PhD Thesis

UPC – Program on Environmental Engineering

ANAEROBIC DIGESTION OF ANIMAL BY-PRODUCTS
Pre-treatments and co-digestion

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A mis padres, Paco y Tati.

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RESUMEN

El sector cárnico lleva asociada la generación de grandes cantidades de subproductos animales no destinados al consumo humano (SANDACH). La demanda de fuentes de energía renovables y la reutilización de los residuos requieren soluciones tecnológicas tales como la digestión anaerobia (DA), proceso incluido en el reglamento europeo actual como uno de los métodos permitidos para valorizar estos subproductos. Debido a su composición rica en grasa y proteína, los SANDACH son considerados buenos sustratos para la DA, dado su elevado rendimiento teórico de producción de metano, aunque se han notificado tasas de hidrólisis lentas y procesos inhibitorios. Con un pretratamiento adecuado, que mejore la solubilidad de los materiales particulados, y/o un proceso de codigestión con residuos complementarios, la DA se podría mejorar.

El objetivo de la presente tesis es evaluar la viabilidad de diferentes SANDACH para la DA, centrándose principalmente en el efecto de los pretratamientos sobre la materia orgánica, el rendimiento y la tasa de producción de metano. Para ello se emplearon pretratamientos térmicos (pasteurización y esterilización) y de altas presiones (200, 400 y 600 MPa). Posteriormente, se utilizaron técnicas termogravimétricas y de espectroscopía (DTG-MS y FTIR), además de una caracterización clásica, para determinar los efectos sobre la materia orgánica, mientras que los efectos sobre la producción de metano, incluyendo los parámetros de desintegración, se obtuvieron por medio de ensayos discontinuos con diferentes ratios inoculo/sustrato. La idoneidad de los SANDACH para la DA fue confirmada con residuos de mataderos avícola y porcino, pero los resultados mostraron que el rendimiento de metano dependía de la composición relativa (proteínas, grasas e hidratos de carbono) de cada sustrato, especialmente en el caso de los pretratamientos térmicos. Se observó que cuando el residuo contenía una elevada concentración de hidratos de carbono y proteínas, se generaban compuestos nitrogenados recalcitrantes durante el pretratamiento térmico que afectaban negativamente a la tasa de producción de metano. Por el contrario, cuando el residuo tenía una concentración elevada de proteína y grasa, tanto la tasa de producción como el rendimiento de metano aumentaron tras la pasteurización y esterilización. Estos resultados fueron confirmados mediante el estudio de la cinética de desintegración. En último lugar, el pretratamiento a altas presiones, aplicadas a residuo de matadero de cerdo, no modificó la biodegradabilidad ni el rendimiento de metano del residuo.

En paralelo, se estudió la codigestión en continuo de SANDACH pasteurizado con purín porcino y glicerina, optimizándose la producción de biogás mediante el control de la composición de la mezcla de residuos a tratar. Se observaron cambios en las poblaciones microbianas (monitorizados mediante DGGE) y se comprobó que las eubacterias fueron la comunidad dominante, aunque también más sensible a los cambios operacionales que las arqueas. En conclusión, la producción de biogás a partir de SANDACH se ha mostrado factible, mejorando su DA mediante codigestión con otros residuos que permitan equilibrar su composición y un pretratamiento térmico, siendo la eficacia de este muy dependiente de la composición en proteínas e hidratos de carbono.

ABSTRACT

The meat sector is associated with the generation of large quantities of animal by-products not intended for human consumption (ABPs). The increasing demand of renewable energy sources and reuse of wastes require good technological solutions for energy production such as anaerobic digestion (AD), which is included in the current European regulation as one of the allowed methods to valorize ABPs.

Due to their composition, with high fat and protein content, ABPs can be considered good substrates for the AD process, according to the high potential methane yield. Although, slow hydrolysis rates and inhibitory process have been reported, with a suitable pre-treatment to improve particulate materials solubility and/or co-digestion process of complementary materials the anaerobic digestion can be improved.

Hence, the aim of this thesis was to evaluate the feasibility of different ABPs for anaerobic digestion. Emphasis was placed on the effect of pre-treatments on the organic matter, methane yield and methane production rate. Within this scope, thermal (pasteurization and sterilization) and high pressure pre-treatments (200, 400 and 600 MPa) were applied. Thermogravimetric and spectroscopy techniques (DTG-MS and FTIR), where used to determine the effects on the organic matter besides to a classical characterization. The effects on the methane yield and methane production rates, including the disintegration parameters, were obtained by means of batch test with different inoculum to substrate ratios (ISR).

The suitability of ABP for anaerobic digestion was confirmed with samples from different origin (poultry and piggery slaughterhouses) but the results showed that methane yield depends on the relative substrate composition (proteins, fats and carbohydrates), especially when a thermal pre-treatment is applied. The thermal pre-treatment produced inhibitory nitrogen-related compounds when there was a high carbohydrate and protein concentration that affected the methane potential rate. On the other hand, thermal pre-treatments (pasteurization and sterilization) increased the methane production rate and methane production yield in the case of a waste with high protein and fat concentration. The results of the disintegration kinetics determination underline these positive effects on the methane production rate being increased in the after pasteurization. The high pressure pre-treatments were tested with piggery ABP without obtaining any effect on the methane production or methane production rate.

In parallel, continuous co-digestion of pasteurized ABP with pig manure and glycerin was studied, optimizing the production of biogas by controlling the composition of the mixture of wastes to be treated. Changes in the microbial populations (monitored by DGGE) were determined and it was also observe that eubacteria was the dominant community but more sensitive to operational changes than archaea. In conclusion, the production of biogas from SANDACH has proved to be feasible by improving their DA by means of co-digestion with other wastes that balance the composition and thermal pretreatment being its effectiveness highly dependent on the composition of proteins and carbohydrates.

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NOMENCLATURE

A	acidogenesis index (%)
AB	anaerobic biodegradability
ABP	animal by-product
amu	unified atomic mass units
BMP	biological methane potential
CHP	combined heat and power
CH_4	methane
COD	chemical oxygen demand
COD_{ch}	COD from carbohydrate fraction
COD_{fat}	COD from fat fraction
COD_{prot}	COD from protein fraction
COD_s	soluble chemical oxygen demand
COD_t	total chemical oxygen demand
$COD_s \cdot COD_t^{-1}$	ratio soluble chemical oxygen demand and total
CSTR	continuously stirred tank reactor
C/N	carbon to nitrogen ratio
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DTG	differential thermogravimetry
FID	flame ionization detector
Fig.	figure
FT-IR	Fourier transform infrared spectroscopy
F:P:C	fat to protein to carbohydrate ratio
G	glycerin
GC	gas chromatography analysis
HEM	n-hexane extractable material
HRT	hydraulic retention time (days)

IR	infrared spectroscopy
LCFA	long chain fatty acid
M	methanization index (%)
mcrA	methyl-coenzyme M reductase sububit α
MPP	methane production potential
MPR	maximum methane production rate
MS	mass spectroscometry
Msa	Methanosaeta
Msr	Methanosarcina
$m \cdot z^{-1}$	ratio mass to charge
NA	not analyzed
$N\text{-}NH_4^+$	ammonium nitrogen
$N\text{-}NH_3$	ammonia nitrogen
ND	not detected
OLR	organic loading rate ($kg_{VSadded} \cdot m^{-3} \cdot d^{-1}$ or $kg_{CODadded} \cdot m^{-3} \cdot d^{-1}$)
PCR	polymerase chain reaction
PM	pig manure
PTI	pasteurized poultry waste
PTII	pasteurized piggery waste
rpm	revolutions per minute
rDNA	ribosomal deoxyribonucleic acid
STII	sterilized piggery waste
t	time
TCD	thermal conductivity detector
TGA	thermogravimetric analysis
TGA-MS	TGA coupled to mass spectrometry
TI	poultry slaughterhouse waste used in thermal experiment
TII	piggery slaughterhouse waste used in thermal experiments

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TIII	piggery slaughterhouse waste used in high pressure experiments
TN	total nitrogen
TS	total solids
TSS	total suspended solids
UASB	up-flow anaerobic sludge blanket
VFA	volatile fatty acid
VS	volatile solids
VSS	volatile suspended solids
%S	degree of solubilization
%TAN	ratio of the ammonium nitrogen to total nitrogen concentrations

Chapter 1. Context, objectives and thesis outline

This Chapter introduces the importance of treating anaerobically solid animal by-products to avoid environmental and health problems and to recover energy. The current European legislation framework related to this topic is taken into account. Finally, the objectives and the thesis outline are depicted.

1.1. CONTEXT

The meat sector is one of the most important in the European Union and in the case of Spain it is one of the top five industries. It is placed after the vehicle industry, the oil and fuel industry and the production and distribution of electricity, while revenues match with the chemical industry (Blancafort, 2009). This sector is associated with the generation of large quantities of materials from animal origin, not intended for human consumption because of their nature or by decision of the operator (for commercial reasons or defects), and being denominated animal by- products not intended for human consumption (ABPs).

In Spain, the meat sector is comprised of slaughterhouses (700), cutting plants (2400) and processing industries (5170) (Cruz, 2012), with a structure constituted mostly of small and medium companies.

It has been estimated that the population in Europe consumes about 68% of the chicken's body, between 62-75% of the pig, a 54-60% of the cow and 52-58% of the goat and / or sheep (MEMO/04/107, 1999). Therefore each year, only in Europe, there is about 16 million tons of meat not intended for human consumption although derived from healthy animals (Kirchmayr *et al.*, 2010). In the case of Spain, the meat production in 2011 was more than 5 million tons (Figure 1.1) and having into considerations the percentage of animal consumption mentioned before, the yearly ABP production is estimated around 2.5 million tons.

The composition of ABPs generated depends largely on whether slaughterhouses are specialized, if they are centralized or not, or if one or more types of animals are processed (Alvarez and Liden, 2008) but, in general, the slaughterhouse by-products are characterized by their consistency, with a high solids content, comprising proteins, lipids, carbohydrates, minerals, vitamins, and water in various proportions (Madrid, 1999). Depending of the ABP type, its characteristics are different (Table 1.1).

Until the appearance of the bovine spongiform encephalopathy disease (BSE), the usual fate of the ABPs was the transformation into meat and bone mill, used in animal feeding and produced by a thermal process called rendering, which consists of grinding, cooking and pressing the animal by-products. Solid and liquid parts are separated, water is evaporated and fat is separated from proteins and bones (Woodgate and Van der Veen, 2004). The finished fat and the solid protein are pressed into cake for processing into crude animal feed.
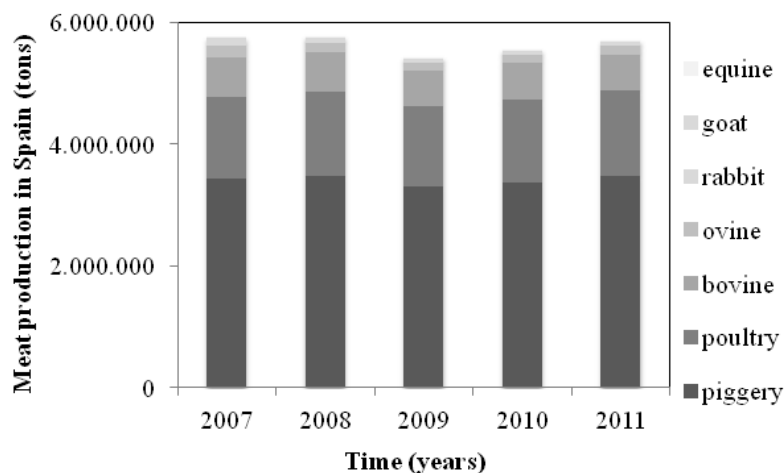
Figure 1.1. *Meat produced for human consumption in Spain. Graph elaborated with data from Cruz (2012).*

Table 1.1. *Characterization of different animal by-products (Palatsi et al., 2011). n.d.: not determine; Protein and COD are estimated.*

	TS	VS	NTK	NH$_4^+$	Protein	Fat	COD
	%	%	g·kg^{-1}	g·kg^{-1}	g·kg^{-1}	g·kg^{-1}	g·kg^{-1}
Cow meat and fat	88.6	85.4	3.2	0.1	19.1	762.8	2294.5
Pig meat and fat	56.6	55.7	13.8	0.4	83.8	467.3	1473.5
Confiscates	24.5	22.0	26.3	1.5	155.0	46.6	385.4
Pig stomach	18.3	18.0	12.4	1.6	67.1	86.6	376.9
Rumen content	11.7	10.9	1.3	0.1	7.9	18.4	152.0
Waste blood	19.7	18.4	31.7	0.1	197.3	n.d	262.7
DAF sludge	9.6	8.3	5.9	0.8	31.3	50.3	192.4
Wastewater	0.1	0.1	0.15	0.1	0.4	0.1	0.9

After this health crisis, new European regulations were established as the legal framework (Nº 1774/2002, Nº 92/2005, Nº 1069/2009 and Nº 142/2011) to enhance bio-security and to limit possible environmental problems associated with animal by-products. According to these regulations, ABPs and their process products and wastes are classified into three categories, depending on the risk they pose towards human, animals and environment. These European regulations also determine their potential use and define the corresponding treatment.

The categories, described in European Regulation Nº 1069/2009, are:

- **Category 1**, for materials with a higher risk and usually disposed of by incineration or co-incineration. There are also other options for treatment such

as burial in licensed landfill after sterilization at high pressures, but its use are very limited. Examples of this category are wastes with specified risk materials derived from animals that have been administered banned substances or catering waste from means of transport operating internationally.

- **Category 2**, used for those materials that present an intermediate risk. Uses of these materials are different from animal feed (except fur animals). Examples include manure and digestive tract content, animals or parts of animals that died without being slaughtered or killed for consumption, including the eradication of diseases, fetuses, dead poultry in the egg etc. The materials in this category, depending on type, can be incinerated, co-incinerated, deposited in landfills, composted, or used to produce biogas and even being applied directly to soil (manure). It is necessary to note that many of these options require a pre-processing method (such as thermo-chemical pre-treatment, high pressure and high temperature process or sterilization).

- **Category 3,** where ABPs with a lower risk are included. Hence, the uses are broader than the other two categories, including in some cases the manufacture of feed for farm animals and pets. Depending on the material and end use, there are also necessary pre-processing methods with heat involved (mainly pasteurization or sterilization). Some of these materials, included in category 3, are the carcasses and parts of slaughtered animals or carcasses or parts of animals killed, in the case of hunted animals that are for human consumption but not intended for commercial reasons. Other ABPs that belong to this category are blood, placenta, wool, feathers, horns, hooves cuts, nails, hooves and raw milk from live animals that do not show signs of diseases communicable to humans or animals, sludge centrifuge or separation resulting from the processing of dairy products and many other materials (Nº 1069/2009).

The slaughterhouses will get daily a several slaughterhouse by-products such as blood, bones, hooves, contents of the rumen, stomach, intestines, manure etc. Around 84% of the national production of ABPs in Spain is included in category 3, 2% in the category 2 and 14% in the 1, the highest risk category (SANDACH white book, 2012). Regardless of the specific situation in each process (slaughterhouse, cutting and cured meat products etc.), the main environmental issues associated with the meat industry are the energy and water consumption, and also the generation of wastewater and wastes.

Due to the ABPs composition, they are energy-rich materials and can be good substrates for anaerobic digestion, one of the energy recovery processes included in the current European Regulations (Nº 1069/2009).

Anaerobic digestion is a promising and sustainable technology that mineralizes the organic matter in the waste and, at the same time, recovers energy in the form of methane and recovers nutrients when the digestion effluents (digestate) is used as source of fertilizer for agricultural crops (Salminen and Rintala, 2002).

Only materials from categories 2 and 3 can be anaerobically digested if sanitation treatment is included before or after the biogas process in order to guarantee the hygienic quality of the digestates (Nº 142/2011). Materials of category 2 must be sterilized (at 133 ºC and 3 bar for 20 min, with a particle size < 50 mm) and materials of category 3 must be pasteurized (at 70 ºC for 60 minutes with a particle size < 12 mm).

The limitations for the use of ABPs in anaerobic digestion are the slow hydrolysis of the particulate matter, the presence of some hardly degradable components, the inhibition by ammonia nitrogen and long chain fatty acids (LCFA). These inconveniences can be solved with a suitable pre-treatment helping to decrease the particle size and solubilization of solid materials, carrying out co-digestion with one or several materials, dilution the inhibitors concentration, or other strategies, like adaptation of the biomass.

Although in the last years many works about anaerobic digestion of slaughterhouse waste have been done, there is still a lack of information in areas such as the effect of thermal pre-treatments on the organic matter and its biodegradability, on the kinetics of disintegration and hydrolysis, co-digestion and the dynamics of the microbial communities involved in ABP anaerobic digestion.

1.2. OBJECTIVES

The main objective of the present work was to evaluate the feasibility of methane production from solid slaughterhouse wastes through anaerobic digestion by means of pre-treatments and co-digestion.

The partial or intermediate objectives were:

i)   To evaluate the effect of different pre-treatments on the solubilization, biodegradability and methane yields of animal by-products (ABPs) with

different compositions.

ii)   To study deeply the influence of the thermal pre-treatments on the organic matter by means of new methodologies as thermogravimetric analysis and Fourier transform spectroscopy.

iii)   To determine whether the thermal pre-treatment affects the disintegration kinetics constants of particulate and total fractions of treated ABPs.

iv)   To study mesophilic co-digestion of pre-treated ABP with pig slurry under semi-continuous operation using completely stirred tank reactors (CSTRs) and to evaluate the effect of glycerin addition on methane production.

v)   To investigate by culture independent molecular methodology the microbial biomass population changes during operation of the CSTR.

1.3. THESIS OUTLINE

A short explanation focusing the topic, the objectives and the thesis outline is introduced in this **Chapter 1.**

A general overview of every point of the thesis (anaerobic digestion process, pre-treatments, benefits of co-digestion, disintegration kinetics etc.) and the state of the art are presented in **Chapter 2**.

In **Chapter 3** two animal by-products, coming from a piggery and a poultry slaughterhouse with different composition related to fat, protein and carbohydrate concentrations are exhaustively characterized, and their methane production yields and rates are determined. Also the effects of thermal pre-treatments (sterilization and pasteurization) on the organic matter bioavailability, methane yield and methane production rate of these two animal by-products are described. The selected pre-treatments are allowed by European Regulations and could be considered as a hygienization method. The formation of Maillard compounds is proposed to explain the different behavior between piggery and poultry samples, where the main difference is the carbohydrate concentration.

A new COD determination methodology for complex solid wastes such as ABPs is extensively detailed in the attached information at the end of this document.

The hypothesis that thermal pre-treatments in some cases, depending on the substrate characteristics, can affect negatively the anaerobic digestion because of some nitrogen-

related new compounds (from Maillard reactions) is studied more deeply in **Chapter 4**. To reach this target, new methodologies in the anaerobic digestion field, such as thermal analysis (DTG), mass spectroscometry (MS) and Infrared technique (FTIR) are used.

Chapter 5 is dedicated to a novel high-pressure pre-treatment, as a previous step to anaerobic digestion of piggery animal by-products. The effects of 200, 400 and 600 MPa application, at environment temperature during 15 minutes, on the organic matter solubilization, bioavailability, methane yield and production rate are studied.

Several times in this thesis, the idea that thermal pre-treatment could improve the methane production rate is emphasized. To check it, in **Chapter 6**, the total and particulate fraction of untreated and pasteurized ABPs, from fishery and poultry facilities, are used in batch tests with different inoculum to substrate ratios to obtain a methane-accumulated curve with a logistic shape, in order to estimate the global disintegration-hydrolysis constant. A COD fractioning is also done. Other solid organic wastes (pig manure, sugar beet and coffee by-product) are used to contrast the method applied.

In **Chapter 7** the feasibility of anaerobic co-digestion of thermally pre-treated slaughterhouse wastes with manure, and in some periods with glycerin in order to improve the C/N ratio, is assessed under semi-continuous mesophilic operation in a completely stirred tank reactor. The behavior of the microbial community (eubacterial and archaeal populations) during all the changes in the operational parameters is determined by means of PCR-DGGE technique.

Finally, general conclusions and perspectives for further research in this topic are exposed in **Chapter 8.**

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Chapter 2. Anaerobic digestion of animal by-products

In this chapter, anaerobic digestion of animal by-products is introduced as the process to be enhanced by pre-treatments, being the main topic in this thesis. Basic concepts of pre-treatments and co-digestion are shown as methodologies to improve the anaerobic digestion process. A brief description of disintegration kinetics and dynamics of the anaerobic population were also highlighted. The results of this thesis were obtained from analytical determinations, batch and continuous reactors carried out in mesophilic range and from specific analyses such as DGGE, FTIR and TGA-MS.

2.1. ANAEROBIC DIGESTION PROCESS

Anaerobic digestion is a biological process where the organic matter is decomposed by different communities of microorganisms in the absence of oxygen, finally producing a gas with a high energy content called biogas and a liquid or semi-solid digestate. The digestate of the anaerobic digestion can be applied to agricultural areas as valuable fertilizer helping to recycle the most important nutrients for agricultural production, which marks an additional environmental benefit (Waltenberger *et al.,* 2010).

Such as other food industry processes, the slaughterhouses facilities accumulate different types of organic wastes and since the places where ABPs are produced and disposed are not the same, large quantities of energy are needed. Biogas production technology appears to be a good solution capable to integrate environmental protection and a method for saving energy. Since biogas can be utilized to generate thermal energy (heat) or electrical power through cogeneration in a combined heat and power plant (CHP), this explains the interest that anaerobic digestion raises over other alternatives of organic waste treatments (Flotats and Sarquella, 2008).

The produced biogas could be used also as vehicle fuel and/or injected to natural gas network to replace fossil fuels. The reduction of greenhouse gas emissions and the use of renewable energy interest are positive side effects of anaerobic digestion. Therefore, the use of ABPs in anaerobic digestion not only offers the possibility of sustainable removal of this type of waste, but at the same time recovers the energy in the form of methane and nutrients, when the digestate is used as source of fertilizer for agricultural crops (Salminen and Rintala, 2002).

Literature shows different results that have been obtained in the last decade related to the anaerobic digestion of ABP. Salminen *et al*. (2000) obtained yields of 0.55-0.67 $m^3_{CH4}·kg_{VS}^{-1}$ added in batch tests with mixture of bones, remains of cuts, blood and feathers and between 0.52- 0.55 $m^3_{CH4}·kg_{VS}^{-1}$ in a CSTR with an organic loading rate (OLR) of 0.8 $kg_{VS}·m^{-3}·d^{-1}$ and long hydraulic retention time (HRT), of 50-100 days, working with poultry solid waste as a substrate (Salminen and Rintala, 2002). At higher OLR and lower HRT the system was inhibited by accumulation of volatile fatty acids (VFA) and long chain fatty acids (LCFA). With the same mixture but in discontinuous experiments, Salminen *et al*. (2003) obtained results of 0.5-0.7 $m^3_{CH4}·kg_{VSadded}^{-1}$. This Finnish research group has also conducted experiments of co-digestion, digesting ABPs

from poultry slaughterhouse with food waste in mesophilic and thermophilic range. With these mixtures they obtained a methane production of 0.33 $m^3_{CH4} \cdot kg_{VS}^{-1}$, after improving the C/N ratio, at an OLR of 4.6 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$ and a HRT of 18 days (Salminen and Rintala, 1999). Digesting anaerobically pre-treated feathers they reached a gas productions of 0.21 $m^3_{CH4} \cdot kg_{VS}^{-1}$ (Salminen *et al.*, 2003).

In other experiments, Banks and Wang (1999) obtained values of 0.27 $m^3_{CH4} \cdot kg_{TS}^{-1}$ with rumen contents and blood in a two-phase anaerobic digestion process. Chen and Shyu (1998) investigated the application of the two-step anaerobic digestion process (UASB reactor and leach-bed) using as substrate dead animals (chickens), achieving a reduction of 86% total solids and methane yields of 0.20 $m^3_{CH4} \cdot kg^{-1}$ in wet weight of the animal. A negative result was obtained by Chen and Huang (2006) when digested anaerobically poultry solid waste (dead chickens) in thermophilic conditions with an UASB and leach-bed reactor. They had problems with the starting up of the device and there was inhibition of the methanogenesis due to the high VFA concentration.

2.1.1. Anaerobic digestion steps

The anaerobic decomposition of organic matter requires the combined activity of several groups of microorganisms with different metabolic capabilities (Zinder, 1984). The whole process can be divided into four steps, carried out by various microbial communities (Figure 2.2). These steps are disintegration and hydrolysis (1), acidogenesis (2), acetogenesis (3) and methanogenesis (4).
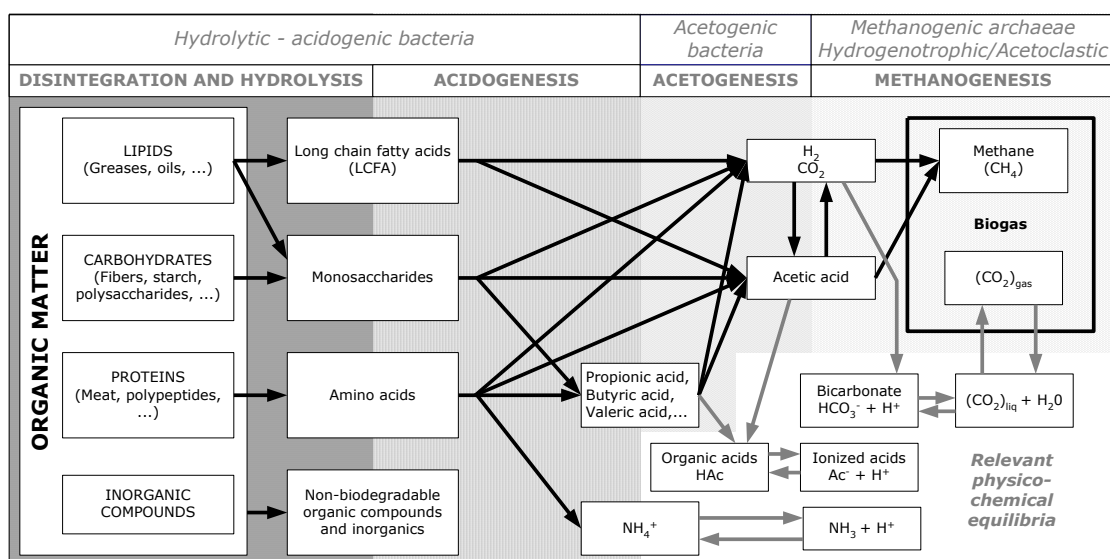
Figure 2.2. *Anaerobic digestion scheme with the different stages and communities involved (modified from Flotats et al., 2011).*

The **hydrolytic step** is the first stage of anaerobic digestion process and consists in the disintegration and hydrolysis of complex organic polymers such as lipids, proteins and carbohydrates to soluble compounds as simple sugars, long chain fatty acids, amino acids and alcohols, which can pass through the cell membrane (Batstone *et al*., 2000). These reactions are catalyzed by the action of extracellular enzymes (proteases, lipases and cellulases), excreted by hydrolytic fermentative bacteria.

Several factors can affect the degree and rate whereby the substrate is hydrolyzed (Lettinga, 1996), such as composition of the substrate (lignin content for example), the particle size, pH and temperature. The concentration of hydrolytic biomass is also important (Pavlostathis and Giraldo-Gomez, 1991).

Microorganisms from different genera are responsible for the reactions that occur in the hydrolysis step, as Propionibacterium, Bacteroides, Lactobacillus, Sporobacterium, Megasphaera, Sphingomonas and Bifidobacterium (Deublein and Steinhauser, 2011).

With complex substrates, hydrolysis can be the rate limiting step of the whole process (Miron *et al*., 2000; Massé *et al*., 2001; Pavlostathis and Giraldo-Gomez, 1991; Vavilin *et al*., 2008) and in some cases it is necessary to apply an initial pre-treatment to the residue to decrease the particle size, increasing the surface area for adsorption of hydrolytic enzymes. By means of pre-treatments lower hydraulic retention times and smaller reactor volumes could be achieved. The rate of hydrolysis, generally, is also increased with increasing temperature (Pavlostathis and Giraldo-Gomez, 1991; Veeken and Hamelers, 1999).

