Lienen, T., Kleyböcker, A., Verstraete, W., Würdemann, H. (2014): Foam formation in a downstream digester of a cascade running full-scale biogas plant: Influence of fat, oil and grease addition and abundance of the filamentous bacterium Microthrix parvicella. - *Bioresource Technology*, *153*, pp. 1—7.

DOI: http://doi.org/10.1016/j.biortech.2013.11.017

Foam formation in a downstream digester of a cascade running full-scale biogas plant: Influence of fat, oil and grease addition and abundance of the filamentous bacterium *Microthrix parvicella*

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List of abbreviations

DGGE, denaturing gradient gel electrophoresis; FOG, fat, oil and grease; qPCR, quantitative polymerase chain reaction; LCFA, long chain fatty acids; TS, total solids; VFA, volatile fatty acids; VS, volatile solids; WWTP, wastewater treatment plant

Abstract

The microbial community composition in a full-scale biogas plant fed with sewage sludge and fat, oil and grease (FOG) was investigated over a 15-month period, including two foam formation events. Addition of FOG as a substrate in the biogas plant together with high abundances of *Microthrix parvicella* were found to promote foam formation in the downstream digester of a cascade of two biogas digesters. Genetic fingerprinting and quantitative PCR (qPCR) indicated a higher abundance of *M. parvicella* in the digester, when the digestion process was accompanied by excessive foaming relative to the reference digesters without disturbance. The creation of foam depended on the introduced proportion of FOG and the abundance of *M. parvicella*. Furthermore, shifts in the abundance of *M. parvicella* in the biogas plant were observed within the 15-month monitoring period corresponding to its seasonal abundance in the sludge of the wastewater treatment plant (WWTP).

Keywords

Full-scale biogas plant, foam, FOG addition, *Microthrix parvicella* threshold abundance

1. Introduction

The use of renewable energy sources is increasing in Germany. As a storable and decentralized energy source, the production of biogas from organic residues is regarded as a very important method for substituting fossil fuels with renewable energy sources (Weiland 2010).

Full-scale biogas plants are often affected by serious process failures. Over-acidification, foaming and the creation of floating layers lead to operational failures or even to complete process interruptions, which results in considerable financial losses (Balussou et al. 2011). From a microbiological point of view, most biogas plants are still operated as so-called "black boxes". Although they are crucial for the biogas production process, the identities of the microorganisms of the complex ecosystem in the sludge and their interactions are mostly unknown. Changes in the operation of the plant can lead to significant alterations in the composition of the microbial community. Therefore, better knowledge about these complex processes is essential to improve the efficiency and process stability of biogas plants and to reduce the risk of process failure. Early warning indicators for over-acidification based on the chemical composition of the digestate have been developed (Kleyböcker et al. 2012). However, effective warning indicators for foam formation in co-digesters are not currently available. Among other problems, foam formation in anaerobic digesters leads to increased liquid levels that reduce the effective digester volume and block gas tubes (Pagilla et al. 1997, Westlund et al. 1998). Furthermore, it increases costs for anti-foaming agents and personnel as well as reduces the electricity generated from biogas (Barber 2005, Ganidi et al. 2009). A survey of biogas plant operators revealed that 12 out of 15 biogas producers have problems with foam formation in their digesters (Moeller et al. 2012). Excessive foaming can be caused by organic overloading and the subsequent increase of the concentration of volatile fatty acids (Ross and Ellis 1992, Ganidi et al. 2011) or by the introduction of a large amount of hydrophobic materials (Westlund et al. 1998). The formation of foam in wastewater treatment plants (WWTPs) is known to be caused by filamentous bacteria, such as *Gordonia* or *Microthrix* species (Blackall et al. 1988, de los Reyes et al. 2002, Martins et al. 2004, Rossetti et al. 2005). Sewage sludge is widely used as a substrate for anaerobic digesters; thus, large amounts of *Gordonia* spp. and *Microthrix parvicella* can be introduced into the digesters (Pagilla et al. 1997, Westlund et al. 1998, Marneri et al. 2009). Alterations in the microbial community reflect changes of the complex biogas system and might be crucial indicators for foaming in anaerobic digesters. Differences in cell numbers or gene copies of organisms involved in process disturbances can indicate the start of process failure. Molecular biology approaches,

such as genetic fingerprinting, quantitative polymerase chain reaction (qPCR) and fluorescence *in situ* hybridization (FISH), are rapid techniques for monitoring the digestate, especially for indicator organisms. In addition to FISH, which is often used for monitoring the filamentous bacteria in activated sludge systems (de Los Reyes et al. 2002), Kaetzke et al. (2005) and Kumari et al. (2009) established qPCR for monitoring the abundance of *Microthrix parvicella* in WWTPs. This rapid, highly sensitive and reliable DNA-based method is more exact than FISH because incomplete permeability of the cell wall to the probe and low rRNA levels due to low metabolic activity might lead to erroneous quantification results (Kaetzke et al. 2005).

