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Monitoring anaerobic digestion of animal slurry during inhibition and recovery phases

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Ph.D. Thesis

Monitoring anaerobic digestion of animal slurry during inhibition and recovery phases

Control de la digestión anaerobia de purines durante periodos de inhibición y recuperación

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Abstract

This PhD thesis is focused on studying the dynamics of inhibition and recovery in the anaerobic digestion process of animal slurry to find indicators to predict process failures, minimize methane (CH_4) losses, and evaluate best management practices at biogas plant level.

To fulfill this objective, five trials were designed and conducted. Firstly, an experiment was designed to monitor physicochemical changes and gas emission of two types of aged pig slurry during 15 consecutive weeks of storage in summer conditions. Secondly, different sulphate (SO_4^{2-}) concentrations in pig and cattle slurry were monitored in terms of CH_4 yield and physicochemical changes in a batch assay. Thermophilic anaerobic degradation of organic matter (OM) and the inhibitory SO_4^{2-} dose were investigated. Thirdly, the effects of including SO_4^{2-} acidified pig slurry in an anaerobic co-digestion process with conventional slurry on process performance in two scale studies (laboratory and full scale) were determined. Key process indicators were identified. Fourthly, a combination of two methods, quantitative real-time polymerase chain reaction (qPCR) and qualitative scanning electron microscopy (SEM) was used to evaluate changes in the microbial population of anaerobic sludge digesters during the addition of pig slurry. Finally, the CH_4 yield, physicochemical composition and microbiological community structure and dynamics were evaluated during the start-up of anaerobic digestion of pig slurry in a laboratory scale. The tested strategies were feedless and non-feedless, followed by a gradual or an abrupt addition of pig slurry

The results presented in this PhD thesis allowed concluding that in the anaerobic degradation of OM from aged pig slurry, there is a relevant transformation of the more degradable into soluble OM during the first three weeks of storage. However, production of CH_4 did not occur until five weeks of storage. Regarding the use of acidified slurry in anaerobic digestion, added SO_4^{2-} concentrations in pig and cattle slurries for anaerobic digestion higher than 2000 mg SO_4^{2-} L^{-1} and 1500 mg SO_4^{2-} L^{-1} respectively, decreased CH_4 yield. However, SO_4^{2-} concentration of 500 mg SO_4^{2-} L^{-1} in pig slurry resulted in an increase in CH_4 yield. Additionally, the process state indicators to detect process failure in anaerobic digestion using acidified pig slurry were: SO_4^{2-} content of the slurry, alkalinity parameters, total volatile

fatty acids (VFA), especially acetic and butyric acids. Pig slurry addition to unadapted anaerobic digesters caused a deterioration of the anaerobic digestion process and the sludge characteristics lowering the pH, increasing VFA concentration, decreasing volatile solids degradation and reducing CH_4 yield in all digesters. During the adaptation period to pig slurry in anaerobic thermophilic conditions, an increase in total bacteria and archaea was observed through qPCR, as well as a change in microbial morphotypes through SEM. The change in microbial morphotypes was attributable to the addition of pig slurry. Regarding the start-up strategies, the strategy which best minimized CH_4 yields losses was non-feedless subjected to an abrupt change in substrate, although differences in microbial population among treatments were low. Moreover, hydrogenotrophic methanogenesis was the main metabolic route in CH_4 formation during the recovery period after star-up. *Methanomicrobiales* first and *Methanobacteriales* second, were the dominant archaeal orders throughout the recovery period, being useful process indicators

This PhD thesis provides useful practical information for monitoring anaerobic digestion of animal slurry during inhibition and recovery phases.

Resumen

Esta tesis doctoral se centra en la dinámica de la inhibición y la recuperación del proceso de digestión anaerobia de purines de cerdo para encontrar indicadores, predecir fallos del proceso, minimizar las pérdidas de metano (CH_4) y evaluar las mejores prácticas de gestión a nivel de la planta de biogás.

Para cumplir con este objetivo, cinco ensayos fueron diseñados y ejecutados. En primer lugar, se diseñó un experimento para controlar los cambios físicoquímicos y de emisión de gas de dos tipos de purines envejecidos durante 15 semanas de almacenamiento en condiciones de verano. En segundo lugar, diferentes concentraciones de sulfato (SO_4^{2-}) fueron evaluadas en digestión anaerobia termofílica de purines de cerdo y vacuno controlando la producción de CH_4 y los cambios fisicoquímicos en discontinuo. Así mismo, la degradación anaeróbica de la materia orgánica (MO) y el límite de inhibición de SO_4^{2-} fueron investigados. En tercer lugar, los efectos de incluir purines de cerdo acidificados con SO_4^{2-} en un codigestión anaerobia con purines de cerdo convencionales se estudió a dos escalas (a escala laboratorio y a gran escala), donde se evaluó el rendimiento del proces, identificando los indicadores clave del proceso. En cuarto lugar, una combinación de dos métodos, reacción en cadena de la polimerasa cuantitativa en tiempo real (qPCR) y microscopía electrónica de barrido cualitativa (SEM) fueron utilizados para evaluar los cambios en la población microbiana de los digestores anaerobios durante la adición de purines. Finalmente, el rendimiento de CH_4, la composición fisicoquímica y la estructura y dinámica de la comunidad microbiológica fueron evaluadas durante la puesta en marcha de la digestión anaerobia de purines de cerdo a escala de laboratorio. Se evaluaron cuatro estrategias de puesta en marcha: inanición y no inanición, seguida de una adición gradual o brusca de los purines.

Los resultados presentados en esta tesis doctoral permitieron concluir que en la degradación de la MO de purines envejecidos, hay una transformación de MO más fácilmente degradable en MO soluble durante las tres primeras semanas de almacenamiento. Sin embargo, la producción de CH_4 no se produjo hasta las semana cinco. En cuanto al uso de purines con SO_4^{2-} en digestión anaerobia, concentraciones de SO_4^{2-}en purines de cerdo superiores

2000 mg $SO_4^{2-}L^{-1}$ y concentraciones superiores a 1500 mg $SO_4^{2-}L^{-1}$ en purines de vacuno produjeron disminuciones importantes en la producción de CH_4. Sin embargo, concentraciones de 500 mg $SO_4^{2-}L^{-1}$ aumentaron la producción de CH_4. Adicionalmente, en la digestión anaerobia de purines acidificados con SO_4^{2-}, los parámetros más importantes a controlar fueron: la concentración de SO_4^{2-}, los parámetros de alcalinidad y el contenido total de ácidos grasos volátiles (AGV), especialmente acético y butírico. La adición de purines de cerdo a digestores anaerobios no adaptados causó un deterioro del proceso de digestión anaerobia disminuyendo el pH, aumentando la concentración de AGV, disminuyendo la degradación de los sólidos volátiles y reduciendo la producción de CH_4. Durante la adaptación al cambio de sustrato en condiciones termofilicas, un aumento en el total de bacterias y arqueas se observó a través de qPCR, así como un cambio en morfotipos microbianos a través de SEM. En cuanto a las estrategias de puesta en marcha, la estrategia que mejor minimizó las pérdidas de CH_4 fue la no inanición con un cambio brusco de sustrato, aunque las diferencias en la población microbiana entre las estrategias fueron bajas. Por otra parte, la principal vía metabólica de producción de CH_4 durante la puesta en marcha y recuperación del proceso de digestión anaerobia fue la ruta hidrogenotrófica con todas las estrategias utilizadas. De hecho, en primer lugar las especies de *Methanomicrobiales* y posteriormente especies de *Methanobacteriales* fueron los órdenes de arqueas dominantes durante todo el período de recuperación, estos dos órdenes resultaron valiosos indicadores del proceso.

Esta tesis contiene información práctica y útil para el seguimiento de la digestión anaerobia de purines durante periodos de inhibición y recuperación del proceso.

Resum

Aquesta tesi doctoral se centra en la dinàmica de la inhibició i la recuperació del procés de digestió anaeròbia de purins de porc per trobar indicadors, pronosticar errors del procés, minimitzar les pèrdues de metà (CH_4) i avaluar les millors pràctiques de gestió a nivell de la planta de biogàs.

Per complir amb aquest objectiu, cinc assaigs van ser dissenyats i executats. En primer lloc, es va dissenyar un experiment per controlar els canvis fisicoquímics i d'emissió de gas de dos tipus de purins envellits durant 15 setmanes d'emmagatzematge en condicions d'estiu. En segon lloc, diferents concentracions de sulfat (SO_4^{2-}) van ser avaluades en digestió anaeròbia termofílica de purins de porc i boví controlant la producció de CH_4 i els canvis fisicoquímics. Així mateix, la degradació anaeròbica de la matèria orgànica (MO) i el límit d'inhibició de SO_4^{2-} van ser investigats. En tercer lloc, els efectes d'incloure purins de porc acidificats amb SO_4^{2-} en codigestió amb purins de porc convencionals es va estudiar a dues escales (a escala laboratori i a gran escala), on es va avaluar el rendiment del procés, identificant els indicadors clau del procés. En quart lloc, una combinació de dos mètodes, reacció en cadena de la polimerasa quantitativa en temps real (qPCR) i microscòpia electrònica de rastreig qualitativa (SEM) van ser utilitzats per avaluar els canvis en la població microbiana dels digestors anaerobis durant un l'addició de purins de porc. Finalment, el rendiment de CH_4, la composició fisicoquímica i l'estructura i dinàmica de la comunitat microbiològica van ser avaluades durant la posada en marxa de la digestió anaeròbia de purins de porc a escala de laboratori. Es van avaluar quatre estratègies de posada en marxa: inanició i no inanició, seguida d'una addició gradual o brusca dels purins.

Els resultats presentats en aquesta tesi doctoral permeten concloure que en la degradació anaeròbia de la MO de purins envellits, hi ha una transformació de MO més fàcilment degradable en MO soluble durant les tres primeres setmanes d'emmagatzematge, no obstant, la producció de CH_4 no es va produir fins a les cinc setmanes d'emmagatzematge. En el cas de l'ús de purins acidificats amb SO_4^{2-} en digestió anaeròbia, concentracions de SO_4^{2-} en purins de porc superiors 2000 mg SO_4^{2-}-L^{-1} i concentracions superiors a 1500 mg SO_4^{2-}-L^{-1} en purins de boví produïren disminucions importants en la producció de CH_4. No obstant, concentracions de 500 mg

$SO_4^{2-}L^{-1}$ en purins augmentaren la producció de CH_4. Addicionalment, en la digestió anaeròbia de purins acidificats amb SO_4^{2-}, els paràmetres més importants a considerar van ser: la concentració de SO_4^{2-}, els paràmetres d'alcalinitat i el contingut total d'àcids grassos volàtils (AGV), especialment acètic i butíric. L'addició de purins de porc a digestors anaerobis no adaptats va causar un deteriorament del procés de digestió anaeròbia disminuint el pH del fang, augmentant la concentració d'AGV, disminuint la degradació dels sòlids volàtils i baixant la producció de CH_4. Durant el període d'adaptació al canvi de substrat en condicions termofíliques, un augment en el total de bactèries i arqueges es va observar a través de qPCR, així com un canvi en morfotipus microbians mitjançant SEM. En el estudi d'estratègies de posada en marxa, l'estratègia que millor va minimitzar les pèrdues de CH_4 va ser la no inanició amb un canvi brusc de substrat, tot i que les diferències en la població microbiana entre les estratègies van ser baixes. D'altra banda, la principal via metabòlica de producció de CH_4 durant la posada en marxa i recuperació del procés de digestió anaeròbia va ser la ruta hidrogenotrófica en totes les estratègies utilitzades. De fet, en primer lloc espècies de *Methanomicrobiales* i posteriorment espècies de *Methanobacteriales* van ser els ordres d'arqueges dominants durant tot el període de recuperació, aquests dos ordres van resultar valuosos indicadors del procés.

Aquesta tesi conté informació pràctica i útil per al seguiment de la digestió anaeròbia de purins durant períodes d'inhibició i recuperació del procés.

Contents

Chapter 1

General introduction

1.1 Anaerobic digestion in Europe: state-of-the-art

Biogas recovering systems result in an efficient and cost-effective method for treating organic materials, mainly due to the low sludge generation and the production of renewable energy in the form of biogas. EurObserv'Er (2010) classified biogas recovering systems into three major groups: landfills (26.8% of total European production in 2010), urban and industrial wastewaters (9.8% of total European production in 2010), and the so-called "other sources" (63.4% of total European production in 2010), which comprise anaerobic digesters treating urban solid wastes and agro-industrial by-products. Agro-industrial by-products are treated in anaerobic plants as a single substrate or as multi-product substrate in co-digestion. These plants usually use products from the agricultural and food industry as dairies, slaughterhouses and farms.

Figure 1.1 shows the energy production from biogas in the European Union in 2009 (EurObserv'Er, 2010). As shown in this figure, Germany presents the highest biogas production in the European Union, representing nearly 50% of Europe's biogas electricity output. Most anaerobic plants in Germany are small (50-500 KW) and decentralized plants that use mainly animal manure in co-digestion with energy crops. As shown in Figure 1.1, United Kingdom ranks second with more than 85% of their total biogas coming from landfills. Spain ranks sixth in the European countries producing biogas, with an annual production in 2009 of 183.7 ktoe (Kilo Tonnes of oil equivalent) mainly from landfill and wastewaters and to a lower extent from agro-industrial by-products.

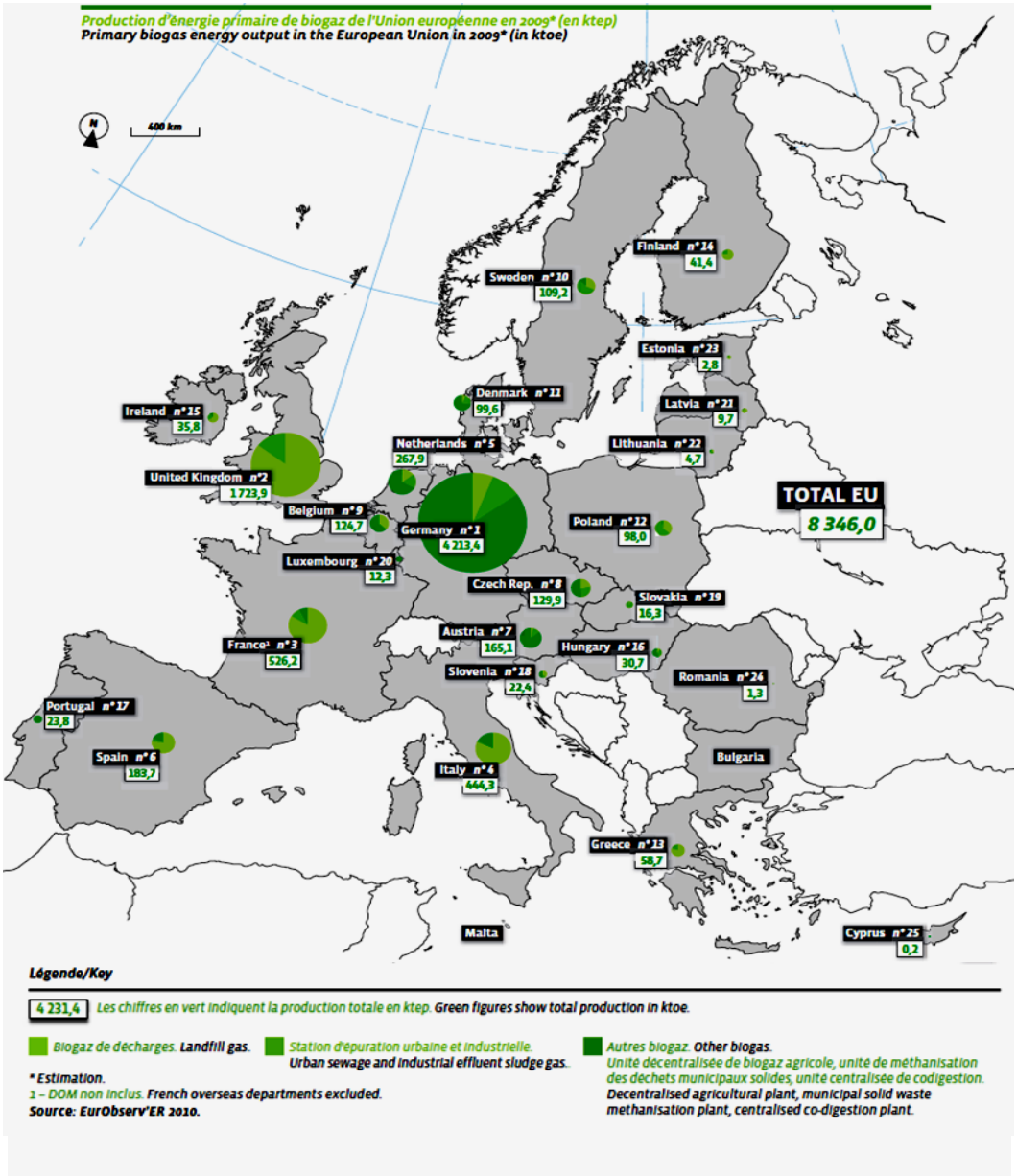

Figure 1.1- Primary biogas energy output in the European Union in 2009 (ktoe). Source: Biogas Barometer (EurObserv'Er, 2010).

Agriculture and food-industry are the main source of organic by-products in Europe. In countries with a strong tradition and use of biogas, like Germany, Austria, Belgium, Denmark and the Netherlands, agro-industrial biogas production represents more than 60% of their total production. However, biogas production from these substrates remains largely untapped in other European countries such as Spain, France, United Kingdom and Italy among others. The future of the biogas production in those countries requires the use of agro-industrial by-products since the use of other sources (such as landfills and wastewaters) for producing biogas is limited.

The current situation of agro-industrial biogas in certain countries in the European Union such as Spain is not as promising as it might be expected, and very few new biogas plants have been installed in recent years. This could be explained by several factors such as the high financial and administrative requirements necessary for the construction of new biogas plants and the lack of governmental support. In fact, the growth in the biogas production from agricultural by-products in Germany has been mainly due to governmental promotion with the implementation of a tariff that combines different premiums to promote biogas production (EurObserv'Er, 2010).

Another limitation for the implementation of new agro-industrial biogas plants is the fact that anaerobic digestion presents some management difficulties during stressful periods like the start-up, changes in operational parameters and substrate composition (Jantsch and Mattiasson, 2004). This limitation especially affects small and decentralized agro-industrial biogas plants making farmers generally reluctant to use this technology. Anaerobic digestion is a complex biological process in which several groups of microorganism are involved and in which the presence and activity of each group affects the equilibrium of the entire process. It is therefore difficult to efficiently monitor this process, since it is necessary to know not only the microbial dynamics and their metabolic routes, but also the substrate composition and the physicochemical parameters involved.

1.2 Methane production processes in anaerobic digestion

Biogas, mainly composed of carbon dioxide (CO_2) and methane (CH_4), is produced from the anaerobic degradation of organic matter (OM). This process takes place naturally in any anoxic and rich in OM environment such as rice paddies, lakes or marine sediments and gastrointestinal tracts of some animals (Lange and Ahring, 2001); but also in artificial anaerobic environments like controlled anaerobic digesters. In this controlled process, biogas is recovered to produce renewable energy.

1.2.1 Microbial community and organic matter degradation dynamics

The success of the anaerobic digestion process, and therefore CH_4 production, depends on the structure and the activity of the microbial groups involved. Figure 1.2 shows a schematic diagram of the anaerobic OM degradation process and the microbial groups involved. Hydrolysis is considered the first step in OM degradation. It is conducted by extracellular hydrolytic enzymes from the **primary fermentative bacteria**, also called acidogens, which convert the complex polymers constituting the OM (polysaccharides, proteins, lipids, etc.) into their respective monomeric constituents (sugars, amino acids, fatty acids, etc.).

After hydrolysis, primary fermentative bacteria transform these monomers into hydrogen (H_2), CO_2, ethanol, lactate, acetate and other volatile fatty acids (VFA), in a process named acidogenesis. Some of these fermentation products, especially acetate, H_2, CO_2 and other one-carbon compounds can be converted directly by methanogenic archaea into CH_4 and CO_2 (Schink, 1997).

Thereafter, the **secondary fermentative bacteria**, also called acetogens, transform the VFA produced in the previous step into acetate, H_2 and CO_2 in a process named acetogenesis. As shown in Figure 1.2, homoacetogenesis is the process in which acetate is oxidized to H_2 + CO_2 or the reverse reaction, acetate is produced from H_2 + CO_2. It is thought that both reactions can be carried out by the same group of bacteria, homoacetogens (Demirel and Scherer, 2008). The direction of

this reaction resulting in either the production of acetate or in its oxidation depends on the partial pressures of H_2 in the digester (Demirel and Scherer, 2008). However, the role of homoacetogens in the overall anaerobic digestion process is still not well understood.

Finally, **methanogenic archaea**, the last group of microorganisms involved in the anaerobic digestion process, produce CH_4 and CO_2 from H_2, CO_2, acetate, simple methylated compounds and alcohols. Acetate is considered the main precursor for CH_4 production during anaerobic digestion of OM in anaerobic digesters (Karakashev et al., 2006). Acetate can be transformed into CH_4 through two routes: aceticlastic methanogenesis, by which acetate is directly transformed into CH_4 and CO_2; and hydrogenotrophic methanogenesis in which acetate is first oxidized to H_2 and CO_2 by homoacetogens, and thereafter these products are converted into CH_4 by hydrogenotrophic methanogenic archaeas.

Each microbial group described in Figure 1.2 involves a large number of species that currently are not fully identified. This high number of microbial species involved is due to the wide range of anaerobic environments in which anaerobic degradation of OM can take place and also to the variability in the composition of the OM to be degraded. In fact, it is thought that the microbial diversity found in anaerobic environments mainly depends on the substrate composition and environment conditions (Liu et al., 2002). Throughout the trophic chain of OM degradation, the number and complexity of organic substances formed is simplified, and thus the diversity of microorganisms involved decreases, finishing in CO_2 and CH_4 produced simply by methanogenic archaea (Snell-Castro et al., 2005). Therefore methanogenic archaea generally show lower diversity than primary and secondary fermentative bacteria in anaerobic environments (Godon et al., 1997; Fernández et al., 1999).

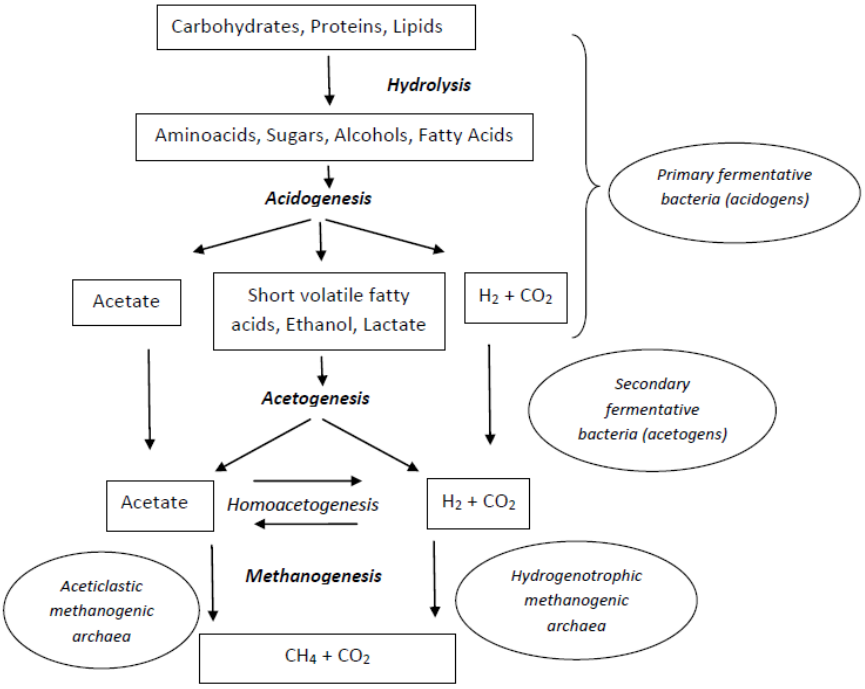

Figure 1.2- Schematic diagram of the anaerobic conversion of organic matter into methane and the microbial groups involved in this process. Adapted from Liu and Whitman (2008) and Demirel and Scherer (2008).

All microbial groups involved in the anaerobic degradation of OM live in an equilibrium called syntrophism. Syntrophism is a special case of symbiotic cooperation between metabolically different types of microorganisms in which one type depends on the other to completely degrade a substrate (Schink, 1997). Particularly, the secondary fermentative bacteria (acetogens), homoacetogens and methanogens live in syntrophism because the formation of acetate is only possible at a low H_2 pressure that is achieved due to the presence of hydrogenotrophic methanogens (Liu and Whitman, 2008). Furthermore, methanogens need the metabolites (mainly acetate and H_2) produced by acetogens and homoacetogens to develop correctly. In addition, the activity of the microorganisms using H_2 (homoacetogens and hydrogenotrophic methanogens) can alter primary fermentative bacteria (acidogens), since

at low H_2 partial pressure (<10 Pa) the fermentation patterns can shift into the production of more acetate, CO_2 and H_2 rather than ethanol, butyrate and lactate (Zeikus, 1977; Schink, 1997).

Nevertheless, this syntrophism can be altered causing process imbalances and even the complete process failure. This contributes to consider anaerobic digesters, and the overall process, as a black box hard to understand and to manage. In this context, methanogens are considered the most disadvantaged microorganisms because they are highly sensitive to inhibitors and grow at a slower rate than the rest of microorganism involved in the process (Ferrer et al., 2010). If methanogenic archaea are altered or negatively affected, the accumulation of intermediate metabolites, such as acetate and H_2, can occur. This may affect the rest of the microbial groups involved in the anaerobic digestion process and even fully stop the process. Acetogens, for instance, are very sensitive to the accumulation of their own metabolites (H_2 and acetate) and to pH fluctuations (Griffin et al., 1998). For this reason, although methanogens act in the terminal stages during the anaerobic degradation of OM, they are considered key microorganisms in the production of CH_4 (Nettmann et al., 2010).

1.2.2 Methanogenic archaea diversity

The anaerobic microorganisms capable of conducting methanogenesis belong to the *Euryarchaeota* kingdom in the *Archaea* domain (Woese et al., 1990). Methanogens are found within five phylogenetic orders. Table 1.1 describes four of the five phylogenetic orders most commonly found in anaerobic digesters: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales*. The fifth order, *Methanopyrales*, only shows a single hyperthermophilic specie *Methanopyrus kandleri* (84-110 ºC) and has not been considered in this PhD thesis. Very recently, a new sixth order, *Methanocellales*, has been proposed and phylogenetically placed between orders *Methanosarcinales* and *Methanomicrobiales* (Sakai et al., 2008). *Methanocellales* order includes a unique specie *Methanocella paludícola*

isolated from rice paddy soils by Sakai et al. (2008), thus has not been considered in this PhD thesis.

The different methanogenic orders show different cell morphology, size, cell wall structure, lipid composition, physiology and other biological properties (Liu and Whitman, 2008). The four main methanogenic orders are further divided into ten families. Table 1.1 shows the families, morphology, size and physiology of the four most relevant methanogenic orders. Although as stated above, two major routes to produce CH_4 are involved in the anaerobic digestion process (aceticlastic and hydrogenotrophic methanogenesis), another metabolic pathway exists, **Methylotrophic methanogenesis.** In this pathway, methylated one-carbon compounds (methanol, methylamine, trimethylamine, dimethylsulfide, etc.) are used as carbon sources and converted into CH_4. This metabolic pathway is consider at a lower extension in this thesis since methylated compounds are thought to be mainly formed during the anaerobic degradation of OM in marine brackish environments (Welander and Metcalf, 2008).

Concerning standard change of free energy for methanogenesis, the most favorable reaction is the acetate oxidation (hydrogenotrophic methanogenesis), and the least favorable is the transformation of acetate into CH_4 (aceticlastic methanogenesis) (García et al., 2000). Therefore, natural selection pressure has led to the development of more hydrogenotrophic methanogens than aceticlastic methanogens (García et al., 2000). Only methanogens from one order (*Methanosarcinales)* are aceticlastic (Table 1.1). In addition, only methanogens from one family within this order (*Methanosaetaceae*) are strict aceticlastic. *Methanosarcinaceae* species are also capable of using the H_2 from the oxidation of acetate (Lovley and Ferry, 1985), and *Methermicoccaceae*, the most recently discovered methanosarcinal family described by Cheng et al. (2007), is comprised only by strict methylotrophic archaea.

Table 1.1- Methanogenic phylogenetic orders commonly found in anaerobic digestion. Adapted from Angelidaki et al. (2011).

Order	Family	Cell morphology	Physiology
Methanobacteriales	*Methanobacteriaceae*	Filaments, short rods or shells lanceolate, round cells in pairs, irregular long rods (0.3-7μm)	Hydrogenotrophic and methylotrophic; H_2, formate, CO and alcohols as electron donor. Mesophilic or thermophilic. From Gram + to variable
	Methanothermaceae	Straight or slightly curved rods singly or in pairs but never in long filaments (0.3-3 μm)	
Methanococcales	*Methanococcaceae*	Irregular cocci (0.5-4μm)	Hydrogenotrophic; H_2 or formate as electron donor. Mesophilic or thermophilic. Gram +
	Methanocaldococcaceae		
Methanomicrobiales	*Methanomicrobiaceae*	Rods, mobile polar flagellum, irregular cocci, flat cells shaped crystals (0.7-3 μm)	Hydrogenotrophic, H_2, formate or secondary alcohols as electron donor. Mesophilic. Gram -
	Methanocorpusculaceae	Small irregular shells (0.4- 2μm)	
	Methanospirillaceae	Curved rods and long filaments (0.4-10μm)	
Methanosarcinales	*Methanosarcinaceae*	Sarcina-like cells (2-3μm)	Aceticlastic or hydrogenotrophic; H_2 and acetate as electron donor. Mesophilic or thermophilic. From Gram + to variable
	Methanosaetaceae	Rods or filaments (0.8-3μm)	Strict aceticlastic; acetate as electron donor. Mesophilic or thermophilic. From Gram + to variable
	Methermicoccaceae	Small mobile cocci (0.7-1μm)	Strict methylotrophic; thermophilic. From Gram + to variable.

Although thermodynamically the most favorable reaction is the hydrogenotrophic methanogenesis, the degradation rate of acetate has been found to be lower when hydrogenotrophic methanogens are involved compared to aceticlastic methanogenesis (Schnürer et al., 1999). When acetate is oxidized, less energy is available to form CH_4, since the energy of the acetate must be shared between the two microbial groups involved (homoacetogens and hydrogenotrophic methanogens), compared with the one-only-organism involved in the aceticlastic pathway. Therefore, when hydrogenotrophic methanogens are dominating an anaerobic digester, the promotion of aceticlastic methanogens like *Methanosarcinales* species, especially *Methanosaetaceae,* could be an interesting option to optimize CH_4 production (Karakashev et al., 2006).

Hydrogenotrophic methanogens, however, are favored at high temperatures, at high concentrations of VFA and free ammonia (Karakashev et al., 2005), making them more prone to resist adverse situations. In fact, the key factor determining the dominance between hydrogenotrophic and aceticlastic methanogens in anaerobic digesters is likely the existence of high levels of inhibitory ions, which in general, have higher severe negative effects on aceticlastic methanogens than on hydrogenotrophic methanogens (Shin et al., 2010). The knowledge about the effect of inhibitory substances on hydrogenotrophic methanogens' survival would contribute to elucidating the ideal environments for hydrogenotrophic, in where they can dominate over aceticlastic methanogens.

The dominance of one physiologic methanogenic group over the other in anaerobic digesters is a dynamic process. Literature shows that the quantitative structure of the methanogenic population can vary continuously in anaerobic digesters, during the start-up and with changes in substrate composition (Angenent et al., 2002; Hori et al., 2006; Lee et al., 2009). Therefore, an understanding of the methanogenic community structure, as well as their dynamics is essential to aid in the prediction and the effective control of process operations (Lee et al., 2009).

1.2.3 Competition of methanogenic archaea with other microorganisms

As described previously, methanogens obtain energy from oxidation of the intermediate metabolites produced in the anaerobic degradation of OM (mainly acetate and H_2) using CO_2 as electron acceptor. However, intermediate metabolites can also serve as electron source for other microorganisms involved or not in the anaerobic digestion process. This might cause competences among microorganisms, since the availability of intermediate metabolites in the anaerobic digestion process is limited by the rate at which insoluble biopolymers are decomposed.

Regarding the microorganisms involved in the anaerobic digestion process, H_2, and acetate are consumed by methanogens, but also by homoacetogens. Nevertheless, it seems that homoacetogens do not compete with methanogens for H_2 in anaerobic digesters. In fact, Schink (1997) and Liu and Whitman (2008) stated that homoacetogenesis from H_2 is thermodynamically less favorable than hydrogenotrophic methanogenesis at temperatures above 20ºC.

Concerning competition with microorganisms not involved in the anaerobic digestion process, there are microorganisms which use other energetically more favorable electron acceptors than CO_2, like nitrate, ferric iron or sulfate, which can compete with methanogens by the intermediate metabolites (VFA and H_2) (Chidthaisong and Conrad, 2000). This competition occurs in almost all anaerobic environments, especially in nitrate and sulfate rich environments, affecting CH_4 production.

For instance, it has been demonstrated that nitrate reducing bacteria successfully compete with methanogens for H_2 and acetate, inhibiting CH_4 emission when nitrate is added to rice field soils (Chidthaisong and Conrad, 2000). In addition to the competition for intermediate metabolites, the metabolic products of nitrate reducing bacteria, nitrogen monoxide (NO) and nitrous oxide (N_2O) can inhibit methanogenic activity (Klüber and Conrand 1998).

The microorganisms most widely studied as competitors of methanogens in anaerobic digesters are sulfate-reducing bacteria (SRB). Sulfate reducing bacteria, can compete not only with methanogens for electron sources such as acetate or H_2, but also with acetogens for propionate in sulfate rich waste-waters (Rintala and Lettinga, 1992). Literature shows that SRB have a higher affinity for acetate and H_2 than methanogens (Bhattacharya et al., 1996; Omil et al. 1998). Additionally, the metabolic product of SRB, hydrogen sulfide (H_2S) can also inhibit methanogenic activity itself. Interaction between SRB and methanogens in anaerobic digesters treating animal slurry could become a practical problem in countries like Denmark, where sulfuric acid (H_2SO_4) is added to slurry in commercial farms to lower slurry pH and prevent ammonia (NH_3) emissions (Eriksen et al., 2008).

Nevertheless, competition in OM rich environments is difficult to predict, since it is affected by several mechanisms such as antagonisms, synergisms, acclimatization and complexation (Chen et al., 2008, Siles et al., 2010). The competition between SRB or nitrate reducing bacteria and methanogens has been mainly evaluated in the literature using pure anaerobic cultures and synthetic appropriate substrates as VFA and ethanol (Klüber and Conrad, 1998; O'Flaherty et al., 1998; van Bodegom et al., 2004). Interactions between methanogens and microorganisms not involved in the anaerobic digestion process have seldom been evaluated in real anaerobic digestion conditions. The study of these interactions could be especially relevant in anaerobic digestion of animal slurry, which is composed of a pool of OM and nutrients with a high biological activity which could affect the competition process.

1.3 Anaerobic digestion of animal slurry: State indicators.

Livestock confinement facilities generate large amounts of animal slurry with a high OM and nutrient content. At farm level, animal slurry management can create serious environmental problems if it reaches in rivers, streams or groundwater supplies. Additionally during slurry storage, OM is aerobically and anaerobically degraded, resulting in

emission of CH_4 and other gases like CO_2 and NH_3 (Møller et al., 2004a). Methane is a greenhouse gas with a global warming potential 21 times higher than that of CO_2. European governments are nowadays promoting the reduction of CH_4 emission from the livestock sector, since it represents a relevant share (between 5-10%) in the global CH_4 emissions (Steed and Hasimoto, 1994). In Spain, CH_4 emitted from manure management constitutes around 2.3% of the total emission of CO_2 equivalent of which, around 89% are due to pig slurry management (UNFCC, 2010).