In the **acidogenic step**, soluble compounds obtained in the previous phase are converted by acidogenic bacteria into different fermentation products as volatile fatty acids (VFA), alcohols, ammonium, carbon dioxide and hydrogen. For example, LCFA are degraded to volatile fatty acids and hydrogen via β-oxidation. Because of the β-oxidation of LCFA is an endothermic reaction, the process is very dependent of the symbiotic action of H_2 consumer microorganisms. In methanogenic environments, where the only electron acceptors are protons and CO_2, interspecies hydrogen transfer between microorganisms plays a central role in LCFA degradation. In such conditions, these compounds are degraded by obligatory syntrophic consortia of proton-reducing acetogenic bacteria, converting LCFA to acetate and hydrogen, and hydrogen-consuming methanogenic archaea, reducing bicarbonate to methane (Schink, 1997). Another example is glycerol

that is degraded mainly to acetate, lactate and 1,3-propanediol by fermentative bacteria (Biebl *et al*., 1999).

Many of the bacteria that are able to perform this stage are also involved in the hydrolysis (Deublein and Steinhauser, 2011) and they belong to the taxonomic groups of Clostridia, Bacilli, Bacteroidetes and Actinobacteria (Souidi *et al*. 2007; Krause *et al*. 2008).

In the **acetogenic step**, the intermediate compounds that were generated in the acidogenic phase are oxidized by proton-reducing acetogenic bacteria to an appropriate substrate for microorganisms of the methanogenic stage, such as acetic acid, hydrogen and carbon dioxide.

Hydrogen partial pressure should be low in order to preserve favorable thermodynamics for the conversion of VFAs to acetate. This requirement is achieved by syntrophic association with hydrogenotrophic methanogenesis that maintain the hydrogen partial pressure at low levels, allowing syntrophic acetogenesis to be active. The most common representatives of this group belong to these orders: *Syntrophomonas, Syntrophobacter, Clostridium* and *Acetobacterium* (Hattori, 2008; Weiland, 2010). If hydrogen is not consumed, acetogenesis is inhibited, causing accumulation of degradation intermediates (VFA), followed by decreasing pH and methanogenesis inhibition. As examples of acetogenic bacteria, it can be mentioned *Syntrophobacter wolinii* that decomposes propionic acid and *Syntrophomonas wolfei*, which decomposes butyric acid (Boone and Bryant, 1980).

A special type of acetogenic microorganisms is the homoacetogenics, consuming H_2 and CO_2, to produce acetate. Two examples are *Acetobacterium woodii* and *Clostridium aceticum* (Espinosa, 2011).

The **methanogenic step** is the final stage of the anaerobic digestion process in which the methanogenic bacteria, using a limited number of substrates such as acetic acid, hydrogen, carbon dioxide, formic acid, methanol, methylamines and carbon monoxide can produce methane. According to their affinity for the substrate, methanogenic microorganisms are divided into two main groups: acetoclastics, methane-forming microorganisms from acetic acid or methanol, and hydrogenotrophic, which produce methane from hydrogen and carbon dioxide.

This last step of the anaerobic digestion could also be considered limiting in not complex wastes.

The methanogens, the most sensitive group of microorganisms in the anaerobic digestion process, could be classified in one of these orders: *Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanococcales* and *Methanopyrales* (Garrity and Holt 2001), having the microorganisms of the order Methanosarcinales the widest range of substrate utilization. This taxonomic group is divided into two families, Methanosarcinaceae and Methanosaetaceae. Methanosaeta may have lower yields and be more sensitive to pH compared with Methanosarcina. Methanosarcina has a higher growth rate, while Methanosaeta need higher solids retention times but can operate at lower acetate concentrations (De Lemos, 2007), because it has a higher affinity for the substrate (Deublein and Steinhauser, 2011).

The acetate-utilizing methanogens have been suggested to be responsible for 70-80% of the methane produced (Zinder, 1984). But despite being the most important way, only organisms of the genera Methanosarcina and Methanothrix are capable of producing methane from acetate.

The genera most frequently determined of hydrogenotrophic bacteria are *Methanobacterium, Methanoculleus* and *Methanospirillum* (Hori *et al*., 2006; Leclerc *et al*., 2004).

There is another alternative way to produce methane, which is activated to high levels of ammonium (Schnürer and Nordberg, 2008). The acetate is converted to hydrogen and carbon dioxide by syntrophic acetate oxidizers (SAO), followed by carbon dioxide reduction by methanogens using hydrogen. The development of SAO community occurs when ammonium concentrations are reached inhibitory values to the acetate-utilizing methanogens (Ek *et al*., 2010). The generation time of these oxidizers is approximately 28 days (Schnürer *et al*., 1994) much longer than the 2-12 days for acetatoclastic methanogens (Jetten *et al*., 1992).

2.2. SPECIFIC FACTORS FOR THE ANAEROBIC DIGESTION OF ABPs

There are several factors affecting the anaerobic digestion like the characteristics of the waste to be treated and the operational parameters of the process such as the hydraulic retention time (HRT), temperature, pH and concentration of substrates, products and inhibitors.

In the specific case of ABP, as mentioned previously, due to the elevated content of

biodegradable organic matter of these materials, these wastes might be efficiently treated by anaerobic digestion. Although inhibitory effects depend on the buffering capacity and degree of adaptation of the microorganisms in the digestion process the high amount of proteins and lipids may cause inhibition of the digestion process due to high ammonia and long chain fatty acids concentrations that could appeared at high organic loads (Salminen and Rintala, 2002; Edström *et al*., 2003; Bayr *et al*., 2012). Other degradation intermediates as VFA and H_2 can also cause inhibition. Inhibitory effects depend on the buffering capacity and degree of adaptation of the microorganisms in the digestion process.

2.2.1. Nitrogen-related compounds

Processes in which protein-rich material are degraded (as ABPs or manure), they tend to yield high ammonia level and could result in reduced biogas production and quality (Poggi-Varaldo *et al*., 1997; Hansen *et al*., 1998; Pechan *et al*., 1987; McCarty and McKinney, 1961; De Baere *et al*., 1984; Zeeman *et al*., 1985; Angelidaki and Ahring, 1993; Kayhanian, 1999; Sung and Liu, 2003). During the anaerobic digestion process, the ammonia produced by the biological degradation of the nitrogenous matter, mostly proteins (Kayhanian, 1999), but also nucleic acids are present in the form of ammonium ions $N-NH_4^+$ and ammonia gas NH_3 (Gerardi, 2003).

The presence of ammonia is beneficial for the system as it can act as a buffer for pH changes and as a major nitrogen source for microorganisms, being ammonia nitrogen concentrations bellow of $0.2 \ g \cdot l^{-1}$ beneficial to the anaerobic process (Liu and Sung, 2002). Nevertheless, free ammonia has been suggested to be inhibitory at high concentrations (Angelidaki and Ahring, 1994; Hansen *et al*., 1998; Angelidaki and Ahring, 1993; Kadam and Boone, 1996) since it can easily diffuse through the cell membrane disrupting their normal functions (Kadam and Boone, 1996). Several mechanisms for ammonia inhibition have been proposed, such as a change in the intracellular pH, increase of maintenance energy requirement and inhibition of specific enzyme reactions (Whittmann *et al*., 1995).

The relative amount of ammonia depends on pH and temperature (Hobson and Wheatley, 1993; Kayhanian, 1999) that affects the dissociation constant of ammonia nitrogen and concentration of free ammonia in the process. The higher is the temperature higher is the concentration of free ammonia (Kayhanian, 1999). At acid pH, the equilibrium is shifted

toward ammonium and at basic pH the equilibrium is shifted towards free ammonia, which is the inhibitor (De Lemos, 2007).

Methanogens are the least tolerant to free ammonia of all microorganisms involved in anaerobic digestion and the most easily inhibited to high ammonia concentrations in the system (Koster and Lettinga, 1988; Robbins *et al.*, 1989; Angelidaki and Ahring, 1993; Braun *et al.*, 2010). This inhibition could be responsible of low degradation rates, poor biogas yields and also an accumulation of volatile fatty acids. An abrupt change in the ammonium concentration causes a decrease in the rate of growth of methanogenic organisms, but not in the growth rate of the acidogenic or acetogenic (Koster and Lettinga, 1988; Robbins *et al.*, 1989). It has been reported that the aceticlastic methanogens are most sensitive to ammonia toxicity than hydrogenotrophic methanogens (Angelidaki and Ahring, 1993; Hansen *et al.*, 1998).

As mentioned previously, there is an alternative pathway for methane production that is activated at elevated levels of ammonia (Schnürer and Nordberg, 2008), where acetate is converted to H_2 and CO_2 by SAO. After that, the reduction of carbon dioxide to methane by a hydrogen utilizing methanogens is produced. When the ammonia inhibits acetate-utilizing methanogens and the solid retention time is high enough, the SAO pathway is favored.

There is not a fixed ammonia and ammonium nitrogen concentration reported as toxic or inhibitive, the inhibitory values are different depending on the previous exposure of the biomass to ammonia and it has been already widely demonstrated that the anaerobic process could be adapted to high ammonium/ammonia concentrations by gradually increasing its content in the process (Hashimoto, 1986; Koster and Lettinga, 1988; Angelidaki and Ahring, 1993; Hansen *et al.*, 1998; Gallert *et al.*, 1998; Álvarez and Liden, 2008).

Several examples were reported in literature to cause a reduction in methane production as a function of pH, temperature and biomass adaptation. This inhibitory range could vary from 1.5 till 5.6 $g_{N-NH4+} \cdot l^{-1}$ (Van Velsen, 1979; Koster and Lettinga, 1984; Hashimoto, 1986; Buendía *et al.*, 2009; Benabdallah El Hadj *et al.*, 2009; Pechan *et al.*, 1987; Hansen *et al.*, 1998; Krylova *et al.*, 1997) or even as high as to 14 $g \cdot l^{-1}$ (Chen *et al.*, 2008a).

Related to NH_3, also a wide range, between 0.02–2.0 $g \cdot l^{-1}$, have been reported to be inhibitory (Hashimoto, 1986; Angelidaki and Ahring, 1993; Angelidaki and Ahring,

1994; Kayhanian, 1999; Braun *et al*., 1981; Hansen *et al*., 1998). As for NH_4^+, it is important to consider experimental temperature, kind of substrate and adaptation of the microorganisms.

Kirchmayr *et al*. (2007) and Resch *et al*. (2006) found out that ammonium concentrations exceeding 5 $g_{N-NH4+}·l^{-1}$ still allow stable operation by mean of experiences with full scale bio-waste and slaughterhouse waste digesters.

2.2.2. Sulphide

When ABPs with high protein concentration are anaerobically degraded not only produce ammonia, the proteins could also be a source of sulphide during their degradation. In the presence of sulphate the methanogenic archaea compete with sulphate-reducing bacteria by useful substrates, showing the latest, thermodynamic and kinetic advantages over the methanogenic bacteria, both hydrogen consuming and acetoclastics (Hulshoff Pol *et al*., 1998). The increased concentration of sulphides in the digester lead to higher concentrations of corrosive H_2S in the biogas and can further lead to sulphide inhibition of the methanogens (Ochieng' Otieno, 1996; Chen *et al*., 2008a).

2.2.3. Volatile fatty acids and hydrogen

The volatile fatty acids (VFA) are the main intermediate species produced during the anaerobic digestion process. Imbalances in the anaerobic process, where the different microbial communities are linked, result in VFA accumulation and are usually a sign of an overloaded digestion process. If the VFA are accumulated and the system is low buffered, a pH drop could be produced and consequently a failure in the process (Murto *et al*., 2004). The toxic effects are dependent on acids composition.

The most important intermediate and process indicator is acetate (Pind *et al*., 2003). Its accumulation reduces the metabolic activity of butyrate and propionate degrading bacteria. Although some authors have reported propionate as the main inhibitor (Nielsen *et al*., 2007) and others consider inhibitory not only the excess of propionate also of butyrate (Mata-Alvarez, 2003). The concentration of propionate is especially important as concentrations as low as 30 mM are known to have an inhibitory effects on methanogenesis (Barredo and Evison, 1991).

Different anaerobic processes are adapted to different concentrations of VFAs. Some inhibiting levels for total VFAs are 2.2-4.9 $g·l^{-1}$ (Kalle and Menon, 1984; Siegert and Banks, 2005; Climent *et al*., 2007).

The two parameters most frequently used to monitor digester stability are alkalinity and the VFA concentration (Ripley *et al*., 1986; Hill *et al*., 1987; Ahring *et al*., 1995). For substrates high in ammonia, such as ABP or pig slurry, the VFA can accumulate in the anaerobic digester due to methanogenesis inhibition, therefore making it difficult to distinguish if hydrolysis is inhibited by VFA or ammonia (Zeeman, 1991).

Hydrogen is also an important intermediary of the anaerobic digestion which accumulation can inhibit the acetogenesis phase (Fukuzaki *et al*., 1990), with the consequent VFA accumulation, especially propionic (Harper and Pohland, 1986; Boone and Xun, 1987; Fukuzaki *et al.*, 1990) and inhibition of all the anaerobic digestion process.

2.2.4. Long chain fatty acids

In some previous works with ABPs, long chain fatty acids (LFCA) were speculated to be responsible for process failures in continuous digesters fed with poultry slaughterhouse wastes (Cuetos *et al*., 2010), pig slaughterhouse waste and manure (Hejnfelt and Angelidaki, 2009) or rendering products (Bayr *et al*., 2012).

During anaerobic digestion, lipids are hydrolyzed by extracellular lipases to long chain fatty acids and glycerol. Then, LCFA are oxidized to acetate and hydrogen through the β-oxidation pathway (Cirne *et al*., 2007; Mata-Alvarez, 2003). Palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids are the most abundant saturated or unsaturated LCFA, present in organic waste and wastewater (Hwu *et al*., 1998). LCFA are abundant in ABPs and have been described as inhibitory species (Hwu *et al*., 1997) and reported to inhibit acetogenesis and/or methanogenesis (Angelidaki and Ahring, 1992; Hanaki *et al.*, 1981). Methanogen microorganisms were reported to be more susceptible to LCFA inhibition compared to acidogens (Lalman and Bagley, 2002; Mykhaylovin *et al*., 2005; Pereira *et al*., 2003).

The inhibition is dependent on the type of microorganism, the carbon chain length, the saturation degree of LCFA (Hwu *et al*., 1996; Salminen and Rintala, 2002) the temperature (higher inhibition in thermophilic than in mesophilic range) and the sludge type (Hwu *et al*., 1997). Inhibitory concentration depends on the individual LCFAs (Lalman and Bagley, 2000, 2001). Oleic acid may be inhibitive already in the concentration of 0.03-0.3 $g \cdot l^{-1}$ (Broughton *et al*., 1998; Alves *et al*., 2001; Lalman and Bagley, 2001).

The most inhibiting LCFAs are saturated fatty acids with 12-14 carbon atom chains (lauric acid, myristoleic acid) and unsaturated acid with 18 carbon atoms (oleic acid).

Nevertheless, a system inhibited by LCFA is able to recover activity and microorganisms can adapt to high levels of these intermediaries, being LCFA inhibition temporal, but recovery time might be long (Cirne *et al*., 2007). After a lag phase, the inhibited microorganisms are able to use the accumulated LCFA (Pereira *et al*., 2004).

Adsorption of LCFA on the microbial surface has been suggested as the mechanism of inhibition, affecting transport of nutrients through the cell membranes (Pereira *et al.*, 2005). Palatsi *et al*. (2009) after testing and evaluating several recovery strategies found out that dilution of the reactor content with inoculum to increase the biomass/LCFA ratio and the addition of adsorbents were the best strategies.

2.3. PRE-TREATMENTS

Disintegration and hydrolysis of the solid organic substrates are considered to be the rate-limiting steps for the digestion of particulate material, such as ABPs. This stage can be improved disintegrating solid materials by means of certain pre-treatments that affect the physicochemical properties of the waste.

The pre-treatments may be physical (such as grinding, milling or shearing), chemical (using ozone or acidic and alkaline solutions), biological (enzymes, fungi, compost), by means of radiation or combination of some of them, such as thermo-chemical treatments. Many studies have been published about different pre-treatment methods (Chandra *et al*., 2007; Chen *et al*., 2008b; Hendriks and Zeeman, 2009).

Pre-treatments can affect the solubilization of the material, reducing particle size and making it more accessible to microorganisms. They could be useful, especially with slow biodegradable material because the pre-treatment could facilitate the material availability, reducing the digestion time and increasing the production of biogas (Bougrier *et al*., 2006).

These effects of pre-treatments on the organic matter can be evaluated by mean of several parameters, being the most used the degree of COD solubilization, which can be defined as the ratio of soluble to total COD (Chulhwan *et al*., 2005). Although an effective pre-treatment could enable process intensification (increasing OLR, reducing HRT and/or

decreasing digester volume) (Alvarez and Liden, 2008; Rosenwinkel and Meyer, 1999) the effectiveness of the selected pre-treatment will not only depends on the waste, also on the selection of the reactor design and operation conditions (Chandra *et al.*, 2007).

The applicability is generally limited to large-scale biogas plants due to the high costs involve in pre-treatments (Deublein and Steinhauser, 2011). The implementation of pre-treatments is possible with many organic materials. In the case of ABP, another reason for the application of pre-treatments is disinfection. The current legislation that applies to the ABP uses and treatments (Nº 1069/2009) requires, depending on the category and type of waste, to carry out pre-treatments as a step to apply the allowed recovery processes, such as anaerobic digestion.

Among all the existing pre-treatments, various types have been applied to the meat industry wastes, both solid and liquid. For example, Wang and Banks, (2003) used a thermal pre-treatment to evaluate a two phases anaerobic digester that was fed with a mixture of wastewater from slaughterhouse. Massé *et al.* (2001) and Dalev (1994) studied the effects of chemical and biological pre-treatments on the fat particles in slaughterhouse wastewater and waste of feathers, respectively. Mendes *et al.* (2006) studied the effect of the enzymatic hydrolysis of lipid-rich slaughterhouse wastewater on anaerobic digestion. Valladao (2007) focused on the enzymatic hydrolysis of poultry slaughterhouse waste, previous to its anaerobic digestion.

2.3.1. Thermal pre-treatment

Thermal pre-treatment is a physical treatment method based mainly in heat application to the waste. Several temperature ranges and application times have been already studied with different wastes such as waste activated sludge, sewage sludge, cattle manure and biowaste (Bougrier *et al.*, 2006; Paavola *et al.*, 2006).

The first trials of thermal pre-treatment applied to improve dewaterability in the case of sludge were performed between 150-180 º C and a partial solubilization of the material was observed (Haug *et al.*, 1978; Fisher and Swanwick, 1971). Müller (2001) also observed that after thermal pre-treatment of sludge at elevated temperature (100-275 ºC) there was a significant increase in the disintegration and solubilization of sludge solids, improving the sludge stabilization.

Some researchers concluded that the optimum conditions of thermal pre-treatments are temperatures between 160-180 °C and treatment times between 30-60 minutes (Carrere,

2009). Some proposed the application of temperature only for 60 seconds (Dohanyos *et al*, 2004) while another proposed the application of the pre-treatment at low temperature (70 °C) but for several days (Gavala *et al*., 2003; Ferrer *et al*., 2008). Gavala *et al*. (2003), Climent *et al*. (2007) and Ferrer *et al*. (2008) have found that temperatures under 100ºC were more effective in increasing biogas production from waste activated sludge, food industry wastewater and sewage sludge than higher temperatures. Beside from recalcitrant compounds formation, the most significant drawback of thermal pre-treatment is probably its energy requirements and it is difficult to operate (Skiadas *et al*., 2005). For this reason low temperature and short time exposures are preferred (Luste, 2011).

There are few works in the literature about the effects of thermal pre-treatments on ABPs, and those works have divergent results regarding anaerobic digestion of thermally pre-treated solid slaughterhouse waste. One study was performed by Edström *et al*. (2003), who observed that biogas yield increases from 0.31 to 1.14 $m^3_{biogas} \cdot kg_{VS}^{-1}$ as a result of pasteurization as a pre-treatment before anaerobic digestion of ABPs. Another important contribution was done by Hejnfelt and Angelidaki (2009), who applied pasteurization, sterilization and alkaline hydrolysis to mixed pork waste and observed no improvements in the anaerobic methane yields, in contrast to the results of Edström *et al*. (2003). They also observed an inhibition due to long chain fatty acids and ammonia and concluded that the mesophilic process was more stable than the thermophilic.

Luste *et al*. (2009) studied the effect of pasteurization on the hydrolysis and methane production of 4 animal by-products, obtaining positive results on the increase in the methane production potential only when this pre-treatment was applied to drum-sieve waste and dissolved air flotation sludge. Pitk *et al*. (2012) observed that the degradation of ABPs from category 2 and 3 was improved after sterilization (dry rendering process) reaching near the 90% of methane potential in the first 10 days.

However thermal pre-treatments may have negative effects too. Some researchers have observed that high temperatures applied to different types of organic waste can produce compounds that are recalcitrant, toxic and/or inhibitory for the anaerobic process (Haug *et al*., 1978; Stuckey and McCarty, 1984; Owen *et al*., 1979; Martins *et al*., 2001; Ajandouz *et al*., 2008; Dwyer *et al*., 2008). This could be due to Maillard reactions, which are non-enzymatic glycosylation of proteins where carbohydrates and amino acids react to form melanoidins, very difficult to biodegrade (Bougrier *et al*., 2008; Martins *et al*., 2001).

During heating, dehydration, retroaldolizations, isomerization and further condensations take place, which allow the formation of brown nitrogen polymers and copolymers in the final stage, known as melanoidins (Martins, 2001). These reactions depend on temperature, pH and water activity (Ajandouz *et al*., 2008) and have been observed to occur at 100ºC or lower temperatures (Mersad *et al*., 2003; Martins and Boekel, 2005; Ajandouz *et al*., 2008). The reactivity of different sugars is given by the availability of carbonyl groups (Ajandouz *et al*., 2008). Maillard reactions are connected with the aroma, flavor and color of the roasted coffee, toast or cooked meat, because of these brown compounds are known to decrease the digestibility of these foods, to form toxic and mutagenic compounds, but also produce antioxidant products (Martins, 2001). Suyama *et al*. (2007) suggested that Maillard reactions could be employed for the decontamination of some ABPs such as bone and bone meal.

2.3.2. High pressure pre-treatment

High pressure technology is a physical method normally used in food processing, where the sample is subjected to elevated pressures, between 100 and 800 MPa (Kadam *et al*., 2012), with or without the addition of heat, to achieve microbial inactivation or to alter the food attributes in order to achieve consumer-desired qualities (Raventós, 2005). High technology pressure was developed initially for ceramics, superalloys or artificial diamond application, although this technology was commonly used in the food industry to preserve and modify foods since the 80´s (Cheftel and Culioli, 1997).

The effects of pressure in the structure and texture of the organic materials are highly variable. For example, changes in muscle enzymes and proteolysis of meat, modifications of ultrastructure of the muscle, changes in myoglobin and meat color, influence on lipid oxidation, inactivation of pathogens and combined with moderate temperature can have similar results as pasteurization at elevated pressure (Cheftel and Culioli, 1997).

It has been also observed that high pressure process can inactivate enzymes, germinate or inactivate some bacterial spores, extend shelf life, reduce the potential for food borne illness, promote ripening of cheese and minimize oxidative browning (Kadam *et al*., 2012). Commercial high pressure, low temperature methods achieve inactivation of vegetative microorganisms by subjecting vacuum-sealed food in flexible packaging to treatment at hydrostatic pressures of 600 MPa (or less) and initial temperatures lower than 40°C for one to fifteen minutes depending upon the product application (Barbosa-

Cánovas and Juliano, 2008).

In general, high pressures at low or moderate temperature cause destruction of microbial cells and inactivation of enzymes. However, the resistance of the microorganisms is very variable depending on the strain and the meat matrix to be treated. The efficacy of the treatment also depends on the achieved pressure, on the treatment temperature and on the exposure time (Hugas *et al*., 2002).

The high pressure can affect the conformation of proteins and may produce the protein denaturation, aggregation or gel formation, depending on the protein, the pressure applied and the temperature and duration of the pre-treatment. The effects on proteins are specifically related to the breakdown of non-covalent interactions and subsequent reformation of intra and intermolecular bridges within or between protein molecules (Messens *et al*., 1997).

Several food biochemical studies indicate that pressures close to 100-200 MPa often cause, at room temperature, the dissociation of oligomeric structures in their subunits, partial unfolding and denaturation of monomeric structures (in the most cases irreversible) and protein aggregation or gel formation (probably as a result of unfolding) whenever the pressure and protein concentrations are high enough (Cheftel and Culioli, 1997).

The melting temperature of lipids (triglycerides) increases, in a reversible manner, more than 10 °C per 100 MPa. Therefore, the lipids will crystallize under pressure (Cheftel and Culioli, 1997). Many pressure treatments have little effect on the oxidation below 300 MPa but increases proportionally to pressures from 300 to 400 MPa, which seems to be the range of critical pressures for change in lipids.

No previous information about the use of high pressure pre-treatment at environmental temperature on ABP samples before anaerobic digestion has been found.

2.3.3. New techniques for studying the effect of pre-treatments

Thermogravimetric analysis (TGA), Fourier Transform infrared technology (FTIR) and mass spectrometry (MS) can be used to characterize raw and pre-treated wastes, besides all previous parameters used for wastes characterization. These analyses are not usual in the field of anaerobic digestion and can be useful to provide further information on the effects of pre-treatments.

Thermogravimetric analysis procedure measures a physical property of a substance as a function of temperature, while subjected to a controlled temperature program. Thermogravimetry reports the gain or loss of mass of a sample, quantifying this reaction. This technique has been used previously for Conesa *et al*. (2001) and Dell´Abate *et al*. (1998) to characterize organic material.

Mass spectrometry is an analytical tool very versatile in their applications that can provide valuable information about the qualitative composition and in some cases quantitative, about organic and inorganic analytes in complex samples, the structures of a wide variety of complex molecular species and the isotope ratios of the atoms in the samples. The mass spectra are obtained by conversion of components of a gaseous sample ions move rapidly and are separated according to their mass / charge ratio. When quantitative analysis could not be performed, a comparison of the intensity of the peaks obtained from the different samples can be made using the normalization procedure described by Arenillas *et al*. (1999). Some signals $m \cdot z^{-1}$ (mass·charge^{-1}) that can be correlate the weight loss with the thermal degradation of the sample, were: 1) $m \cdot z^{-1}$ 18, representing H_2O emission; 2) $m \cdot z^{-1}$ 41, for the release of light hydrocarbon products; 3) $m \cdot z^{-1}$ 44, for CO_2 emission, and 4) $m \cdot z^{-1}$ 46, for NO_2 emission.

Infrared spectroscopy (IR) is a quantitative and qualitative analysis that relies on the interaction of the infrared light with the material and is sensitive to the presence of functional chemical groups being useful to identify organic compounds. The principle of monitoring by IR reactions is based on the conversions of functional groups by chemical reactions or by the appearance or disappearance of functional groups during the reaction. The analysis of the functional groups of direct mode allows the qualitative analysis of every step of the reaction throughout the entire process of synthesis (Dal Cin *et al*., 2002). More in detail, the IR spectrum of a compound is a graphical representation of the wave number values versus values of transmittance or absorbance (Cuetos *et al.*, 2007). Fourier Transform (FTIR) is a specific technique to determine the IR spectra with weak signals. FTIR allows for example distinguishing between different functional groups of nitrogen in organic matter (Calderon, 2005).

Cuetos *et al.* (2007) used FTIR and thermogravimetric analysis coupled to mass spectroscopy to determine the degree of stabilization reached by the organic matter of ABP once the anaerobic digestion finished. She observed that some complex nitrogen polymers were formed with the pre-treatment applied to the ABP and also an increase in

the aromatic degree coupled to a decrease in biodegradable materials, as stabilization of the waste takes place.

2.4. DISINTEGRATION AND HYDROLYSIS KINETICS

The kinetics of the different anaerobic digestion stages depend on the characteristics of the substrate, being the disintegration and hydrolytic phase a slow step when complex wastes are digested, as ABP.

The hydrolysis process and its velocity depend on several factors as pH, temperature, concentration of hydrolytic biomass, type of particulate organic matter (Pavlostathis and Giraldo-Gomez, 1991) and particle size (Hills and Nakano, 1984). This process can be described following a first order kinetic (Eastman and Ferguson, 1981), it has been found to be a good simplification of this complex step (Batstone, 2006) and used to fit most experimental data quite accurately (Batstone *et al*., 2002; Sanders, 2001; Vavilin *et al*., 2008).

