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A balanced microbiota efficiently produces methane in a novel high-rate horizontal anaerobic reactor for the treatment of swine wastewater

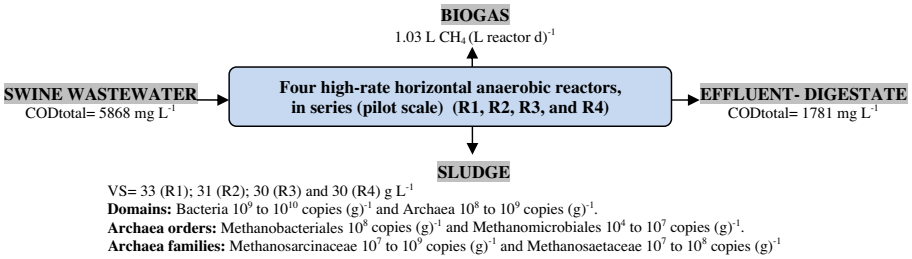
Rose Maria Duda [a,d,*], Juliana da Silva Vantini [a], Larissa Scattolin Martins [a], Alessandro de Mello Varani [b], Manoel Victor Franco Lemos [c], Maria Inês Tiraboschi Ferro [b], Roberto Alves de Oliveira [a]

[a] Faculty of Agricultural and Veterinary Sciences, Univ Estadual Paulista, Campus of Jaboticabal, Department of Rural Engineering, Laboratory of Environmental Sanitation, 14884-900, Jaboticabal, SP, Brazil
[b] Faculty of Agricultural and Veterinary Sciences, Univ Estadual Paulista, Campus of Jaboticabal, Department of Technology, Laboratory of Biochemistry and Molecular Biology, 14884-900, Jaboticabal, SP, Brazil
[c] Faculty of Agricultural and Veterinary Sciences, Campus of Jaboticabal, Department of Applied Biology, Bacterial Genetics Laboratory, 14884-900, Jaboticabal, SP, Brazil
[d] Faculty of Technology Jaboticabal, 14883-130, Jaboticabal, SP, Brazil

HIGHLIGHTS

- Introducing a novel structurally simple high-rate horizontal anaerobic reactors.
- Methane production of 0.30 L CH_4 (g removed COD_{tot})$^{-1}$.
- 94% of the sludge microbial diversity related to Bacteria and Archaea.
- Methane-producing Archaea observed.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel combination of structurally simple, high-rate horizontal anaerobic reactors installed in series was used to treat swine wastewater. The reactors maintained stable pH, alkalinity, and volatile acid levels. Removed chemical oxygen demand (COD) represented 68% of the total, and the average specific methane production was 0.30 L CH_4 (g removed COD_{tot})$^{-1}$. In addition, next-generation sequencing and quantitative real-time PCR analyses were used to explore the methane-producing Archaea and microbial diversity. At least 94% of the sludge diversity belong to the Bacteria and Archaea, indicating a good balance of microorganisms. Among the Bacteria the Proteobacteria, Bacteroidetes and Firmicutes were the most prevalent phyla. Interestingly, up to 12% of the sludge diversity belongs to methane-producing orders, such as Methanosarcinales, Methanobacteriales and Methanomicrobiales. In summary, this system can efficiently produce methane and this is the first time that horizontal anaerobic reactors have been evaluated for the treatment of swine wastewater.

1. Introduction

Pig farming contributes approximately 37% of the animal protein consumed worldwide (McGlone, 2013). Over the past decades, leading pork producers such as China, USA, and Brazil have seen a decrease in the number of individual farmers while the population of animals per farm has increased (Deng et al., 2014). This scenario has resulted in a higher concentration of waste in small areas,

* Corresponding author at: Faculty of Agricultural and Veterinary Sciences, Univ Estadual Paulista, Campus of Jaboticabal, Department of Rural Engineering, Laboratory of Environmental Sanitation, 14884-900, Jaboticabal, SP, Brazil.
E-mail address: rosemariaduda@hotmail.com (R.M. Duda).

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which urgently requires the improvement, or development, of easy-to-implement, locally adaptable solutions. Among the potential solutions, anaerobic digestion deserves special attention.

Anaerobic digestion systems represent a viable technology for tropical and subtropical countries where the weather permits the operation of reactors at air temperature. These robust systems require low investment, have low running costs, and have recently been improved to increase the removal of organic matter and the production of methane gas, reducing the sludge output, and requiring smaller areas (Duda and de Oliveira, 2011; Shin et al., 2011).

Horizontal anaerobic reactors with sludge blanket (HARSB) or fixed bed (HARFB) provide advantages, such as low structural complexity and area requirements. Zaiat et al. (1996) first proposed the use of high-rate horizontal anaerobic reactors, with tubular geometry and horizontal flow containing biomass inside a stationary support made of polyurethane foam. These reactors were extensively studied for the treatment of domestic sewage, industrial wastewater and toxic waste, and, more recently, for the treatment of wastewater from agricultural and agro-industrial practices, such as wastewater from cassava processing plants (Colin et al., 2007). The low concentration of total suspended solids in swine wastewater supports the utilization of HARSB and HARFB. In some Asian countries (Yang et al., 2015) and in Brazil (Duda and de Oliveira, 2011) a large amount of water is used in pig farming to cool down the animals – pigs have deficient thermoregulatory systems (Lima et al., 2011) – and to clean the floors. Thus, this practice produces wastewater in large volumes with a low concentration of nutrients and total solids, approximately 1–2%. In fact, wastewater from pig finishing farms in China contain, on average, six times less dry matter (DM) than described for the same activity in Europe.

Anaerobic treatment of swine wastewater involves a biochemical process with multiple stages and many different microorganisms. During treatment, complex organic matter contained in waste is hydrolyzed, fermented in the intermediate stages of the process, and reduced to methane and carbon dioxide (Kim et al., 2012). The use of in-series anaerobic reactors for the treatment of swine wastewater removes suspended solids, chemical oxygen demand (COD), metals, and coliform bacteria, while reducing hydraulic retention time (HRT) and increasing system stability.

Nowadays the knowledge of the microbial diversity in anaerobic reactors may be accessed by molecular approaches. For instance, next generation sequencing (NGS) may provide details about the structure and composition of anaerobic microbial communities, and could allow the identification and functional description of previously unknown microorganisms (Pervin et al., 2013). Indeed, the study of microbial diversity can provide insights of the operations of such reactors. For instance, a recent study indicated that the substrate and temperature may have a strong effect on the bacterial diversity found in anaerobic reactors (Dohrmann et al., 2011).

