relatives from the genus *Dechloromonas*, which were also detected by the *Rhodocyclales*-specific genetic fingerprinting, were indeed shown to assimilate acetate under anaerobic conditions, while phosphate accumulation under aerobic conditions was not observed (Ahn et al. 2007). Therefore, most likely *Accumulibacter phosphatis* and *Dechloromonas* sp. competed for acetate, but the phosphate contained in the aggregates was released only by *Accumulibacter phosphatis*.

Even when utilizing a substrate containing 87% VS of fat fraction, the methane yield accounted on average 86% of the expected value despite the high loading rate and the relatively rapid increase in the OLR to 9.5 kg VS m^{-3} d^{-1}. According to Bischofsberger *et al.* (2005), von Felde *et al.* (2005), Röske and Uhlmann (2005), Janke (2008), and DWA (2009), typical OLRs for completely mixed digesters range between 1 and 4.5 kg VS m^{-3} d^{-1}. Thus, the OLR in the presented experiment was more than two times higher than the typical maximal OLR. The fat fraction of 87% VS was approximately twice as high than in the experiments of Luostarinen *et al.* (2009). Their investigations showed that the digestion of sewage sludge with grease trap sludge was on a high performance until an OLR of approximately 3.5 kg VS m^{-3} d^{-1} and a maximal fat fraction of 46% VS. It should be noted that Luostarinen *et al.* (2009) did not use any

additives. Davidsson and Loevstedt (2008) also investigated co-fermentation of sewage sludge with grease trap sludge without using additives. The OLR was much lower with 2.5 kg VS m^{-3} d^{-1} than the OLR in the presented experiment, while the fat fraction was only between 10% and 30% VS. Despite the lower OLR, the methane yield was 10% to 30% below its expected value. It should be noted that the hydraulic residence time was 13 days in the experiments of Davidsson and Loevstedt (2008). According to Angelidaki and Ahring (1992) and Luostarinen *et al.* (2009), the complete degradation process of fat lasts between 25 and 30 days. This might be the reason for the lower methane yields at low OLRs compared to our experiments, in which the hydraulic residence time was 20 days.

Ganesh *et al.* (2013) investigated the effect of increasing the OLR in solid waste co-digestion. At a maximal OLR of 7.5 kg VS m^{-3} d^{-1}, the loss in the methane yield was 20%. In our experiment, at an OLR of 9.5 kg VS m^{-3} d^{-1}, the methane yield still increased to 94% of its expected value. Elango and Pulikesi (2007) used NaOH as additive to adjust the pH, when increasing the OLR during co-digestion of municipal solid waste and domestic wastewater. Despite the addition of additives, the process failed due to over-acidification after the OLR was raised to 3.5 kg VS m^{-3} d^{-1}. Thus, we achieved higher OLRs and higher methane yields by the application of the EWI-VFA/Ca and the addition of CaO compared to the results of the other authors. Furthermore, the addition of CaO prevented the system against a process failure at high OLRs.

In our study, the interval between two OLR rises was on average only 38% of one HRT for high OLRs. Gallert *et al.* (2003) also conducted experiments to raise OLR from 3 to 13 kg VS m^{-3} d^{-1} at a full-scale biogas plant fed by biowaste suspension and applied intervals of two HRTs between two increments. Despite the longer intervals between the OLR rises, propionic acid accumulated up to 3,400 mg L^{-1} and the system had to be stabilized by periodic feed stops. According to Röske and Uhlmann (2005) the state of equilibrium between acid production and consumption after OLR increase is reached at the best after 20 days and at the worst after 6 months. However, in our experiment, the state of equilibrium between acid production and acid consumption in the fluid could already be reached after one week due to the regulation of the LCFA availability by the addition of CaO.

