**Application of a real-time qPCR method to measure the methanogen concentration during anaerobic digestion as an indicator of biogas production capacity.**

# UNIVERSITÀ DEGLI STUDI DI TORINO

**TITLE PAGE:**

# Application of a Real-time qPCR method to evaluate methanogen load as a high-efficiency process bio-indicator for wet anaerobic co-digestion of organic wastes after different pre-treatments.

**Author names:**

D. Traversi[1], S.Villa[1], E. Lorenzi[2], R. Degan[1], G. Gilli[1]

[1] Department of Public Health and Microbiology,

University of the Study of Turin, via Santena 5 bis, 10126 Turin, Italy

phone +390116705810

fax +390112605810

e-mail: deborah.traversi@unito.it

[2] SMAT S.p.A., corso XI Febbraio 14, 10152 Torino, Italy

***Corresponding author:** e-mail: deborah.traversi@unito.it

**KEY WORDS:**

methanogen, anaerobic digestion, biogas production, renewable energy, Archea communities

**ABSTRACT:**

Biogas is a biofuel with a large energy value and primarily consists of methane. It is a renewable energy source and produced via anaerobic digestion of various organic materials, including waste water sludge and organic urban wastes. In reactors, anaerobic microorganisms can degrade the waste organic matter into two different products: digestate and biogas. If the microbial community is optimised, then the methane production rate could be improved, and the digestate could be used as a soil fertiliser. Methanogen is the major microbiologic group responsible for methane production. We detected the methanogen load during two wet digestion processes fed with pre-treated urban organic waste and waste water sludge. Two different pre-treatments were involved in the experimental digestions: pressure-extrusion and turbo-mixing. The applied methodology is based on Real Time quantitative PCR (RT-qPCR) using an *mcrA* target. First, we evaluated the validity of the analyses, including standard calibration, specificity, accuracy and precision. Next, we applied this method to 50 digestate samples. A positive and significant correlation between the biogas production rate and methanogen abundance was observed ($r=0.579$, $p<0.001$). The difference in the methanogen load between the two digestions is significant ($F=41.190$, $p<0.01$). Moreover, considering all the collected data and using 0.6 $m^3$/Kg TVS as the 'cut-off', representing an optimal production, a higher methanogen load mean was observed with the optimal production rates ($F= 7.053$; $p<0.05$). This suggests a role for methanogen load as diagnostic tool in digestion pre-treatment and optimisation.

40 **1. INTRODUCTION**

41 Two serious environmental and public health problems our society faces today include, first, reduction
42 and treatment of waste, especially in high-density areas, and, second, finding an answer to the energetic
43 request to limit use of conventional fuels (das Neves et al., 2009). In urban communities, these goals
44 have no clear resolution, though renewable energy sources are likely a key strategy (Balat and Balat,
45 2009). The anaerobic digestion process for organic waste combines the removal of organic pollutants,
46 the reduction of organic waste volume, and energy conservation via biogas production (Rozzi and
47 Remigi, 2004). Numerous organic wastes are subject to anaerobic digestion, such as wastewater sludge,
48 pre-treated organic household waste (Bouallagui et al., 2005; Schievano et al., 2009). Biogas production
49 is the consequence of a series of metabolic interactions among bacterial and Archeal micro-organisms
50 (Ward et al., 2008). A particular ecosystem is present in an anaerobic reactor where several groups of
51 microorganisms work interactively in the conversion of complex organic matter into biogas. The
52 following four stages compose the digestion process: hydrolysis, acidogenesis, acetogenesis and
53 methanogenesis (Lozano et al., 2009). In each step, various microorganisms are involved. During the first
54 stage a group of micro-organisms secrete enzymes which hydrolyse polymers to monomers to convert
55 particulate materials into dissolved materials (Whitman et al., 2006). They remove the small amounts of
56 $O_2$ present and create anaerobic conditions. Subsequently, the acidogenic phase includes the action of a
57 large and diverse group of fermentative bacteria, usually belonging to the Clostridia class and the
58 Bacteroidaceaea family. These bacteria hydrolyse and ferment the organic materialsand they produce
59 organic acids, $CO_2$ and $H_2$. Next, in the third phase, the acetogenic bacteria convert these monomers to
60 $H_2$ and volatile fatty acids. Aceticlastic methanogens perform the final step in biogas production,
61 primarily *Methanosarcina* in a high-acetate environment (>$10^{-3}$M) and *Methanosaeta* which grows only
62 by the aceticlastic reaction, as well as hydrogenotrophic methanogens. Methanogenesis is considered
63 the rate-limiting step. Moreover, this phase is most vulnerable to either temperature or pH variations
64 and toxic chemicals (Liu and Whitman, 2008b). Low methanogen activity produces an accumulation of $H_2$