Conversely, controlling specific processes with accuracy requires the knowledge of absolute abundance of determined microbial groups (Yu et al., 2006). Therefore, current studies have sought not only to elucidate bacterial identity, but also to unveil expression patterns and monitor biological responses to different stimuli, including changes in operational and environmental conditions, in substrate composition, and in reactor configuration. These changes directly affect gene expression of the microbial group, allowing for the quantification of the transcriptional levels of specific genes (Franke-Whittle et al., 2014; Zamorano et al., 1996), and for comparisons between induced and repressed genes under variable metabolic conditions (Brown and Botstein, 1999). Conversely, quantitative real-time PCR (qPCR) allowed a exact and reproducible quantification of gene expression and abundance of different microorganisms, representing a current method to unravel the microbial diversity involved in the treatment of wastewater. Therefore the quantitative analysis of reactor microbiota provides a useful tool for understanding processes of anaerobic digestion, and for making comparisons between systems (Merlino et al., 2013).

Next-generation sequencing and real-time qPCR have not been used in combination to advance our knowledge of the anaerobic processes involved in swine wastewater treatment. This combination of methods may ultimately lead to a wider, more precise, and reproducible quantification of microbial groups. These advances should pave the way for better high-rate horizontal reactors and contribute to the solution of environmental issues such as waste management and bioenergy production. Thus, in this work were analyzed the methane production and sludge microbiota of four in-series high-rate horizontal anaerobic reactors for the treatment of swine wastewater. This is the first time that such reactors have been evaluated for the treatment of swine wastewater.

2. Methods

2.1. Operation of reactors

The anaerobic treatment system consisted of one HARSB (R1) and three HARFB (R2–4) installed in series as illustrated in Fig. 1. Electrical tubing made of corrugated plastic with 87% empty space provided the support medium for fixed-bed reactors R2–4. Reactors were built with 6 m PVC tubes with internal diameters of 249, 200, 150, and 100 mm and volumes of 292, 164, 91, and 41 L for R1–4, respectively. The HRTs applied were 12.0, 6.72, 3.60, and 1.68 h for R1–4, respectively. Swine wastewater used as affluent for the reactor system was collected daily from confined growing-finishing hogs fed a corn- and soybean-based feed supplemented with vitamins and minerals. The average temperature during reactor operation was 22.6 °C, with a variation coefficient of 15%.

The reactors R1–4 were inoculated, respectively, with 88, 49, 27 and 12 L of sludge from an upflow anaerobic sludge blanket (UASB) reactor used in swine wastewater treatment. These amounts of sludge corresponding to 30% of the volume of each reactor. Total solids (TS), volatile solids (VS) and SV/ST ratio in the inoculum were 61.2 g L^{-1}, 41.9 g L^{-1} and 0.69, respectively. The reactors (R1–4) received inoculum with the same characteristics.

To assess experimental unity performance, were sampled affluent and effluent wastewaters twice weekly. The composite samples of affluent (inlet of R1) and effluents (output of R1, R2, R3 and R4) were composed of single samples of 200 ml, which were collected every 30 min during 6 h.

Tested parameters included total COD (CODtot) and dissolved COD (CODdis), total suspended solids (TSS), volatile suspended solids (VSS), pH, total, partial, and intermediate alkalinity (TA, PA, and IA) and thermotolerant coliforms according to the methodologies described in APHA (2005) and Ripley et al. (1986). Biogas production volume was assessed daily in the gasometers, and biogas composition was analyzed weekly by gas chromatography, as described in APHA (2005). The results of the methane production were reported at standard temperature and pressure (STP, 101.325 kPa, 273.15 K).

The theoretical electrical energy were calculated using the methodology described by Kythreotou et al. (2012). The following assumptions were considered: efficiency of energy generator of 35%, and methane energy of 55.6 MJ kg^{-1} and density of methane of 0.6556 MJ m^3.

2.2. Sample information and genomic DNA extraction

Sludge samples analyzed in this study were collected from a tap positioned in the middle of each horizontal reactor after 196 days of continuous operation. In this operating time, the reactors showed the best conditions of stability, with variation coefficient

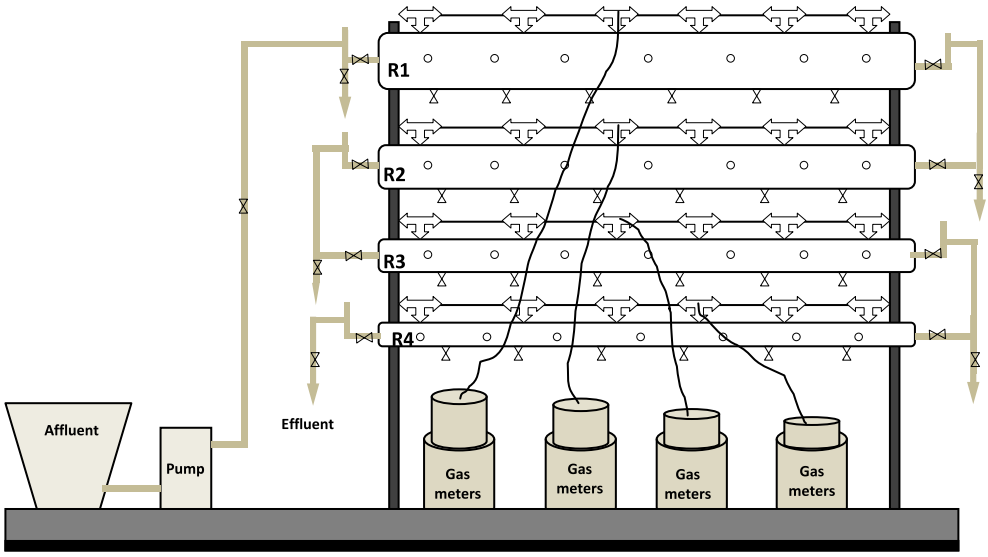

Fig. 1. Anaerobic treatment system comprising four high-rate horizontal reactors installed in series (R1, R2, R3, and R4).

(VC) minor than 30% to the volumetric production of methane in the reactors system (R1 + R2 + R3 + R4), and VC minor than 20% to the total alkalinity and TVA and 4% to the pH, in the effluent of R4. The VS concentrations of sludge were 33, 31, 30, and 30 g L^{-1} in the R1, R2, R3, and R4, respectively. Genomic DNA was extracted, in triplicate, from 0.35 mg of sludge collected from each of the reactors and of inoculum using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA), and stored at −20 °C until the PCR assay was conducted. Sample DNA quality was analyzed by NanoDrop-1000 (Thermo Scientific, Wilmington, DE, USA) and 1% agarose gel with 1× TAE buffer, and the quantity of the genomic DNA extracted was assessed using the Qubit® dsDNA HS Assay Kit 100 assays, 0.2–100 ng (Invitrogen, Carlsbad, California, USA).

