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Polyphasic Analyses of Methanogenic Archaeal Communities in Agricultural Biogas Plants[▽]

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Knowledge of the microbial consortia participating in the generation of biogas, especially in methane formation, is still limited. To overcome this limitation, the methanogenic archaeal communities in six full-scale biogas plants supplied with different liquid manures and renewable raw materials as substrates were analyzed by a polyphasic approach. Fluorescence *in situ* hybridization (FISH) was carried out to quantify the methanogenic *Archaea* in the reactor samples. In addition, quantitative real-time PCR (Q-PCR) was used to support and complete the FISH analysis. Five of the six biogas reactors were dominated by hydrogenotrophic *Methanomicrobiales*. The average values were between 60 to 63% of archaeal cell counts (FISH) and 61 to 99% of archaeal 16S rRNA gene copies (Q-PCR). Within this order, *Methanoculleus* was found to be the predominant genus as determined by amplified rRNA gene restriction analysis. The aceticlastic family *Methanosaetaceae* was determined to be the dominant methanogenic group in only one biogas reactor, with average values for Q-PCR and FISH between 64% and 72%. Additionally, in three biogas reactors hitherto uncharacterized but potentially methanogenic species were detected. They showed closest accordance with nucleotide sequences of the hitherto unclassified CA-11 (85%) and ARC-I (98%) clusters. These results point to hydrogenotrophic methanogenesis as a predominant pathway for methane synthesis in five of the six analyzed biogas plants. In addition, a correlation between the absence of *Methanosaetaceae* in the biogas reactors and high concentrations of total ammonia (sum of NH_3 and NH_4^+) was observed.

During the last decade the production of biogas from organic materials and residues has increased continuously in order to reduce the greenhouse gas emission resulting from the use of fossil energy sources. The energy-bearing substance of biogas is methane, which is produced as an end product of microbial anaerobic degradation of organic substrates, such as energy crops like maize, grains, grasses, or beets. Research for optimization of biogas production from renewable materials was initially focused on the evaluation of substrate eligibility and on the development and optimization of technical systems. However, biogas formation primarily depends on the structure and activity of the microbial community (28).

The key microorganisms in the biogas formation process are the methane-generating microorganisms (methanogens). The capacity for methanogenesis is limited to members of the domain *Archaea* and, within this domain, on the phylum *Euryarchaeota*. With respect to the main metabolic precursors used, methanogens are usually divided into two groups: the aceticlastic methanogens that strictly metabolize acetate and the hydrogenotrophic methanogens that use H_2 or formate as an electron donor and CO_2 as a carbon source for their metabolism. Besides these major groups, certain methanogens are also able to convert methyl groups, methylamines, or methanol to methane (23, 40). The substrates for the methanogens are provided by several physiological groups of bacteria which degrade organic matter, sometimes in close syntrophic interaction with the methanogens (1).

Several studies on the microbial diversity present in lab-scale biogas reactors supplied with renewable raw material (7, 57) have been recently published. However, analyses under laboratory conditions do not necessarily reflect conditions in full-scale reactors (35). Therefore, further research on the methanogenic community in full-scale biogas reactors is crucial.

Generally, studies regarding the microbial community structure in full-scale biogas reactors have focused on different systems for wastewater treatment or classical biogas plants based on manure digestion (32, 38, 43). In most systems, approximately 70% of the carbon fixed in methane was derived from acetate. Only minor amounts, up to approximately 30%, were deduced from CO_2 (1, 42). Together with the presence of huge assemblages of *Methanosarcina* sp., it was assumed by some authors that aceticlastic methanogenesis was the predominant pathway for methane formation. Moreover, as shown by other studies, the relative contribution

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of H_2/CO_2 versus acetate as metabolic precursors for methanogens can be quite different in other anaerobic environments (10, 33, 37). However, the methanogenic microfloras in full-scale biogas reactors supplied with energy crops as a primary or sole substrate have rarely been studied (35, 37, 45).

The aim of this study was to gain insight into the diversity of methane-producing *Archaea* in six full-scale biogas plants supplied with renewable raw material and different types of liquid manure as substrates. Therefore, a polyphasic approach with three different culture-independent techniques (fluorescence *in situ* hybridization [FISH], quantitative PCR [Q-PCR], and 16S rRNA gene analysis) to analyze methanogen diversity was carried out to overcome the known limitations of each single approach (15, 46). To analyze potential effects of different process parameters on the methanogenic archaeal community, the reactor performances were correlated with the apparent archaeal diversity.

MATERIALS AND METHODS

Reactor operation and sampling. Six full-scale biogas plants (R1 to R6) located in northeastern Germany (Brandenburg, Mecklenburg-Vorpommern, and Sachsen-Anhalt) were chosen for polyphasic analysis of the composition of the methane-producing *Archaea* in biogas plants supplied with a mixture of different liquid manures and renewable raw materials as substrates (Table 1). All reactors were operated at mesophilic temperatures and under wet fermentation conditions. The substrates of reactors R1 to R3 and of reactors R5 and R6 consisted of mixtures of animal manure and renewable raw materials. Reactor R4 was fed exclusively with renewable raw material.

The biogas plants were sampled once in a time period from August 2006 to July 2007. At the date of sampling all biogas plants had been operated for at least 1 year. From each of the six biogas plants, four samples of 5 liters each were taken from the stirred reactor contents in time intervals of 15 min. From each individual sample 500 ml was pooled and stored at room temperature for a maximum of 12 h until further processing. Corresponding operating parameters are summarized in Table 1. Total ammonia nitrogen is defined as the sum of NH_3-N and NH_4^+-N. The NH_3-N (ammonia nitrogen) concentration was calculated with following formula after Anthonisen et al. (4) and Gallert and Winter (22): (total ammonia-N $\times$ 10^{pH})/(K_b/K_w + 10^{pH}), where N (nitrogen) concentration is in g liter^{-1}, K_b/K_w is $e^{(6344/273+T)}$, and T is the temperature in °C.