The objective of this study was to identify the reasons for two foam formation events occurring in a sewage sludge and fat, oil and grease (FOG) co-digesting full-scale biogas plant. The plant consisted of four digesters and was run as a cascade of two digesters. Only one of the two downstream digesters that was additionally fed with FOG was affected by foaming. The microbial community composition in the digestates was investigated by denaturing gradient gel electrophoresis (DGGE) and qPCR. The FOG addition and process performance were correlated with changes in the microbial community of the digesters. Furthermore, the microbial community composition was analyzed with respect to the ambient temperature because the digested sewage sludge originated from a WWTP and seasonal changes in the microbial community composition of activated sludge has been shown in previous studies (Eikelboom 1994, Mamais et al. 2006, Kumari et al. 2009).

2. Material and Methods

2.1 Biogas plant scheme

The full-scale biogas plant consisted of four 8000 m^3 digesters that were operated mesophilically (Figure 1). Digesters 1 and 3 as well as digesters 2 and 4 were operated in line-forming cascades. Cascade I ran from the upstream digester 1 to the downstream digester 3, and cascade II ran from digester 2 to 4 (Heinzmann and Engel, 2006). The hydraulic retention time of digesters 1 and 2 was approximately 10 days, and the hydraulic retention time of digesters 3 and 4 was approximately 15 days. Digesters 1 and 2 were fed with sewage sludge from a nearby WWTP, and digesters 3 and 4 were charged with the digestates of the upstream reactors. To avoid excessive foaming in wintertime, the organic loading rate (OLR) was reduced to below the regular rate from December to May to 2.5 – 3 kg VS m^{-3} d^{-1}, whereby from June to November, the OLR was raised to 4 – 4.5 kg VS m^{-3} d^{-1}. Digester 3 was additionally fed with FOG from the food industry. From December 2009 to March 2010, approximately 60 – 80 kg FOG m^{-3}

digestate were charged. Furthermore, 25 kg FOG m^{-3} digestate were added in April 2010, 10 kg FOG m^{-3} digestate were fed in November 2010 and 2 kg FOG m^{-3} digestate were charged in December 2010. From May to October 2010 as well as in January and February 2011 no FOG was added due to a technical defect. The temperature in the digesters was maintained by heating the substrate before dosage. Online measurements of the plant operators revealed temperatures of 38 – 42 °C in the digesters. A pump was used (800 m^3/h) to recirculate the digestate.

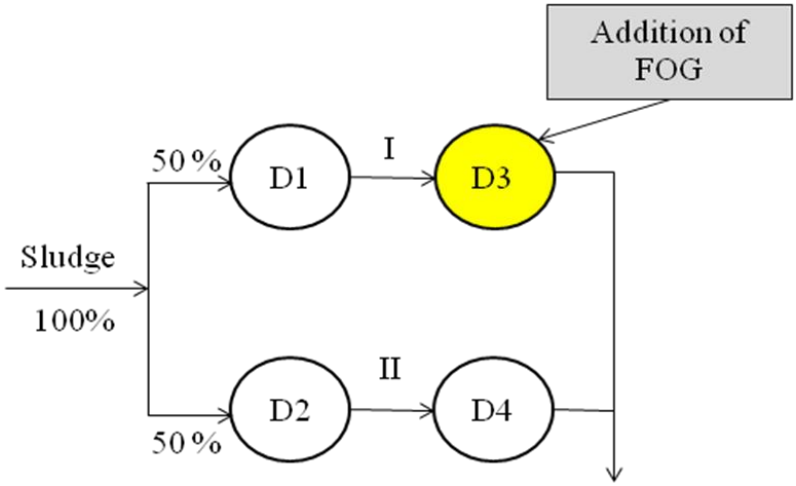

Figure 1: Process scheme for the biogas plant. Cascade I ran from digester 1 to 3 (D1, D3). Cascade II ran from digester 2 to 4 (D2, D4). All digesters were fed with sewage sludge. Digester 3 was additionally charged with FOG. A foam formation event occurred in digester 3 in December 2009 and January 2010.

2.2 Sample collection, chemical analyses and DNA extraction

The four digesters of the biogas plant were sampled monthly from September 2009 to February 2011. In December 2009 and January 2010, foaming occurred in the downstream digester 3. From each of these digesters, 1000 mL samples, corresponding to approximately 1000 g, were withdrawn at the outlet of the recirculation pump and additionally at the drain of every reactor. Furthermore, in December 2009, a foam sample was taken directly at the overflow of digester 3. The temperature and pH values of the digestates were measured with a WTW pH 340i, using a Sen Tix 41 pH electrode. The maximum ambient temperature variations were recorded by a nearby weather station (wind.met.fu-berlin.de).