and short chain fatty acids with a consequent decrease in pH. Therefore, enhancing methanogenesis is a promising method for improving anaerobic reactor performance. There are three primary useful substrates for methanogens. Acetate is the predominant intermediate in the anaerobic food chain, and as much as two-thirds of biologically generated methane is produced from this molecule (Liu and Whitman, 2008a). Although every pathway begins differently, they all end with the same step, the reaction of methyl-coenzyme M (HS-CoM) with a second thiol coenzyme (coenzyme B) that forms methane and a mixed disulphide between coenzyme M and coenzyme B. This reaction is catalysed by methyl-coenzyme M reductase (Mcr), thus, Mcr is the key enzyme in methanogenesis (Friedrich, 2005). In its active site, this enzyme contains a unique prosthetic group, which is a nickel (Ni) porphinoid referred to as coenzyme F430 (Hedderich and Whitman, 2006). As HS-CoM has been found in all methanogens examined, it has been proposed as a sensitive biomarker for quantitative and qualitative methanogen identification in different anaerobic environments. Mcr is exclusive to methanogens, with the exception of methane-oxidising Archaea (Knittel and Boetius, 2009; Whitman et al., 2006), and specific primers have been developed for the Mcr α-subunit gene sequence (*mcrA*) (Franke-Whittle et al., 2009; Luton et al., 2002; Steinberg and Regan, 2008). *McrA* analysis can be used either in conjunction with or independently of the *16S rRNA* gene (Bergmann et al., 2010a; Bergmann et al., 2010b; Cardinali-Rezende et al., 2009; Nettmann et al., 2008); this gene is absent in all non-methanogens, with the exception of the anaerobic methane-oxidizing Archea (Steinberg and Regan, 2008). The aim for the work herein was to apply a recently proposed methodology for determining methanogen abundance using Real-Time quantitative PCR (RT-qPCR) targeting the *mcrA* gene to evaluate methanogen load during the digestion process and identify its correlation with the biogas production efficiency. The purpose is to develop a useful Archea bio-indicator for process performance. The analyses involved two different digestion sessions that differed only in pre-treatment of the organic material input. We observed methanogen modulation during the digestions referring to the recorded methane production rate.

## 2. METHODS AND MATERIALS

### 2.1 Digestion processes

Two pilot reactors were fed with a pre-treated organic fraction of municipal solid waste (OFMSW) and waste-water sludge. The pre-treatment methods used in this study included a pressure-extrusion (A) and a turbo-mixing (B) system. In method A, a specially designed extruder press separates the input waste into two fractions: dry, which is sent to thermal conversion, and semi-solid. The municipal waste, fed into the extruder press, is exposed to high pressure (280 bar) in a perforated extrusion chamber that results in a fluid organic portion (food residues, various putrescible fractions). The organic fluid is pushed out by the difference in pressure between the interior and exterior of the chamber, yielding a more homogeneous sample. This is a consequence from separating the more mechanically resistant portions (paper residues, cellulose tissues, wood and lignin parts, etc.). The pressure-extrused OFMSW, obtained using the previously described procedure, was then diluted with waste-water sludge (1:3). In contrast, method B (the turbo-mixing system) is a wet process that operates with a TS content lower than 8%. Mixing and treating occur via a rotating plate, with hummers at the bottom of the turbo-mixing chamber that, rotating at high velocity, induces the suspension to shear and crush. After 20 minutes of mixing, the turbo-mixer was empty and a vessel was filled with the mixture, where density separated the contents. The particles that weigh more than water precipitate to the bottom, where they are taken up using a screw and collected in an external vessel. The same procedure was applied to the particles that weigh less than water and float on the surface. The remaining suspension is the organic fraction, which is pumped to a storage basin after a passage through a shredding pump. In this case, the OFMSW was directly turbo-mixed with waste water sludge. Full control of the final TS content suspension is impossible in the turbo-mixing system. The primary physical-chemical characteristics for each type of feed used herein, immediately prior to the reactor, are shown in Table 1.