The ADM1 model was developed to describe the anaerobic digestion process of sludge from domestic wastewater treatment plant. Later it has been improved several times to simulate anaerobic digestion with different types of substrates, (Fezzani and Cheikh, 2009; Galí *et al*., 2009). The hydrolysis rate constants have been updated for different substrates, according to experimental data (Derbal *et al*., 2009). In the ADM1 first order kinetics are considered for disintegration, hydrolysis and decay processes, while Monod type expressions are used for the rest of bioconversions.

As mentioned previously, the pre-treatments can influence the solubilization of the organic material and the particle size modifying the disintegration and hydrolysis rate. If pre-treatments positively modify the characteristics of the substrate, it can be observed in two ways: 1) increasing the biodegradability of the substrate, and/or 2) increasing the disintegration of hydrolysis first order constant values. In the first case, it can be measured using biodegradability assays; in the second case, it can be measured estimating the disintegration or the hydrolysis constant if a first order process is ensured (Vavilin *et al*., 2008).

2.5. CO-DIGESTION OF ANIMAL BY-PRODUCTS

The term co-digestion refers to the anaerobic digestion of two or more substrates from different origins simultaneously, mainly to obtain synergy of the mixture and to compensate lacks of each substrate separately. It could be a good strategy to prevent inhibition and optimize methane production in anaerobic digesters (Mata-Alvarez *et al*., 2011). The combined treatment of organic wastes from different sources has also other advantages, such as reducing costs and energy expenses as result of a more integrated waste management (Alatriste-Mondragon *et al*., 2006).

There are several cases where the effectiveness of this process has been demonstrated, such as co-digestion of livestock waste and industrial organic waste, which gave good results when using mixed systems both at mesophilic and thermophilic range (Brinkman, 1999).

Co-digestion allows the progressive acclimatization of the bacteria to specific inhibitors such as ammonia (Angelidaki and Ahring, 1993; Edström *et al*., 2003) and/or LCFA (Broughton *et al*., 1998), thus facilitating the control of the anaerobic process. The implementation of an adequate co-digestion regime in industrial plants lays in accurate selection of co-substrates. For example, in the case of digesting blood or manure, which both have high contents of nitrogen, is preferable to co-digest them with waste characterized by a low concentrations of nitrogen, such as fruit and vegetable waste (Alvarez and Liden, 2008).

The co-digestion is one of the strategies to improve the production of biogas from slurry (Hartmann *et al*., 2006) while other strategies are the application of thermal pre-treatments (Bonmatí *et al*., 2001) or the change of the digestion temperature (Angelidaki *et al*., 1993). The advantages of pig slurry and other livestock wastes as co-substrates are that they are produced steadily throughout the year, contain a higher water content than many other wastes such as industrial waste, higher buffer capacity, provide a wide variety of nutrients necessary for the growth of anaerobic microorganisms (Angelidaki *et al*., 1997; Hartmann *et al*., 2006) and they usually have small amounts of pollutants such as heavy metals, organic compounds and residues of antibiotics (Canet *et al*., 2006). Although, on the other hand, they have a low biogas production potential due to the poor organic matter content. The relatively low methane production potential of cattle slurry can be improved with co-digestion with other feed materials (Mata-Alvarez *et al*., 2000).

Several positive experiences have been described on the co-digestion of pig manure with different substrates, as sewage sludge in mesophilic and thermophilic range (Wong, 1990; Flotats *et al*., 1999), vegetal wastes, obtaining improvements in the production of biogas (Dar and Tandon, 1987), tomato waste (Trujillo *et al*., 1993), fruit and vegetable wastes (Campos *et al*., 1999; Callaghan *et al*., 1999), residues from the manufacture of milk (Gavala *et al*., 1996; Desai and Madamwar, 1994), residues from the manufacture of canned fish and sludge from the brewing industry (Callaghan *et al.*, 1999). Other positive examples have been observed co-digesting sewage sludge and organic fraction of municipal waste (OFMW) (Di Palma *et al*., 1999; Hamzawi *et al*., 1998), the mixture of OFMW with urban wastewater (Edelmann *et al*., 1999), and co-digestion of sewage sludge with waste fruit and vegetables (Dinsdale *et al*., 2000). Other organic wastes such as recovered glycerin from the biodiesel production process from energy crops have been mixed with nitrogen rich-substrates like manure, in order to balance the C/N ratio (Amon *et al*., 2006; Alatriste-Mondragon *et al*., 2006).

Related to the anaerobic co-digestion of ABP, Rosenwinker and Meyer (1999) co-digested stomach contents with intestines of pigs and cows with sludge as co-substrate, reaching at pilot scale yields of 0.44 $m^3 \cdot kg_{TS}^{-1}$ for stomach contents and between 0.60 and 0.80 $m^3 \cdot kg_{TS}^{-1}$ for the mixture. On an industrial scale, a mixture of both residues achieved a biogas yield of 0.47 $m^3 \cdot kg_{VS}^{-1}$. Solid slaughterhouse waste with manure and vegetable and fruit waste were co-digested mesophilically by Alvarez and Liden (2008), who observed that the co-digestion of mixtures gave better results than anaerobic digestion of separate substrates in 9 of the 10 mixtures. Good results have been also obtained for livestock waste mixtures with various meat industry and slaughterhouse wastes, rich in fat, getting high methane yields in the order of 47 $m^3_{CH4} \cdot t^{-1}$ of added residue (Brinkman, 1999). Cuetos *et al*. (2008) co-digested poultry waste with urban solid waste in mesophilic range while diminishing the HRT once the inoculum was acclimated to ammonia and high fat concentrations, the optimum conditions found for co-digestion were 25 days of HRT and 3.7 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$ of OLR, where fat removal reached 83%.

Several rendering and slaughterhouse by-products were co-digested and evaluated in base to their methane and fertilizer production by Kaparaju *et al*. (2010). They obtained a stable process for a mixture of rendering plant wastes and slaughterhouse by-products only during mesophilic digestion and until a maximum load of 1.5 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$. In thermophilic conditions, the same organic load of 1.5 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$ made the system

unstable. High ammonification efficiency (70-80%) was reached facilitating nitrogen recover. Pitk *et al.* (2012) observed that addition of melt (one fraction of the ABP after rendering) with manure in proportions of 2.5% and 5% per wet weight increased the methane production by 1.75 and 2.70 times respectively, compared to manure digestion alone.

Luste (2011) co-digesting separately an ABP mixture with sewage sludge and with cattle slurry observed significantly higher methane production: 400 and 410 $m^3_{CH4} \cdot t_{VSadded}^{-1}$, respectively, than digesting sewage sludge alone: 220-270 $m^3_{CH4} \cdot t_{VSadded}^{-1}$ (Ferrer *et al.*, 2008; Luostarinen *et al.*, 2009; Salsabil *et al.*, 2009) or slurry without the ABP mixture: 130-240 $m^3_{CH4} \cdot t_{VSadded}^{-1}$ (Angelidaki and Ahring, 2000; Ahring *et al.*, 2001; MØller *et al.*, 2004; Nielsen *et al.*, 2004; Amon *et al.*, 2006; Mladenovska *et al.*, 2006).

Another study was carried out by Murto *et al.*, (2004), who co-digested pig manure, slaughterhouse waste, vegetal residue and various types of industrial waste, resulting in a highly buffered system thanks to the manure. They set up three reactors with different OLR obtaining biogas productions of 1.0 $m^3 \cdot kg_{VS}^{-1}$ for the reactor at the highest OLR (3.7 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$).

Bayr *et al.* (2012) co-digested rendering and slaughterhouse wastes at mesophilic and thermophilic range using CSTR reactors. In mesophilic conditions, average methane yields of 0.73 and 0.72 $m^3_{CH4} \cdot kg_{VS}^{-1}$ were obtained with OLRs of 1 and 1.5 $kg_{VS} \cdot m^{-3} \cdot d^{-1}$ respectively at HRT of 50 d. At thermophilic range, the process was unstable due to accumulating ammonia, VFAs and probably also LCFAs. Since methane yields were higher and the process more stable in mesophilic conditions, they concluded that mesophilic range was more feasible for solid slaughterhouse wastes co-digestion than the thermophilic.

Digestion of ABP materials alone may not be feasible due to inhibitive concentrations of intermediates (Rosenwinkel and Meyer, 1999; Buendia *et al.,* 2008; Luste *et al.*, 2009), its high concentration of solids that can lead to mechanical problems, low buffering capacity and the need for dilution of some possible inhibitors (NH_4^+, H_2, VFA, LCFA). Although these results there are few examples of successful mono-digestion, as the work done by Prechtl *et al.* (2003), who previously applied to the residue a pressure thermal hydrolysis process (10-40 bars and temperatures >160 °C) before anaerobic digestion. They noted that digestion was developed in a more stable and faster way than a

conventional digestion process. Another experience at bigger scale as the work carried out by Waltenberger *et al.* (2010), who operated the first European biogas plant that utilized slaughterhouse wastes in monodigestion.

In summary, the co-digestion of ABP with other co-substrate is a good choice for solving some of the inhibitions mentioned above, since the content of nutrients can be balanced and the negative effects of the toxic components can be decreased by dilution, increasing biogas production (Alvarez and Liden, 2008). As main substrate for co-digestion with the ABPs, slurry could be used because its high water content and high buffer capacity.

2.6. MOLECULAR BIOLOGY TECHNIQUES

Over the last decade, an increasing number of studies have been aimed at opening the black box of the anaerobic digestion processes because only a small fraction of all bacteria have been isolated and characterized (Wayne *et al.*, 1987; Ward *et al.*, 1992). This improvement in microbial ecology knowledge is due to some new molecular biology tools for monitoring the microbial diversity in biological systems. Traditional examinations of the microbial communities were based on cultivation-based methods that attempted to isolate and identify species in pure culture. However, since a large percentage of microbial species present in the environment are impossible to culture using current methods, during last years the molecular biology analysis became more commonly used because they are faster, more refined and less expensive than the previous methodology.

Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified ribosomal DNA fragments (16S rDNA genes) is an electrophoretic method to distinguish among DNA sequences having the same length but differing in the base composition (Muyzer *et al.*, 1993). Cloning and sequencing of 16S rDNA genes provide information about the genetic diversity and phylogenetic relationships between microorganisms present in a particular ecosystem, after comparative analysis of the retrieve 16S rDNA gene sequences with 16S rDNA gene databases (Amann *et al.*, 1995; Godon *et al.*, 1997).

DGGE is a robust, rapid and relatively simple technique very useful for the characterization of complex bacterial and archaeal communities DGGE can also be described as a genetic fingerprinting technique that provides a pattern or profile of the

genetic diversity in a microbial community (Muyzer and Smalla, 1998).

This technology has been used widely in to study diversity and the relative abundance shifts in the populations and for describing ecological communities by using diversity indexes and species abundance (Muyzer, 1999; Briones and Raskin, 2003). DGGE technique has been also used with samples from complex systems, including manure (Leung and Topp, 2001) and anaerobic bioreactors (Liu *et al*., 2002; Roest *et al*., 2005; Connaughton *et al*., 2006; Miura *et al*., 2007). For example, Pereira *et al*. (2002) using a combination of different molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), cloning and sequencing techniques to analyze the microbial diversity of eubacteria and archaea in two bioreactors inoculated with granular and suspended sludge, observed that reactors fed with LCFA had a high diversity of genotypes related to *Syntrophomonas spp*. Calli *et al*. (2005) used DGGE to study the diversity of methanogenic populations in anaerobic reactors subjected to extremely high ammonia level. Dearman *et al*. (2006) studied changes in microbial community structure in four food waste-fed anaerobic digesters during start-up or at steady state, and inhibited with high VFA using DGGE and statistical analyses. This study is one of the first illustrating the usefulness of statistical multivariate analyses in the microbiology of anaerobic digesters. Microbial diversity of mesophilic anaerobic sludge acclimated to oleate or palmitate was studied by clone library analysis and monitored by DGGE by Sousa *et al*. (2007). Civit (2009) by means of 16S rDNA DGGE technique characterized the microbial community structure during the continuous operation of thermophilic anaerobic digesters that were fed with manure and exposed to successive inhibitory pulses of LCFA. The population profiles of eubacterial and archaeal revealed that no significant shift on microbial community composition took place upon biomass exposure to LCFA and DNA sequencing of predominant DGGE bands showed close phylogenetic affinity to ribotypes characteristic from specific ß-oxidation bacterial genera (Syntrophomonas and Clostridium), while a single predominant syntrophic archaea was related with the genus Methanosarcina (Civit, 2009).

Sometimes, changes in community structure may occur without detectable changes in bioreactor performance (Fernández *et al*., 1999), but may eventually result in severe process disruption. Hence, the better understanding of the microbial interactions in anaerobic digesters can provide control, diagnostic and prevision tools for enhanced process monitoring and can help also to avoid failure, to predict eventual instability

problems, and also to evaluate the reactor efficiency and biogas yield (Sousa *et al*., 2007).

2.7. REFERENCES

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Chapter 3. Effects of thermal pre-treatments on solid slaughterhouse waste methane potential

The effects of thermal pre-treatments on the biogas production potential of two solid slaughterhouse waste types (poultry and piggery slaughterhouse by-products) were assessed by means of batch experiments. Both animal by-products were characterized in terms of fat, protein and carbohydrate concentrations. The selected thermal pre-treatments, pasteurization (70 ºC for 60 min) and sterilization (133 ºC and 3 bars for 20 min), are included in the current European regulations for the disposal or use of animal by-products. The pre-treatments produced notable improvements in organic matter solubilization, but had different effects on the anaerobic bioavailability of the treated substrates. The methane yield of the initial volatile solids did not increase significantly after pre-treatment when carbohydrate concentration was high, reaching a maximum of 0.48 $m^3_{CH4} \cdot kg_{VS}^{-1}$ for the pasteurized poultry waste. However, this yield increased by up to 52.7% after pasteurization and 66.1% after sterilization for the lower carbohydrate concentration sample (piggery waste), reaching 0.88 and 0.96 $m^3_{CH4} \cdot kg_{VS}^{-1}$, respectively. The maximum methane production rates, measured as the maximum slope of the accumulated methane production curve, per unit of initial biomass content, were also different. While this rate increased by 52.6% and 211.6% for piggery waste after pasteurization and sterilization, respectively, it decreased by 43.8% for poultry waste after pasteurization with respect to untreated waste. Compounds with low biodegradability that are produced by Maillard reactions during thermal pre-treatment could explain the low bioavailability observed for waste with a high carbohydrate concentration.

3.1. INTRODUCTION

Solid slaughterhouse waste is characterized by a high solid content that is mainly composed of protein and fat with different amounts of carbohydrate and inorganic compounds, depending on waste management and sorting techniques. Because of its composition, solid slaughterhouse waste is considered a good substrate for anaerobic digestion. However, some inhibition processes may take place owing to N-NH4, from protein decomposition, or to long-chain fatty acids, from fat (Angelidaki and Ahring, 1993; Edström et al., 2003; Hanaki et al., 1981; Hansen et al., 1998; Luste et al., 2009; Palatsi et al., 2010; Salminen et al., 2003).

During the processing and valorization of this type of animal by-product (ABP) it is essential to avoid potential risks to human and animal health. The European regulations (European Community Regulation (EC) N° 1774/2002 and N° 92/2005) classify ABPs in three categories depending on the hazard level, and require a minimum sanitation standard, depending on category, before any biological treatment (anaerobic digestion or composting) or disposal. The use of category 1 waste (the most hazardous) for biological treatment is severely restricted. Category 3 waste, which is the 84% of the total ABP produced in Spain (MAPA, 2007), must be pasteurized (60 min at 70 ºC) or sterilized at a high temperature (20 min at 133 ºC and 3 bars) before anaerobic digestion; while category 2 waste can only be sterilized. Other pre-treatments, such as chemical or high-pressure and high-temperature treatments, are also allowed (European Community Regulation N° 92/2005).

Although the configuration of a biogas plant must include provision of pre-treatment processing for ABP sanitation, pre-treatments can also be applied to increase the bioavailability of the protein and fat content of the waste and to improve the energy and the economical balance of full scale slaughterhouse facilities (Kirchmayr *et al*., 2009). Bioavailability is defined as the availability of the organic material to microorganisms. An indirect measure of the increase in bioavailability is the degree of solubilization of particulate organic matter. This increment due to thermal pre-treatment, with positive effects on anaerobic digestion performance, has been reported for many different types of organic waste, i.e. for sewage sludge (Climent *et al*., 2007; Haug *et al*., 1978) and pig manure (Bonmatí *et al*., 2001). Thermal pre-treatments can also improve the stabilization

of sewage sludge, its dewatering capacity and the reduction of its pathogenic charge (Gavala *et al*., 2003).

However, thermal pre-treatments can also have negative effects. Some researchers have observed that high temperatures applied to different types of organic waste can produce compounds that are recalcitrant, toxic and/or inhibitory for the anaerobic process (Haug *et al*., 1978; Stuckey and McCarty, 1984; Owen *et al*., 1979; Martins *et al*., 2001; Ajandouz *et al*., 2008; Dwyer *et al*., 2008). Such compounds are products of Maillard reactions, where carbohydrates react with amino acids to form melanoidines, which are difficult to degrade (Bougrier *et al*., 2008; Martins *et al*., 2001). It is well known that Maillard products decrease food digestibility due to the formation of toxic and mutagenic compounds, although antioxidant products can also be generated (Martins *et al*., 2001), and Maillard reactions can be employed for the decontamination of ABPs such as bone and bone meal (Suyama *et al*., 2007). These reactions depend on temperature, pH and water activity (Ajandouz *et al*., 2008) and have been observed to occur at 100 ºC or lower temperatures (Mersad *et al*., 2003; Martins and Boekel, 2005; Ajandouz *et al*., 2008).

There is little work in the literature, and what there is has contrasting results, regarding anaerobic digestion of thermally pre-treated solid slaughterhouse waste, and none of the work relates the composition of the waste (relative fractions of fats, proteins and carbohydrates) with the bioavailability of the substrate and yields. One study was performed by Edström *et al*. (2003) who observed that biogas yield increases from 0.31 to 1.14 $m^3_{biogas} \cdot kg_{VS}^{-1}$ as a result of pasteurization as a pre-treatment before anaerobic digestion of ABPs. Another important contribution have been done by Hejnfelt and Angelidaki (2009), who applied pasteurization, sterilization and alkaline hydrolysis to mixed pork waste and observed no improvements in the anaerobic methane yields, in contrast to the results of Edström *et al*. (2003). Luste *et al*. (2009) observed an improvement of the methane yield in some cases though not all, after five pre-treatments (thermal, ultrasound, acid, base and bacterial product), when the anaerobic process was not inhibited by the apparition of toxic hydrolysis products.

The aim of the present work is to study bioavailability during anaerobic digestion of two types of solid slaughterhouse waste characterized by different proportions of fat, protein and carbohydrate (F:P:C) after thermal pre-treatments, pasteurization or sterilization, in order to elucidate the importance of substrate composition for the methane yield obtained.

3.2. MATERIAL AND METHODS

3.2.1. Slaughterhouse wastes

The selected ABPs were sampled at poultry and piggery slaughterhouse facilities located in Lleida and Barcelona (Spain), respectively (Figure 3.1). The fractions collected from the poultry slaughterhouse (named TI) were a mixture of wings, necks, internal organs and heads; while in the piggery slaughterhouse (TII), a mixture of internal organs (kidneys, lungs, livers and hearts), reproductive organs and piggery fatty waste was collected. All the waste samples were minced (4 mm maximum particle size) and mixed to produce two mixtures with different protein, fat and carbohydrate concentrations. Equal amounts (20% of the total weight) of each ABP were used in the case of poultry waste, while twice the amount of fat was used to prepare the piggery waste mixture. The mixtures obtained (TI and TII) were lyophilized before characterization in order to improve their homogeneity.

Figure 3.1. *Examples of the selected ABP from piggery and poultry facilities before mincing (a and b respectively) and after mincing (c and d).*

3.2.2. Thermal pre-treatments

Thermal pre-treatments were performed under the conditions laid out in the ABP European regulation (No 1774/2002 and No 92/2005). The effects of pasteurization were tested on both ABP mixtures (TI and TII) at 70 ºC for 60 min, while sterilization at 133 ºC and 3 bars for 20 min was only applied to the piggery mixture (TII). Each pre-treatment was performed in triplicate on 500 g of waste in a high pressure and temperature reactor with a working volume of 2 l (Figure 3.2), allowing maximum operating conditions of 232 ºC and 151 bars (Iberfluid Instruments, Spain). Temperature was increased by means of a heating jacket before the indicate treatment time, and after the treatment time had been completed, the temperature was rapidly decreased using an inner heat exchanger fed with cold water. The reactor was watertight and condensate losses were not significant.
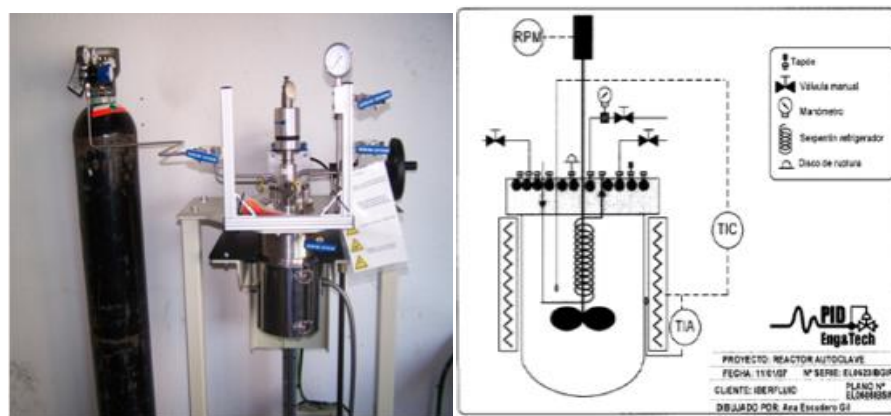
Figure 3.2. *Picture and diagram of the high pressure and temperature reactor.*

3.2.3. Analytical methods

A complete characterization of the ABP mixtures was performed before and after thermal pre-treatment. Total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), pH, total and soluble chemical oxygen demand (COD_t, COD_s) and total and ammonium nitrogen (TN, NH_4^+) where determined according to standard methods (APHA, AWA, WEF, 2005).

Volatile fatty acids (VFAs) were determined by a modified standard method (APHA, AWA, WEF, 2005) protocol, following Campos *et al*. (2008). A CP-3800 gas chromatograph (Varian, USA), fitted with a Tecknokroma TRB-FFAP capillary column (30 m x 0.32 mm x 0.25 lm) and FID detection were used. The VFAs measured (with detection limit given in mg·l^{-1}) were: acetic (10.0), propionic (5.0), iso-butiric (1.0), n-butiric (1.5), iso-valeric (1.5), n-valeric (1.0), iso-caproic (1.5), n-caproic (1.0) and heptanoic (1.0).

Protein concentration was estimated from the organic nitrogen content using a factor of 6.25 $g_{protein}$·g_{Norg}^{-1}, as suggested by AOAC (2003). The fat content was analyzed using Soxtec™ 2050 extraction equipment (Foss, Denmark) following the recommendations for n-hexane extractable material (HEM) from sludge, sediments and solid samples (EPA, 1998, Method 9071b). The COD_t was also estimated by elementary analysis (Leco, USA) using the empirical formulas of each sample (Angelidaki and Sanders, 2004). The protein-COD (COD_{prot}) and fat-COD (COD_{fat}) were estimated using the factors 1.42 g_{COD}·g_{prot}^{-1} and 2.90 g_{COD}·g_{fat}^{-1}, respectively (Angelidaki and Sanders, 2004). The

carbohydrate-COD (COD_{ch}) concentration was estimated by subtracting the COD_{prot} and COD_{fat} values from COD_t.

Methane (CH_4) content in the biogas produced was determined by gas chromatography using a CP-3800 (Varian, USA) fitted with Hayesep Q 80/100 Mesh (2 m x 1/800 x 2.0 mSS) packed column (Varian, USA) and TCD detection, as described elsewhere (Campos *et al.*, 2008).

3.2.4. Anaerobic biodegradability assay

The anaerobic biodegradability (AB) of both the untreated and treated samples was determined according to Field *et al.*, (1988), Soto *et al.* (1993) and Angelidaki *et al.* (2009). Anaerobically digested sewage sludge from the mesophilic anaerobic digester of the wastewater treatment plant at La Llagosta (Barcelona, Spain) was used as inoculum. The inoculum was maintained in an incubation chamber (35 ºC) for 7 days to decrease the amount of residual COD_t. The methanogenic activity of the inoculum was determined in triplicate following Soto *et al.* (1993), obtaining 42 ± 2 $mg_{CH4\text{-}COD}\cdot g_{VSS}^{-1}\cdot d^{-1}$.

The AB was determined in triplicate. Glass flaks of 1200 ml were filled with 500 g of a solution composed of the inoculum, macronutrients and micronutrients, substrate and bicarbonate (1 $g_{NaHCO3}\cdot g_{CODadded}^{-1}$), giving an initial inoculum concentration of 5 $g_{VSS}\cdot l^{-1}$ and a substrate concentration of 5 $g_{CODt}\cdot l^{-1}$ (Soto *et al.*, 1993). The pH was adjusted to neutrality using HCl or NaOH. The flasks were stirred and bubbled with a N_2 gas in order to remove O_2 before they were closed with rubber bungs. A reducing solution was finally added (5 ml of 10 $g_{Na2S}\cdot l^{-1}$). The flasks were continuously shaken (100 rpm) during incubation at 35 ºC for 31 days. The time course of the methane production was monitored by gas chromatography (Campos *et al.*, 2008), sampling the head space periodically. The gas volume was expressed at normal conditions (0 ºC and 1 atm.). Three flasks with the inoculum but without any substrate (blanks) were tested, to monitor the methane production of the residual COD. Net methane and biogas production potential or yield was calculated by subtracting the blank methane and biogas production. Periodic samples were collected to analyze the evolution of VFAs and NH_4^+.

3.2.5. Evaluation of the results

Substrate bioavailability after the thermal pre-treatments was evaluated by the degree of solubilization (%S) of the chemical oxygen demand (COD), defined as the ratio of soluble to total COD ($COD_s \cdot COD_t^{-1}$) (Chulhwan *et al.*, 2005) and by the ratio of the

ammonium nitrogen to total nitrogen concentrations (%TAN). These ratios were related to the variation of the anaerobic biodegradability, the methane production potential or yield (MPP) and the maximum methane production rate (MPR). The MPR ($l_{CH4} \cdot kg_{VSS}^{-1} \cdot d^{-1}$) was estimated as the maximum slope of the accumulated methane production curve, per unit of initial biomass content (VSS of the inoculum), obtained during anaerobic biodegradability assays.

3.3. RESULTS AND DISCUSSION

3.3.1. Waste characterization

The characteristics of raw ABP mixed substrates (TI and TII) are summarized in Table 3.1. The untreated piggery slaughterhouse mixture (TII) presented a higher solid content than the untreated poultry mixture (TI). The solid content was 30.7 and 50.7% TS for TI and TII, respectively. The ash content was higher in TI (4.1% of inorganic solids with respect to total substrate) than in TII (1.8% with respect to total substrate), due to the bone fraction in the poultry by-products. The pH of both initial samples was close to neutral (Table 3.1).

Table 3.1. Characterization (mean ± standard deviation) of untreated poultry and piggery wastes (TI and TII respectively). Nomenclature: (1) volatile solids with respect to the total substrate; (2) F-fat, P-protein and C-carbohydrate expressed in % of COD$_t$; (3) estimated value from elemental analysis.

Parameter	Units	TI	TII
TS (w·w⁻¹)	%	30.7±0.4	50.7±0.4
VS (w·w⁻¹) (1)	%	26.6±0.6	48.9±0.1
F:P:C (2)	%:%:%	33:33:34	82:13:04
COD$_t$ (3)	g·kg⁻¹	653.5	1275.0
COD$_s$	g·kg⁻¹	66.3±3.7	52.2±0.5
pH	-	6.4	6.9
Total N	g·kg⁻¹	26.3±0.5	20.7±0.9
NH$_4^+$	g·kg⁻¹	2.1±0.1	1.4±0.0
Est. Protein	g·kg⁻¹	151.3±3.6	120.9±5.7
COD$_{prot}$	g·kg⁻¹	214.0±5.1	170.9±8.0
COD$_{VFA}$	g·l⁻¹	1811±42	2439±87
Fat	g·kg⁻¹	74.7±1.0	363.4±0.6
COD$_{fat}$	g·kg⁻¹	215.8±2.9	1050.3±1.7
COD$_{ch}$	g·kg⁻¹	223.7±8.0	53.8±9.7

The raw ABP materials had different fat, protein and carbohydrate ratios (F:P:C), related to initial CODt content. The different organic fractions in TI were quite similar (33:33:34), while the fat fraction was the main component of TII waste, with little carbohydrates (82:13:4).