All organic acids including volatile fatty acids (VFA) were calculated as acetic acid equivalents (HAc eq.).

FISH. A total of 25 ml of pooled reactor sample was mixed with 1 volume of 96% ethanol and kept at −20°C for a maximum of 12 h. The fixation of the samples with 3.7% formaldehyde was carried out according to a slightly modified protocol published by Daims et al. (19). To disperse the extracellular polymeric substances (EPS) which promote the adhesion of cells to plant particles and the formation of cell aggregates, an enzyme mixture of α-glucosidase, β-galactosidase, and lipase (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used according to a modified protocol developed by Böckelmann et al. (8). A total of 100 μl of each formaldehyde-fixed sample was washed twice with 1× phosphate-buffered saline ([PBS] 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 5.7); the pellet was mixed with 2 U of α-glucosidase, 2 U of β-galactosidase, 10 U of lipase, and 2 mM $MgCl_2$. PBS (1×; pH 5.7) was added to a final volume of 500 μl. After incubation for 1 h (at 30°C and 1,000 × *g*), a two-step ultrasonic treatment (Sonoplus GW2070; Bandelin, Berlin, Germany) for 30 s each at pulse level 1 with 50% power (approximately 35 W) was carried out to support the dispersion of EPS. For each reactor sample, a dilution series (100-, 500-, and 1,000-fold) was performed.

For identification of total archaeal and bacterial populations the ARC915 (58) and the EUB223 (2) probes, respectively, were used in experiments performed as triplicates. Specific probes for *Methanomicrobiales* (MG1200) (50), *Methanobacteriales* (mix of MB310 and MB1174) (50), *Methanosarcinaceae* (Ms821) (50), and *Methanosaetaceae* (Mx825) (18, 50) were applied only for biogas plants R1, R4, R5, and R6 in duplicates. The oligonucleotide probes were used under optimal stringency conditions as described in the probeBase data bank (41). All probes were Cy3 labeled and purchased from metabion (Martinsried, Germany). 4′,6′-Diamidino-2-phenylindole (DAPI) was used for total cell detection.

As positive controls for the specific probes the following reference cultures were used: *Methanoculleus marisnigri* (DSM1498), *Methanospirillum hungatei* (Mh1), *Methanosaeta concilii* (DSM6752), *Methanobacterium formicicum* (DSM1525), *Methanobrevibacter arboriphilus* (DSM1125), and *Methanothermobacter thermautotrophicus* (DSM1053).

Prior to hybridization each of the 10 wells of a Teflon-coated slide was treated with 0.1% gelatin and 0.01% $CrK(SO_4)_2$. Hybridizations and washing procedures were performed according to a modified protocol of Daims et al. (19). The hybridization buffer as described by Daims et al. (19) was supplemented with 10× Denhardts reagent (20) to speed up the hybridization and to prevent nonspecific binding of the probes. Hybridization was performed in a buffer-saturated humidity chamber at 46°C for 2 h in a hybridization oven (HL-2000 HybriLinker; UVP Laboratory Products, Cambridge, United Kingdom).

Prior to microscopic analysis, 5 μl of the antifading reagent Citifluor AF1 and 0.1 μl of DAPI (70 μg ml^{-1}) were added to each sample. Fluorescence was detected by a Nikon Optiphot-2 microscope (Nikon, Düsseldorf, Germany) fitted for epifluorescence microscopy with a 100-W mercury high-pressure bulb (HBO 103W/2) and filter sets for DAPI (DAPI AMCA) and Cy3 (HQ:Cy3). Digital images of the samples were taken with a Nikon Digital Sight DS-2Mv (Nikon, Düsseldorf, Germany) and the software NIS-Elements, version 2.2. For determination of total cell counts, approximately 1,000 DAPI-stained cells from independent, randomly chosen microscopic fields were counted at a magnification of ×630. In the case of aggregate-forming *Methanosaeta* spp. and *Methanosarcina* spp., the area of cell aggregates was determined using the software NIS-Elements, version 2.2. Based on aggregate area, the corresponding cell numbers were estimated as follows: members of the genus *Methanosarcina* have an average diameter of 2 μm (23), which corresponds to an area of 3.1415 μm^2. A *Methanosaeta* sp. cell is 1.05 μm wide and 4.5 μm long on average (23), resulting in a two-dimensional image with an average area of 4,725 μm^2.

Total cell counts, total bacteria, and total archaea per ml of reactor contents were calculated by following formula (49): $A_{well}/A_{count} \times X_m \times v$, where A_{well} is the effective area of well surface (35.26 mm^2), A_{count} is the surface area of a microscopic field (1.44 mm^2), X_m is the average cell number per microscopic field, and v is the dilution factor (1 ml^{-1}). The sum of the average cell counts determined by specific FISH probes served as a basis for the calculation of percent distribution of the four main methanogenic groups.

DNA extraction and purification. From the pooled reactor samples (total volume, 2 liters) four parallel subsamples of 40 ml each were taken and processed separately. The genomic DNA was extracted and purified as described previously (45). This included sample purification by centrifugal steps; enzymatic cell lysis with lysozyme, proteinase K, and sodium dodecyl sulfate (SDS); purification steps with cetyl-trimethylammonium-bromide (CTAB) and chloroform-isoamylalcohol (24:1); and a subsequent isopropyl alcohol precipitation.

For the biogas plants R1 and R6, an additional purification step was necessary to remove PCR inhibitors. A purification technique based on low-melting point (LMP) agarose (AppliChem GmbH, Darmstadt, Germany) was carried out according to a modification of the protocol of Moreira (44).