We compared the analytical results obtained from feed samples pre-treated with methods A (pressure-extrusion) and B (turbo-mixing). OFMSW pre-treated with press-extrusion has a higher percentage of

115 volatile solids that may possibly transform into biogas in feed pre-treated with method B where the
116 organic matter value is lower. Further, the C/N ratio for the feed mixtures was evaluated. It has been
117 shown that optimum values for the carbon-to-nitrogen (C/N) ratio are within the 20 to 70 range for the
118 anaerobic digestion process (Burton and Turner, 2003), but even lower values (12 to 16) have also been
119 reported (Mshandete et al., 2004) and, recently, more widely studied (Mata-Alvarez et al., 2011). The
120 C/N values herein were better after pre-treatment A than pre-treatment B.
121 Anaerobic co-digestion was examined using a reactor, illustrated in Figure 1, with a 15 L volume capacity
122 and a 10 L working volume. The temperature was mesophilic and maintained at 38±2°C using a water
123 recirculation system connected to a thermostatic valve. The biogas produced was collected and
124 measured in a calibrated gasometer, and a mixing system with the re-circulated biogas from the
125 anaerobic digestion process was used. The reactors were equipped with two openings, one at the top
126 for feeding and one below to collect effluent discharge. Every day, 500 ml of digestate was removed
127 from each reactor before an additional 500 ml of fresh feed was added. The reactors were operated at a
128 constant organic load rate of 4.5±0.3 kg TVS /$m^3$*day for OFMSW pressure-extrusion and 1.7±0.5 kg TVS
129 /$m^3$*day, as a mean value, for the OFMSW pulper pre-treatment. The pre-treatments were examined
130 over two 20-day consecutive hydraulic retention time periods for each organic loading rate. The first
131 period would ensure the highest replacement parts of the waste material inside the reactors, and in the
132 second 20 days, we analysed the process under stable conditions, as all the feed had replaced the
133 inoculum content.
134 Every 2 days, representative samples from the anaerobic reactor effluent were analysed. The following
135 parameters were analysed three times per week in accordance with Standard Methods (APHA, 1995):
136 pH, total solids (TS), total volatile solids (TVS), alkalinity, acidity, N, and total carbon. Daily biogas
137 production was measured using a liquid displacement system connected to the digester. The biogas
138 volume was corrected using standard temperature and pressure conditions. The biogas composition
139 (methane and carbon dioxide percentages) was analysed once per week using a portable analyser and

140 confirmed via gas chromatography. For methanogen analysis, the samples were collected three times
141 per week in a 50 ml sterile tube and frozen at -20°C until extraction. More than 80 digest effluent
142 samples were collected from more than a year of digestion operations beginning March 2009.
143
144 **2.2 DNA extraction and purification**
145 The digestate aliquots were thawed at 4°C overnight and centrifuged at 3000 g for 20 minutes; the
146 supernatant was then removed, and the semi-dry aliquots were used for the following steps. The total
147 DNA was extracted from 0.25 g of particulate matter (residue humidity was 31±5%) using the PowerSoil
148 DNA Isolation Kit followed by UltraClean Soil DNA Kit (MoBio Laboratories). The DNA quantity extracted
149 was 3.51±1.53 ng/µl (mean), and its quality was evaluated using gel electrophoresis before the chain
150 reaction. Only the samples over 1 ng/µl and of sufficient DNA quality, tested by gel electrophoresis,
151 were used for the following step. Lower quantity or a bed quality of the DNA makes the successive
152 quantification difficult so this kind of DNA quality cut-off was applied. 50 samples were sufficient in
153 distinct DNA quantity and quality.
154
155 **2.3 RT-qPCR analysis**
156 After DNA extraction and purification, total methanogen was quantified using methanogen-specific
157 short primers for an *mcrA* sequence described by Steinberg and Regan (Steinberg and Regan, 2008) and
158 synthesised by ThermoBiopolymer. For the reactions, we used a standard super-mix (Bio-Rad SsoFast™
159 EvaGreen SuperMix), the RT-PCR Chromo4 (Bio-Rad) and the Opticon Monitor 3 Software. The reaction
160 conditions were previously described (Steinberg and Regan, 2008). The standard reference was a
161 *Methanosarcina acetivorans* mcrA sequence of approximately 470 base pairs (Lueders et al.,
162 2001)included in a pCR21 vector (Invitrogen) supplied by L.M. Steinberg and J.M. Regan of the
163 Pennsylvania State University. This plasmid was amplified by transforming *Escherichia coli* Top10 cells
164 according to the manufacturer's instruction. The transformed cells were selected on LB agar in presence