2.3. Sequencing and data analysis

The genomic DNA from the sludge of each horizontal anaerobic reactor (R1, R2, R3, and R4) and inoculums were extracted and sequenced using the Illumina HiScanSQ platform, using a 2 × 100 bp paired-end libraries. The sequences were annotated and analyzed with MG-RAST platform (http://metagenomics.anl.gov/).

2.4. qPCR: absolute quantification

Quantitative real-time PCR (qPCR) was conducted with DNA samples isolated from the inoculum and sludge collected from each reactor after 196 days of continuous operation. The main microorganisms identified were quantified. The Archaea and Bacteria Domains, the orders Methanobacteriales and Methanomicrobiales, and two families, Methanosarcinaceae and Methanosaetaceae, belonging to the order Methanosarcinales, had regions of 16S rDNA amplified using specifics primers (Song et al., 2010; Lee et al., 1996). Initially, primers had their specificity confirmed by Silva TestPrime (http://www.arb-silva.de/search/testprime/). These regions were amplified by conventional PCR with specific primers (Table 1) and cloned using the pGEM ®-T Easy Vector System I (Promega, Wisconsin, USA) and inserted into *Escherichia coli* DH10β competent cells. Positive clones were selected among DH10β transformed cells. The DNA sequencing was performed at the CREBIO (Center for Genomic Biology and Biological Resources) of FCAV/UNESP (University of São Paulo State) in Jaboticabal, São Paulo, Brazil. The analysis was performed using the BLAST tool (Altschul et al., 1997), a public database provided by NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). An *e*-value lower than 10^{-08} was considered acceptable. The purified plasmids containing the insert were quantified using fluorometry.

The qPCR reactions were conducted in the *Applied Biosystems* 7500 Real-Time PCR apparatus. Reactions included 6.25 µL of Power SYBR Green® MasterMix (Applied Biosystems), primers at different concentrations depending on the micro-organism (300 nM for Methanobacteriales, Methanosaetaceae, and Methanosarcinaceae; 100 nM for Archaea and Bacteria; 600 nM for Methanomicrobiales), 10 ng of metagenomic DNA, and ultrapure water to bring the reaction volume to 12.5 µL. All the reactions were conducted in 96-well plates in an Applied Biosystems 7500 Real-Time PCR System® apparatus (Applied Biosystems) with the following settings: 2 min at 50 °C; 10 min at 95 °C; and 40 cycles at 95 °C for 15 s, 1 min at specific annealing temperatures (Table 1), and 30 s at 78 °C. After these steps, there was a step with increasing temperature (60–95 °C) to obtain the dissociation curve of the reaction products.

The copy number of each sample was determined by serial dilution of the standard curve from the linearized plasmid, 3×10^8 to 3×10^2 (1:10), except the order Methanobacteriales, 3×10^8 to 2×10^4 (1:5). For each standard curve, we determined whether efficiency was close or equal to 100%. The Ct was determined for the samples and compared to standard curves to determine the number of copies in 10 ng of metagenomic DNA. Taking into account the original mass of starting material, the DNA extraction yield and PCR template dilution, the number of 16S rRNA gene targets was determined per gram (wet weight), according (Lunedo et al., 2014).

3. Results and discussion

3.1. Physical and chemical parameters

Physical and chemical parameters of reactors affluent and effluent materials were evaluated (Supplementary data – Table S1). Average CODtot and CODdis applied into R1 were, respectively, 5868 and 1507 mg L^{-1}, resulting in average organic loading rates

Table 1
Specific primers.

Name	Role	Microorganisms	Sequence (5′–3′)	Tm* (°C)
Song et al. (2010)				
ARC787F	Primer F	Archaea	ATTAG ATACC CSBGT AGTCC	62
	Primer R		GCCAT GCACC WCCTC T	
MBT857F	Primer F	Methanobacteriales	CGWAG GGAAG CTGTT AAGT	57
	Primer R		TACCG TCGTC CACTC CTT	
MMB282F	Primer F	Methanomicrobiales	ATCGR TACGG GTTGT GGG	64
	Primer R		CACCT AACGC RCATH GTTTA C	
Msc380F	Primer F	Methanosarcinaceae	GAAAC CGYGA TAAGG GGA	57
	Primer R		TAGCG ARCAT CGTTT ACG	
Mst702F	Primer F	Methanosaetaceae	TAATC CTYGA RGGAC CACCA	62
	Primer R		CCTAC GGCAC CRACM AC	
Lee et al. (1996)				
DB1	Primer F	Domínio Bacteria	CGGYCCAGACTCCTACGGG	62
DB2	Primer R	Domínio Bacteria	TTACCGCGGCTGCTGGCAC	

* Tm: annealing temperature.

(OLR) of 12 (R1), 11 (R2), 18 (R3), and 33 (R4) g CODtot $(L\,d)^{-1}$. The affluent CODtot remained at a level similar to that previously described by Yang et al. (2015), of 5692 mg L^{-1}. These relatively low values result from the utilization of large amounts of water to ensure animal welfare while animals are under high temperatures, and to clean farm facilities. The average affluent concentration of total volatile acids (TVA), 1014 mg L^{-1}, represented 72% of CODdis and the VSS (2781 mg L^{-1}) represented 72% of TSS (3862 mg L^{-1}) in the affluent. Shin et al. (2011) observed similar values in swine wastewater. According to these authors, the range of values indicates the existence of a heterogeneous mixture of substrates readily available for acidogenic and methanogenic microorganisms, as well as other compounds that depend on prior hydrolysis.

Removal efficiencies of CODtot, CODdis, TSS, and VSS were, respectively, of 47%, 7%, 50%, and 48% in R1 alone and efficiencies of 68%, 41%, 72%, and 75%, for the entire reactor system R1 + R2 + R3 + R4 (Fig. 2). Reactor R1 removed a large portion of the organic material, possibly reflecting the 12 h HRT applied, which equaled the sum of HRTs of the other three reactors. Despite the reduced removal efficiencies of R2, R3, and R4, these reactors contributed to the stability of organic matter removal in the system.