Microscopic verification of cell lysis. Before and after the cell lysis procedure, microscopic verification of complete cell lysis was conducted: the samples were examined by phase-contrast light microscopy (Nikon Optiphot-2 with a 40× objective; Nikon, Düsseldorf, Germany) and UV fluorescence microscopy (Nikon Optiphot-2 with a 40× objective and filter block DM430; Nikon, Düsseldorf, Germany). Fluorescence microscopy was used to detect hydrogenotrophic methanogens by their coenzyme F_{420} autofluorescence (56). In all samples only very few unlysed cells were visible by phase-contrast microscopy, which indicated a sufficient treatment for cell lysis. Accordingly, the samples showed none of the F_{420} fluorescence signals that are characteristic for many methanogens, indicating lysis of the majority of methanogenic cells.

Q-PCR. For Q-PCR a 5′ nuclease assay (TaqMan assay) was applied and performed on an ABI 7300 System (Applied Biosystems, Darmstadt, Germany). Primer sets and TaqMan probes, including their PCR conditions, for the domains *Archaea* and *Bacteria*, the orders *Methanomicrobiales* and *Methanobacteriales*, and the families *Methanosarcinaceae* and *Methanosaetaceae* as published by Yu et al. (64, 65) were applied as described by Nettmann et al. (45). Standard amplification curves were constructed according to Nettmann et al. (45). The Q-PCR results were analyzed with the 7300 Real-Time PCR System Sequence Detection Software, version 1.3 (Applied Biosystems, Darmstadt, Germany). The 16S rRNA gene copies in the samples were calculated as described previously (45).

Construction and analysis of 16S rRNA gene clone libraries. Construction of 16S rRNA gene clone libraries and the subsequent library screening by amplified rRNA gene restriction analysis (ARDRA) was carried out as described previously (45). The following 16S rRNA gene primers were used: Arch16S-Forw2 (5′-YGAYTAAGCCATGCRAGT-3′) modified after (24) and Univ16S-Rev5

TABLE 1. Biogas plants and operating conditions[a]

Biogas plant	Feedstock components (% fresh wt of total feedstock)[b]	Reactor vol (m^3)	Organic loading rate ($kg_{ODS}\ m^{-3}\ day^{-1}$)	Retention time (days)	Total biogas production ($m_{BG}^3\ m^{-3}\ day^{-1}$)[d]	CH_4 production ($m_{CH4}^3\ m^{-3}\ day^{-1}$)[d]	Operation temp (°C)	pH	Total ammonia (g $liter^{-1}$)[e]	NH_3-N (g $liter^{-1}$)	NH_4^+-N (g $liter^{-1}$)	VFA ($g_{HAc\ eq.}\ liter^{-1}$)
R1[c]	Cattle dung (18%), cattle sewage (41%), maize silage (38%), water (2.5%)	3,600	3.8	41	2.90	1.5	36.8	7.6	1.6	0.08	1.52	1.4
R2	Pig liquid manure (50%), maize silage (39%), turkey dung (9%)	1,300	2.9 ± 0.2 (2.5)	30.2 ± 3.1 (35)	3.6 ± 0.2 (3.4)	1.9 ± 0.2 (1.8)	44.6 ± 0.4 (44.0)	8.2 ± 0.1 (8.3)	4.3 ± 0.0 (4.3)	1.15 ± 0.1 (1.23)	3.11 ± 0.1 (3.03)	7.6[f]
R3	Pig liquid manure (57%), maize silage (40%)	2,700	3.9 ± 0.2 (3.9)	47 ± 1.5 (48)	2.5 ± 0.1 (2.7)	1.3 ± 0.1 (1.4)	38.5 ± 0.3 (38.7)	7.7 ± 0.1 (7.6)	2.0 ± 0.5 (1.5)	0.12 ± 0.04 (0.08)	1.85 ± 0.41 (1.42)	2.5 ± 0.5 (2.8)
R4[c]	Maize silage (82%), barley grain (12%), water (6%)	3,674	3.4	108	2.6	1.4	44.7	7.5	2.6	0.37	2.23	2.1
R5	Cattle liquid manure (64%), maize silage (37%), pig liquid manure (6%), turkey dung (2%)	2,640	4.2 ± 0.7 (4.0)	36.0 ± 3.2 (34.0)	NA	NA	41.0 ± 0.1 (41.0)	7.9 ± 0.1 (7.8)	4.0 ± 0.0 (4.0)	0.45 ± 0.04(0.38)	3.55 ± 0.04(3.62)	2.4[f]
R6	Cattle liquid manure (76%), maize silage (13%), grass silage (5%), cattle dung (4%), grains (2%)	1,950	2.5 ± 0.3 (2.4)	46.9 ± 1.4 (47.0)	1.3 ± 0.1 (1.3)	0.7 ± 0.0 (0.7)	39.9 ± 0.8 (39.3)	7.9 ± 0.3 (7.4)	1.7 ± 0.0 (1.7)	0.20 ± 0.08(0.06)	1.50 ± 0.08(1.64)	1.5[f]

[a] Data are shown are average values with standard deviations of a time period of 4 weeks before sampling (data of the sampling day are in parentheses). ODS, organic dry substance; BG, biogas; HAc eq., acidic acid equivalent of all organic acids; NA, not analyzed.
[b] For components representing ≥2% of the total.
[c] One-time sampling from biogas plant at the beginning of a measurement period.
[d] Data normalized with 273.15 K and 1,013.25 hPa.
[e] Sum of NH_3-N and NH_4^+-N.
[f] Data from day of sampling.