of ampicillin, and the plasmid was extracted using a plasmid DNA purification kit (NucleoSpin Plasmid, Macherey-Nagel). The standard curve had six points (figure 2) and was calculated using the threshold cycle method; for the highest yield, 2,3 ng of plasmid was amplified (~$4.5*10^8$ plasmid copies). Between each standard curve point there is a 1:10 dilution. The standards and samples were tested in triplicate. The triplicate value was accepted only if the coefficient of variation was below 20%. The correlation coefficient was considered sufficient if above 0.990. PCR efficiency was 97%, and the resolution limit for the method was $4.5*10^3$ copies of *mcrA.* We used in the sample amplification 2 µl of a 1:10 dilution for each DNA extract. This quantity was evaluated as the best among various tested quantities for a standard curve and with an acceptable PCR efficiency. The 1:10 dilution limited the effect of inhibiting substances present in this type of sample. Figure 3 shows the quantification precision obtained, beginning with the same two samples re-extracted 10-fold. This test illustrated that the sampling procedure does not affect the final analysis. The variation coefficient was below 10% for sample 1 and below 20% for sample 2. The 50 samples were sufficient in DNA quantity and quality.

### 2.4 Statistics

Statistical analyses were performed using the SPSS Package, version 17.0 for Windows. A Pearson correlation coefficient was used to assess the relationship between the variables. A T-test of independent variables was used in the mean evaluations. The differences and correlations were considered significant at $p<0.05$ and highly significant at $p<0.01$.

## 3. RESULTS AND DISCUSSION

Table 2 shows the primary process-control factors that were monitored, such as biogas production parameters, solid reduction data and acidity values. Pre-treatment A not only yields more relevant quantities of biogas, but the biogas production was also double the value observed in treatment B. Thus, treatment A provides better input preparation likely due to a reduction in particle size, increasing the

190 available specific surface and enhancing the contact surface between bacteria. This was further
191 confirmed via a %TVS reduction. The digestion process fed with pre-treatment A material performs
192 better with respect to pre-treatment B, despite the unfavourable input pH (table 1).
193 The correlation between methanogen abundance and biogas production is positive and statistically
194 significant ($p<0.01$), as shown in figure 4. This correlation holds both considering all the collected data
195 (first and second digestions) and separating the two data sets. As a measure of the correlation, Pearson's
196 rho is 0.579 ($p<0.01$). In figure 4, the correlations are expressed for each collected data set. Further, the
197 single digestion showed a positive and significant correlation between methanogen quantification and
198 biogas production rate.
199 Even if biomolecular methodology for methanogen analyses during anaerobic processes became
200 nowadays diffused, this is one of first studies to analyse and correlate methanogen load with the biogas
201 production during full digestion sessions. Further, this are very few studies that monitored by such
202 biological method different process and that observed a significant correlation using a *mcrA*-based PCR
203 method. Although, anaerobic methanotrophs that are phylogenetically related to methanogens have an
204 Mcr-like protein that catalyses methane oxidation (Nunoura et al., 2008), the abundance of this
205 microbial population is likely negligible with respect to the methanogen community, as deduced from
206 the scarcity of methanotroph products such as $N_2$ and $HS^-$ (Balat and Balat, 2009).
207 Figure 5 shows a marked and statistically significant difference between the methanogen load present in
208 the digester feed from pre-treated A and pre-treated B organics. This trend is similar to biogas
209 production (table 2), even if only the methanogen load difference is statistically significant ($p<0.01$).
210 Setting a 'cut-off' for optimal production generated an additional measure of correlated activity. As also
211 observed in the literature, production rate ranged between 0.3 to 0.8 $m^3$ biogas/kg TVS in base of
212 various parameters such as input characteristics, process temperature, organic load, and so on. Optimal
213 production can be defined as 0.6 $m^3$ biogas/kg TVS (Cuetos et al., 2009; Gomez et al., 2006). Shown in
214 figure 5, the methanogen load is lower in the samples with sub-optimal production rates compared with