For the total solids content (TS) and the volatile solids content (VS) approximately 50 g of the digestates were dried at 105 °C in a Memmert drying chamber for 24 h and then ashed at 550 °C (Nabertherm Controller B170). The weight of the samples was determined using a Sartorius CP220S-OCE laboratory balance (scale ± 0.01 g). The TS

and VS were analyzed according to German guideline DIN 38409-1.

For the analysis of volatile fatty acids (VFAs) and for DNA extraction, approximately 60 g of the samples were centrifuged 10 min at 12,857 x g (Eppendorf Centrifuge 5804) to pellet solid substances. Pellets were transferred into 1.5 mL tubes and stored at -20 °C for later DNA extraction. The supernatant was centrifuged again for 10 min at 12,857 x g. In the second supernatant, volatile fatty acids were measured photometrically (Hach-Lange DR2800). To compare the diversity of the microbial community compositions, the total genomic DNA was extracted from approximately 350 mg of the pellets using a commercial DNA isolation kit according to the manufacturer's instructions (MP Fast DNA Spin Kit for Soil).

2.3 PCR-DGGE analysis

The partial 16S rRNA genes of the bacterial community were amplified by polymerase chain reaction (PCR) in 50 µL reactions with 1 µL of 1:10 diluted template using the primer pair 341F-GC/907R (Muyzer et al. 1993; Amann et al. 1992) (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). Fifty-microliter 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^{-1}, Genecraft), 28.3 µL RNA/DNA-free water (Fermentas) and 1 µL of 1:10 diluted template. Amplicons were subsequently purified using a Fermentas GeneJET PCR Purification Kit.

Denaturing gradient gel electrophoresis (DGGE) was performed afterwards with equal concentrations of the amplicons and a gradient of 35 % to 65 % urea and 6 % acrylamide (Biorad DCode System). The DGGE gel was run for 17 hours at 110 V and 60 °C. Bands of interest were excised and transferred into a 0.5 mL tube containing 50 µL of sterile H_2O. The tube was shaken for 1 h at 37 °C. Reamplification was carried out using 4 µL template and 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). PCR products were purified using the Avegene Gel/PCR DNA fragment extraction kit. DNA concentrations were measured fluorimetrically (BMG Labtech FLUOstar OPTIMA). PCR products were sequenced by GATC Biotech AG. Sequence homologies were examined by BLAST (Basic Local Alignment Search Tool). Based on the DGGE profiles, a graphical representation of the bacterial community evenness was developed using Pareto–Lorenz (PL) distribution curves as previously described by Wittebolle et al. (2008). GelQuant.NET software provided by biochemlabsolutions.com was used to

determine the band intensities. The band intensities for every DGGE lane were ranked from high to low, and the cumulative band intensities were used as the y-axis. The cumulative normalized number of bands was set as the x-axis. Evaluation of the curves was conducted by comparison to a vertical 20 % x-axis line. The theoretical perfect evenness line was set as a 45° diagonal.

2.4 Quantitative real-time PCR

Quantification of *M. parvicella* was determined by quantitative real-time PCR (qPCR) using the 16S rRNA gene primer set S-S-M.par-0828-S-21/S-S-M.par-1018-A-17 (Kaetzke et al. 2005) (95 °C 10 min, 95 °C 15 sec, 53 °C 20 sec, 72 °C 20 sec) and SYBR Green as the fluorescent dye. All reactions were performed in triplicate. Amplicon length (190 bp) was checked by gel electrophoresis, and PCR products were sequenced. The amplification was carried out in 20 µL reactions containing 1 µL of a 1:100 dilution of extracted DNA, 10 µL SYBR-Green (Applied Biosystems PowerSYBRGreen), 0.5 µL forward primer (10 mM), 0.5 µL reverse primer (10 mM), 0.5 µL BSA (20 mg ml^{-1} Fermentas) and 7.5 µL RNA/DNA-free water (Fermentas). A standard curve using dilutions of *M. parvicella* PCR products (10^{-3} to 10^{-9}) was created for absolute quantification. Gene copy numbers of *M. parvicella* 16S rRNA were calculated relative to the standard curve and DNA concentrations (gene copies μL^{-1} = DNA concentration [g μL^{-1}] / (190 x 660) x 6.022 x 10^{23}). Melting curve (72 °C to 95 °C) analysis was conducted afterwards to determine the specificity of amplification. The amplification efficiency of the specific qPCR primer set was 98 % with an R^2 of 0.998 for the standard curve. The melting curve analyses showed one specific peak at 83 °C.

3. Results and Discussion

This study presents the chemical and molecular biological investigation of a foaming-prone digester in a full-scale biogas plant that failed in operation due to two foaming events. Process performance and the microbial community composition in the sewage sludge and FOG-digesting biogas plant were monitored over a 15-month period including two foam formation events. The FOG co-fermenting digester 3 was termed as a foaming-prone digester by the biogas plant operator, and therefore, this digester was chosen for molecular biological long-term monitoring over one year.