3.3.2. Effect of the selected pre-treatments on solubilization

The characteristics of the pre-treated ABP (PTI, PTII and STII) are summarized in Table 3.2. Although the reactor was watertight, there was a slight increase in the total and volatile solids concentration of the samples after the pre-treatments. This was produced by some water evaporation when the reactor was opened after heating and cooling; however, the effect on the COD_t of the pre-treated samples was almost negligible (less than 4% for PTI, 3% for PTII and 0% for STII).

Table 3.2. *Characterization (mean ± standard deviation) of pasteurized poultry waste (PTI), pasteurized piggery waste (PTII) and sterilized piggery waste (STII). Nomenclature: (1) volatile solids with respect to the total substrate; (2) estimated value from elemental analysis.*

Parameters	Units	PTI	PTII	STII
TS (w·w^{-1})	%	34.4 ± 0.0	55.2 ± 0.3	49.5 ± 0.1
VS (w·w^{-1}) (1)	%	29.9 ± 0.0	54.3 ± 0.2	47.8 ± 0.1
COD_t (2)	g·kg^{-1}	680.3	1318.0	1273.2
COD_s	g·kg^{-1}	151.6 ± 2.4	175.4 ± 7.5	155.3 ± 9.1

The effect of the thermal pre-treatments on organic material solubilization was clear for both types of waste (Tables 3.3 and 3.4), with an increase with respect to the untreated samples of 119.5% for PTI, and 225.0% and 206.4% for PTII and STII, respectively.

The thermal processes led to increased protein decomposition in both types of waste, measured as an increase in % TAN (Tables 3.3 and 3.4). Although the protein concentration was higher in TI, protein decomposition was bigger in TII after pasteurization. After sterilization pre-treatment, transformation of organic N into ammonia N was higher than after pasteurization for TII. The lower ammonification for TI could be related to Maillard reactions, which could produce sugar-amino acid compounds with low biodegradability (Stuckey and McCarty, 1984; Owen *et al.*, 1979).

The pre-treatments studied had little effect on the decomposition of fats, with very little variation in COD_{fat} and VFA concentration values (see Tables 3.3 and 3.4).

Table 3.3. Comparison between the untreated (TI) and pasteurized (PTI) poultry waste. Increments are referred to the untreated waste.

Parameters	Units	TI	PTI
Solubilization (S)	$\%COD_s \cdot COD_t^{-1}$	10.2	22.3 ± 0.3
S increment	%	-	119.5
%TAN	$\%NH_4^+ \cdot TN^{-1}$	7.8 ± 0.3	8.4 ± 0.0
%TAN increment	%	-	6.8
COD_{fat}	$g_{COD} \cdot kg^{-1}$	215.8 ± 2.9	217.5 ± 0.9
COD_{fat}t increment	%	-	0.8
VFA	$mg_{COD} \cdot l^{-1}$	1811 ± 42	1875 ± 166
VFA increment	%	-	3.5
MPP	$m^3_{CH4} \cdot kg_{VS}^{-1}$	0.46 ± 0.01	0.48 ± 0.01
MPP increment	%	-	2.6
AB	$\% COD_t$	55.2 ± 7.0	61.8 ± 1.3
AB increment	%	-	12.0
MPR	$l_{CH4} \cdot kg_{VSS}^{-1} \cdot d^{-1}$	31.4 ± 0.6	17.6 ± 0.3
MPR increment	%	-	-43.8

Table 3.4. Comparison between the untreated (TII) and pre-treated (pasteurized: PTII and sterilized: STII) piggery waste. Increments are referred to the untreated waste.

Parameters	Units	TII	PTII	STII
Solubilization (S)	$\%COD_s \cdot COD_t^{-1}$	4.1	13.3 ± 1.8	12.5 ± 0.6
S increment	%	-	225.0	206.4
%TAN	$\%NH_4^+ \cdot TN^{-1}$	6.5 ± 0.3	7.4 ± 0.7	9.6 ± 0.4
%TAN increment	%	-	13.7	47.8
COD_{fat}	$g_{COD} \cdot kg^{-1}$	1050.3 ± 1.7	1123.5 ± 6.7	1003.0 ± 18.3
COD_{fat} increment	%	-	7.7	-4.5
VFA	$mg_{COD} \cdot l^{-1}$	2439 ± 87	2474 ± 154	2251 ± 86
VFA increment	%	-	1.5	-7.7
MPP	$m^3_{CH4} \cdot kg_{VS}^{-1}$	0.58 ± 0.03	0.88 ± 0.01	0.96 ± 0.01
MPP increment	%	-	52.7	66.1
AB	$\% COD_t$	76.6 ± 8.6	94.3 ± 3.0	98.7 ± 1.3
AB increment	%	-	23.2	28.8
MPR	$l_{CH4} \cdot kg_{VSS}^{-1} \cdot d^{-1}$	24.7 ± 1.4	37.6 ± 2.2	76.8 ± 5.0
MPR increment	%	-	52.6	211.6

3.3.3. Effect of the pre-treatments on anaerobic biodegradability

In the batch anaerobic tests, untreated and pre-treated substrates reached stable methane production before 25 days (Figs. 3.3 and 3.4). From the accumulated methane curves for untreated and pasteurized TI and TII substrates (especially for TII, where there was a higher fat concentration), a ''sigmoid-type'' methane production curve was observed, probably because the inoculum was not previously exposed and adapted to substrates with a high fat content (Palatsi *et al*., 2010), or because the particles disintegration and hydrolysis processes were affected by the microbial growth, being the rate limiting steps of the anaerobic digestion process (Vavilin *et al*., 2008). In sterilized TII, although the fat concentration was similar to that of the untreated and pasteurized TII, the methane production curve advanced respect to the others, suggesting that sterilization produced a significant thermal particles disintegration and hydrolysis, releasing more readily biodegradable compounds.

The methane production potentials (MPP) obtained for both untreated types of waste (0.46 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ and 0.58 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ for TI and TII, respectively) were in the range of those obtained by Hejnfelt and Angelidaki (2009), between 0.23 and 0.62 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$, with the maximum value corresponding to the mixed pork waste. Salminen *et al*. (2003) also obtained 0.6 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ for various samples from a poultry slaughterhouse. Palatsi *et al*. (2011) obtained higher values for mixed cattle–pig waste, between 0.63 and 0.78 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$. The MPP of the pre-treated waste was 0.48 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ for PTI, and 0.88 and 0.96 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ for PTII and STII, respectively.

The MPP results confirm in both cases the results obtained by Edström *et al*. (2003), who observed an improvement in the yield after pre-treatment.

The AB of untreated waste was 55.2% COD_t for TI and 76.6% COD_t for TII, while it was 61.8% COD_t for PTI and 94.3% and 98.7% COD_t for PTII and STII, respectively. The non-biodegradable fraction decreased in thermally pre-treated samples compared to untreated waste, from 44.8% to 38.2% COD_t in TI and from 23.4% in untreated TII to 5.3% and 1.3% COD_t after pasteurization and sterilization, respectively.

Cartilaginous materials in TI, although composed of proteins, could be responsible for the lower biodegradability of this waste, confirming results obtained with feathers by Salminen *et al*. (2003).
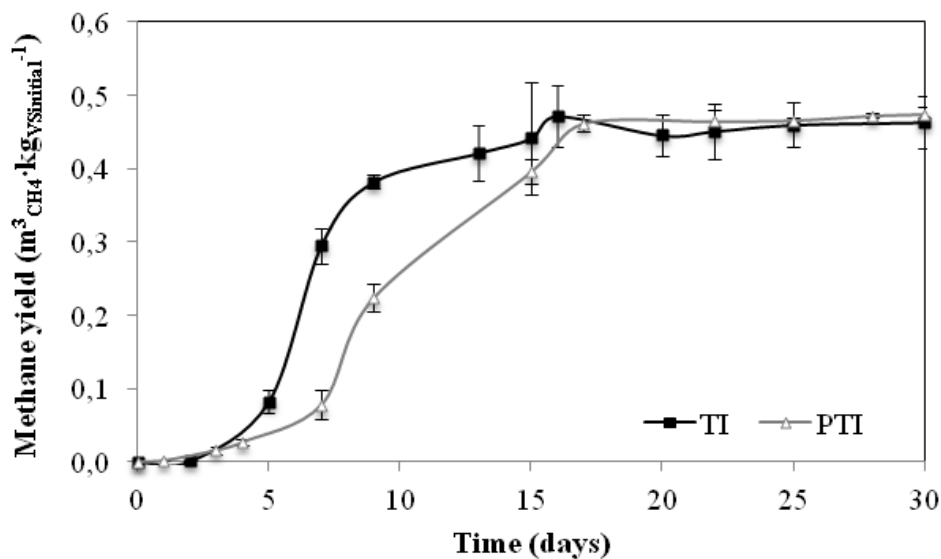
Figure 3.3. *Methane evolution related to initial volatile solids during the anaerobic biodegradability assay of untreated and thermally pre-treated with TI samples.*
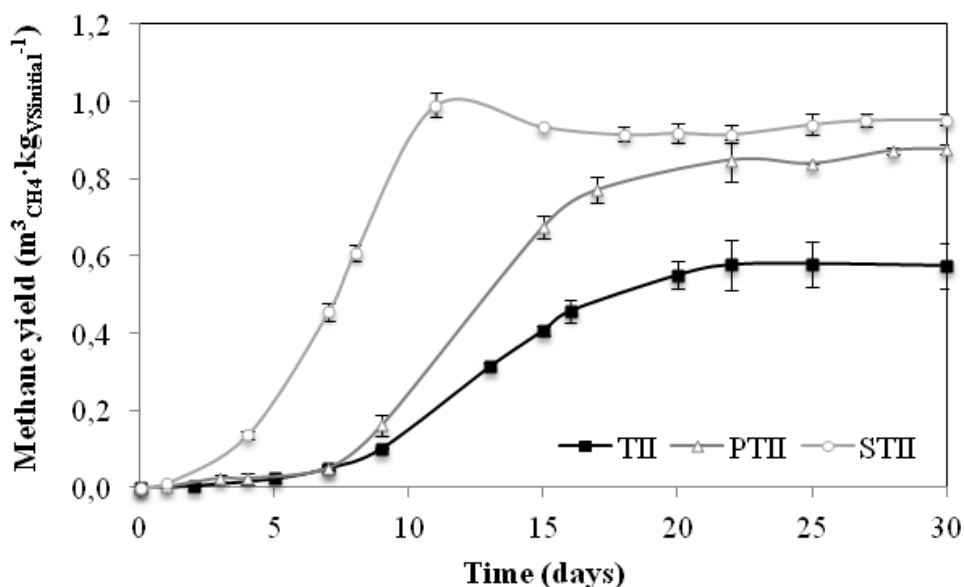
Figure 3.4. *Methane evolution related to initial volatile solids during the anaerobic biodegradability assay of untreated and thermally pre-treated with TII samples.*

AB and MPP are not significantly higher after pasteurization for TI waste, meaning that this pre-treatment is not contributing to a significant increase in biodegradable material in poultry waste. Contrarily, for TII waste the two pre-treatments tested produced significant higher values of AB and MPP, suggesting that the different compositions of TI and TII are the responsible of this different behavior, since all experimental conditions were the same. The methane production rate (MPR) increased after both pre-treatments for TII (by 52.6% for PTII and 211.6% for STII) (Table 3.4), while this rate decreased by 43.8%

after pasteurization for TI waste (Table 3.3). These values are consistent with the evolution of VFA shown in Fig. 3.3, where the higher accumulation of VFA at seventh day was found for PTI and the lower for STII. Estimated MPR values were similar to those obtained by Palatsi *et al*. (2011) studying anaerobic biodegradability of different mixtures of slaughterhouse waste, except for PTII assay, which were significantly lower. The effect of thermal pre-treatment decreasing particle size, decreasing the relative importance of the biological disintegration and hydrolysis as rate limiting steps of anaerobic digestion process, and releasing soluble highly biodegradable compounds could explain the increase of AB and MPR values for TII waste. The MPR decrease for PTI respect to TI, maintaining similar final MPP and AB values, could be explained by the presence of inhibiting compounds produced during the thermal pre-treatment.

Maximum ammonia concentrations found during batch assay were around 600 mg NH_4^+-$N \cdot l^{-1}$, and specifically lower for PTI (Fig. 3.5), which are below inhibitory concentrations (Chen *et al*., 2008; Hansen *et al*., 1998). The high VFA concentrations found on the seventh day for PTI (Fig. 3.5, Table 3.5) can be considered a consequence of a lower methanogenic activity due to the presence of some toxic rather than VFA inhibitory values and, indeed, VFA were completely consumed by the end of the assays. The low initial methanogenic activity of the inoculum, around 42 $mg_{CH4-COD} \cdot gvss^{-1} \cdot d^{-1}$, could also explain the temporary VFA accumulation.

Although LCFA were not analytically determined, the initial fat concentration for TI waste (33% of the total COD) were significantly lower than that used by Palatsi *et al*. (2011), studying LCFA inhibition to the anaerobic digestion of slaughterhouse waste and obtaining a fast inoculum adaptation to increasing initial substrate COD concentrations up to 15 $g_{COD} \cdot l^{-1}$, with 80% fat content. Since initial fat concentration for TI and PTI waste were much lower than for TII waste, for which inhibition were not measured after pre-treatments, it is considered that LCFA were not inhibiting the process for PTI assay.

Table 3.5. *Maximum values of VFAs measured during the batch assays (7th day).*

VFA (mg l^{-1})	TII	PTII	STII
Acetic	490	870	60
Propionic	265	325	5
i-Butyric / n-Butyric	66/ 78	52/ 84	2/ 3
i-Valeric/ n-Valeric	126/ 29	66/ 33	3/ 1
i-Caproic/ n-Caproic	2/ 61	27/ 79	6/ 1
Heptanoic	2	3	0
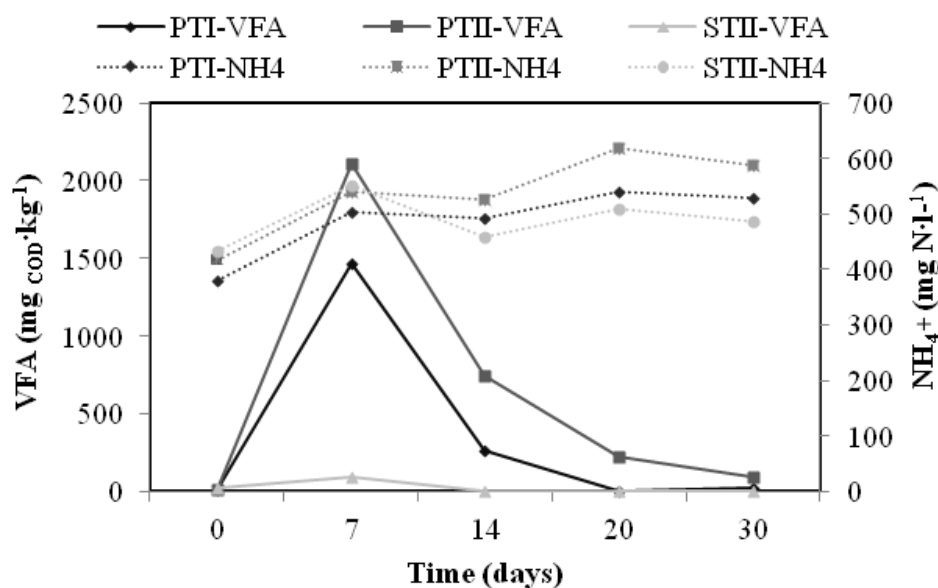
Figure 3.5. *Evolution of VFA and NH_4^+ concentration during the anaerobic biodegradability assay of thermally pre-treated TI and TII waste.*
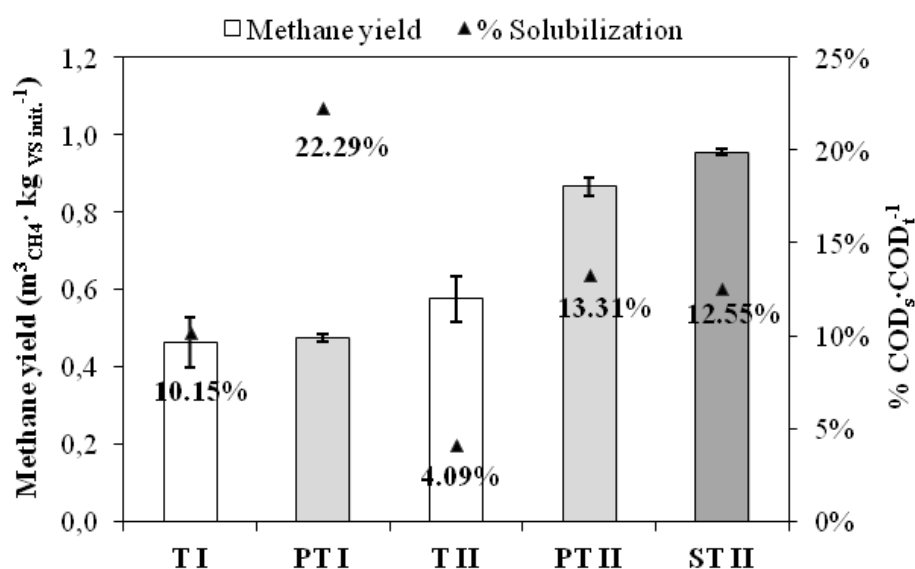
Figure 3.6. *Relation between solubilization (dots) and methane yield (columns). Nomenclature: TI: untreated poultry waste; PTI: pasteurized poultry waste; TII: untreated piggery waste; PTII: pasteurized piggery waste; STII: sterilized piggery waste.*

Fig. 3.6 summarizes the relation between solubilization of COD and the methane yields obtained for all the substrates. While the increase in solubilization implied a large increase in biodegradability, reaction rate and methane yield for TII (with a low sugar content), the increase in solubilization did not similarly affect TI (Table 3.3). The presence of carbohydrates in the TI waste suggests the occurrence of Maillard reactions during thermal pre-treatment, since melanoidins or Maillard's products form at ambient

temperatures, but are promoted by its rise around 70-75 ºC, and they develop intensely in a moderate basic medium (Mersad *et al*., 2003).

These compounds have been documented to be soluble organic compounds recalcitrant to anaerobic digestion (Ajandouz *et al*., 2008; Dwyer *et al*., 2008; Martins *et al*., 2001; Stuckey and McCarty, 1984).

Stuckey and McCarty (1984) found that thermal pre-treatment of carbohydrates alone produced toxicity to methanogenic microorganisms, illustrating that small changes in compound structure can have significant effects on anaerobic process behavior. Similar results were found by Ajandouz *et al*. (2008), who studied the kinetics of Maillard reactions, caramelization and protein reticulation processes, which can occur simultaneously at different temperature conditions, making it difficult to generalize about the relative importance of every process.

Based on current data we cannot conclude whether Maillard reactions between amino acids and sugars produced inhibitory compounds that could decrease the reaction rate, or whether an increase in the biodegradability of some compounds was compensated by a decrease in others. However, our results suggest that the presence of carbohydrates in the TI substrate could be responsible for the observed behavior.

In general, pre-treatments conducted to decrease particle sizes led to an increase of the accessible active sites at which exocellular enzymes can attach and cleave complex macromolecules to simpler and more biodegradable constituents (Vavilin *et al*., 2008).

This is the case of thermal pre-treatments, for which Stuckey and McCarty (1984) found that the above could be the case for temperatures up to 175 ºC. These authors indicated that the optimum bioconvertibility appears to be a net result of two competing mechanisms: an increase due to the thermal hydrolysis of refractory particulate organics to soluble degradable ones, and a decrease due to the thermal degradation of soluble organics to undefined refractory compounds. Further studies conducted to evaluate the changes in compounds structure after thermal pre-treatments, using tools such as thermal analysis or infrared spectrometry (Su *et al*., 2010) will help to elucidate the relative importance of the initial substrate concentration in the distribution and structure of compounds produced and their bioavailability to anaerobic microorganisms.

Results indicate that pasteurization of pig by-products prior to anaerobic digestion could present a positive energy balance, complying also with sanitary regulations. This

interesting result cannot be generalized, since the energy balance values depend on the specific waste composition.

3.4. CONCLUSIONS

The thermal pre-treatments described produced a significant solubilization of particulate COD in the two types of slaughterhouse waste tested. However, the different results related to protein decomposition, AB, methane production potential and maximum methane production rates, suggest the importance of the influence of composition on the anaerobic bioavailability of treated substrates.

While the thermal pre-treatments produced a significant increase in the methane production rate, AB and methane yield in the piggery waste, this increment was not significant for the poultry waste. For the piggery by-products tested, the methane yield increased from 0.58 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ to 0.88 and 0.96 $m^3_{CH4} \cdot kg_{VSadded}^{-1}$ after pasteurization and sterilization, respectively, increasing also the methane production rate, while for poultry by-products methane yield was 0.46 $m^3_{CH4} \cdot kg_{VS\ added}^{-1}$, and increased only 2.6% after pasteurization. In this case, methane production rate decreased significantly.

The presence of carbohydrates and the possible occurrence of Maillard reactions are thought to be the main reasons for the low pre-treatment efficiency observed for poultry by-product, although the cartilage and other solid compounds could have had some influence.

3.5. ACKNOWLEDGEMENTS

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Chapter 4. Study of thermal pre-treatment effects on anaerobic digestion of solid animal by-products by TGA-MS and FTIR spectroscopy

Thermogravimetric analysis coupled to mass spectrometry (TGA-MS) and Fourier-transform infrared spectroscopy (FTIR) were used to describe the effect of pasteurization as hygienization pre-treatment of animal by-products over biogas production. Piggery and poultry wastes were used as substrates for assessing the anaerobic digestion under batch conditions at mesophilic temperatures. Poultry wastes were characterized by high protein and carbohydrate content, while piggery wastes presented a major fraction of fat and lower carbohydrate content. Results from anaerobic digestion tests showed a lower methane yield for the pre-treated poultry sample. TGA-MS and FTIR spectroscopy permitted the qualitative identification of recalcitrant nitrogen-containing compounds in the pre-treated poultry sample. These components were produced by Maillard reactions during pasteurization and explained the distinctive behavior of the pre-treated waste in terms of anaerobic biodegradability and methane yield, with lower values after the pre-treatment. In the case of piggery waste, the recalcitrant compounds were not detected and its biodegradability test reported higher methane yield and production rate. TGA-MS and FTIR spectroscopy demonstrated to be a useful tool for explaining results obtained by anaerobic test and describe the presence of recalcitrant components.

4.1. INTRODUCTION

A huge amount of animal by-products (ABPs) from meat-processing industry are produced daily, around 17 million tons per year in Europe (Woodgate and Van der Veen, 2004). ABPs are characterized by a high protein and fat content, which makes them suitable substrates for the anaerobic digestion process although their hydrolysis may be a limiting step (Eastman and Ferguson, 1981; Vavilin *et al*., 2008) and some N-NH$_4^+$ or LCFA inhibition phenomena may occur (Angelidaki and Ahring, 1993; Edström *et al*., 2003; Hanaki *et al*., 1981; Hansen *et al*., 1998; Luste *et al*., 2009; Palatsi *et al*., 2010; Salminen *et al*., 2003). In order to improve biodegradability of such complex wastes, different pre-treatment options are available. However, after the bovine spongiform encephalopathy disease, the valorization of ABPs through anaerobic digestion must be combined with thermal hygienization (European Community Regulation (EC) N° 1069/2009 and N° 142/2011), which may have contradictory effects over the methane yield. This is the case of results reported by Hejnftelt and Angelidaki (2009) who observed lower methane yields in a mixture of thermally pre-treated ABPs compared to untreated materials, while Edström *et al*. (2003), obtained an improvement in methane yield after pre-treatment. In the Chapter 3 of this PhD thesis these differences were attributed to the change in the chemical nature of the organic wastes after the thermal treatment, since the formation of recalcitrant molecules depends on the composition of the untreated waste.

Thermogravimetry (TGA) and Differential Thermogravimetry (DTG) analysis are fast characterization methods widely used in chemical industry, i.e., in polymer production, or in combustion or pyrolysis studies of a variety of materials such as coal or scrap tyre (Idris *et al*., 2010; Otero *et al*., 2007; Yanfen and Xiaoqian, 2010). These techniques are based on programmed heating under controlled atmospheres. The identification of two main decomposition processes (disregarding water release) considers: (1) oxidation of carbohydrates, proteins and the loss of carboxyl groups, giving information about labile structures and (2) decomposition of complex and recalcitrant compounds, as polyphenolic molecules or condensed structures such as lignin (Flaig *et al*., 1975; Rovira *et al*., 2008). Thermogravimetric analysis (TGA) has recently been used in the study of pyrolysis of biomass (Huang *et al*., 2011; Sánchez *et al*., 2007) and in the study of the degradation of organic matter under biological stabilization (Gómez *et al*., 2007; Cuetos *et al*., 2010). The use of TGA coupled to mass spectrometry (TGA-MS) allows the identification of

evolved gaseous species emitted by a sample during thermo-oxidative degradation at a determined atmospheric condition (Raemaekers and Bart, 1997).

Another useful technique is Fourier-transformed infrared (FTIR) spectroscopy, which gives valuable information related to chemical bonds (by its vibrations and rotations movements). This technique has been used to study changes undergone by the organic matter during biological degradation in composting, for a better understanding of the humification process and the formation of fulvic structures (Xiaosong *et al*., 2001; Jouraiphy *et al*., 2008) for the developing of a model to predict important compost parameters (Meissl *et al*., 2007) or under anaerobic digestion (Cuetos *et al*., 2009; Cuetos *et al*., 2010; Marcato *et al*., 2008). Infrared techniques have already been used to determine the methane potential of wastes and positive results have been obtained, such as the work carried out by Lesteur *et al*. (2011), who found that near infrared spectroscopy (NIR), characterized by using infrared light between 4,000 ~ 14,000 cm^{-1}, was a useful tool to predict the BMP value of municipal solid wastes.

Considering the premise that the chemical characteristics of a substrate may determine its degradation pattern under anaerobic digestion, and thus the expected methane production potential, it may be reasonable to assume that modifications in the chemical composition of the substrates will lead to modifications in biogas yield. The objective of this study was to obtain a characterization of the selected slaughterhouse samples by means of TGA-MS and FTIR spectroscopy and to test the hypothesis that the thermal pre-treatment can modify the structure and composition of the samples, which will affect the bioavailability of the organic matter and therefore the uses of TGA-MS and FTIR results may aid in the interpretation of digestion test results.

4.2. MATERIAL AND METHODS

4.2.1. Slaughterhouse wastes

ABPs were obtained from poultry and piggery slaughterhouse facilities located in Lleida and Barcelona (Spain), respectively. The collected fractions of the poultry slaughterhouse wastes (denoted as TI) were composed of a mixture of wings, necks, internal organs and heads. Piggery slaughterhouse wastes (denoted as TII) were composed of a mixture of internal and reproductive organs and fats. All sampled wastes were minced (4 mm maximum particle size) and mixed. The mixtures (TI and TII) were lyophilized and

grounded again only for chemical characterization, to ensure an improved homogeneity of the samples.

4.2.2. Analytical methods

Total and volatile solids (TS, VS) of untreated and pre-treated wastes were measured in accordance with Standard Methods (APHA, AWA, WEF 2005). Total COD (COD_t) was estimated by elementary analysis (LECO, USA), using the empiric formulas of each sample in accordance with the methodology proposed by Angelidaki *et al.* (2009) and checked following new COD solid determination (Noguerol-Arias *et al.*, 2012, annex-1). Methane (CH_4) content in produced biogas was determined by gas chromatography CP-3800 (Varian, USA) fitted with Hayesep Q 80/100 Mesh (2 m $\times$ 1/8" $\times$ 2.0 mm SS) packed column (Varian, USA) and TCD detector, as described elsewhere (Campos *et al.*, 2008). Deeper information about chemical composition of the samples (NH_4^+, VFA, COD_t, etc.) can be found in Chapter 3.

