Real-Time qPCR method to study methanogen communities during wet anaerobic co-digestion of organic wastes

REAL-TIME qPCR METHOD TO STUDY METHANOGEN COMMUNITIES DURING WET ANAEROBIC CO-DIGESTION OF ORGANIC WASTES.

D. TRAVERSI*, R. DEGAN*, E. LORENZI** AND G. GILLI*

** Department of Public Health and Microbiology, University of the Study of Turin, via Santena 5 bis, 10126 Torino, Italy*
*** SMAT S.p.A., corso XI Febbraio 14, 10152 Torino, Italy*

SUMMARY: Biogas is a biofuel with a large energy value and is mostly consisting of methane. It is a renewable energy source, as a substitute for natural gas, and is produced by anerobic digestion of various organic materials. Among which there are agricultural residues, waste water sludges and organic urban wastes. In the reactors anaerobic microrganisms can degrade the waste organic matter and its pollutants in two different products: digested sludge and biogas. If the microbial community is optimised the outing digested sludge could be used as a soil fertilizer and the methane production rate could be improved. Methanogen population is liable to the biogas production. We detected the methanogens during a wet digestion process fed by pre-treated urban organic wastes and waste water sludges. Applied methodology is a real-time quantitative PCR (qPCR) based on *mcrA* target. We observed a positive and significant correlation between the biogas production rate and the methanogen abundance ($r=0.498$, $p<0.001$). Moreover methanogen abundance could be proposed as a diagnostic tool in methane produce optimization.

1. INTRODUCTION

Two serious environmental and public health problems characterise our society today, the first is to reduce and treat the produced waste, especially in high demographic density areas, and the second is to answer at the energetic request limiting the use of conventional fuels (das Neves et al., 2009). In urban communities these goals have no clear resolution but renewable energy sources are probably one of the key strategies (Balat and Balat, 2009). Anaerobic digestion process of organic waste combines the removal of organic pollutants reducing the organic waste volumes and contemporary produces energy conservation in the form of biogas production (Rozzi and Remigi, 2004). To the wet anaerobic digestion can be addressed numerous organic wastes such as wastewater sludge, pre-treated organic household waste, food processing wastes, agro-zootechnic waste, working refuse, and selected crops (Bouallagui et al., 2005; Schievano et al., 2009).The biogas production is the consequence of a series of metabolic interactions among bacterial and archeal micro-organisms (Ward et al., 2008).

Proceedings Venice 2010, Third International Symposium on Energy from Biomass and Waste
Venice, Italy; 8-11 November 2010

At the end, methanogenic Archea produce mainly CH_4 and CO_2 converting H_2, formate and acetate. Methanogens are difficult to study through culture-based methods although the methanogenesis represents the critical step in biogas production in anaerobic reactors (Liu and Whitman, 2008). During the last years culture-independent techniques were developed (Hughes et al., 2001). They have been based on phylogenetic markers like the 16S rRNA or methyl coenzyme M reductase genes (Nunoura et al., 2008; Rastogi et al., 2008). The 16S rRNA gene is the most widely used target for gene surveys (Nayak et al., 2009) while the Mcr is exclusive to the methanogens with the exception of the methane-oxidizing Archaea (Knittel and Boetius, 2009, Whitman et al., 2006), today are present specific primers for the gene sequence of the α-subunit of the methyl coenzyme M reductase (*mcrA*) (Luton et al., 2002, Steinberg and Regan, 2008).

The *mcrA* analysis can be used in conjunction with, or independently of the 16S rRNA gene and it minimizes potential problems with non-specific amplification (Steinberg and Regan, 2008).

The scientific aim is mainly to study methanogen population on which there are a limited knowledge and also, with a more applicative approach to propose a biologic indicator assuring the good performance of the biogas producing process.