In this framework, anaerobic digestion could be an interesting process to treat animal slurries because besides decreasing atmospheric CH_4 emission from its storage, the CH_4 produced in the controlled anaerobic digestion process can be used as a renewable energy source. In addition, the manure resulting from the anaerobic digestion process (sludge) presents advantages compared with those obtained with other animal slurry treatments such as a low generation of sludge, conservation of nutrients and reduction of odors. According to Directive 2009/28/CE, biogas production from agricultural by-products such as animal slurry has a great potential, not only from an environmental standpoint, but also because it can contribute to the development of rural areas offering farmers new income opportunities.

However, the use of animal slurry in anaerobic digestion plants faces several challenges which make its use difficult. In the anaerobic digestion of animal slurry there are three main aspects that should be taken into account for the design, implementation and monitoring of the process:

1) The low OM content in slurries leads to low CH_4 yields. For this reason, there is need for strategies to increase the amount of OM and thus CH_4 production from animal slurry. Some of these strategies focus on increasing the volatile solids (VS) content by substituting the liquid fraction by the solid fraction of animal slurry from solid to liquid separation (Møller et al., 2007), or on co-digestion with easily-fermentable carbohydrates (Ward et al., 2008).

2) The high relative amount of components of the fiber fraction (cellulose, hemicelluloses and lignin) in animal slurries results in low CH_4 yields, as well. These compounds are degraded at a low rate (slowly biodegradable compounds), being hydrolysis the limiting step (Gonzalez-Fernández et al., 2008). Therefore the availability of intermediate metabolites in anaerobic digesters treating these slowly biodegradable substrates will be limited by the low hydrolysis rate.

3) The high VFA and NH_3 content in animal slurries can inhibit the anaerobic digestion process, especially in cases of non-adapted microbiota (Stamatelatou et al., 2004). The control of these parameters could avoid instability problems or help to solve them by using countermeasures to compensate the instability.

Once the dynamics of the anaerobic digestion process and the peculiarities of animal slurry to be considered in anaerobic digestion plants have been presented, some of the parameters frequently used to monitor this process will be reviewed below: both, physicochemical and microbiological indicators. Their role in the overall process and their limitations will be discussed.

1.3.1. Methane yield

At biogas plant level, the parameter CH_4 yield is widely used. Methane yield indicates the CH_4 produced per unit of a variable. It can be measured in terms of OM loaded, generally as VS, or chemical oxygen demand (COD) (expressed as L CH_4 Kg $VS_{destroyed}^{-1}$ or L CH_4 Kg $COD_{destroyed}^{-1}$), in terms of VS included in the digester (expressed as L CH_4 Kg VS_{added}^{-1}), in terms of volume (expressed as L CH_4 m^{-3}_{slurry}) or in terms of livestock unit (LU) production (expressed as m^3 CH_4 LU^{-1}). Methane yield measured in terms of VS added to the digesters (L CH_4 Kg VS_{added}^{-1}) is the most common and standardized measure used in anaerobic digesters since it allows comparing digester performance with the ultimate CH_4 yield (B_0).

Ultimate CH_4 yield is defined as the maximal CH_4 production from a substrate obtained in optimal batch conditions for an indefinite degradation time, following procedures described by Owen et al. (1979) and Angelidaki and Sanders (2004). Published values for B_0 in animal slurries vary from 250 to 480 mL CH_4 gVS^{-1} for pig slurries and from 100 to 240 mL CH_4 gVS^{-1} for cattle slurries (Møller et al., 2004b; Amon et al., 2007; Vedrenne et al., 2008). These wide ranges depend mainly on differences in slurry composition.

Knowing CH_4 yield in terms of VS added to the digesters (L CH_4 Kg VS_{added}^{-1}) and in volumetric terms (L CH_4 m^{-3}_{slurry}) is essential for the economy of biogas plants. The amount of biogas that can be produced is proportional to the CH_4 yield in terms of VS added, and the volumetric CH_4 yield affects the total energetic production of the plant (Møller et al., 2004b).

1.3.2. Sludge's physicochemical indicators

Standardized methodologies, generally from the *Standard Methods for the examination of water and wastewater* (APHA, 2005), are used to determine the sludge's physicochemical parameters in anaerobic digestion. Therefore, the procedures employed to determine each physicochemical parameter will not be discussed in the present document.

1.3.2.1. Removal efficiency of volatile solids

The percentage of VS removal from the influent is widely used to control the process in anaerobic digestion plants. It is calculated as the difference between the VS content in the influent and effluent with respect to the VS of the influent. This parameter is closely related with CH_4 yield in terms of VS added to the digesters and it mainly depends on the digester conditions and substrate composition. The combination of CH_4 yield and VS removal is considered as the most useful indicator to estimate efficiency in the digestion process (Hill and Bolte, 2000).

In thermophilic conditions with a high hydraulic retention time (HRT), VS removal will be higher than at mesophilic conditions with lower

retention times. Among different substrates in the same conditions, VS removal mainly depends on the amount of carbohydrates, lipids, proteins and lignin and the accessibility of these nutrients to the anaerobic microorganisms. Carbohydrates, especially the fiber fraction, show the lowest VS removal and thus the lowest CH_4 yield in terms of VS added (Angelidaki and Sanders, 2004).

1.3.2.2. pH and alkalinity

The optimal pH for the microorganisms involved in the anaerobic digestion process is near neutrality (pH=7). Among the microbial groups involved in the anaerobic digestion process, methanogens are likely the most severely affected by pH changes (Chen et al., 2008). Monitoring pH is necessary in anaerobic digesters, particularly when a stable bacterial population has not already been established and during periods of increasing organic loads (Barredo and Evison, 1991; Ahring et al., 1995). Failing to maintain pH within an appropriate range might cause digester failure and thus important reductions in CH_4 yield (Chen et al., 2008).

Therefore, pH is commonly used as a process indicator in anaerobic digesters. However, the effectiveness of pH as a control parameter is strongly dependent on the buffering capacity of the sludge (Ahring et al., 1995). The buffering capacity of an aqueous sample is measured as its resistance to pH changes, due to the equilibrium between weak acids and its conjugate bases or weak bases and its conjugate acids. In anaerobic digesters, the buffering capacity depends mainly on carbonate components ($CO_2/HCO_3^-/CO_3^{2-}$), VFA and NH_3, and is measured as the alkalinity. Total alkalinity of a sample is the sum of partial alkalinity, which is correlated with carbonate components, and intermediate alkalinity, which is correlated with VFA and ammonium (NH_4^+)/NH_3 components (Ripley et al., 1986; Jantsch and Mattiasson, 2004).

Animal slurries show a high buffering capacity probably due to their high NH_3 levels, which gives them high pH stability even when significant increases in VFA are observed (Ripley et al., 1986; Pind et al., 2003). Thus in the anaerobic digestion of animal slurry, generally no

acidification problems caused by accumulation of intermediate metabolites such as VFA appear. Therefore, the pH uniquely does not assure an efficient control of the process. Other parameters like alkalinity, or NH_3 and VFA, should be monitored together with pH to efficiently control the anaerobic digestion process of animal slurry.

1.3.2.3. Volatile fatty acids

Volatile fatty acid concentration has been widely used in the literature as an indicator of anaerobic process imbalances (Ahring et al., 1995; Pind et al., 2003; Ferrer et al., 2010). The relation between VFA accumulation and reduction in CH_4 yield in anaerobic digesters is very close, because VFA accumulation implies the impossibility of acetogens and methanogens to efficiently consume the VFA produced by acidogens. Generally accumulation of VFA in anaerobic digesters happens during stress periods, due to differences in growth velocities and sensitivity to environmental conditions among the microbial groups involved in the anaerobic digestion process. Within individual VFA, acetate, butyrate, and acetate/propionate ratio seem to be the best correlated with CH_4 yield (Ahring et al., 1995; Pind et al., 2003; Ferrer et al., 2010).

However, it is still not clear whether a high VFA concentration itself or the associated H_2 accumulation and pH drop cause the inhibition of the anaerobic digestion process because it is difficult to separate these effects. Some authors reported a specific inhibition of VFA themselves in anaerobic digestion. Their hypothesis is that undissociated VFA can permeate well through the cell membrane causing an acidification of the cytoplasm (Barredo and Evison, 1991; Strick et al., 2006). The excess of protons in the cytoplasm would require an extra energy consumption (an extra hydrolysis of adenosine-5'-triphosphate, ATP) to maintain a functional proton gradient, meaning a decrease in ATP availability for the cell growth and metabolism (Fukuzaki et al., 1990). In this regard, acetic acid itself has been described as an inhibitor for the acetogenic populations of *Clostridium thermoaceticum* (Baronofsky et al., 1984).

During VFA accumulation in anaerobic digesters, due to imbalances or animal slurry addition, changes in the microbial groups involved in the process take place, especially among methanogens. In fact, it has been reported that *Methanosarcinaceae* have a higher affinity for acetate than *Methanosaetaceae* (Hori et al., 2006). However, as stated above, hydrogenotrophic methanogenesis is favored *vs.* aceticlastic methanogenesis at high concentrations of VFA (Karakashev et al., 2005). Within hydrogenotrophic methanogens, *Methanobacteriales* have been reported to be the dominant order during the accumulation of VFA, especially propionate (Hori et al., 2006). However, although hydrogenotrophic methanogens are dominant during process imbalances in anaerobic digesters, available information about the role and activity of these microorganisms in the literature is lower than the information reported about aceticlastic methanogens (Demirel and Sherer, 2008). In fact is still not clear if hydrogenotrophic dominance in digesters working with animal slurry is due to high VFA concentration or to high NH_3 levels (Nettmann et al., 2008; Song et al., 2010; Zhu et al., 2011).

1.3.2.4. Ammonia

Ammonia, which is usually high in anaerobic digesters working with animal slurries, is formed from the hydrolysis of urea and proteins (Sung and Liu, 2003). Although as stated before, NH_3 is an important buffer in the anaerobic digestion process and an essential nutrient for microorganisms, a high NH_3 concentration is one of the major causes of operational failure during the anaerobic treatment of animal slurries (Calli et al., 2005).

The unionized fraction of NH_3 (also called free ammonia, FA) has been suggested to be the main cause of inhibition, since it can permeate well through the cell membrane (Chen et al., 2008). This fraction (FA) may diffuse passively into the cell, causing proton imbalance and potassium deficiency in microorganisms (Gallert et al., 1998). The amount of FA in the sludge depends mainly on three parameters: total NH_3 concentration, temperature and pH (Hansen et al., 1998). Free NH_3 increases with

temperature and decreases with the pH. Therefore, the anaerobic digestion process becomes more sensitive towards NH_3 in thermophilic digesters with high pH.

It is thought that the inhibitory effects of NH_3 in anaerobic digesters influence mainly the terminal methanogenesis phase. Hansen et al. (1998) stated that the activity of hydrolytic enzymes and fermentative bacteria were not affected by FA concentration in anaerobic digestion of pig and cattle slurry at thermophilic conditions. In the work carried out by Hansen et al. (1998), aceticlastic methanogens were the first microorganisms to be inhibited at 1.1 g FA L^{-1}. However, Calli et al. (2005) suggested that acetogens (specifically propionate degrading bacteria) could be inhibited by lower NH_3 concentration than methanogens. However, little is known about acetogenesis inhibition by high NH_3 concentration.

Nowadays research on NH_3 inhibition in anaerobic digesters is focused on the evolution of methanogenic populations with increasing NH_3 concentrations. On this regard, Calli et al. (2005) suggested that aceticlastic species might be more sensitive than hydrogenotrophic ones to FA. Among aceticlastics, *Methanosaetaceae* species seems to be more sensitive to FA accumulations than *Methasorsarcinaceae,* which has been found to be the dominant aceticlastic order at high NH_3 concentration (4.1 g N-NH_3 L^{-1}) (Karakashev et al. 2005). Calli et al. (2005) obtained similar results when evaluating the effect of methanogenic diversity in anaerobic digesters fed with synthetic wastewater exposed to a gradual increase in NH_3 levels (ranging from 1000 to 6000 mg/L). In this work, *Methanosaetaceae* species dominated the sludge before NH_3 addition and lost their activity during the adaptation period to NH_3. On the contrary, *Methasorsarcinaceae* species were abundantly detected during the experiment. Although traditionally studies have focused on *Methanosaetaceae vs. Methasorsarcinaceae* dominance during high NH_3 levels, generally, hydrogenotrophic methanogenesis dominates in digesters working with high NH_3 concentration (Karakashev et al., 2005; Song et

al., 2010). However, the influence of NH_3 concentration on hydrogenotrophic methanogens has been evaluated to a lower extent.

Methanogens have been reported to show the ability to adapt to high NH_3 concentration (Hansen et al., 1998; Sung and Liu 2003). However, this adaptation has not always been followed by a complete recovery of the original CH_4 yield of digesters. In the anaerobic digestion of animal slurry, it is recommended to use an inoculum adapted to high NH_3 levels during the start-up period (Calli et al., 2005).

1.3.3. Microbial indicators

As stated above, anaerobic digestion process imbalances are mainly due to differences in growth velocities among the microbial groups involved. Therefore, monitoring microbial dynamics might give practical information about process stability. This information would be especially useful if through monitoring it were possible to detect the microbial groups responsible for each critical step (Calli et al., 2005). However, microbiological parameters have only been recently linked to the efficiency of anaerobic digesters (Sanz and Klöching 2007; Song et al., 2010) and therefore their potential as tools for designing or monitoring anaerobic digestion process is still being assessed.

Different methodologies are used nowadays to monitor microbial groups in anaerobic digesters. The most relevant microbiological factors which can be used in anaerobic digesters are detailed below; additionally the microbiological techniques for their determination are also discussed.

1.3.3.1. Quantitative microbial abundance

Quantitative information about the microbial population in an anaerobic digester has a special interest because it can help to elucidate the bio-kinetic parameters, which are regarded as key factors in the process design, operation and control (Song et al., 2010). This information can also help to detect dominant microbial groups during stressful periods and to expand the knowledge about the role of hydrogenotrophic *vs.* aceticlastic methanogens during VFA and NH_3 accumulations.

Due to the incomplete knowledge about the physiological needs of the microorganisms involved in the anaerobic digestion process and the complex syntrophic relationship among them, the use of conventional microbiological quantification techniques based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays is difficult (Sanz and Köchling, 2007). Nucleic acid-based molecular methods, however, can provide rapid, specific, and high sensitivity analysis of the microbial communities present without the need of culturing.

Within nucleic acid-based molecular methods, quantitative real-time polymerase chain reaction (qPCR) is commonly used. Other nucleic acid-based molecular methods such as fluorescence *in situ* hybridation (FISH) have also been used to determine quantitative microbial abundance in anaerobic digesters (Karakashev et al., 2005; Hori et al., 2006).

Nevertheless, the quantification of microbial abundance in anaerobic digesters using molecular methodologies has certain limitations. Using molecular methodologies, it is not possible to distinguish among living, non-living, dormant or extremely slow-growing cells (physiological state) and free DNA present in samples (Solera et al., 2001). This fact might cause an overestimation of the microbial population counts. In addition, the quantification through qPCR is directed to certain microbiological groups using selected primers; therefore the existence of other microbial groups will not be determined. However; total bacteria, total archaea and total eukarya can also be quantified by qPCR using universal primers for the three domains.

For this reason, the critical step in designing a molecular methodology is to select primer set targeting genes of interest which cover special taxonomic or functional groups (Zhang, 2010). The majority of published studies using molecular methods in anaerobic digestion are focused on the four main methanogenic orders (Liu et al., 2002; Karakashev et al., 2005; Zhu et al., 2011) due to their pivotal importance for the stability of the process. Concerning qPCR, specific primers for

the qPCR assay with specific TaqMan probes have been developed by Yu et al. (2005) for: *Archaea* domain, *Bacteria* domain, *Eukarya* domain, *Methanobacteriales* order, *Methanosarcinales* order, *Methanomicrobiales* order, *Methanococcales* order, *Methanosarcinaceae* family and *Methanosaetaceae* family. These primers and probes have been applied in anaerobic digesters working with swine wastewaters (Bergmann et al., 2010; Song et al., 2010).

1.3.3.2. Spatial distribution of microbial community

Due to the clear syntrophism among microbial groups involved in the anaerobic digestion process, acetogens and methanogens tend towards a self-immobilization to form aggregates (McLeod et al., 1990). Aggregates, also called flocks, facilitate the metabolite transfer between acetogens and methanogens by reducing their diffusion distance and creating associations. Aggregation of anaerobic microorganisms is thus critical for the energetic efficiency and kinetics of the overall substrate conversion in anaerobic digestion (Guiot et al., 1992). This aggregation process has been widely described in anaerobic up-flow systems (Guiot et al., 1992; McHugh et al., 2003; Hulshoff Pol et al., 2004; Picioreanu et al., 2005). However, to our knowledge, the aggregation process in continuous stirred tanks reactors (CSTR), the most widely anaerobic digester used with animal slurry, is still unclear.

Two main methodologies are used in the literature to evaluate microbial aggregation: FISH (Karakashev et al., 2005; Hori et al., 2006), and scanning electron microscopy (SEM).

Scanning electron microscopy has widely been used to evaluate the layered structure and the granulation process in upflow anaerobic digesters (McLeod et al., 1990; Guiot et al., 1992; Veiga et al., 1997). In addition to evaluate aggregation among the microbial groups involved in the anaerobic digestion process, SEM can also reveal ultra structural features in anaerobic environments. For instance, the use of SEM to explore ruminal content has allowed extending the knowledge about the

dynamics of anaerobic degradation of OM, especially in the hydrolysis step (Akin and Amos, 1975).

The information obtained by microscopic techniques in CSTR observations could be useful in anaerobic digestion of slowly-biodegradable suspended solids in which hydrolysis is the limiting step, as in the case of animal slurry and other substrates with a high fiber content (Song et al, 2005). In fact, factors like biofilms, complex associations of microorganisms, and microbial products attached to the solids surface have been shown to be of pivotal importance in hydrolysis of organic material in most anaerobic environments (Song et al., 2005). These factors can be evaluated more easily through microscopy techniques than using nucleic acid-based molecular methods.

1.3.3.3. Composition of the microbial community

The acclimatization of an anaerobic sludge to specific conditions or substrate compositions may involve a change in microbial population to a population better adapted to these new conditions. The determination of changes in microbial diversity is interesting to increase the knowledge about the microbial aspects related with adaptation.

Fingerprint methods such as denaturing gradient gel electrophoresis (DGGE) (Liu et al., 2002; Shin et al., 2010) and terminal restriction fragment length polymorphism (t-RFLP) (Kobayashi et al., 2009; Wang et al., 2009) are widely used to evaluate microbial diversity in anaerobic digesters. However, these methodologies are limited to the sequences of the bands obtained, which if they correspond to short DNA fragments, it could difficult the establishment of the phylogenetic relations (Sanz and Klöching, 2007). Additionally in cases of a clear dominance, the detected bands can correspond only to the predominant species in the original sample (Sanz and Klöching, 2007).

Microscopy can be also used to examine the morphological microbial diversity in an anaerobic sludge. This methodology however, has been seldom used to this end. Only recent studies have evaluated

morphological changes in anaerobic sludge after a change in operational conditions like when adding vegetable substrate in co-digestion with pig slurry in CSTR (Molinuevo-Salces et al., 2012) or to evaluate the toxicity of an anaerobic sludge to a high long chain fatty acids concentration (Pereira et al., 2004).

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Chapter 2

Objectives and PhD thesis outline

This PhD thesis is focused on studying the dynamics of inhibition and recovery in the anaerobic digestion process of animal slurry. It addresses the search for physicochemical and microbiological state process indicators which predict process failures and provides best management practices at biogas plant level to minimize methane (CH_4) losses. There were three main objectives in this PhD thesis:

1. To explore the dynamics of the anaerobic degradation of organic matter (OM) from pig slurry in terms of sludge's physicochemical composition and gas emission.
2. To evaluate sulfate inhibition thresholds in thermophilic anaerobic digesters with acidified pig and cattle slurry and to identify the physicochemical process state indicators useful for predicting process failure.
3. To evaluate the effect of pig slurry addition in thermophilic anaerobic digesters and the implementation of different start-up strategies on physicochemical and microbiological process state indicators useful for predicting process failure.

To fulfill these objectives, five trials were designed and conducted. Each trial corresponds to a different chapter in this PhD thesis.

Chapter 3 is focused on exploring the dynamics of the degradation of OM from pig slurry during storage conditions in terms of physicochemical changes and gas emissions. For this purpose, an experiment was designed to monitor gas emission (CH_4, carbon dioxide, nitrous oxide, ammonia and water vapor) and the physicochemical composition of two types of aged pig slurry during 15 consecutive weeks of storage in summer conditions.

Chapters 4 and **5** are focused on evaluating sulfate inhibition thresholds in thermophilic anaerobic digestion process of acidified pig and cattle slurry and to identify the physicochemical process state indicators useful for predicting process failure. In **Chapter 4**, different non-inhibitory and inhibitory sulfate concentrations in pig and cattle slurry were monitored

in terms of CH_4 yield and physicochemical changes in order to study thermophilic anaerobic degradation of OM, the inhibitory sulfate dose and its effects on the monitored parameters. The experiment conducted in **Chapter 5** aimed at investigating the effects of including sulfate acidified pig slurry in an anaerobic co-digestion process with conventional pig slurry in a two scale study (laboratory and full scale) to find the optimum ratio which can optimize process performance in practical conditions.

Chapters 6 and **7** are focused on evaluating CH_4 yield, physicochemical and microbiological changes and start-up strategies in thermophilic anaerobic digesters during a change in substrate composition by adding pig slurry. The main objective of **Chapter 6** was to determine microbial population changes in thermophilic anaerobic sludge during the adaptation to pig slurry. In **Chapter 7**, the CH_4 yield, physicochemical composition and methanogenic community structure and dynamics were evaluated with different start-up strategies in anaerobic digestion of pig slurry.

The main results of this thesis concerning physicochemical and microbiological process state indicators and the best management practices to be used during periods of stress at a practical scale are discussed in **Chapter 8**. The main conclusions of this PhD thesis are drawn in **Chapter 9**.

Chapter 3

Evolution of chemical composition and gas emission from aged pig slurry during outdoor storage with and without prior solid separation

Evolution of chemical composition and gas emission from aged pig slurry during outdoor storage with and without prior solid separation

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Abstract. Chemical composition and gas emissions from two types of pig slurry were evaluated: the liquid fraction of mechanical solid-liquid separated slurry (SS), and raw slurry (RS). The slurry was obtained at the end of a pig fattening period and stored in 100 L vessels for 15 weeks simulating outdoor storage conditions. During this period, representative samples were taken and analyzed for chemical composition. Methane, carbon dioxide, ammonia, water vapor and nitrous oxide emissions were recorded. The results showed a high biological degradation during the first five weeks of outdoor storage in SS and RS slurries, as a result of an increase in the dissolved chemical oxygen demand, volatile fatty acids and carbon dioxide emission observed in this period. However, methanogenic activity was not evident until week 6 of storage in both slurries, confirmed by volatile fatty acids accumulation and the negligible methane emissions during the first five weeks of storage. The results showed that differences in the initial slurry organic matter content, influenced by solid separation process, affects the evolution pattern of the organic matter degradation and that the storage time can considerably affect the biodegradability of organic matter in pig slurry.

Keywords: chemical composition, gas emission, aged pig slurry, solid-separation, storage conditions.

3.1. Introduction

The anaerobic degradation of organic matter (OM) takes place during the storage of animal slurries like in any anoxic and rich in OM environment such as rice paddies, the rumen or the hind gut of monogastrics. This is a complex process in which different groups of microorganisms interact to convert OM into carbon dioxide (CO_2) and methane (CH_4). Primarily, hydrolytic enzymes from the fermentative bacteria convert complex polymeric biomass (polysaccharides, proteins, lipids, etc.) into their respective monomeric constituents (sugars, amino acids, fatty acids, etc.). The acidogenic fermentative bacteria transform these monomers into H_2, CO_2 and volatile fatty acids (VFA). The VFA are then converted by the acetogenic bacteria into acetic acid, which is the main product utilized by the methanogenic archaea, the last group of microorganisms to be established in the anaerobic degradation process (Angelidaki et al., 1999).

During animal slurry storage, all of these microorganisms coexist in equilibrium with other groups responsible for processes such as aerobic degradation of OM (Møller et al., 2004), nitrogen nitrification, denitrification and urea mineralization (Cortus et al., 2008). This high microbial activity results in the emission of gases related with climate change and detrimental environmental effects such as ammonia (NH_3) and greenhouse gases: CO_2, CH_4 and nitrous oxide (N_2O). Besides gas emissions, bacterial fermentation processes can also lead to a reduction not only in the fertilizer value of manure due to nitrogen losses (Muck and Steenhuis, 1982), but also as energy value to produce biogas due to fermentable OM losses (Møller et al., 2004).

Storage conditions, slurry composition and age are key influencing factors in the performance of these microorganisms. Storage conditions affect the anaerobiosis degree of the slurry, limiting the establishment of anaerobic *vs.* aerobic microorganisms. Furthermore, slurry composition affects the establishment of microorganisms in the slurry not only because some components, as nitrogen and biodegradable carbon are

sources of energy for them, but also because, as stated Fangueiro et al. (2008), the higher contents of OM, especially solids with low density such as fibers, could facilitate anaerobic conditions and thus a better development and establishment of anaerobic microorganisms. Therefore, treatments such as solid-liquid separation where high contents of fibers are separated from liquid to solid phases could have a relevant effect on anaerobic conditions and thus on CH_4 emissions.

Slurry composition depends not only on well known factors such as diet or slurry management (Cahn et al., 1997; Béline et al., 1999; Panetta el al., 2006) but also on its age. The OM in slurry is formed by degradable and non-degradable volatile solids, during storage, the degradation of the most degradable OM by microorganisms causes an increase in the fibrous content of the slurry (Sommer et al., 2004), since this fraction is unaffected by microbial activity. In addition, during the degradation of slurry there is an accumulation of compounds as metabolic products of the fermentative bacteria (such as VFA) and mineralization products of nitrogen as NH_3 and N_2O (Béline et al., 1998). Consequently, gas emissions derived from aged slurry are expected to differ over time from these obtained from fresh slurry, thereby affecting its subsequent management.

Monitoring gas emissions and slurry composition during storage might help elucidate the variation of microbial activity with time. Methane emission is produced only by methanogenic archaea and NH_3 is produced in the mineralization of organic nitrogen. However, CO_2 is produced by anaerobic and aerobic microorganisms and is also related with urea mineralization. There are several works reported in the literature in which fresh slurry is monitored for gas emission and composition over time at different temperatures (Béline et al., 1997; Møller et al*.,* 2004; Sommer et al., 2007), identifying temperature and slurry composition as the most influencing factors affecting gas emission. However, gas emissions and slurry composition in aged slurry stored over long periods in warm temperature conditions (> 20°C) have been studied to a lesser extent and this could provide useful information to

develop best management practices to reduce environmental impact caused during aged slurry storage. This information is particularly relevant in Mediterranean counties, such as Spain, where the management of pig slurry consists of a pre-storage below slatted floor during the fattening period (3-4 months) and a further outdoor storage occurs until the slurry is applied to agricultural land. In this context, mechanical solid separation treatment techniques are often applied to reduce the capacity of the outdoor storage lagoons and facilitate slurry transport and field application.

The aim of this study was to monitor gas emissions (CH_4, CO_2, N_2O, NH_3 and H_2O) and the chemical composition of two types of aged fattening pig slurry during 15 consecutive weeks under summer conditions, and to study the effect of initial slurry chemical composition on these parameters by applying the mechanical solid separation process.

3.2. Material and methods

3.2.1. Experimental setup

Pig slurry from a complete fattening period (19 weeks) carried out with 128 female pigs (initial weight 20.85 ± 2.80 Kg), was obtained from the Animal and Technology Research Centre (CITA) in Segorbe, Castellón, Spain. The animals were fed a diet containing, on average 2,425 Kcal net energy Kg^{-1}, 15.1% crude protein, 5.8% crude fat and 3.9% crude fiber. Animals were housed in whole-slatted pens. At the end of the fattening period, the slurry under the pit was mixed in order to avoid stratification and a representative sample (2000 L) was taken. Approximately half of the total amount of collected slurry was immediately subjected to a mechanical solid separation process via a mechanical screen separator, with a screen pore diameter of 0.5 mm, commonly used in commercial farms. This slurry was designated separated slurry (SS). The rest was not modified and remained as raw slurry (RS).

For each treatment three 100 L polyethylene vessels were filled with slurry until they reached 80% of their total capacity. A headspace of 130 mm was left between the slurry surface (0.104 m^2) and the top of each

vessel. During 15 consecutive weeks in summer, vessels were stored in a roofed space. Slurry and ambient temperature were continuously registered using data loggers (*HOBO®U12-013,* Onset Computer Corporation, MA, USA).

3.2.2. Chemical analyses

At the beginning of the experiment, and fortnightly, a representative sample of the slurry from each vessel was taken. The samples were collected using a device for layered liquids sampling (Eijkelkamp©, Eijkelkamp Agrisearch Equipment BV, Germany) that allows sampling the complete vertical profile of the slurry without agitation. After collection, the samples were homogenized and the pH was measured with a pH meter (Crison Basic 20+, Crison, Barcelona, Spain). After pH measurements, samples were frozen at -30ºC.

Total solids (TS), volatile solids (VS), total and dissolved Kjeldhal nitrogen (TKN_t and TKN_d), and dissolved chemical oxygen demand (COD_d) were determined according to APHA (2005). Volatile fatty acids concentration was determined by gas chromatography following the method described by Jouany (1982) with the addition of an internal standard (4-metil valeric). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to the Van Soest procedure (Van Soest, 1991). The nitrogen and fiber content were only determined in weeks 0, 9 and 15.

3.2.3. Gas emissions

From the filling of the vessels and during the 15 weeks of storage, gas emissions were measured treating the vessels as dynamic chambers. The gas measurements were performed weekly (3 days per week). On each measuring day, the emissions from two vessels, one from SS treatment and one from RS treatment, were registered during 24 h. During week 11 of the study, no gas measurements were conducted due to equipment malfunctioning.

The dynamic chambers were set up by sealing the vessels containing the slurry with hermetic lids. Three holes were left in the lid for air inlet by depression (inlet holes). The air was sucked from each headspace by a

pump (38 L min^{-1} and 7.5 KPa (outlet), Ilmivac, Ilmenau, Germany). Inlet and outlet holes were on opposite sides of the lid to promote air mixing in the headspace. When vessels were not being measured for gas emissions, they remained open to simulate natural conditions in outdoor storage.

Inlet and outlet concentrations of CH_4, CO_2, N_2O, water vapor (H_2O) and NH_3 were analyzed every two hours using a photoacoustic gas monitor (INNOVA1412, Air Tech Instruments, Ballerup, Denmark).

The airflow rate was measured daily in the outlet using a flow meter (Aalborg instruments and Controls INC., NY, USA) and modified if necessary to keep concentrations in the measuring range of the equipment; therefore, airflows in this study ranged from 0.30 $m^3\ h^{-1}$ at the beginning and 1.03 $m^3\ h^{-1}$ at the end of the measuring period.

3.2.4. Calculations and data analyses

Gas emission rates (E, mg h^{-1}) were calculated by multiplying the airflow rate times the difference between the gas concentrations in the output and input holes of each vessel for each measured gas, using Equation 3.1.

$$E = F \times (C_{out} - C_{in}) \quad (3.1)$$

where: F is the airflow rate through the vessel ($m^3\ h^{-1}$), C_{out} is the gas concentration in the output (mg m^{-3}), and C_{in} is the gas concentration in the input (mg m^{-3}).

The evolution of slurry chemical composition and gas emission at different moments over the storage period was analyzed using a repeated measures analysis (PROC MIXED) of SAS® (2001). The relationship between chemical parameters and pH was studied using a correlation analysis (PROC CORR) of SAS®.

3.3. Results

Figure 3.1 shows the evolution of the hourly environmental and slurry temperature pooled by treatment. Environmental temperature showed a clear diurnal fluctuation at hourly intervals. However the environmental

temperature during the experiment was similar among weeks, except for the final week (from week 13 to 15) in which a decrease of the environmental temperature was observed. The average environmental temperature recorded 24.9 ± 2.90 ºC, ranged from 15.3 ºC to 30.3 ºC. As regards the slurry temperature, these diurnal fluctuations were less marked than for environmental temperature, being the average slurry temperature equal to 23.9 ± 1.85 ºC, ranged from 18.9 ºC and 26.2 ºC.

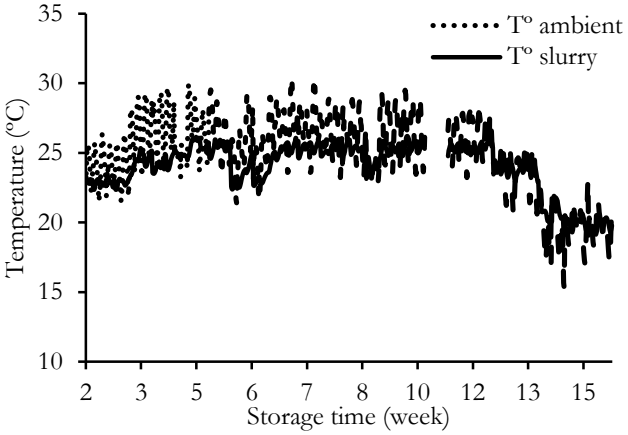

Figure 3.1- Evolution of the hourly environmental (Tº ambient: dotted line) and slurry (Tº slurry: continuous line) temperature.

3.3.1. Result of storage time on slurry chemical composition

Figure 3.2 shows the evolution of TS and VS in SS and RS over the 15-week storage period. Raw slurry showed a higher content of TS ($p < 0.05$) and VS ($p < 0.01$) than SS slurry at the beginning and throughout the storage period. At the beginning of the storage period (week 1), the concentration of TS was 31.3±1.93 g Kg^{-1} in SS and 37.1±1.93 g Kg^{-1} in RS; at this time the concentration of VS was 27.1±1.68 g Kg^{-1} in SS and 35.1±1.68 g Kg^{-1} in RS. At the end of the storage period (week 15), differences in TS and VS between treatments increased ($p<0.001$) compared with those observed at the beginning of the storage period ($p<0.05$). Regarding TS and VS evolution over the 15-week period, both TS and VS concentration showed a marked decrease during the first three weeks of storage, being this especially relevant for VS. From this

point onwards, TS and VS concentration remained constant or slightly increased, showing an increment in both slurries by the end of the study.

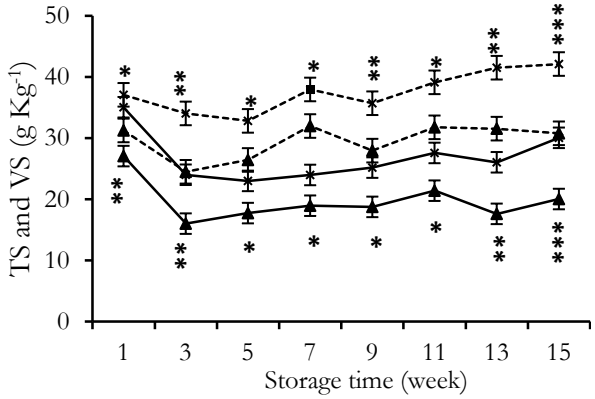

Figure 3.2- Evolution of the total solids (TS: dotted line) and volatile solids (VS: continuous line) of the separated (▲) and raw aged fattening pig slurry (×). Error bars indicate standard error (n =3). The statistical differences between treatments are marked as follows: *** p <0.001, **p <0.01 and *p <0.05.

Table 3.1 shows the chemical composition of manure in terms of TKN and fibrous components on weeks 0, 9 and 15 of the study. As for TS and VS, nitrogenous compounds (TKN_t and TKN_d values) in RS were higher than those obtained for SS throughout the storage period. However, the differences between treatments were only statistically significant at the end of the storage period (week 15) and only in the case of TKN_t. Concerning TKN_t and TKN_d evolution, both of them showed a slight decrease over the storage period.

Regarding fiber content, NDF and ADF were significantly higher in RS compared to SS slurry on weeks 9 ($p < 0.05$) and 15 ($p < 0.001$) of the study. On week 15, the ADL content was also significantly higher ($p < 0.001$) in RS than in SS. Over the storage period, NDF, ADF and ADL concentrations decreased from week 0 to week 9 and increased from week 9 to 15 of the storage period in both treatments, this increase was more pronounced in RS compared to SS.

Table 3.1- Chemical manure composition from separated and raw aged fattening pig slurries in outdoor storage conditions at different storage times in high temperature conditions.