The reactor system R1–4 had a somewhat lower removal efficiency of CODtot with an HRT of 24 h if compared to the 74% obtained by (Song et al., 2010), utilizing a UASB with 6.4-fold greater HRT and OLR of 2.3 g CODtot $(L\,d)^{-1}$. Thus, high-rate horizontal anaerobic reactor systems should provide an excellent alternative for the treatment of swine wastewater, with good levels of organic matter removal, low HRT, and high OLR.

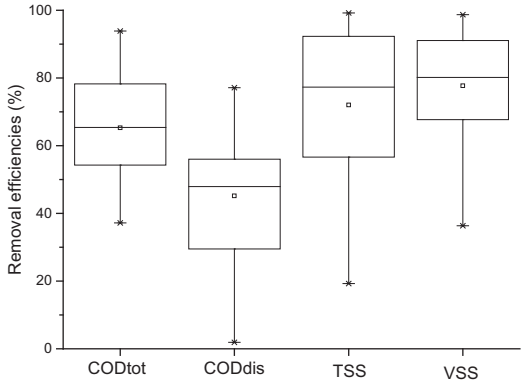

Fig. 2. Average removal efficiencies of total chemical oxygen demand (CODtot), dissolved chemical oxygen demand (CODdis), total suspended solids (TSS), and volatile suspended solids (VSS) in a treatment system comprising four high-rate horizontal reactors installed in series (R1 + R2 + R3 + R4).

The TVA concentrations decreased of 754, 595, 546, and 528 mg L^{-1} in R1, R2, R3, and R4, respectively. The affluent had a 6.9 average pH, whereas in the effluents, pH values were 7.1 (R1) and 7.2 (entire system), indicating the buffering capacity of reactors, even with increasing OLR resulting from the conversion of volatile acids to methane. Effluent pH values remained within the optimal range for anaerobic digestion of 7.2–7.8 (Ward et al., 2008).

Average TA, PA, and IA values in the affluent were 1968, 1095, and 872 mg L^{-1}, respectively. The TA and PA values in the effluent of reactor R4 increased to 2368 and 1707 mg L^{-1}, respectively. The higher PA values in the effluent in comparison to the affluent indicate an increase in alkalinity, further showing the buffering capacity of the system. The increase in TA occurred due to increasing concentrations of bicarbonate, which can be observed through the sharp increases in average PA values in the effluents of R1 = 1465 mg L^{-1}, R2 = 1451 mg L^{-1}, R3 = 1664 mg L^{-1}, and R4 = 1707 mg L^{-1}, in comparison to an affluent value of 1095 mg L^{-1}. The IA, which is an indicator of volatile fatty acids, changed from a value of 872 mg L^{-1} in the affluent to 743, 946, 700, and 661 mg L^{-1} in the effluents of each reactor, indicating the production and gradual consumption of these fatty acids.

Methane concentration in biogas was approximately 79% for each reactor, and average volumetric methane production levels were 1.15 (R1), 0.91 (R2), 0.88 (R3), 1.00 (R4), and 1.03 (R1 + R2 + R3 + R4) L CH_4 $(L\,d)^{-1}$, as shown in Fig. 3. These results correspond to the last 108 days of continuous operation, when the reactors R1–4 showed the best conditions of stability, with variation coefficients minor than 30% to methane volumetric production.

Specific methane production levels were 0.25 (R1); 0.24 (R2); 0.22 (R3); 0.18 (R4); and 0.30 (R1 + R2 + R3 + R4) L CH_4 (g removed $COD tot)^{-1}$. The lower specific methane production observed in R4 may have resulted from an increase in the fraction of resistant organic matter in its affluent, as well as from low HRT and an increase in average OLR to 33 g CODtot $(L\,d)^{-1}$.

Reactors R2–4 also produced methane, contributing to the conversion of COD to the gas and indicating that the in-series system functions well. Values for specific methane production near the theoretical maximum of 0.35 L CH_4 (L removed $COD)^{-1}$ further validated the horizontal serial system, indicating the possibility of high conversion rates even with low HRTs and a consequently small system. Song et al. (2010) treated swine wastewater in a UASB reactor, and obtained 0.28, 0.33, 0.31, and 0.29 L CH_4 (g removed $COD tot)^{-1}$, with higher HRTs than those used in this study: 7.0, 6.4, 5.0 and 3.5 d, respectively.

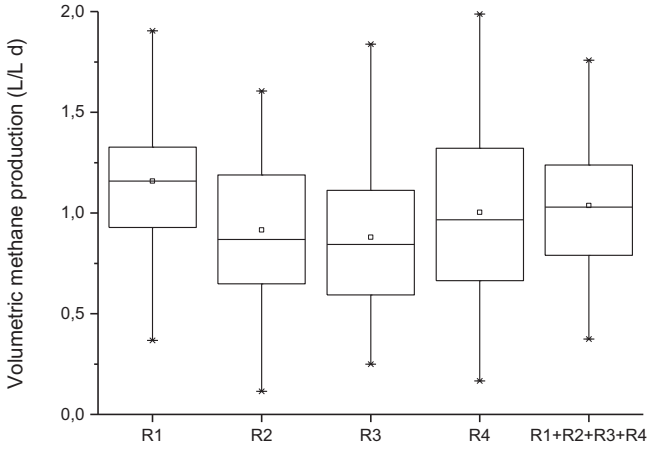

Fig. 3. Volumetric methane production by each of the four high-rate horizontal anaerobic reactors installed in series (R1, R2, R3, and R4) and by the entire system (R1 + R2 + R3 + R4).

3.2. Theoretical electrical energy generation

Theofanous et al. (2014) observed methane production (0.0015–0.017 L CH_4 $(L\,d)^{-1}$) in biodigesters used with swine waste, can generate electricity with yields of 0.004–0.06 Wh $(L\ reactor\ d)^{-1}$. Indeed the electricity yields are much smaller than those obtained with horizontal anaerobic reactors in series. According with the methodology described by Kythreotou et al. (2012), the methane produced in the reactors system (R1–4) can generate electricity with yields of 3.6 Wh $(L\ reactor\ d)^{-1}$ or 1.06 Wh $(g\ removed\ CODtot)^{-1}$.

For instance, in the Southeast, South and Midwest regions in Brazil, the swine population is approximately of 30 million, corresponding to 82% of the country herd (IBGE-SIDRA, 2015). Most of these animals are in feedlots with waste management and intensive use of water. Under these conditions, the volume of swine wastewater is of approximately 7–15 L $(swine\ d)^{-1}$ with COD of 25–50 g L^{-1}. Therefore, the theoretical daily electricity generation from methane produced in horizontal anaerobic reactors with wastewater from these herd is estimated in 3784–7569 MW h d^{-1}.