2. FUNDAMENTALS OF METHANOGENESYS

2.1 Anaerobic Digestion Microbiology

A particular ecosystem are present in an anaerobic reactor where several groups of microorganisms work interactively in the conversion of complex organic matter into biogas. This is composed mainly by methane (~ 60% by volume) and carbon dioxide (~ 40% by volume) and than there are trace of hydrogen sulfide, molecular nitrogen, molecular hydrogen, molecular oxygen and ammonia (~ 0,5%, ~ 2%, ~ 0,5%, ~ 1%, ~ 0,5% by volume respectevely) (Balat and Balat, 2009). In the digestion process four stages take place: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Lozano et al., 2009). The first group of micro-organisms secretes enzymes which hydrolyze polymers to monomers so particulate materials are converted into dissolved materials by the action of exoenzymes excreted by the hydrolitic fermentative bacteria such as *Bacillus* and *Pseudomonas* (Whitman et al., 2006). This group includes both obligate and facultative anaerobes, and may occur up to 10^8-10^9 cells/ml of sewage sludge digesters. They remove the small amounts of O_2 present and create anaerobic conditions. Subsequently acidogenic phase includes the action of a large and diverse group of fermentative bacteria, usually belong to the clostridia group and the family Bacteroidaceaea. These bacteria hydrolyze and ferment the organic materials, e.g., cellulose, starch, proteins, sugars, lipids, etc., and produce organic acids, CO_2 and H_2. They were species that often form spores that surviving in adverse environment. Then acetogenic bacteria convert these monomers to H_2 and volatile fatty acids. The final phase of the biogas production is carried out by aceticlastic methanogens - mainly *Methanosarcina* with high acetate level (>10^{-3}M) and *Methanosaeta* with lower acetate level - and hydrogenotrophic methanogens. Methanogenesis is considered the rate-limiting step moreover this phase is most vulnerable to temperature or pH variations and toxic chemicals (Liu and Whitman, 2008). A low activity of the methanogens conduct to accumulation of H_2 and short chain fatty acids with a consequent decrease of the pH, therefore enhancement of methanogenesis is a major route for improving the performance of anaerobic digestors.

2.2 Methanogen biochemistry

The useful substrates for methanogens are substatially three: CO_2, methyl-group containing compounds and acetate. Methanogens acquire energy from esoergonic biochemist reactions (from -31,0 to -135,6 kJ/mol CH_4) (Whitman et al., 2006).The acetate is a major intemediate in the anerobic food chain and as much as two-thirds of the biologically generated methane is produced from this molecule (Liu and Whitman, 2008). There are many novel coenzymes that are associated with the methane synthesis most of them are also involved in the eubacteria biosynthetic reactions.

Among which there are methafuran, tetrahydromethanopterin, 7-mercaptoheptanoylthreonine phosphate, methyl coenzyme M and coenzyme F_{430} .The first previous coenzymes are expressed also in Eubacteria (Liu, 2008). Although every pathway starts out differently, they all end with the same step, the reaction of methyl-coenzyme M (HS-CoM) with a second thiol coenzyme, (coenzyme B), to form methane and the mixed disulfide of coenzyme M and coenzyme B. This reaction is catalyzed by methyl-coenzyme M reductase (Mcr), making Mcr the key enzyme in methanogenesis (Friedrich, 2005). In its active site, this enzyme contains a unique prosthetic group, which is a nickel (Ni) porphinoid called coenzyme F430 (Hedderich and Whitman, 2006). Since HS-CoM has been found in all methanogens examined, it has been proposed as a sensitive biomarker for the quantitative and qualitative identification in different anaerobic environment. Also anaerobic methanotrophs that are phylogenetically related to methanogens have Mcr-like protein that catalyzes methane oxidation (Nunoura et al., 2008). Moreover the abundance of this microbial population is probably negligible respect to the methanogen community this is deduced from the scarcity of the methanotroph products such as N_2 and HS^-(Balat and Balat, 2009).