TABLE 2. Cell and 16S rRNA gene copy concentrations in the six biogas reactors determined by FISH and Q-PCR

Biogas plant	Total cell count (ml^{-1})[a]	Bacterial abundance determined by:		Archaeal abundance determined by:	
		FISH (cells ml^{-1})	Q-PCR (16S rRNA gene copies ml^{-1})	FISH (cells ml^{-1})	Q-PCR (16S rRNA gene copies ml^{-1})
R1	$(8.5 \pm 5.6) \times 10^8$	$(6.2 \pm 2.7) \times 10^8$	$(2.5 \pm 0.4) \times 10^{11}$	$(6.5 \pm 5.8) \times 10^7$	$(2.0 \pm 0.3) \times 10^{10}$
R2	$(3.1 \pm 2.5) \times 10^8$	$(9.6 \pm 7.0) \times 10^7$	$(9.3 \pm 2.8) \times 10^{11}$	$(1.7 \pm 1.4) \times 10^7$	$(3.1 \pm 0.2) \times 10^{10}$
R3	$(7.6 \pm 0.0) \times 10^8$	$(4.6 \pm 2.3) \times 10^8$	$(9.6 \pm 3.7) \times 10^{10}$	$(3.0 \pm 1.2) \times 10^7$	$(5.0 \pm 0.8) \times 10^9$
R4	$(1.9 \pm 5.4) \times 10^8$	$(1.0 \times 0.2) \times 10^9$	$(1.2 \pm 0.6) \times 10^{11}$	$(7.9 \pm 2.9) \times 10^7$	$(3.0 \pm 0.9) \times 10^9$
R5	$(1.1 \pm 1.0) \times 10^8$	$(5.6 \pm 4.1) \times 10^8$	$(4.1 \pm 0.9) \times 10^{11}$	$(7.8 \pm 6.0) \times 10^7$	$(2.9 \pm 0.9) \times 10^{10}$
R6	$(1.9 \pm 8.7) \times 10^8$	$(9.8 \pm 5.3) \times 10^8$	$(2.5 \pm 0.2) \times 10^{11}$	$(1.3 \pm 0.9) \times 10^8$	$(2.1 \pm 0.7) \times 10^{10}$

[a] Detected by DAPI staining.

(5′-TGCTCCCCCGCCAATTCCT-3′), modified according to Fernandez et al. (21). Subsequently, the amplified DNA fragments (860 bp) were digested with the restriction enzymes BsuRI and Hin6I (Fermentas, St. Leon-Rot, Germany). Clones with similar ARDRA patterns were taken as operational taxonomic units (OTUs). For DNA sequencing (MWG Biotech AG, Martinsried, Germany) and subsequent phylogenetic classification, one clone representative of a group of clones with identical ARDRA patterns was chosen.

Phylogenetic analysis. All obtained 16S rRNA gene sequences were checked for chimeric artifacts by the Chimera Check software tool (14). Alignments of 16S rRNA gene sequences and reference sequences from NCBI GenBank were performed with the software package Mega, version 4.0 (61), and ClustalW, version 1.6 (62), using a neighbor-joining algorithm (51) with the Kimura-2 parameter (34) as a distance correction model, applying standard settings. For direct comparison of determined 16S rRNA gene nucleotide sequences (approximately 860 bp) with individual reference sequences, the uncorrected *p*-distances (Mega, version 4.0, software package) were calculated as indicators of nucleotide sequence dissimilarities.

Statistical analyses of 16S rRNA gene clone libraries. Rarefaction analyses to obtain richness curves were performed with the software Analytical Rarefaction, version 3.1 (http://www.uga.edu/~strata/software/Software.html). The interpolating rarefaction method evaluates how the number of OTUs in a sample changes with the number of individuals (29) and reflects the OTU richness of a clone library. Additionally, the extrapolating Chao I index (11) was calculated with the software EstimateS, version 8.0.0 (R. K. Colwell, University of Connecticut, Storrs [http://viceroy.eeb.uconn.edu/EstimateS]). This nonmetric estimate index considers the OTUs represented by one or two clones in the sample. The 95% confidence intervals represent the significance of measuring points.

The following formula described by Good (26) was used to calculate the coverage of the clone libraries: $1 - (n/N)$, where n is the number of OTUs represented by one clone, and N is the total number of clones.

In order to quantify the diversity of archaea, the Shannon index (H) was calculated (55). This index gives the proportional abundance of species and reacts sensitively to rare species. Evenness (E) was computed to describe the uniformity of the distribution of the individuals over the number of OTUs (47). These analyses were performed using the software package PAST, version 1.75b (P. D. Ryan, University of Oslo, Oslo, Norway [http://folk.uio.no/ohammer/past]).

Comparison of archaeal communities in the biogas plants. Similarities between archaeal communities and archaeal OTU abundances in the biogas plants were calculated by the Chao-Jaccard (CJ) similarity index (12). This index is slightly sensitive to the sample size because it accounts for the unseen shared species. The calculation of the CJ index is based upon the diversity of methanogens as determined by the abundance of OTUs and ARDRA patterns. All CJ similarity values were computed with the software EstimateS, version 8.0.0 (see above). The distances for multidimensional scaling (MDS), calculated by the software program PERMAP (R. B. Heady and J. L. Lucas, University of Louisiana, Lafayette [http://www.ucs.louisiana.edu/~rbh8900/]), were used to provide a two-dimensional illustration of the diversity similarities between the samples. To analyze potential effects of main substrates on methanogenic biocoenosis, these parameters were correlated with the determined archaeal diversities.

Nucleotide sequence accession numbers. All nucleotide sequences obtained in this study have been deposited in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers EU447678, EU636905, FJ222201 and FJ222202, FJ222204 and FJ222205, FJ222208 to FJ222225, FJ222228 and FJ222229, FJ222231 to FJ222236, and FJ356063 to FJ222266.

RESULTS

Reactor performance. All biogas plants were sampled during a stable operating phase, where the biogas and methane production rates were maintained at constant rates. All biogas plants were operated in a mesophilic temperature range between 36.8 (R1) and 44.6°C (R2). The pH values of all six biogas reactors varied from pH 7.4 (R6) to 8.2 (R2) and were thus in the lower alkaline range (Table 1). Biogas production rates of the biogas plants ranged between 1.3 (R6) and 3.6 m^3 m^{-3} day^{-1} (R2) (Table 1). Methane yields amounted to between 51 and 52% (vol/vol) of total biogas yield. These yields were comparable to production rates and methane yields, respectively, of other agricultural biogas plants (9).