3.1 Foam formation related to FOG addition and substrate degradation

Chemical analyses of the digested sludge from December 2009 to February 2011 showed a decrease in TS and VS values between the upstream digesters 1 and 2 and the downstream digesters 3 and 4 (Table 1). While the average TS value in digesters 1 and

2 was approximately 35 g L^{-1}, the TS in the downstream digesters was approximately 32 g L^{-1}. The VS values of digesters 1 and 2 were at 25 g L^{-1}, while the VS values of digesters 3 and 4 were approximately 22 g L^{-1}.The concentration of volatile fatty acids (VFAs) in digester 3, which was charged with FOG, was at least 17 % higher than in the other digesters, with a value of approximately 790 mg L^{-1}. This was most likely caused by the higher VFA levels in the fed FOG. Pagilla et al. (1997) and Westlund et al. (1998) identified the accumulation of acetic acid as a foaming cause; however, it was unclear whether the accumulation of VFAs is a cause or the consequence of foaming (Ross and Ellis 1992). FOG addition to the foaming-prone digester was most likely a source of lipids and oleate. Frigon et al. (2006) showed that high lipid-loading rates are a factor for foam promotion in WWTPs, while Boe et al. (2012) observed that the highest potential to create foam in manure digestion systems is due to Na-oleate addition. Remarkably, the addition of FOG in the months of February and March did not provoke foaming; the digester was only affected by FOG addition in December and January. Therefore, the addition of FOG alone did not cause the foam formation in this case.

Table 1: Digester (D1-D4) performance of the biogas plant. Parameters were measured between December 2009 and February 2011 calculating average values and standard deviations. Total solid content (TS), volatile solid content (VS), volatile fatty acid concentration (VFA). [a]Months with FOG addition, [b]Months without FOG addition.

Reactor	pH	TS [g L^{-1}]	VS [g L^{-1}]	VFA [g L^{-1}]
D1	7.2 ± 0.1	35.7± 3.1	25.3 ± 1.9	535 ± 113
D2	7.2 ± 0.1	35.0 ± 3.2	24.8 ± 1.9	543 ± 134
D3	7.3 ± 0.1	33.2 ± 2.2	22.5 ± 2.0	790^{a} ± 50
				585^{b} ± 50
D4	7.3 ± 0.1	32.1 ± 2.0	21.8 ± 2.0	652 ± 62

3.2 Microbial community composition

Almost identical diversities in the genetic fingerprints indicated a similar microbial community composition in the four digesters (Figure 2). Close relatives of the genus *Ruminococcus* and the phylum *Spirochaetes* were identified in the genetic fingerprinting of both cascades (Table 2). Members of the genus *Ruminococcus* are

known to ferment cellulose, a typical component of sewage sludge, to acetate in the presence of methanogens (Zhang et al. 2009). A recent study on the physiology of members of the phylum *Spirochaetes* indicated the capability to oxidize acetate syntrophically (Lee et al. 2013). Most likely, the *Spirochaetes* bacteria provide hydrogen for hydrogenotrophic methanogenesis by the oxidation of acetate. Phylogenetic affiliation of the dominant bands revealed sequences assigned to the classes *Proteobacteria* and *Clostridia* in the foaming-prone digester over the one-year monitoring (Figure 3, Table 2), which are typically identified in anaerobic digesters (Regueiro et al. 2012). Furthermore, relatives of Candidatus *Cloacamonas acidaminovorans* and Candidatus *Microthrix parvicella* were detected in the four digesters. Because the abundance of these microorganisms differed in the foaming digester and foam, they are discussed in detail in the next section.

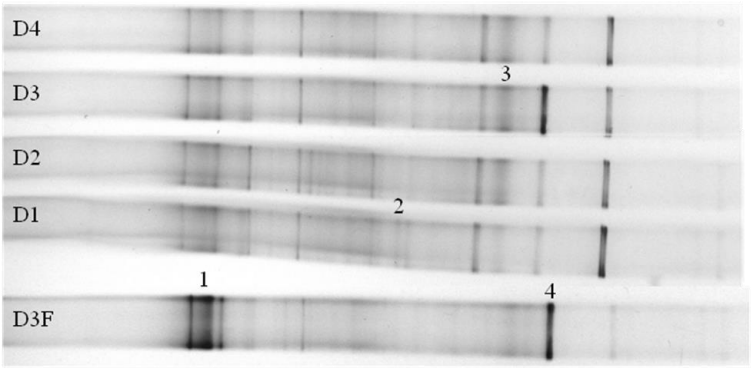

Figure 2: Comparative DGGE-analysis of 16S rRNA gene fragments using bacterial DNA from sludge samples taken from the digesters 1 to 4 (D1-D4) and a foam sample (D3F) taken at digester 3 (D3) of the biogas plant in December 2009. Numbered bands were sequenced. Higher band intensities of Candidatus *Cloacamonas acidaminovorans* (band 1) and Candidatus *Microthrix parvicella* (band 4) were detected in the foam sample.