Storage time (weeks)		0	9	15
Total Kjeldhal Nitrogen g Kg^{-1} [FM][1]	Separated Slurry	3.5	3.2	2.9
	Raw Slurry	4.1	3.6	3.5
	SEM[2]	0.23	0.23	0.23
	Significance	ns[3]	ns	$p<0.05$
Dissolved Kjeldhal Nitrogen g Kg^{-1} [FM]	Separated Slurry	2.6	2.5	2.2
	Raw Slurry	3.1	2.8	2.6
	SEM	0.18	0.18	0.18
	Significance	ns	ns	ns
Neutral Detergent Fiber g Kg^{-1} [FM]	Separated Slurry	4.23	2.22	4.12
	Raw Slurry	6.34	5.31	10.6
	SEM	0.761	0.761	0.761
	Significance	ns	$p<0.05$	$p<0.001$
Acid Detergent Fiber g Kg^{-1} [FM]	Separated Slurry	1.50	0.754	1.62
	Raw Slurry	2.44	2.12	4.52
	SEM	0.314	0.314	0.314
	Significance	ns	$p<0.05$	$p<0.001$
Acid Detergent Lignin g Kg^{-1} [FM]	Separated Slurry	0.60	0.32	1.76
	Raw Slurry	0.83	0.78	3.3
	SEM	0.153	0.153	0.153
	Significance	ns	ns	$p<0.001$

[1]FM: Fresh matter
[2]SEM: standard error (n =3)
[3]ns: no significant differences between treatments ($p >0.05$)

Figure 3.3 shows the evolution of the COD_d in the SS and RS slurries. During the first three weeks of storage, the COD_d content increased in RS being the COD_d levels in week 3 and 5 significantly higher in RS than in SS ($p<0.05$). Thereafter COD_d decreased reaching the minimum values in week 13 of storage. After week 13, there was a similar increase in COD_d content in both slurries, RS and SS.

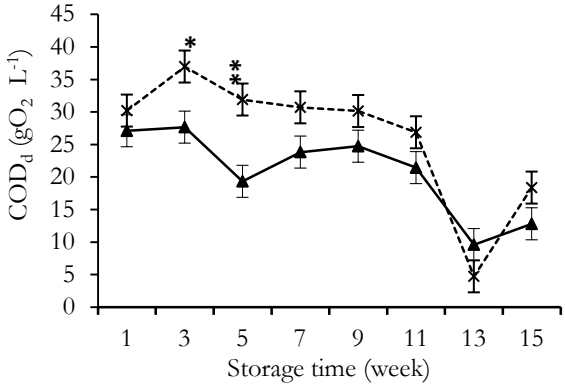

Figure 3.3- Evolution of the dissolved chemical oxygen demand (COD_d) of the separated (continuous line and ▲) and raw aged fattening pig slurry (dotted line and ×). Error bars indicate standard error (n =3).The statistical differences between treatments are marked as follows: *** p <0.001, **p <0.01 and *p <0.05.

Figure 3.4 shows the total VFA content and the individual VFA (acetic, propionic, butyric and isobutyric acids) concentration during the storage period. As for the COD_d, total VFA content in the slurry increased within the first three weeks of the storage period in RS and until the fifth week in SS slurry. An increase in total VFA was observed on week 11 for RS. The VFA content was higher in RS than in SS at the beginning of the storage period ($p < 0.05$). Acetate evolution showed a similar trend than the total VFA, also peaking in week 11 in RS. During the first 11 weeks of storage, acetate comprised approximately 50% of the total VFA in both slurries, declining thereafter until 38% in RS and 32% in SS at the end of the storage period.

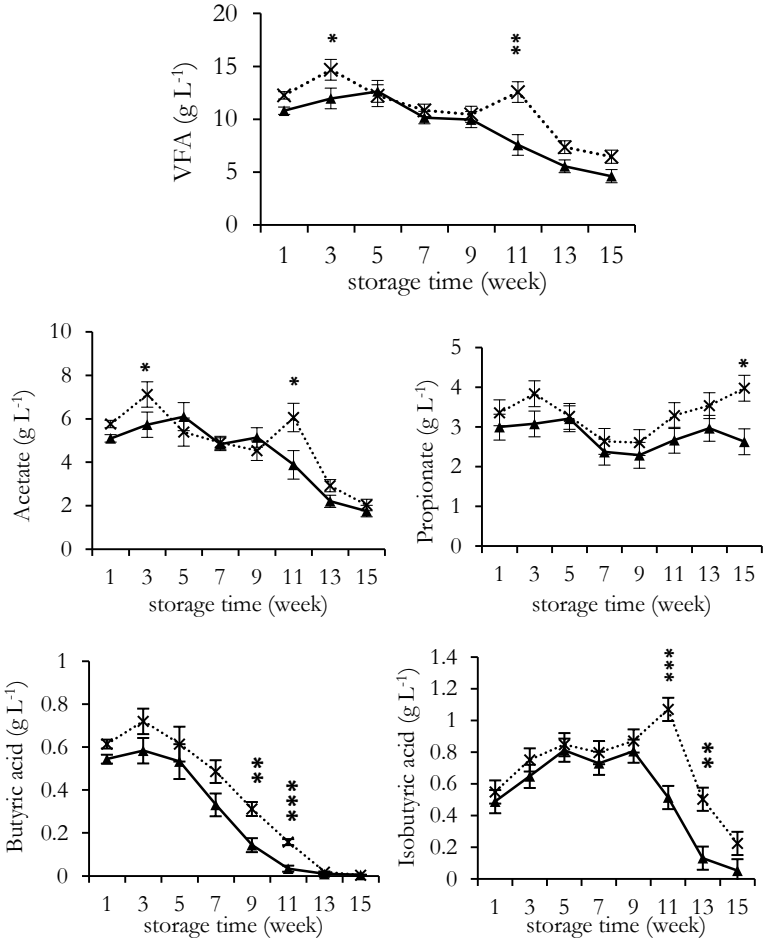

Figure 3.4- Evolution of the total volatile fatty acids (VFA) content and the profile of VFA concentration during the storage time of the separated (continuous line and▲) and raw aged fattening pig slurry (dotted line and ×). Error bars indicate standard error (observation =3). The statistical differences between treatments are marked as follow: *** p <0.001, **p <0.01 and *p <0.05.

There were no statistically significant differences between treatments in the evolution of propionate until the end of the experimental period (week 15). Propionate followed the same trend as total VFA during the first nine weeks in both slurries, thereafter its concentration in both slurries increased, contrary to total VFA evolution, being higher in RS

compared to SS slurry during almost the whole storage period. At the end of the storage period propionate comprised 57% in RS and 62% in SS of the total VFA. Concerning butyrate, its concentration increased during the first three weeks of storage and decreased thereafter reaching negligible levels. The values for butyrate obtained for RS were higher than those obtained for SS during almost all the storage period. However, the concentration of isobutyrate increased during the first 9 weeks (SS) and 11 weeks (RS) in the storage period, and decreased thereafter.

Figure 3.5 shows the evolution of the pH of both slurries. Contrary to total VFA, the pH of both slurries decreased during the first three weeks and increased thereafter until week 15. There were differences between treatments in weeks 9 and 11 of the study, being the pH in SS slurry significantly higher than that of RS ($p < 0.05$) at these moments.

When pH was correlated with VFA it was obtained that VFA content explained 80% of the variation in pH ($R^2=0.80$, $p<0.001$) and the relationship between these two variables, in the range of the pH variation in this experiment, was linear and negative, indicating that the higher levels of VFA the lower pH values.

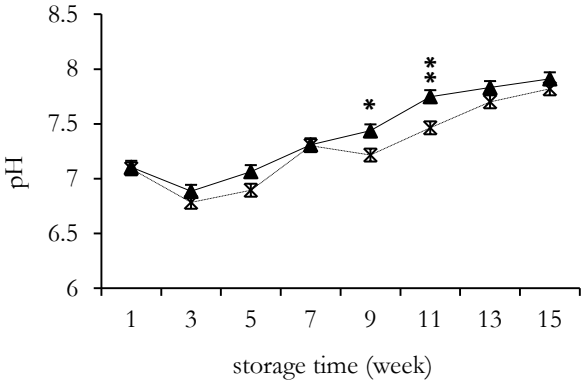

Figure 3.5- Evolution of the pH of the separated (continuous line and▲) and raw aged fattening pig slurry (dotted line and ×). Error bars indicate standard error (observation =3). The statistical differences between treatments are marked as follow: *** $p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

3.3.2. Effect of storage time on gas emissions

The emissions of H_2O and N_2O over the storage period were similar and followed a similar pattern among them (data not shown). The minimum emission rates of H_2O and N_2O were recorded at the beginning of the storage period and the maximum levels were observed in week 10 for both gases (H_2O: RS = 86.41 g h^{-1} m^{-2} and SS = 81.46 g h^{-1} m^{-2} and N_2O: RS = 1.98 mg h^{-1} m^{-2} and SS =1.59 mg h^{-1} m^{-2}). Only during week 3, there were statistical significant differences between treatments ($p < 0.05$), being N_2O and H_2O emissions higher in RS than in SS slurry (H_2O: RS = 49.58 g h^{-1} m^{-2} and SS = 32.64 g h^{-1} m^{-2} and N_2O: RS =1.64 mg h^{-1} m^{-2} and SS = 0.94 mg h^{-1} m^{-2}).

Figure 3.6 shows the evolution of the weekly average CO_2 and NH_3 emissions over the 15-week storage period. During the first three weeks of storage, there was an increase in CO_2 emission in RS being CO_2 emission in weeks 2 and 3 higher ($p < 0.001$) in RS than in SS. The maximum CO_2 emission rate was observed in week 10 in both slurries (RS = 11.18 g h^{-1} m^{-2} and SS = 9.92 g h^{-1} m^{-2}). In week 12, CO_2 emission was again higher ($p < 0.001$) in RS than in SS slurry.

Ammonia emission increased with time showing emission rates of 0.2-0.3 g h^{-1} m^{-2} at the beginning of the storage period and approximately 0.4 g h^{-1} m^{-2} at the end. Differences in NH_3 emissions between treatments were found in week 3, in which NH_3 emission was higher ($p<0.05$) in RS than in SS slurry.

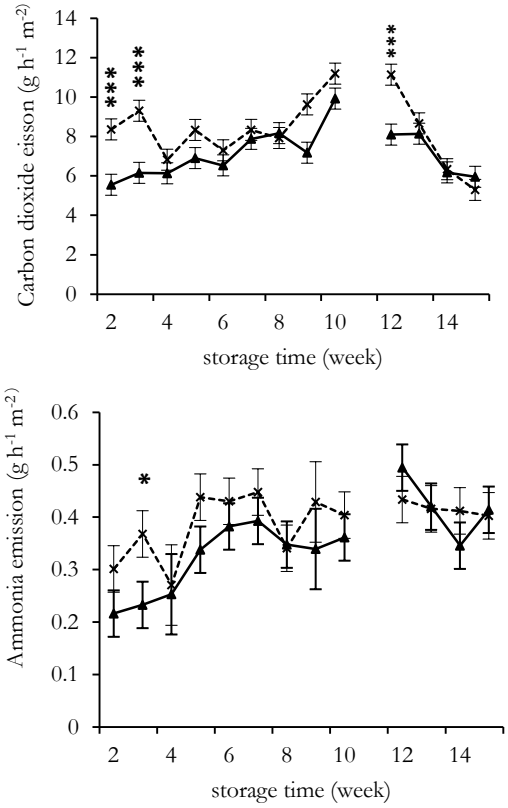

Figure 3.6- Ammonia y carbon dioxide emission from separated (continuous line and ▲) and raw aged fattening pig slurry (dotted line and ×). All registrations are average from 12 observations from three vessels, error bars indicate standard error. The statistical differences between treatments are marked as follow: *** p <0.001, **p <0.01 and *p <0.05. Missing data on week 11 are due to equipment malfunction.

The evolution of the weekly average CH_4 emissions over the 15-week storage period is shown in Figure 3.7. Methane emission was very low during the first six weeks of storage in both treatments, however during this period statistical significant differences ($p < 0.05$) were observed, being CH_4 emission higher in RS than in SS slurry. From week 6 onwards, CH_4 emission increased in both slurries. The maximum measured CH_4 emission was reached before in SS slurry than in RS

slurry. Maximum measured CH_4 emission was reached in week 10 for SS (3.08 g h^{-1} m^{-2}) and in week 12 for RS (4.72 g h^{-1} m^{-2}).

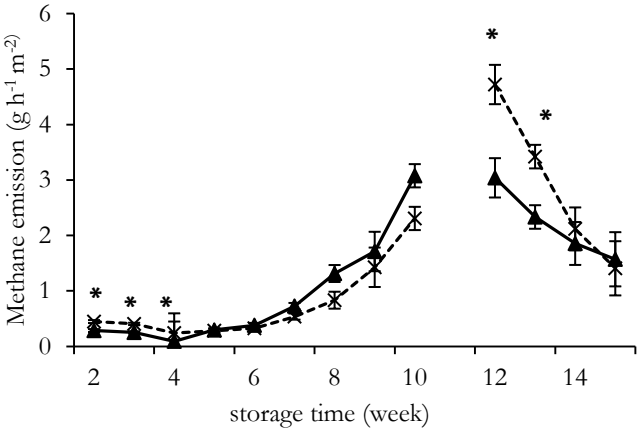

Figure 3.7- Methane emission from separated (continuous line and▲) and raw aged fattening pig slurry (dotted line and ×). All registrations are average from 12 observations from three vessels, error bars indicate standard error. The statistical differences between treatments are marked as follow: *** $p<0.001$, **$p<0.01$ and *$p<0.05$. Missing data on week 11 are due to equipment malfunction.

Fractions of C-CH_4 emissions to total carbon emission [C-CH_4/(C-CO_2+C-CH_4)] were also calculated. The C-CH_4(C-CO_2+C-CH_4) ratio during the peak of CH_4 production (week 10-12) increased from 0.12 to 0.50 in SS and from 0.12 to 0.54 in RS.

3.4. Discussion

The anaerobic degradation of OM from the initial breakdown of organic polymers to the production of CH_4 is a long process that comprises different stages. Our results support the stages defined by Angelidaki et al. (1999), where OM is fermented by the acidogenic and acetogenic bacteria leading first to the formation of intermediate VFA and finally to the production of CH_4.

In our study, during the first stages of the storage period (first five weeks), there was a relative transformation of the more degradable OM into soluble OM as shown by the decrease in TS, VS, NDF, ADF and ADL concentrations and the increase in COD_d, VFA concentration and

CO_2 emission during this period. Then, COD_d and VFA concentration decreased coinciding with the increase in the CH_4 production in both slurries as the final step of the anaerobic OM degradation.

Similar trends been observed in other studies when pig fresh slurry was used. Møller et al. (2004) found a similar increment of total VFA content during the first weeks of storage in pig slurry stored at 20ºC followed by an increment in CH_4 emission and a drop of VFA concentration. However, our results show further differences in the OM degradation process between the solid-separated (SS) and the non-separated (RS) slurries. The COD_d is usually used as an indicator of the degree of OM degradation, since during the first steps of the degradation process; fermentative bacteria hydrolyze and convert the suspended solids into dissolved solids to obtain a continuous food supply for their growth (Zhu et al., 2000). These dissolved solids (composed of soluble organic compounds) are represented by the COD_d content. The higher COD_d content observed in RS in week 3 in our results compared with SS, might indicate a higher hydrolytic bacteria activity at the beginning of the storage period in RS compared to SS. These results could be related to the higher OM content of RS compared to SS. In fact, the OM concentration is one of the most relevant parameters in the kinetics of its degradation (Vavilin et al*.,* 1996; Vavilin and Angelidaki, 2004). In addition, as suggested by Fangueiro et al. (2008), the higher OM content in RS slurry, especially the higher fiber content, may have promoted better anaerobic conditions in this slurry and thus enhanced anaerobic bacteria establishment.

The hypothesis that there is a higher bacterial activity in RS during the first weeks is also supported by the higher CO_2 emission at this moment in RS compared with SS slurry. The two main sources of CO_2 emission from slurry are the microbial degradation of OM and the urea mineralization process by the enzyme urease, which also leads to NH_3 volatilization (Cortus et al*.,* 2008). The higher CO_2 emission rates observed in RS compared to SS in week 3 could have been related with these two processes. As stated above, this could be explained by a higher hydrolytic, acidogenic and acetogenic activity, as shown by the increase in COD_d and VFA during OM degradation during the first three weeks

of storage, but also by a higher rate of organic nitrogen mineralization and denitrification, as shown by a higher NH_3 and N_2O emission in RS at this time (week 3).

The initial VFA content in both slurries was higher compared to values reported in the literature (Møller et al., 2004) in which fresh slurry was used. However, in this study the maximum VFA, which was reached on weeks 3 (RS) and 5 (SS), was lower than that obtained in the works in which fresh slurry was used, probably due to the lower content of biodegradable OM in aged pig slurry as regards to fresh slurry. Concerning the individual VFA, at the beginning of the OM degradation process, acetate was the main VFA produced in both slurries. However, at the end of the storage period, the production of propionate was higher, especially in the RS slurry. Accumulations of propionate in slurry storage have been observed also by other authors such as Møller et al. (2004) and Nozhevnikova et al. (2000). These authors suggested that, in outdoor storage conditions, propionate is accumulated as an intermediate product because it is degraded at a lower rate than butyrate and acetate.

Concerning gas emission, CO_2 emissions obtained in this work were in a similar range that those obtained by Dinuccio et al. (2008) for a liquid fraction and untreated pig slurry stored at 25ºC (5-15 mg CO_2 h^{-1} m^{-2}). However, the NH_3 emission obtained by Dinuccio et al. (2008) in the liquid fraction and in the untreated pig slurry at 25ºC was slightly higher (300-700 mg NH_3 h^{-1} m^{-2}) that those obtained in this work, probably because these authors used fresh pig slurry. As stated by Béline et al. (1998), a large part of the nitrogen organic is mineralized during the first two weeks of storage in fresh slurries, therefore low and stable NH_3 emissions over time are expected in aged slurries instead of the observed increase in NH_3 emissions during the storage period obtained in this study. However, this increase could be related with the increment in the pH of both slurries because as stated Muck and Steenhuis (1982) and Canh et al. (1998) the pH of the slurry is one of the most important factors influencing NH_3 emission.

N_2O emission obtained in this work was lower compared than those obtained by Amon et al. (2006) using untreated pig slurry at 10ºC. However, it was similar to that obtained by Dinuccio et al. (2008) at 25ºC. These authors registered negligible N_2O emission in untreated slurry and in liquid phase slurry; and significant N_2O emission only in the solid fraction during the first 25 days of the storage period.

Our results showed that CH_4 was not emitted from pig slurry until week 6 after the slurry was removed from the storage pit. This delay in CH_4 emission detected in the present study has been observed in other studies (Møller et al., 2004; Sommer et al., 2007). The equilibrium of methanogenic bacteria is generally achieved more slowly than the equilibrium of the rest of bacterial populations that inhabit the slurry (Vavilin and Angelidaki 2004). Additionally, Vavilin and Angelidaki (2004) also suggested that the slow growth of methanogenic bacteria may be related to the formation of specific bacterial morphological aggregates or flocks. In the present study, aged slurry which could presumably have already established methanogenic bacteria was used and this could have accelerated the production of CH_4. However, an important delay in CH_4 emission was observed probably due to the change in slurry conditions from the pit under slatted floor and the tanks, together with the vigorous mixing of the slurry at the beginning of the study to promote homogenization. These facts could have disrupted the anaerobic conditions presumably already established under slatted floor and the structure of the bacterial flocks, thus delaying the onset of methanogenic activity.

The understanding of the CH_4 emission pattern during aged slurry storage is useful in order to recommend a maximum period for outdoor storage to prevent significant losses of CH_4, applicable to Mediterranean conditions where aged pig slurry is stored generally without covers during long periods. From our results, the recommended time of storage in summer time in order to minimize CH_4 losses from aged fattening pig slurry to the atmosphere could be established between 30 to 35 days (week 4 to 5). This recommendation could be applicable in those slurry management systems which consist on a pre-storage below slatted floor during the whole fattening period followed by outdoor storage until its

application to agricultural land. This is specially the case in those areas where the use of livestock manure as fertilizer is restricted to specific periods of the year (i.e. vulnerable areas under the European Nitrates Directive, 91/676/EC), and therefore slurry is stored in outdoor storage lagoons for long periods of time. Moreover, under the European Nitrates Directive storage lagoons must have a minimum storage capacity of 3-4 months. During this time, and taking into account the results obtained in the present study, major CH_4 emissions to the atmosphere could be expected. According to our results, in storage periods longer than five weeks, the use of gas collection systems in such storage installations to avoid CH_4 losses could be recommended. Although a wide range of management systems are used for pig rearing and slurry handling worldwide, our results are valuable to characterize the evolution of aged slurry, representative to a large extent of outdoor storage in Mediterranean areas and in those cases where pre-storage under pits is expanded throughout the whole of the fattening period and is not mixed with slurries from animals that are in other physiological states.

The results obtained in this study concerning $C\text{-}CH_4/(C\text{-}CO_2+C\text{-}CH_4)$ ratio show that, under our experimental conditions, during the peak of CH_4 emission, decomposition of OM was dominated by methanogenic microbial community and thus, at this time, the biogas produced could be used as energy source. However, the $C\text{-}CH_4/[C\text{-}CO_2+C\text{-}CH_4]$ ratio during the peak of CH_4 production obtained in the present study (0.50 - 0.54) was lower compared to other experiments. Sommer et al. (2007) obtained a ratio between 0.50 - 0.65 during the CH_4 production peak and Møller et al. (2004) obtained a ratio between 0.60 -0.70. This difference could be attributable to the use of aged slurry in our study. Sommer et al. (2007) and Møller et al. (2004) worked with fresh slurry, however the slurry used in this work was obtained after 19 weeks of storage under the pit. The VS biodegradability in the slurry after long pre-storage times is lower than that of the fresh slurry because the degradable *vs.* non-degradable fraction increases with the age of the slurry (Sommer et al., 2004).

3.5. Conclusions

From our results concerning 15-week storage period in summer conditions of two types of aged fattening pig slurry: separated slurry (SS) and raw slurry (RS), we can conclude that:

- There is relevant transformation of the more degradable OM into soluble OM during the first weeks of aged fattening pig slurry storage. This transformation is more pronounced in the slurry with a higher initial OM concentration (RS) than in separated slurry (SS), indicating a higher hydrolytic, acidogenic and acetogenic activity, as well as higher rate of urea mineralization and nitrogen denitrification rate at the beginning of the storage period in RS than in SS.
- In aged fattening pig slurry stored under Mediterranean summer conditions, the establishment of all bacterial groups involved in the anaerobic degradation process does not occur until week 5, shown in our results by the VFA accumulation and the negligible CH_4 emission during the first five weeks of storage in both treatments.
- Slurry storage time and thus, the age of the slurry can decrease the biodegradability of OM, since the non-degradable fraction of OM increases over storage time. Storage time can considerably affect the biodegradability of OM in pig slurry.

3.6. Acknowledgements

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Chapter 4

The inhibiting effect of sulfate on thermophilic anaerobic digestion of cattle and pig waste slurry

The inhibiting effect of sulfate on thermophilic anaerobic digestion of cattle and pig waste slurry

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Abstract. Slurry acidification with sulfuric acid is a commonly used measure to reduce ammonia emissions from animal waste in Denmark. However, high sulfate content is an inhibiting factor in anaerobic digestion. The objective of this study was to investigate the sulfate concentration thresholds for inhibition of thermophilic anaerobic digestion of animal slurry. We conducted a batch assay for 114 days using two types of slurry (pig and cattle) and ten different sulfate concentrations per type of slurry (0-5000 mg SO_4^{2-} L^{-1}). Added sulfate concentrations higher than 2500 mg SO_4^{2-} L^{-1} in pig slurry and higher than 2000 mg SO_4^{2-} L^{-1} in cattle slurry caused reductions of more than 40% in methane production and accumulations of volatile fatty acids throughout the study. The biogas produced contained large amounts of hydrogen sulfide (3.2% in pig slurry and 1.9% in cattle slurry) with concentrations peaking in the first 20 days, and subsequently decreasing. Waste properties can affect the inhibitory threshold of the sulfate in anaerobic digestion. Our results provide specific sulfate inhibition thresholds for pig and cattle slurries, which differ from other sulfate-rich wastewaters.

Keywords: acidification, sulfate, methane production, pig slurry, cattle slurry.

4.1. Introduction

The acidification of slurry by manipulation of the balance between ammonia and ammonium is an effective measure of reducing ammonia emissions from animal slurry (Berg et al., 2006). The technique is currently being used in around 90 Danish pig and cattle farms (Infarm® Company, personal communication, 2012). Sulfuric acid (H_2SO_4) is used as acidifying agent for economic reasons, since it is a cheap acid and because of the added benefit of the fertilizer value of sulfur (Ottonsen et al., 2009). In practice, sulfuric acid is usually added at a rate of 5000 mg SO_4^{2-} per L of slurry (Kai et al., 2008).

Because slurry acidification can also reduce methane (CH_4) emissions from the manure during storage, pre-treatment of slurry with H_2SO_4 could potentially increase the CH_4 yield in the anaerobic digestion of slurry (Berg et al., 2006). However, the full implementation of this technique is problematic due to the activity and growth of sulfate-reducing bacteria (SRB) during anaerobic digestion (O'Reilly and Colleran, 2006). When sulfate is present, SRB use this as the terminal electron acceptor during the oxidation of organic matter, resulting in the production of sulfide which is distributed among hydrogen sulfide (H_2S) in the gas phase and total inorganic dissolved sulfides species (TIDSS: H_2S, HS^- and S^{2-}) in the liquid phase, depending on the sample's pH (Isa et al., 1986). Typically, biogas is composed of 55-80% CH_4 and 20-45% CO_2, with trace amounts of H_2S ranging between 0.005 and 1%, depending on the composition of the feed substrate (Pipatmanomai et al., 2009). Therefore higher H_2S concentration in the biogas could confirm the presence of SRB. Hydrogen sulfide is an odorous gas which is dangerous at high levels. Its negative impacts in anaerobic digesters are its corrosive effect on combined heat and power engines (Cirne et al., 2008); additionally TIDSS have an inhibitory effect on anaerobic bacteria. The accumulation of TIDSS in the anaerobic reactors can result in inhibition of the digestion process and even in its total failure (Hulshoff Pol et al., 1998). Consequently, high sulfide content might be a limiting factor for the anaerobic process.

The inhibitory effect of TIDSS on anaerobic bacteria is presumably caused by the non-ionized form (H_2S), since the cell membrane has a higher permeability to neutral molecules. Different inhibitory H_2S thresholds have been proposed in the literature (Koster et al., 1986; McCartney and Oleszkiewicz, 1991; O'Flaherty et al., 1998). Hulshoff Pol et al. (1998) found that the concentration of H_2S in anaerobic reactors should not exceed the critical value of 150 mg H_2S L^{-1} and Chen et al. (2008) reported a wide range of inhibition thresholds from 100–800 mg L^{-1} TIDSS or approximately 50–400 mg L^{-1} of undissociated H_2S. This wide range is due to the biological nature of the anaerobic digestion process and the strong dependence of temperature and pH on the inhibition process (Hulshoff Pol et al., 1998; Cirne at al., 2008).

In addition, SRB can compete with methane-producing bacteria (MPB) for electron sources such as acetate or hydrogen and with the obligate hydrogen-producing bacteria for propionate (Rintala and Lettinga 1992). Literature shows that the SRB have a higher affinity for acetate and hydrogen than MPB (Bhattacharya et al., 1995; Ciccoli et al., 2010). In fact, Omil et al. (1997) reported that SRB are thermodynamically and kinetically more advantageous than MPB for volatile fatty acid oxidation and molecular hydrogen consumption.

Nevertheless, previous research on bioreactors has shown that MPB can co-exist with SRB (Isa et al., 1986; Yoda et al., 1987; McCartney and Oleszkiewicz, 1991). O'Reilly and Colleran (2006) reported that MPB were responsible for hydrogen utilization at an influent COD/SO_4^{2-} ratio of 16, and were still dominant when the ratio was reduced to 4. It was only when the COD/SO_4^{2-} ratio was reduced to 2 that the SRB assume a more influential role.

As a result of the complex interactions among all the variables involved in the anaerobic digestion of sulfuric acid-treated animal waste, the process is highly complex. Even with our current knowledge on anaerobic digestion and its inhibitors, it is still difficult to predict its behavior and to draw reliable conclusions on a single inhibition threshold, because the phenomenon of inhibition is affected by several mechanisms such as antagonisms, synergisms, acclimatization and complexation (Chen et al., 2008; Siles et al., 2010). Furthermore, studies

of methane inhibition in sulfate-rich wastewater have focused on sulfate concentration, pH and temperature regardless of the type of substrate (Rintala and Lettinga, 1992; Hulshoff Pol et al., 1998; Siles et al., 2010). Animal slurry is composed of a pool of organic and inorganic constituents and it also has high microbiological activity; these factors could affect SRB activity and hence the inhibition process. Consequently, how the physicochemical composition of animal slurry can influence inhibitory concentration thresholds is of particular interest, both from a research and practical perspective. It is also of special interest because it would enable co-digestion of acidified and non-acidified manure in ratios that will not reduce the CH_4 yields significantly.

In the present work we sought to establish sulfate concentration thresholds for the inhibition of thermophilic anaerobic digestion in pig and cattle slurry, and we furthermore examined the change of slurry composition during inhibitory and non-inhibitory conditions in order to develop anaerobic digestion with acidified slurries.

4.2. Materials and Methods

4.2.1 Substrates

To test the sulfate concentration threshold of anaerobic digestion, we used pig and cattle slurry as the two substrates. Both slurries were obtained from the experimental farms at Research Centre Foulum (Tjele, Denmark). The initial age and composition of each slurry is shown in Table 4.1. Besides raw slurry, inoculum was collected from the full-scale biogas digester located at Research Centre Foulum and incubated at 55°C for 15 days to ensure a minimal endogenous biogas production from the inoculum (endogenous biogas production). The initial composition of the inoculum is also shown in Table 4.1. Total volatile fatty acid (VFA) content was calculated from fresh and dried slurry. Total VFA loss from the slurry drying process was calculated after drying the slurry at 103ºC for 24 h (APHA, 2005) (2540-B procedure) and rehydrating to initial total solid content.

Table 4.1- Initial chemical composition of the substrates and inoculum used in the study. Replications per treatment: 1.

	Pig slurry	Cattle slurry	Inoculum[1]
pH	7.51	8.21	7.99
Initial slurry age, days	14	2	15
Total solids, g Kg^{-1}	44.7	93.4	23.0
Volatile solids, g Kg^{-1}	33.4	77.6	14.2
Sulfide, mgS^{-2} L^{-1}	1.19	0.33	0.79
Sulfate, $mgSO_4^{2-}$ L^{-1}	286	670	330
Total volatile fatty acids before drying, g L^{-1}	13.36	5.29	0.19
Total volatile fatty acids after drying, g L^{-1}	2.95	5.08	0.19
Total volatile fatty acids lost during drying, %	77.92	3.97	0

[1]Inoculum samples were taken after incubation at 55°C for 15 days

4.2.2 Batch assays

The experiment was conducted in a factorial design in which two types of slurry (pig and cattle) and ten different sulfate concentrations for each slurry were tested. Each tested combination was conducted in a batch assay (0.5 L bottles). Sulfuric acid was added to obtain the varying sulfate concentrations. Table 4.2 shows the different treatments and substrates tested, the sulfate concentration added to bottles and the resulting total sulfate concentration from the mixture of animal slurry, inoculum and sulfuric acid. As shown Table 4.2, differences in total sulfate concentration between slurries were <70 mg SO_4^{2-} L^{-1} in each treatment. These differences ranged from less than 20% in treatment 1 (0 mg SO_4^{2-} L^{-1} added) to less than 1.3% in treatment 10 (5000 mg SO_4^{2-} L^{-1} added).

Average volatile solids (VS) contents were 5.95±0.049 g per bottle and the ratio inoculum: substrate was 0.93±0.015 on a VS basis. Four bottles per treatment were prepared: three of them were used to measure CH_4, carbon dioxide (CO_2) and H_2S concentration in the biogas, and the fourth bottle was used to measure chemical composition. In addition, four bottles with 200.80±1.10 mL of inoculum were used as blanks, their

biogas production (endogenous biogas production from the inoculum) was subtracted from the biogas production of the experimental bottles.

After filling, each bottle was sealed with butyl rubber stoppers and aluminum crimps and the headspace was flushed with pure N_2 for two minutes. Bottles were then incubated at 50°C for 114 days.

Table 4.2- Description of substrates, sulfate concentrations added and total sulfate concentration estimated in bottles used in this study.

Treatment	Substrate	Added sulfate concentration, mg SO_4^{2-} L^{-1}	Total sulfate concentration in bottles, mg SO_4^{2-} L^{-1}
1	pig slurry	0	316
2		200	516
3		500	816
4		1000	1316
5		1500	1816
6		2000	2315
7		2500	2816
8		3000	3316
9		4000	4316
10		5000	5316
11	cattle slurry	0	385
12		200	586
13		500	886
14		1000	1386
15		1500	1888
16		2000	2387
17		2500	2886
18		3000	3386
19		4000	4386
20		5000	5386

The total volume of biogas produced per bottle was measured on days: 2, 5, 8, 12, 17, 23, 30, 37, 44, 51, 62, 92 and 114. During the first 25 days, a maximum interval of five days between measurements was maintained thereafter, the measurement interval was increased until day 60. From day 60 until day 114 only two measurements were taken since the yield from all bottles was very low. The measurement of biogas was done by inserting a needle connected to a tube with inlet to a column filled with

acidified water (pH<2) through the butyl rubber. The biogas produced was calculated by the water displaced until the two pressures (column and headspace in bottles) were equal.

Biogas and CH_4 production in this study were expressed at standard conditions (STP, Temperature = 273.15 K, Pressure = 100 KPa) according to the recommendations of Angelidaki and Sanders (2004). The amount of biogas produced was measured in this study with the water displacement method immediately after bottles were taken out from the incubators at a temperature of 50 ºC and with the gas temperature only slightly below this during volume measurement. The difference in volume between gases at 50ºC and at standard conditions (0ºC) is 15% according to the ideal gas law.

Methane, carbon dioxide and H_2S concentration from three bottles per treatment were analyzed every time the total volume of biogas was measured. Methane and carbon dioxide were measured on an Agilent 7890A gas chromatograph (Agilent technologies, USA) using a thermal conductivity detector (TCD) and a flame photometric detector (FPD) as described by Raju et al. (2011). The H_2S in the biogas was determined colorimetrically with Kitagawa gas detector system comprised of a 100 cc air sampling pump (AP-20, Kogyo K.K., Japan) and Kitagawa colorimetric gas detector tubes (Kogyo K.K., Japan). Two H_2S detector tubes were used depending on the H_2S concentration in biogas, namely, tube number 120SF (measurement range: 100 to 2000 ppm and accuracy: 10%) and tube number 120SH (measurement range: 1000 to 40000 ppm and accuracy: 10%).
Initial nitrogen concentration in the headspace was not considered and CH_4 and CO_2 concentrations obtained by chromatography were corrected to assume that CO_2, CH_4 and H_2S were the only gases present in the headspace.

4.2.3 Chemical analyses

At the beginning of the experiment and at the same time of the biogas sampling, a representative liquid sample was taken from the fourth bottle (in which biogas measurements had not been performed). This was

analyzed for pH, total solids (TS) and VS) as described per APHA (2005) (2450-B and 2450-E procedures).

Total inorganic dissolved sulfide species (TIDSS: H_2S + HS^- + S^{2-}) in the slurry was determined with photometric kits (Spectroquant kit, Merck, USA) and VFA with gas chromatography equipped with a flame ionization detector (HP 68050 series Hewlett Packard) according to APHA (2005) (5560-D procedure). The VFAs determined were: acetic acid, valeric acid, butyric acid, propionic acid, 2-methyl propionic acid, and methyl butyric acid + isovaleric acid.

4.2.4 Calculations and data analyses

Treatments effect on biogas and CH_4 production over the 114-d study was determined using repeated measures ANOVA (PROC MIXED) in SAS Version 9.0 (SAS, 2001). If at least one treatment was different, Tukey's test was performed for pairwise comparison. Statistical significance level was set at 5% ($\leq$ 0.05). Inhibition by sulfate in a treatment was considered to be taking place when the CH_4 production was significantly lower than the CH_4 production in the treatment with zero sulfate addition.