The VFA concentrations in biogas plants R2 (7.6 g $liter^{-1}$), R3 (2.8 g $liter^{-1}$), and R5 (2.4 g $liter^{-1}$), supplied with pig liquid manure (R2 and R3) and cattle liquid manure (R5), were higher than in R1 (1.4 g $liter^{-1}$) and R6 (1.5 g $liter^{-1}$), fed with cattle liquid manure, and in R4 (2.1 g $liter^{-1}$), fed with maize silage (Table 1). The total ammonia concentration (sum of NH_3 and NH_4^+-nitrogen) in reactors R2, R4, and R5 ranged between 2.6 and 4.3 g $liter^{-1}$ and was higher than the concentrations in reactors R1, R3, and R6, with total ammonia concentrations between 1.6 and 2.0 g $liter^{-1}$ (Table 1). The calculated NH_3-N concentrations ranged from 0.01 g $liter^{-1}$ (R4) to 1.15 g $liter^{-1}$ (R2) (Table 1).

Methanogenic archaeal communities in agricultural biogas reactors. For the detection and quantification of main methanogenic groups in six agricultural biogas plants, two different techniques, microscopic analysis by FISH and 16S rRNA gene quantification by Q-PCR analysis, were applied.

The concentrations of bacteria determined by FISH (EUB338 probe) for the six biogas plants ranged between $9.6 \times 10^7 \pm 7.0 \times 10^7$ (R2) and $1.0 \times 10^9 \pm 0.2 \times 10^9$ (R4) cells per ml of reactor content (Table 2), which corresponds roughly to 51% ± 4% (R2) and 65% ± 12% (R1) of total cell counts determined by DAPI staining. The low percentage of bacteria detected by FISH probe EUB338 might be caused by the high background fluorescence in the samples, which interferes with probe signal. Archaeal abundances ranged between $1.7 \times 10^7 \pm 1.4 \times 10^7$ (R2) and $1.3 \times 10^8 \pm 0.9 \times 10^8$ (R6) cells per ml of reactor content (Table 2). The percentage of archaea determined by FISH ranged between 3% ± 0.4% (R6) and 7% ± 3.9% (R1) of total cell counts.

The detected numbers of bacterial and archaeal 16S rRNA gene copies, respectively, were higher than the counted bacte-

TABLE 3. Percentage of *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcinaceae*, *Methanosaetaceae*, and unclassified *Euryarchaeota* calculated on basis of the total archaeal community as determined by ARDRA, Q-PCR, and FISH

Reactor and analysis method	Distribution of the indicated methanogenic group (%)[a]				
	MM	MB	Ms	Mx	NCE
R1					
ARDRA	75	4	ND	21	ND
Q-PCR	61	2	<1	36	ND
FISH	68	11	ND	21	ND
R2					
ARDRA	99	1	ND	ND	ND
Q-PCR	99	<1	ND	ND	ND
FISH	NA	NA	NA	NA	NA
R3					
ARDRA	98	ND	1	1	ND
Q-PCR	85	3	ND	12	ND
FISH	NA	NA	NA	NA	NA
R4					
ARDRA	96	1	ND	ND	3
Q-PCR	83	16	<1	ND	ND
FISH	64	36	ND	ND	ND
R5					
ARDRA	93	1	4	ND	2
Q-PCR	97	2	<1	ND	ND
FISH	60	10	30	ND	ND
R6					
ARDRA	49	5	ND	40	6
Q-PCR	24	3	<1	72	ND
FISH	30	6	ND	64	ND

[a] MM, *Methanomicrobiales*; MB, *Methanobacteriales*; Ms, *Methanosarcinaceae*; Mx, *Methanosaetaceae*; NCE, not classified *Euryarchaeota*; ND, not detected; NA, not analyzed.

rial and archaeal cell densities, respectively, as determined by FISH (Table 2). Because of different 16S rRNA gene copy numbers in microbial genomes, the results of Q-PCR show only relative abundances of the microorganisms.

Table 3 shows the percent distribution of the main methanogenic groups based on results of ARDRA, Q-PCR, and FISH analyses of the six biogas reactors. The hydrogenotrophic *Methanomicrobiales* represented the dominant order in reactors R1, R4, and R5, determined as 60 to 64% of archaeal cell counts (FISH) and 61 to 97% of archaeal 16S rRNA gene copies (Q-PCR). In addition, the Q-PCR analyses showed a dominance of *Methanomicrobiales* in R2 with 99% and in R3 with 85% of archaeal 16S rRNA gene copies (Table 3).

The aceticlastic *Methanosaetaceae* were detected in reactors R1 and R6, with 21% and 64% of archaeal cell counts (FISH), respectively. Q-PCR analysis resulted in 36% (R1) and 72% (R6) *Methanosaetaceae* of archaeal 16S rRNA gene copies, respectively. In reactor R3 *Methanosaetaceae* were assigned to 12% of archaeal 16S rRNA gene copies by Q-PCR analysis. However, this archaeal group was not detected in reactors R2, R4, and R5 (Table 3).

In reactors R1, R4, R5, and R6 *Methanosarcinaceae* were detected with less than 1% of archaeal 16S rRNA gene copies by Q-PCR. With FISH analysis this family was detected with 30% of archaeal cell counts only in reactor R5 (Table 3).

Methanobacteriales seemed to be underrepresented by the Q-PCR analysis in contrast to FISH analysis: the Q-PCR values of reactors R1 to R6 ranged between <1 and 16% of archaeal 16S rRNA gene copies, whereas the FISH values in R1, R4, R5, and R6 ranged between 8 and 36% of archaeal cell counts (Table 3).