optimal samples (T-test, $p<0.05$).

However, we would also discuss in this paper whether, overall, the data support methanogen abundance, determined with the previously described RT-qPCR method, decrease as a biomarker for methanogenesis efficiency. The stability parameters typically measured when monitoring digestions, such as pH, alkalinity and acidity of the mixture as well as split volatile fatty acid concentration in the reactor, were not sufficient early predictors during this process. A variation of these parameters correlates with a contemporaneous decrease in production. In this instance, corrective steps to support a productive balance in the reactor are ineffective. As shown in figure 6, prediction may only be relevant at certain times during the process, such as between the 3$^{rd}$ and 17$^{th}$ of April, 8$^{th}$ and 15$^{th}$ of May and 22$^{nd}$ and 29$^{th}$ of May. On certain days, we observed a decrease in the *mcrA* copies just before a decrease in the specific biogas production rate; however, this event was not regular during the digestion process.

Recent studies show that methanogen population dynamics and community structures can vary during the digestion processes (Lee et al., 2009a; Lee et al., 2009b; Yu et al., 2006), as can volatile fatty acid concentrations (Wang et al., 2009). All of these studies discussed the microbial communities' capacity for responding to changes in anaerobic environments, such as altered feeding (Kovacik et al., 2010) and different temperature (Sasaki et al., 2011), among others.

## 4. CONCLUSIONS

Despite the environmental complexities inherent in the work herein, we conducted a representative sampling procedure and a valid DNA extraction. The results show proper quantitation for the majority of the collected samples (63%). The experiments conducted during the digestion process in the reactors demonstrate a good method for determining methanogen concentration with respect to the biogas production. The hypothesis, that methanogen community abundance and composition are strictly related to the methane production, is confirmed.

In the last few years, the research community has enhanced our knowledge regarding Archea microorganisms from both an ecological standpoint (Leigh et al., 2011; Liu and Whitman, 2008b) and a human health perspective (Johnston et al., 2010). The economic costs are really onerous for these analyses (Kalia and Purohit, 2008) moreover the proposed PCR method herein is a useful tool for studying methanogen populations and their modulation related to biogas production rate (Lee et al., 2009a; Patil et al., 2010), even if the results herein cannot yet provide a clear activity prediction.

McrA gene-based methods could be even more diffused methods (Zhu et al., 2011), also relating with digestion performance (Bialek et al., 2011) and feeding matrices, which currently pose a relevant concern for waste management and public health (Cardinali-Rezende et al., 2009). A fundamental step includes analysis of, at least, the different families of methanogens to identify a better early bio-indicator among the total methanogen population (Steinberg and Regan, 2009; Vavilin et al., 2008). From these studies, an alarm threshold for micro-organism levels could be a useful, fundamental process-control parameter but the introduction of this analysis must be economically sustainable. Prediction ability with respect to biogas loss over several days might also be interesting from an economic standpoint. The problems related with arrested methanogenesis are one of the largest obstacles to anaerobic digestion diffusion. In the approach proposed herein, it is essential to examine the community composition and the genus contribution to optimise the digestion process and maximise $CH_4$ yield.