2.3 Methanogen determinations

Despite their key role as the terminal oxidizers in a complex microbial community very little is known about the methanogen community structure. Probably only a fraction of the methanogens in nature have been described and most of the species description are based on the examination of few strains so the phenotypic characterization is far to be complete. Moreover, the possibility to grow *in vitro* this kind of microrganisms is no very common in the laboratories. It is due mainly to the necessity of strictly anaerobic conditions but also to the lack research attention on this field until the last twenty years. All this factors conduced researchers to develop various biomolecular methods to identify methanoges sub-populations such as ribosomal RNA sequence analysis (Whitman et al., 2006). qPCR is an alternative technique capable of determining the copy number of a particular gene present in the DNA extracted from an environmental sample. Only few studies have used qPCR for quantitative examine of methanogen communities, and most of these studies have exclusively targeted the 16S rRNA gene (Freitag and Prosser, 2009; Rizzi et al., 2006). Moreover in the last years methods based on mcrA diversity was proposed (Freitag and Prosser, 2009). Methanogens are classified into five orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales) further divided into 10 families and 31 genera. Anaerobic digestors are one typical habitat especially for the following genera: *Methanobacterium, Methanothermobacter, Methanomicrobium, Methanoculleus, Methanofollis, Methanospirillum, Methanocorpusculum, Methanosarcina* and *Metanosaeta* (Liu and Whitman, 2008). The genera frequently isolated are *Methanobacterium, Methanospirillum* and *Methanobrevibacter*. In a mesophilic biogas plant 84% of all detected methanogens were affiliated with the Methanomicrobiales, while only 14% belonged to the Methanosarcinales and 2% to the Methanobacteriales order (Bergmann et al., 2010).

3. EXPERIMENTAL STUDY

In this paper we apply a methodology for determining methanogen gene copy numbers through the use of real-time quantitative PCR (RT-qPCR) targeting the *mcrA*.

3.1 Digestion process and digestate sampling

More than 40 digestion effluent samples were collected during a semester of digestion (March-July 2009) from two pilot reactors fed with pre-treated household organic waste, waste-water sludge and caw sewage (the last only in the start-up mixture). The Figure 1 represents both the equal equipment for each reactor and the mainly reactor parameters. During the digestion the feeding organic fraction was increased in three steps from ~4 to ~10% in one of the reactor (P1) - VS% varied from 3.16±0.49 to 8.68±0.69 - while in the other (P2) the organic load is quite constant (~10%) for the period of the sampling, VS% was 8.68±0.69. The volatile solids represented more than 84% of the total solids. The feeding pH decreased with the enhance of the organic load from 4.72±0.69 to 4.39±0.27. The outing digestates pH was ~7.40 and the VS reduction percentage varied from 67 to 77%. The gas yield was meanly of 0,71 and 0,64 m^3/kg VS added respectively in the reactor P1 e P2. The samplings were collected three times a week in 50ml sterile tube and frozen at -20°C until the extraction session.

3.2 DNA extraction and purification

The digestate aliquots were unfreeze at 4°C over night, then they are centrifugate at 3000 g for 20 minutes, the supernatant was removed and the semi-dry aliquots were used for the following steps. Total DNA was extracted from 0,25g of this particulate matter (residue humidity 32%) using the PowerSoil DNA Isolation Kit following by UltraClean Soil DNA Kit (MoBio Laboratories). The DNA quantity extracted varied from 2,84 ng/µl to 6,40 ng/µl, the DNA quality was evaluated by gel electrophoresis.

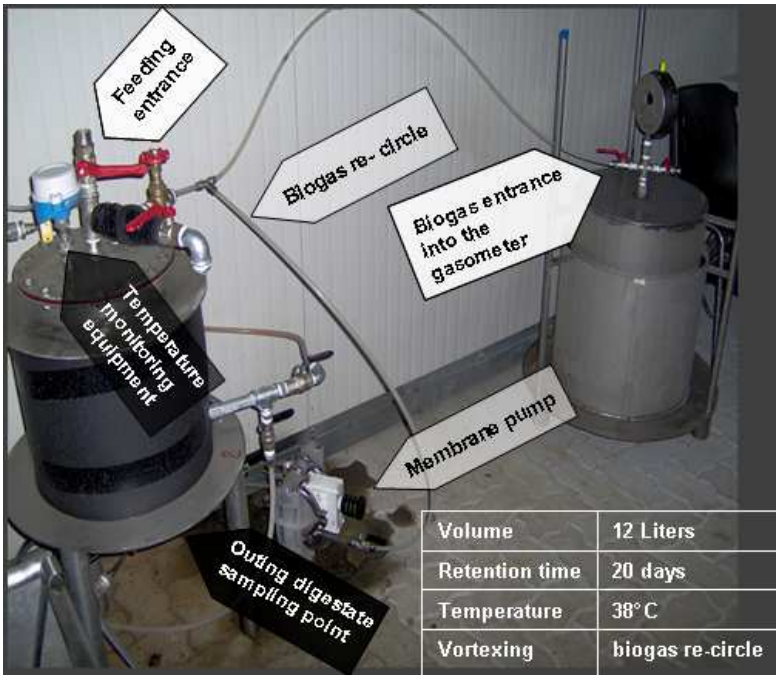

Figure 1. The P1 and P2 hardware description is illustrated. Two reactor with the same characteristics are used during this research activities.