The functional organization of the bacterial community in the four digesters (Figure 4a) as well as over the one-year monitoring period (Figure 4b) was analyzed by Pareto-Lorenz distribution patterns. Although the genetic fingerprinting revealed almost the same diversity for both cascades, the band intensities differed between the cascades. In both digesters of cascade I, more than 75 % of the band intensities derived from 20 % of the bands. Therefore, only few bacteria dominated the community in high abundance, whereas the other microorganisms were only present in low abundance. Remarkably, the banding pattern in cascade I did not change in the downstream digester, although

FOG was added to this stage. However, the genetic fingerprints showed different intensified bands in digester 3 than in digester 1 that indicated a change in the microbial abundance in the downstream digester. Cascade II showed a different distribution pattern. The microbial community composition of digester 2 was more even than the others, with approximately 50 % of the band intensities derived from 20 % of the microorganisms, whereas the downstream digester 4 was characterized by a more uneven community. In the upstream digester, the availability of organic substrates was higher than in the downstream digester due to previous fermentation in the upstream stage and the residence time. Therefore, other microorganisms prevailed in the first stage. The Pareto-Lorenz distribution over the one-year monitoring period revealed differences in the functional organization between spring and winter on the one hand and summer on the other hand. The patterns in March and December showed even microbial community compositions with 45 % and 30 % band intensities derived from 20 % of the bands. In contrast, the distribution pattern in August was more uneven, with 75 % of the cumulative band intensity derived from 20 % of the bands. Obviously, the FOG introduction, and thus the higher availability of lipids, in March and December influenced the microbial ecosystem so that more bacteria co-dominated in the digester. Furthermore, the lower temperatures in the spring and winter months most likely affected the bacterial composition in the fed sewage sludge; therefore, a more evenly distributed microbial population was introduced to the digester than in the summer.

3.3 Microbial community composition related to foam formation

Using the universal bacterial 16S rRNA gene primer pair, DGGE bands with higher intensities indicating a greater abundance of Candidatus *Cloacamonas acidaminovorans* were detected in the foam (Figure 2). Genome sequencing of this strain revealed genes for amino acid fermentation and degradation of proteins (Pelletier et al. 2008). Because high protein concentrations are known to promote foaming, *C. acidaminovorans* might serve as indicator for high protein concentrations. However, the physiology of this organism has not been studied so far, and the protein concentrations in the foam were not measured in the present study so that our assumptions regarding an indicator function of *C. acidaminovorans* were not supported. In addition, the highest DGGE band intensities of the filamentous bacterium *Microthrix parvicella* were detected in the digestate of the foaming digester and in the foam itself indicating higher abundance (Figure 2). Previous studies in anaerobic digesters have already shown the involvement of *M. parvicella* in foam formation (Pagilla et al. 1997, Westlund et al. 1998, Marneri et al. 2009). In addition to forming a filamentous network, relatives of *M. parvicella* have

a hydrophobic cell surface and thus a strong affinity towards the air/liquid interface. Biogas bubbles are trapped by the filaments carrying the microorganism at the surface of the digester, thus stabilizing the foam (Westlund et al. 1998). Furthermore, hydrophobic substances are released by *M. parvicella* that also stabilize the foam (Barber et al. 2005). Since another foam causing bacterium such as *Gordonia* spp. was not detected by DGGE analyses as being a dominant member of the microbial community, the focus of this study was on the investigation of digester foaming caused by *M. parvicella*. Absolute quantification using qPCR and the *M. parvicella* specific 16S rRNA gene primer set showed the highest *M. parvicella* 16S rRNA gene copy numbers per µg DNA in digester 3 during the foam formations, with values of more than 2 x 10^8 (Figure 5). Therefore, the specific qPCR results supported the previous DGGE analyses with the universal bacterial 16S rRNA gene primer pair.