4.2.3. Thermal pre-treatments and anaerobic biodegradability assay

Pasteurization at 70 ºC for 60 minutes was applied to mixtures TI and TII. Pre-treated samples were named PTI for pasteurized poultry waste and PTII for pasteurized piggery waste. The methodology of anaerobic biodegradability test was described in detail in Chapter 3. Digested sewage sludge was used as inoculum. This inoculum was obtained from the digester of the wastewater treatment plant at La Llagosta (Barcelona, Spain). The assays were performed in triplicate using glass flaks of 1.2 l filled with 500 g of a solution containing an inoculum concentration of 5 $g_{VSS}.l^{-1}$ and a substrate concentration of 5 $g_{CODt} \cdot l^{-1}$ (Soto *et al.*, 1993). The pH was adjusted to neutrality and 5 ml of a solution of Na_2S (10 $g \cdot l^{-1}$) were added. The flasks were bubbled with a mixture of N_2 before closing with rubber bungs. The flasks were continuously shaken (100 rpm) during incubation at 35 °C for 31 days. The methane production was monitored by gas chromatography (Campos *et al.*, 2008) sampling the head space periodically. The gas volume was expressed at normal conditions (0 °C and 1 atm). Three flasks containing only inoculum were tested as blanks to monitor the residual methane production of the inoculum.

Net methane and biogas production were calculated by subtracting from results of anaerobic biodegradability tests data obtained from blank tests. These data were used to calculate anaerobic biodegradability (AB) expressed as % COD_t. Methane production

potential (MPP) was expressed as $m^3_{CH4} \cdot kg_{VS}^{-1}$ and methane production rate (MPR) was measured as the maximum slope of the accumulated methane production curve per unit of initial biomass content and expressed as $l_{CH4} \cdot kg_{VSS}^{-1} \cdot d^{-1}$, at standard conditions (0 ºC and 1 atm).

4.2.4. TGA-MS and FTIR spectroscopy

TG analysis was performed using a TA Instruments SDT 2960 thermobalance (Figure 4.1a and b). The heating rate applied was 10 ºC·min^{-1}, from ambient temperature up to 900 ºC, with a flow-rate of 100 ml·min^{-1} of synthetic air. DTG curves obtained from the first derivative of TG profiles were used to characterize the organic material in accordance with Conesa *et al*. (2001) and Dell´Abate *et al*. (1998). Thus DTG profiles represent the velocity of mass change respect to time. Identification is easier with DTG profiles, mainly because small changes in TG curves are magnified in the DTG peaks.

The mass-spectrometry apparatus (Quadrupole MS BalzersThermostar GSD 300 T Pfeiffer Vacuum, D-35614 Asslar) was equipped with an electron ionization source, a Faraday cup and a SEM detector (channeltron TM) (Figure 4.1c). The mass range was 0-200 amu. The apparatus was used in line with the thermal analysis equipment to monitor the gas obtained from the combustion process (Figure 4.1d). It was connected through a capillary filament at 200 ºC. Although a full quantitative analysis could not be performed, a comparison of the intensity of the peaks obtained from the different samples was made using the normalization procedure described by Arenillas *et al*. (1999). This normalization procedure is based on the use of a factor calculated as the ratio between the sample mass analyzed and the maximum value of the signal, both recorded by the mass-spectrometry apparatus. For comparison purposes, signal registered was also normalized following the procedure of Meissl *et al*. (2007). The selected signals m·z^{-1} (mass·charge^{-1}), to correlate the weight loss with the thermal degradation of the sample, were: 1) m·z^{-1} 18, representing H_2O emission; 2) m·z^{-1} 41, for the release of light hydrocarbon products; 3) m·z^{-1} 44, for CO_2 emission, and 4) m·z^{-1} 46, for NO_2 emission.

FTIR analysis was performed using 2 mg of lyophilized milled samples, ground up with 200 mg KBr (FTIR grade) and homogenized in an agate mortar to later produce pellets. These pellets were compressed at a pressure of 6000 kg·cm^{-1} for 10 min (Figure 4.2a). Infrared spectra were recorded using an FTIR Perkin-Elmer 2000 spectrophotometer, over the 4000-400 cm^{-1} range at a rate of 0.5 cm·s^{-1} (Figure 4.2b). Fifty scans were

collected, averaged for each spectrum and corrected against ambient air as background. Spectra vector was normalized for comparison purposes following the procedure suggested by Meissl *et al*. (2007).
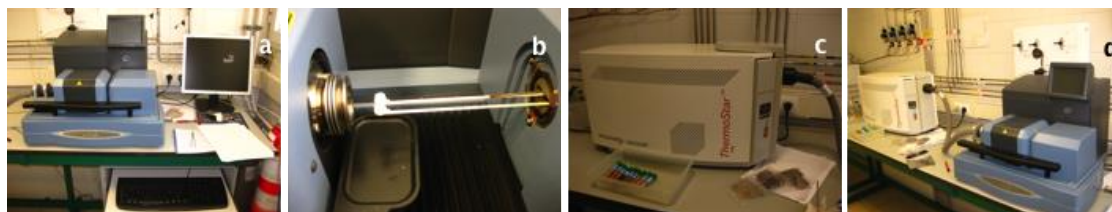
Figure 4.1. *Pictures of the thermal analyzer (a and b), mass-spectrophotometer (c) and thermal analyzer-mass spectophotometer connected in line (d).*

Figure 4.2. *Pictures of the compressor to create the pellets (a) and FTIR Perkin-Elmer 2000 spectrophotometer (b).*

4.3. RESULTS AND DISCUSSION

4.3.1. Digestion tests: poultry and piggery wastes

The basic characteristics of substrates (TI and TII) are summarized in Table 4.1. The ash content, mainly due to the presence of bone fractions, was higher for TI sample, although low values were reported for both samples. ABP materials were characterized by different fat, protein and carbohydrate ratios (F:P:C), based on the initial COD_t content: the COD_t composition of TI waste was balanced for proteins, fats and carbohydrates, while the fat fraction was the major component of TII sample, with a low amount of carbohydrates being reported.

The anaerobic biodegradability of untreated wastes was 55.2 and 76.6% COD_t, for poultry and piggery wastes, respectively, while it was increased to 61.8 and 94.3% COD_t for pre-treated samples. The AB increase obtained due to the pre-treatment in the case of the poultry samples was not significant in comparison with results obtained from piggery samples. A discussion of these results is reported in Chapter 3. Figure 4.3 shows the

evolution of methane production potential during AB tests. The increase in the biodegradability of substrates was not directly related to the increase in methane yields (see Table 4.2).

Table 4.1. *Characterization of initial samples of poultry waste (TI) and piggery waste (TII). Nomenclature: (1) F-fat, P-protein and C-carbohydrate expressed in % of COD_t; (2) estimated value from elemental analysis.*

Parameters	Units	TI	TII
TS (w·w^{-1})	%	30.7 ±0.4	50.7 ±0.4
VS (w·w^{-1})	%	26.6 ±0.6	48.9 ±0.1
F:P:C(1)	%:%:%	33:33:34	82:13:4
COD_t(2)	g·kg^{-1}	653.5	1275.0

Table 4.2. *Results of the methane production potential (MPP) and methane production rate (MPR) of raw (TI and TII) and pasteurized wastes (PTI and PTII). Increments are referred to the initial waste.*

Parameters	Units	TI	PTI	TII	PTII
MPP	m$^3_{CH4}$·kg$_{VS}^{-1}$	0.46 ±0.01	0.48 ±0.01	0.58 ±0.03	0.88 ±0.01
MPP increment	*%*	-	2.6	-	52.7
MPR	l$_{CH4}$·kg$_{VSS}^{-1}$·d^{-1}	31.4±0.6	17.6 ±0.3	24.7±1.4	37.6±2.2
MPR increment	*%*	-	- 43.8	-	52.6
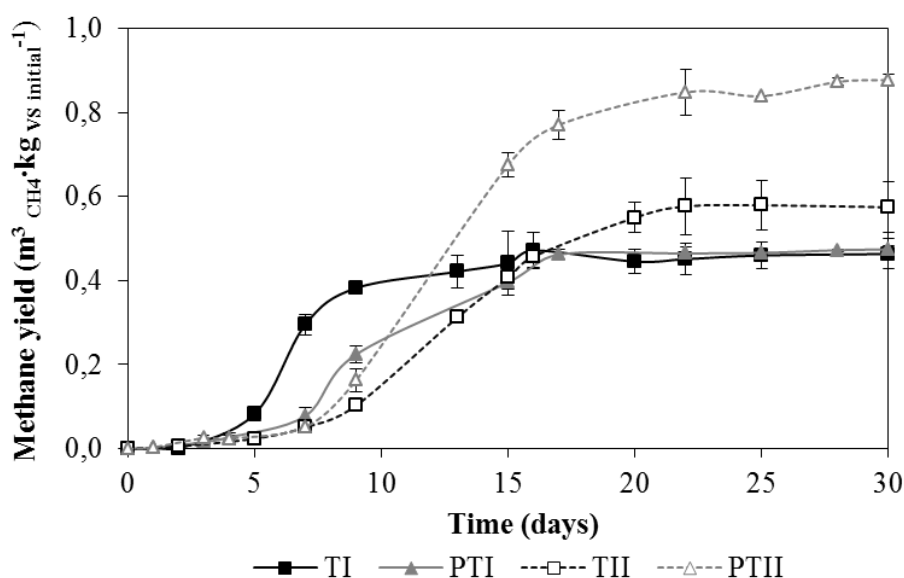
Figure 4.3. *Methane production yield during the AB test of initial and pre-treated TI and TII.*

A lag phase was observed during the batch assay in all cases. However, thermal pre-treatment caused a significant change in the lag phase experienced by poultry wastes (Fig.

4.3). A steep increase in the rate of methane production of TI was observed at day 5, while the pre-treated sample (PTI) presented this increase at day 8. This modification may be explained by a major degree of complexity of the pre-treated organic material since inhibitions due to accumulation of $N-NH_4^+$ or VFA were not reported (Chapter 3). Results from the anaerobic degradation of piggery wastes are also presented in Fig. 4.3. In this case, the methane production started at about the same time for untreated and pre-treated samples. An increase in the methane production potential and also in the methane production rate was observed for the pre-treated sample.

4.3.2. TGA-MS results of poultry ABP

The DTG profiles obtained from poultry samples are shown in Figure 4.4. DTG curves reached a value of zero at the end of the profile, thus indicating that reactions can be considered completed (Dell'Abate *et al.*, 1998). Thermal pre-treatment did not affect the onset temperature at which volatilization and mass loss due to oxidation reactions take places. However, the profile of the poultry waste sample was considerably modified by the pre-treatment. When considering the temperature range of 150-350 ºC, two peaks were reported for PTI sample, while only one peak was obtained from the poultry sample (TI) thermogram. This oxidation pattern may indicate the presence of labile fractions that should be easily degraded by microorganisms, as reported by Gómez *et al.* (2007). This statement should translate into a shortening of the lag phase for methane production when considering AB test of the pre-treated sample. However, this was not the case, and results obtained from AB tests reported an increase in the lag phase (Fig. 4.3).
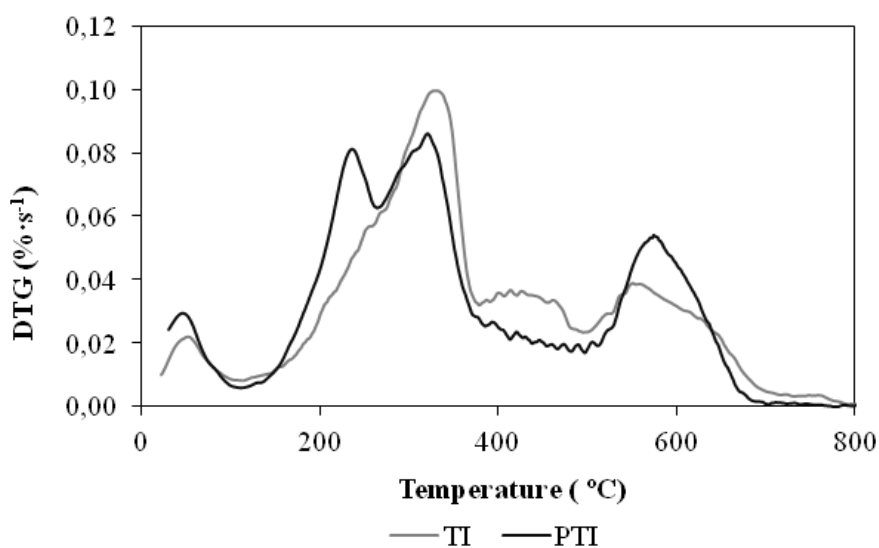
Figure 4.4. *Evolution of the weight-loss profiles (TGA-DTG) of the initial and pre-treated poultry wastes (TI and PTI).*
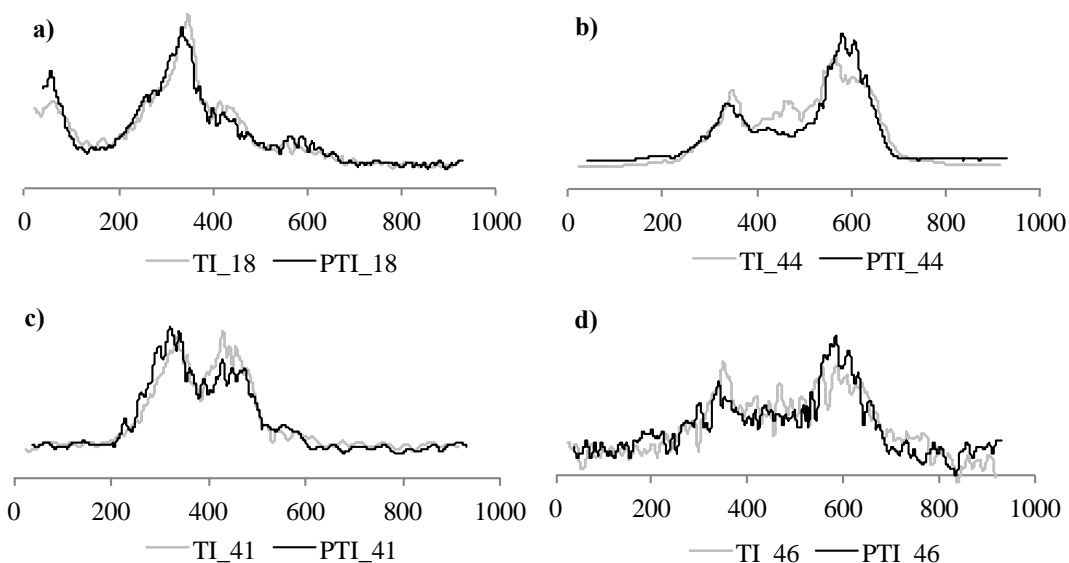
Figure 4.5*. Emission profiles for mass/charge ($m \cdot z^{-1}$) signals in an oxidizing atmosphere the initial (TI) and pasteurized (PTI) poultry waste: a, b, c, d denote $m \cdot z^{-1}$ 18 (H_2O), 44 (CO_2), 41 (light hydrocarbons) and 46 (NO_2) respectively.*

When considering the high temperature range of the spectra, two main changes are observed from the thermal profile of TI and PTI samples. The first difference is the lower intensity of the peak at 400-500 ºC and the second one is the higher intensity of the peak recorded at about 600 ºC for the PTI sample. The continuous weight loss at around 350-450 ºC may be associated with the combustion of char formed at lower temperatures. This process has been reported by Font *et al*. (2001) and Zhu *et al*. (2007) in the thermogravimetric study of sewage sludge and landfill sludge. In this sense, the pre-treatment of the sample resulted in a decrease of organic components, which are prone to suffer pyrolysis. On the other hand, regarding the peak at 600 ºC, the increase registered in its intensity may be interpreted as an increase of complex organic materials containing nitrogen (Cuetos *et al*., 2010).

Signals obtained for evolved gases for poultry wastes are presented in Fig. 4.5. The signal at $m \cdot z^{-1}$ 18 was ascribed to water release (Fig. 4.5a). An important release of water was observed during the combustion of labile structures, indicating high content of H atoms in these compounds. This signal presented a continuous decrease in its intensity with the increase in the oxidation temperature. This behavior may indicate that materials suffering thermal degradation at higher temperatures had a lower content in H atoms and thus a greater complexity. These variations are in agreement with those observed in the intensity of peaks registered in DTG profiles at about 400-500 ºC for TI and PTI. The mass loss

obtained in this temperature range was rationalized by the combustion of pyrolysis products, which are characterized by high carbon content.

In this same line, signal $m \cdot z^{-1}$ 44 ascribed to CO_2 release (Fig. 4.5b) presented an increasing trend at temperatures above 300 ºC, with a major intensity around 600 ºC, thus corroborating the assumption of the lower tendency of the pre-treated sample to suffer pyrolysis but, at the same time, the high complexity of components formed due to the pre-treatment. CO_2 evolved at this high temperature reflected the presence of stable ether structures, quinones and oxygen-bearing heterocycles or even carbonate decomposition (Arenillas *et al*., 1999), with this latter being explained by the presence of bones in the poultry wastes.

With regard to signals ascribed to light hydrocarbons ($m \cdot z^{-1}$ 41) and NO_2 ($m \cdot z^{-1}$ 46) release (Fig. 4.5c and 4.5d), PTI sample presented a higher relative intensity of light hydrocarbon emissions at the low temperature range but a greater relative intensity in the high temperature range when considering N compounds. These observations evidenced that, although pre-treatment at low temperature improved the decomposition of organic matter, it may also carry out the formation of complex N containing forms when proteins are an important constituent of the organic matter. This fact may explain the lower methane rate obtained from PTI AB test when compared to the non-treated sample.

4.3.3. FTIR results of poultry ABP

The FTIR spectra of poultry wastes are presented in Fig. 4.6. Previously, in order to identify different compounds, an information review was done and shown in Table 4.3. One of the broadest bands can be observed at 3430-3300 cm^{-1}, assigned to the stretching of OH bond (Padmavathy *et al*., 2003). This band presented an asymmetry caused by the presence of N-containing components (N-H stretching band around 3300 cm^{-1}; Won *et al*., 2006), which increased after pre-treatment and was also reported in thermographs and mass-spectrometry analysis. The increase in these N-containing compounds may explain the results reported AB tests as responsible of the higher lag phase experienced. Bands at 3000-2800 and cm^{-1}were ascribed to aliphatic groups by Réveillé *et al*. (2003), agreeing with Ilani *et al*. (2005), who ascribed 2965-2850 cm^{-1} bands to aliphatic component (C–H stretch of $–CH_2$ and $–CH_3$) and also with Smidt and Schwanninger (2005) who considered bands from 2925 to 2855 cm^{-1} due to stretching vibration of aliphatic methylene as fatty acids.
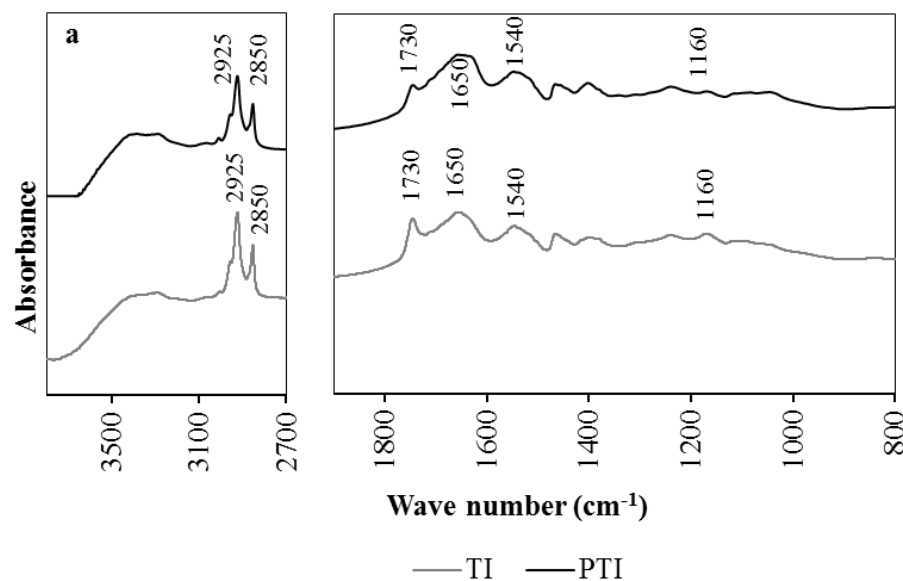
Figure 4.6. *FTIR spectra for the initial (TI) and pasteurized (PTI) poultry waste.*

Table 4.3. *The main absorbance bands in FTIR spectra and their assignments.*

Bands (cm^{-1})	**Functional group or component**	**References**
1170-950	Polysaccharides and phosphodiesters	Hesse *et al*. (1999); Naumann *et al*. (1996)
1384	Nitrate and fatty acids	Smith (1999); Smidt *et al*. (2002); Zaccheo *et al*. (2002).
1425	COO- stretch of carboxylic acids and C-O stretch of carbonate	Hesse *et al*. (1999); Smith (1999); Smidt and Schwanninger (2005)
1540	N-H in plane bending of the amide II	Smidt *et al*. (2002)
1607-1463	Compounds derived from Maillard reactions	Yaylayan and Kaminsky (1998)
1647	C=N stretching vibration attributed to Maillard reaction	Su *et al*. (2010)
1650	Aromatic structures, C=O stretching in quinones, ketonic acids and primary amides	Senesi *et al*. (1996)
1730	Carboxylic bands	Haberhauer *et al*. (1998)
1740	C=O stretch of aldehydes, ketones, acid carboxylic and esters	Smidt and Schwanninger (2005)
1745	Carbonyl compounds in aliphatic ester structures	Moreno *et al*. (1999)
2925, 2855	Methylene groups of aliphatics	Chefetz *et al*. (1998); Smidt and Lechner (2002); Révéille *et al*. (2003)
3000-2800	Aliphatic groups	Réveillé *et al*. (2003)
3500-3300	O-H vibrations of the hydroxyl groups of phenols, alcohols and carboxyl N-H vibrations from amides and amines	Padmavathy *et al*. (2003); Won *et al*. (2006)

The peak observed at 1730 cm^{-1} and 1160 cm^{-1}, which both decreased after thermal pre-treatment, are due to the carboxylic bands (Haberhauer *et al*., 1998) and polysaccharides (Hesse *et al*., 1999; Naumann *et al*., 1996) respectively. Partial degradation of these compounds due to pasteurization may have led to an increase in MPR, however this was not the case, and the reason may be associated to the presence on inhibitory compounds.

Signals recorded at 1790-1500 cm^{-1} were assigned to nitrogen containing compounds (Réveillé *et al*., 2003). The amide bond is the responsible for the linkage of successive amino acids to form peptides and proteins (Farhat *et al*., 1998). In the infrared spectrum of poultry samples, the peaks due to amide I (1690-1630 cm^{-1}) and II (1570-1540 cm^{-1}) (Farhat *et al*., 1998) are the peaks with higher intensity in the region 1900-800 cm^{-1}. The pre-treatment of poultry waste caused a relative increase of signal registered at 1650 cm^{-1} in comparison to untreated sample. This signal was ascribed to C=O vibration in primary amides (Senesi *et al*., 1996). The N-H bending in secondary amides also increased lightly (peak at 1535 cm^{-1}). Su *et al*. (2010) observed that the band at 1647 cm^{-1} indicates C=N stretching vibration that they attributed to Maillard reactions. The presence of this N-complex components may be associated with the formation of compounds derived from Maillard reactions, as may be inferred from the relative increase in the signal intensity in the regions 1607-1463 cm^{-1} (Yaylayan and Kaminsky, 1998). The nature of these N species may be responsible of the lower methane rate observed during the anaerobic digestion of PTI samples.

4.3.4. TGA-MS results of piggery ABP

DTG profiles obtained from piggery samples are shown in Figure 4.7. Profiles are characterized by a main peak at around 350 ºC, with a higher intensity being registered for PTII sample. This behavior is associated with the volatilization and combustion of fatty compounds, which are the main components of TII and PTII (Table 4.1).

Peaks with a lower relative intensity, when compared to the main peak, are also observed at around 400 ºC and at 500-600 ºC. As stated previously, the formation of char at low temperature range resulted in the combustion of pyrolysis products at higher temperatures. This was probably the case for peaks registered at this higher temperature range. As in the previous case, pre-treatment of TII also resulted in a decrease of the fraction prone to suffer pyrolysis at low temperatures. However, in this case the different chemical composition of TII sample, with lower protein content, may have influenced the

conversion of the organic matter and thus explained the absence of a major peak observed in poultry samples around 600 ºC.

Results obtained from evolved gases analyses are presented in Fig. 4.8. The main oxidation of labile compounds with a high H content is corroborated and a CO_2 signal of higher relative intensity in the low temperature range is also presented (Fig. 4.8a and 4.8b). As in the previous case, the pre-treatment decreased the formation of pyrolysis products suffering oxidation in the 400-500 ºC range, which is in accordance with the higher relative intensity of $m \cdot z^{-1}$ 41 signal at low temperature (Fig. 4.8c).
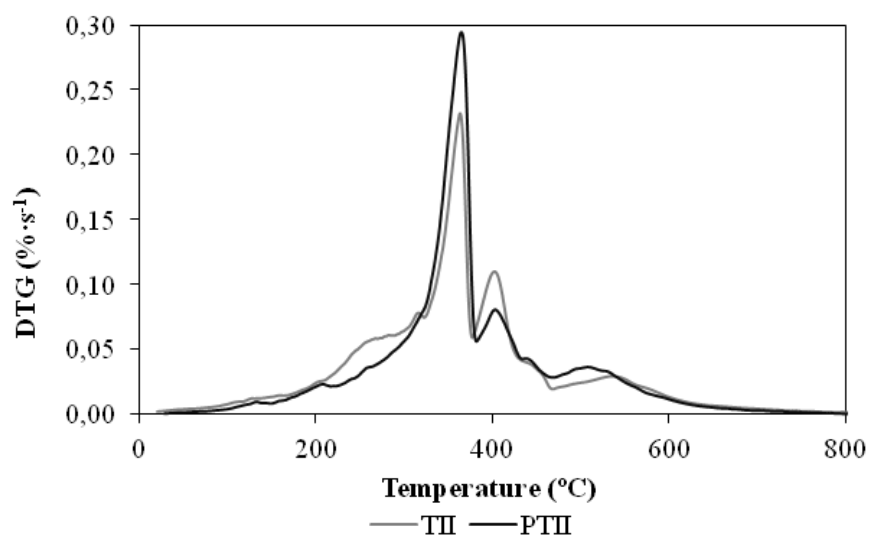
Figure 4.7. *Evolution of the weight-loss profiles (TGA-DTG) of the initial and pre-treated piggery wastes (TII and PTII).*
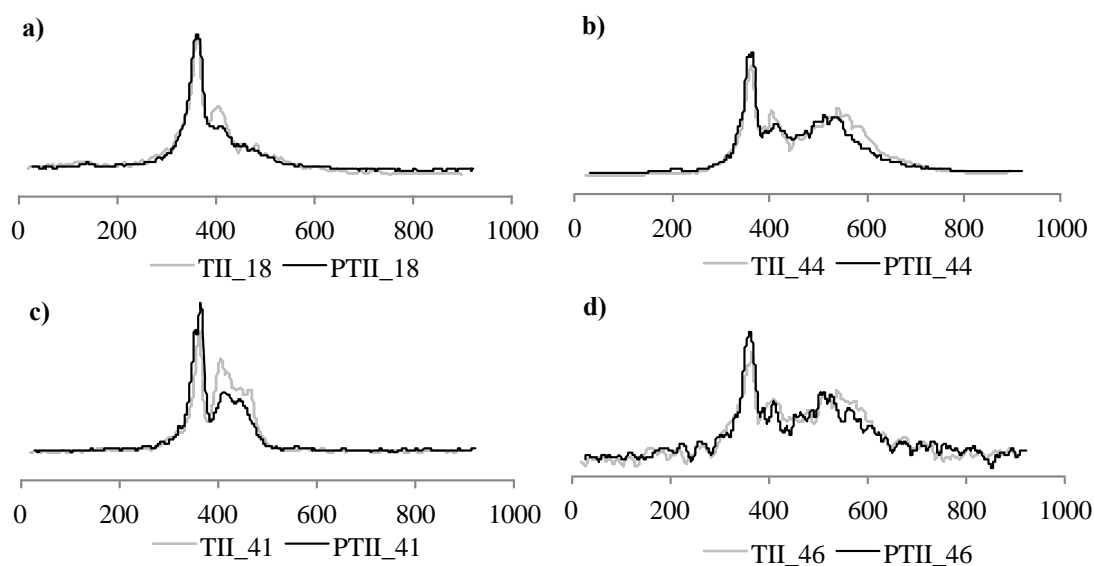
Figure 4.8. *Emission profiles for mass/charge ($m \cdot z^{-1}$) signals in an oxidizing atmosphere the initial (TII) and pasteurized (PTII) piggery waste: a, b, c, d are $m \cdot z^{-1}$ 18 (H_2O), 44 (CO_2), 41 (light hydrocarbons) and 46 (NO_2) respectively.*

Contrarily to the thermal behavior observed from the poultry system, in the case of piggery waste no major contribution was observed from the NO_2 signal at high temperatures (Fig. 4.8d), with the main release being associated with the thermal oxidation of labile structures. In this line, the lower content of protein in this sample may be the main factor contributing to the low presence of N complex compounds. Considering once again the cumulative methane curves obtained from the piggery ABP (Fig. 4.3), it may be reasonable to assume that the increase in the methane yield observed in the pre-treated sample keeps a straight relation with the steep mass loss occurred at about 350 ºC. The applied pre-treatment allowed a faster and higher degradation of organic matter by increasing the content of labile degradable components.