Table 2
Taxonomic distribution in phyla (%) of microorganisms from the Bacteria and Archaea domains found in samples of inoculum sludge (I) and the sludge from four high-rate horizontal anaerobic reactors (R1, R2, R3, and R4) installed in series for the treatment of swine wastewater.

	I	R1	R2	R3	R4
Proteobacteria	35.6	44.8	40.6	40.6	44.4
Bacteroidetes	13.9	13.1	11.2	12.5	13.8
Firmicutes	14.5	9.0	13.7	11.4	8.5
Actinobacteria	6.0	7.8	7.1	6.2	7.2
Euryarchaeota	12.3	6.1	5.7	5.9	5.3
Others	17.7	19.2	21.7	23.4	20.8

Percentage results based on next-generation sequencing (Illumina).

3.3. Next-generation sequencing analysis

Metagenomic analyses utilizing the Illumina HiScanSQ platform allowed the detection of a large number of Bacteria and Archaea microorganisms in the sludge of reactors R1–4. Bacteria displayed the largest microbial diversity (Fig. 4). This large diversity probably occurs because bacteria perform the hydrolysis, acidogenesis, and acetogenesis of complex and simple compounds present in wastewater, and these distinct processes require a variety of species.

The amount of organic suspended solids in swine wastewater further promoted the large variety of bacteria in horizontal anaerobic reactors. Bacteria act on these compounds by hydrolyzing proteins, carbohydrates, and lipids into amino acids, sugars, and fatty acids. These metabolites will be converted by other bacterial species into long-chain fatty acids, volatile fatty acids, alcohols, ketones, and, finally, acetate, CO_2, and H_2, which will ultimately be used by methanogenic archaea to form biogas.

The domain Bacteria comprises 26 phyla according to the Taxonomy Browser NCBI 2014 (http://www.ncbi.nlm.nih.gov/guide/taxonomy/). Some of these phyla consist of one or a few species, but others include a wider variety of microorganisms, such as the phyla Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria, which were found in sludge samples from the inoculum sludge and from reactors R1, R2, R3, and R4 (Table 2).

Proteobacteria had the widest range of sequences in inoculum and reactor sludge. Liu et al. (2015) reported a predominance of

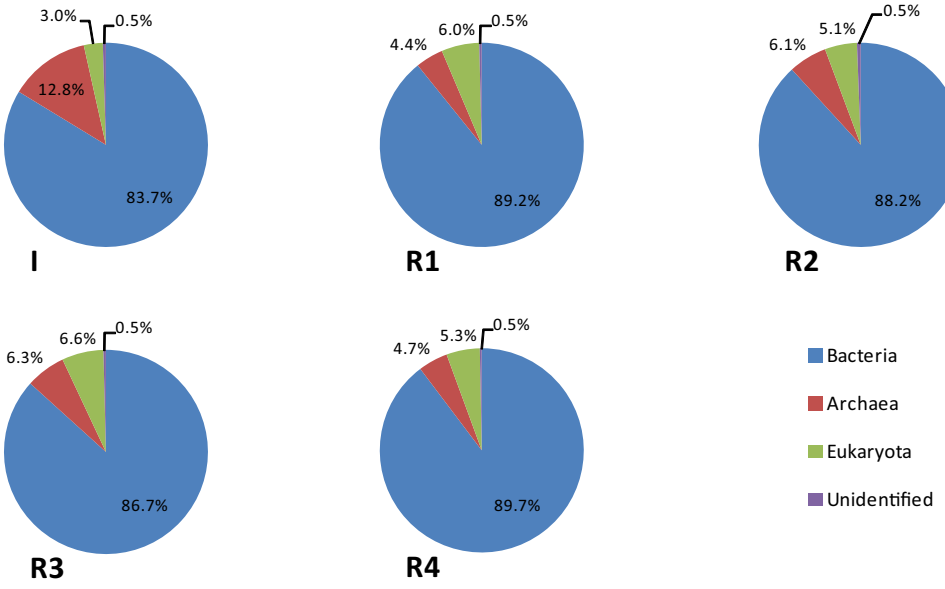

Fig. 4. Taxonomic distribution of DNA sequences found in inoculum sludge (I) and in sludge from the four high-rate horizontal anaerobic reactors (R1, R2, R3, and R4) installed in series for the treatment of swine wastewater.

Proteobacteria in swine wastewater, because this phylum represents the most common group of microorganisms in the digestive tract of pigs and in the farm environment. Consequently, among these bacteria, the strict and facultative anaerobes remain active in the sludge of anaerobic reactors. Usually, Proteobacteria grow in environments with high nutrient and organic matter content, such as mammal feces, and they decompose these contents (Kopecky et al., 2011). The taxonomic distribution of the phylum Proteobacteria increased when comparing inoculum and reactor sludge; this change possibly results from the increase in organic load and decomposition activity in the reactors.

The phyla Firmicutes, Bacteroidetes, and Actinobacteria were represented in the inoculum and in reactors R1–4 (Table 2). The phylum Firmicutes includes many bacteria in the class Clostridia that break down complex organic material (Wirth et al., 2012), and ferment lactic as well as acetic acids (Jang et al., 2014). Typically, bacteria in the phylum Bacteroidetes produce lytic enzymes and acetic acid during the degradation of organic material (Jang et al., 2014), whereas Actinobacteria produce propionic acid (Gao and Gupta, 2005).

A previous study has reported similar taxonomic profiling in anaerobic environments (Kim et al., 2013). Table 2 shows that reactors R1–4 displayed a similar distribution of bacterial phyla, despite an OLR of up to 33 g CODtot $(L\,d)^{-1}$ in R4.

Among the most representative phyla also found the Euryarchaeota, which belongs to the Archaea domain, and includes microorganisms that produce methane. Acetoclastic and hydrogenotrophic archaea produce methane in different environmental and operational reactor conditions (Franke-Whittle et al., 2014). Thus, although displaying lower genetic diversity than bacteria, the domain Archaea was present in the inoculum and in all reactors, which kept high rates of methane production. The main orders identified included Methanosarcinales (families Methanosarcinaceae and Methanosaetaceae), Methanobacteriales (family Methanobacteriaceae), and Methanomicrobiales (family Methanomicrobiaceae). Among genera, the most representative were Methanosarcina (8%), Methanosaeta (>2%), and Methanothermobacter, Methanobrevibacter, and Methanoculleus (<2%) (Fig. 5). This wide distribution of the Archaea domain shows that keeping a stable environment is fundamental to maintain a population of acetoclastic and hydrogenotrophic microorganisms in the sludge, producing a balanced level of methane from acetic acid, H_2 and CO_2 (Song et al., 2010; Franke-Whittle et al., 2014).