For the differentiated capture of methanogenic diversity, one 16S rRNA gene clone library with approximately 100 clones was constructed for each biogas reactor (Table 4). In total, 643 clones of six clone libraries were analyzed by ARDRA; 29 clones considered to be possible chimeras were excluded from subsequent analysis. By 16S rRNA gene sequencing, 35 OTUs representative of 488 clones were found to be of archaeal origin. The remaining 126 clones were of bacterial origin.

The sample sizes of reactors R2, R4, R5, and R6 were sufficient for analysis of the diversity of the major methanogenic groups, as shown by a rarefaction analysis of the six clone libraries (data not shown). In the case of R1 and R3 the rarefaction curves showed no asymptotic progression. Hence, the Chao I index was additionally calculated to estimate a sufficient sample size (Table 4). The Chao I values of R1, R4, and R6 showed only small differences from the actual OTU number of detected archaea, indicating that the sample size of the clone libraries was sufficient and confirming the results from the rarefaction analysis. In contrast, the results of the Chao I analyses of R2, R3, and R5 revealed higher OTU estimation values than actual OTU numbers. However, the lower 95% confidence intervals were near the actual sample size (Table 4), indicating that the majority of methanogens in the samples were detected. The coverage values (75 to 96%) of all clone libraries supported this assumption (Table 4).

Biogas reactors R1 and R6 showed the highest methano-

TABLE 4. Statistical analyses of 16S rRNA gene clone libraries

Reactor	Total no. of clones	No. of archaeal clones	No. of archaeal OTUs	Shannon index	Evenness	Chao I index[a]	Coverage (%)[b]
R1	102	28	7	1.60	0.71	7 (1–15)	75
R2	108	99	11	0.96	0.24	39 (18–116)	92
R3	103	88	10	1.26	0.35	25 (13–78)	93
R4	100	80	9	1.43	0.47	10 (9–18)	96
R5	113	105	11	1.04	0.29	32 (16–97)	90
R6	117	88	13	1.99	0.56	16 (13–35)	94

[a] Numbers in parentheses represent the 95% confidence intervals.
[b] Based on the formula of Good (26).

genic diversity, which was determined by Shannon index values (*H*) of 1.60 and 1.99, respectively. The lowest diversity was found in R2 (*H* of 0.96). The OTUs determined by ARDRA of all biogas plants were assigned to the orders *Methanomicrobiales*, *Methanobacteriales*, and *Methanosarcinales* belonging to the phylum *Euryarchaeota*. The calculation of uncorrected *p*-distances (see the data at http://www.atb-potsdam.de/veroeffent/AEM_Nettmann_et_al_2010_Supplement.pdf) between nucleotide sequences determined in this study and NCBI GenBank reference sequences resulted in similarity values between 83 and 100%. In accordance with FISH and Q-PCR analyses, the majority of all detected archaeal clones in five of six biogas plants was allocated to the hydrogenotrophic order *Methanomicrobiales* (Table 3) and within this order to the genera *Methanoculleus* (97 to 100% nucleotide similarity) and *Methanospirillum* (95% nucleotide similarity) (see the data at the URL mentioned above). This was supported by the low evenness values (0.24 to 0.56) of the analyzed reactors, except for reactor R1 (*E* of 0.71), which indicated the existence of some dominant OTUs (Table 4).

In R1 (21% of archaeal clones), R3 (1% of archaeal clones), and R6 (4% of archaeal clones), the family *Methanosaetaceae* were detected (Table 3), which is known to be the only methanogenic group exclusively utilizing acetate for methanogenesis. The uncorrected *p*-distance analysis of detected OTUs and reference sequences resulted in a nucleotide similarity of 97% with known *Methanosaeta* species (see data at the URL mentioned above).

In addition, in reactors R3 (1% of archaeal clones) and R5 (4% of archaeal clones), OTUs were detected that were assigned to the genus *Methanosarcina* (see the data at the URL mentioned above). This genus utilizes acetate as well as methyl compounds and CO_2 with H_2 or formate as electron donors.

With 1 to 5% of archaeal clones (Table 3), the hydrogenotrophic order *Methanobacteriales*, represented by the genera *Methanobacterium* (91 to 94% nucleotide similarity) and *Methanobrevibacter* (94 to 95% nucleotide similarity) (see the data at the URL mentioned above), was detected in all digesters, except in R3.

Detection of hitherto uncultivated potential methanogens. In reactors R4 and R5 OTUs were detected by ARDRA which could not be assigned to known species. The calculation of the uncorrected *p*-distances between the 16S rRNA gene sequence of these OTUs and known reference sequences showed only 83% similarity to DNA sequences of the CA-11 cluster (see the data at the URL mentioned above). Members of this cluster were first found in a fluidized-bed reactor fed by wine distillation waste (25).

Furthermore, the 16S rRNA gene sequence of one OTU detected in reactor R6 showed 98% nucleotide sequence similarity to a hitherto unclassified member of the ARC-I cluster (see the data at the URL mentioned above). Members of the ARC-I cluster were first found in an anaerobic slugged digester by Chouari et al. (13).