Genetic fingerprinting with DGGE was used to observe shifts in the diversity of the biocenosis and dominance of species due to changes in the environmental conditions. The microbial community diversity did not significantly change due to the increased OLRs and the addition of CaO, members of the phylum *Firmicutes* dominated the community. Other studies already indicated their participation and dominance in the degradation of LCFAs (Sousa *et al.*, 2007a; Sousa *et al.*, 2008). Furthermore, Baserba *et al.* (2012) showed the importance of members from the phylum *Firmicutes* for the degradation of oleate. A comparison of the DGGE band intensities served as a semiquantitative approach since the DNA concentrations were set equally before separating sequences by DGGE. Particularly at OLRs higher than 7.6 kg VS m^{-3} d^{-1}, the abundances of *Syntrophomonas* and *Methanobacterium* affiliated organisms increased, which was indicated by stronger band intensities. Bacteria of the genus *Syntrophomonas* were shown to take part in the degradation of LCFA in which they live in close syntrophic relation to hydrogenotrophic methanogens such as *Methanobacterium formicicum* (Sousa *et al.*, 2007b) or *Methanospirillum hungatei* (Hatamoto *et al.,* 2007). As the hydrogen partial pressure was too high for propionic acid degradation, presumably, *Syntrophomonas* was at an advantage compared to other bacteria due to the hydrogen consumption of its syntrophic partner *Methanobacterium formicicum* and probably they were located in microhabitats formed by aggregation. The presence of methanogenic organisms affiliated to the genus *Methanosarcina* was detected with high band intensities over the whole OLR range. *Methanosarcina*-like organisms were already shown to dominate the methanogenic community in case of high loading rates and LCFAs concentrations (Sousa *et al.*, 2007a, 2009; Palatsi *et al.* 2010; Lerm *et al.*, 2012). *Methanosarcina* prefers high acetate concentrations and as the acetate content yielded up to 450 mg L^{-1} during the experiment, *Methanosarcina* probably outcompeted other acetoclastic methanogens such as *Methanosaeta* because of its higher growth rate (Jetten *et al.*, 1992). Beside *Methanobacterium*- and *Methanosarcina*-affiliated methanogens, a close relative of *Methanoculleus* was identified. *Methanoculleus* was recently assumed to be a major contributor in methanogenesis in swine manure storage tanks (Barret *et al.*, 2012) and anaerobic sludge digestion (Hori *et al.*, 2006). The codominance of this hydrogenotrophic

methanogen in this study supports the assumption that *Methanoculleus* contributes to the methane production in anaerobic digesters even at high OLRs.

5. Conclusions

The EWI-VFA/Ca indicated any imbalance in process performance sufficiently early to take action in successful countermeasures, thereby allowing the stepwise increase of OLR and minimizing the risk of process failure. Calcium oxide served as an effective additive for process stabilization. The application of the EWI-VFA/Ca to control the process of biogas formation and to regulate additive dosage was shown to be an adequate procedure to maintain process stability. The precipitation of LCFAs stimulated the formation of aggregates and offered microhabitats for its efficient degradation. Despite LCFA precipitation and subsequent aggregate formation, the methane yield was only 10-20% lower than expected, indicating that decomposition of the aggregates occurred continuously. Precipitation reduced inhibitory effects of LCFAs and thereby regulated availability to allow for an efficient degradation. PAOs were present and may have supported buffering of slight imbalances during the OLR increase by the uptake of fatty acids. The application of EWI-VFA/Ca and additive addition will help biogas plant operators to react flexibly either to the substrate availability on the market or to the requirements of energy production and supply.

Acknowledgements

Thanks to the German “Federal Ministry for the Environment, Nature and Nuclear Safety” for funding the project “Optgas” (03KB018) and to Dr. Andrea Vieth-Hillebrand and her team (Section for Organic Geochemistry at GFZ) for analyzing short chain fatty acids by ion chromatography and Sebastian Teitz for technical support.

References

Ahn, J., Schroeder, S., Beer, M., McIlroy, S., Bayly, R.C., May, J.W., Vasiliadis, G., Seviour, R.J. (2007) Ecology of the microbial community removing phosphate from wastewater under

continuously aerobic conditions in a sequencing batch reactor. Applied and Environmental Microbiology 73 (7), 2257-70.