Biogas and CH_4 production was expressed as the cumulative CH_4 production (mL) at standard conditions per gram of VS from the slurry introduced to the bottles (VSi) including the VFA content lost by drying. Following Table 4.1, the VFA content lost by drying in this study was 80% in pig slurry and 0% in cattle slurry. Differences between slurries in VFA content lost by drying were probably due to the lower pH value of pig slurry compared with cattle slurry (Derikx et al., 2004; Rico et al., 2007).

Ultimate CH_4 yield (B_0) was calculated as the cumulative CH_4 produced in bottles with zero sulfate addition after 114 days. Methane inhibition in percentage was calculated following Equation 4.1:

$$\%\, Inhibition = 100 - \left(\frac{CH_4 produced}{B_0} x100\right) \quad (4.1)$$

where CH_4produced (L CH_4 gVS^{-1}) is the cumulative CH_4 produced in each treatment in 114 days. Sulfur loss (%S loss) was calculated as shown in Equation 4.2:

$$\%\, S\ loss = \frac{H_2Sbiogas}{initial\ S} x\ 100 \quad (4.2)$$

where H_2Sbiogas is the cumulative sulfur (S-H_2S) loss (mg) in the biogas and initial S is the initial total inorganic dissolved sulfur content (mg) in the slurry (SO_4^{2-} + H_2S + HS^- + S^{2-}).

4.3. Results and Discussion

4.3.1 Methane production

Ultimate CH_4 yield in pig slurry was 295 ± 9.6 mL CH_4 gVS^{-1} and 225 ± 14.4 mL CH_4 $gVSi^{-1}$ in cattle slurry. Published values for B_0 vary from 250 to 480 mL CH_4 gVS^{-1} for pig slurries and from 100 to 240 mL CH_4 gVS^{-1} for cattle slurries (Møller et al., 2004a; Amon et al., 2007; Vedrenne et al., 2008). Ultimate CH_4 yield is a key parameter, not only since it is indispensable in assessing design, economic and management issues for the full-scale implementation of anaerobic digestion processes (Møller et al., 2004b; Angelidaki et al., 2009), but also because of the wider environmental implications of CH_4 emissions. Furthermore, countries that report on their greenhouse gas emissions under the Kyoto Protocol use the B_0 parameter to calculate CH_4 emissions (IPCC, 2006). Ultimate CH_4 yields obtained in this work for pig and cattle slurry were within the range of values reported in the literature. However, the range of B_0 values reported is extremely wide, due in part to differences in slurry composition, but also to differences in B_0 methodology. Methodologies currently used are adapted from Owen et al. (1979), but generally, results are not expressed at standard conditions, and VFA losses during drying are not considered in VS determination, although these parameters can considerably affect B_0 results (Vedrenne et al., 2008).

Cumulative biogas production in, respectively, pig and cattle slurry is shown in Figure 4.1. The figure shows a clear threshold in biogas production in both pig and cattle slurry between 2000 and 2500 mg

SO_4^{2-} L^{-1}. Statistical significant differences between treatments in pig slurry were observed from the first measurement, but for cattle slurry differences did not appear until 20 days into the incubation period. These differences persisted throughout the experiment (statistical data evolution not shown). Cumulative biogas production was lower than those obtained in the blank bottles (endogenous biogas production of inoculum) in bottles that exhibited a decrease in cumulative biogas production on time (bottles with 2500, 3000, 4000 and 5000 mg SO_4^{2-} L^{-1} in cattle slurry).

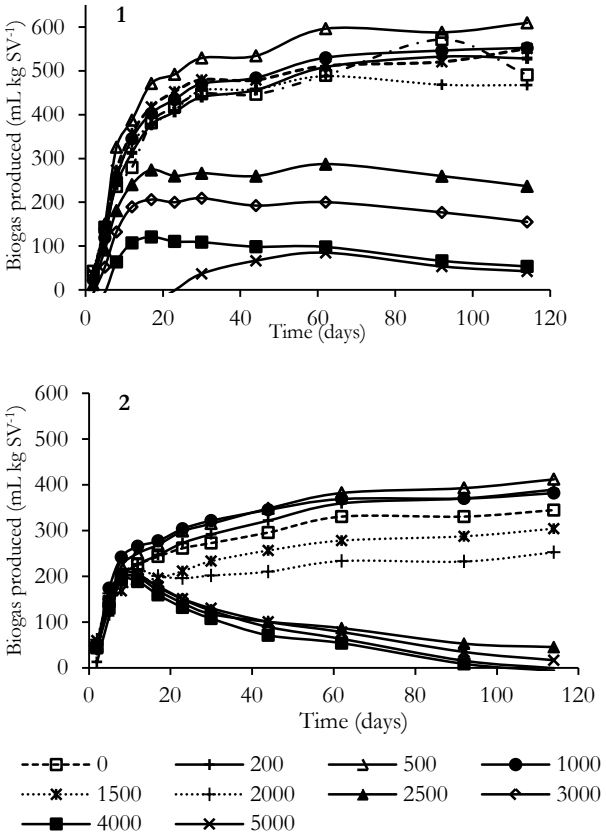

Figure 4.1- Cumulative biogas production with increasing sulfate concentrations (mg SO_4^{2-} L^{-1}) for (1) pig slurry and (2) cattle slurry at standard conditions in mL per Kg of VS including the VFA content lost by drying and under thermophilic conditions.

Table 4.3 shows the final, cumulative CH_4 production (after 114 days) from pig and cattle slurry, respectively, with increasing sulfate concentrations. In pig slurry, the largest CH_4 production was in bottles with 500 mg SO_4^{2-} L^{-1} additions and this was also significantly higher ($P<0.05$) than B_0. The CH_4 production from 500 and 1000 mg additions was similar (no significant differences). During the experimental period, there were no significant differences in CH_4 and biogas production at 0, 200, 1000, 1500 and 2000 mg SO_4^{2-} L^{-1} additions, with no inhibition taking place at these sulfate concentrations. In pig slurry, sulfate inhibition started with the 2500 mg SO_4^{2-} L^{-1} addition. The inhibition for 2500, 3000, 4000 and 5000 mg SO_4^{2-} L^{-1} additions was 58%, 78%, 93% and 94%, respectively, after 114 days.

Similar to pig slurry, the highest CH_4 production in cattle slurry was for the 500 mg SO_4^{2-} L^{-1} addition. In this case, however, there were no significant differences among 0, 200, 500, and 1000 mg SO_4^{2-} L^{-1}. In cattle slurry, sulfate inhibition started from the 2000 mg SO_4^{2-} L^{-1} addition, since CH_4 production from this treatment was significantly lower than B_0. However, no significant differences were found among 1500 mg SO_4^{2-} L^{-1} and 2000 mg SO_4^{2-} L^{-1}. The inhibition in cattle slurry with 2000 mg SO_4^{2-} L^{-1} was 47%. For 2500, 3000, 4000 and 5000 mg SO_4^{2-} L^{-1} additions there was complete inhibition, CH_4 production in these bottles was lower than that in bottles used as blanks (endogenous biogas production of inoculum).

In cattle slurry, sulfate inhibition started at a lower sulfate concentration than in pig slurry (2000 mg SO_4^{2-} L^{-1} *vs.* 2500 mg SO_4^{2-} L^{-1}). Differences in inhibition threshold between slurries could reflect differences in initial sulfate concentrations, but at 2000 mg SO_4^{2-} L^{-1} the difference in the total sulfate concentrations was less than 3% (Table 4.2). The higher CH_4 production at 500 mg SO_4^{2-} L^{-1} in both cattle and pig slurry compared with no addition could be explained by sulfur being a nutrient for organisms, and by methanogenic bacteria needing low concentrations of sulfide and sulfate to survive (Daniels et al., 1986). However, the levels and the forms of sulfur needed by methanogenic bacteria living in the slurry have not been sufficiently explained in the literature.

Table 4.3- Cumulative methane production and standard error after 114 days from pig and cattle slurry with increasing sulfate concentrations. Replications per treatment: 3.

Sulfate concentration added (mgSO42- L-1)	Pig slurry		Cattle slurry	
	Average CH4 production (mL CH4 gVSi-1)	Standard error	Average CH4 production (mL CH4 gVSi-1)	Standard error
0	295 b[1]	9.6	225 ab	14.4
200	310 b	15.8	212 ab	51.1
500	360 a	41.5	242 a	21.5
1000	322 ab	12.4	235 ab	16.5
1500	322 b	10.0	159 bc	9.2
2000	290 b	16.1	120 c	18.0
2500	123 c	3.8	CI[2]	-
3000	66 d	6.5	CI	-
4000	21 e	2.1	CI	-
5000	18 e	14.2	CI	-

[1]Statistically significant differences ($P < 0.05$) between treatments, using the repeated measures analysis of variance ANOVA (Tukey's test), are indicated by different superscript letters within columns.
[2]CI: complete inhibition; when CH_4 production in treatment bottles was lower than the CH_4 production in blank bottles (endogenous CH_4 production of inoculum).

The results obtained herein primarily provide comparable inhibition thresholds of sulfate in anaerobic digestion of pig and cattle slurries. The inhibitory thresholds of sulfate presented in our study are higher than values reported in the literature. Siles et al. (2010) reported decreases in biogas production with 1400 mg SO_4^{2-} L^{-1} in a glucose solution under thermophilic conditions. Differences could be explained by factors such as the OM: sulfate ratio in substrates, antagonism, synergism, and the acclimatization of bacteria populations (Siles et al., 2010). Slurry is inhabited by a variety of microorganism as well as organic compounds and minerals such as nitrates and nitrites, which can interact in the metabolism of SRB (Hulshoff Pol et al., 1998; Cirne et al., 2008; Moreno et al., 2010). This indicates that differences in the physicochemical and microbiological characteristics of substrates could play a key role when determining the inhibition thresholds of sulfate. Further research is necessary to identify the substrate-specific effect in the sulfate inhibition thresholds.

4.3.2 Hydrogen sulfide concentration in the biogas

Our results show that in addition to a reduction in CH_4 and biogas production with increasing sulfate concentrations, there was also an increase in H_2S concentrations. The H_2S concentration in pig and cattle slurry without sulfate inhibition (< 2000 mg SO_4^{2-} L^{-1}) remained below 0.5% (5000 ppm) (data not shown). However, higher H_2S concentrations were found at higher sulfate concentrations in both slurries. In both slurries, the highest H_2S concentrations were obtained in the first 20 days of digestion. The highest H_2S concentration in biogas was found in pig slurry on day 12 with 4000 mg SO_4^{2-} L^{-1} added (3.2% H_2S). In cattle slurry the highest concentration was 1.9% H_2S on day 17 with 5000 mg SO_4^{2-} L^{-1} added. The H_2S was probably generated by SRB, which compete with methanogens and acetogenic bacteria for VFA and H_2. The SRB utilize these substrates as electron donor and sulfate as electron acceptor, producing H_2S (Ranade et al., 1999). In natural conditions, biogas is composed of 55-80% CH_4, 20-45% CO_2 with trace amounts of H_2S ranging between 0.005 and 1%, depending on the composition of the feed material (Pipatmanomai et al., 2009). The eventually highest H_2S in the biogas obtained in this work could confirm the presence of SRB in bottles.

Figure 4.2 shows the calculated sulfur loss in bottles. As shown in this figure, in pig slurry, the highest removal of sulfur at nearly 10% was obtained in bottles containing 0, 3,000, 4,000, and 5,000 mg SO_4^{2-} L^{-1}. In cattle slurry, the recovery of sulfur was lower than in pig slurry, with the highest in bottles added: 0, 200 and 2,000 mg SO_4^{2-} L^{-1}.

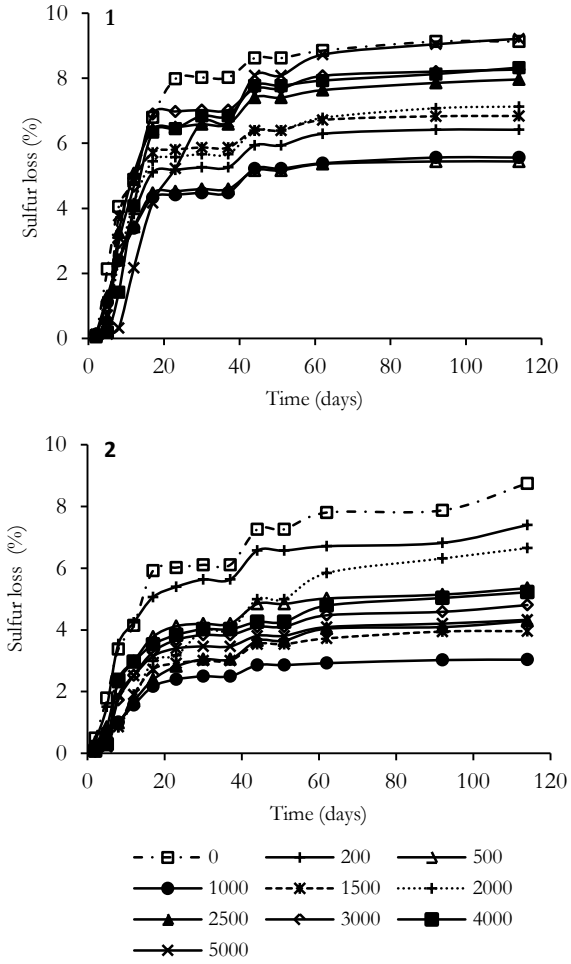

Figure 4.2- Calculated sulfur loss (expressed as % sulfur (S) removal from the initial sulfur content in the slurry) in bottles containing pig slurry (1) and cattle slurry (2) with increasing sulfate concentrations (mg SO_4^{2-} L^{-1}) in thermophilic conditions.

Sulfur loss calculated in this study were lower than in the literature (Isa et al., 1986; Bhattacharya et al., 1996; Damianovic and Foresti, 2009), indicating that SRB have not completely colonized the substrate. This could be because SRB growth is affected by the organic compounds used as a carbon source, as suggested by Damianovic and Foresti (2009).

In fact, Damianovic and Foresti (2009) found that sulfate is more efficiently removed when SRB use ethanol as the sole source of OM rather than VFA generated in an anaerobic digester.

The higher H_2S production in the biogas in the initial phase of the incubation period may indicate a higher SRB activity during the first 20 days, which subsequently decreased, probably because high sulfide concentrations can also be toxic for SRB (O'Flaherty et al., 1998). As stated by O'Flaherty et al. (1998), the sulfide inhibition thresholds for SRB are not clear, because they depend on several factors such as pH, temperature and substrate.

4.3.3 Slurry chemical composition

The minimum concentration of TIDSS during the experimental period was 26 mg S^{2-} L^{-1}, measured in bottles containing pig slurry with 200 mg SO_4^{2-} L^{-1} on day 8. The maximum concentration was 92.5 mg S^{2-} L^{-1} for pig slurry on day 62 at 4000 mg SO_4^{2-} L^{-1} (data not shown). Mizuno et al. (1994) obtained similar values for TIDSS (9 to 68 mg S^{-2-} L) in an effluent with a sulfate concentration of 1600 mg SO_4^{2-} L^{-1} in which methanogenic archaea population was inhibited. Damianovic and Foresti (2009) also obtained similar TIDSS concentration (40-70 mg L^{-1}) in a horizontal-flow, anaerobic, immobilized biomass digester treating a substrate composed exclusively of acetate, in which SRB bacteria were not well established. The low TIDSS concentration in bottles can be explained by the sulfur originating from SO_4^{2-} reduction escaping with the biogas; in fact, there were more evident differences between treatments in the H_2S content in the biogas than in the TIDSS concentrations in slurries.

Table 4.4 shows the average pH and the standard error of the average pH measured in bottles. The pH per treatment was obtained by averaging 13 measurements obtained from the same bottle during 114 days. The pH throughout the experiment was very stable, as shown by the low standard error for each treatment. For the first ten days, lower pH values were observed in bottles with high sulfate concentrations compared to bottles with low sulfate concentrations, but the differences

were less pronounced over time. During the incubation period, the pH from pig slurry was slightly lower than the pH from cattle slurry.

In the sulfate methanogenic inhibition process, pH is critical in the liquid phase, where the TIDSS are present in the non-ionized form (H_2S) and in the unionized form ($HS^- + S^{2-}$). The acid dissociation constant (pKa) is around 7 in aqueous solutions, meaning that at low pH a large percentage of the TIDSS is present as H_2S (Blunden and Aneja, 2008). The slightly lower pH observed in pig slurry compared with cattle slurry could therefore explain the higher H_2S concentration in the biogas from this slurry. Small variations in the pH range actually affect H_2S concentration which, as suggested by several authors, is the toxic form for the methanogenic archaea (Hulshoff Pol et al., 1998; Cirne at al., 2008).

Table 4.4- Average pH and standard error from pig and cattle slurry with increasing sulfate concentrations. The pH per treatment was obtained by averaging ten measurements obtained from the same bottle during 114 days.

Sulfate concentration added ($mgSO_4^{2-}$ L^{-1})	Pig slurry		Cattle slurry	
	Average pH	Standard error	Average pH	Standard error
0	8.02	0.06	8.07	0.07
200	7.93	0.05	8.08	0.07
500	7.98	0.07	8.06	0.07
1000	7.97	0.07	7.96	0.06
1500	7.83	0.05	8.02	0.06
2000	7.90	0.06	7.97	0.07
2500	7.88	0.07	7.97	0.07
3000	7.68	0.12	7.94	0.07
4000	7.53	0.17	7.78	0.13
5000	7.59	0.15	7.72	0.13

Koster et al. (1986) and O'Flaherty et al. (1998) found that at a pH between 6.8 and 7.2, the concentration of H_2S required to cause 50% inhibition was constant. Above a pH of 7.2, however, the H_2S concentration necessary to cause inhibition decreased. There are two possible explanations: firstly, at pH levels above 7.2, the inhibition could be caused by the two forms, HS^- and H_2S, rather than only by H_2S form. In this case, there would be two inhibition pathways, one for H_2S and another for HS^-. Secondly, at high pH levels, H_2S might become more

toxic for the methanogenic archaea, and thus the methanogenic archaea could have different inhibition thresholds for H_2S depending on the pH. Animal slurry has a higher pH than other sulfate-rich wastewaters that have been used in the literature to determine sulfate inhibition thresholds in anaerobic digestion (Bhattacharya et al., 1996; O' Flaherty et al., 1997). The pH value in this study was, in fact, higher than 7.5 in all bottles from day 17 of the incubation period, and thus specific inhibition thresholds in animal wastes might be expected.

Figure 4.3 shows the evolution of VFA produced from pig slurry and cattle slurry with increasing sulfate concentrations. As shown in this figure, VFA concentrations in pig slurry were higher than in cattle slurry, because of the higher initial VFA content in pig slurry (Table 4.1). Slurries with low VFA concentrations are correlated with lower storage age. Møller et al. (2004a) stated that the total content of VFA in stored cattle and pig manures increased within the first few weeks and decreased thereafter due to bacterial activity. Cattle slurry used in this work had a lower storage age than pig slurry (Table 4.1), due to the lower periodicity of the pit discharges in cattle buildings. from day 18 of the incubation period. In cattle slurry, the increase in VFA was also observed in bottles with additions $\geq$2000 mg SO_4^{2-} L^{-1} from day 18 of the incubation period. However, at the end of the incubation period, a drop in VFA concentration in cattle slurry was observed for the 2000, 2500 and 3000 mg SO_4^{2-} L^{-1} additions.

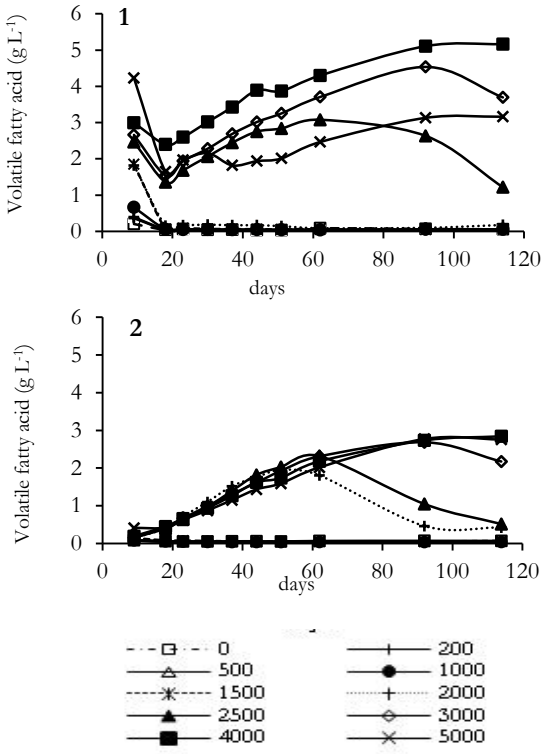

Figure 4.3- Total Volatile fatty acids (VFA) produced from pig slurry (1) and cattle slurry (2) with increasing sulfate concentrations (mg SO_4^{2-} L^{-1}) in thermophilic conditions. Replications per treatment: n =1.

The concentration of VFA has been widely used in the literature as an indicator of anaerobic process imbalance (Marchaim and Krause, 1993; Pind et al., 2002; Ferrer et al., 2010). This is related to the inhibition of the methanogenic bacteria, which are unable to assimilate the VFA at the rate they are produced by the acidogenic bacteria. In addition, VFA accumulation, especially acetic, is also related to incomplete oxidation by SRB (Damianovic and Foresti, 2009). In this study, the accumulation in VFA could probably be explained by the fact that concentrations of SO_4^{2-} did not cause inhibition of the fermentative bacteria, or at least to a lesser extent, as it did inhibit methanogens, syntrophic propionate-degrading bacteria and SRB.

This VFA accumulation due to methanogenic inhibition in bottles with high sulfate concentrations was also found by Ottonsen et al. (2009). Ottonsen et al. (2009) explained that high concentrations of protonized short-chain volatile fatty acids in acidified slurry can act as an uncoupling agent of the cell membrane potential and in this way arrest anaerobic microbial metabolism during slurry storage. Therefore, the lowest CH_4 emission could be expected during storage and the highest CH_4 potential could be expected with acidified slurries rather than normal slurries.

Figure 4.4 shows the distribution of VFA content in pig and cattle slurry at 9, 42 and 114 days of incubation. Differences between treatments were observed from the beginning of the incubation period, mainly in pig slurry.

Bottles with sulfate additions higher than 1500 mg SO_4^{2-} L^{-1} showed a high variability in VFA and this remained constant until the end of the experiment. An accumulation of propionic acid over time was also observed in both slurries in bottles with 2000 and 2500 mg SO_4^{2-} L^{-1}, the percentage of propionic acid of the total VFA was 71% in pig slurry receiving the 2500 mg SO_4^{2-} L^{-1} and 78% and 48% in cattle slurry receiving 2000 and 2500 mg SO_4^{2-} L^{-1}, respectively, at the end of the incubation period. The individual VFA accumulations meant that not only acetate-utilizing methanogenic archaea activity was inhibited in slurries with a high SO_4^{2-} concentration, but also the activities of SRB and hydrogen-producing bacteria were inhibited as stated by O'Reilly and Colleran (2006).

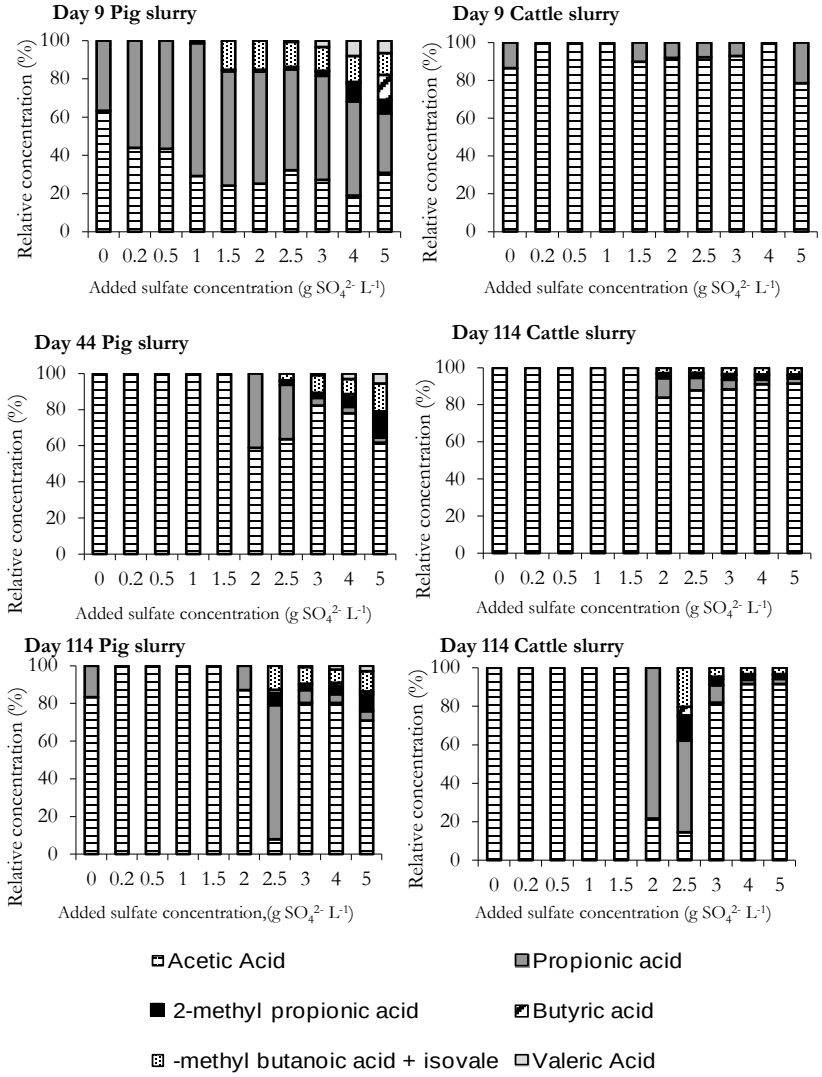

Figure 4.4- Distribution of the volatile fatty acids contents in pig and cattle slurry at different storage times with increasing additions of sulfate concentrations in thermophilic conditions. Replications per treatment: n = 1.

4.4. Conclusion

From our results with a batch assay conducted over 114 days using two types of slurry (pig and cattle) and ten different sulfate concentrations (0-5000 mg SO_4^{2-} L^{-1}) per type of slurry, we can conclude that:

- Added sulfate concentrations higher than 2500 mg SO_4^{2-} L^{-1} in pig slurry and higher than 2000 mg SO_4^{2-} L^{-1} in cattle slurry caused reductions of more than 40% in CH_4 production and an accumulation of VFA throughout the 114-day incubation period. For higher sulfate slurries, co-digestion with non-acidified slurry or other substrates would be needed to maintain the sulfate concentration in reactors below the inhibitory sulfate thresholds.
- An added sulfate concentration of 500 mg SO_4^{2-} L^{-1} in pig slurry resulted in a higher CH_4 production compared with slurry without additional sulfate.
- Biogas produced with sulfate additions > 2000 mg SO_4^{2-} L^{-1} contained >3 % H_2S in pig slurry and > 2% H_2S in cattle slurry during the first 20 days of incubation, decreasing thereafter.
- Our results provide specific sulfate inhibition thresholds for pig and cattle slurries, which differ from other sulfate-rich wastewaters. These differences are attributable to the physicochemical composition and pH of the substrates. Further research on the substrate-specific effect on sulfate inhibition thresholds is still necessary.

4.5. Acknowledgements

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Chapter 5

Process performance of anaerobic co-digestion of raw and acidified pig slurry

Process performance of anaerobic co-digestion of raw and acidified pig slurry

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Abstract: The effect of incorporating different ratios of acidified pig slurry on methane yield was evaluated in two scales of anaerobic digesters: Thermophilic (50°C) pilot scale digester (120 L), operating with an average hydraulic retention time of 20 days and thermophilic (52°C) full-scale digesters (10 and 30 m^3), operating with an average hydraulic retention time of 30 days. In the lab-scale digester, different inclusion levels of acidified slurry (0-60%) were tested each 15 days, to determine the maximum ratio of acidified to non-acidified slurry causing inhibition and to find process state indicators helping to prevent process failure. In the full-scale digesters, the level of inclusion of the acidified slurry was chosen from the ratio causing methane inhibition in the pilot scale experiment and was carried on in a long-term process of 100 days. The optimal inclusion level of acidified pig slurry in anaerobic co-digestion with conventional slurry was 10%, which promoted anaerobic methane yield by nearly 20%. Higher inclusion levels caused methane inhibition and volatile fatty acids accumulations in both experiments. In order to prevent process failure, the most important traits to monitor in the anaerobic digestion of acidified pig slurry were found to be: sulfate content of the slurry, alkalinity parameters (especially partial alkalinity and the ratio of alkalinity) and total volatile fatty acids (especially acetic and butyric acids).

Keywords: acidification, sulfur, methane yield, pig slurry.

5.1 Introduction

Slurry acidification by manipulating the balance between ammonia (NH_3) and ammonium is an effective measure to reduce NH_3 emissions from animal slurry (Berg et al., 2006; Kai et al., 2008). Different strategies to acidify slurry have been used in the last decades such as: the incorporation of additives in the slurry (McCrory and Hobbs, 2001), modifications in the formulation of feeds for animals (Canh et al., 1998) and changes in manure management and animal housing conditions at the farm level (Sommer and Hutchings, 1995). Nevertheless, most of these techniques have not a commercial application. Sulfuric acid (H_2SO_4) is currently used in Denmark to acidify slurry with success. Kai et al. (2008) reported reductions in NH_3 emission from pig houses by 70% when adding H_2SO_4 to the slurry, compared to untreated pig slurries.

Nevertheless, the full implementation of this technique is limited due to the increased activity and growth of sulfate reducing bacteria (SRB) and its negative effects on anaerobic digestion (O'Reilly and Colleran, 2006). Hydrogen sulfide (H_2S) produced by SRB during the degradation of organic matter (OM) can result in an inhibition of the anaerobic digestion process or even its total failure (Hulshoff Pol et al., 1998). In addition, SRB themselves can compete with methanogenic archaea for electron sources such as acetate or hydrogen, and with the obligate hydrogen producing bacteria for propionate (O'Reilly and Colleran 2006).

However, some research on bioreactors has shown that methanogenic archaea can co-exist with SRB (Isa et al., 1986; Yoda et al., 1987; McCartney and Oleszkiewicz, 1993) under some specific conditions that depend on digester operational parameters like temperature, digester characteristics, the tolerance of anaerobic bacteria to sulfide, sulfate concentration and substrate composition (Visser et al., 1996; O'Flaherty et al., 1998; O'Reilly and Colleran, 2006).

Substrate composition is an important factor to take into account in the relationship between these two groups of microorganisms and thus, in methane (CH_4) production. The decrease of CH_4 production during anaerobic digestion of sulfate rich wastewaters using pure anaerobic cultures and synthetic appropriate substrates as volatile acids and ethanol has been widely studied in the literature (Isa et al., 1986; Yoda et al., 1987; McCartney and Oleszkiewicz, 1993; O'Flaherty et al., 1998). Concerning acidified animal slurry, solids from solid-liquid separation of acidified dairy cow manure has been tested in anaerobic co-digestion with non-acidified cow slurry with success (Sutaryo et al., 2012). However, to our knowledge, there are no studies concerning the effects of using the entire fraction of acidified animal slurries in anaerobic digestion. Eriksen et al. (2008) and Ottonsen et al. (2009) reported that the activity of SRB in acidified pig slurries during storage is low, despite the presence of high amounts of acetate and sulfate, which are essential components for SRB development. Thus, the effects of high sulfate concentrations on the anaerobic digestion of slurry might differ from results achieved when digesting sulfate rich wastewaters.

The aim of this study was to investigate the effects of including acidified pig slurry in an anaerobic co-digestion process with conventional slurry used as the basic substrate, and to find the optimum ratio not having detrimental influence on the process performance. For this purpose two experiments were designed: a pilot scale experiment in which different sulfate concentration levels were added in short periods to find the range of sulfate concentration causing inhibition and the chemical process state indicators that could potentially be used for predicting process failure; and a full scale experiment in which we tested the results obtained in the pilot scale in a long term study to achieve the steady state conditions.

5.2 Materials and methods

5.2.1. Substrates

Conventional (raw) and acidified pig slurry were obtained from commercial pig farms in Denmark. In the farm where the acidified slurry was collected, acidification was performed by using the technology provided by Infarm® Company. Acidification was carried out in a process tank outside the fattening buildings in which concentrated H_2SO_4 was added at a ratio of approximately 7-8 Kg per ton of slurry. The amount of H_2SO_4 added was controlled by a pH-sensor with the aim to reach a pH level of 5.5. Simultaneously to the acidification process, slurry was aerated by injecting compressed air to prevent the conversion of sulfate ion into hydrogen sulfide. The acidified pig slurry was used to flush the waste pits to ensure that pH under the slatted floor was always kept low.
Inoculum for starting up the digesters in this study was obtained from the thermophilic anaerobic digesters of the Research center in Foulum, Denmark. The digesters were completely filled with this inoculum before starting up the experiments.

5.2.2. Pilot-scale digesters experiment

This experiment was conducted using a continuously stirred tank with a working volume of 120 L. The digester was operated at 50°C and with an average hydraulic retention time of 20 days. Mixing was performed by a central shaft with a propeller at the bottom, rotating at 60 rpm and programmed to work for 10 min every half hour. Therefore the sludge retention time can be considered equal to the hydraulic retention time under the steady-state conditions. The tank temperature was controlled by means of heating the tubes placed in the bottom of the digester and the body of the digester was provided with an insulating jacket for ensuring stable operational temperature.

In order to measure the volume of the biogas produced, the digester was continuously connected to a gas collection cylinder placed in an oil solution. Gas production was measured by the volume displacement

method. Feeding and unloading the digester was performed automatically using electric pumps.

During one month the digester operated with normal pig slurry with the purpose of reaching steady state conditions. After the digester was stabilized, it was operated with 10% acidified pig slurry and 90% normal pig slurry. Every two weeks, the percentage of acidified pig slurry was increased. The experiment had a total duration of 90 days and the acidified slurry percentages tested were: 10%, 20%, 40% and 60%.

5.2.3. Full-scale digesters experiment

This experiment was conducted using two continuously stirred tanks with a working volume of 10 and 30 m^3. These two digesters were very similar in design, they were constructed in stainless steel and heated by an external water jacket; the two digesters were operated in thermophilic (52°C) conditions with an average hydraulic retention time of 30 days. Continuous mixing in both digesters was performed by a central shaft with a propeller at the bottom, rotating at 60 rpm. Gas production was measured with a differential pressure transmitter device (EJX110A Yokogawa, Japan). Feeding and unloading the digesters was performed automatically using electric pumps and the exact amount of slurry feed and unload was measured with weighing cells.

The 30 m^3 digester was filled with inoculum and fed with a mixture of 30% acidified slurry + 70% raw slurry from the beginning of the experiment. The level of inclusion of the acidified slurry in this case was decided from the results obtained in the pilot scale experiment. In this experiment it was observed that the production of CH_4 was inhibited when acidified pig slurry was included in a ratio between 20 and 40%, thus, a 30% of acidified slurry inclusion level was tested in this experiment. The 10 m^3 digester was fed with raw pig slurry as control.

5.2.4. Chemical analyses

Samples for chemical analyses were taken from all digesters once a week in both experiments. In the pilot scale experiment, where the percentage of acidified pig slurry was increased each two weeks, two sludge samples were collected in each period: three and ten days after the increase of acidified slurry.

After sample collection, pH, total solids (TS) and volatile solids (VS) were measured as described in APHA (2005) (methodology number: 2450-B and 2450-E). Alkalinity (ALK) was determined by titration method according with APHA (2005) (methodology number: 2320-B) with the titration equipment Mettler DL21 (Mettler Toledo). The method consists of a two steps titration, the first one at pH of 5.75 (Partial alkalinity, PA) and the second at 4.3 (total alkalinity, TA). The intermediate alkalinity (IA) was then estimated as the difference between TA and PA.

Total ammonia (TAN), sulfate (SO_4^{2-}) and total inorganic dissolved sulfide species (TIDSS: dissolved $H_2S + HS^- + S^{2-}$) were determined with photometric kits (Spectroquant® kit, Merk, USA). Volatile fatty acids (VFA) were analyzed using a gas chromatograph (5560-D of APHA, 2005) equipped with a flame ionization detector (HP 68050 series Hewelt Packard).