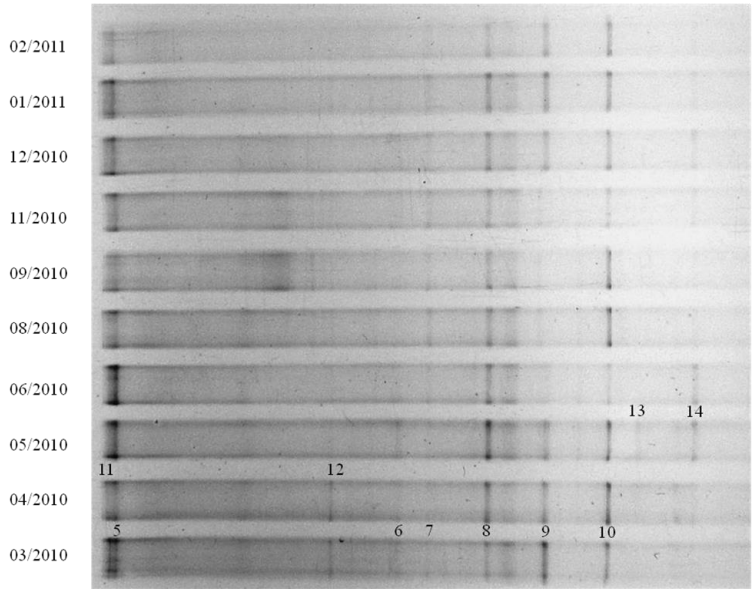

Figure 3: Comparative DGGE-analysis of 16S rRNA gene fragments using bacterial DNA from sludge samples taken from digester 3 (D3) of the biogas plant during a one-year sampling campaign (March 2010 – February 2011). Numbered bands were sequenced. The band intensities of Candidatus *Microthrix parvicella* (band 9) were higher in the wintertime and decreased in the summer.

The DGGE band intensity as well as the 16S rRNA gene copy numbers, which serve as a quantitative measure of *M. parvicella* abundance, changed during the year and showed the highest abundance of *M. parvicella* in wintertime (Figure 3, Figure 5).

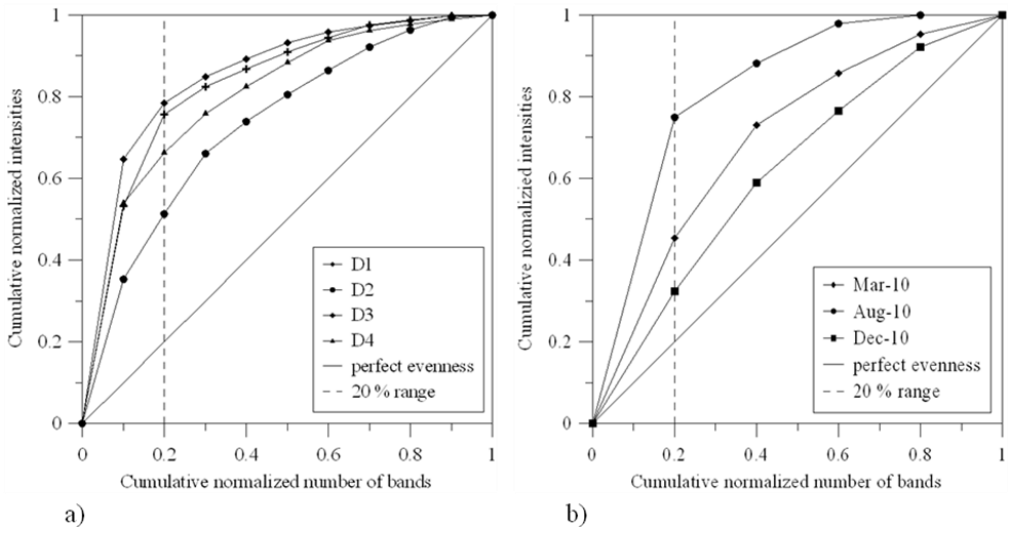

Figure 4: a) Pareto-Lorenz distribution curves based on the DGGE profiles of the digesters 1 to 4 (D1-D4) in December 2009. b) Pareto-Lorenz distribution curves based on DGGE profiles of digester 3 (D3) in the months March 2010, August 2010 and December 2010. Perfect evenness is illustrated as straight line. A dashed vertical line is plotted to evaluate the range of the Pareto value.

The ambient temperatures showed an opposing trend to the gene copy numbers. With increasing temperatures, the abundance of *M. parvicella* decreased and vice versa. Between June and September, the maximum ambient temperature measured at the nearby weather station reached a value of more than 25 °C, while the 16S rRNA gene copy numbers per µg DNA decreased by a factor of 10-100 to less than 1 x 10^6. With the decrease in temperatures from September to November, the 16S rRNA gene copies per µg DNA increased again to more than 1 x 10^7. The same seasonal trend with increasing *M. parvicella* gene copy numbers in the wintertime was detected for the incoming feed of the biogas plant. At this, in March, the 16S rRNA gene copy number per µg DNA amounted to 2.0 x 10^8 and decreased in September to 6.6 x 10^5. In November, the abundance of *M. parvicella* increased by a factor of 40, rising further to 2.9 x 10^8 16S rRNA gene copies per µg DNA by February. Several studies showed the influence of temperature on the abundance of *M. parvicella* in activated sludge (Eikelboom 1994, Mamais et al. 2006, Kumari et al. 2009). Foam formation in sludge digesters mostly occurred in the winter and spring months, when the abundance of *M. parvicella* in the incoming sewage sludge was high due to its low temperature optimum and its high affinity for LCFA. In consequence, higher amounts of *M. parvicella* were introduced to the digesters in the winter season. Mamais et al. (2006) observed a positive correlation between the presence of fatty acids and the amount of *M. parvicella*

during the winter periods in WWTP. Under aerobic, anaerobic and anoxic conditions, *M. parvicella* is able to store long chain fatty acids (LCFAs) as a carbon and energy source (Nielsen et al. 2002, Noutsopoulos et al. 2012).