**Acknowledgements**

The Authors wish to thank the Piedmont Region for funding; this work is a portion of a larger project referred to as DigestedEnergy, and it was founded for the 2006 call for Pre-competitive Development and Industrial Research. This funding scheme was promoted by the Piedmont Region with European Community resources. Special acknowledgments are due to L. Steinberg and J. Regan for the plasmid standard supply. Finally, the Authors thank all of the numerous collaborators employed in each of the

265 involved institutions: Università degli Studi del Piemonte Orientale “A. Avogadro”, Politecnico di Torino,
266 SMAT S.pA., Amiat S.p.A., Ansaldo FC S.p.A., Acsel Susa S.p.A., VM-press s.r.l., Federsviluppo, E.R.A.P.R.A
267 Piemonte, and Università degli Studi di Torino.
268

**FIGURE CHAPTERS**

**Figure 1** – The pilot hardware description is illustrated. The same reactor, in different six-month fermentation sessions, was used with two different feed pre-treatments.

**Figure 2** – Example of a standard curve obtained during experiment. The same curve is included in all analysis sessions, and the samples are placed within the standard points.

**Figure 3** - RT-PCR quantification of 10 different, consecutive extractions for the same two samples. Different samples collected on different days were used. Cross-shaped points represent each different extract quantification. For each set, the last point (score) represents the mean from all the analyses. For each data point, the standard deviation is shown.

**Figure 4** - Linear regression model for RT-PCR quantification samples collected from reactors and the logarithm of the target gene copies for each µl of DNA sample. (A) circles represent pressure-extrusion pre-treatment data, $y=1.0303x+6.5118$ (correlation=0.418, $p<0.05$); (B) rhombus represent the turbo-mixing pre-treatment data, $y=2.5629x+4.3782$ (correlation=0.622, $p<0.05$).

**Figure 5** - *McrA* abundance evaluated during the digestion process, subdivided by input material pre-treatment (T-test $p<0.01$) and optimal biogas production (0.6 $m^3$/kg SV) (T-test $p<0.05$).

**Figure 6 -** Temporal trends of *mcrA* abundance (squares) and biogas production (rhombus) during digestion of the pressure-extruded input for the reactor.

294

295 **TABLES**

296

| | Pre-treatment A | Pre-treatment B |
|---|---|---|
| **pH** | 4.4 ± 0.3 | 6.0 ± 0.7 |
| **Total Solids (TS) (%)** | 9.9 ± 0.7 | 4.6 ± 1.1 |
| **Total volatile solids (TVS) (%)** | 8.7 ± 0.7 | 3.3 ± 1.1 |
| **TVS/ST (%)** | 86.8 ± 0.2 | 70.6 ± 4.9 |
| **Carbon (C) (%ST)** | 46.0 ± 0.9 | 37.0 ± 3.4 |
| **Nitrogen (N) (%ST)** | 3.1 ± 0.2 | 3.5 ± 0.3 |
| **C/N ratio** | 15.2 ± 1.1 | 10.4 ± 1.5 |

297

298 **Table 1:** Characteristics of the pre-treated inputs from the two different methods used in the anaerobic
299 co-digestion processes.

300

| **Parameters** | **Pre-treatment A** | **Pre-treatment B** |
|---|---|---|
| Daily biogas production (L/die) | 27.08 ± 3.01 | 4.87 ± 2.46 |
| Specific biogas production ($m^3$/kg $VS_{added}$) | 0.64 ±0.07 | 0.30 ± 0.13 |
| TS reduction (%) | 64.44 ± 7.57 | 31.67 ± 6.23 |
| TVS reduction (%) | 73.84 ± 5.87 | 38.13 ± 6.70 |
| pH | 7.36 ± 0.34 | 6.82 ± 0.52 |
| Ac./Alc. ratio | 0.37 ± 0.18 | 2.47± 2.41 |
| $CH_4$ (%) | 60.60 ± 2.90 | 57.50 ± 6.10 |
| $CO_2$ (%) | 37.70 ± 3.20 | 41.00 ± 6.44 |

301

302 **Table 2:** Evaluation of relevant parameters for the co-digestion processes subdivided by pre-treated
303 method.