3.3 qRT-PCR analysis

After the DNA extraction and purification, methanogens were quantified in total using methanogen-specific primers described by (Steinberg and Regan, 2008) and synthesized by ThermoBiopolymer. The reactions were conducted with EVA green super mix (Bio-Rad SsoFast^{TM} EvaGreen SuperMix) using the RT-PCR Chromo4 (Bio-rad) and the Opticon Monitor Software. The reaction conditions are previously described (Steinberg and Regan, 2008). We used 2 µl of a 1:9 dilution for each sample. This quantity of sample is the best tested in order to obtain a good quantification respect to the standard curve and limiting the effect of inhibition substances present in this kind of samples. The reaction efficiency is >0,75. The standard reference is a *Methanosarcina acetivorans* mcrA sequence included in pCR21 vector (Invitrogen) supplied by L.M. Steinberg and J.M. Regan of the Pennsylvania State University. This plasmid is amplified transforming Escherichia Coli Top10 cells according to the manufacturer's instruction. 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 and it is calculated with the threshold cycle method, in the highest standard 2,3 ng of plasmid was amplified (~ 4.5 $*10^8$). Between each following standard curve point there is a 1:10 dilution. Standard curve and samples are tested in triplicates. Resolution Limit of the method is $5*10^3$ copies of *mcrA.*

3.4 Data validation

The Figure 2 shows the quantifications obtained beginning from the same two samples re-extracted 10 folds. This test illustrated as the sampling procedure doesn't affect the final determination. The variation coefficient is below of 10% for the sample 1 and below of 20% for the sample two. Statistical analyses were performed using the SPSS Package, version 17.0 for Windows. A Spearman correlation coefficient was used to assess the relationships between the variables. The mean differences and correlations were considered significant at $p<0.05$.