Biogas samples were taken twice a week in both experiments by flushing a 22 mL sample bottle with 300 mL of biogas. In the pilot scale experiment, where the percentage of acidified pig slurry was increased each two weeks, four biogas samples were collected in each period: at 0, 3, 7 and 10 days after the increase of acidified slurry. Methane and carbon dioxide (CO_2) concentration in the biogas were analyzed on a Perkin Elmer Clarus 500 gas chromatograph equipped with a thermal conductivity detector according with Sutaryo et al. (2012). The temperatures of injection port, oven, filament and detector were 110ºC, 40ºC, and 150 ºC, respectively. The carrier gas was Helium with a flow rate of 30 mL min^{-1}.

Additionally, H_2S concentration in the biogas samples was determined colorimetrically using precision gas detector tubes (Kogyo K.K., Kitagawa, Japan). Hydrogen sulfide in the biogas was analyzed in the full-scale experiment from day 70 and onwards.

5.2.5. Calculations and statistical analyses

In anaerobic digestion, sulfate is reduced by SRB bacteria to sulfide which is distributed between H_2S in the gas phase and H_2S, HS^- and S^{2-} in the liquid phase (Isa et al., 1986). In this work the percentage of SO_4^{2-} reduced was calculated following Equation 5.1:

$$SO_4\ reduced\ (\%) = \frac{S\_SO_4\ fed\ slurry - S\_SO_4 sludge}{S\_SO_4\ fed\ slurry} x\ 100 \quad (5.1)$$

The percentage of VS degraded from the slurry during the anaerobic digestion process (removal efficiencies) was also calculated. During the drying process for VS determination, important losses of VFA can be expected depending of the sample's pH (Derikx et al., 1994; Rico et al., 2007). Derikx et al. (1994) estimated that more than 80% of the VFA present in the slurry were lost during the drying process in slurries with a pH around 7.5 and that the percentage of VFA lost during drying process increased as the pH decreased. Thus, in this study VFA content in the slurry were added to VS fraction when calculating the removal efficiency in the following manner: 100% of VFA was added to VS in slurries with a pH below 7, in slurries with pH between 7 and 8, 80% of VFA were added and in slurries with a pH higher than 8, 10% of VFA were added to VS.

Methane yield was expressed as the CH_4 production (L) per Kg of VS introduced in the digesters. The inhibition of CH_4 yield in the pilot scale experiment was calculated following Equation 5.2:

$$Inhibition\ (\%) = 100 - \left(\frac{Average\ CH_4 produced\ using\ the\ mix\ with\ acidified\ slurry}{Average\ CH_4 produced\ using\ raw\ slurry} x100\right) \quad (5.2)$$

The correlation between all the compositional variables and gas production determined in the pilot-scale experiment was assessed using SAS System® Software (Version 9.0, SAS Inst. Inc., Cary, NC) (2001)

using PROC CORR procedure. In all cases, statistical significance level was set at 5% (P-value ≤ 0.05).

5.3 Results

5.3.1 Chemical composition of acidified and raw slurries

Table 5.1 shows the average chemical composition of acidified and raw slurries used in the two experiments. Sulfate concentration was nearly twenty times higher in the acidified slurries compared to raw slurries in both experiments. However SO_4^{2-} content was slightly higher in the acidified slurry used in the pilot-scale experiment compared to that used in the full-scale experiment.

Sulfide and VFA concentrations were lower in the acidified slurry than in the raw slurry in both experiments, especially in the full-scale experiment. As expected, pH was lower in the acidified slurry than in the raw slurry and TAN concentration was higher in the acidified slurry than in raw slurry. As shown in Table 5.1, ALK parameters were lower in the acidified slurry than in the raw slurry in both experiments.

Table 5.1- Initial chemical composition of slurries used to feed the pilot-scale and the full-scale digesters.

Parameter	Units	Pilot-scale experiment		Full-scale experiment	
		Acidified Slurry	Raw slurry	Acidified Slurry	Raw slurry
Total Solids	%	5.70	7.10	3.76	5.41
Volatile Solids	%	4.12	5.29	2.59	4.29
Sulfate	g SO_4^{2-} L^{-1}	11,83	0.63	10.74	0.32
Sulfide	mg S^{2-} L^{-1}	32.85	64.22	37.71	56.33
Total volatile fatty acids	g L^{-1}	10.90	13.43	3.20	12.47
Acetic	g L^{-1}	3.94	6.69	2.00	6.15
Propionic	g L^{-1}	1.79	2.42	0.23	2.44
Butyric	g L^{-1}	3.02	2.42	0.56	1.99
pH		5.77	6.85	5.94	6.48
Total ammonia	g NH_4 L^{-1}	2.97	2.16	2.54	2.41
Total alkalinity	g $CaCO_3$ L^{-1}	11.92	19.19	5.11	13.58
Partial alkalinity	g $CaCO_3$ L^{-1}	5.74	9.33	2.42	5.99
Intermediate alkalinity	g $CaCO_3$ L^{-1}	6.17	9.86	2.69	7.59

5.3.2 Pilot-scale digester experiment

5.3.2.1. Sludge and biogas composition

Table 5.2 shows the chemical composition of the sludge used during anaerobic digestion with increased concentrations of acidified slurry. The pH remained almost constant until the period in which 60% of acidified slurry was added; the average pH value until then was 8.15. However, when 60% of acidified slurry was added (7.50 gSO_4^{2-} L^{-1} of fed slurry) the pH value dropped until 7.53. Volatile fatty acids concentration in the sludge increased in the last two periods, when 40% and 60% of acidified slurry were added (Table 5.2). When adding 60% acidified slurry, acetic, butyric and total VFA were respectively 3.53, 21.6 and 3.15 times higher than at the beginning of the experimental period using no acidified slurry.

Partial alkalinity decreased and SO_4^{2-} increased proportionally with increases in the acidified slurry in the digester. The calculated percentage of SO_4^{2-} reduced was almost negligible at the beginning when no acidified slurry was added, and increased with the addition of acidified slurry to the digester.

Removal efficiency calculated as the degradation of VS is shown in Table 5.2, at the beginning of the experiment with up to 10% of acidified slurry in the mixed substrate, the removal efficiency was stable at around 52%. However the removal efficiency was reduced significantly when the digester was fed with a proportion of 20 and 60% of acidified slurry.

Table 5.2- Average chemical and biogas composition during anaerobic digestion of increasing concentrations of acidified pig slurry in the pilot-scale experiment. For each period of 15 days (each percentage of acidified slurry), in chemical parameters n=2 (at 3 and 10 days) and in biogas parameters n=4 (at 0, 3, 7 and 10 days).

Parameter	Units	% Acidified slurry				
		0%, 0.4 gSO_4^{2-} l fed^{-1}	10% 1.84 gSO_4^{2-} l fed^{-1}	20% 2.82 gSO_4^{2-} l fed^{-1}	40% 4.70 gSO_4^{2-} l fed^{-1}	60% 7.50 gSO_4^{2-} l fed^{-1}
Total Solids	%	5.31	5.69	4.14	3.22	4.09
Volatile Solids	%	3.74	3.93	2.79	2.04	2.71
pH		8.16	8.18	8.17	8.14	7.53
Ammonia	g NH_4 L^{-1}	2.00	2.77	2.55	2.16	3.23
Acetic	g L^{-1}	1.23	1.06	0.73	2.19	4.34
Propionic	g L^{-1}	1.46	2.95	3.12	2.83	2.47
Butyric	g L^{-1}	0.06	0.02	0.04	0.38	1.30
Total volatile fatty acids	g L^{-1}	3.19	4.60	4.20	6.60	10.06
Total alkalinity	g $CaCO_3$ L^{-1}	19.77	20.75	18.84	19.51	18.56
Partial alkalinity	g $CaCO_3$ L^{-1}	16.24	16.30	15.25	13.96	11.15
Intermediate alkalinity	g $CaCO_3$ L^{-1}	3.53	4.45	3.59	5.54	7.41
Sulfate	g SO_4^{2-} L^{-1}	0.39	0.68	0.73	1.74	2.51
Total dissolved sulfide	mg S^{2-} L^{-1}	45.92	44.62	46.13	32.59	34.95
Calculated Sulfate S-SO_4^{2-} reduced	%	1.61	64.42	74.26	63.47	62.33
H_2S biogas	%	0.27	0.29	1.01	0.91	< 1.2
Calculated removal efficiency	%	51.99	52.28	25.87	64.98	39.06
Methane yield	L CH_4 Kg VS^{-1}	220.24	262.87	179.92	62.91	6.96
Calculated methane inhibition	%		-19.36	18.91	71.43	96.84
Methane in the biogas	%	54.20	63.61	63.35	51.83	29.51

The CH_4 yield increased by nearly 20% when the inclusion of acidified pig slurry increased from 0 to 10% of the total slurry, however, when the proportion of the acidified slurry was increased by 20%, 40% and 60%, CH_4 yield was reduced by 18%, 70% and 96%, respectively. Methane concentration in the biogas was maintained constant (around 50-60%) until the addition of 40% of acidified slurry and decreased in the last period with addition of 60% of acidified slurry (7.50 gSO_4^{2-} L fed^{-1}); in this period CH_4 concentration reached levels below 30%.

Figure 5.1 shows the evolution of daily biogas production (L day^{-1}) measured in the pilot scale experiment with increasing concentrations of acidified slurry. Biogas production was linearly reduced with increasing sulfate concentrations in the slurry. As in CH_4 yield, Figure 5.1 shows that the inhibition of biogas production started when the proportion of acidified slurry in the mixture was 20%. The drop in biogas production was almost linear between 20% and 40% periods showing values close to zero at the end of the period in which the percentage of acidified slurry reached 40% of the mixture.

In the full-scale experiment we decided to test the effects of including a 30% of acidified slurry in co-digestion with raw slurry on CH_4 production for a long period of time. Both, the degree of CH_4 inhibition and to evaluate a likely adaptation of methanogenic bacteria to these conditions with the time were evaluated.

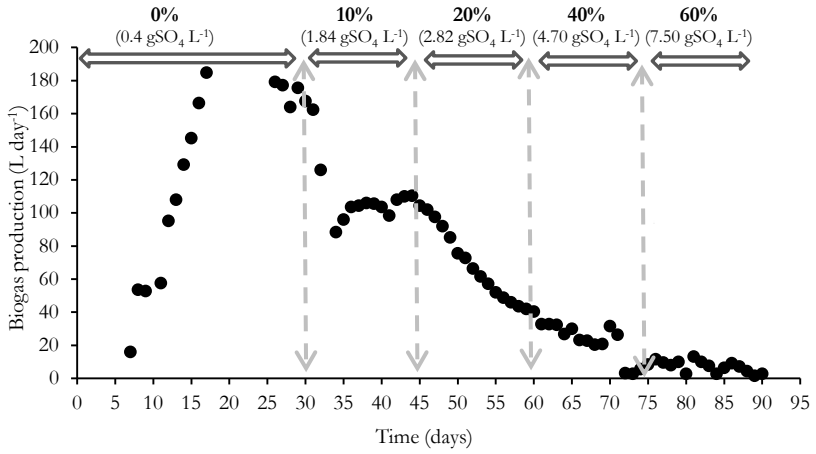

Figure 5.1- Daily biogas production (liters per day) from the digester of the pilot-scale experiment.

5.3.2.2. Relationship between slurry composition and methane yield

The correlation among process state indicators and CH_4 yield was studied in order to find the most important traits to be taken into account to detect a process failure in anaerobic digestion when acidified pig slurry is used as substrate. The most significant results of the correlation analysis performed are shown in Table 5.3. The trait most closely related to CH_4 yield was the SO_4^{2-} content in the slurry, explaining a 79% of the variation in CH_4 yield (R=-0.79, P-value<0.05). The relationship between these two variables was negative, indicating that SO_4^{2-} was the main source of CH_4 inhibition in this study.

Alkalinity parameters were also well correlated with CH_4 yield, mainly PA (R=0.77) and the ratio of alkalinity (RA=IA/TA) (R=-0.73). The relationship between CH_4 and PA in this study was positive indicating that CH_4 yield was reduced when PA decreased. Nevertheless RA was negatively correlated with CH_4 yield. Volatile fatty acids in the digester were negatively correlated with CH_4 yield in this study, especially total VFA, acetic and butyric acids.

Table 5.3 also shows the correlation among the compositional variables. The results concerning the correlation between VFA and IA indicated that total VFA (R= 0.98) and acetic acid (R= 0.98) were highly and positively correlated with IA. Figure 5.2 shows the positive and linear relationship between VFA and IA indicating that higher levels of VFA and acetic acid are correlated with higher IA values. Concerning RA (IA/TA) (Figure 5.2), this parameter correlated well with total VFA (R^2= 0.98) and butyric acid (R^2= 0.96), probably due to the relationship existing between IA and VFA. The IA/PA ratio showed a low correlation with VFA (data not shown) being this only significantly correlated with butyric acid (R^2= 0.55). The relationship between PA and VFA was also studied (Figure 5.2). Partial alkalinity was highly correlated with total VFA (R^2=0.86) and acetic acid (R^2=0.86) showing a linear and negative relationship.

Table 5.3- Sample coefficient of correlation (R) of the chemical parameters measured during anaerobic digestion of increasing concentrations of acidified pig slurry in a pilot-scale experiment (n=26).

	Methane yield	Acetic	Propionic	Butyric	Total volatile fatty acids	Total alkalinity	Partial alkalinity	Intermediate alkalinity	Ratio of Alkalinity
Acetic.	-0.50								
Propionic									
Butyric	-0.71	0.78							
Total volatile fatty acids	-0.46	0.86	0.52	0.88					
Total alkalinity									
Partial alkalinity	0.77	-0.93		-0.96	-0.93				
Intermediate alkalinity	-0.67	0.98		0.94	0.98		-0.93		
Ratio of Alkalinity	-0.73	0.99		0.98	0.99		-0.85	0.99	
Sulfate concentration	-0.79	0.98		0.86	0.98		-0.97	0.92	0.97

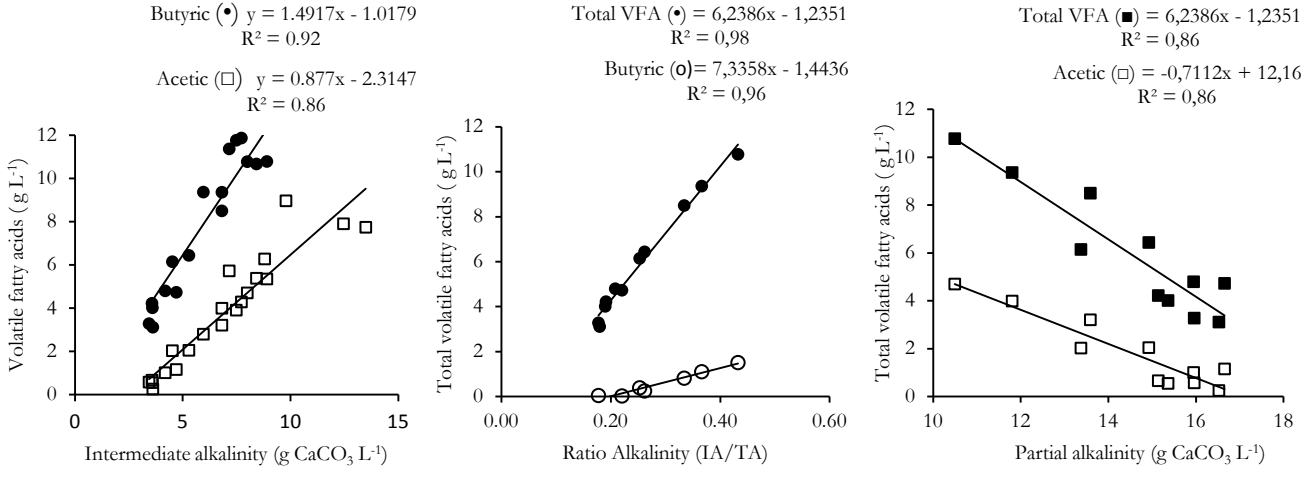

Figure 5.2- Correlation between the concentration of volatile fatty acids (acetic and butyric acids) with intermediate alkalinity (IA), the ratio of alkalinity (IA/total alkalinity (TA)) and partial alkalinity.

5.3.3 Full-scale digesters experiment

In the full-scale experiment, TS and VS remained stable over the 100 days with levels ranging between 4.0% and 2.5%. Ammonia was also stable and slightly higher in the acidified digester (1.89 g NH_4 L^{-1}) than in the non-acidified digester (1.57 g NH_4 L^{-1}). Table 5.4 shows the chemical composition (initial and final) in terms of pH, VFA, alkalinity traits and SO_4^{2-} and TIDSS content of the acidified and non-acidified digesters working in the full-scale experiment. The pH was stable in both digesters throughout the experiment. The concentration of VFA at the end of the study was higher than at the initial point in both digesters. This increment was more marked in the acidified digester compared to the non-acidified; in fact, total VFA in the non-acidified digester remained lower than 1.7 g L^{-1} during the experimental period. In the acidified digester the increase in total VFA started from day 40 of the experimental period (data not shown) and at the end of the study reached 5.48 g L^{-1}. This VFA concentration was almost four times higher than that observed in the non-acidified digester. Regarding individual VFA, as expected from the pilot-scale experiment, all of them were higher in the digester treating acidified slurry compared to the digester treating non-acidified slurry at the end of the experiment.

Alkalinity parameters in the non-acidified digester were stable over the experimental period. Total and partial ALK remained lower in the acidified digester than in the non-acidified digester until day 60 (data not shown). From day 60, TA and PA in the acidified digester started to increase until they almost reached the values obtained in non-acidified digester. At the end of the experimental period, IA was higher in the acidified digester than in the non-acidified digester.

Table 5.4- Initial and final composition of the digestate in the digesters used in the full-scale experiment after a 100-day working period.

Parameter	Units	Acidified digester		Non-Acidified digester	
		Initial	Final	Initial	Final
pH		8.06	7.89	7.91	8.02
Acetic	g L^{-1}	0.25	1.87	0.32	0.88
Propionic	g L^{-1}	0.02	1.93	0.07	1.00
Butyric	g L^{-1}	0.00	0.24	0.00	0.05
Total volatile fatty acids	g L^{-1}	0.28	5.48	0.39	2.16
Total alkalinity	g $CaCO_3$ L^{-1}	12.17	17.47	16.51	17.53
Partial alkalinity	g $CaCO_3$ L^{-1}	9.86	13.42	14.01	14.56
Intermediate alkalinity	g $CaCO_3$ L^{-1}	2.31	4.05	2.00	2.98
Sulfate	g SO_4^{2-} L^{-1}	0.58	0.41	0.39	0.45
Total dissolved sulfide	mg S^{2-} L^{-1}	44.02	45.28	41.42	43.32
H_2S in biogas	%		1.85		0.05

Regarding SO_4^{2-} and TIDSS content in the non-acidified digester, SO_4^{2-} concentration was stable and close to 0.40 gSO_4^{2-} L^{-1}. In the acidified digester, SO_4^{2-} concentration was stable and close to 0.55 gSO_4^{2-} L^{-1} until day 64; from this day, SO_4^{2-} content fell to reach non-acidified digester levels (0.40 gSO_4^{2-} L^{-1}) and remained at this concentration until the end of the experimental period. Total inorganic dissolved sulfide species in the non-acidified digester were stable and close to 43 mg S^{2-} L^{-1}. However, in the acidified digester, there was a slight increment in TIDSS concentration from day 40 until the end of the experimental period. The average H_2S concentration in the biogas of the acidified digester at the end of the experimental period was higher than that obtained in the non-acidified digester (1.85 *vs.* 0.05 %).

Over 100 days of full-scale experiment, the average CH_4 yield in the digester operating with raw slurry was 221.80±7.65 L CH_4 Kg^{-1} (data not shown). This production was similar to that obtained in the pilot-scale experiment when the digester was operated with non-acidified slurry (220.24±13.20 L CH_4 Kg^{-1}). The average CH_4 yield in the digester operating with 30% acidified slurry in this experiment was 154.65±6.23 L CH_4 Kg^{-1}.

Figure 5.3 shows the CH_4 yield in the digesters over the experimental period. A varying CH_4 yield over time was observed in both digesters. However, it was observed that CH_4 yield from the digester fed with 30%

of acidified slurry was lower at the beginning (days 30-50) of the experimental period, increasing thereafter to reach a similar CH_4 yield to that of the non-acidified digester. From day 75 of study, the acidified digester showed again a reduction in CH_4 yield. The CH_4 concentration in the biogas produced by the acidified digester was stable (60.52±0.91%) during the experimental period and close to that obtained in the non-acidified digester (60.80±01.14%) and in the pilot-scale digester.

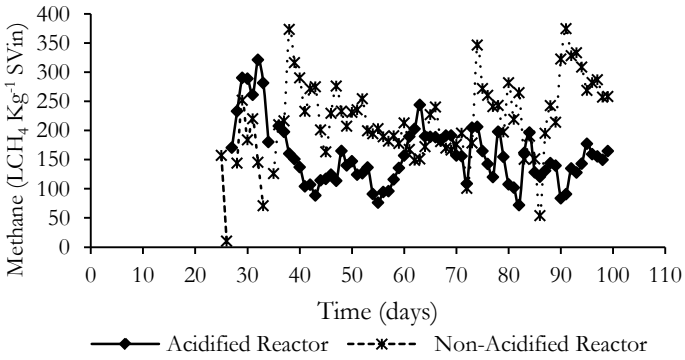

Figure 5.3- Methane yield per Kg of volatile solids fed in two digesters, one operating with 30% of acidified slurry (3.45 gSO_4^{2-} L^{-1} in feed slurry) (Acidified Digester) and other operating with non-acidified slurry (Non-Acidified Digester).

5.4 Discussion

Remarkable differences in acidified slurries between experiments were found in SO_4^{2-}, TS and VS content, probably due to variability among seasons and sources. Important differences between acidified and non-acidified slurries were also found in the case of VFA, TAN and ALK parameters. In addition to natural variability on composition in slurries coming from different farms, VFA differences between acidified and non-acidified slurries could also be caused by a reduced biological activity in the acidified slurry during storage. Ottonsen at al. (2009) observed a reduction greater than 98% in the biological activity of the acidified slurry compared to untreated slurries during storage. This

diminution of the biological activity could decelerate the OM degradation process and thus VFA production and sulfate reduction. Therefore, as Berg et al. (2006) suggested, acidification might increase slurry potential for biogas production since it slows down the process of OM degradation, maintaining its initial chemical composition. Total ammonia nitrogen was also different between acidified and non-acidified slurries in both experiments, probably due to lower NH_3 losses to the environment in acidified slurries because the lower pH compared to raw slurries. Similar differences between acidified slurries and conventional pig slurries concerning pH and NH_3 were also observed by Kai et al. (2008).

Concerning differences in ALK parameters between acidified and non-acidified slurries, slurry acidification could have decreased ALK because the acid added consumes buffer carbonate capacity resulting in a reduction of PA. In addition, the lower VFA concentration found in acidified slurries can be the cause of the lower IA in these slurries. In fact, TA of a sample is the sum of PA, which is correlated with HCO_3^- components, and IA which is correlated with VFA and TAN components (Ripley et al., 1986; Jantsch and Mattiasson, 2004). Concerning digesters, the addition of H_2SO_4 to the digester may have consumed buffer carbonate capacity decreasing PA in the pilot-scale experiment. However IA increased with SO_4^{2-} addition to the digester, probably because of increments in VFA and TAN concentration in the digester. In this study, results from the lab-scale experiment (Figure 5.2) indicated that PA was highly correlated with total VFA (R^2= 0.86) and acetic acid (R^2= 0.86) showing a linear and negative relationship and IA was linear and positively correlated with total VFA (R^2= 0.96) and acetic acid (R^2= 0.96). Similar correlations between VFA and ALK parameters were obtained by Ferrer et al. (2010). Total alkalinity in the pilot-scale experiment was stable probably because the reduction of PA might have compensated by increments in IA.

In addition to the good correlation between VFA and ALK, ALK parameters also resulted in high correlation with CH_4 yield in this study. The relationship between ALK and CH_4 yield has been widely reported in the literature (Ahring et al., 1995; Ferrer et al., 2010). A variation in

ALK traits might indicate a failure in the anaerobic digestion process due to an overloading since, as observed in this study, IA was positively correlated with total VFA and VFA were negatively correlated with CH_4 yield (Table 5.3), especially total VFA, acetic and butyric acids ($R > -0.4$, $P<0.05$).

Volatile fatty acids accumulations are associated with anaerobic process imbalances. Increments in VFA concentration are related with a decoupling between the bacterial groups involved in anaerobic digestion (Ahring et al., 1995), mainly due to the higher growth velocity of the fermentative and acidogenic bacteria compared with methanogenic bacteria. Volatile fatty acids are one of the most important precursors of CH_4 production in anaerobic digesters and the accumulation of VFA indicates that they are not being converted to CH_4, and thus some of the potential production is lost with the effluent from the digester. In the present study, VFA concentration in the sludge was increased when adding acidified slurry in both experiments. This accumulation could have been caused by a higher fermentative and acidogenic bacteria tolerance to high sulfate and sulfite concentration than the methanogenic bacteria. Within individual VFA, butyric acid was more strongly correlated with CH_4 yield than other VFA ($R=-0.71$) and thus, butyric acid is the best predictor of instability in the anaerobic digestion process. Ahring et al. (1995) found increments in butyric acid concentration up to 875% within 2 days after a perturbation in anaerobic thermophilic digesters. In the present study, increments of butyric acid above 2000% were observed in the digester fed with 60% acidified slurry compared with the digester fed with 100% raw slurry.

The sulfate added to the digester in both experiments was reduced and transformed into H_2S. Total dissolved sulfide concentration in the digesters remained low and stable in both experiments, which means that almost all sulfur originated by SBR activity from SO_4^{2-} reduction must have been emitted to the gas phase as H_2S. In fact, the H_2S concentration in the biogas was higher when digesters treated acidified slurries in both experiments. Thus, a likely increase in biological activity of the SRB compared to other anaerobic bacteria populations might have occurred when acidified slurry is used in anaerobic digestion. This

increase in the SRB activity could also explain the increase in ALK parameters found at the end of the full-scale experiment in the acidified digester. Sulfate reducing bacteria can use propionate and butyrate, among other organic compounds, as carbon source and electron donor producing CO_2 (Hirasawa et al., 2008; Sabumon, 2008). Carbon dioxide production and VFA consumption can promote a rise in PA. However the VFA accumulations at the end of the full-scale experimental period might indicate that the SRB were not well established. Accumulations of VFA, especially acetic (Damianovic and Foresti, 2009) and butyric (Briones et al., 2009) acids indicates an incomplete oxidation of OM by SRB that could probably be explained by the fact that H_2S is also toxic for SRB (O'Flaherty et al., 1998).

The increment in the SO_4^{2-} reduction in the digester fed with acidified slurry that took place from day 60 in the full-scale experiment, probably caused by higher SRB activity, could also explain the reduction in CH_4 yield that was observed from day 76 in this digester. McCartney and Oleszkiewicz (1993) also observed that SRB and methanogenic archaea can compete for the substrate. These authors suggested that SRB are more efficient than methanogenic archaea in utilizing OM. Additionally, in the present study CH_4 yield was not improved after 100 days of slurry anaerobic co-digestion with a 30% of acidified slurry, thus indicating that methanogenic bacteria could not be completely adapted to these conditions even after a long anaerobic digestion period. Probably, a new equilibrium between methanogenic archaea and SRB has been reached.

Regarding CH_4 production, the average CH_4 yield in the digester operating with 30% acidified slurry in the full-scale experiment was approximately 30% lower than CH_4 yield in the non-acidified slurry. This result is in accordance with that obtained in the pilot scale digester where CH_4 inhibition increased from 18.91% to 71.43% when the percentage of acidified slurry included increased from 20% to 40%. Thus, as stated for sulfate rich wastewaters (McCartney and Oleszkiewicz, 1993; O'Flaherty et al., 1998) the inclusion of acidified slurry in an anaerobic digestion process reduce CH_4 production. However, in the pilot-scale experiment, at low dose of acidified slurry CH_4 yield increased by nearly 20% when the inclusion of acidified pig slurry increased from 0 to 10%

of the total slurry, probably due to the conservative effect of acid in terms of chemical composition of the slurry as suggested by Ottonsen et al. (2009). In addition, it has been reported that sulfur is a nutrient for the anaerobic bacteria that can promote biological activity at low levels (O'Flaherty et al., 1998; Briones et al., 2009). However, this effect should be further tested in a long term anaerobic digestion.

5.5 Conclusions

From our two experiments concerning the inclusion of acidified pig slurry in an anaerobic co-digestion process with conventional slurry we can conclude that:

Acidified slurry inclusion at a level higher than 40% in a pilot-scale experiment and 30% in a full-scale experiment, caused an increased in acetic, butyric, and total VFA contents.

From our results, the most important traits to be taken into account to detect a process failure in the anaerobic digestion using acidified pig slurry were: SO_4^{2-} content of the slurry, alkalinity parameters (especially PA and the ratio of alkalinity), total VFA, acetic, and butyric acids.

Alkalinity parameters like intermediate alkalinity and the ratio of alkalinity are good predictors of total VFA and acetic acid. Variations in ALK traits indicated VFA accumulations and these parameters can predict anaerobic digestion failure.

The inclusion of a 30% of acidified slurry in co-digestion with raw pig slurry caused a reduction of around 30% in CH_4 yield. Nevertheless at a level lower than 10%, acidified slurry could promote CH_4 yield by nearly 20%.

5.6 Acknowledgements

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Chapter 6

Microbial examination of anaerobic sludge adaptation to pig slurry

Microbial examination of anaerobic sludge adaptation to pig slurry

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Abstract. The objective of this study was to evaluate changes in the microbial population of anaerobic sludge digesters during the addition of pig slurry using a combination of methods: quantitative real-time polymerase chain reaction (qPCR) (to monitor total bacteria, total archaea and total fungal populations) and qualitative scanning electron microscopy (SEM) (to evaluate microbial and organic matter morphological structure and diversity). Moreover, the relationship between microbial parameters and sludge physicochemical composition (volatile fatty acids, pH, total ammoniacal nitrogen and removal efficiency) and methane yield was examined. Three identical continuously stirred tanks with a working volume of 5.5 L were used, set up to work at thermophilic conditions (50ºC). All three digesters were initially fed with a commercial broiler feed during 20 weeks. After this period, two of the reactors were fed with pig slurry for 13 weeks and the third digester remained fed with broiler feed. Results showed that the addition of pig slurry to an unadapted thermophilic anaerobic digester caused an increase in volatile fatty acids concentration a decrease in removal efficiency and methane yield. Additionally, increases of total bacteria and total archaea were observed, however not differences in total fungal population were observed. Scanning electron micrographs provided a general overview of the sludge's cell morphology, morphological diversity and degree of organic matter degradation. A change in microbial morphotypes from homogeneous cell morphologies to a higher morphological diversity, similar to that observed in pig slurry, was observed with the addition of pig slurry by SEM. The combination of qPCR and SEM allows expanding the knowledge about the microbial adaptation to pig slurry in thermophilic anaerobic digesters.

Keywords: Anaerobic digestion, animal slurry, scanning electron microscopy, quantitative real-time polymerase chain reaction.

6.1 Introduction

The main microorganisms involved in the anaerobic digestion of organic matter (OM) have been traditionally described and classified into three groups: acidogens, acetogens and methanogens. The first two belong to the *Bacteria* domain, whereas the last group belongs to the *Archaea* domain (Angelidaki et al., 2011). Each group, however, involves several species. The role of the different groups of microorganisms responsible for biodegradation of OM to form methane (CH_4) and carbon dioxide (CO_2), and the microbial composition in terms of species present in anaerobic environments are not fully understood (Cirne et al., 1997; O'Flaherty et al., 2006). In fact, new microbial groups such as *Planctomyces*, *Synergistes*, *Crenarcheota* (belonging to *Bacteria* and *Archaea* domains) and several *Eukarya* organisms like *Hemiascomycetes* and *Phreatamoebis* have been identified in anaerobic environments (Godon et al., 1997; O'Flaherty et al., 2006). Their functions during the anaerobic digestion process, however, are still partially unknown.

Variations in substrate composition, concerning physicochemical parameters, can impact on the entire microbial community structure and the metabolic stability in anaerobic digesters (Keyser et al., 2006). In addition, the new substrate can have potentially toxic compounds for the original biomass. After a change in substrate composition, the organisms that will eventually predominate will be those that can survive and compete under the new conditions (Keyser et al., 2006).

On this regard, high concentrations of inhibitory ions present in animal slurry (mainly ammonia, NH_3 and volatile fatty acids, VFA) have shown to have severe negative effects on aceticlastic methanogens, especially *Methanosaetaceae* species (Angenent et al., 2002; Song et al., 2010; Zhu et al., 2011). The evaluation of changes in substrate composition in anaerobic digesters have been traditionally solely focused on substrate's physicochemical composition, without considering substrate's microbial composition. Pig slurry on its own contains a high bioburden (Snell-

Castro et al., 2005; Peu et al., 2006). This could affect digester's microbial community.

Nowadays, there is considerable interest in elucidating not only the microbial composition and metabolic diversity involved in anaerobic digesters, but also in setting up an applied and less costly method to trace microbial structure in anaerobic digesters (Liu et al., 2009). Nucleic acid-based molecular methods, like quantitative real-time polymerase chain reaction (qPCR) or fluorescence *in situ* hybridation (FISH) are being used to expand the current knowledge about microbial population dynamics in anaerobic digesters, especially methanogens (Hori et al., 2006; Song et al., 2010). However, due to recent application of these molecular techniques, the relationship between microbial composition and digester performance is still not well established (Sanz and Köchling, 2007). In addition, the lack of knowledge on the specific microbial species involved in the process prevents from quantifying a target group of microorganisms or quantifying at species-level. Consequently, molecular techniques are generally carried at a domain, order or family level.

Techniques which do not require identification of the key microbial groups *a priori* might be useful to gain knowledge on novel microbial groups involved in the anaerobic degradation of complex OM like animal slurry. This information can be obtained using qualitative scanning electron microscopic (SEM) techniques through visual observations of the anaerobic sludge. Observed microbial structures can be compared to known standards or qualitatively classified into distinctive groups by morphology (size and type). Additionally, information concerning digestate's structure (degree of OM degradation) might also be obtained using microscopic techniques. Combining quantitative and qualitative methodologies can add value to molecular-only PCR-based methodologies to elucidate microbial composition involved in the anaerobic digestion process.

The objective of this study was to evaluate changes in the microbial population of anaerobic sludge digesters during the addition of pig slurry

using a combination of methods: quantitative qPCR and qualitative SEM methods. The relationship among microbial parameters, physicochemical composition and CH_4 yield of anaerobic digesters during the adaptation to pig slurry was also examined.

6.2 Materials and Methods

6.2.1 Digester operational parameters

Three identical continuously stirred tanks with a working volume of 5.5 L were used. Digesters were started with a sludge coming from an anaerobic plant treating urban wastewaters in Valencia (Sagunto, Spain). During the entire experimental period, all three digesters were set up to work at thermophilic conditions (50ºC) with an organic loading rate of 1 g volatile solids (VS) L^{-1} d^{-1}. Temperature was controlled by a thermostatically regulated water bath and it was continuously registered using dataloggers (*HOBO®U12-013,* Onset Computer Corporation, MA, USA). The digesters mixing system consisted in an intermittent mechanical stirring (RZR1 77W Heidolph) working during 15 minutes every 60 minutes.

Before the addition of pig slurry, the three digesters were fed with a commercial broiler feed during 20 weeks. After this period, two of the digesters were fed with pig slurry (PS digesters) and the third digester remained fed with broiler feed (BF digester). After the addition of pig slurry, the three digesters were monitored during 13 weeks. The total duration of the experimental period was 33 weeks: 20 weeks before pig slurry addition and 13 weeks after.

The commercial broiler feed used was mixed with water to reach a 10% in total solids. Pig slurry was obtained from the slurry pit at the of a fattening period carried in the Animal and Technology Research Centre (CITA) in Segorbe (Castellón, Spain) at the end of a fattening period in summer conditions.