Similarities in the methanogenic archaeal community structure between the analyzed biogas plants. The pairwise diversity similarities based on the OTU abundance of archaeal origin in the 16S rRNA gene clone libraries were calculated by the CJ index (data not shown). The results of the CJ calculation were illustrated by multidimensional scaling (Fig. 1). The highest

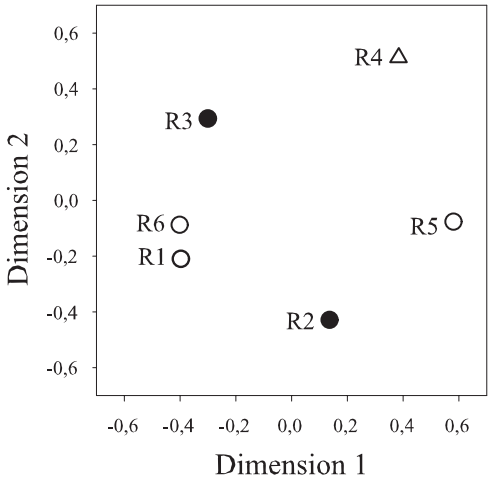

FIG. 1. MDS of CJ similarity values for archaeal diversity determined in biogas plants R1 to R6. The following symbols represent the main substrates of the biogas plants: ○, cattle liquid manure; ●, pig liquid manure; and △, maize silage. CJ values were calculated on the basis of OTUs determined by ARDRA. The relative distance between the points correlates with their dissimilarity.

similarity of methanogenic diversity was found in the 16S rRNA gene clone libraries of reactors R1 and R6. These biogas plants were fed with cattle liquid manure as the main substrate. However, the methanogenic community of reactor R5, also fed with cattle liquid manure, was nonconforming with the communities of reactors R1 and R6. Also the two reactors R2 and R3, which were operated with pig slurry as a main substrate, showed a lower similarity in the structure of their methanogenic biocoenosis.

DISCUSSION

Several studies have been published regarding the community structure of methanogenic *Archaea* in biogas reactors. However, the structure of microbial biocoenosis in full-scale biogas reactors supplied with renewable energy crops was not sufficiently known. Thus, to enlarge the recently acquired knowledge of methanogenic consortia in this kind of biogas plant, a polyphasic approach was applied to study methanogenic biocoenosis in six agricultural biogas plants.

Each of the biogas plants analyzed showed an individual methanogenic community structure. However, the hydrogenotrophic order *Methanomicrobiales* was predominant in five of the six biogas plants as determined by FISH, Q-PCR, and ARDRA. In one biogas plant only (R6) the aceticlastic family *Methanosaetaceae* was determined to be predominant methanogenic group (Table 3).

This finding stands in contrast to the common opinion which favors aceticlastic methanogenesis as the dominant methane generation pathway also in agricultural biogas plants. Therefore, possible influencing factors on the structure of methanogenic consortia were included in the analysis. To compare the methanogenic communities within the six biogas plants with consideration of the main substrate supplied, CJ similarity indices were computed and visualized by MDS (Fig. 1). Analysis of the MDS illustration showed no direct dependence of

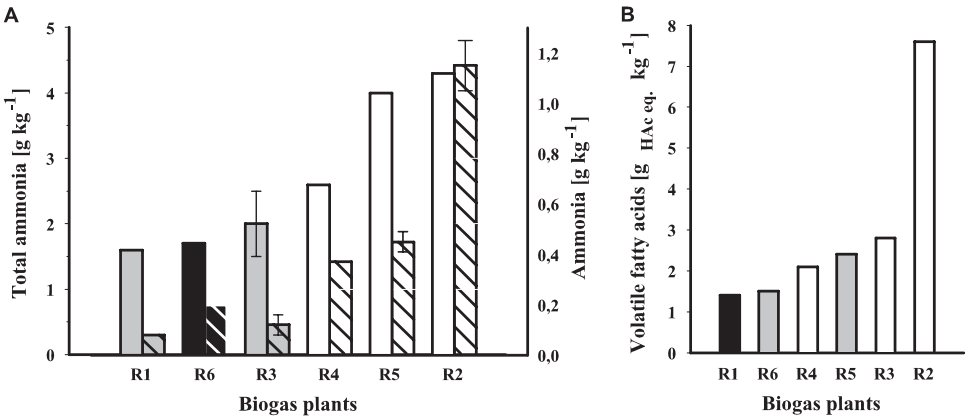

FIG. 2. Influence of total ammonia nitrogen (NH_4^+-N + NH_3-N) and calculated ammonia nitrogen (NH_3-N) (A) and volatile fatty acids (acidic acid equivalents) (B) on methanogenic community composition in biogas reactors R1 to R6. White bars, only hydrogenotrophic methanogens were detected; gray bars, hydrogenotrophic methanogens dominated aceticlastic methanogens; black bars, aceticlastic methanogens dominated hydrogenotrophic methanogens. In panel A, plain bars represent data of total ammonia nitrogen, and striped bars represent data of calculated NH_3-N.

methanogenic diversity on the main substrate supplied. Although R1, R5, and R6 were operated with cattle liquid manure as the main substrate, methanogenic biocoenoses only of R1 and R6 showed close similarities, as illustrated in Fig. 1. In addition R2 and R3, which were supplied with pig slurry, also showed low similarity in their methanogenic consortia (Fig. 1). Reactor R4, fed only with maize silage, showed no conformity in its structure of methanogenic diversity with the remaining biogas plants (Fig. 1).

Interestingly, reactors R2 and R5 possessed the highest total ammonia nitrogen (sum of NH_3-N + NH_4^+-N) concentrations, with 4.3 and 4.0 g liter^{-1}, respectively (Table 1). These high contents of total ammonia nitrogen concentrations might result from the supply of nitrogen-rich poultry feces as the substrate for the fermentative process in these reactors (Table 1). However, biogas plant R4, which was supplied with maize silage as the sole substrate, also showed high values of total ammonia nitrogen (Table 1). In these three biogas reactors high temperatures between 41 and 44.6°C were measured (Table 1), which in combination with high pH values influence the dissociation equilibrium of NH_4^+/NH_3. The calculated NH_3-N concentrations were 0.37 (R4), 0.45 (R5), and 1.15 g liter^{-1} (R2), the highest values of the analyzed biogas reactors. This could be an explanation for the absence of *Methanosaetaceae* in these three biogas plants (Table 3). It is well known that the growth of *Methanosaeta* spp. is inhibited by high total ammonia nitrogen concentrations, whereas *Methanosarcina* spp. and the hydrogenotrophic methanogens also prosper at high concentrations (3, 17, 32). The critical total NH_4^+-N concentrations may range between 2.5 and 8.0 g liter^{-1}, depending on the supplied substrates (48). For pure culture of *Methanosaeta concilii* a maximum concentration of less than 1.1 g liter^{-1} NH_4^+ (corresponding to 0.047 g liter^{-1} NH_3) at 35°C and pH 7.6 was determined (60). In full-scale biogas reactors supplied with wastewater sludge or manure, *Methanosaetaceae* were detected just at total ammonia concentrations below 1.5 g liter^{-1}, whereas in reactors with total ammonia concentrations between 2.1 and 4.1 g liter^{-1}, *Methanosarcinaceae* and not *Methanosaetaceae* were found (32).