4.3.5. FTIR results of piggery ABP

FTIR spectra obtained from piggery ABP are shown in Fig. 4.9. Untreated and treated samples spectra are characterized by huge bands ascribed to aliphatic components (between 2850 and 3000 cm^{-1}), in accordance with the high fat content of TII and PTII. The band at 1745 cm^{-1} also presents an important contribution to both spectra. This band was assigned to carbonyl compounds in aliphatic ester structures (Moreno *et al*., 1999).
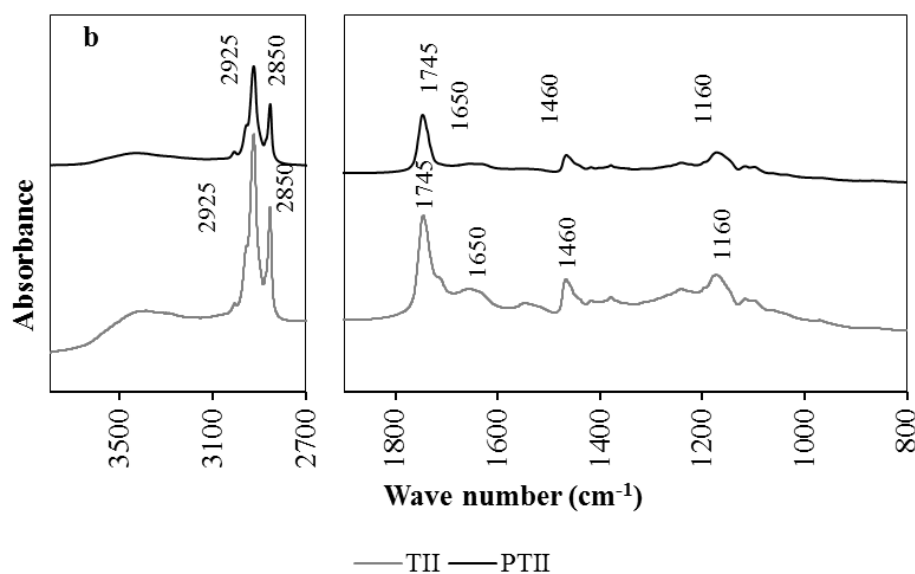
Figure 4.9. *FTIR spectra for the initial (TII) and pasteurized (PTII) pig waste.*

In untreated sample there is a small shoulder at 1730 cm^{-1} that is due to carboxylic compounds (Haberhauer *et al*., 1998). Other important bands, described in Table 4.3, are at 1650 cm^{-1}, 1460 cm^{-1} and 1160 cm^{-1} that did not change significantly after pre-treatment. In opposition to the poultry case, the bands ascribed to N-compounds did not

registered increases in their intensity, being in accordance to TGA-MS analysis and when comparing the spectra obtained from piggery and poultry samples, the first shows the absence of asymmetry (3300 cm^{-1}), which is characteristic of high protein content.

The transformation of the organic matter due to the thermal pre-treatment of piggery wastes allowed the partial oxidation of aliphatic components resulting in an increase in the methane yield and production rate of the pre-treated sample (Fig. 4.3). On the other hand, the lack of formation of N-complex components due to the pre-treatment may be closely related to the relative content of protein, fat and carbohydrate in the sample.

4.4. CONCLUSIONS

The present study, where TGA-MS and FTIR techniques were applied on untreated and thermally pre-treated poultry and piggery ABP was useful to characterize deeply the organic matter and to demonstrated the existence of changes in the organic matter after this thermal pre-treatment. It can explain the different behaviors in methane production depending on the fat, protein and carbohydrate composition.

With these new techniques was also possible to observe that thermal pre-treatment decomposed labile compounds of poultry wastes but that at the same time favored the formation of complex N containing molecules, resulting in a lower value of methane production rate. The apparition of a new band in FTIR profile related to Maillard compounds can explain the lower methane rate observed during the anaerobic digestion of the pre-treated poultry waste. In the case of piggery wastes, an increase in the content of labile degradable compounds after the pasteurization that improved the biological conversion into methane was observed thanks to TGA-MS and FTIR. The lack of N-complex components may be closely related to the relative low content of protein and carbohydrate in the raw waste.

4.5. ACKNOWLEDGEMENTS

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Chapter 5. Study of the effects of high pressure pre-treatment on animal by-products

High pressure application on animal by-products (ABP) as a new physical pre-treatment was evaluated in this chapter. The objective of this work was to study its effects on the solubilization and the anaerobic digestion of ABP at mesophilic range. Three different pressures, 200, 400 and 600 MPa, were applied at room temperature for 15 minutes. The effect of this pre-treatment on the organic fraction solubilization was higher at 200 MPa although during the biochemical potential test the protein degradation was much higher with 600 MPa pre-treated sample. Related to the methane yield and the anaerobic biodegradability (in %COD degradation), 200 and 400 MPa pre-treatment gave similar results, improving slightly all the studied parameters compared to the untreated ABP, while the most severe pre-treatment (at 600 MPa) produced a decrease in all of them. Methane production rate was not improved significantly by this pre-treatment at any pressure. As conclusion, the high pressure pre-treatment did not produce significant improvements or negative effects on the anaerobic digestion of the slaughterhouse waste samples studied.

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5.1. INTRODUCTION

High pressure application is a physical method of food processing where the sample is subjected to elevated pressures, between 100 and 800 MPa (Kadam *et al*., 2012), with or without the addition of heat, to achieve microbial inactivation or to alter the food attributes in order to achieve consumer-desired qualities (Raventós, 2005). In published experimental results using high pressures with several food products, it has been observed that high pressure process can prevent spoilage caused by organisms, inactivate enzymes or bacterial spores, extend shelf life, reduce the potential for food borne illness, promote ripening of cheese and minimize oxidative browning (Kadam *et al*., 2012). However, the resistance of the microorganisms is very variable depending on the strain and the meat matrix to be treated. The efficacy of the treatment also depends on the achieved pressure, on the treatment temperature and on the exposure time (Hugas *et al*., 2002).

Until now there are no previous studies about the effects of high pressures on substrates and on methane production by anaerobic digestion. Considering that some of the applications of high pressures affect the myofibrillar proteins, the lipids and their gel-forming properties, as well as cause a partial sanitation, there are interesting possibilities for its use as a pre-treatment of the anaerobic digestion of animal waste.

The aim of this chapter is to study the effects of three high pressure pre-treatments on the solubilization and anaerobic digestion of ABP, in order to evaluate a new method for pre-treating solid animal by-products.

5.2. SPECIFIC MATERIAL AND METHODS

5.2.1. Slaughterhouse waste

The selected ABP was sampled at one piggery slaughterhouse facility located in Barcelona (Spain). The fractions collected from the piggery slaughterhouse (TIII) were a mixture of internal organs (kidneys, lungs, livers and hearts), reproductive organs and piggery fatty wastes. All the waste samples were minced (4 mm maximum particle size) and mixed. The mixture obtained (called TIII in Table 5.1) was lyophilized before characterization in order to improve their homogeneity (see Material and Methods of Chapter 3 for the analytical determination methodology).

5.2.2. High pressure pre-treatment

The experiments were performed in triplicate with TIII in a hyperbaric reactor (NC Hyperbaric 6000/120 wave model, Burgos) with capacity of 60 kg and with the possibility of regulating the temperature from 10-12 °C to 30-35 °C. The reactor was placed at CENTA-IRTA Research Centre (Monells, Girona, Spain).

Figure 5.1. *Hyperbaric reactor (NC Hyperbaric 6000/120 wave model).*

The three selected pressures were 200, 400 and 600 MPa (Table 5.1), the operation time was 15 minutes and the experiments were carried out at room temperature (13 ºC), although temperature increments between 3-15 ºC were observed (Table 5.1). The samples were vacuum packed in flexible and watertight plastic bags and during the hydrostatic pressure treatment they were placed in a cylinder of 300 mm diameter and immersed in a bath of 120 litters of water. The final cooling phase was instantaneous after the pressure was removed.

The nomenclature of the untreated and treated samples and the increments in the temperature are summarized in Table 5.1.

Table 5.1. *Nomenclature of the raw and pre-treated ABP samples and the temperature increment during experiments.*

ABP	Nomenclature	Pre-treatment	Pressure (MPa)	Temperature increment (ºC)
Piggery	TIII	High pressures	200 MPa	3.0
			400 MPa	9.7
			600 MPa	14.6

5.2.3. Evaluation of the results and anaerobic biodegradability assay

The effects of the high pressure pre-treatments on the substrate was evaluated by the degree of solubilization (%S) of the COD_t, defined as the ratio between soluble and total COD ($COD_s \cdot COD_t^{-1}$) (Chulhwan *et al.*, 2005) and other parameters as the ammonium

nitrogen to total nitrogen concentrations ratio (% TAN), the VFA and fat concentrations. These ratios were evaluated together with the results obtained in the biochemical methane potential tests (BMP).

Following the same methodology for BMP tests than in the Chapter 3, the degradation of organic matter was deeply evaluated in terms of COD to obtain the anaerobic biodegradability (AB) of the waste, taking into account the fraction converted to methane (% M), the fraction in form of volatile fatty acids (% A) and also the fraction employed in cell growth by methanogenic (Y_M =*0.028 $g_{CODcel}·g_{CODelim}$*$^{-1,}$ from Field *et al.*, 1988) and acidogenic populations (Y_A =*0.064 $g_{CODcel}·g_{CODelim}$*$^{-1}$, it is an average value obtained from Batstone *et al.,*2002).

Methanization index (%M)

$$M = 100 \cdot \frac{COD_{CH4}}{COD_{ini}}$$

Acidogenesis index (%A)

$$A = 100 \cdot \frac{\left(COD_{CH4} + COD_{VFAfinal}\right)}{COD_{initial}}$$

Anaerobic Biodegradability (%AB)

$$AB = A + \frac{Y_A}{(1 - Y_A)} \cdot \left(A - 100 \frac{COD_{VFAini}}{COD_{ini}}\right) + \frac{Y_M}{(1 - Y_M)} \cdot M$$

With the data obtained of the BMP test the methane production potential (MPP) and methane production rate (MPR) were also determined.

5.3. RESULTS AND DISCUSSION

5.3.1. Wastes characterization

The characteristics of raw ABP substrate (TIII) are shown in Table 5.2. The TIII is characterized by a high organic matter content (1205.1 g·kg^{-1} as COD$_t$). The total solids and the inorganic fraction were 45.1 and 4.5% respectively of the total fresh matter. The main component of the TIII was fat, having also similar amounts of protein and carbohydrate related to initial COD$_t$ content. The F:P:C ratio was 72:15:12.

Table 5.2. *Characterization, mean ± standard deviation, of untreated piggery ABP (TIII). Nomenclature: (1) volatile solids with respect to the total substrate; (2) F-fat, P-protein and C-carbohydrate expressed in % of COD_t; (3) estimated value from elemental analysis.*

Parameters		TIII
TS $(w \cdot w^{-1})$	%	45.1±0.6
VS $(w \cdot w^{-1})$ (1)	%	43.1±0.7
F:P:C (2)	%:%:%	72:15:12
COD_t (3)	$g \cdot kg^{-1}$	1205.1
COD_s	$g \cdot kg^{-1}$	16.4±2.1
Total N	$g \cdot kg^{-1}$	19.3±0.7
NH_4^+	$g \cdot kg^{-1}$	1.3±0.1
Est. Protein	$g \cdot kg^{-1}$	112.8
COD_{prot}	$g \cdot kg^{-1}$	186.1±3.7
COD_{VFA}	$g \cdot l^{-1}$	2.3±0.0
Fat	$g \cdot kg^{-1}$	302.4±1.4
COD_{fat}	$g \cdot kg^{-1}$	870.9±7.7
COD_{ch}	$g \cdot kg^{-1}$	148.1±10.1

5.3.2. Effect of the high pressure pre-treatments on the organic matter

The characteristics of the pre-treated ABP (200, 400, 600 MPa) are summarized in Table 5.3. In this case, like after the thermal pre-treatment, there was a slight increase in the total and volatile solids concentration, similar in all the high pressure pre-treatments due to some water loss during the process but the COD_t was nearly the same than in the untreated sample (having into account the standard deviations, the differences were negligible).

The effect of the high pressure pre-treatments on organic material solubilization was clear in all the cases (Table 5.4), with an increase with respect to the untreated samples of 87.6% for 200 MPa, 68.5% for 400 MPa and 59.7% for 600 MPa.

The highest solubilization was achieved with the less severe pre-treatment (200 MPa). The $NH_4^+ \cdot TN^{-1}$ ratio increased after the application of 200 and 400 MPa pre-treatments (16.6 and 20.8% respectively) meaning some effect on the protein fraction. This ratio was barely modified in the case of 600 MPa. VFA concentration (in COD units) increased

after all the pre-treatments, being higher after the 400 MPa pre-treatment, reaching 34.2%.

Table 5.3. *Characterization, mean ± standard deviation, of pre-treated wastes at 200, 400 and 600 MPa. Nomenclature: (1) volatile solids with respect to the total substrate; (2) estimated value from elemental analysis.*

Parameter		200 MPa	400 MPa	600 MPa
TS (w·w^{-1})	%	47.6±1.1	47.1±1.3	47.9±0.4
VS (w·w^{-1}) (1)	%	46.0±1.4	44.9±2.1	46.1±1.0
COD$_t$ (2)	g·kg^{-1}	1190.3±16.6	1195.6±17.6	1198.3±10.6
COD$_s$	g·kg^{-1}	38.6±0.1	34.0±1.2	32.8±1.1

Table 5.4. *Comparison between the untreated sample (ABP) and the pre-treated samples at 200, 400 and 600 MPa. Increments (incr.) are referred to the untreated waste and evaluated with the average values.*

Parameter	Units	ABP	200 MPa	400 MPa	600 MPa
Solubilization (S)	% COD$_s$·COD$_t$$^{-1}$	1.6±0.6	3.0±0.4	2.7±0.3	2.6±0.3
S incr.	%	-	87.6	68.5	59.7
%TAN	% NH$_4$$^+$·TN^{-1}	6.7±0.7	7.8±0.8	8.1±0.1	6.6±1.6
%TAN incr.	%	-	16.6	20.8	-0.5
COD$_{VFA}$	g·l^{-1}	2.3±0.1	2.6±0.1	3.0±0.5	2.5±0.2
COD$_{VFA}$ incr.	%	-	13.2	34.2	11.0

5.3.3. Effect of the high pressure pre-treatments on anaerobic biodegradability

Results of methanization, acidogenesis, anaerobic biodegradability, methane yield (MPP) and methane production rate (MPR) of raw and thermally pre-treated ABPs are shown in Table 5.5. The initial methanogenic activity of the inoculum was around 54 mg$_{CH4-COD}$·g$_{VSS}$$^{-1}$·d^{-1} a bit higher than the activity of the sludge used in the thermal pre-treatment experiments of Chapter 3 (42 mg$_{CH4-COD}$·g$_{VSS}$$^{-1}$·d^{-1}).

Anaerobic biodegradability of the untreated ABP was high; achieving 85.6% of the COD$_t$ and reaching also elevate methane yields of 0.3 m^3$_{CH4}$·kg$_{COD}$$^{-1}$ and 0.9 m^3$_{CH4}$·kg$_{VS}$$^{-1}$, this last value was in the high range of most of the values observed in literature (between 0.2-0.9 m^3$_{CH4}$·kg$_{VS}$$^{-1}$, Hejnfelt and Angelidaki, 2009; Salminen *et al.*, 2003; Bayr *et al.*, 2012; Chapter 3 of this thesis).

Table 5.5. Comparison between the untreated sample (TIII) and the pre-treated samples at 200, 400 and 600 MPa. Increments (incr.) are referred to the untreated waste and evaluated with the average values.

Parameter	Units	TIII	200 MPa	400 MPa	600 MPa
MPP	$m^3_{CH4} \cdot kg_{VS}^{-1}$	0.9±0.1	0.9±0.0	0.9±0.0	0.8±0.1
MPP incr.	%	-	-1.5	0.4	-10.0
%M	% COD_t	76.5±7.6	81.5±3.9	80.6±1.5	74.1±3.2
%M incr.	%	-	6.5	5.4	-3.1
%A	% COD_t	78.4±7.3	82.4±4.0	83.7±1.6	75.3±3.2
%A incr.	%	-	5.1	6.7	-4.0
AB	% COD_t	85.6±8.0	90.0±4.3	91.3±1.7	82.2±3.5
AB incr.	%	-	5.2	6.7	-4.0
MPR	$l_{CH4} \cdot kg_{VSS}^{-1} \cdot d^{-1}$	71.6±9.5	59.3±5.4	71.1±2.0	62.8±6.1
MPR incr.	%	-	-17.1	-0.6	-12.3

The methane yields (MPP) were barely affected after 200 and 400 MPa pre-treatments, reaching almost the same final yields than in TIII (0.9 $m^3_{CH4} \cdot kg_{VS}^{-1}$). In the case of 600 MPa a small decrease in the MPP was observed (10.0 %). The methanization (M), the acidogenesis (A) and the anaerobic biodegradability (AB) were slightly augmented for 200 and 400 MPa pre-treatments, but without significant differences and being negatively affected by the 600 MPa treatment. These AB values were too low compared with the results obtained applying a thermal pre-treatment to a similar sample, 23.2-28.8% increment in AB related to the untreated waste (Chapter 3).

Figure 5.2 shows methane evolutions during BMP tests. A lag-phase in the untreated or pre-treated samples was not observed, although the fat fraction was considerable high (Table 5.2). The high pressure pre-treatment did not improve the methane production rate in any of the studied pressures. In the case of 200 and 600 MPa the MPR decreased 17.1 and 12.3% respectively and in 400 MPa experiment it was almost the same (there was only a decrease of 0.6%). It means that this pre-treatment at the selected pressures make some organic fractions more slowly degrade.

Increments in the ammonia concentrations, comparing initial and final NH_4^+ values during batch assays were observed in all the cases, from 15.9% in the untreated waste until 19.1, 28.4 and 32.9 % in 200, 400 and 600 MPa respectively (Fig. 5.3), so the most

intense pre-treatment, carried out at 600 MPa, seems to influence the protein degradation during the BMP test. The VFA were also measured at the beginning and at the end of the BMP (data not shown), observing that the VFA were totally consumed at the end of the batch test, being the end values under detection limits.

The effect of the high pressures on the organic fraction solubilization was higher with the lower pressure studied (200 MPa) and produces some positive effects increasing VFA and NH_4^+ concentration after 200 and 400 MPa. Related to the biogas and methane yield, the methanogenesis (%), the acidogenesis (%) and the anaerobic biodegradability (%), 200 and 400 MPa pre-treatment gave similar results, affecting all the studied parameters slightly compared to the untreated ABP, while the most severe pre-treatment (at 600 MPa) produces decreases in all of them. The methane production rate was not improved in any pre-treatment, meaning that high pressures make some organic fraction more slowly to degrade.

Figure 5.2. *Methane evolution related to the initial volatile solids (VS$_{initial}$) during the anaerobic biodegradation of untreated (ABP) and pre-treated at the indicated pressures.*

Figure 5.3. *Initial (white columns) and final NH_4^+ (grey columns) concentrations during the anaerobic biodegradability assay of TIII and 200, 400 and 600 MPa pre-treated samples. In % the increments of NH_4, considering initial and final BMP samples, are shown.*

5.4. CONCLUSIONS

Although the selected high pressure pre-treatments increased the solubilization of the solid fraction in all the cases and affected the protein degradation during the BMP tests, the batch anaerobic digestion performance indicate that these pre-treatments does not provide significant positive or negative differences respect to the methane yield and methane production rate of the raw substrate. Probably, the high initial anaerobic biodegradability of the tested slaughterhouse waste did not allow obtaining considerable improvements.

5.5. ACKNOWLEDGEMENTS

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Chapter 6. Study of the disintegration kinetic of raw and thermally pre-treated ABP and other solid organic wastes

Disintegration and hydrolysis phases can be considered the rate-limiting step for the anaerobic digestion process of solid substrates as animal by-products (ABP) from piggery slaughterhouse, characterized by high particulate matter content. Based on the structure of the ADM1, it is not easy to identify the corresponding coefficient (k_{dis}) without the knowledge of the parameters defining the disintegration products hydrolysis. In the present work, solutions of the differential equations expressing the dynamics of disintegration and hydrolysis and experimental conditions for the approximation of k_{dis} have been studied, concluding that the observed value k'_{dis} could tend to the true k_{dis} value performing batch experiments with particulate matter as substrate and with a high inoculum to substrate ratio (ISR), in order to obtain an accumulated methane production following a logistic-type curve. Several biochemical methane potential tests with different ISR were conducted to determine a global k'_{dis} and to check the effect of thermal pre-treatment on this parameter. With an ISR of 4, k'_{dis} values have been approximated for raw and thermally pre-treated ABP and for other solid substrates. A complete characterization of the particulate and total fraction of the selected samples was carried out to obtain a COD fractionation and to make a COD balance at the beginning and at the end of the batch tests. Finally, it was not possible to distinguish between k_{dis} and the different hydrolysis constants (of proteins, lipids and carbohydrates), due to the extreme difficulty to accomplish the chemical determinations of every fraction. Model values fitted perfectly the experimental results in all the cases and k'_{dis} values obtained indicated an increase of disintegration and hydrolysis overall rates after thermal pre-treatment with piggery ABP, explaining results obtained previously with these kind of substrates.

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6.1. INTRODUCTION

A major part of the animal by-products (ABP) obtained during the slaughtering process are solid substrates, characterized by high particulate matter content, mainly proteins and lipids (Palatsi *et al*., 2011). To avoid animal, human and environmental risks, some specific pre-treatments included in European regulations (No 1069/2009 and No 142/2011) should be applied to hygienize the ABP. These pre-treatments could also decrease the particle size and solubilize the material, improving in many cases the bioavailability of the proteins, lipids and carbohydrates (Edström *et al*., 2003; Chapter 3).

The extracellular biological and non-biological processes responsible for the breakdown and solubilization of complex organic material affect the disintegration and the hydrolysis phases, which are usually the rate-limiting steps of the anaerobic digestion of many solid organic wastes, such as the ABP. Numerical simulations of the overall anaerobic process, for a given waste or a mixture of them in anaerobic co-digestion plants, require reliable parameter values of the disintegration constant. While the proposed kinetic parameters in ADM1 (Batstone *et al*., 2002) for acidogenesis, acetogenesis and methanogenesis are proven to be applicable in a wide range of situations, parameters for disintegration and hydrolysis depend on the specific characteristics of a given substrate and, therefore, must be precisely identified. For example, the first order hydrolysis constant (k_h) is strongly dependent on the experimental conditions such as pH, temperature, particle size, stirring conditions or the applied ISR (Neves, 2009). As an example, the high variability of the protein hydrolysis coefficients for a first order kinetics is shown in Table 6.1.

Disintegration of composites (X_c) is expressed in ADM1 as a first order reaction with a constant k_{dis}. Products of this process are proteins (X_p), lipids (X_{li}), carbohydrates (X_{ch}), which follow a subsequent hydrolysis process, usually expressed also as a first order reaction, and particulate and soluble inerts, X_I and S_I respectively (Batstone *et al*., 2002). If acidogenesis of the hydrolysis products and the subsequent acetogenesis and methanogenesis are fast enough, the curve of the accumulated methane production obtained in batch experiments contains information about the kinetics of disintegration and hydrolysis. In this case, considering these two processes being the rate limiting steps defining the general kinetics of methane production, the dynamics of the system can be simplified and expressed with the differential equations defining the evolution of X_c, X_p, X_{li}, X_{ch} and the products of hydrolysis, considered to be methane (CH_4) if the uptake of soluble compounds is fast enough.

Table 6.1. *Kinetic coefficients values (k_h) of the first order rate of hydrolysis, based on Neves (2009) and Vavilin et al. (2008).*

Substrate	k_h (day^{-1})	T (ºC)	Reference
Proteins	0.015−0.075	55	Christ *et al.*, 2000
Proteins	0.25−0.28	-	García−Heras, 2003
Proteins	0.25−0.8	35	García−Heras, 2002
Proteins	0.14 -0.67	15-35	O'Rourke, 1968; Sanders, 2001
Proteins	6.2	30	Palenzuela−Rollón, 1999
Proteins	0.015 - 0.075	55	Christ *et al.*, 2000
Proteins	0.02 - 0.03	-	Gujer and Zehnder, 1983
Gelatine	0.65	55	Flotats *et al.*, 2006
Gelatine	0.60	-	Pavlostathis and Giraldo-Gómez, 1991
Domestic sewer prot.	0.2	35	Boon, 1994
"Excess of prot."	0.24	37	Neves, 2009
Casein	0.35	-	Pavlostathis and Giraldo-Gómez, 1991
Maize protein	0.04	-	Pavlostathis and Giraldo-Gómez, 1991

With first order reaction rates, the set of the five differential equations has an analytical solution, making possible its analysis. The general trend of the CH$_4$ production curve for any value of k_{dis}, of the hydrolysis constants ($k_{h,p}$, $k_{h,li}$, $k_{h,ch}$) and of the coefficients of the transformation of X_c to its products, f_p, f_{li}, f_{ch}, is a sigmoidal-type curve, tending to a logistic-type curve when k_{dis} value is much less or much higher than $k_{h,p}$, $k_{h,li}$, and $k_{h,ch}$ values (see ANNEX). In this case, the logistic-type curve can be fitted by:

$$F = B_0 \cdot (1 - e^{-k'_{dis} \cdot t})$$

(1)

where F is the accumulated methane production at time t, B_0 is the asymptotic methane production, and k'_{dis} is the observed constant, a global k_{dis}, tending to the true k_{dis} value when $k_{dis} <<< k_{h,p}$, $k_{h,li}$ and $k_{h,ch}$, while tending to the weighted harmonic mean of the hydrolysis constants in the opposite case. Equation 1 has been used for the approximation of hydrolysis constants (Vavilin *et al.*, 2008) or the disintegration constants assuming that higher values for the hydrolysis constants are considered (Galí *et al.*, 2009).

Several substrates have been used to obtain the disintegration and hydrolysis kinetics for calibrating the ADM1 model such as proteins, carbohydrates, lipids, bio-waste, sludge, kitchen and food waste, cattle manure, cellulose, fish processing wastewater, bark, grass, etc. (Christ *et al.*, 2000; Liebetrau *et al.*, 2004; Siegrist *et al.*, 2002; Vavilin *et al.*, 2008; Greco *et al.*, 1983; Palenzuela-Rollón, 1999; Veeken and Hamelers, 1999), but no information have been found related to k_h and k_{dis} of ABP.

General conditions for the use of Equation 1 were also applied to other solid organic wastes, such as pig manure, coffee by-product and sugar beet, in order to generalize methods used and to contrast results. The aim of this work was to study the effect of thermal pre-treatment of ABP (piggery and fishery wastes) on the kinetics of disintegration and hydrolysis processes, identifying the related constants, if possible, or the observed k'_{dis} value.

6.2. MATERIALS AND METHODS

6.2.1. Samples nomenclature and preparation

Raw and pasteurized animal by-products (ABP) from piggery and fishery industries, sugar beet, pig manure and coffee by-product were chosen for this work. The nomenclature used is shown in Table 6.2. The piggery ABPs consisted in the same slaughterhouse by-product used in Chapter 3 but from a different mixture. Fishery ABPs were composed by whole healthy fishes (trout) as surpluses from a fishing farm located in Lleida (Spain). Both ABPs were grinded, mixed and homogenized before any use. The pasteurization of ABP samples was performed following the methodology applied in Chapter 3. The sugar beet was a by-product from a sugar factory located in Cádiz (Spain). The pig manure was obtained from a centralized pig manure treatment facility located in Lleida (Spain) and the coffee by product was taken from a coffee factory of Girona (Spain).