Table 2: Phylogenetic affiliation of partial bacterial 16S ribosomal RNA gene sequences from DGGE-profiles of sludge samples from the biogas plant.

Band	Closest relative (Reference genbank accession number)	Phylum	Genbank accession number	Similarity
1	Candidatus *Cloacamonas acidaminovorans* (CU466930.1)	WWE1	KF356037	99 %
2	*Ruminococcus flavefaciens* (AM748742.1)	*Firmicutes*	KF356038	98 %
3	Uncultured *Spirochaetes* bacterium (CU922923.1)	*Spirochaetes*	KF356039	98 %
4	Candidatus *Microthrix parvicella* (FJ638889.1)	*Actinobacteria*	KF356040	99 %
5	Uncultured *Firmicutes* bacterium (AB780954.1)	*Firmicutes*	KF356041	89 %
6	Uncultured *Deltaproteobacteria* bacterium (CU926802.1)	*Proteobacteria*	KF356042	93 %
7	Uncultured *Syntrophaceae* bacterium (JX505414.1)	*Proteobacteria*	KF356043	94 %
8	Uncultured *Desulfofaba* sp. (GQ183225.1)	*Proteobacteria*	KF356044	95 %
9	Candidatus *Microthrix parvicella* (FJ638889)	*Actinobacteria*	KF356045	97 %
10	Uncultured Unclassified bacterium (CU917887.1)	n.d.	KF356046	95 %
11	Uncultured *Bacteroidetes* bacterium (CU922385.1)	*Bacteroidetes*	KF356047	91 %
12	Uncultured *Clostridiales* bacterium (JX456421.1)	*Firmicutes*	KF356048	96 %
13	Uncultured *Clostridiales* bacterium (JN173135.1)	*Firmicutes*	KF356049	89 %
14	Uncultured *Pelobacter* sp. (GU271649.1)	*Proteobacteria*	KF356050	97 %

However, because the upstream digesters 1 and 2 that were directly charged with the sewage sludge and the second downstream digester were not affected by foam formation, it is assumed that the FOG addition to digester 3 also favored foam formation. The dependence on foam-stabilizing materials and the abundance of filamentous bacteria with respect to foaming were also shown by Marneri et al. (2009). The comparatively low abundance of *M. parvicella* in the non-foaming month of March 2010 was consistent with a lowered foam formation risk. Only if FOG was added and the abundance of *M. parvicella* exceeded a threshold of 2×10^8 16S rRNA gene copies per µg DNA, as in December 2009 and January 2010, foam formation occurred. The *M. parvicella* threshold of 2×10^8 16S rRNA gene copies per µg DNA was shown to exceed the foaming threshold for mycolata in activated sludge treatment plants, which was given as 2×10^6 mycolata cells per mL liquor (Davenport et al. 2008). Furthermore, the absolute threshold of the present study complements the relative results of Kaetzke et al. (2005) and Kumari et al. (2009) on activated sludge that showed that even a relative *M. parvicella* population of 3-4 % might cause foaming problems.