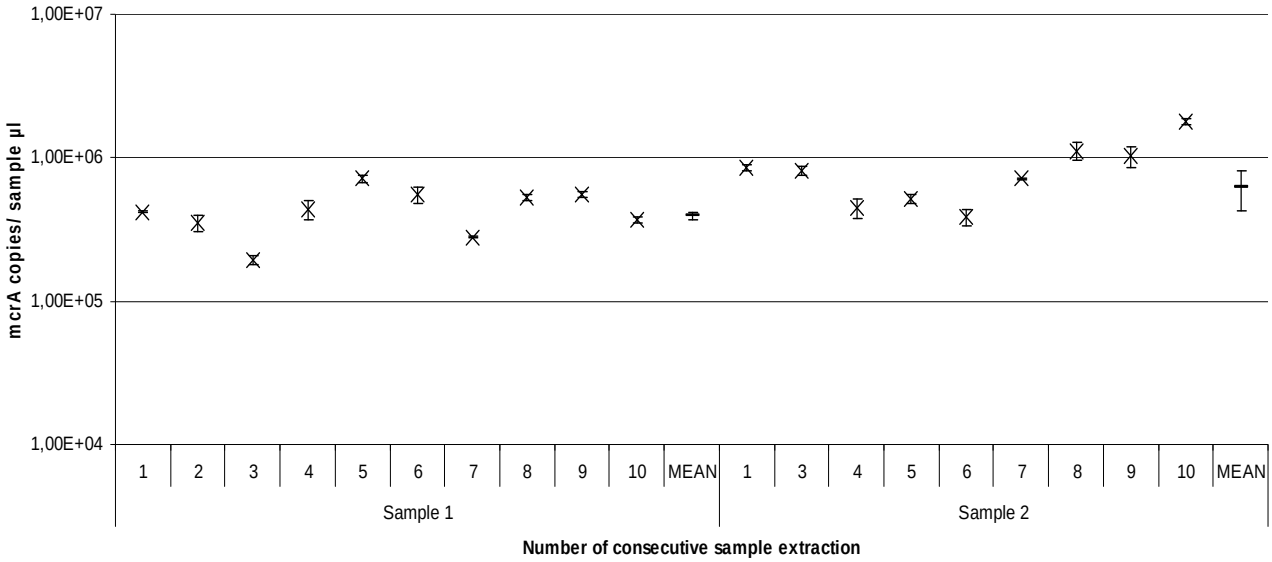

Figure 2. RT-PCR quantification of 10 different and consecutive extractions of the semi-dry sample. Two different samples collected in different data were used. The last point (score) for each sample set represents the mean. For each determination the standard deviation is showed.

4. RESULTS AND DISCUSSION

The first experimental session conducted on the digestor P1 would consider the relationship between methanogen community and organic load. As described on the Figure 3 this correlation is not present. The increase of organic load probably influences mainly the first steps of anaerobic digestion process, as previously described (Cardinali-Rezende et al., 2009), and not so much the last step in which the methanogens are involved.

On the second digestor the sample collection for methanogen determination is conducted since the attainment of the process stability at a constant organic load of ~10% VS. As showed on the figure 4 the correlation between methanogen abundance and biogas production has a statistical high significant ($p<0.001$) and the Spearman's rho is equal to 0.498.

On the other hand the experimentation is proposed mainly to identify a microbiological indicator of the good health of the digestion process. The stability parameters usually utilized in the digestion monitoring such as pH, alkalinity and acidity of the mixture and even the splitted volatile fatty acid concentrations in the reactor are not sufficient early predictors during the process. A variation of this parameters reaches quite simultaneously with a decrease of the production. When this happens it is too late to promote a corrective action conducing to a productive balance in the reactor. So we would understand if a decrease of the methanogen abundance, determined with this RT-qPCR method, is useful as biomarker of sufferance methanogenesis process. As showed on the figure 5 there isn't a clear prediction prospective even if sometimes during the process it could be observed that some days after (from 2 to 7) the decrease of the *mcrA* abundance it is recorded also a decrease of the production.

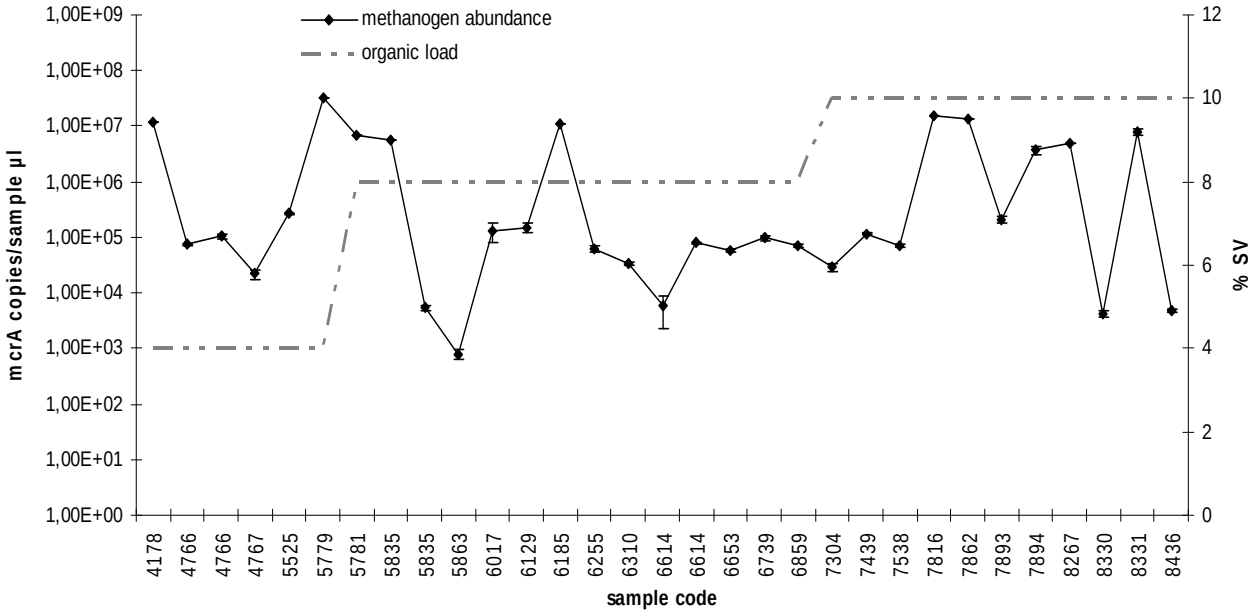

Figure 3. RT-PCR quantification samples collected from digestor P1 and relationship with organic load increase. Each mrcA RT-qPCR data is expressed as mean of the triplicate and is equipped with standard deviation on the triplicate.