3.4. Real-time qPCR

Results obtained with next-generation sequencing and the analyses performed with MG-RAST platform allowed the identification of methane-producing microorganisms. Among the Bacteria and Archaea domains, two orders of hydrogenotrophic methanogenic organisms (Methanobacteriales and Methanomicrobiales), and two acetoclastic families (Methanosarcinaceae and Methanosaetaceae) of the Archaea domain had their 16S rDNA quantified through real-time qPCR (Fig. 5).

The qPCR data allowed for the quantification of Bacteria (1.9×10^9 to 1.0×10^{10} copies/g) and Archaea (6.3×10^8 to 1.9×10^9 copies/g) (Fig. 6). The Archaea domain displayed lower diversity than the Bacteria domain, as indicated by the next-generation sequencing results, but total quantities of both types of microorganisms were similar as indicated by qPCR (Fig. 6).

Anaerobic reactor stability requires high concentrations of methanogenic archaea, because these organisms have lower growth rates and utilize substrate less efficiently than bacteria. In high-rate horizontal anaerobic reactors reached such concentrations even at high OLR and high quantities of acetic acid, H_2 and CO_2 produced by bacteria. Imbalances in the proportion of bacteria to archaea can affect the entire system, leading to a reduction in the amount of methane produced (Akuzawa et al., 2011). This effect did not occur with high-rate horizontal anaerobic reactors, which maintained high levels of methane output (Fig. 2) and COD removal (Fig. 3) in face of similar numbers of bacteria

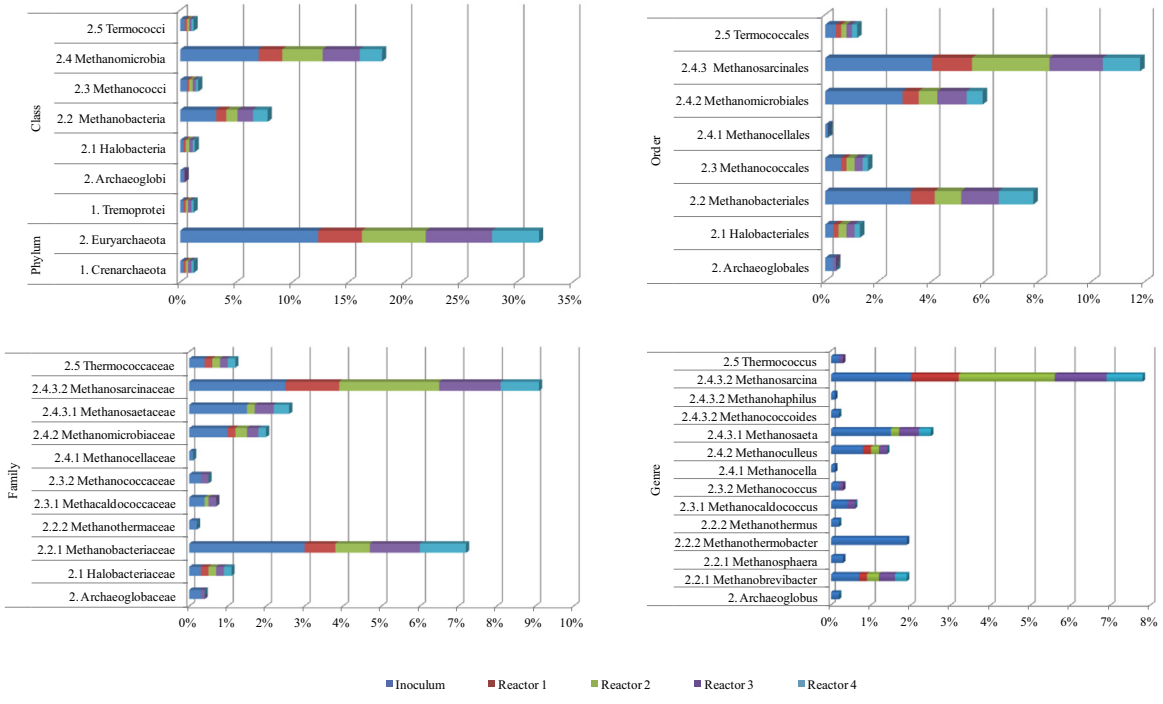

Fig. 5. Taxonomic diversity of Archaea domain microorganisms found in samples from inoculum sludge and sludge from four high-rate horizontal anaerobic reactors (R1, R2, R3 and R4) installed in series for the treatment of swine wastewater.