3.4 Indication of anaerobic growth of Microthrix parvicella

The investigated biogas plant was operated as a cascade of two digesters. During the foam formations, the digestates of digester 3 showed a 71 % increase in *M. parvicella* 16S rRNA gene copy numbers in December 2009 and a 106 % increase in *M. parvicella* 16S rRNA gene copy numbers in January 2010 in relation to the upstream digester 1 of cascade I (Figure 5). *M. parvicella* was characterized as being able to reduce nitrate only to nitrite (Slijkhuis et al. 1983; Nielsen et al. 2002), and recently, genome sequencing of *M. parvicella* revealed a nitrate reductase but not a nitrite reductase (Muller et al. 2012). The foaming downstream digester was assumed to be nitrate depleted due to a preceding denitrifying step in the WWTP and the upstream digester. However, higher band intensities in the genetic fingerprinting as well as the increased *M. parvicella* specific 16S rRNA gene copy numbers in the downstream digester of the cascade indicated anaerobic growth, since microbial growth leads to higher amounts of DNA and gene copy numbers, respectively. Chacin et al. (1994) also showed that the growth of *M. parvicella* was possible in the complete absence of molecular oxygen or inorganically bound oxygen. However, it is unclear what metabolism was involved. It has to be taken into consideration that DNA studies deliver no direct information about microbe viability and that even inactive *M. parvicella* cells may promote foaming (de Los Reyes et al. 2002). Fluorescence *in situ* hybridization (FISH) might have been useful in this case, but it was shown that qPCR is more exact for quantification because an incomplete permeability of the cell wall and low rRNA levels due to low metabolic activity might lead to erroneous measurements (Kaetzke et al. 2005). Furthermore, it has to be considered that the effect of the hydraulic retention time as well as short cuts in the digesters might also result in a higher *M. parvicella* abundance in the downstream digester. However, increased gene copy numbers were determined in the downstream digester 3 in January 2010 compared to the upstream digester 1 in December 2009, which was the feedstock of digester 3. Thus, if anaerobic growth did not take place, digester 3 would have shown the same abundance of *M. parvicella* in January 2010 as digester 1 in December 2009. In addition, besides the foaming events, increased 16S rRNA gene copy numbers of *M. parvicella* were also detected in the downstream digester in May, June, September and November 2010. Compared to the upstream digester 1, the *M. parvicella*-specific gene copies per µg DNA in the downstream digester 3 increased by 130 % in May, by 75 % in June, by 50 % in September and by 2 % in November (Figure 5). Therefore, a retention time effect as a cause for an increased abundance of *M. parvicella* in the downstream digester can be excluded.

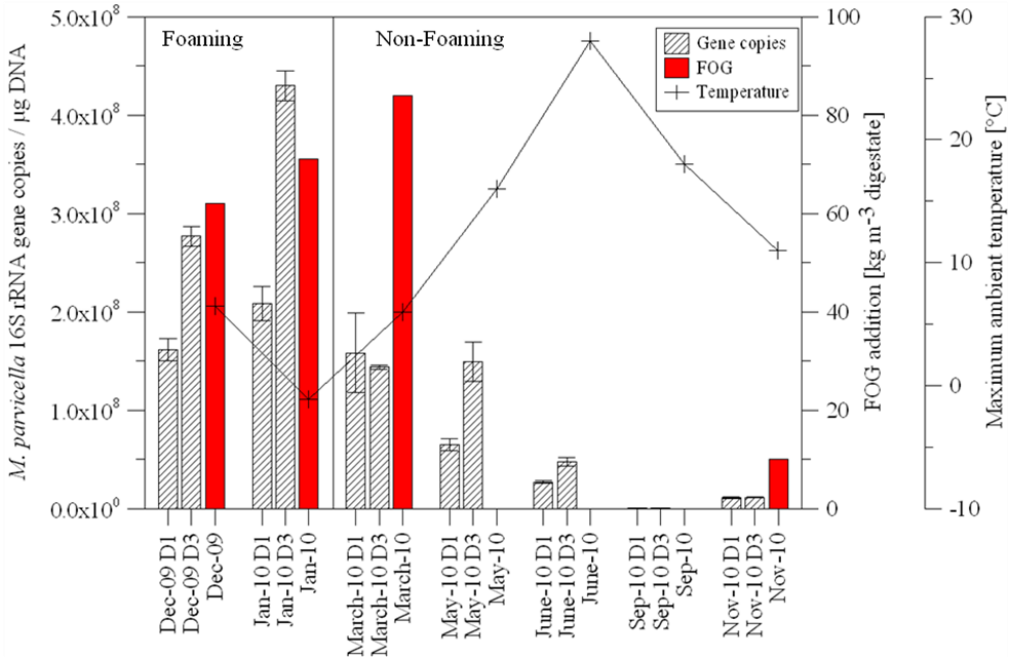

Figure 5: Abundance of *Microthrix parvicella* based on the 16S rRNA gene in sludge samples taken at digester 1 (D1) and digester 3 (D3) of the biogas plant from December 2009 to November 2010 with respect to FOG addition and maximum ambient temperatures.

4. Conclusions

Mesophilic digesters that are fed with activated sludge rich in *M. parvicella* (> 2 x 10^8 gene copies per µg DNA) are prone to intensive foam formation if the digesters are additionally loaded with FOG. In this case, the abundance of *M. parvicella* was shown to increase in the downstream digester of a cascade by a factor of 2. The indication of growth of the filamentous organism in the digester warrants further elucidation. As a matter of early warning, the operator can monitor the abundance of *M. parvicella* in the incoming feed in addition to the amount of FOG introduced.

Acknowledgements

The authors wish to thank the German "Federal Ministry for the Environment, Nature and Nuclear Safety" for funding the project "Optgas" (FKZ 03KB018), the plant operator for the collaboration and the Microbial GeoEngineering group at the GFZ in Potsdam for their valuable help in the analytical work. Willy Verstraete was supported by the MRP Biotechnology for a sustainable economy (01 MRA 510 W).

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