Angelidaki, I., Ahring, B., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Applied Microbiolology and Biotechnology 37, 808-812.

ATV-DVWK, 2002. M 363 „Herkunft, Aufbereitung und Verwertung von Biogasen“, Hennef

Barret, M., Gagnon, N., Morissette, B., Topp, E., Kalmokoff, M., Brooks, S.P., Matias, F., Massé, D.I., Masse, L., Talbot, G., 2012. *Methanoculleus* spp. as a biomarker of methanogenic activity in swine manure storage tanks. FEMS Microbiology Ecology 80 (2), 427-440.

Baserba, M. G., Angelidaki, I., Karakashev, D. (2012) Effect of continuous oleate addition on microbial communities involved in anaerobic digestion process. Bioresource Technology 106, 74-81

Bischofsberger, W., Dichtl, N., Rosenwinkel, K., Seyfried, C., Böhnke, B., 2005. Anaerobtechnik, Springer-Verlag, Berlin Heidelberg

Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D., Blackall, L.L., 2000. Identification of polyphosphate accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. Applied and Environmental Microbiology 66: 1175–1182.

DWA, 2009. M 380 „Co-Vergärung in kommunaler Klärschlammfaulbehältern, Abfallvergärungs-anlagen und landwirtschaftlichen Biogasanlagen, Hennef

Davidsson, A., Loevstedt, C., 2008. Co-digestion of grease trap sludge and sewage sludge. Waste Management 28, 986-992.

Elango, D., Pulikesi, M., 2007. Production of biogas from municipal solid waste with domestic waste sewage. Journal of Hazardous Materials 141, 301-304.

FNR, BMELV (2005): Ergebnisse des Biogas-Messprogramms, Bundesforschungsanstalt für Landwirtschaft, Gülzow

Fukushima T., Uda N., Okamoto M., Onuki M., Satoh H., Mino T., 2007. Abundance of Candidatus Accumulibacter phosphatis in Enhanced Biological Phosphorus Removal Activated Sludge Acclimatized with Different Carbon Sources. Microbes and Environments

22, 346-354.

Ganesh, R., Torrijos, M., Sousbie, P., Steyer, J., Lugardon, A., Delgenes, J., 2013. Anaerobic co-digestion of solid waste: Effect of increasing organic loading rates and characterization of the solubilised organic matter. Bioresource Technology 130, 559-569.

Gallert, C., Henning, A., Winter, J., 2003. Scale-up of anaerobic digestion of the biowaste fraction from domestic wastes. Water Research 37, 1433-1441.

Harper, S., Pohland, F., 1986. Recent developments in hydrogen management during anaerobic biological wastewater treatment. Biotechnology and Bioengineering 28, 585-602.

Hartmann, H., Ahring, B., 2005. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of codigestion with manure. Water Research 39, 1543-1552.

Hatamoto, M., Imachi, H., Fukayo, S., Ohashi, A., Harada, H. (2007) *Syntrophomonas palmitatica* sp. nov., an anaerobic, syntrophic, long-chain fatty-acid-oxidizing bacterium isolated from methanogenic sludge. International Journal of Systematic and Evolutionary Microbiology 57, 2137-2142.

Hesselmann, R.P., Werlen, C., Hahn, D., van der Meer, J.R., Zehnder, A.J., 1999. Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphate removal in activated sludge. Systematic and Applied Microbiology 22 (3):454-465.

Hori, T., Haruta, S., Ueno, Y., Ishii, M.Y., 2006. Dynamic Transition of a Methanogenic Population in Response to the Concentration of Volatile Fatty Acids in a Thermophilic Anaerobic Digester. Applied and Environmental Microbiology 72 (2): 1623–1630.

Hwu, C., Tseng, S., Yuan, C., Kulik, Z., Letting, G., 1998. Biosorption of long-chain fatty acids in UASB treatment process. Water Research 32, 5, 1571-1579.

Jäkel, K., 2003. Managementunterlage “Landwirtschaftliche Biogaserzeugung und -verwertung”, Sächsische Landesanstalt für Landwirtschaft.