304

## BIBLIOGRAFY


**APHA, Standard methods for the examination of water and wastewater. American Water Works Association, Water Environment Federation, Washington, DC, USA,, 1995.**

**Balat, M., Balat, H., 2009. Biogas as a Renewable Energy SourceA Review. Energy Sources Part a-Recovery Utilization and Environmental Effects. 31, 1280-1293.**

**Bergmann, I., et al., 2010a. Influence of DNA isolation on Q-PCR-based quantification of methanogenic Archaea in biogas fermenters. Systematic and Applied Microbiology. 33, 78-84.**

**Bergmann, I., et al., 2010b. Determination of methanogenic Archaea abundance in a mesophilic biogas plant based on 16S rRNA gene sequence analysis. Canadian Journal of Microbiology. 56, 440-444.**

**Bialek, K., et al., 2011. Quantitative and qualitative analyses of methanogenic community development in high-rate anaerobic bioreactors. Water Research. 45, 1298-1308.**

**Bouallagui, H., et al., 2005. Bioreactor performance in anaerobic digestion of fruit and vegetable wastes. Process Biochemistry. 40, 989-995.**

**Burton, C. H., Turner, C., 2003. Anaerobic treatment options for animal manures. UK: Silsoe Research Institute, Silsoe, Bedford.**

**Cardinali-Rezende, J., et al., 2009. Molecular identification and dynamics of microbial communities in reactor treating organic household waste. Applied Microbiology and Biotechnology. 84, 777-789.**

**Cuetos, M. J., et al., 2009. Anaerobic co-digestion of poultry blood with OFMSW: FTIR and TG-DTG study of process stabilization. Environmental Technology. 30, 571-582.**

**das Neves, L. C. M., et al., 2009. Biogas Production: New Trends for Alternative Energy Sources in Rural and Urban Zones. Chemical Engineering & Technology. 32, 1147-1153.**

**Franke-Whittle, I. H., et al., 2009. Design and testing of real-time PCR primers for the quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of uncultured methanogens. Canadian Journal of Microbiology. 55, 611-616.**

**Friedrich, M. W., 2005. Methyl-Coenzyme M reductase genes: unique functional markers for methanogenic and anaerobic methane-oxidizing Archea. Methods in Enzymology, 428-442.**

**Gomez, X., et al., 2006. Anaerobic co-digestion of primary sludge and the fruit and vegetable fraction of the municipal solid wastes - Conditions for mixing and evaluation of the organic loading rate. Renewable Energy. 31, 2017-2024.**

338 **Hedderich, R., Whitman, W. B., 2006. Physiology and Biochemistry of the methane-producing**
339 **Archeae. Prokaryotes Volume 2, 1050-1079.**

340 **Johnston, C., et al., 2010. A real-time qPCR assay for the detection of the nifH gene of**
341 **Methanobrevibacter smithii, a potential indicator of sewage pollution. J Appl**
342 **Microbiol. 109, 1946-56.**

343 **Kalia, V. C., Purohit, H. J., 2008. Microbial diversity and genomics in aid of bioenergy. J Ind**
344 **Microbiol Biotechnol. 35, 403-19.**

345 **Knittel, K., Boetius, A., 2009. Anaerobic Oxidation of Methane: Progress with an Unknown**
346 **Process. Annual Review of Microbiology. 63, 311-334.**

347 **Kovacik, W. P., et al., 2010. Microbial dynamics in upflow anaerobic sludge blanket (UASB)**
348 **bioreactor granules in response to short-term changes in substrate feed.**
349 **Microbiology-Sgm. 156, 2418-2427.**

350 **Lee, C., et al., 2009a. Quantitative analysis of methanogenic community dynamics in three**
351 **anaerobic batch digesters treating different wastewaters. Water Research. 43, 157-**
352 **165.**

353 **Lee, C., et al., 2009a. Quantitative and qualitative transitions of methanogen community**
354 **structure during the batch anaerobic digestion of cheese-processing wastewater.**
355 **Applied Microbiology and Biotechnology. 87, 1963-1973.**

356 **Lee, M., et al., 2009b. Comparative performance and microbial diversity of hyperthermophilic**
357 **and thermophilic co-digestion of kitchen garbage and excess sludge. Bioresour**
358 **Technol. 100, 578-85.**

359 **Leigh, J. A., et al., 2011. Model organisms for genetics in the domain Archaea: methanogens,**
360 **halophiles, Thermococcales and Sulfolobales. FEMS Microbiol Rev.**