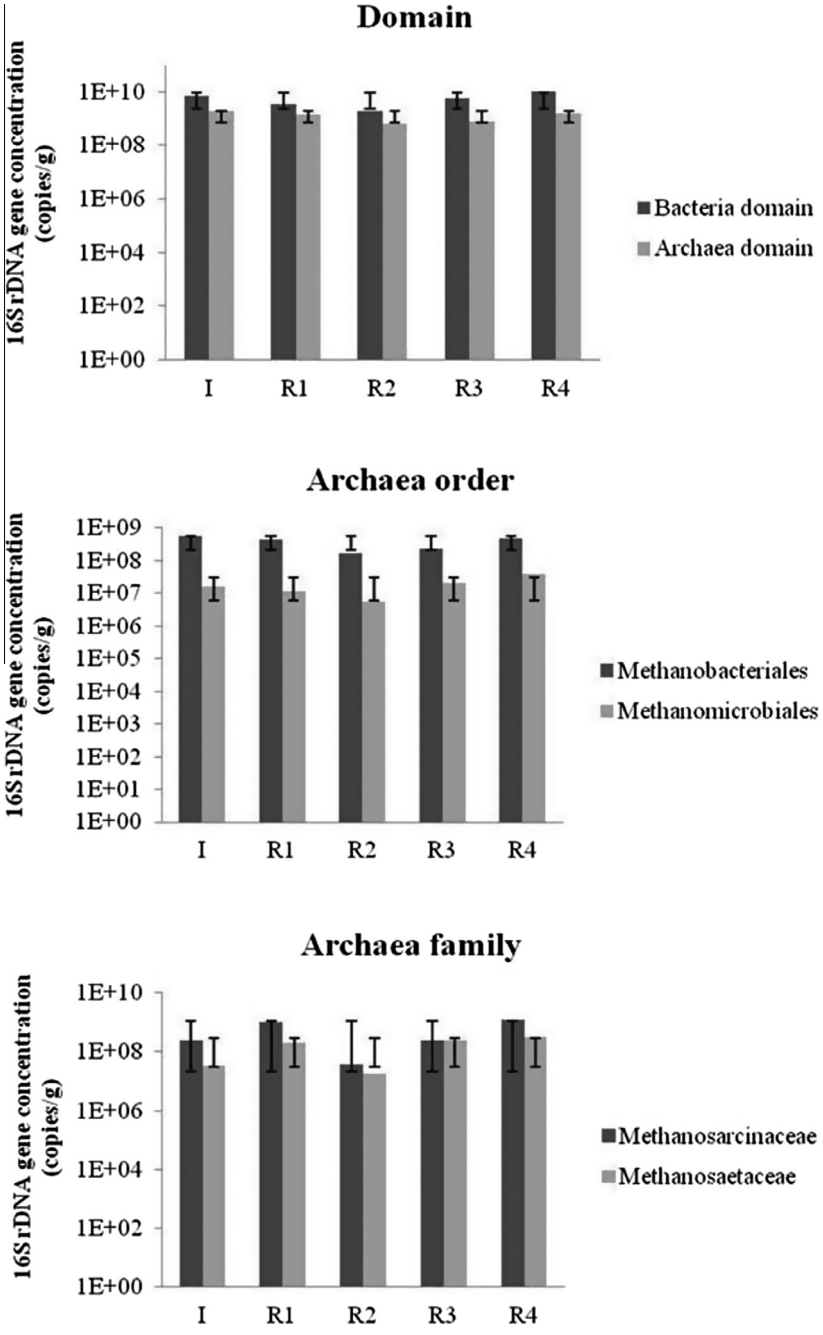

Fig. 6. Quantitative real-time PCR analyses of microorganisms in the Bacteria and Archaea domains, two orders (Methanobacteriales and Methanomicrobiales), and two families (Methanosarcinaceae and Methanosaetaceae) of the Archaea domain present in samples from inoculum sludge (I) and the sludge from four high-rate horizontal anaerobic reactors (R1, R2, R3, and R4) installed in series for the treatment of swine wastewater after 196 days of operation.

(1.9×10^9 to 1.0×10^{10} copies/g) and archaea (6.3×10^8 to 1.9×10^9 copies/g).

Gene copies of 16S rDNA per gram of sludge of the hydrogenotrophic methanogenic orders Methanobacteriales and Methanomicrobiales were 1.7×10^8 to 5.5×10^8 and 8.0×10^4 to 3.7×10^7, respectively. These measurements were obtained with VS of 33, 31, 30, and 30 g L^{-1}, in reactors R1, R2, R3, and R4, respectively. The larger predominance of the Methanobacteriales order agree with next-generation sequencing results (Fig. 5). Thus, the system requires similarly high concentrations of hydrogenotrophic methanogenic and acetotrophic methanogenic organisms (1.8×10^7 to 1.2×10^9 copies/g) in the anaerobic sludge of reactors for the efficient interspecific transfer of hydrogen (H_2) and its utilization with CO_2 for methane production. Consequently, maintaining a low H_2 partial pressure in the reactors promotes the equilibrium of fermentation reactions for the formation of a more oxidized final product (acetate). Methanogenic acetoclastic archaea consume acetate to produce high levels of methane in all the reactors.

Ju and Zhang (2014), working with the seed sludge from digester of organic solid residues in anaerobic batch reactors utilizing phenol as substrate, indicated that hydrogenotrophic methanogenic archaea represent 11.2–15.7% of all Archaea in the sludge. These microorganisms consume H_2 and help maintain low levels of this molecule. Song et al. (2010) observed a higher expression of hydrogenotrophic methanogenic archaea, specifically of the order Methanobacteriales, while working with sludge granules

from UASB reactor used for the treatment of swine wastewater. The authors suggested that hydrogenotrofic methanogenesis, with the syntrophic oxidation of volatile fatty acids, was the main pathway for methane production.

According to next-generation sequencing results, Methanosarcinales represented the most common Archaea order; thus, its two main families Methanosarcinaceae (3.5×10^7 to 1.2×10^9 copies/g) and Methanosaetaceae (1.8×10^7 to 3.0×10^8 copies/g) were quantified by q-PCR. The genera Methanosarcina and Methanosaeta, highly predominant according to next-generation sequencing (Fig. 5), belong to the families Methanosarcinaceae and Methanosaetacea, respectively. These acetoclastic methanogenic organisms account for approximately 70% of all methane produced in anaerobic reactors (Franke-Whittle et al., 2014). According to Ju and Zhang (2014), 71.4–76.5% of methanogenic archaea are acetoclastic (Methanosaeta) in the sludge from anaerobic batch reactors utilizing phenol as substrate. These results contrast with ours, and possibly indicate the influences of reactor type, substrate, and operational conditions. The Methanosarcinaceae family displayed greater diversity if compared to the Methanosaetaceae, according to the Illumina metagenomic analysis, but absolute quantities of both families were similar in the inoculum and the four reactors. Thus, in-series high-rate horizontal anaerobic reactors require an equilibrium between hydrogenotrophic and acetoclastic methanogens, as well as between the genera Methanosarcina and Methanosaeta, with counts ranging from 10^7 to 10^9 copies/g of sludge.

4. Conclusions

This study introduced a new configuration and combination of high-rate, horizontal anaerobic reactors installed in series with high volumetric and specific methane production. The microbial diversity was assessed by NGS and real-time qPCR. A good microbial diversity balance between the Archaea and Bacteria domains were observed. Noteworthy hydrogenotrophic and acetoclastic methanogenic archaea, belonging to the Methanosarcinales, Methanobacteriales and Methanomicrobiales orders were identified in the reactors sludge. Further functional analysis of this Archaea diversity is underway. This system can efficiently produce methane and this is the first time that horizontal anaerobic reactors have been evaluated for the treatment of swine wastewater.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.08.004.

References

Akuzawa, M., Hori, T., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y., 2011. Distinctive responses of metabolically active microbiota to acidification in a thermophilic anaerobic digester. Microb. Ecol. 61, 595–605.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21th ed. American Public Health Association, Washington DC, USA.

Brown, P.O., Botstein, D., 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. 21, 33–37.