Experiments with piggery ABP (raw and pre-treated samples), sugar beet and pig manure were conducted with the total and the particulate fractions. Experiments with fishery ABP (raw and pre-treated) and coffee by-product were carried out only with the total fraction.

To separate the particulate fraction, the whole sample was centrifuged for 10 minutes at 8000 rpm. After centrifugation, the liquid fraction was removed and placed separately. This procedure was repeated four times with the solid part from the previous centrifugation phase. All the liquid fractions collected in the previous step were also centrifuged to recover solids that could remain in those fractions. The sum of particulate fractions were placed over a 0.45 µm filter and washed with distilled water to remove the possible soluble material. The same was done with the liquid fraction, to be sure that all the solid particles were recovered. Summarizing, in this final step all the samples that did

not pass through the 0.45 µm filter were considered particulate fraction and used in this work.

6.2.2. Anaerobic biodegradability assay

The anaerobic biodegradability (AB) of the total and particulate samples was determined by mean of biochemical methane potential tests (BMP) following the methodology of Field *et al*., (1988), Soto *et al*. (1993) and Angelidaki *et al*. (2009). Anaerobically digested sewage sludge from the mesophilic anaerobic digester of the wastewater treatment plant (WWTP) at La Llagosta (Barcelona, Spain) was used as inoculum (denoted as I in Table 6.2). The inoculum was maintained in an incubation chamber (35 ºC) for 7 days to decrease the amount of its residual COD. The inoculum was characterized in terms of the specific methanogenic activity following Soto *et al*. (1993) methodology. The obtained value was 48 $mg_{CH4-COD} \cdot g_{VSS}^{-1} \cdot d^{-1}$.

Table 6.2. Source, substrate, fraction and nomenclature of the selected substrates and inoculum.

Source	Substrate	Fraction	Nomenclature
Piggery slaughterhouse	Solid ABP	total	P-ABP$_t$
		particulate	P-ABP$_p$
	Pasteurized solid ABP	total	PP-ABP$_t$
		particulate	PP-ABP$_p$
Fishing farm	Solid ABP	total	F-ABP$_t$
	Pasteurized solid ABP	total	FP-ABP$_t$
Sugar factory	Sugar beet by-product	total	SB$_t$
		particulate	SB$_p$
Pig manure treatment facility	Pig manure	total	PM$_t$
		particulate	PM$_p$
Coffee factory	Solid coffee by-product	total	C-BP$_t$
WWTP	Sewage sludge	-	I

The AB was determined in triplicate. Glass flaks of 1200 ml were filled with 500 g of a solution composed of the inoculum, macronutrients and micronutrients, substrate and bicarbonate (1 $g_{NaHCO3} \cdot g_{CODadded}^{-1}$). These tests were performed with different initial inoculum to substrate ratio (ISR=1, 2 and 4 $g_{VSS} \cdot g_{COD}^{-1}$) in order to obtain the ratio for which the concentration of microorganisms is not limiting the process and logistic-type curves are obtained. The pH was adjusted to neutrality using HCl or NaOH. The flasks were stirred and bubbled with a N_2 in order to remove O_2 before they were closed with rubber bungs. A reducing solution was finally added (5 ml of 10 $g_{Na2S} \cdot l^{-1}$). The flasks were continuously shaken (100 rpm) during incubation at 35 ºC for 31 days. The time

course of the methane production was monitored by gas chromatography (Campos *et al.*, 2008), sampling the head space periodically. The gas volume was expressed at normal conditions (0 ºC and 1 atm). Three flasks with the inoculum but without any substrate (blanks) were tested, to monitor the methane production of the residual COD. Net methane and biogas production potential or yield was calculated by subtracting the blank methane and biogas production.

6.2.3. Analytical methods

A complete characterization of all the samples was performed. Total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), pH, soluble chemical oxygen demand (COD_s) and total and ammonium nitrogen (TN, NH_4^+) where determined according to standard methods (APHA, AWA, WEF, 2005).

Volatile fatty acids (VFAs) were determined by a modified standard method (APHA, AWA, WEF, 2005) protocol, following Campos *et al.* (2008). A CP-3800 gas chromatograph (Varian, USA), fitted with a Tecknokroma TRB-FFAP capillary column (30 m x 0.32 mm x 0.25 lm) and FID detection were used. The VFAs measured (with detection limit given in $mg \cdot l^{-1}$) were: acetic (10.0), propionic (5.0), iso-butiric (1.0), n-butiric (1.5), iso-valeric (1.5), n-valeric (1.0), iso-caproic (1.5), n-caproic (1.0) and heptanoic (1.0).

Protein concentration was estimated from the organic nitrogen content using a factor of 6.25 $g_{protein} \cdot g_{Norg}^{-1}$, as suggested by AOAC (2003). The fat content was analyzed using Soxtec™ 2050 extraction equipment (Foss, Denmark) following the recommendations for n-hexane extractable material (HEM) from sludge, sediments and solid samples (EPA, 1998, Method 9071b). The COD_t was also estimated by elementary analysis (Leco, USA) using the empirical formulas of each sample (Angelidaki and Sanders, 2004). The protein-COD (COD_{prot}) and fat-COD (COD_{fat}) were estimated using the factors 1.42 $g_{COD} \cdot g_{prot}^{-1}$ and 2.90 $g_{COD} \cdot g_{fat}^{-1}$, respectively (Angelidaki and Sanders, 2004). The carbohydrate-COD (COD_{ch}) concentration was estimated by subtracting the COD_{prot} and COD_{fat} values from COD_t.

Methane (CH_4) content in the biogas produced was determined by gas chromatography using a CP-3800 (Varian, USA) fitted with Hayesep Q 80/100 Mesh (2 m x 1/800 x 2.0 mSS) packed column (Varian, USA) and TCD detection, as described elsewhere (Campos *et al.,* 2008).

6.2.4. Calculation methods

When methane evolution obtained in the BMP tests followed a logistic type curve, equation 1 were used to fit experimental values and to estimate the constants k'_{dis} (d^{-1}) and B_0 (l$_{CH4}$·kg$_{CODadded}$$^{-1}$) by non-linear regression using the Marquardt algorithm. Correlation matrix, standard deviation and 95% confidence intervals of the estimated values have been obtained calculating the error covariance matrix. The *Students't test* has been also performed in order to evaluate the statistical significance of the obtained values.

6.3. RESULTS AND DISCUSSION
6.3.1. Substrates and inoculum characterization

The characteristics of the substrates under study and the inoculum used in the batch test are summarized in Table 6.3.COD fractioning for total and particulate piggery ABP (P-ABP), pasteurized piggery ABP (PP-ABP) and sugar beet (SB) was done (Table 6.3).

The piggery ABP materials (raw and pre-treated) had different fat, protein and carbohydrate ratios (F:P:C) related to initial COD$_t$ content, but in all of them the fat fraction was the main component, with little carbohydrates. In the case of sugar beet and pig manure, the initial COD fractioning was the opposite, being the carbohydrates fraction the bigger one and the fat fraction the smaller.

6.3.2. Anaerobic biodegradability assay and disintegration constants

Figure 6.1 shows in the case of the pig manure sample: the methane production curve had different shapes depending on the studied ISR (1, 2 and 4 g$_{VSS}$·g$_{COD}$$^{-1}$). This behavior was generalized for the rest of selected substrates in this work. The results obtained in the anaerobic biodegradability tests with an ISR value of 4 g$_{VSS}$·g$_{COD}$$^{-1}$, which tended closely to a logistic-type curve, were chosen for further analysis.

For solving the differential equations expressing the dynamics of disintegration and hydrolysis, a deeply COD fractioning is required. However, due to the small amount of substrate added initially in every vial of the batch assays, was not feasible to determine accurately the COD fractioning and to have the COD balance through the BMP tests (sampling at the initial and final step). So, since it was not possible to analytically determine the fractionation of the COD, the general analytical solution of the proposed

equations defining the first order disintegration and hydrolysis processes could not be studied nor the meaning of the observed k'_{dis} values when using equation 1.

The experimental data from the methane yields on COD concentration base and the model predicted data are shown in Figure 6.2 with dots and lines, respectively. As can be seen, the model predicts accurately the results for BMP tests.

In general, BMP test results indicated that in all the cases, the refractory non-biodegradable COD that remained at the end of the BMP were quite low, obtaining high methane potentials yields. In many cases as early as the 10th day, the maximum yield could be considered achieved, except in the case of C-BP$_t$ that continued producing methane until around the 45th day. The slow methane production in the case of the coffee waste could be due to the high number of phenol heterocyclic compounds that may appear during the roasting process.
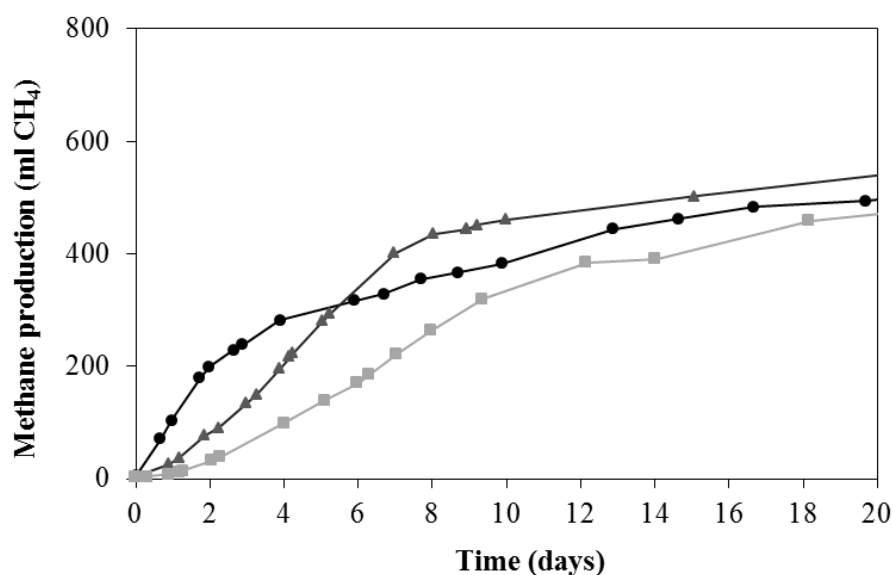
Figure 6.1. *Time course of the experimental methane production for the total fraction of pig manure with different ISR ($g_{VSS} \cdot g_{COD}^{-1}$). Notes: the light grey line denotes ISR= 1; the dark grey line denotes ISR= 2; the black line denotes ISR= 4.*

Related to the particulate piggery ABP, P-ABP$_p$ in Figure 6.2a, the standard deviations were increasing unusually from approximately the 14th day of the BMP test, achieving high values at the end of the assay. During the first 10 days, the behavior of the vials was almost the same, producing quite similar methane potential yields and methane production rates. So, since the sample was homogenized previously to be added in every vial and because of the close initial behavior, the differences between replicates could be mainly related to some experimental problems, such as a leakage of biogas through the

rubber cork. If only the first 10 days of the BMP test with P-ABP$_p$ are taken into account, it is possible to say that the model matches perfectly with the experimental values, as with the rest of the studied substrates.

As Table 6.4 indicates, among the different wastes used in this study, the raw and pre-treated piggery ABP (P-ABP$_t$ and PP-ABP$_t$) produced the highest methane yield (356.7 and 353.6 l$_{CH4}$·kg$_{COD}$$^{-1}$ respectively), followed by fishery ABP with 306.2 and 398.0 l$_{CH4}$·kg$_{COD}$$^{-1}$for F-ABP$_t$ and FP-ABP$_t$, respectively. These piggery results were as expected, since similar P-ABP were analyzed in Chapter 3. Table 6.4 also shows the estimated parameter values, the determination coefficient (r^2) for the best global fitting and 95% confidence intervals. t-Student values indicate that all estimated values are significant in the model.

Disintegration rate constants of the studied substrates varied from 0.06 to 0.47 d^{-1}, being inside the referenced interval of 0.005-7.8 d^{-1} for hydrolysis constants for wastes with different sources (Pavlostathis *et al.*, 1988; Sanders, 2001; Vavilin *et al.*, 2008; Veeken and Hamelers, 2000; Batstone *et al.*, 2002). The assays with higher carbohydrates and protein presented higher k'_{dis} constants when compared to the assays containing high amount of fat fraction such as P-ABP, except for pig slurry. The lowest values were obtained for the coffee by-product indicating some component difficult to degrade.

Observed disintegration coefficients k'_{dis} had higher values for the total waste tests than for the particulate fractions, according with the results obtained in the theoretical analysis, shown in the ANNEX. The determination coefficients (r^2) were higher than 0.96 (Table 6.4) in general, except for P-ABP$_p$, that was 0.93. Although this relative low r^2 value for P-ABP$_p$, the significance t-Student test indicates that estimated parameters of Table 6.4 are significant in the model, with significance values close to 100% in all the cases.

For the studied animal by-products (piggery and fishery ABP), it can be seen that the results are different depending on initial sample composition (Table 6.4 and Fig. 6.3). In the case of piggery ABP, the estimated k'_{dis} value was significantly increased with pasteurization, and no significant difference was found for B_0, when comparing the pair with same initial fraction. These results corroborate the conclusions reported in Chapter 3. Related to fishery by-products, the thermal pre-treatment improved slightly the estimated k'_{dis} value as can be seen in Table 6.4 and in Fig. 6.3.
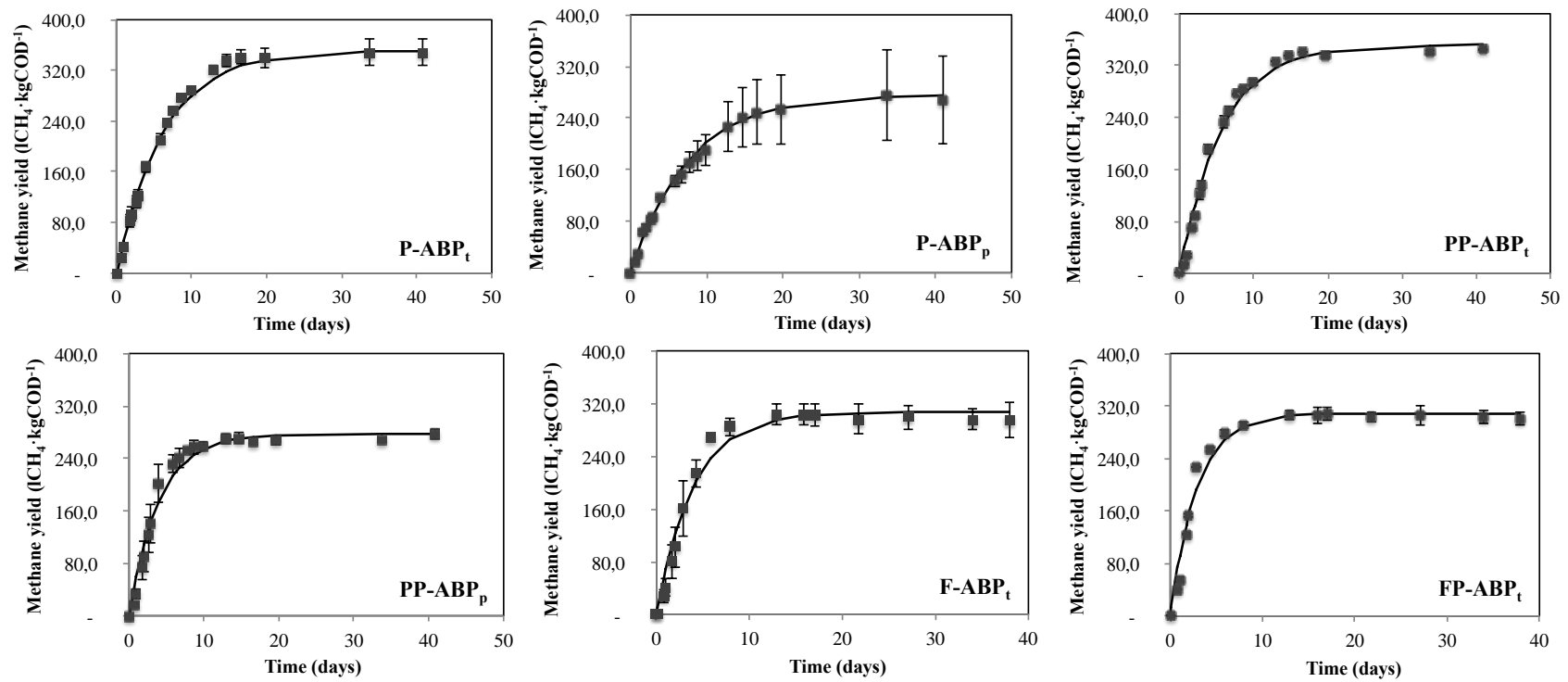
Figure 6.2.a. *Time course of the experimental and predicted methane production for the ABP substrates (□experimental; – model equation 1).*
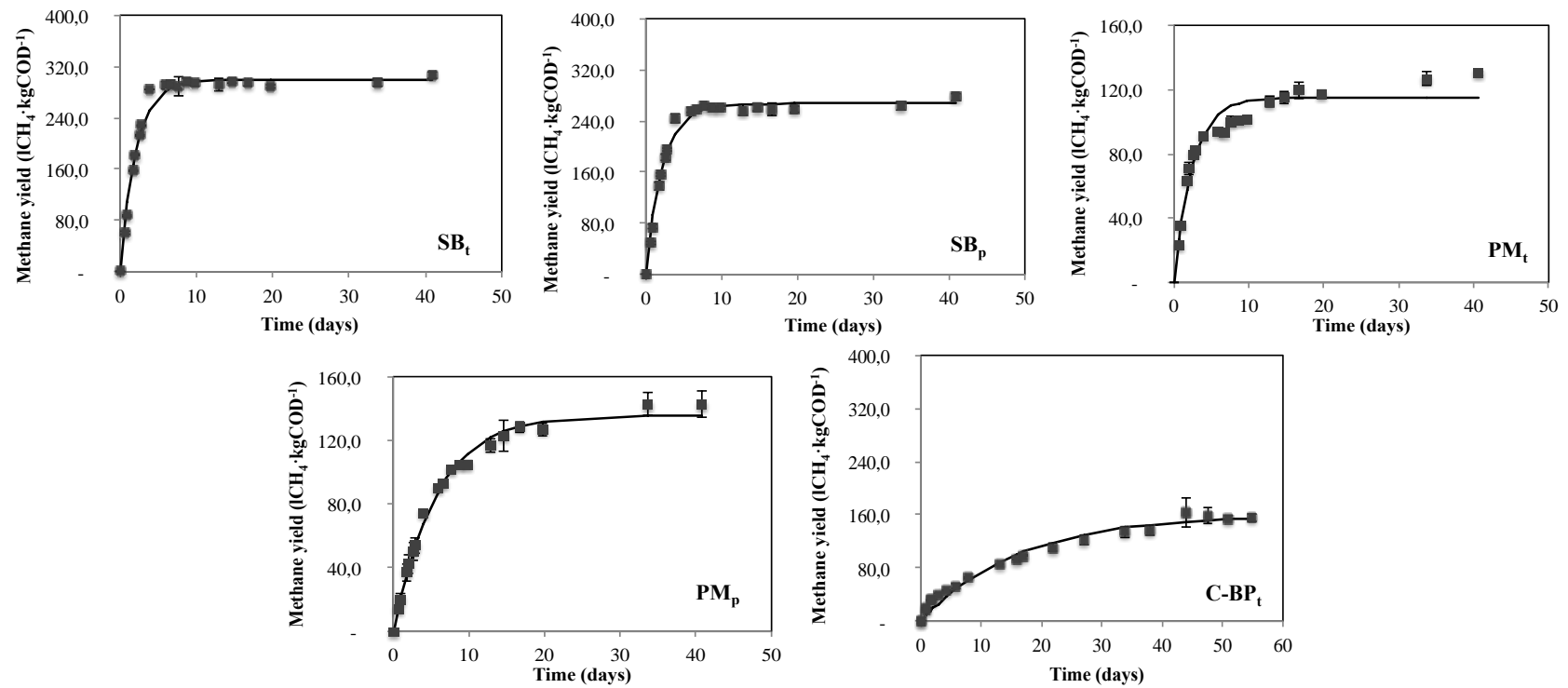
Figure 6.2.b. *Time course of the experimental and predicted methane production for pig manure, sugar beets and coffee wastes (□experimental; – model equation 1).*

Table 6.3. Characterization of all the studied substrates and inoculum. Nomenclature: (1) volatile solids with respect to the total substrate; (2) F-fat, P-protein and C-carbohydrate expressed in % of COD_t; (3) estimated value from elemental analysis.

Parameter	Unit	P-ABP$_t$	PP-ABP$_t$	P-ABP$_p$	PP-ABP$_p$	F-ABP$_t$	FP-ABP$_t$	SB$_t$	SB$_p$	PM$_t$	PM$_p$	C-BP$_t$	I
TS (w·w^{-1})	%	47.3	50.8	57.6	35.9	29.1	28.5	21.0	21.6	4.1	23.5	39.4	2.4
VS (w·w^{-1})$_{(1)}$	%	46.4	50.0	56.9	34.9	27.1	26.4	20.1	20.5	2.1	17.3	39.0	1.4
COD$_{t\,(3)}$	g·kg^{-1}	1116.6	1173.5	1403.5	714.1	521.2	500.6	201.5	202.6	46.9	281.6	752.7	21.3
COD$_s$	g·kg^{-1}	77.6	101.6	-	-	-	-	-	-	8.3	-	-	-
F:P:C $_{(2)}$	%:%:%	78:15:7	83:16:0	82:15:3	44:32:24	-	-	3:9:88	3:9:87	3:18:78	8:47:45	-	-
Total N	g·kg^{-1}	20.3	22.9	23.6	27.0	-	-	2.4	2.4	3.3	15.1	-	1.8
NH$_4^+$	g·kg^{-1}	1.3	1.5	-	-	-	-	0.1	-	2.3	-	-	1.0
Est. Protein	g·kg^{-1}	118.6	133.7	147.5	162.0	-	-	13.9	15.3	6.1	94.4	-	-
COD$_{prot}$	g·kg^{-1}	167.7	189.0	208.6	229.2	-	-	19.7	21.6	8.7	133.5	-	-
Fat	g·kg^{-1}	303.0	338.8	399.2	108.0	-	-	2.7	2.6	0.5	7.7	-	-
COD$_{fat}$	g·kg^{-1}	875.5	979.2	1153.5	312.1	-	-	7.7	7.6	1.5	22.2	-	-
COD$_{ch}$	g·kg^{-1}	73.4	5.3	41.3	172.8	-	-	243.8	204.9	36.7	126.0	-	-

Table 6.4. r^2 *determination coefficient for the best experimental values fitting and 95% confidence intervals for the estimated parameter values, for the indicated solid waste tested.*

Sample	r^2	Estimated parameters	
		Bo ($l_{CH4} \cdot kg_{COD}^{-1}$)	k'_{dis} (d^{-1})
P-ABP$_t$	0.9908	356.7 ±10.7	0.161 ±0.013
PP-ABP$_t$	0.9875	353.6 ±12.1	0.176 ±0.017
P-ABP$_p$	0.9314	274.1 ±25.5	0.131 ±0.030
PP-ABP$_p$	0.9639	279.0 ±13.5	0.250 ±0.039
F-ABP$_t$	0.9665	306.2 ±15.2	0.252 ±0.045
FP-ABP$_t$	0.9791	308.0 ±10.7	0.348 ±0.042
SB$_t$	0.9830	299.7 ±6.7	0.469 ±0.043
SB$_p$	0.9839	266.6 ±6.0	0.444 ±0.040
PM$_t$	0.9488	113.9 ±4.7	0.405 ±0.064
PM$_p$	0.9878	135.8 ±4.3	0.175 ±0.015
C-BP$_t$	0.9697	160.2 ±10.8	0.061 ±0.011
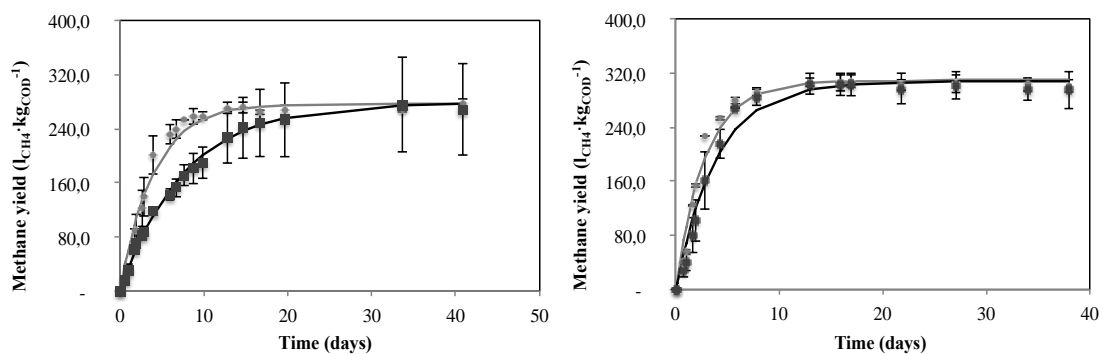
Figure 6.3. *Time course of the experimental and predicted methane production (dots and lines respectively) for particulate P-ABP and PP-ABP in the left side and total F-ABP and FP-ABP in the right side. The values of the pasteurized fraction are shown in grey.*

It has been observed in literature that previous results, related to the effect of pre-treatments on kinetics, depend on substrate characteristics and type of pre-treatment (physical, chemical, biological). Souza *et al.* (2013) also obtained positive effects on CH$_4$ production when they applied an auto-hydrolysis pre-treatment to sludge. On the other hand, Masse *et al.* (2003) observed that the first-order hydrolysis rates were around 0.50 d^{-1} and 0.63 d^{-1}, respectively, after pre-treating biologically with enzymes slaughterhouse wastewater and using those pre-treated and raw substrates for feeding anaerobic sequencing batch reactors. Eskicioglu *et al.* (2006), studying microwave irradiation and conventional heating at 96 ºC, were successful in disrupting the complex waste activated

sludge floc structure but also realized that both pre-treatments resulted in slightly lower rate degradation constants.

6.4. CONCLUSIONS

Biochemical methane potential test is a useful tool to obtain data for calibrating AD models. If inoculum concentration is high enough for obtaining a logistic-type curve, and if the test is applied to the particulate fraction of a substrate, observed k'_{dis} coefficient is an approximation informing about the rates of disintegration and the hydrolysis processes. In all the studied substrates, the correlation between the model and the experimental results was satisfactory. The COD fractioning methodology used in this study was not appropriate to determine COD fractions at the end of the batch test, to do a COD balance and to study deeply the solutions of the differential equations expressing the dynamics of disintegration and hydrolysis. For piggery animal by-products, the obtained k'_{dis} values indicate that pasteurization increases significantly the overall disintegration and hydrolysis rates.

6.5. ACKNOWLEDGEMENTS

6.6. REFERENCES

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Annex

Mathematical analysis of the system

1. Hypothesis

In batch experiments with very high inoculum concentration, the rate limiting steps of the overall anaerobic digestion process of solid organic wastes are disintegration and hydrolysis. The processes, following Monod kinetics, are very fast since microorganism's concentration is very high and methane production can be expressed as result of disintegration and hydrolysis processes.

2. Equations

The system can be expressed with differential equations representing disintegration, hydrolysis of proteins, lipids and carbohydrates, and final recovery of methane:

$$\frac{dX_c}{dt} = -k_{dis} X_c \quad,$$

$$\frac{dX_p}{dt} = k_{dis} f_p X_c - k_{h,p} X_p \quad,$$

$$\frac{dX_{li}}{dt} = k_{dis} f_{li} X_c - k_{h,li} X_{li} \quad, \tag{1}$$

$$\frac{dX_{ch}}{dt} = k_{dis} f_{ch} X_c - k_{h,ch} X_{ch} \quad,$$

$$\frac{dF}{dt} = k_{h,p} X_p + k_{h,li} X_{li} + k_{h,ch} X_{ch} \quad,$$

where F is the accumulated methane production. All components being expressed in COD units.