361 **Liu, Y. C., Whitman, W. B., 2008a. Metabolic, phylogenetic, and ecological diversity of the**
362 **methanogenic archaea. Incredible Anaerobes: From Physiology to Genomics to Fuels.**
363 **1125, 171-189.**

364 **Liu, Y. C., Whitman, W. B., Metabolic, phylogenetic, and ecological diversity of the**
365 **methanogenic archaea. In: J. Wiegel, et al., Eds.), Incredible Anaerobes: From**
366 **Physiology to Genomics to Fuels. Blackwell Publishing, Oxford, 2008b, pp. 171-189.**

367 **Lozano, C. J. S., et al., 2009. Microbiological characterization and specific methanogenic**
368 **activity of anaerobe sludges used in urban solid waste treatment. Waste**
369 **Management. 29, 704-711.**

370 **Lueders, T., et al., 2001. Molecular analyses of methyl-coenzyme M reductase alfa-subunit**
371 **(mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic**
372 **phenotype of a novel archaeal lineage. Environmental Microbiology. 3, 194-204.**

**Luton, P. E., et al., 2002. The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. Microbiology-Sgm. 148, 3521-3530.**

**Mata-Alvarez, J., et al., 2011. Codigestion of solid wastes: A review of its uses and perspectives including modeling. Critical Reviews in Biotechnology. 31, 99-111.**

**Mshandete, A., et al., 2004. Anaerobic batch co-digestion of sisal pulp and fish wastes. Bioresource Technology. 95, 19-24.**

**Nettmann, E., et al., 2008. Archaea diversity within a commercial biogas plant utilizing herbal biomass determined by 16S rDNA and mcrA analysis. Journal of Applied Microbiology. 105, 1835-1850.**

**Nunoura, T., et al., 2008. Quantification of mcrA by fluorescent PCR in methanogenic and methanotrophic microbial communities. Fems Microbiology Ecology. 64, 240-247.**

**Patil, S. S., et al., 2010. Microbial community dynamics in anaerobic bioreactors and algal tanks treating piggery wastewater. Applied Microbiology and Biotechnology. 87, 353-363.**

**Rozzi, A., Remigi, E., 2004. Methods of assessing microbial activity and inhibition under anaerobic conditions: A literature review. Reviews in Environmental Science and Biotechnology. 3, 93-115.**

**Sasaki, K., et al., 2011. Bioelectrochemical system accelerates microbial growth and degradation of filter paper. Applied Microbiology and Biotechnology. 89, 449-455.**

**Schievano, A., et al., 2009. Substituting energy crops with organic wastes and agro-industrial residues for biogas production. Journal of Environmental Management. 90, 2537-2541.**

**Steinberg, L. M., Regan, J. M., 2008. Phylogenetic Comparison of the Methanogenic Communities from an Acidic, Oligotrophic Fen and an Anaerobic Digester Treating Municipal Wastewater Sludge. Applied and Environmental Microbiology. 74, 6663-6671.**

**Steinberg, L. M., Regan, J. M., 2009. mcrA-Targeted Real-Time Quantitative PCR Method To Examine Methanogen Communities. Applied and Environmental Microbiology. 75, 4435-4442.**

**Vavilin, V. A., et al., 2008. Methanosarcina as the dominant aceticlastic methanogens during mesophilic anaerobic digestion of putrescible waste. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology. 94, 593-605.**

**Wang, Y. Y., et al., 2009. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass & Bioenergy. 33, 848-853.**

**Ward, A. J., et al., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresource Technology. 99, 7928-7940.**

**Whitman, W. B., et al., 2006. The Methanogenic Bacteria. Prokaryotes. 3, 165-207.**

**Yu, Y., et al., 2006. Use of real-time PCR for group-specific quantification of aceticlastic methanogens in anaerobic processes: Population dynamics and community structures. Biotechnology and Bioengineering. 93, 424-433.**

**Zhu, C. G., et al., 2011. Diversity of methanogenic archaea in a biogas reactor fed with swine feces as the mono-substrate by mcrA analysis. Microbiological Research. 166, 27-35.**