6.2.2 Sludge physicochemical composition and methane yield

At the beginning of the study, physicochemical composition of the inoculum, broiler feed and pig slurry was analyzed according to APHA (2005). The analyzed parameters were: total solids (TS), VS, pH, VFA, total ammoniacal nitrogen (TAN), fiber, fat and protein. The composition of the inoculum, broiler feed and pig slurry used in this study is shown in Table 6.1.

Table 6.1- Physicochemical composition of inoculum, broiler feed and pig slurry.

Parameter	Units	Inoculum	Broiler feed	Pig slurry
Total solids	g L^{-1}	2.22	10.11	3.93
Volatile solids	g L^{-1}	1.36	9.49	2.68
pH	-	7.48	n.a.	7.84
Total ammonia	mg $N\text{-}NH_3$ L^{-1}	990	n.a.	1870
Total volatile fatty acids	mg L^{-1}	1580	n.a.	1190
Acetic acid	mg L^{-1}	1360	n.a.	800
Propionic acid	mg L^{-1}	50	n.a.	330
Butyric acid	mg L^{-1}	90	n.a.	10
Neutral detergent fiber	g Kg DM^{-1}[2]	n.a.[1]	9.06	27.53
Acid detergent fiber	g Kg DM^{-1}	n.a.	2.47	15.80
Acid detergent lignin	g Kg DM^{-1}	n.a.	n.d.	5.58
Protein	g Kg DM^{-1}	n.a.	195.54	156.25
Fat	g Kg DM^{-1}	n.a.	65.74	62.49

[1]n.a.: not analyzed
[2]D.M.: dry matter

Additionally, during the 33 weeks of the experimental period, representative samples from each digester were taken weekly to determine: pH, TS, VS, TAN and VFA concentration according with the APHA (2005). Removal efficiency was calculated with the percentage of VS removed from the influent.

Biogas production from each digester was measured three times per week during the experimental period by determining total volume collected in aluminum bags and measuring it through a gas meter-Precision Wet Test Gas Flow Meters (RITTER ®, Germany). Biogas

samples were taken by flushing a 15 mL sample bottle with 60 mL of biogas.

Methane concentration in biogas samples collected from each digester was analyzed on a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The separation was performed in a GS-Q capillary column (J&W Scientific, USA) (30 m x 0.32 mm i.d.). The carrier gas was helium at a constant flow of 1 mL min^{-1}. The samples were injected with a split ratio of 1/100. The initial oven temperature was set at 50 ºC held for 1 min and increased to 150 at 50 ºC min^{-1} and finally maintained at that temperature for 2 min. Both detector and injector temperatures were set at 200 ºC. An external standard (41.21% CO_2 and 58.79 % CH_4) was employed for quantification of CH_4 content in samples. Methane yield was expressed as the CH_4 produced (L) per kilogram of VS introduced to the digesters.

6.2.3 Quantitative PCR assays

On week 20 (before the addition of pig slurry) and on weeks 30 and 33 (after the addition of pig slurry) of the experimental period, duplicate samples from each digester were taken during agitation and transferred into 20 mL sterile Falcon tubes. Samples were immediately frozen with liquid nitrogen and stored at -80ºC. Additionally, samples from the pig slurry used to feed PS digesters were taken on week 20 from the pit containing pig slurry.

Total DNA was extracted from 250 μL of each sample after thawing using QIAamp DNA Stool Mini kit (Qiagen, Spain). The DNA concentrations present within the different extracted DNA preparations were determined with NanoDrop 2000C Spectrophotometer (Fisher Scientific, Spain). The extracted DNA was diluted until reaching a standardized concentration of 0.5-1.0 ng DNA μL^{-1}.

6.2.3.1. Total bacteria and total archaea population

Copy numbers of 16SrRNA genes of the total bacteria (*Bacteria* domain) and total archaea (*Archaea* domain) were quantified with qPCR. Real-time qPCR assays were performed using a StepOne Plus (Applied Biosystems, Spain) thermocycler. Amplification was carried out in a final volume of 12 μL containing 2 μL of template, 6 μL of Master Mix solution (Applied Biosystems, Spain), 0.6 μL of each primer (final concentration 0.5 μM) and 0.18 μL of the TaqMan probe (final concentration 0.15 μM). Control reactions without DNA were used in all reaction series to ensure that contaminating DNA was not present. The specific primer sets used were those described by Yu et al. (2005). The qPCR conditions were modified from those proposed by Yu et al. (2005) in this work as follow:

- Bacteria-set: one step at 95 ºC for 10 min followed by 45 cycles of 95 ºC for 10 s, 60 ºC for 30 s and 73 ºC for 30 s .
- Archaea-set: one step at 95 ºC for 10 min followed by 45 cycles of 95 ºC for 10 s and 60 ºC for 60 s.

Standard curves were generated as described previously by Song et al. (2010). The PCR products were serially diluted in the range of 10^{-4}-10^{-11} copies μL^{-1} and directly used as a template for qPCR with the corresponding primer and probe sets in duplicate. Standard curves were amplified in each qPCR plaque. Slopes and intercepts used to determine 16S rRNA gene concentration of target microbial orders in the samples were:

- Bacteria, slope -3.405 and intercept -45.282 (R^2=0.995).
- Archaea, slope: -3.145 and intercept: -49.991 (R^2=0.992).

Data acquisition and analysis were performed using StepOne Plus 2.0 (Applied Biosystems, Spain).

6.2.3.2. Total Fungal population

Copy numbers of ITS1 rRNA genes of the total fungi population (*Fungi* kingdom) were also quantified with qPCR technique. The analysis was

carried out using Brilliant II SYBR® Green qPCR Master Mix (Stratagene, La Jolla, CA) in a Real Time PCR System MX3000P (Stratagene) following the protocol described by Prenafeta-Boldú et al. (2012).

The standard curve was carried by using a single DGGE band (Gen-Bank accession nº N982550) cloned onto the PGEM plasmid vector using PGEM-T Easy Vector System I (Promega, Madison, WI). This reference gene was quantified by Quant-iT™ PicoGreen ® dsDNA Reagent and then, serially diluted in the range of 10^1-10^8 copies/μL for developing the standard curve. Each sample was analyzed in duplicated with two independent DNA extracts. A negative control was added in each reaction to ensure any external DNA contamination.

The qPCR efficiency of the fungal ITS1 rRNA gene amplification were 101.5%; the Pearson Correlation Coefficient (R^2) of the standard curve was 0.998 and the slope was 3.286.

Results for total fungal community were processed by means of MxProTM QPCR Software.

6.2.4 Microbial structure and dynamics using scanning electron microscopic observations

Samples from the pig slurry used to feed PS digesters (on week 20) and from each digester (every four weeks from week 20 to week 33) were taken and placed into a sterile 50 mL Falcon tubes. Samples were taken during agitation from the pit containing pig slurry and from digesters, respectively. Immediately after collection, samples were prepared following the methodology indicated in Thiele et al. (1988) to separate the free flora and the flock fraction, by successively centrifuging and suspending samples in phosphate buffer.

After sample preparation, they were examined under the cryo-scanning electron microscopy (CT1500C by Oxford Instruments attached to a JEOL JSM 5410 SEM, Japan) for cell morphology (size and type),

morphological diversity and digestate's structure (degree of OM degradation).

One mL of each fraction (free flora and flock fraction) was pipetted on top of a clean polycarbonate filter (Whatman, 25 mm, 0.2 μm pore size). A 0.5 cm^2 piece of the polycarbonate filter was cut and mounted onto a sample holder and immediately introduced into slush nitrogen at -210 ºC. This process caused a fast conversion of the liquid samples into a glassy-like amorphous solid without any ice crystals forming (vitrification). The fast vitrification process avoids crystallization of water to ice and associated volume changes that can alter structures.

Each sample was then transferred under vacuum to the cryostage and from here to the microscope sample stage, where the condensed surface water was sublimed by controlled warming to -90 ºC for 20 min. Then, the sample was transferred again to the cryostage in order to gold coat it by sputtering. Finally, the sample was introduced again in the microscope sample stage to be viewed at an accelerating voltage of 15 kV.

Each polycarbonate filter sample was firstly examined to ensure homogeneous sample preparation. Then, following the diagonal along the filter sample, from 40 to 50 photomicrographs were obtained and analyzed. Photomicrographs were taken at 5000x and 1500x magnifications.

6.3 Results and Discussion

6.3.1. Physicochemical composition and methane yield

Digesters' temperature during the experimental period was constant in all digesters (49.7± 0.96 ºC), except in BF digester from weeks 23 to 26, where due to technical problems it worked at mesophilic conditions (36.8 ± 1.43 ºC).

Table 6.2 shows the evolution of digesters' performance during the 33-weeks experimental period in terms of physicochemical parameters. As shown in Table 6.2, pH was high (on average 8.1) and stable over time

during the experimental period in all digesters, slightly increasing towards the end of the experimental period.

From weeks 1 to 20 before pig slurry addition, total VFA in all digesters was similar and below 1000 mg L^{-1}. From this moment, VFA concentration increased with pig slurry addition in PS digesters reaching on average 2000 mg VFA L^{-1} on week 24 (data not shown). From this week, VFA decreased reaching on average 893 mg VFA L^{-1} between weeks 30 to 33. In BF digester, total VFA was constant until week 23, from this moment, total VFA concentration increased exceeding 5,000 mg VFA L^{-1} at the end of the experimental period, probably due to the decrease in temperature which occurred from weeks 23 to 26.

Concerning individual VFA, acetic acid was the most abundant individual VFA present in PS digesters. The percentage of propionic acid, however, increased over time being its concentration the highest between weeks 24 to 28, where more than 30% of the total VFA was due to propionic acid concentration in PS digesters. In BF digester, propionic was the most abundant individual VFA. More than 50% of the total VFA was due to propionic acid concentration from week 24 to 33 in BF digester. This could be explain because propionate oxidation is the individual VFA most sensitive to a sudden change in temperature (Palatsi et al., 2009) as that occurred in BF digester.

During the first 20 weeks of the experimental period, removal efficiency was similar in all digesters (on average 80%). In PS digesters, the removal efficiency decreased drastically with the addition of pig slurry from weeks 22 to 30 (on average equal to 25%). In BF digester, removal efficiency was stable over time (on average 82%) despite changes in temperature.

At the beginning of the experimental period (weeks 1-20), TAN content in BF digesters was around 1200 mg $N\text{-}NH_3$ L^{-1} and increased until 2000 mg $N\text{-}NH_3$ L^{-1} on week 28 (data not shown), four weeks after pig slurry addition. In BF digester, TAN was also higher from week 20 to 33 (on average 1700 mg $N\text{-}NH_3$ L^{-1}) than from week 1 to 20.

Concerning CH_4 yield, during the first 20 weeks, before the addition of pig slurry, CH_4 yield was similar in all digesters (average 472 L CH_4 Kg VS^{-1}). Pig slurry addition caused a decreased in CH_4 yield in PS digester reaching the minimal value on week 24 where CH_4 yield was below than 80 L CH_4 Kg VS^{-1} (data not shown). From this period, CH_4 yield in PS digesters increased slightly, reaching around 151 L CH_4 Kg VS^{-1} at the end of the experimental period (between weeks 30 to 33). This value however, did not reach initial CH_4 yield, probably because the biodegradability of the OM in aged pig slurry would be lower than broiler feed. In BF digester, CH_4 yield slightly decreased from week 24 and remained constant (on average 310 L CH_4 Kg VS^{-1}) from this moment until the end of the experimental period. The decrease in CH_4 yield observed in BF digester could be related with VFA accumulation in the sludge. In fact, VFA accumulations indicate the impossibility of methanogens to convert VFA to CH_4, and thus some of the CH_4 potential production is lost with the effluent from the digester (Moset et al., 2012). Methane concentration in the biogas was stable and around 60% in all digesters during the experimental period (data not shown).

Table 6.2- Evolution of digester performance during the experimental period.

		Period (Weeks)		
		1-20	20-30	30-33
pH	PS digester[1]	7.9 ±0.20	8.1 ±0.16	8.4 ±0.04
	BF digester[2]	7.8 ±0.32	8.0 ±0.20	8.2 ±0.13
Volatile fatty acids (mg L^{-1})	PS digester	718 ± 332.0	1825 ± 452.8	893 ± 271.5
	BF digester	961 ± 269.3	2856 ± 1457.8	5622± 2119.5
Removal efficiency (%)	PS digester	81 ± 0.1	27 ± 8.6	23 ± 6.67
	BF digester	80 ± 3.3	83± 2.3	82 ± 3.0
Total ammonia (mg N-NH_3 L^{-1})	PS digester	1203 ± 64	2,060 ± 182	2077 ± 61
	BF digester	1209 ± 83	1,702 ± 52	1674 ± 108
Methane yield (L CH_4 $KgVS^{-1}$)	PS digester	486 ± 67.8	149 ± 39.6	151 ± 8.37
	BF digester	458 ± 52.2	304 ± 53.5	315 ± 31.0

[1]PS digester: Digester fed with broiler feed from week 1 to 20 and with pig slurry from week 20 to 33.
[2]BF digester: Digester fed with broiler feed from week 1 to 33

6.3.2. Molecular quantification by qPCR of total microbial populations: archaea, bacteria and fungi

Table 6.3 shows the gene copy numbers of 16SrRNA of total bacteria and archaea from the samples of pig slurry used to feed PS digester, and from biomass samples of the digesters taken on the weeks 20, 30 and 33 of the experimental period. As shown in Table 6.3, before pig slurry addition (week 20), the target gene content of total bacteria was similar in BF and PS digesters and in pig slurry (on average 12.5 log copies mg DNA^{-1}). On week 30, there was an increase in gene concentration of total bacteria in BF and PS digesters. This could be related with the increment in VFA concentration in BF and PS digester at this time (Table 6.2). In fact, a close relationship between VFA and the quantity of total bacteria population has been previously reported during process imbalances like acidifications (Liu et al., 2002) and changes in temperature (Griffin et al., 1998).

Concerning the total archaeal population, on week 20, similar values were obtained in PS and BF digesters and also in pig slurry (on average 10.6 log copies mg DNA^{-1}). Total archaea gene content increased in PS digester on week 30 until the end of the experimental period, probably due to the decrease in VFA observed in the sludge from PS digester at this time. However, in BF digester, total archaea gene content was stable (on average 10.8 log copies mg DNA^{-1}) slightly decreasing towards the end of the experimental period.

Gene copy number of total fungi population was very low during the experimental period in BF and PS digesters (on average 6.3 log copies mg DNA^{-1}). This indicates that anaerobic fungi were not abundant in the digesters, even though they could play a relevant role in a variety of anaerobic environments like in animal's rumen (Griffith et al., 2009). The presence of fungi structures in anaerobic digesters represents quite a remarkable feature, even if they play a relatively minor role, since these organisms primarily display an aerobic metabolism. The fungi determined in this work might be related to fermentation of hydrolyzed

intermediates, or to the hydrolysis of cellulosic matter. However, molecular identification would be necessary to infer on the potential function and importance of fungi in anaerobic digesters.

Overall, the bacterial community dominated over total archaea and fungal population during the experimental period. These results are in accordance with the literature, where generally less than 10% of the total DNA determination in anaerobic sludge samples is due to methanogenic archaea, the rest mainly belonging to Bacteria domain, especially during stressful periods (Liu et al., 2002; Shin et al., 2010). The dominance of total bacteria over total archaea population in anaerobic reactors is due to the fact that methanogenic archaea occupy the last position in the methanogenic trophic chain. Despite the bacteria dominance during process imbalances, little works in the literature, consider the dynamics of adaptation of the bacteria involved in anaerobic digestion processes. Most studies have focused only on the evolution of methanogenic population by means of the detection and quantification of the four main methanogenic orders involved in CH_4 production (Liu et al., 2002; Calli et al., 2005; Karahashev et al., 2005).

Likewise, it has been reported that *Bacteria* domain presents the highest diversity in anaerobic digesters (Godon et al., 1997; Fernández et al., 1999), especially in anaerobic digesters treating complex OM substrates like animal slurry. Due to the simplification of organic compounds (both in number and complexity) through the trophic chain, *Archaea* domain presents lower diversity than *Bacteria* domain (Snell-Castro et al., 2005).

Table 6.3- Evolution of the total bacteria, total archaea 16S rRNA gene concentration and total fungi ITS1 gene concentration (log copies mg DNA^{-1}) in pig slurry, BF and PS digesters. Nº replicates=2.

	Pig slurry	Pig slurry digester (PS)			Broiler feed digester (BF)		
	20[2]	20[2]	30[2]	33[2]	20	30	33
Total bacteria	12.64	12.31	13.25	13.36	12.46	13.52	13.22
Total archaea	10.49	10.56	11.40	11.34	10.84	10.97	10.52
Total fungi	n.a.[1]	6.47	6.68	6.29	5.95	6.82	5.67

[1]n.a., not analyzed

6.3.3. Microbial structure and dynamics using scanning electron microscopy

6.3.3.1. Pig slurry

Figure 6.1 shows the most representative cell-morphotypes in the free living fraction of the pig slurry used to feed PS digesters in this study. A wide variability of cell-morphotypes was present in the pig slurry, mainly rod-shaped microorganism. Rod-shaped cell-morphologies varied in size from 1 to 5 μm and could be classified into four types: large-wide rod-shaped cells (*Rod.* in Figure 6.1A and 6.1B), small curved rods (*Curv.* in Figure 6.1B), chain-forming rods (*Ch.* in Figure 6.1A) and aggregates with an angular cell structure (*Ang.* in Figure 6.1B).

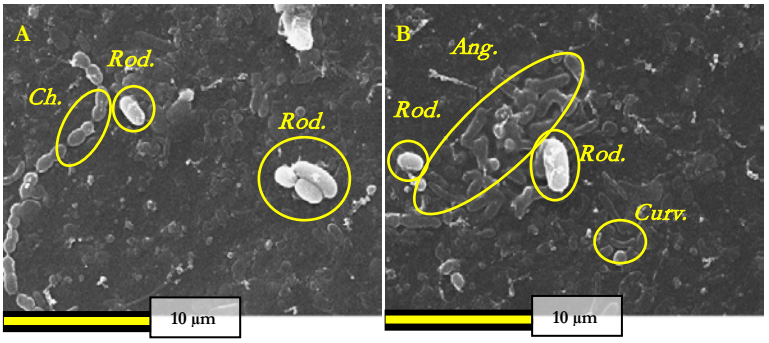

Figure 6.1- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in the free living fraction of the pig slurry used to feed pig slurry digesters, being: *Rod.*, large-wide rod-shaped cells; *Curv.,* small curved rods; *Ch.,* chain-forming rods; *Ang.,* aggregates with an angular cell structure.

Figure 6.2 shows the most representative cell-morphotypes in the flock fraction of the pig slurry used in this study. The most representative morphotypes observed were clumps of cells showing the characteristic sarcina morphology, which could presumably belong to Methanosarcinaceae family (*Meth.* in Figure 6.2A and Figure 6.2B), and large-wide rod-shaped cells (from 3 to 5 μm in size) (*Rod.* in Figure 6.2A).

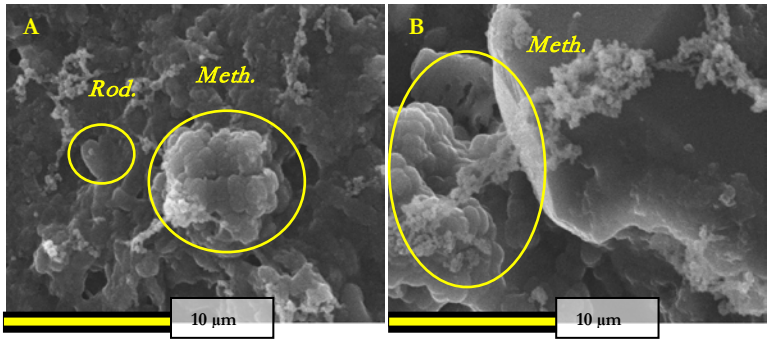

Figure 6.2- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in flock fraction of the pig slurry used to feed pig slurry digesters, being: *Meth.*, methanosarcina-like cells; *Rod.*, large-wide rod-shaped cells.

Aged pig slurry from the pit has been reported to show a high microbial biodiversity (Snell-Castro et al., 2005). The most represented bacterial groups in pig slurry at the end of a storage period in terms of phylotype and clone abundance have been reported to be *Clostridium*, *Bacillus-Lactobacillus-Streptococcus*, *Mycoplasma*, and *Flexibacter-Cytophaga-Bacteroides* (Snell-Castro et al., 2005). The majority of the cell-morphologies of these genuses could match with rod-shaped cells, as those observed by SEM in the present work, except for the aggregates with an angular cell structure (*Ang.* in Figure 6.1B) and methanosarcina-like cells (*Meth.* in Figure 6.2A and 6.2B). The aggregates with an angular cell structure could resemble fungi or actinomycete structures due to their short branched filaments forming right angles. Little is known about the presence or function of these structures in aged pig slurry.

6.3.3.2. Broiler feed digester

Figure 6.3 shows the most representative cell-morphotypes in the free living fraction of BF digester on two weeks over the experimental period, week 20 (Figure 6.3A) and week 33 (Figure 6.3B). Predominant cell-morphotypes were long-slender rod-shaped cells with a slight curvature (*Sld.*, Figure 6.3A and 6.3B). These cell structures dominated in the free living fraction during all the experimental period. Long-slender

rod-shaped cells varied in size from 1 to 20 μm, and generally appeared as individual cells (*Sld.*, Figure 6.3A and 6.3B), although rods-shaped chain-forming cells were also observed (*Ch-sld.*, Figure 6.3A). Occasionally some single sheathed rods with sharp ends were also distinguished (*Shp.*, Figure 6.3A and 6.3B).

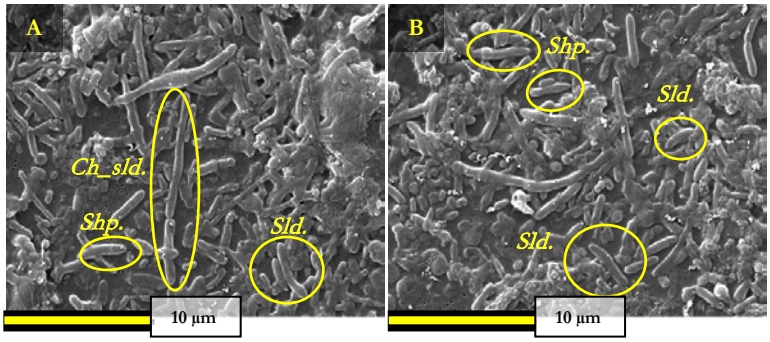

Figure 6.3- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in the free living fraction of broiler feed digester on week 20 (Figure 6.3A) and 33 (Figure 6.3B), being: *Sld*, long-slender rod-shaped cells with a slight curvature; *Ch_sld*, rod-shaped chain-forming cells; *Shp*, single sheathed rods with sharp ends.

Figure 6.4 shows the most representative cell-morphotypes in the flock fraction in BF digester on two weeks during the experimental period, week 20 (Figure 6.4A) and week 33 (Figure 6.4B). Predominant cell morphotypes in the flock fraction on weeks 20 and 33 were large cocci-shaped organisms (>1 μm in size) aggregated in homogeneous large granules (*Cc.* in Figure 6.4A and 6.4B).

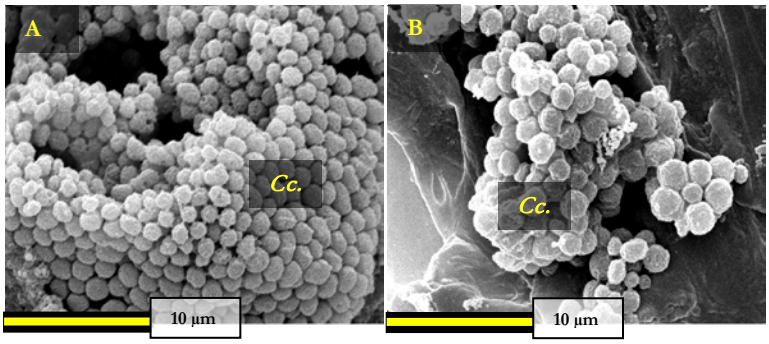

Figure 6.4- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in flock fraction of broiler feed digester on week 20 (Figure 6.4A) and 33 (Figure 6.4B), being: *Cc.*, large cocci-shaped organisms aggregated in homogeneous large granules.

Overall, BF digester showed fewer cell-morphotypes than pig slurry, being one type mainly dominating in free living fraction (long-slender rod-shaped cells), and one dominating in flock fraction (large cocci-shaped cells). These cell morphologies dominated over the experimental period, despite the fact that at the end of the experimental period, an accumulation of VFA occurred in BF digester. The presence of only few cell-morphotypes in BF digester could be due to the substrate composition which remained constant over time. In these conditions, the existent microorganisms were well adapted to degrade the substrate. In addition, no external biomass was added to BF digester. A wide difference in microbial morphotypes was observed between pig slurry (Figs 6.1 and 6.2) and BF digesters (Figs 6.3 and 6.4), not only because in pig slurry a higher morphological diversity was observed, but also because the long-slender, rod-shaped cells and large cocci-shaped organisms dominating in BF digester were not observed in pig slurry. The rod-shaped cells, methanosarcina-like cells and the aggregates with an angular cell structure present in pig slurry were neither observed in BF digester.

Figure 6.5 shows the most representative OM structures in BF digester on two weeks during the experimental period, week 20 (Figure 6.5A) and week 33 (Figure 6.5B). Figure 6.5A shows long (>150 µm in size) angular

grooves or tubes structures resembling a tubular junction of rings (*Fibr.* in Figure 6.5A). This structure could be attributable to partly degraded fiber fractions, presumably vegetables structures like xylem cells with lignified walls. Molinuevo-Salces et al. (2012) reported similar structures in an anaerobic digester fed with pig slurry in co-digestion with vegetable wastes, attributing their presence to vegetable structures. In our study, these structures probably came from the vegetable ingredients found in broiler feed. Additionally large pieces of OM (>100 μm in size) showing a crack or fragmented surface were also observed on week 20 of the experimental period (*OM.* in Figure 6.5A). Figure 6.5B shows walls of polygonal rings with amorphous aggregates slightly degraded, observed on week 33 in BF digester (*Pol.* in Figure 6.5B). These could also belong to vegetable cells walls.

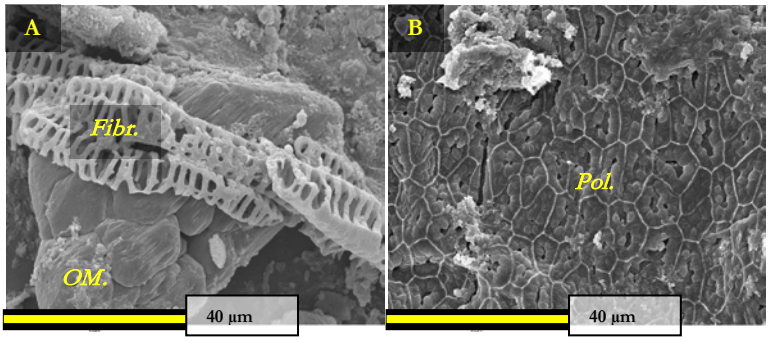

Figure 6.5- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in organic matter structures in flock fraction of broiler feed digester on week 20 (Figure 6.5A) and 33 (Figure 6.5B), being: *Fibr.*, structures composed by angular grooves or tubular junction of rings; *O.M.,* large pieces of organic matter showing a crack or fragmented surface; *Pol.*, walls of polygonal rings with amorphous aggregates slightly degraded.

6.3.3.3. Pig slurry digester

Figure 6.6 shows the most representative cell-morphotypes in the free living fraction of PS digesters on two weeks during the experimental period, on week 20 (Figure 6.6A), before the addition of pig slurry, and on week 33 (Figure 6.6B). Before the addition of pig slurry (Figure 6.6A), predominant cell-morphotypes were long-slender rod-shaped cells with a

slight curvature (from 1 to 20 μm in size) similar to those observed in BF digester (*Sld.* in Figure 6.3A). After the addition of pig slurry, at the end of the experimental period, in addition to the long-slender rod-shaped cells initially observed (*Sld.* in Figure 6.6B), other cell-morphotypes were observed such as large-wide rod-shaped cells (<2 μm in size) (*Rod.* in Figure 6.6B), chain-forming organism consisting of 10 or more cells (*Ch.* in Figure 6.6B), and aggregates with an angular cell structure (*Ang.* in Figure 6.6B). These new cell-morphotypes were similar to those observed in pig slurry (Figure 6.1).

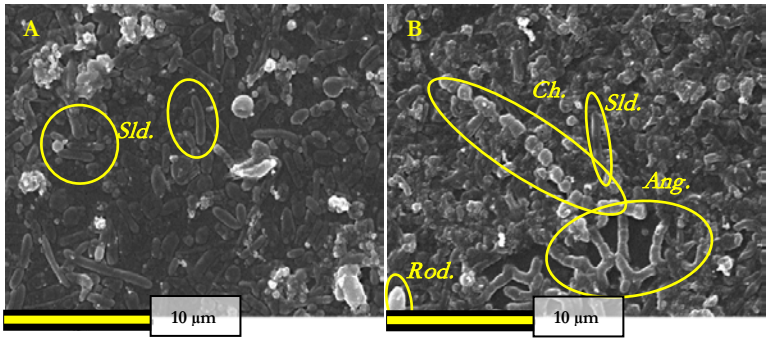

Figure 6.6- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in the free living fraction of pig slurry digesters on week 20, before the addition of pig slurry, (Figure 6.6A) and on week 33 (Figure 6.6B), being: *Sld.*, long-slender rod-shaped cells with a slight curvature; *Rod.*, large-wide rod-shaped cells; *Ch.*, chain-forming rods; *Ang.*, aggregates with an angular cell structure.

Figure 6.7 shows the most representative cell-morphotypes in the flock fraction of PS digester on two days during the experimental period, before pig slurry addition on week 20 (Figure 6.7A), and on week 33 (Figure 6.7B). Before the addition of pig slurry (week 20), very few distinguishable cell-morphotypes were observed (Figure 6.7A). Predominant morphologies were large-wide individual rod-shaped cells (from 1 to 2 μm in size) (*Rod.* in Figure 6.7A) and aggregates of small particles probably composed of OM. Cell-morphotypes changed in PS digesters with the addition of pig slurry. On week 33, new cell-morphotypes and a higher morphological diversity were observed. On week 33, a well aggregated and diverse structure of cell-morphotypes was

observed in the flock fraction. Predominant cell-morphotypes were clumps of cells showing the characteristic sarcina morphology attached to the OM (*Meth.* in Figure 6.7B), similar to those observed in pig slurry (*Meth.* in Figure 6.2); aggregates with an angular cell structure (*Ang.* in Figure 6.7B), and large-wide individual rod-shaped cells (>3 μm in size) (*Rod.* in Figure 6.7B). In Figure 6.7B, a microbial colony surface composed by methanosarcina-like cell constituted by multicellulars lobes attached to each other and anchored to the OM can be distinguished.

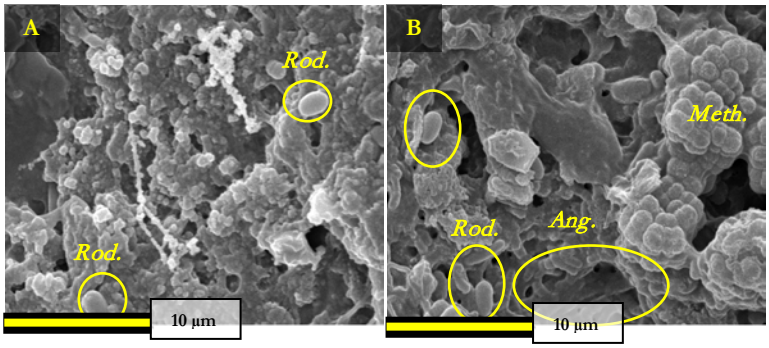

Figure 6.7- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in flock fraction of broiler feed digesters on week 22, before the addition of pig slurry (Figure 6.7A), and on week 33 (Figure 6.7B), being: *Rod.*, large-wide rod-shaped cells; *Meth*, *Methanosarcina*-like cells; *Ang.*, aggregates of rods-shaped cells with an angular cell structure.

Aggregates with an angular cell structure, similar to those observed in pig slurry (Figure 6.1), were observed in the free living (*Ang.* in Figure 6.6B) and in the flock fraction (*Ang.* in Figure 6.7B) at the end of the experimental period in PS digesters. Although these structures could resemble mycelia of fungi, gene concentration of total fungi was found very low during the experimental period in PS digesters through qPCR analyses (Table 6.3) and quantifications of gene concentration of total fungi did not follow a clear trend over time.

Cirne et al. (1997) reported that the two main phyla within *Bacteria* domain responsible to degrade lignocellulosic materials in anaerobic digesters are *Actinomycetales* and *Clostridiales*. The aggregates with an

angular cell structure could therefore belong to *Actinomycetales* phyla. To confirm this hypothesis, samples from PS digester on week 33 were cultured in actinomycete isolation agar (AIC, 12168 - BD Difco™, USA), incubated at mesophilic (28ºC) and thermophilic (42ºC) temperatures in anaerobic jars during one week. Isolated colonies were observed. For this, gram staining was performed previously and fresh samples were observed with a phase contrast microscopy (Nikon H600L, Japan). Figure 6.8 shows the Gram staining of a colony isolated with AIC. As shown in this figure, microorganisms present a structure with right angle branching typical of actinomycete, and could thus indicate their presence in PS digesters after pig slurry addition.

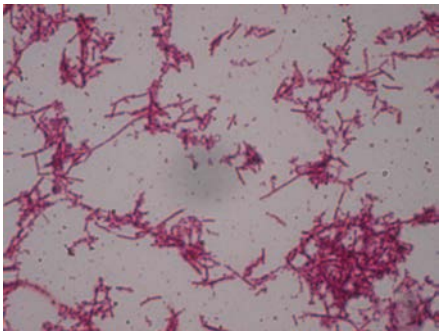

Figure 6.8- Gram staining of a colony isolated with actinomycete isolation agar (100x magnification).

Figure 6.9 shows the most representative OM structures in PS digester on two weeks during the experimental period, week 20 (Figure 6.9A) and week 33 (Figure 6.9B). As shown in Figure 6.9A, OM structure in PS digesters before pig slurry addition was similar to those observed in BF digester (Figure 6.5), which was dominated by long fibrous structures (>200 μm in size), long angular grooves or tubular structures (*Fibr.* in Figure 6.9A). However, at the end of the experimental period (on week 33), the fiber structures observed presented a higher degradation degree. On week 33, OM was mainly composed of structures showing a crack or fragmented surface (*OM.* in Figure 6.9B). Additionally, at the end of the experimental period, aggregates of methanosarcina-like cells (*Meth.* in Figure 6.9B) were present attached to the OM structures. After pig slurry

addition, fiber structures presented a more degradable structure (*Fibr.* in Figure 6.9B), probably because during pig slurry storage, anaerobic degradation of OM takes place in which suspended OM is transformed into soluble OM (Moset et al., 2012).

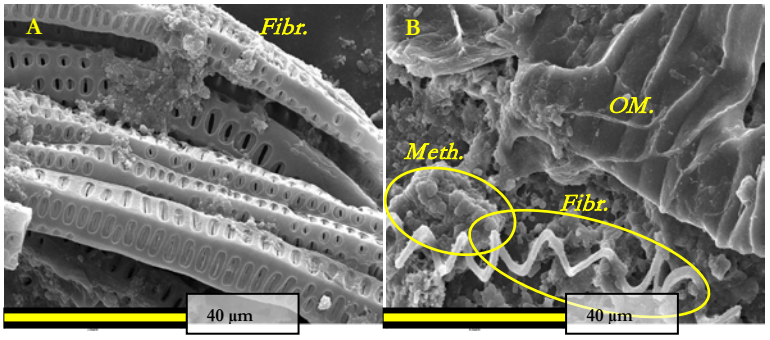

Figure 6.9- Cold-stage scanning electron microscopy micrographs of the most representative cell-morphotypes in organic matter structures in flock fraction of pig slurry digester on week 20, before the addition of pig slurry (Figure 6.9A) on week 33 (Figure 6.9B), being: *Fibr.*, angular grooves or tubes structures resembling a tubular junction of rings; *O.M.,* large pieces of organic matter showing a crack or fragmented surface; *Meth*, methanosarcina-like cells.