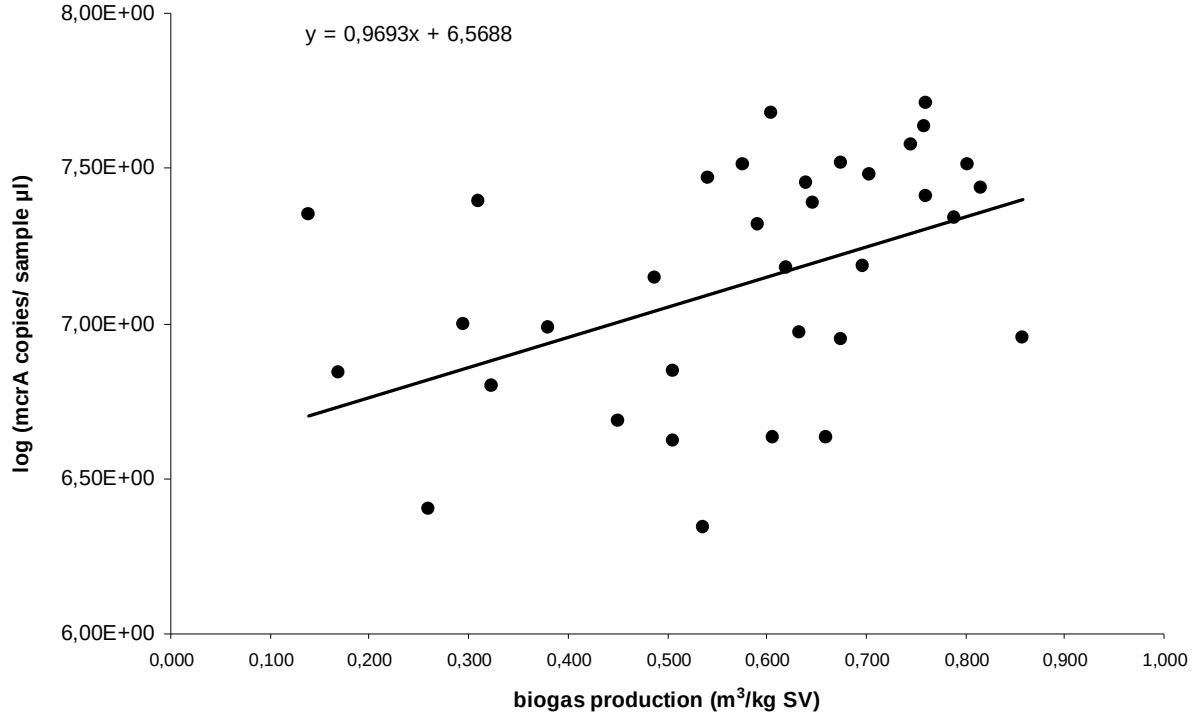

Figure 4. Linear regression model between RT-PCR quantification samples collected from digestor P2 and the logarithm of the target gene copies for each µl of DNA sample.