Colin, X., Farinet, J.L., Rojas, O., Alazard, D., 2007. Anaerobic treatment of cassava starch extraction wastewater using a horizontal flow filter with bamboo as support. Bioresour. Technol. 98, 1602–1607.

Deng, L., Li, Y., Chen, Z., Liu, G., Yang, H., 2014. Separation of swine slurry into different concentration fractions and its influence on biogas fermentation. Appl. Energy 114, 504–511.

Dohrmann, A.B., Baumert, S., Klingebiel, L., Weiland, P., Tebbe, C.C., 2011. Bacterial community structure in experimental methanogenic bioreactors and search for pathogenic clostridia as community members. Appl. Microbiol. Biotechnol. 89, 1991–2004.

Duda, R.M., de Oliveira, R.A., 2011. Tratamento de águas residuárias de suinocultura em reator UASB e filtro anaeróbio em série seguidos de filtro biológico percolador. Eng. Sanit. Ambient. 16, 91–100.

Franke-Whittle, I.H., Walter, A., Ebner, C., Insam, H., 2014. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag. 34, 2080–2089.

Gao, B., Gupta, R.S., 2005. Conserved indels in protein sequences that are characteristics of the phylum Actinobacteria. Int. J. Syst. Evol. Microbiol. 55, 2401–2412.

IBGE - SIDRA [WWW Document], n.d. URL <http://www.sidra.ibge.gov.br/bda/pecua/default.asp?t=2&z=t&o=24&u1=1&u3=1&u4=1&u5=1&u6=1&u7=1&u2=1> (accessed 7.15.15).

Jang, H.M., Kim, J.H., Ha, J.H., Park, J.M., 2014. Bacterial and methanogenic archaeal communities during the single-stage anaerobic digestion of high-strength food wastewater. Bioresour. Technol. 165, 174–182.

Ju, F., Zhang, T., 2014. Novel microbial populations in ambient and mesophilic biogas-producing and phenol-degrading consortia unraveled by high-throughput sequencing. Microb. Ecol. 68, 235–246.

Kim, W., Shin, S.G., Cho, K., Lee, C., Hwang, S., 2012. Performance of methanogenic reactors in temperature phased two-stage anaerobic digestion of swine wastewater. J. Biosci. Bioeng. 114, 635–639.

Kim, W., Cho, K., Lee, S., Hwang, S., 2013. Comparison of methanogenic community structure and anaerobic process performance treating swine wastewater between pilot and optimized lab scale bioreactors. Bioresour. Technol. 145, 48–56.

Kopecky, J., Kyselkova, M., Omelka, M., Cermak, L., Novotna, J., Grundmann, G.L., Moënne-Loccoz, Y., Sagova-Mareckova, M., 2011. Actinobacterial community dominated by a distinct clade in acidic soil of a waterlogged deciduous forest. FEMS Microbiol. Ecol. 78, 386–394.

Kythreotou, N., Tassou, S.a., Florides, G., 2012. An assessment of the biomass potential of Cyprus for energy production. Energy 47, 253–261.

Lee, D.H., Zo, Y.G., Kim, S.J., 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl. Environ. Microbiol. 62, 3112–3120.

Lima, A.L., de Oliveira, R.F.M., Donzele, J.L., Fernandes, H.C., Campos, P.H.R.F., Antunes, M.V.D.L., 2011. Resfriamento do piso da maternidade para porcas em lactação no verão. Rev. Bras. Zootec. 40, 804–811.

Liu, A., Chou, C., Chen, L., Kuo, C., 2015. Bacterial community dynamics in a swine wastewater anaerobic reactor revealed by 16S rDNA sequence analysis. J. Biotechnol. 194, 124–131.

Lunedo, R., Fernandez-Alarcon, M.F., Carvalho, F.M.S., Furlan, L.R., Macari, M., 2014. Analysis of the intestinal bacterial microbiota in maize- or sorghum-fed broiler chickens using real-time PCR. Br. Poult. Sci. 55, 795–803.

McGlone, J.J., 2013. The future of pork production in the world: towards sustainable, welfare-positive systems. Animals 3, 401–415.

Merlino, G., Rizzi, A., Schievano, A., Tenca, A., Scaglia, B., Oberti, R., Adani, F., Daffonchio, D., 2013. Microbial community structure and dynamics in two-stage vs single-stage thermophilic anaerobic digestion of mixed swine slurry and market bio-waste. Water Res. 47, 1983–1995.

Pervin, H.M., Batstone, D.J., Bond, P.L., 2013. Previously unclassified bacteria dominate during thermophilic and mesophilic anaerobic pre-treatment of primary sludge. Syst. Appl. Microbiol. 36, 281–290.

Ripley, L.E., Boyle, W.C., Converse, J.C., 1986. Improved alkalimetric monitoring for anaerobic digestion of high-strength wastes. J. Water Pollut. Control Fed. 58, 406–411.

Shin, S.G., Yoo, S., Hwang, K., Song, M., Kim, W., Han, G., Hwang, S., 2011. Dynamics of transitional acidogenic community along with methanogenic population during anaerobic digestion of swine wastewater. Process Biochem. 46, 1607–1613.

Song, M., Shin, S.G., Hwang, S., 2010. Methanogenic population dynamics assessed by real-time quantitative PCR in sludge granule in upflow anaerobic sludge blanket treating swine wastewater. Bioresour. Technol. 101, S23–S28.

Theofanous, E., Kythreotou, N., Panayiotou, G., Florides, G., 2014. Energy production from piggery waste using anaerobic digestion: current status and potential in Cyprus. Renew. Energy 71, 263–270.

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99, 7928–7940.

Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol. Biofuels 5, 41.
Yang, D., Deng, L., Zheng, D., Liu, G., Yang, H., Wang, L., 2015. Separation of swine wastewater into solid fraction, concentrated slurry and dilute liquid and its influence on biogas production. Fuel 144, 237–243.
Yu, Y., Kim, J., Hwang, S., 2006. Use of real-time PCR for group-specific quantification of aceticlastic methanogens in anaerobic processes: population dynamics and community structures. Biotechnol. Bioeng. 93, 424–433.
Zaiat, M., Cabral, a.K.a., Foresti, E., 1996. Cell wash-out and external mass transfer resistance in horizontal-flow anaerobic immobilized sludge reactor. Water Res. 30, 2435–2439.
Zamorano, P.L., Mahesh, V.B., Brann, D.W., 1996. Quantitative RT-PCR for neuroendocrine studies. A minireview. Neuroendocrinology 63, 397–407.