At the end of the experimental period, microbial morphology was constituted by heterogeneous cell morphologies and well-attached microbial aggregates to the OM. Both cell morphotypes present in BF digester as well as those observed in pig slurry were present.

Associations between microbial groups in anaerobic digesters has been widely described in up-flow anaerobic digesters (McLeod et al., 1990; Guiot et al., 1992; McHugh et al., 2003) but in CSTR, aggregation has been considered to have little importance due to the wash-up of microorganisms which occurs because there is no attachment surface in CSTR. However, the microbial associations in anaerobic digester are important since the syntrophic relationships needed for the OM degradation, are facilitated by the formation of mixed microbial aggregates (McHugh et al., 2003).

It is widely assumed that variations in substrate composition can impact on the entire microbial community structure and the metabolic stability in anaerobic digester (Keyser et al., 2006). Several authors have suggested that substrate's microbial composition can be an important factor affecting the final microbial composition in anaerobic digesters (Shin et al., 2010; Zhu et al., 2011). However, few studies consider the effect of substrate's microbial composition on the final microbial composition in anaerobic digesters, taking into account all microbial groups involved in the process, methanogens and non-methanogens.

With the addition of pig slurry, cell-morphotypes different from those observed in BF digester were present in this work. These cell-morphotypes probably come from the pig slurry as they resembled those present in pig slurry in size and type. Therefore, the addition of pig slurry increased the morphological diversity initially observed in PS digesters. The fact that these new structures started to grow in PS digester could be due to the shift in physicochemical changes after pig slurry addition, such as increases in VFA and TAN concentration. Microorganism coming from the pig slurry will most probably be best adapted to these environmental conditions.

6.4 Conclusions

The addition of pig slurry to an unadapted thermophilic anaerobic digester caused an increase in VFA concentration a decrease in removal efficiency and CH_4 yield.

The addition of pig slurry in anaerobic thermophilic conditions caused an increase in total bacteria (from 12.3 log copies mg DNA^{-1} to 13.3 log copies mg DNA^{-1}) and in total archaea (from 10.6 log copies mg DNA^{-1} to 11.4 log copies mg DNA^{-1}) determined by qPCR. The gene content of total fungi, however, remained comparatively low (on average 6.3 log copies mg DNA^{-1}) during the experimental period. The total bacterial content dominated over total archaea and total fungi over the experimental period.

Scanning electron micrographs provided a general overview of the sludge's cell morphology, morphological diversity and degree of OM degradation. A change in microbial morphotypes from homogeneous cell morphologies to a higher morphological diversity, similar to that observed in pig slurry, was observed with the addition of pig slurry by SEM.

The combination of different microbial methodologies, qPCR and SEM, allows expanding the knowledge about the microbial adaptation to pig slurry in thermophilic anaerobic CSTR.

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Chapter 7

Start-up strategies for thermophilic anaerobic digestion of pig slurry

Start-up strategies for thermophilic anaerobic digestion of pig slurry

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Abstract: Sludge physicochemical composition, methane (CH_4) yield, and methanogenic community structure and dynamics (by quantifying: total bacteria, total archaea, and four archaeal orders, *Methanobacteriales*, *Methanosarcinales*, *Methanococcales* and *Methanomicrobiales* by quantitative real-time polymerase chain reaction) were determined after start-up of anaerobic digestion of pig slurry in eight thermophilic continuous stirred anaerobic digesters. Four management strategies were investigated: a feedless period followed either by a gradual or an abrupt addition of pig slurry and a non-feedless period followed either by a gradual or an abrupt addition of pig slurry. During the first 43 days, a deterioration of the digesters performances concerning sludge physicochemical composition and CH_4 yield was observed; after this period, the recovery of the original parameters started, being the last parameter to be re-established propionic acid. The best start-up strategy was non-feedless digester with an abrupt addition of pig slurry; however differences in microbial population among treatments were not evident. Hydrogenotrophic methanogenesis, *Methanomicrobiales* first and *Methanobacteriales* second, was the dominant metabolic pathway in all groups of digesters. Although qPCR conditions have been improved in this work, molecular tools have to be still clarified to allow greater accuracy in the determination of the microbial populations involved in the anaerobic digestion process.

Keywords: anaerobic digestion, pig slurry, real-time qPCR, start-up strategies

7.1 Introduction

Anaerobic digestion of OM is a complex process which can present operational difficulties under certain stressful conditions such as changes in operational parameters (temperature or organic loading rate, OLR) or in substrate composition. Generally, after these stressful conditions, an adaptation period is necessary in which methane (CH_4) production is low and economical losses can be expected. To avoid this situation, strategies that improve microbial adaptation to new conditions and speed up digesters recovery are essential. Different strategies based on feeding patterns, have been addressed to improve digester performance and to overcome changes in temperature (Palatsi et al., 2009a), or to avoid inhibition by long chain fatty acids (Palatsi et al., 2009b). However, little information is available in the literature reporting start-up strategies for anaerobic digestion of pig slurry in continuous stirred tank reactors (CSTR) not adapted to this substrate.

Variations in substrate composition can impact on the entire microbial community structure and on the metabolic stability in anaerobic digesters, affecting digester performance (Keyser et al., 2006). For instance, in substrates with a high proportion of slowly biodegradable OM (fiber) such as in animal slurry, hydrolysis has been reported to be the rate limiting step (Vavilin and Angelidaki, 2005). With easy-degradable substrates, however, methanogenesis is considered the rate limiting step, due to the lower growth velocity of methanogens compared with the rest of microbial groups involved in the process (Murto et al., 2004). In addition, animal slurry has high ammonia (NH_3) and volatile fatty acids (VFA) contents which can further inhibit the anaerobic digestion process at low concentrations, especially with non-adapted microbiota (Stamatelatou et al., 2004).

Physicochemical parameters like CH_4 yield, VFA, pH, alkalinity or volatile solids (VS) degradation rates have been traditionally used to evaluate the overall stability of the anaerobic digestion process (Ahring et al., 1995; Ferrer et al., 2010). Although these indicators are useful to monitor anaerobic digester's performance, they do not provide

information about the composition and the physiological state of the biomass contained in the digesters. Microbiological parameters such as diversity, structure and dynamics of the microbial populations, using molecular methods, have been recently linked to the performance and efficiency of anaerobic digesters (Sanz and Klöching, 2007; Song et al., 2010). However, due to the recent application of molecular biology in anaerobic digestion, the relationship between the physicochemical parameters routinely used and the microbial diversity, structure and dynamics is still not well established. Therefore further research is needed in laboratory conditions to assess the molecular methods' potential in monitoring the anaerobic digestion process on full-scale digesters. This information will be valuable to identify microbial indicators which could add value to the physicochemical ones in reducing CH_4 losses during stressful periods.

The objective of this work was to evaluate CH_4 yield, sludge physicochemical composition and methanogenic community structure and dynamics, after the start-up of anaerobic digestion of pig slurry in eight anaerobic thermophilic CSTRs. Four start-up strategies were investigated: a feedless period followed either by a gradual or an abrupt addition of pig slurry and a non-feedless period followed either by a gradual or an abrupt addition of pig slurry.

7.2 Materials and methods

7.2.1 Experimental design and substrates

The experiment was conducted using eight identical CSTR with a working volume of 5.5 L each, at thermophilic conditions (50°C). The temperature was controlled by a thermostatically regulated water bath. The digester's mixing system consisted in an intermittent mechanical stirring (RZR1 77W Heidolph, Schwabach Germany) which worked for 15 minutes every 60 minutes.

The total duration of the experiment was 126 days in where digesters were subjected to four start-up strategies and thereafter they were allowed to recover reactor performances. The four start-up strategies

were: feedless + abrupt addition of pig slurry (F abrupt), feedless + gradual addition of pig slurry (F gradual), non-feedless + abrupt addition of pig slurry (Non-F abrupt), non-feedless + gradual addition of pig slurry (Non-F gradual). Four of the eight digesters were subjected to feedless strategy, which consisted in no feed being supplied during 10 days. The other four digesters remained working with broiler feed (non-feedless reactors). After these 10 days of feedless period, pig slurry was added to all digesters following two strategies: an abrupt addition consisting in feeding the digesters exclusively with pig slurry from the beginning; or a gradual addition, which consisted in a gradual and incremental addition of pig slurry (from 0% to 100% during 20 days with an increase of 5% of the proportion of VS added per day coming from pig slurry). Therefore the 126-days experimental period comprised three phases: 10 days of **feedless period** (from day 0 until day 10 of the experimental period), 20 days of **substrate change period** (from day 10 until day 30 of the experimental period) and 96 days of **recovery period** (from day 30 until day 126 of the experimental period) in which digesters were allowed to recover reactor's performances.

Before start-up strategies, digesters were working with broiler feed at stable conditions, the broiler feed used was mixed with water until 10% in total solid to facilitate its incorporation into the digesters.

The pig slurry used in this study was obtained from a completed pig fattening period in summer conditions carried on in the Animal and Technology Research Centre (CITA, Segorbe, Spain) belonging to Valencian Institute for Agricultural Research (IVIA). Table 7.1 shows broiler feed and pig slurry composition.

The organic loading rate was constant (1 g VS L^{-1} d^{-1}) during all the experimental period, except during the feedless period in F reactors. The hydraulic retention time (HRT) was adjusted to maintain the OLR constant, and thus it was decreased with the addition of pig slurry from 90 to 30 days. In all cases, HRT was higher that the minimal required for the growth and development of all microbial groups involved in the

thermophilic anaerobic digestion process reported in the literature (Miron et al., 2000; de la Rubia et al., 2006).

Table 7.1- Broiler feed and pig slurry composition used as digester's substrate.

Parameter	units	Broiler feed	Pig slurry
Total Solids	g L^{-1}	101.1	39.3
Volatile solids	g L^{-1}	94.9	26.8
pH		n.a.[1]	7.84
Total ammonia Nitrogen	mg N-NH_3 L^{-1}	n.a.	1870
Total volatile fatty acids	mg L^{-1}	n.a.	1190
Acetic acid	mg L^{-1}	n.a.	800
Propionic acid	mg L^{-1}	n.a.	330
Butyric acid	mg L^{-1}	n.a.	10
Neutral detergent fiber	g Kg DM^{-1}[2]	9.06	27.53
Acid detergent fiber	g Kg DM^{-1}*	2.47	15.80
Lignin	g Kg DM^{-1}*	n.d.[3]	5.58
Protein	g Kg DM^{-1}*	195.54	156.25
Fat	g Kg DM^{-1}*	65.74	62.49

[1]n.a.: not analyzed
[2]D.M.: dry matter
[3]n.d. not determined

7.2.2 Physicochemical analyses

Representative samples of each digester were taken on day 0, before start-up strategies (initial composition). Additionally, weekly samples were obtained during the recovery period. Total solids (TS), VS, pH and total ammonia nitrogen (TAN) were analyzed as described in APHA (2005) and VFA concentration was determined by gas chromatography equipped with flame ionization detector (HP 68050 series Hewelt Packard, USA).

Biogas production from each digester was measured three times per week by determining total volume collected in aluminum bags and measuring it through a gas meter-Precision Wet Test Gas Flow Meters (RITTER ®, Germany). Biogas samples were taken by flushing a 15 mL sample vacutainers with 60 mL of biogas.

Methane concentration in biogas samples collected from each digester was analyzed on a Focus Gas Chromatograph (Thermo, Italy) equipped with a split injector and a flame ionization detector. The separation was performed in a GS-Q capillary column (J&W Scientific, USA) (30 m x 0.32 mm i.d.). The carrier gas was helium at a constant flow of 1 mL min^{-1}. The samples were injected with a split ratio of 1/100. The initial oven temperature was set at 50 ºC held for 1 min and increased to 150 ºC at a rate of 50 ºC min^{-1} and finally maintained at that temperature for 2 min. Both detector and injector temperatures were set at 200 ºC. An external standard (41.21% CO_2 and 58.79 % CH_4) was employed for quantification of CH_4 content in samples.

7.2.3 Methanogenic community structure and dynamics

7.2.3.1. DNA extraction and purification

On days 17, 31, 45, 59, 73, 85, 99, 114 and 126 of the experimental period, two samples of sludge from each digester were taken during agitation into 20 mL sterile Falcon tubes. After sampling, samples were immediately frozen with liquid nitrogen and stored at -80ºC.

Total DNA from each sample was extracted after thawing from 250 μL of anaerobic sludge using QIAamp DNA Stool Mini kit (Qiagen, Germany). The DNA concentrations present within the different extracted DNA preparations were determined with NanoDrop 2000C Spectrophotometer (Fisher Scientific, Spain). The extracted DNA was diluted with nuclease-free water (Ambion, USA) until a final concentration of 0.5 to1.0 ng DNA μL^{-1}.

7.2.3.2. DNA quantification using real-time quantitative chain reaction

Copy numbers of 16S rRNA genes of total bacteria (*Bacteria* domain), total archaea (*Archaea* domain) and of the four main archaeal orders: *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanoccocales* were quantified with quantitative real-time TaqMan polymerase chain reaction (qPCR) using a StepOne Plus thermocycler (Applied Biosystems, Spain).

Amplification was carried out in a final volume of 12 μL containing 2 μL of template, 6 μL of Master Mix solution (Applied Biosystems, Spain), 0.6 μL of each primer (final concentration 0,5 μM) and 0.18 μL of the TaqMan probe (final concentration 0,15 μM). The specific primer sets used for total bacteria, total archaea, *Methanobacteriales, Methanosarcinales*, *Methanomicrobiales* and *Methanoccocales* were those described by Yu et al. (2005). The qPCR conditions of these authors were modified in this work as follow:

- Total bacteria, *Methanosarcinales* and *Methanoccocales*: one step at 95 ºC for 10 min followed by 45 cycles of 95 ºC for 10 s, 60 ºC for 30 s and 73 ºC for 30 s.
- Total archaea: one step at 95 ºC for 10 min followed by 45 cycles of 95 ºC for 10 s and 60 ºC for 60 s.
- *Methanomicrobiales*: one step at 95 ºC for 10 min followed by 45 cycles of 95 ºC for 10 s, 63 ºC for 30 s and 73 ºC for 30 s.
- *Methanobacteriales*: one step at 95 ºC for 10 min followed by 45 cycles of 95ºC for 10 s, 57 ºC for 30 s and 73 ºC for 30 s.

Each qPCR reaction was performed in duplicate tubes. Additionally control reactions without DNA were used in all amplification series to ensure that contaminating DNA was not present.

Standard curves were generated as previously described by Song et al. (2010). Described strains were purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany). Genomic DNA was extracted from each strain and the target 16S rRNA gene sequence was amplified by conventional PCR with the corresponding primers. The resulting PCR products were purified and the corresponding copy concentration was calculated using Equation 7.1.

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\left(\frac{\text{copy}}{\text{mole}}\right) \times \text{DNA amount (g)}}{\text{DNA Length (bp)} \times 660\ \left(\frac{\text{g}}{\frac{\text{mole}}{\text{bp}}}\right)} \qquad (7.1)$$

The PCR products were serially diluted in the range of 10^{-4}-10^{-11} copies μL^{-1} and directly used as a template for qPCR with the corresponding

primer and probes sets in triplicate. Standard curves were amplified in each qPCR run. Average slope and intercepts used for 16S rRNA genes concentration of target microbial orders in the samples were:

- Total bacteria, average slope -3.19 ± 0.165, average intercept -44.44± 1.337 ($R^2 > 0.994$).
- Total archaea, average slope -3.14 ± 0.162, average intercept -49.99± 0.196 ($R^2 > 0.992$).
- *Methanobacteriales*, average slope -3.29 ± 0.163, average intercept -45.30 ± 3.088($R^2 > 0.994$).
- *Methanosarcinales*, average slope -3.48 ± 0.101, average intercept -46.83± 3.145 ($R^2 > 0.993$).
- *Methanomicrobiales*, average slope -3.22 ± 0.132 average intercept -50.83± 3.595 ($R^2 > 0.991$).
- *Methanoccocales*, average slope -3.13 ± 0.273 average intercept -52.06 ± 2.036 (R^2>0.997).

Data acquisition and analysis were performed using StepOne Plus 2.0 machine (Applied Biosystems, Spain).

7.2.4 Calculations

The removal efficiency, the percentage of degraded VS, before and weekly after the application of each strategy was calculated taking into account VFA losses during drying following the procedure described by Moset et al. (2012).

Total free ammonia (NH_{3f}) was calculated before and weekly after the application of each strategy using the equations described by Budavari (2001) as a function of the temperature, pH and TAN as shown in Equation 7.2:

$$[NH_3]_f = TAN\ x\ \frac{ratio}{ratio+1} \quad (7.2)$$

where $[NH_3]_f$ is free ammonia concentration and ratio, is the ratio of distribution between ammonia and ammonium in the slurry. This ratio depends of pH and temperature of the slurry as shown in Equation 7.3:

$$ratio = 10^{\left[\frac{-pOH}{1{,}77\ x\ 10^{-5}\ x\ e^{\left(\frac{62}{8.31}\right)x\left(\frac{1}{T(K)}x\frac{1}{T_{STP}(K)}\right)}}\right]} \quad (7.3)$$

where T, is the temperature in the sludge (Kelvin) and T_{STP} is the temperature in standard conditions.

To evaluate microbial composition in each digester, the percentage of 16S rRNA gene concentration of total archaea from the sum of total bacteria and archaea; and the percentage of 16S rRNA gene concentration of each methanogenic archaeal order, from the sum of the gene concentration of the four methanogenic archaeal orders, was determined.

7.3 Results

7.3.1 Initial digesters' performance

Table 7.2 shows the initial (day 0) digester's performance in terms of physicochemical parameters, CH_4 yield and CH_4 concentration in biogas. Initial parameters were similar among strategies. The pH was around 7.8 and total VFA was lower than 400 mg L^{-1} in all digesters. From the total VFA, acetic acid represented more than 80% in all digesters. Total ammonia concentration was on average 1,500 mg NH_3 L^{-1} and free ammonia was lower than 50 mg NH_3 L^{-1} in all digesters except in non-F abrupt digesters in which free ammonia was slightly higher, equal to 60 mg NH_3 L^{-1}.

Removal efficiency was on average 72% in all digesters. The percentage of CH_4 in biogas was around 70% in all digesters. Methane yield was slightly higher in non-F digesters, ranging from 415 to 460 mL CH_4 g VS^{-1} than in F digesters, in which CH_4 yield ranged from 312 to 318 mL CH_4 g VS^{-1}.

Table 7.2- Initial (day 0) digesters' physicochemical composition and performance at the beginning of the experimental period. Replications per treatment: 2.

Parameter	Units	Feedless + abrupt change in substrate	Feedless + gradual change in substrate	Non-Feedless+ abrupt change in substrate	Non-Feedless gradual change in substrate
pH		7.8 ± 0.06	7.8 ± 0.01	7.9 ± 0.04	7.7 ± 0.08
Total volatile fatty acids	mg L^{-1}	379 ± 130.4	338 ± 70.9	391 ± 140.0	316 ± 45.1
Acetic acid	mg L^{-1}	315 ± 112.6	294 ± 71.8	335 ± 124.2	253 ± 22.0
Propionic acid	mg L^{-1}	41 ± 8.0	27 ± 2.3	33 ± 10.4	30 ± 4.0
Butyric	mg L^{-1}	3 ± 1.1	2 ± 0.1	4 ± 0.3	1 ± 0.5
Total ammonia	mg $N\text{-}NH_3$ L^{-1}	1512 ± 16.5	1464 ± 26.2	1563 ± 30.5	1520 ± 108.1
Free ammonia	mg $N\text{-}NH_3$ L^{-1}	47 ± 7.2	41 ± 2.0	60 ± 3.3	46 ± 11.1
Volatile solids degraded	%	72 ± 0.3	73 ± 0.1	72 ± 1.4	71 ± 1.9
CH_4 in biogas	%	70 ± 9.3	70 ± 2.6	72 ± 3.0	73 ± 4.6
Methane yield	mLCH_4 g VS^{-1}	312 ± 52.3	318 ± 23.0	415 ± 48.3	460 ± 17.8

7.3.2 Digesters' performance during the recovery phase

During the recovery phase, pH ranged from 7.7 to 8.4 in all digesters, reaching on day 56 the minimal value and at the end of the recovery period (on day 126) the maximal value.

Total ammonia nitrogen concentration in the digesters achieved its maximal level (2016 ± 109.0 mg $N\text{-}NH_3$ L^{-1}) on day 49 of the experimental period in all digesters. From this moment, TAN content in all digesters remained stable until the end of the experimental period. Free ammonia followed the same trend than pH in all digesters; it ranged from 70 (on day 56) to 240 mg $N\text{-}NH_3$ L^{-1} at the end of the experimental period (on day 126).

Figure 7.1 shows total VFA, acetic, propionic and butyric acids evolution during the recovery period. At the beginning of the recovery period, VFA concentration was higher in F reactors than in non-F reactors. Total VFA concentration increased from day 50 of the recovery period reaching maximal values between days 70 to 77 of the experimental period. Among strategies, VFA peak were the highest (3600 mg L^{-1}) in F gradual digesters (on day 70), followed by 2,900 mg L^{-1} in F abrupt digesters (on day 77). The VFA peak in non-F digesters was reached on day 77 approximately. From this point, total VFA levels decreased in all digesters showing similar VFA levels in all strategies at the end of the recovery phase (1150 ± 228.89 mg L^{-1} on average).

Regarding acetic, butyric and propionic acids shown in Figure 7.1, at the beginning of the recovery period, acetic acid was the dominant individual VFA representing around 80% of total VFA in all digesters. Similarly to total VFA, acetic acid concentration peaked between day 70 and 77 in all digesters, except in non-F abrupt digesters in which the peak was reached on day 49. In general, acetic acid concentration was lower in non-F digesters compared with F digesters. Final acetic acid concentration was similar, and on average 615 ± 163 mg L^{-1}, in all digesters.

Concerning butyric acid, as shown in Figure 7.1, its peak coincided with the peak of acetic acid in all digesters (from day 70 to day 77 except for non-F abrupt digesters that peaked on day 49). Final butyric acid concentration was similar among digesters (on average 4 g L^{-1}) and higher than at the beginning of the recovery period in all digesters, except in F abrupt digesters in which final propionic concentration was half of the initial value.

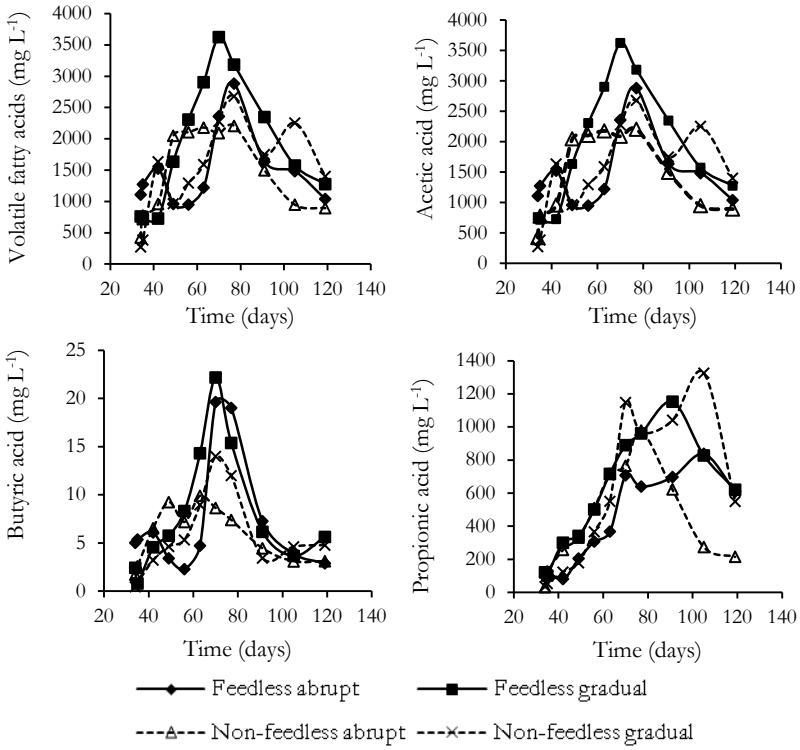

Figure 7.1- Total VFA, acetic butyric and propionic acid evolution during the recovery period after pig slurry addition. Replications per treatment: 2

Concerning propionic acid, as shown in Figure 7.1, the peak was found between 90 to 105 days in all digesters, except in non-F abrupt digesters in which the peak was obtained earlier, on day 77. Final propionic concentrations were similar in all digesters (average 600 mg L^{-1}), except in non-F abrupt digesters in which final propionic concentration were

lower (200 mg L^{-1}). At the end of the recovery period the percentage of propionic acid in the total VFA reached more than 50% in F digesters and around 30% in no-F digesters. Non-F abrupt digesters showed the lowest percentage of propionic acid in the total VFA at the end of the experimental period (<25%).

Removal efficiency at the beginning of the recovery period (on day 35-44 of the experimental period) was lower in digesters with a gradual change in substrate (7% in F and 4% in non-F digesters) compared with digesters subjected to an abrupt change in substrate (30% in F and 40% in non-F digesters). From day 65 to day 70 of the experimental period, removal efficiency was similar in all digesters (average 20%) and slightly increased at the end of the recovery period reaching on average 33% in all digesters. In general, during all the recovery period, digesters with a gradual change of substrate showed lower percentage of degraded VS than digesters with an abrupt change of substrate.

Figure 7.2 shows CH_4 yield evolution during the recovery period. Before day 77, CH_4 yield was low and unstable in all digesters, mostly ranging from 70 to 140 mL CH_4 g VS^{-1}. From day 77, CH_4 yield showed a recovery in all digesters, except F abrupt digesters in which CH_4 yield was not restored until day 84. Average CH_4 yield at the end of the experiment was similar in all digesters (123±19.2 mL CH_4 g VS^{-1} on average).

Cumulative CH_4 production during the 96 days of the recovery period was slightly higher in non-F digesters (59 L CH_4 in non-F gradual and 57 L CH_4 in non-F abrupt) than in F digesters (54 L CH_4 in F gradual and 55 L CH_4 in F abrupt).

Methane concentration in the biogas was stable with time during the recovery period, ranging from 60% (beginning of the recovery period) to 67% (end of the recovery period) in all digesters.

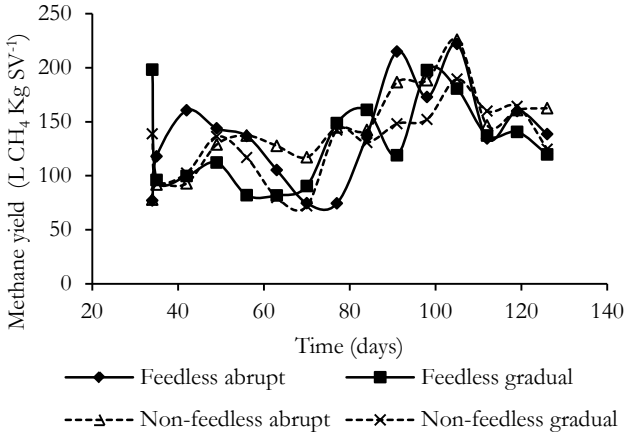

Figure 7.2- Digesters' CH_4 yield (L CH_4 Kg VS^{-1}) during the recovery period after pig slurry addition. Replications per treatment: 2.

7.3.3 Microbial community structure and dynamics

Figure 7.3 shows changes in 16S rRNA gene concentration of total bacteria and archaea expressed as logarithm of the copies mg DNA^{-1} at the beginning of the substrate change period (on day 17) and during the recovery period. The 16S rRNA gene concentration of the total bacteria population was stable until day 59 in all digesters (average of 12.3 log copies mg DNA^{-1}). From this moment, gene concentration of total bacteria increased in all digesters reaching the maximal total bacteria population on day 85 (average of 13.3 log copies mg DNA^{-1}). The 16S rRNA gene concentration of the total bacteria population was similar among treatments overall.

Concerning total archaea, the 16S rRNA gene concentration of the total archaea population was stable until day 59 in all digesters (average of 10.8 log copies mg DNA^{-1}). From this moment, the 16S rRNA gene concentration of total archaea population increased reaching an average of 12.4 log copies mg DNA^{-1} throughout the rest of the recovery period. Differences among strategies in 16S rRNA gene concentration of total archaea were only evident on day 17, where non-F gradual digesters showed higher 16S rRNA gene concentration of total archaea population

than the rest of digesters. Nevertheless, these differences were dumped during the recovery period and differences among treatments did never exceed one order of magnitude during the recovery period.

Overall, total archaea population was lower than total bacteria population in all treatments during the experimental period. Until day 59, the percentage of 16S rRNA gene concentration of total archaea to total bacteria ranged from 2 to 9%. From day 73 onwards, this ratio decreased due to the increase in total bacteria population. At the end of the experimental period, the percentage of 16S rRNA gene concentration of total archaea to total bacteria was around 1% in all digesters.

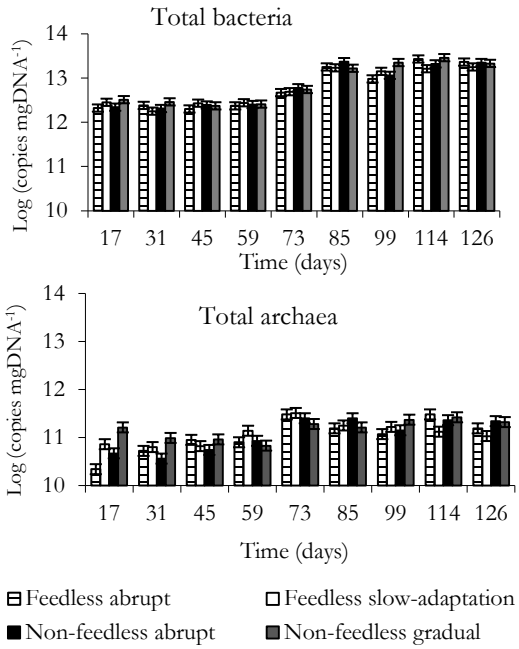

Figure 7.3- Evolution in 16S rRNA gene concentration of total bacteria and total archaea at the beginning of the substrate change period (from day 10 to 34) and during the recovery period (from day 34 to 126). Bars show the standard deviation of the average.

Concerning individual archaeal orders, Figure 7.4 shows changes in 16S rRNA gene concentration of *Methanobacteriales, Methanosarcinales* and

Methanomicrobiales at the beginning of the substrate change period and during the recovery period. The order *Methanococcales* could not be detected in this work in any qPCR reaction and thus data are not shown. At the beginning of the recovery period, the 16S rRNA gene concentration of *Methanobacteriales* was around 9.0 log copies mg DNA^{-1} in all digesters and remained stable until day 73. From this moment it started to increase reaching maximal levels on day 85 in all treatments (average of 10.4 log copies mg DNA^{-1}) and remaining stable until the end of the experimental period. Concerning differences among treatments, these were more pronounced before day 73, leveling off thereafter and showing no clear pattern. During the first days of the recovery period 16S rRNA gene concentration of *Methanosarcinales* was very low; on day 45 *Methanosarcinales* gene copies reached the minimal value (average of 8 log copies mg DNA^{-1}) increasing thereafter until the maximal levels on day 73 (average of 9.4 log copies mg DNA^{-1}). As in the case of *Methanobacteriales*, differences among digesters in gene concentration of *Methanosarcinales* were more evident before day 73 than after.

At the beginning of the recovery period, 16S rRNA gene concentration of *Methanomicrobiales* species was around 9.5 log copies mg DNA^{-1} in all treatments. Gene concentration of *Methanomicrobiales* remained fairly constant in time compared with the rest of archaeal orders determined in this work. Therefore, in general, differences between treatments or time were not marked in the 16S rRNA gene concentration of *Methanomicrobiales*.

Considering these three archaeal orders, *Methanomicrobiales* was the dominant order in terms of 16S rRNA copies at the beginning of the recovery period. In fact, *Methanomicrobiales* represented more than 50% of 16S rRNA gene concentration of total archaeal orders quantified in this work until day 73. From day 85, this percentage decreased to less than 20% in all groups of digesters. From this moment, the number of gene copies of *Methanobacteriales* increased and this order became the dominant order in all groups of digesters; on day 85 more than 80% of the gene copies from the three archaeal orders came from *Methanobacteriales*. The dominance of *Methanobacteriales* remained until the end of the

experimental period. *Methanosarcinales* order contributed less than 15% of the 16S rRNA gene concentration quantified in this work throughout the experimental period.

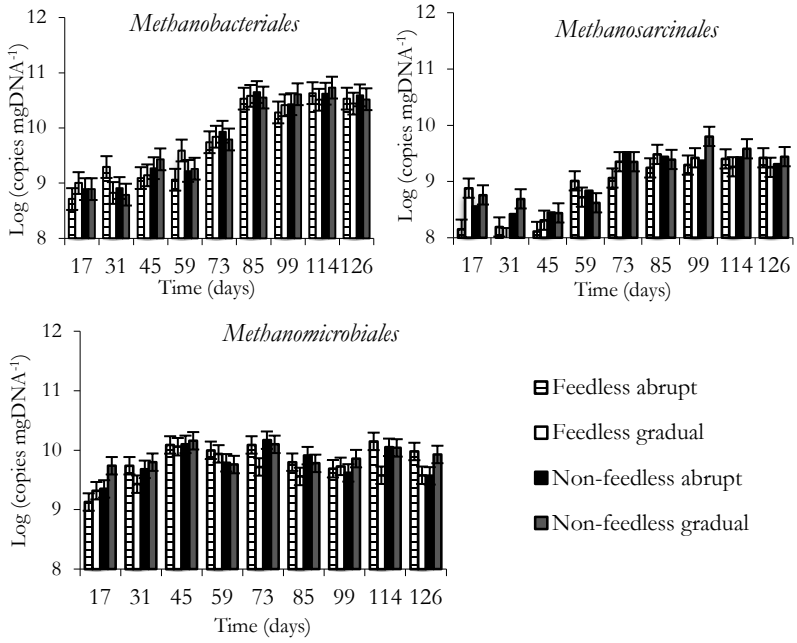

Figure 7.4- Evolution in 16S rRNA gene concentration of *Methanobacteriales*, *Methanosarcinales* and *Methanomicrobiales* order dynamics at the beginning of the substrate period (from day 10 to 34) and during the recovery period (from day 34 to 126). Bars show the standard deviation of the average.

7.4 Discussion

The start-up is generally considered the most critical step in the operation of anaerobic digesters (Griffin et al., 1998). During the first 43 days after the start-up (until day 77 of the experimental period) several alterations were observed in reactors like a decrease in the pH, an accumulation in VFA concentration and a decrease in VS degradation, these factors might have influenced the low CH_4 yield achieved during

this period in all digesters. After day 77, a clear recovery in digesters performance was observed.

This evolution after start-up strategies can be explained by an uncoupling among the three main microbial groups involved in the in the anaerobic degradation of OM process (acidogens, acetogens and methanogens) (Ahring et al., 1995). The accumulation of VFA concentration observed until day 70-77 in all digesters coincides with the lowest 16S rRNA gene concentration of total bacterial and archaeal levels, probably indicating a high activity of the hydrolytic and fermentative bacteria and a low activity of acetogens and methanogenic archaea to convert this VFA into acetic and CH_4 at this time. This apparent lower activity of archaea during the first days of the recovery period, confirmed by the low CH_4 yield achieved at this time, could be due to an inhibition caused by the increase in VFA and TAN content from pig slurry.

From day 77, a decrease in VFA concentration, especially acetic and butyric acids was observed in addition to an increase in VS degradation and CH_4 yield, indicating that presumably acetogens and archaeal populations increased at this moment. In fact, significant increases in 16S rRNA gene concentration of total bacteria, *Methanobacteriales* and *Methanosarcinales* orders were observed in all treatments from day 73 onwards.

However, high levels of propionic acid in digesters remained high for a longer period of time than the rest of the individual VFA, indicating that within acetogens, propionate-consuming acetogenic bacteria could have been the last group of bacteria to be established after a shift in substrate composition caused by the addition of pig slurry. Griffin et al. (1998) and Calli et al. (2005) suggested that propionate-degrading microorganism have a lower specific growth rate than acetate or butyrate-degrading microorganism in anaerobic digesters. Presumably for this reason, propionate accumulations in anaerobic digesters have been widely related with stress periods, either during the first days of acclimatization to changes in temperature from thermophilic from mesophilic conditions

(Ortega et al., 2008; Palatsi et al., 2009a) or during increments in TAN concentration in anaerobic digesters (Calli et al., 2005).