Janke, H., 2008. Umweltbiotechnik: Grundlagen und Verfahren, Verlag Eugen Ulmer Stuttgart

Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B., 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp.. FEMS Microbiology Reviews 88: 181–197.

Kasina, M., Kleyböcker, A., Michalik, M., Liebrich, M., Würdemann, H., in prep. Fast increase of organic loading rate during co-digestion of rape seed oil and sewage sludge in a CSTR - characterization of granules formed due to CaO addition

Kleyböcker, A., Liebrich, M., Verstraete, W., Kraume, M., Würdemann, H., 2012a. Early warning indicators for process failure due to organic overloading by rape seed oil in one-stage continuously stirred tank reactor, sewage sludge and waste digesters. Bioresource Technology 123, 534-541.

Kleyböcker, A., Liebrich, M., Kasina, M., Kraume, M., Wittmaier, M., Würdemann, H., 2012b. Comparison of different procedures to stabilize biogas formation after process failure in a thermophilic waste digestion system: Influence of aggregate formation on process stability. Waste Management 32 (6), 1122-1130.

Lerm, S., Kleyböcker, A., Miethling-Graff, R., Alawi, M., Kasina, M., Liebrich, M., Würdemann, H., 2012. Archaeal community composition affects the function of anaerobic co-digesters in response to organic overload. Waste Management 32 (3), 389-399.

Luostarinen, S., Luste, S., Sillanpää, M., 2009. Increased biogas production at wastewater treatment plants through co-digestion of sewage sludge with grease trap sludge from meat processing plant. Bioresource Technology 100, 79-85.

MacLeod, F., Guiot, S., Costerton, J., 1990. Layered structure of bacterial aggregates produced in an upflow anaerobic sludge bed and filter reactor. Applied and Environmental Microbiology 56 (6), 1598-1607.

Palatsi, J., Illa, J., Prenafeta-Boldú, F.X., Laureni, M., Fernandez, B., Angelidaki, I., Flotats, X. 2010. Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: batch tests, microbial community structure and mathematical modelling. Bioresource Technology 101 (7), 2243-2251.

Rajeshwari, K., Balakrishnan, M., Kansal, A., Lata, K., Kishore, V., 2000. State-of-the–art of anaerobic digestion technology for industrial wastewater treatment. Renewable and Sustainable Energy Reviews 4, 135-156.

Röske, I., Uhlmann, D., 2005. Biologie der Wasser und Abwasserbehandlung, Verlag Eugen

Ulmer Stuttgart

Schüsseler, P., 2008. Zielsetzung des Fachgesprächs, Gülzower Fachgespräche, Fachagentur für Nachwachsende Rohstoffe e. V., 27, 8-16.

Seviour, R., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiology Reviews 27, 99-127.

Sousa, D.Z., Pereira, M. A., Smidt, H., Stams, A.J.M., Alves, M.M., 2007a. Molecular assessment of complex microbial communities degrading long chain fatty acids in methanogenic bioreactors. FEMS Microbiology Ecology 60, 252–265.

Sousa, D.Z., Smidt, H., Alves, M.M., Stams, A.J., 2007b. *Syntrophomonas zehnderi* sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with *Methanobacterium formicicum*. International Journal of Systematic and Evolutionary Microbiology 57, 609-615.

Sousa, D.Z., Pereira, M.A., Alves, J.I., Smidt, H., Stams, A.J.M., Alves, M.M., 2008. Anaerobic microbial LCFA degradation in bioreactors. Water Science and Technology 57 (3), 439-444.

Sousa, D.Z., Pereira, M.A., Alves, J.I., Smidt, H., Stams, A.J.M., Alves, M.M., 2009. Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids. FEMS Microbiology Ecology 68, 257-272.

von Felde, D., Staske, S., Wilms, R., 2005. Co-Vergärung von Fettabscheiderrückständen in Faulbehältern kommunaler Kläranlagen, KA – Abwasser, Abfall. 52 (10), 1151-1156.