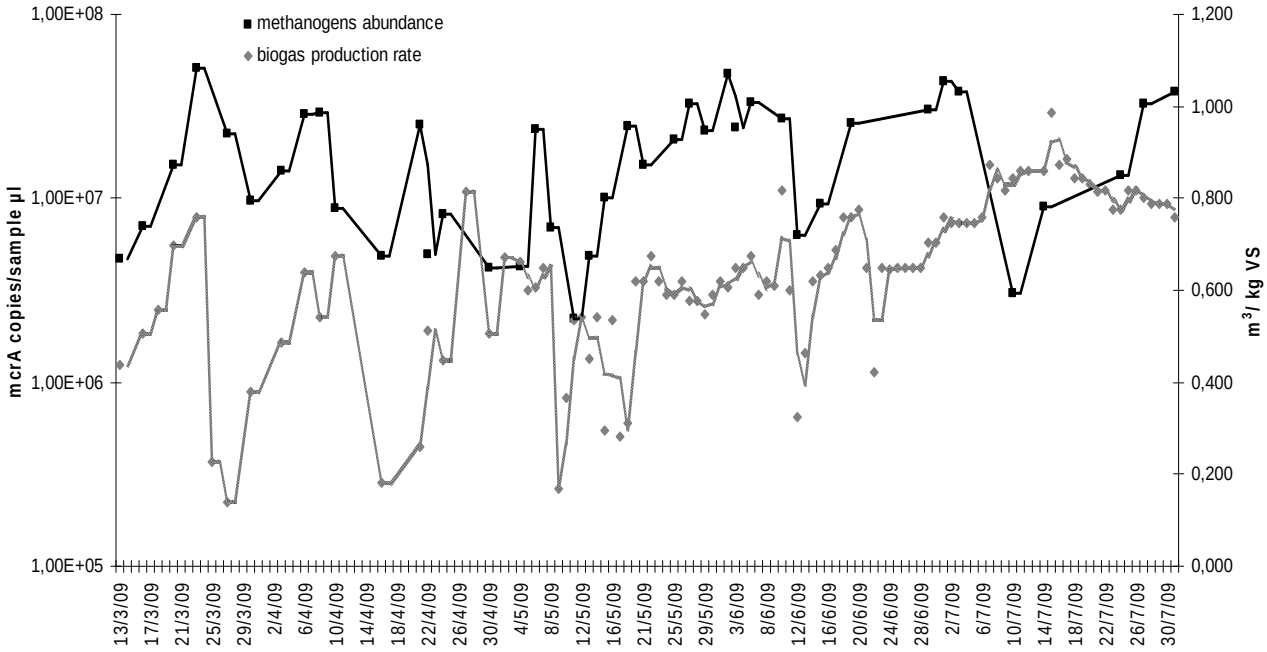

Figure 5. Temporal trends of the mcrA abundance (squares) and biogas production (rhombus point) during the digestion process in the reactor P2. The lines are produced by the mobile mean calculation of two consecutive determinations.

5. CONCLUSIONS

In spite of the troubles due to the complex nature of the environmental matrix in this work seems to be conducted a representative sampling procedure and a valid DNA extraction and analysis modus operandi. The results show a suitable quantification for each sample. The experimental activity conducted during the digestion process in the reactors show a real prospective in the methanogen determination respect to the biogas production. The hypothesis that the methanogen community abundance and composition are strictly related to the methane production is confirmed. Despite the onerous economic costs of this kind of determination (Kalia and Purohit 2008) the proposed method is useful to study methanogen population and its modulation relating to methane production rate but the results can't describe yet a clear predictor activity. A following research step is fundamental to analyse at least the different order, family of methanogens in order to identify a better early bio-indicator among the total methanogens (Steinberg and Regan, 2009; Vavilin et al., 2008). Accordingly to this concept the choose of alarm threshold in micro-organisms levels could be a fundamental control process parameter. The prospective to introduce this kind of analysis must be economically sustainable. A prediction ability respect to sufferance of the digestor that produce biogas losses production in term of two or more days could present an interest also under an economic view. The troubles related to stopped methonogenesis are one of the most obstacle to the anaerobic digestion diffusion. In the approach proposed it could be essential to examine the community composition and the genus contribution in order to optimise the digestion process and in the end to maximise the CH_4 yield.

ACKNOWLEDGEMENTS

The Authors wish to thank the Piedmont Region for the founding supply: this work is part of a big project called DigestedEnergy and it is founded in the area of the 2006 call for Pre-competitive Development and Industrial Research. This founding scheme is promoted by the Piedmont Region with European Community resources. Acknowledgemnts are due to L. Steinberg and J. Regan for the plasmid standard supply. Finally the Authors would thank all the numerous collaborators employed in each involved institutions: Università degli Studi del Piemonte Orientale, Politecnico di Torino, 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 Piemonte, Università degli Sudi di Torino.

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