The predominance of *Methanomicrobiales* and *Methanobacteriales* in this work indicates that hydrogenotrophic methanogenesis was the main metabolic pathway for OM conversion to CH_4 during the recovery periods in all digesters. The predominance of hydrogenotrophic *vs.* aceticlastic methanogenesis has also been observed in anaerobic digesters working with animal slurry in the literature (Nettmann et al., 2008; Song et al., 2010; Zhu et al., 2011). This could be related to the fact that hydrogenotrophic methanogenesis is favored at high concentrations of VFA and FA (Karakashev et al., 2005), which are substances typically found in high concentration in animal slurries.

In the present work, a shift in the dominance from *Methanomicrobiales* to *Methanobacteriales* was observed on day 85, when propionic acid was high in all reactors. Hori et al. (2006) also found a shift in the dominance between *Methanomicrobiales* and *Methanobacteriales* during a stress period caused by digester acidification adding VFA. According to these authors, the predominance of *Methanomicrobiales* was replaced by *Methanobacteriales* in response to propionate accumulations in anaerobic digesters. However, available information about the role and activity of hydrogenotrophic methanogens in anaerobic conversion of complex organic material to CH_4 is still scarce. An understanding of the role of hydrogenotrophic methanogens in anaerobic digestion processes would be useful since as it is shown in this work, archaeal orders belonging to the hydrogenotrophic methanogens are the dominant species during process imbalances in anaerobic digesters.

Concerning differences among treatments, non-F digester showed better performances during the recovery period than F digesters in terms of less VFA accumulation and CH_4 yield recovery. Feedless period is a common strategy used especially in up-flow anaerobic and aerobic digesters to ensure flock constitution (Liu and Tay, 2008). It has been suggested that feedless periods enhance adaptation of the biomass to degrade complex substrates in the sludge (Nadais et al., 2006). However,

Hwang et al. (2010) observed that after four months of feedless period in anaerobic batch digesters working with pig slurry, changes in anaerobic microbial dynamics such as a reduction in *Methanosarcinales* order and methanogenic activity occurred. Additionally Palatsi et al. (2009b) reported that feedless was not an appropriate strategy to overcome long chain fatty acids accumulation, due to the high VFA accumulation and the low CH_4 yield achieved in digesters. In our work, F reactors also showed the highest VFA accumulation although differences in microbial population were less evident.

The rate at which the change in substrate occurred (gradual o abrupt) had practically no effect on reactors and seemed to have a lower influence on the recovery of the digesters than the feedless or non-feedless strategy. The digesters showing the best performances (lower VFA accumulation and higher CH_4 yield) during the recovery period were non-F abrupt digesters; however, results from microbial orders and dynamics did not support clearly this fact.

The quantification of the two domains, total bacteria and total archaea, and the three archaeal orders, *Methanobacteriales, Methanosarcinales* and *Methanomicrobiales* by qPCR, can provide useful information in order to evaluate microbial dynamics and dominances during transitional periods. However, the quantification of microbial abundance in anaerobic digesters using molecular methodologies techniques involves specific limitations as the inability to distinguish among living, non-living, dormant or extremely slow-growing cells (physiological state) and free DNA present in samples (Solera et al., 2001).

Additionally the current primers and described protocols present some drawbacks that have been partially overcome with the modifications established in the present work concerning the qPCR conditions. One of the most important drawbacks which still remain unclear is the fact that the sum of the 16S rRNA gene copy numbers of the three methanogenic orders, estimated in our work by qPCR, was lower than the 16S rRNA gene copy numbers of total archaea. This fact could indicate that or not all *Methanobacteriales*, *Methanosarcinales* and *Methanomicrobiales* have been

detected with the specific primers and set used, or that *Methanococcales*, which were not determined in this work, were present in the anaerobic digesters. In fact, it is stated that matching efficiency of the primers and probe for *Methanococcales* was lower than 60% (Yu et al., 2005). Although several works in the literature have reported low quantifications of *Methanococcales* in anaerobic digesters using real-time qPCR (Song et al., 2010) and fluorescence in situ hybridation (Karakashev et al., 2005); relevant *Methanococcales* species have been determined by phylogenetic analysis (McHugh et al., 2003) and denaturing gradient gel electrophoresis (Liu et al., 2002) in anaerobic digesters treating industrial and agricultural wastewater.

Due to the recent application of molecular biology techniques in anaerobic digestion, little information is available in order to determine the best molecular tools and methodologies to monitor the process. Therefore, further research on this matter is necessary before the use of molecular methodologies to monitor reliably and accurately the anaerobic digestion process on full-scale digesters, in order to find microbial state indicators that could potentially be used for predicting process failure and to improve the system stability.

7.5 Conclusions

After the start-up of anaerobic digestion with pig slurry a lowering of pH, a increasing of VFA concentration, a decreasing of VS degradation and a lowering in CH_4 yield was observed in all digesters. The recovery of the original parameters started 77 days after the start-up. Concerning sludge's physicochemical composition the strategy which best minimized CH_4 losses during this period was non-F abrupt, although differences in microbial population among treatments were very less evident. In all groups of digesters *Methanomicrobiales* first and *Methanobacteriales* second, were the dominant archaeal orders throughout the recovery period. Therefore, in the conditions of this study, hydrogenotrophic methanogenesis is the main metabolic route in CH_4 formation during the recovery period. The use of qPCR have lead to expand the knowledge about the dynamics of microorganism during the start-up period,

however further research are needed in order first clarify the role and activity o hydrogenotrophic methanogens during the recovery start-up period and secondly to find the best molecular tools and procedures to improve the monitoring of microbial populations and dynamics. These procedures seem to be essential to improve the understanding of the dynamics of adaptation of microorganisms and therefore to minimize methane losses during stress periods.

7.6 Acknowledgments

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Chapter 8

General discussion

The use of animal slurry as a substrate for anaerobic digestion not only decreases atmospheric methane (CH_4) emission from livestock, but also produces renewable energy. However, the use of animal slurry in anaerobic digestion plants may lead, under different circumstances, to the appearance of inhibitors or overloads that can produce process imbalances. The present PhD thesis aimed at studying the dynamics of inhibition and recovery in the anaerobic digestion process of animal slurry. It addressed the search for physicochemical and microbiological process state indicators which predict process failures under these circumstances. In this chapter, the main results from this PhD thesis are discussed in a broader context and their implications on best management practices at biogas plant level to minimize CH_4 losses are described.

8.1 Dynamics of anaerobic animal slurry degradation: effect of imbalances

The anaerobic degradation of animal slurry, from the initial breakdown of organic polymers to the production of CH_4, is a complex process which depends on the syntrophic relationship among three groups of microorganisms: acidogens, acetogens and methanogens. In this PhD thesis, the dynamics of these groups of microorganisms has been acknowledged in storage conditions with aged pig slurry (Chapter 3) through different physicochemical parameters and gaseous emissions as indicators.

Our results showed a decrease in total solids (TS), volatile solids (VS) and fiber fractions and an increase in dissolved chemical oxygen demand (COD_d), volatile fatty acids (VFA) concentration, carbon dioxide (CO_2) and ammonia (NH_3) emission during the first five weeks of storage. This means that a relative transformation of the most degradable organic matter (OM) into soluble OM took place during the first five weeks of storage, probably due to microbial activity of acidogens and acetogens. In this thesis we have also observed that a higher initial content of OM in the substrate (expressed as higher VS content in the pig slurry) increased OM degradation rates. The final step of anaerobic OM degradation (methanogenesis) took place 6 weeks after storage. This step

was indicated by a decrease in COD_d and VFA concentration, coinciding with the increase in the CH_4 and CO_2 production. Our results indicated that both physicochemical parameters and gaseous emissions can serve as appropriate indicators of the anaerobic degradation of animal slurry process.

On the other hand, according to our results, in thermophilic batch anaerobic digestion of pig and cattle slurry (Chapter 4) it was more difficult to detect these steps using intermediate metabolites as physicochemical indicators. This can be due to the fact that in anaerobic digesters, an inoculum is added in which presumably all microbial groups involved in the anaerobic digestion process are present and balanced. For this reason, no accumulation of intermediate metabolites such as VFA is generally observed during the regular anaerobic digestion process. Nevertheless, when imbalances occur in the anaerobic digestion process (Chapters 4 to 7), an accumulation of intermediate metabolites occurs probably due to differences in growth velocities and sensitivity to environmental conditions among the microbial groups involved in the process.

In this regard, throughout this thesis, different situations were created in which imbalances of the anaerobic OM degradation process were induced: slurry removal from the pit and a change in the environmental conditions (Chapter 3), the addition of sulfate (SO_4^{2-}) or SO_4^{2-} acidified slurry in anaerobic digesters (Chapters 4 and 5) and the inclusion of animal slurry in non-adapted anaerobic digesters (Chapter 6 and 7). In all these situations, the effects observed in digesters' health and performance were similar during and after process imbalance. These effects include a decrease in CH_4 production, an accumulation of intermediates metabolites (VFA), and a reduction in the removal efficiency of VS.

Therefore, monitoring of both CH_4 production and intermediate metabolites can provide useful information to anticipate to reactors' inhibition and prevent process imbalances.

8.2 Evaluation of process state indicators in anaerobic digestion using animal slurry

8.2.1 Gases

Biogas produced during the anaerobic degradation of animal slurry is composed mainly by CO_2 and CH_4 but also by other gases like NH_3 and hydrogen sulfide (H_2S). In this thesis, different gases have been monitored as indicators of microbial activity. Among these gases, CH_4 is the most clearly related with anaerobic degradation of OM, and specifically with methanogenic activity, since CH_4 is exclusively produced by methanogenic archaea. Carbon dioxide, however, is produced during both aerobic and anaerobic OM degradation and urea mineralization. On the other hand, NH_3 is derived from the hydrolysis of proteins and urea in aerobic and anaerobic conditions and is mainly dependent on temperature and pH (Aarnink et al., 1995).

8.2.1.1 Methane

Methanogens are involved in the last step of the anaerobic degradation of OM hence, CH_4 production entails the establishment of all microbial populations involved in the process. Therefore, monitoring CH_4 production at biogas plant level is useful not only because CH_4 is the economic source in biogas plants, but also because CH_4 production can be used as a process indicator.

Methane production both in storage conditions and in anaerobic digesters depends on the environmental conditions and slurry composition. Throughout the present thesis, differences in slurry composition were appreciable and therefore differences in CH_4 yield were also observed among studies. For instance, relevant differences between pig and cattle ultimate CH_4 yields were obtained in Chapter 4 in this thesis (295±9.6 mL CH_4 gVS^{-1}, pig slurry and 225 ± 14.4 mL CH_4 $gVSi^{-1}$, cattle slurry).

Among the different factors affecting slurry composition, the age of the slurry was presented as an important variation factor in slurry composition affecting CH_4 production in Chapter 3. The VS biodegradability in slurry after long storage times is lower than that of the fresh slurry, because the non-degradable fraction increases with the age of the slurry (Sommer et al., 2004). Thus aged slurries are expected to produce less CH_4 than recently excreted slurries.

Monitoring CH_4 emission pattern during pig slurry storage allowed recommending a maximum period for outdoor storage to prevent significant losses of CH_4 of 6 weeks after the removal of the slurry from the pit and its placement in the experimental containers (Chapter 3). This is applicable to those slurry management systems which consist in a pre-storage below slatted floor during the whole fattening period followed by outdoor storage. Thus, based on our results, the recommended storage time of pig slurry in summer conditions in order to minimize CH_4 losses to the atmosphere could be established in 30 to 35 days (4 to 5 weeks).

Surprisingly, during the start-up of the anaerobic digestion process with pig slurry in thermophilic conditions, a similar period (36 to 43 days) was necessary to recover CH_4 (Chapter 7). However, when the anaerobic digestion process was inhibited by the addition of SO_4^{2-}, CH_4 production did not show signs of CH_4 yield recovery after 100 days. Therefore, our results indicate that both in storage conditions and in thermophilic anaerobic digestion, about 40 days are needed to re-establish the equilibrium among the different microbial groups involved in the methanogenesis process when an imbalance takes place. If CH_4 yield is not re-established after 40-45 of the stress period in thermophilic continuous stirred tank reactor (CSTR), it probably means that total CH_4 yield recovery is not possible.

Although CH_4 yield has shown to be a good indicator of anaerobic digestion process recovery, Ahring et al. (1995) stated that a good process indicator should be able to detect imbalances at an early stage. Therefore, it is interesting to investigate other intermediate metabolites different from CH_4, which are produced at earlier stages, as process

indicators to detect failure and to facilitate the recovery of the process. These indicators should be key factors in the process and easily measured on a routinely basis.

8.2.1.2 Hydrogen sulfide

Hydrogen sulfide is a gas produced by sulfate reducing bacteria (SRB) from the sulfur present in SO_4^{2-} or amino acids in anaerobic environments. Produced hydrogen sulfide (H_2S) is distributed between H_2S in the gas phase and sulfide ions (HS^- and S^{2-}) in the liquid phase depending on the pH (Isa et al., 1986). In the present thesis we have monitored H_2S gas in order to identify SRB activity (Chapter 4 and Chapter 5). Sulfate reducing bacteria can compete with methanogens (Rintala and Lettinga 1992), and therefore their establishment can cause a decrease in CH_4 yield. We have stated that when SO_4^{2-} is present at high concentrations in animal slurry, it is reduced almost entirely and converted into H_2S which escapes from the anaerobic digestion forming part of the biogas (Chapter 5).

From our results (Chapter 4 and Chapter 5) we can state that when no inhibition caused by sulfate occurred, the H_2S concentration in biogas was around 0.05%-0.3%. However in cases of sulfate inhibition, H_2S concentration increased until around 1%-3%. Therefore, monitoring H_2S can be useful to elucidate if SRB populations are established in the digester and provides an indication of process imbalances.

In addition, H_2S resulting from SRB activity is a corrosive gas which can negatively affect heat and power engines (Cirne et al., 2008). Therefore, H_2S should be monitored in the biogas and removed from it prior to its energetic use.

8.2.2 Sludge's physicochemical indicators

8.2.2.1 Removal efficiency of volatile solids

The percentage of VS degraded from the slurry during the anaerobic digestion process (removal efficiency) is considered a key factor in the

design and control of the efficiency of biogas plants. It indicates the ability of microbial populations to convert VS into CH_4 and CO_2. In stable conditions, the removal efficiency obtained in this thesis ranged between 72% in digesters fed with broiler diet to 30% at the end of the recovery period in digesters fed with pig slurry (Chapter 7). Reported values for removal efficiency in anaerobic digestion of animal slurry are around 50% (Angenent et al., 2002). Removal efficiencies depend on substrate composition and its biodegradability. Animal slurry has a low biodegradability rate, especially aged pig slurry (Chapter 3). This fact can explain the lower VS removal efficiency obtained in digesters fed with aged pig slurry (25-30%) at the end of the recovery period (Chapter 6 and Chapter 7) compared with that obtained in Chapter 5 (50-52%) using fresh pig slurry.

During SO_4^{2-} inhibition (Chapter 5), removal efficiency ranged from 26% to 65%. Nevertheless, it did not coincide with the maximum and minimum CH_4 production, respectively. Therefore, removal efficiency was not a useful indicator to detect process failures in this thesis. Differences between removal efficiency fluctuations and CH_4 yield could be due to two main factors. Firstly, in VS determination, VFA losses during drying are generally not taken into account and therefore potential substrates for aceticlastic methanogens, such as acetic acid, may be underestimated. Secondly, the compounds used by hydrogenotrophic populations to produce CH_4, hydrogen (H_2) and CO_2, are not determined in VS and thus, during imbalances it is difficult to find a direct relationship between CH_4 production and degradation of VS.

In this thesis, VS determination has been reconsidered (Chapter 4 and 5) taking into account VFA losses during drying according with Derikx et al. (1994) and Rico et al. (2007). Volatile fatty acid losses during the drying process (24 hours at 100ºC, in accordance with the procedure described by APHA, 2005) were calculated in Chapter 4 and a proposal to include VFA when determining VS removal efficiencies was established in Chapter 5 based on the results obtained in Chapter 4 and the recommendations proposed by Derikx et al. (1994):

- In slurries with a pH below 7: 100% of VFA should be included in VS determination.
- In slurries with a pH between 7 and 8: 80% of VFA should be included in VS determination
- In slurries with a pH higher than 8: 10% of VFA should be included in VS determination.

8.2.2.2 Volatile fatty acids

It is widely assumed that VFA concentration is one of the most important parameters for controlling the anaerobic digestion process. Its accumulation implies an uncoupling among the three main microbial groups involved in the process (acidogens, acetogens and methanogens) (Ahring et al., 1995). However the specific role of the individual VFA in the overall anaerobic process is under debate and is still not completely understood (Pind et al., 2003).

Throughout the present thesis, total and individual VFA were monitored and related with process imbalances and the establishment of the different microbial groups, both during slurry storage conditions and in anaerobic digesters under stressful conditions. Although in all process imbalances induced in this thesis there was an accumulation of total VFA; there were differences concerning individual VFA accumulations among imbalances. In fact, throughout this thesis, a similar behavior was observed between acetic and butyric acid, while propionic acid presented a totally different behavior in all studied cases.

In the case of inhibition by SO_4^{2-} (Chapter 4 and Chapter 5), acetic and butyric acids presented the highest increases compared to the initial values. Within these two individual VFA, butyric acid was more strongly correlated with CH_4 yield ($R=-0.71$, $P<0.05$) than acetic acid (Chapter 5). Therefore, butyric acid could be considered a good predictor of instability in the anaerobic digestion process of acidified slurry. Ahring et al. (1995) reported that butyric acid was the best predictor in thermophilic CSTR after imposed perturbations (alteration of the

hydraulic reaction time, organic load and temperature). Propionic acid concentration, however, was very low in acidified pig slurries. Sulfate reducing bacteria consume propionic acid and this could explain these finding. Little is known, however, about this subject in the literature.

In the case of slurry storage (Chapter 3) and the start-up of anaerobic digestion of animal slurry with unadapted digesters (Chapter 7), propionic acid showed the highest increment and took the longest time to recover compared with acetic and butyric acids. Propionate degrading bacteria have a lower growth rate than the rest of acidogenic and acetogenic bacteria, both in outdoor storage conditions (Nozhevnikova et al., 2000; Møller et al., 2004) and in anaerobic digesters (Griffin et al., 1998; Calli et al., 2005). Therefore, the decrease in propionic acid concentration could be used as an indicator of process recovery when changes in substrate composition using pig slurry occur.

Overall, VFA monitoring can be used as an indicator of process failure. However, it is not feasible to give a VFA threshold in order to detect process imbalances since its concentrations are strongly dependent on the substrate composition. Animal slurry has a naturally high VFA content due to the fact that its OM is partially degraded during slurry storage (Møller et al., 2004). Volatile fatty acid concentration of the different pig slurries used in this thesis ranged from 1.2 (Chapter 6 and 7) to 13.5 g L^{-1} (Chapter 4 and 5). Due to the high content of VFA in animal slurry, a high content of VFA in anaerobic digesters treating this substrate is also expected. For this reason, the inhibitory thresholds reported in the literature for anaerobic digestion process (0.6 g total VFA L^{-1}, provided by Ferrer et al., 2010 and 50 mM provided by Ahring et al., 1995) can easily be exceeded in cases of anaerobic digestion of pig slurry in stable conditions, as shown in this thesis (Chapter 5 and Chapter 7).

8.2.2.3 Alkalinity

Alkalinity (ALK) determination has been suggested as an indirect method to determine VFA accumulations (Ferrer et al., 2010). Alkalinity determination presents some advantages compared to VFA

determination. Volatile fatty acids must be determined by chromatography, this procedure is expensive and not always available at biogas plant level; ALK however is an easily, and inexpensively methodology commonly used at biogas plant level (Ripley et al., 1986).

In this thesis, partial alkalinity (PA) was highly correlated with total VFA and acetic acid showing a linear and negative relationship ($R>-0.93$, $P<0.05$) and intermediate alkalinity (IA) was linear and positively correlated with total VFA, acetic and butyric acids ($R>0.94$, $P<0.05$) in acidified pig slurry (Chapter 5). However, no correlations were found among ALK parameters and propionic acid concentration, probably because propionic acid was stable during this experiment. Poor correlations among propionic acid and ALK parameters were also found by Ferrer et al. (2010) during imbalances caused by a decrease in the sludge retention time in thermophilic anaerobic digesters working with sewage sludge. As obtained in this thesis, Ferrer et al. (2010) found good correlations among IA and total VFA and acetate concentration. Therefore, in cases where there is no availability to determine VFA concentration at biogas plant level, ALK parameters could be used to estimate them.

8.2.2.4 Inorganic sulfur species: Sulfide and Sulfate

In the present thesis we have monitored total inorganic dissolved sulfide species (TIDSS) and SO_4^{2-} content as potential indicators of process failure in cases of anaerobic digestion of acidified slurry. Our results indicate that TIDSS resulting from reduction of SO_4^{2-} is not a good indicator of process failure or inhibition, because in cases where inhibition was observed in anaerobic digesters, very low TIDSS concentration was measured in the digesters (Chapter 4 and 5), probably because as stated above TIDSS escapes with the biogas.

Nevertheless, SO_4^{2-} content in the slurry was the trait most closely related to CH_4 yield explaining 79% of its variation (Chapter 5), due to the high correlation between SO_4^{2-} content and CH_4 yield. Sulfate inhibition thresholds have been determined in this thesis, allowing

predicting the behavior of anaerobic digesters at different SO_4^{2-} concentrations. Therefore SO_4^{2-} content in the slurry seems to be a useful indicator to monitor process imbalances in acidified slurries.

8.2.3 Microbiological indicators

Two different methodologies in order to evaluate microbial population changes have been used in this thesis: a molecular technique based on quantitative real-time polymerase chain reactions (qPCR) (Chapter 6 and Chapter 7), and a microscopic technique, scanning electron microscopy (SEM) (Chapter 6).

8.2.3.1 Quantitative microbial abundance using quantitative real-time polymerase chain reaction

Using molecular techniques, selected primers are needed to identify specific groups of microorganisms. Due to the wide range of microorganism involved in the anaerobic digestion process, most studies focus on the use of specific primers to determine taxonomic hierarchies like orders, families or even domains. In the case of anaerobic digestion plants, as methanogens are considered of pivotal importance for the stability of the process, studies using molecular techniques are mainly focused on four main methanogenic orders: *Methanobacteriales*, *Methanomicrobiales*, *Methanoccocales* and *Methanosarcinales* (Liu et al., 2002; Karakashev et al., 2005; Zhu et al., 2011). Each of these orders can be classified into the hydrogenotrophic or aceticlastic metabolic route to produce CH_4, thus their dynamics and shifts can provide valuable information for understanding the process underlying anaerobic digestion.

In this thesis, we have used specific primers to evaluate the effect of a change in substrate (from broiler feed to pig slurry) in thermophilic anaerobic digestion on total bacteria, total archaea and total fungi microorganisms (Chapter 6) and the four main methanogenic orders within total archaea: *Methanobacteriales*, *Methanomicrobiales*, *Methanoccocales* and *Methanosarcinales* (Chapter 7). These analyses provided information

on the main domains, methanogenic orders and thus metabolic routes involved in the inhibition and recovery phases.

Results showed that although the main works in the literature are focused on methanogenic orders, bacteria dominated in anaerobic digesters during the recovery period after start-up strategies and fluctuated with changes in digester composition, especially with VFA. Liu et al. (2002) also stated important increments in total bacteria population during the start-up of anaerobic digesters working with dairy wastewater.

Concerning differences among the different orders of total archaea, during anaerobic digestion of pig slurry, hydrogenotrophic methanogens dominated over aceticlastic (Chapter 7). Within hydrogenotrophic methanogens there was a shift in the dominance from *Methanomicrobiales* to *Methanobacteriales*. During the period of *Methanobacteriales* dominance, the concentration of total bacteria gene concentration increased and VFA concentration was reduced, probably due to the syntrophic relationship between VFA-oxidizing bacteria and hydrogenotrophic methanogens described in the literature (Angenent et al., 2002). From these results, we suggest that dynamics of total bacteria and *Methanobacteriales* have a pivotal importance for the anaerobic digestion recovery. To further evaluate this hypothesis, studies of hydrogenotrophic population dynamics should be complemented with studies of syntrophic VFA-oxidizing bacteria population dynamics during inhibition and recovery periods. In fact, although the syntrophic relationship between VFA-oxidizing bacteria and hydrogenotrophic methanogens in anaerobic digesters have been described in the literature (Hansen et al., 1999; Karakashev et al., 2006), the role of each of these groups of microorganisms in the recovery processes has been only poorly described. This information could be particularly relevant when choosing an appropriate inoculum for the start-up anaerobic digestion process to minimize CH_4 losses.

Compared with physicochemical parameters traditionally used to detect process imbalances, results from Chapter 7 showed that qPCR presented

less accuracy in detecting changes in anaerobic digesters functionality, especially in determining differences among start-up strategies. This could be partly explained because using qPCR it is not possible to distinguish among living, non-living, dormant or extremely slow-growing cells (physiological state) and free DNA present in samples (Solera et al., 2001). Therefore, the interpretation of results is often ambiguous and changes in microbial populations might be damped by the detection and quantification of non-viable microorganisms or free DNA which are actually not playing any role in the process.

Nevertheless, information obtained with molecular techniques such as qPCR provides essential information on the different orders of archaea and also on their quantitative importance within the sludge. The isolation and quantification of archaea by means of other methodologies different from molecular techniques such as traditional cultures in specific media is not easy (Solera et al., 2001). Thus, the use of qPCR and other molecular techniques are necessary tools to improve the knowledge on the microbial dynamics in anaerobic digestion.

8.2.3.2 Morphological microbial changes evaluated by scanning electron microscopy

Scanning electron microscopy (SEM), a non-molecular technique consisting on the direct visualization of samples *in situ,* was used in this thesis to evaluate OM degradation and morphological microbial changes in anaerobic digesters during stressful periods (Chapter 6). Differences were observed in sludge characteristics during the adaptation to pig slurry; SEM observations were useful to distinguish the differences in dominant microbial morphotypes during the adaptation of an anaerobic digester to pig slurry.

Scanning electron micrographs provided an overview of the sludge evolution over time. By examining SEM micrographs, an adaptation of the original biomass to pig slurry was observed. The adaptation involved a change in morphological sludge's characteristics from homogeneous morphological characteristics composed by individual rods-shaped and large cocci-shaped cells to a higher morphological diversity. Additionally,

non-expected morphological structures resembling yeasts, fungi or actinomyces structures appeared in the anaerobic digester during the adaptation to pig slurry.

Thus, an advantage of this methodology is that there is no need to pre-define the groups of microorganisms and structures to be observed. If a representative examination of the sample is conducted, the most and less dominant morphotypes and structures (both expected and no expected) will be visualized. However, the identification of these structures solely by its morphology is not always possible and this methodology needs to be combined with others such as direct cultures, staining or molecular techniques to achieve microorganism identification.

Moreover, the role and activity of specific microbial groups or species that are currently still not fully described should be more extensively studied. Especially, the microbial groups promoting hydrolysis of slowly degradable OM like lignocelluloses which could have an important role in anaerobic degradation of animal slurry, like *Fungi* species or *Actinomyces*. Non specific qualitative techniques such as SEM could play a role in this field of research.

Morphological microbial changes cannot easily be used as an indicator of the anaerobic digestion process, but combined with molecular techniques, they can add value to quantitative microbial abundance determination using molecular techniques.

8.3 Best management practices in anaerobic digestion during stressful situations

Stressful periods in anaerobic digestion correspond mainly to changes in operational parameters or substrate composition and the appearance of inhibitors or overloads. It is known that special management difficulties exist during these moments and that drops in CH_4 production can be expected (Griffin et al., 1998; Angenenet et al., 2002). In this thesis, the dynamics of different parameters involved during stressful periods (SO_4^{2-} inhibition and a start-up) have been monitored. Also, different management practices have been tested in order to avoid inhibition or to

promote microbial adaptation to new conditions. Although strategies based on feeding patterns, dilution strategies and gradual changes have been evaluated to overcome changes in temperature (Palatsi et al., 2009a) and to avoid inhibition by long chain fatty acids (Palatsi et al., 2009b), little is known about the effects and best management techniques to overcome situations of acidification and changes in substrates in anaerobic digestion. From the results of this thesis, strategies to avoid SO_4^{2-} inhibition in anaerobic digestion of acidified slurries and to promote the start-up anaerobic digestion with pig slurry can be outlined.

8.3.1 Anaerobic digestion of acidified slurries: Sulfate thresholds

Added SO_4^{2-} concentrations higher than 2500 mg SO_4^{2-} L^{-1} in pig slurry and higher than 2000 mg SO_4^{2-} L^{-1} in cattle slurry caused reductions of more than 40% in CH_4 yield and an accumulation of VFA (Chapter 4). These results obtained in a batch assay, are in accordance with those obtained in CSTR working with normal pig slurry in co-digestion with acidified pig slurry (Chapter 5). In Chapter 5, inhibition started in the range from 1840 to 2820 mg SO_4^{2-} L^{-1} (pilot scale experiment), and at 3400 mg SO_4^{2-} L^{-1} (full scale experiment). Inclusion of 30% of acidified slurry in co-digestion with raw pig slurry caused a reduction of around 30% in CH_4 yield. Therefore, according to our results, added SO_4^{2-} concentration in pig and cattle slurries for anaerobic digestion should not exceed 2000 mg SO_4^{2-} L^{-1} (pig slurry) and 1500 mg SO_4^{2-} L^{-1} (cattle slurry).

Additionally in long term full-scale digesters, the drop in CH_4 yield, when slurry was co-digested with 30% of acidified slurry (3400 mg SO_4^{2-} L^{-1}), was not recovered after 100 days of slurry anaerobic co-digestion, indicating that methanogenic activity could not be completely reestablished in these conditions even after a long anaerobic digestion period. At this inclusion level of acidified pig slurry, probably a new equilibrium between methanogens and SRB is reached, in which low CH_4 yield can be expected.

Nevertheless, it seems that low SO_4^{2-} concentration can promote CH_4 production. In this thesis, SO_4^{2-} concentration of 500 mg SO_4^{2-} L^{-1} in a batch assay with pig and cattle slurry (Chapter 4) and a SO_4^{2-} concentration ranging from 400 to 1840 mg SO_4^{2-} L^{-1} in CSTR working with pig slurry (Chapter 5), increased CH_4 production by 20% from initial CH_4 yield. Therefore, an inclusion level between 10-20% of acidified slurry in anaerobic digesters would be recommendable to increase CH_4 yield. The explanation for the increase in CH_4 yield is unknown and further research is needed to confirm this effect in long term anaerobic digestion.

In practical conditions, the necessary SO_4^{2-} concentration in slurry to lower NH_3 emission is more than 10 times higher than the SO_4^{2-} threshold obtained in this work to prevent CH_4 losses (Chapter 4 and Chapter 5). Therefore, the use of acidified pig slurry itself in anaerobic digestion is not recommended. In these cases, mixing a certain percentage of acidified slurry with non-acidified slurry or other substrates in co-digestion is necessary. This would maintain SO_4^{2-} concentration in digesters below the inhibitory SO_4^{2-} threshold suggested in this study.

The co-digestion of acidified slurry with other substrates is an important alternative in anaerobic digestion not only because of the SO_4^{2-} dilution in the final substrate, but also because it can increase the OM concentration of the substrate and this is known to affect SO_4^{2-} inhibition threshold. In fact, O'Reilly and Colleran (2006) reported that methanogens dominated in anaerobic digestion of substrates with a ratio COD/SO_4^{2-} higher than 4, however, when the COD/SO_4^{2-} ratio was reduced to 2 the SRB assumed a more influential role than methanogens. In this regard, Sutaryo et al. (2012) co-digested the solid fraction of acidified slurry with non-acidified slurry with success. Despite alternative solutions have been tested in the literature to decrease SO_4^{2-} reduction by selective inhibition of SRB such as the addition of molybdate to digesters (Isa and Anderson, 2005; Peu et al., 2011), no successful results have been achieved.

The results obtained herein are applicable to pig and cattle acidified animal slurry but not to other SO_4^{2-} rich wastewaters since SO_4^{2-} inhibition threshold vary depending on factors such as the OM content, antagonism, synergism, and the acclimatization of bacteria populations (Siles et al. 2010).

8.3.2 Start-up anaerobic digestion: strategies to improve microbial adaptation to pig slurry

In this thesis, the effects of a feedless strategy and an abrupt *vs.* gradual change in substrate were studied to overcome anaerobic digestion imbalances when a change in substrate is performed (Chapter 7). Feedless resulted in a poor strategy to improve adaptation to pig slurry. In fact, digesters that did not undergo a feedless period showed better performances during the recovery period than digesters subjected to a feedless period. Improved performance consisted in less VFA accumulation, faster CH_4 yield recovery and higher gene copies number of total archaea, *Methanosarcinales* and *Methanomicrobiales*. Hwang et al. (2010) also observed similar affects on microbial populations after a feedless period in batch anaerobic digesters.

The rate at which substrate was changed (abrupt *vs.* gradual) seemed to have a lower effect on the recovery of the digesters than the feedless strategy. Paradoxically, the digesters showing the best performances (lower VFA accumulation and lower CH_4 yield decrease after the change in substrate) during the recovery period were non-feedless digesters with an abrupt change of substrate. Therefore, neither feedless nor gradual change in substrate prevented the inhibition of CH_4 production period after the addition of pig slurry to a digester. Other strategies based on feeding patterns like slurry dilution with active inoculum from digesters could be tested.

8.4 References

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Chapter 9

Conclusions

Under the experimental conditions of this PhD thesis, the following conclusions can be drawn:

1. In the anaerobic degradation of organic matter (OM) from aged pig slurry, there is a relevant transformation of the more degradable OM into soluble OM during the first three weeks of storage. This was stated by decrease in total solids (TS), volatile solids (VS) and fiber fractions and an increase in dissolved chemical oxygen demand (COD_d), volatile fatty acids (VFA) concentration, carbon dioxide (CO_2) and ammonia (NH_3) emission. This transformation was promoted at higher OM levels.

2. Methane (CH_4) production does not occur until five weeks of pig slurry storage in anaerobic conditions. At this time, a reduction in VFA concentration was observed.

3. Added sulfate (SO_4^{2-}) concentration in pig and cattle slurries for anaerobic digestion should not exceed 2000 mg SO_4^{2-} L^{-1} and 1500 mg SO_4^{2-} L^{-1} respectively. Otherwise reductions in CH_4 yield, increases of hydrogen sulfide (H_2S) in biogas and VFA accumulations (acetic and butyric acids) might occur. Although, the use of acidified pig slurry itself in anaerobic digestion is not recommended, a SO_4^{2-} concentration of 500 mg SO_4^{2-} L^{-1} in pig slurry can enhance CH_4 production.

4. The most important physicochemical process state indicators to detect a process failure in the anaerobic digestion using acidified pig slurry are: SO_4^{2-} content of the slurry, alkalinity parameters (especially partial alkalinity and the ratio of alkalinity), total VFA, acetic and butyric acids.

5. The addition of pig slurry to an unadapted thermophilic anaerobic digester caused an increase in VFA concentration a decrease in removal efficiency and CH_4 yield.

6. In terms of microbial changes, the addition of pig slurry in anaerobic thermophilic conditions caused an increase in total bacteria and archaea population determined by quantitative real-time polymerase

chain reaction (qPCR), however no differences were observed in total fungi population. A change in microbial morphotypes from homogeneous cell morphologies to a higher morphological diversity, similar to that observed in pig slurry, was observed by scanning electron microscopy (SEM). The use of combined qPCR and SEM methodologies is recommended for exploring microbial adaptation to new substrates.

7. The start-up strategy in anaerobic thermophilic digestion of pig slurry which best minimized CH_4 losses was non-feedless followed by an abrupt addition of pig slurry, although differences in microbial population among treatments were low.

8. Hydrogenotrophic methanogenesis is the main metabolic route in CH_4 formation after the start-up in anaerobic digestion of pig slurry in thermophilic conditions. *Methanomicrobiales* first and *Methanobacteriales* second, were the dominant archaeal orders throughout the adaptation period. The dynamics of total bacteria and *Methanobacteriales* could serve as microbiological process state indicators for predicting process recovery.

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