Currently, the nature of the influence from ammonia and ammonium, on the anaerobic-digestion microbial community has not bee examined adequately. In the literature a variety of toxicity mechanisms and the possibility of adaptation to high total ammonia concentrations have been discussed (3, 22, 30, 36). Therefore, the effect of high ammonium or ammonia concentrations on methanogenic *Archaea* in full-scale biogas reactors requires further investigation.

Besides total ammonia, acetate is another factor limiting the growth of aceticlastic methanogens: *Methanosaeta* spp. have a lower acetate threshold and, thus, a lower growth rate at high acetate concentrations than *Methanosarcina* spp. (16, 33, 59). Karakashev and coworkers (32) showed that not only acetate but also volatile fatty acids (VFA) have a strong influence on the methanogenic consortia in biogas plants. In this study, a correlation between high VFA (HAc eq.) concentration and the presence of *Methanosaetaceae* was not observed (Fig. 2b). In R4 no aceticlastic *Archaea* were detected although this reactor showed comparatively low VFA concentrations. Hence, high ammonium concentrations might have a higher impact than high VFA concentrations on the aceticlastic *Archaea* (52).

The underrepresentation of aceticlastic methanogens in five of six analyzed biogas reactors supports the conclusion that aceticlastic methanogenesis plays a minor role in the biomethanation process. The fact that aceticlastic methanogens have a lower rate of substrate conversion (8.4 g COD_s g COD_{BM}^{-1} day^{-1}; COD is chemical oxygen demand, S is substrate, and BM is biomass) than hydrogenotrophic methanogens (37.0 g COD_s g COD_{BM}^{-1} day^{-1}) fortify this thesis (6). Moreover, hydrogenotrophic methanogens posses higher growth rates, up to 2.0 day^{-1} than aceticlastic methanogens with 0.4 day^{-1} (6).

This assumption raises the question, which microorganisms utilize the acetate derived from bacterial hydrolysis of organic compounds if aceticlastic methanogens are not present. Under these conditions potential candidates for acetate degradation to CO_2/H_2 are the syntrophic acetate oxidizers (27). As this process is thermodynamically extremely unfavorable ($\Delta G^{0'}$, 104.6 kJ mol^{-1}) the acetate oxidizers need partner organisms such as hydrogenotrophic methanogens (39, 53). Up to now, only a few microbial species which are able to degrade acetate

to H_2 and CO_2 in syntrophy with hydrogenotrophic methanogens have been identified: an acetate-oxidizing, rod-shaped (AOR) bacterium (39), *Clostridium ultunense* strain B (53), *Thermacetogenium phaeum* strain PB (31), *Thermotoga lettingae* strain TMO (5), and *Acetobacterium woodii* (63). Other bacteria also present in biogas reactors (37) oxidize acetate by parallel reduction of sulfur. Examples are members of the orders *Desulfovibrionales*, *Desulfobacterales*, and *Desulfomonadales*. Earlier studies following acetate utilization processes by ^{14}C isotope-labeled acetate revealed that in the absence of aceticlastic methanogens due to high ammonium concentrations, nonmethanogenic acetate oxidation took place (33, 54). Accordingly, in biogas plants with nonmethanogenic acetate oxidation, the hydrogenotrophic genus *Methanoculleus* was observed to be the predominant group of methanogens (54). Members of this genus were also detected to be most prevalent in several lab-scale and full-scale biogas reactors (35, 37, 45).

In this study, members the genus *Methanoculleus* (order *Methanomicrobiales*) were also found to be the predominant methanogen as determined by ARDRA (see data at http://www.atb-potsdam.de/veroeffent/AEM_Nettmann_et_al_2010_Supplement.pdf). This might point to the occurrence of nonmethanogenic acetate oxidation by syntrophic acetate oxidizers in the analyzed biogas plants. However, to solve this question, further investigations of the syntrophic relationships within microbial communities, their influence on the carbon fluxes, and, finally, their influence on the efficiency of the biogas formation process in full-scale biogas reactors are required.

The diversity analysis by ARDRA also resulted in the detection of members of the hitherto unclassified *Euryarchaeota* ARC-I cluster (R6). Additionally, putative new species detected by ARDRA showed closest nucleotide similarity (83%) to members of the CA-11 cluster (R4 and R5). The CA-11 cluster was first determined by Godon et al. (25) in a reactor supplied with wine distillation waste. Members of the ARC-I and CA-11 clusters were also found in lab-scale and full-scale biogas reactors supplied with triticale (35), in sludge samples of a mesophilic wastewater reactor, and in a reactor supplied with wine waste (25). The role of these putative methanogen groups within the microbial consortia and their effect on the biogas formation process in biogas plants remain unclear and require further investigations.

This study provides new insights into the methanogenic community structure present within biogas plants supplied with energy crops and liquid manure. The results imply that hydrogenotrophic methanogenesis is favored for methane formation. However, this study is a snapshot of the microbial community at one moment in time. Further studies will be needed to monitor the microbial population dynamics during ongoing biogas fermentation.

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