The general solution for *Xc=Xco, Xp=Xpo, Xli=Xlio, Xch=Xcho* and *F=0*, at *t=0*, is:

$$X_c = X_{co} e^{-k_{dis}t} \quad,$$

$$X_p = X_{po} e^{-k_{h,p}t} + \frac{k_{dis} f_p X_{co}}{k_{h,p} - k_{dis}} \left(e^{-k_{dis}t} - e^{k_{h,p}t} \right) \quad,$$

$$X_{li} = X_{lio} e^{-k_{h,li}t} + \frac{k_{dis} f_{li} X_{co}}{k_{h,li} - k_{dis}} \left(e^{-k_{dis}t} - e^{k_{h,li}t} \right) \quad,$$

$$X_{ch} = X_{cho} e^{-k_{h,ch}t} + \frac{k_{dis} f_{ch} X_{co}}{k_{h,ch} - k_{dis}} \left(e^{-k_{dis}t} - e^{k_{h,ch}t} \right) \tag{2}$$

$$F = X_{po}(1-e^{-k_{h,p}t}) + X_{lio}(1-e^{-k_{h,li}t}) + X_{cho}(1-e^{-k_{h,ch}t})$$

$$+ X_{co}f_p\left(1 + \frac{k_{dis}}{k_{h,p}-k_{dis}}e^{-k_{h,p}t} - \frac{k_{h,p}}{k_{h,p}-k_{dis}}e^{-k_{dis}t}\right)$$

$$+ X_{co}f_{li}\left(1 + \frac{k_{dis}}{k_{h,li}-k_{dis}}e^{-k_{h,li}t} - \frac{k_{h,li}}{k_{h,li}-k_{dis}}e^{-k_{dis}t}\right)$$

$$+ X_{co}f_{ch}\left(1 + \frac{k_{dis}}{k_{h,ch}-k_{dis}}e^{-k_{h,ch}t} - \frac{k_{h,ch}}{k_{h,ch}-k_{dis}}e^{-k_{dis}t}\right).$$

The particular solution for $X_c=X_{co}$, $X_p=X_{li}=X_{ch}=0$ and $F=0$, at $t=0$, is:

$$X_c = X_{co}e^{-k_{dis}t},$$

$$X_p = \frac{k_{dis}f_pX_{co}}{k_{h,p}-k_{dis}}\left(e^{-k_{dis}t} - e^{k_{h,p}t}\right),$$

$$X_{li} = \frac{k_{dis}f_{li}X_{co}}{k_{h,li}-k_{dis}}\left(e^{-k_{dis}t} - e^{k_{h,li}t}\right),$$

$$X_{ch} = \frac{k_{dis}f_{ch}X_{co}}{k_{h,ch}-k_{dis}}\left(e^{-k_{dis}t} - e^{k_{h,ch}t}\right), \tag{3}$$

$$F = X_{co}f_p\left(1 + \frac{k_{dis}}{k_{h,p}-k_{dis}}e^{-k_{h,p}t} - \frac{k_{h,p}}{k_{h,p}-k_{dis}}e^{-k_{dis}t}\right)$$

$$+ X_{co}f_{li}\left(1 + \frac{k_{dis}}{k_{h,li}-k_{dis}}e^{-k_{h,li}t} - \frac{k_{h,li}}{k_{h,li}-k_{dis}}e^{-k_{dis}t}\right)$$

$$+ X_{co}f_{ch}\left(1 + \frac{k_{dis}}{k_{h,ch}-k_{dis}}e^{-k_{h,ch}t} - \frac{k_{h,ch}}{k_{h,ch}-k_{dis}}e^{-k_{dis}t}\right).$$

For this last particular solution, when $k_{dis}<<< k_{h,p}$, $k_{h,li}$, $k_{h,ch}$, F tends to:

$$F = X_{co}\cdot(f_p + f_{li} + f_{ch})\cdot(1-e^{-k_{dis}\cdot t}), \tag{4}$$

which follows a logistic-type curve, if initial concentration of X_p, X_{li} and X_{ch} is zero.

With high inoculum concentration, if a logistic type curve is obtained, and if equation 4 is used to fit experimental data, the question is: *what is measured with the calculated k_{dis} value?* In the general case, the curve expressed by equation 4 can be expressed also by equation 5:

$$F = B_0(1-e^{-k'_{dis}\cdot t}) \tag{5}$$

where Bo is the asymptotic methane production, or the biodegradability if methane is expressed in COD units and the value is divided by the added COD, and k'_{dis} is the observed constant.

What is the meaning of this constant? In the following figure, the blue line indicates the general solution of the differential equations and the tendency shown by experimental data, while magenta line indicates the logistic-type curve obtained by fitting (equation 5):
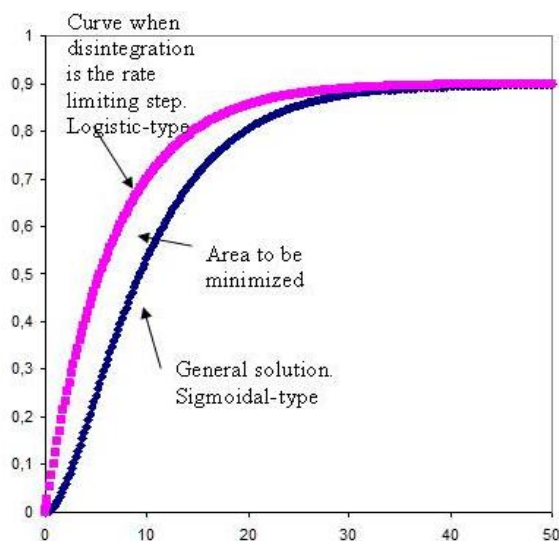
3. Objective

The objective is to minimize the area between the two curves. If the function following the logistic curve as F_{dis}, and the curve following experimental data as F (general solution for F, equation 2), the area between the two curves is:

$$Area = \int_{0}^{+\infty} F_{dis}dt - \int_{0}^{+\infty} Fdt \tag{6}$$

In the general case, Bo is the methane coming from the biodegradable fraction of X_{co}, that is: $X_{co} \cdot (f_p + f_{li} + f_{ch})$, and the methane coming from the initial concentration of proteins, lipids and carbohydrates (X_{po}, X_{lio}, X_{cho}), considering that these three components are biodegradable, following ADM1. Therefore, F_{dis} is:

$$F_{dis} = \left[X_{co} \cdot (f_p + f_{li} + f_{ch}) + X_{po} + X_{lio} + X_{cho} \right] (1 - e^{-k'_{dis} \cdot t}) \tag{7}$$

Solving the improper integrals (equation 6), the area is:

$$Area = \left(\frac{X_{po}}{k_{h,p}} + \frac{X_{lio}}{k_{h,li}} + \frac{X_{cho}}{k_{h,ch}} \right) - \frac{X_{co}(f_p + f_{li} + f_{ch}) + X_{po} + X_{lio} + X_{cho}}{k'_{dis}}$$
$$+ X_{co}f_p \frac{k_{h,p} + k_{dis}}{k_{dis}k_{h,p}} + X_{co}f_{li} \frac{k_{h,li} + k_{dis}}{k_{dis}k_{h,li}} + X_{co}f_{ch} \frac{k_{h,ch} + k_{dis}}{k_{dis}k_{h,ch}} \tag{8}$$

If Area>0 (experimental values following a sigmoidal type curve), it is not possible to calculate k_{dis}, although a good fitting (high r^2 values) of equation 5 could be obtained. The key is to obtain experimentally a logistic type curve, and in this case Area=0.

Case 1: Area=0

When Area tends to zero, it means that a very good fitting is obtained and experimental values follow a logistic-type curve.

Case 1: $X_{co}=0$, and X_{po}, X_{lio}, $X_{cho}>0$

$$k'_{dis} = \frac{X_{po} + X_{lio} + X_{cho}}{\dfrac{X_{po}}{k_{h,p}} + \dfrac{X_{lio}}{k_{h,li}} + \dfrac{X_{cho}}{k_{h,ch}}}$$

(9)

In this case, what is measured by the estimated k'_{dis} obtained with equation 5 is the weighted harmonic mean of the hydrolysis constants, weighted by the initial concentrations of proteins, lipids and carbohydrates.

Case 2: $X_{co}>0$, and X_{po}, X_{lio}, $X_{cho}=0$

$$k'_{dis} = \frac{k_{dis}\left(f_p + f_{li} + f_{ch}\right)}{f_p\left(1 + \dfrac{k_{dis}}{k_{h,p}}\right) + f_{li}\left(1 + \dfrac{k_{dis}}{k_{h,li}}\right) + f_{ch}\left(1 + \dfrac{k_{dis}}{k_{h,ch}}\right)}$$

(10)

Case 2.a: same as case 2, with k_{dis}<<< $k_{h,p}$, $k_{h,li}$, $k_{h,ch}$

$$k'_{dis} = k_{dis}$$

Only in this case the estimated value with equation 5 can be considered the true k_{dis} value.

Case 2.b: same as case 2, with k_{dis}>>> $k_{h,p}$, $k_{h,li}$, $k_{h,ch}$

$$k'_{dis} = \frac{f_p + f_{li} + f_{ch}}{\dfrac{f_p}{k_{h,p}} + \dfrac{f_{li}}{k_{h,li}} + \dfrac{f_{ch}}{k_{h,ch}}}$$

Now, the estimated constant with equation 5 is the harmonic mean of the hydrolysis constants weighted by the fractions of proteins, lipids and carbohydrates contained in X_c.

If it is assumed that equation 10 is applicable to the experimental values, kdis could be calculated based on the estimated value k'_{dis} if the fractionation of X_{co} (f values) is known and the values of the hydrolysis constants are assumed.

Chapter 7. Characterization of microbial community dynamics and its correlation with physic-chemical parameters during the anaerobic co-digestion of thermally pre-treated slaughterhouse wastes

The anaerobic digestion process was studied in a completely stirred mesophilic reactor fed with mixtures of pig manure, pasteurized slaughterhouse waste and glycerin. The co-digestion feeding regime lasted for a 595-day period during which operational parameters (retention time and organic load) were kept constant, while the feed composition was optimized through progressive co-substrate additions. These staged changes were followed by a stability period for microbial adaptation, leading to an increment of the methane production rate and organic matter removal without accumulation of intermediate compounds. The microbial community structure of biomass samples taken at different operational stages was analyzed by DGGE profiling of 16S rRNA genes (*Eubacteria* and *Archaeobacteria*). The composition of the dominant eubacterial populations remained relatively stable, when compared to those in the influent, but the highest changes were observed upon the introduction of glycerin, as depicted by direct gradient multivariate Canonical Correspondence Analysis (CCA). Biodiversity of archaeobacteria along the experiment was restricted to a few representatives of the genera *Methanosaeta* and *Methanosarcina* but *Methanospirillum* sp. was detected only when glycerin was introduced in that mixture, coinciding with the strongest increase in methane yield (from 0.22 to 0.64 $m^3_{CH4} \cdot m^{-3} \cdot d^{-1}$). Interrelations between microbial community dynamics and physic-chemical parameters have been discussed.

7.1. INTRODUCTION

Anaerobic digesters are bioreactors designed for the conversion of residual organic matter into useful biogas by diverse and complex syntrophic microbial communities. The anaerobic digestion process involves a complex series of biochemical reactions that are mediated by microorganisms belonging to three trophic levels: hydrolytic-fermenting and acetogenic hydrogen-producing bacteria, both encompassed within the *Eubacteria* domain, and acetoclastic and hydrogenotrophic methanogens that belong to the *Archaeobacteria* domain (Stams and Zehnder, 1990; Zinder *et al.*, 1984). Knowledge on the metabolic function of the microorganisms involved in each step of the anaerobic digestion pathway, and on how they interact with the physic-chemical parameters governing the process, is a prerequisite for an optimal and stable running of the anaerobic digester. Because methanogens have a relatively low growth rate and live in a very specific set of environmental conditions, the methanogenic activity in an anaerobic digester ultimately relies on operational stability. However, changes in community structure may occur without detectable changes in bioreactor performance (Fernández *et al.*, 1999), but may eventually result in severe process disruption. Hence, the better understanding of the microbial interactions in anaerobic digesters can provide control, diagnostic and prevision tools for enhanced process monitoring. Disturbances in populations from one trophic level affect the entire community and cause an effect on bioreactor performance by a reduced efficiency or accumulation of intermediates (Fernández *et al.*, 1999). The anaerobic digestion process is generally monitored by an exhaustive control of the ammonia and/or fatty acids. This is very important during the anaerobic digestion of complex wastes such as animal by-products (ABP), a highly biodegradable organic residue that is mainly composed by proteins and lipids with variable water content (Álvarez and Liden, 2008). The combined release of ammonia due to protein decomposition and long chain fatty acids (LCFA) because of fat degradation might severely compromise the stability of the whole anaerobic process (Ahring *et al.*, 1995; Salminen and Rintala, 1999; Chen *et al.*, 2008; Galbraith *et al.*, 1971; Hanaki *et al.*, 1981; Koster and Cramer, 1987; Hwu *et al.*, 1996).

Co-digestion is a good strategy to prevent inhibition and optimize methane production in anaerobic digesters (Mata-Alvarez *et al.*, 2011). The combined treatment of organic wastes from different sources also has other advantages, such as the reduction on the

emissions of greenhouse gases (methane and nitrous oxide) and on reducing costs and energy expenses as result of a more integrated waste management (Alatriste-Mondragon *et al.*, 2006). Co-digestion allows the progressive acclimatization of the bacteria to specific inhibitors such as ammonia (Angelidaki and Ahring, 1993; Edström *et al.*, 2003) and/or LCFA (Broughton *et al.*, 1998), thus facilitating the control of the anaerobic process. The implementation of an adequate co-digestion regime in industrial plants lays in accurate selection of co-substrates. Notwithstanding its low biogas production potential due to the poor organic matter content, pig manure has an important buffer capacity and contributes with a wide variety of nutrients that are necessary for the development of anaerobic microorganisms (Angelidaki *et al.*, 1997; Hartmann and Ahring, 2006). Several positive experiences have been described on the co-digestion of pig manure with different substrates, as sewage sludge, vegetal, dairy or fish cannery residues and brewery wastes (Flotats *et al.*, 2009; Dar and Tandon, 1987; Trujillo *et al.*, 1993; Gavala *et al.*, 1996; Desai and Madamwar, 1994; Callaghan *et al.*, 1999). Despite of the generation of potential inhibitions, good results have also been obtained in relation to the co-digestion of ABP and manure, and stable operation has been reach with high biogas yields (0.7-1.0 $m^3 \cdot kg_{VS}^{-1}$) and specific methane yields (0.52-0.55 $m^3_{CH4} \cdot kg_{VS}^{-1}$) (Salminen and Rintala, 2002; Edstrom *et al.*, 2003; Murto *et al.*, 2004). Other organic wastes such as residual glycerin from the biodiesel production process from energy crops have been mixed with nitrogen rich-substrates like manure, in order to balance the C/N ratio (Amon *et al.*, 2006; Alatriste-Mondragon *et al.*, 2006).

Culture independent molecular techniques have increasingly been applied to the analysis of microbial communities in anaerobic digesters (Talbot *et al.*, 2008; Demirel and Scherer, 2008), and have become a useful tool for understanding the reactor performance (Mladenovska *et al.*, 2006). Some works have already been published on the microbial aspects related to the co-digestion of organic substrates, particularly in relation to energy crops, alone or mixed with manure (Krakat *et al.*, 2011; Kröber *et al.*, 2009; Fernández *et al.*, 1999; Palatsi *et al.*, 2011). However, the results are often partial (e.g. only the bacterial domain is often covered), and quantitative studies on the dynamics of specific functional microbial groups are rare. The deeper understanding on the microbial interactions inside an anaerobic digester could be of help for avoiding failure, to predict eventual instability problems, and also to evaluate the reactor efficiency and biogas yield (Sousa *et al.*, 2007). Besides these practical aspects, biomonitoring of digesters using

molecular methods could also lead to the identification of new and functionally relevant species (Tabatabaei *et al.*, 2010).

The present work was aimed to study and optimize the performance of the anaerobic digestion process using animal by-products in combination with pig manure and residual glycerin. The biomass adaptation from pig manure to full co-digestion regime was monitored at the level of microbial community structure by a culture-independent molecular DGGE profiling of 16S rRNA genes from both eubacterial and archaeo bacterial microbial domains. Correlations between microbial dynamics and operational reactor parameters were depicted by multivariate analysis.

7.2. MATERIAL AND METHODS

7.2.1. Selected substrates

The selected ABP comprising solid slaughterhouse residues were classified as category 3 by the European Community Regulations (Nº 1069/2009 and Nº 142/2011) and came from a pig slaughterhouse facility located in Barcelona (Spain). They consisted of a mixture of internal organs (kidney, lungs, livers and hearts, reproductive organs and fatty fractions). All fractions were minced separately at 4 mm maximum particle size and then mixed. Pasteurization was performed at 70 ºC during 60 minutes, as European Community regulations (Nº 1069/2009 and Nº 142/2011) for category 3 ABPs specify, in a high pressure and temperature autoclave of 2 liters (Iberfluid Instruments, Spain). Pig manure was regularly collected from a centralized pig manure treatment facility located in Lleida (Spain). Both the pasteurized slaughterhouse waste (PP-ABP) and pig manure (PM) were frozen at -20ºC until use, in order to prevent biological decay. A total of twelve different fresh manure samples were collected and characterized, in order to account the temporal variability (seasonal fluctuations, changes in pig slurry management, etc.). Residual glycerin (RG), a brown-yellow and viscous liquid from a glycerol-containing waste discharge from a biodiesel factory located in Barcelona (Spain), was also used in the co-digestion experiments. This material was preserved under 4ºC.

7.2.2. Analytical methods

The PP-ABP was lyophilized before characterization in order to improve their homogeneity, while PM and RG were analyzed immediately after collection. Usual parameters were measured according to Standard Methods (APHA, AWA, WEF, 2005): total and volatile solids (TS, VS), pH and alkalinity ratio, total Kjeldahl nitrogen (TKN), ammonium nitrogen (NH_4^+). Total carbon (TC) and total nitrogen (TN) were determined by elemental analysis (Leco, USA). Total chemical oxygen demand (COD) was determined by a modified Standard Methods procedure (Noguerol-Arias *et al.*, 2012) and confirmed by elemental analysis using simple empiric formulas of each waste (Angelidaki and Sanders, 2004). Free ammonia content was calculated using the formula given by Hansen *et al.* (1998). Proteins were calculated by multiplying the organic nitrogen by 6.25 $g_{protein} \cdot g_{Norg}^{-1}$ factor (Gelegenis *et al.*, 2007). The fat content was analyzed by the Soxtec™ 2050 extraction equipment (Foss, Denmark), according to recommendations of n-hexane extractable material for sludge, sediment and solid samples method of EPA (2005). Methane (CH_4) content and volatile fatty acids (VFA) were determined by gas chromatography (Campos *et al.*, 2008), being both expressed in COD equivalents for balance purposes, using the equivalence factors suggested by Angelidaki *et al.* (2009). All gas quantities were normalized at 0ºC and 1atm.

7.2.3. Anaerobic biodegradability assay

The mesophilic anaerobic biodegradability (AB) of every waste, expressed as a percentage of the total COD, was determined by triplicate in glass flaks of 1.2 litres, filled with 500 g of a solution composed by the inoculum, macro and micronutrients, the substrate (with an initial concentration of 5 $g_{COD} \cdot l^{-1}$), and $NaHCO_3$ as a buffer (1 $g_{NaHCO3} \cdot g_{COD}^{-1}$) according to Field *et al.*, (1988), Soto *et al.* (1993) and Angelidaki *et al.* (2009). The AB results were expressed as an average with standard deviation. Anaerobically digested sewage sludge from a mesophilic anaerobic digester of an urban WWTP (Barcelona, Spain) was used as inoculum, with a concentration of 5 $g_{VSS} \cdot l^{-1}$. Three flasks without substrate were performed as blanks to obtain the methane production of the residual COD, although the inoculum was maintained in an incubation chamber (35ºC) during 7 days to reduce the amount of its residual COD. The flasks were continuously shaken at 100 rpm during incubation for 30 days. The time course of methane production was followed, sampling the head space periodically. Net methane

volume, or total accumulated methane from vials less total accumulated methane from blanks, was used to calculate the maximum methane yield ($m^3_{CH4} \cdot kg_{VS}^{-1}$ and $m^3_{CH4} \cdot t^{-1}$).

7.2.4. Continuous experiment set up

A 6 litres continuous stirred reactor (CSTR) without recirculation was used due to its simplicity, as Gavala *et al*. (1999) suggested (Figure 7.1). It was operated at 36±1℃ for 595 days (85 weeks) and inoculated with two mesophilic anaerobic sludges: 4 litres from the digester of a centralized plant, where the PM was also collected, and 1 litre from the digester of an urban WWTP, where the inoculum of the AB tests was taken. An acclimation period (called P0) of the inoculum was done with fresh pig manure, diluted with tap water, with an HRT of 20 days. The feed of the reactor was stirred and pumped 4 times per day, using a temporized control system that ensured a homogenized influent mixture while feeding the digester. The biogas flow was measured by displacement with a Ritter flow-meter, after a silica bed to retain water vapour and a filter to avoid particles in the gas. The reactor inlet and outlet flows, biogas flow and temperature were monitored daily, while pH, alkalinity ratio, COD concentration, N related compounds content, VFA content and biogas composition were measured twice a week. All parameters were expressed as a weekly average.

Figure 7.1. Completely stirred tank reactor used in co-digestion experiments.

The operational parameters selected were two HRT (20 and 33 days), being the organic loading rate (OLR) 0.8 $kg_{COD} \cdot m^{-3} \cdot d^{-1}$ along the acclimation period and between 2.2-3.2 $kg_{COD} \cdot m^{-3} \cdot d^{-1}$ for the others periods. The HRT and OLR values were chosen since they are the design values of anaerobic digesters in centralized manure treatment facilities in Spain (Flotats *et al*., 2009). The performance was divided in 3 stages based on feed composition, and in 6 periods based on HRT and OLR values. For each experimental condition, the specific methane yield ($m^3_{CH4} \cdot kg_{VS}^{-1}$ and $m^3_{CH4} \cdot t^{-1}$), specific methane production rate ($m^3_{CH4} \cdot m^{-3} \cdot d^{-1}$) and COD removal efficiency were used as control parameters, as well as the biogas composition, the ammonia and VFA concentrations in the effluent. The samples for investigating the microbial communities were taken at the end of each period.

7.2.5. Denaturing gradient gel electrophoresis (DGGE) molecular profiling

Influent (i) and effluent (e) samples were collected at the steady-state of each period (from P1 to P6), including the mixture of initial inoculums (P0). Total DNA was extracted from approx. 0.25 g of each sample with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, USA), a protocol based on a bead-beating according to the instructions of the manufacturer. Three primer sets were used to selectively amplify bacterial (F341GC/R907) and archaeal (ArchF0025/ArchR1517; nested ArchF344/ArchR915GC) rRNA fragments. The PCR amplification of hypervariable V3-V5 region from the 16S rRNA gene of both domains and the DGGE profiles were performed as previously reported by Palatsi *et al*. (2010).

Sequencing of the predominant DGGE bands were accomplished using the ABI Prism Big Dye Terminator Cycle-Sequencing Reaction Kit v. 3.1 and an ABI 3700 DNA sequencer (both Perkin–Elmer Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. Sequences were edited using the BioEdit software package v.7.0.9 (Ibis Biosciences, Carlsbad, CA, USA) and aligned with the NCBI genomic database using the BLAST search alignment tool and checked by RDP (Ribosomal Data Project) database.

7.2.6. Statistical data analysis

DGGE profiles were digitized and parameterized by using the image analysis program Gene Tools (Syngene). A numerical output matrix was obtained on the basis of the position and relative intensity of the depicted DGGE bands, which was used as the

"species" data in subsequent multivariate analysis. A second matrix was constructed on relevant physico-chemical parameters of reactor operation. Direct gradient analysis between the environment primary data and the "species" response data was performed by Canonical Correspondence Analysis (CCA), using the CANOCO software version 4.5. Statistical significance between environmental parameters and microbial community dynamics was assessed by Monte Carlo test using 1000 permutations.

7.3. RESULTS AND DISCUSSION

7.3.1. Characterization of organic substrates

In this study, pig manure (PM) and residual glycerin (RG) were used as co-substrates for the co-digestion with pasteurized animal by-product (PP-ABP). These wastes were chosen because of the high PM production in Catalonia (Campos, 2001), while RG and PP-ABP complement and improve the PM characteristics, especially in relation to C/N ratio (Table 7.1). RG and PP-ABP were characterized by high concentrations of COD, in relation to PM (1517.0 and 1318.0 versus 45 $g_{COD} \cdot kg^{-1}$).

Table 7.1. *Waste characterization of pig manure (PM), pasteurized pig waste (PP-ABP) and residual glycerin (RG), including their anaerobic biodegradability (AB) and methane yields (MPP). Nomenclature: nd - not detected, nm – not measured. Note: *Calculated value from elemental analysis.*

Parameter	PM	PP-ABP	RG
TS ($g \cdot kg^{-1}$)	36.7 ±10.2	551.7 ±3.1	926.1 ±0.1
VS ($g \cdot kg^{-1}$)	26.0 ±8.3	542.5 ±2.1	924.4 ±1.2
C/N ($g \cdot g^{-1}$)	5.7 ±2.3	14.1 ±2.3	587.5 ±2.3
CODt ($g \cdot kg^{-1}$)	45.4 ±7.1	1318.0*	1517.0 ±12.9
VFA ($g_{COD} \cdot kg^{-1}$)	8.3 ±4.9	2.5 ±0.1	nd
NH_4^+ ($g \cdot kg^{-1}$)	2.5 ±0.3	1.5 ±0.3	nm
TKN ($g \cdot kg^{-1}$)	3.4 ±0.3	19.2 ±2.3	nm
Protein ($g \cdot kg^{-1}$)	3.1 ±1.2	110.6 ±2.6	nm
Fat ($g \cdot kg^{-1}$)	nm	363.4 ±0.6	nm
SO_4^{2-} ($g \cdot kg^{-1}$)	nd	nd	1.7 ±0.1
AB (%COD_t)	41.0 ±0.7	94.3 ±3.0	65.3 ±4.8
MPP $m^3_{CH4} \cdot kg_{VS}^{-1}$	0.20 ±0.0	0.88 ±0.0	0.35 ±0.0
$m^3_{CH4} \cdot t^{-1}$	6.0 ±0.1	476.3 ±7.2	201.9 ±29.3

On the other hand, PM and PP-ABP presented a relatively high amount of nitrogen, 3.4 and 19.2 $g_{NT} \cdot kg^{-1}$, respectively, being organic nitrogen the predominant form in PP-ABP, while being almost inexistent in RG. This fact is also reflected in the carbon to nitrogen

ratio (C/N of 5.7 and 14.1 for PM and PP-ABP), while the C/N ratio was particularly high in RG (C/N=587.5). The content of volatile solids (VS) was very different in the three substrates but was particularly low in PM (Table 7.1), reason why this substrate was very suited as dilution media for the mixtures.

The anaerobic biodegradability of PM, PP-ABP and RG was 41.0%, 94.3% and 65.3% in terms of COD respectively (Table 7.1). The PM had a maximum methane yield of 0.20 $m^3_{CH4} \cdot kg_{VS}^{-1}$ (6.0 $m^3_{CH4} \cdot t^{-1}$), value that was lower than the range of 0.3-0.5 $m^3_{CH4} \cdot kg_{VS}^{-1}$ reported in the literature for swine slurry (Moller *et al*., 2004; Grebrezgabher *et al*., 2009; Bernet *et al*., 2009; Hashimoto, 1984; Burton and Turner, 2003). This parameter might display a strong variability due to the presence of slowly biodegradable lignocellulosic materials (Moller *et al*., 2004), as well as because of the biodegradation of organic matter during manure storage (Rodriguez and Lomas, 2002) or management (Palatsi *et al*., 2004). PP-ABP had a maximum methane potential yield of 0.88 $m^3_{CH4} \cdot kg_{VS}^{-1}$ (476 $m^3_{CH4} \cdot t^{-1}$), which was higher than the values of 0.23 -0.62 $m^3_{CH4} \cdot kg_{VS}^{-1}$ reported previously by Hejnfelt and Angelidaki (2009), probably because of different fat and water contents. RG had a methane potential yield of 0.35 $m^3_{CH4} \cdot kg_{VS}^{-1}$ (202 $m^3_{CH4} \cdot t^{-1}$), which is lower than 1295 $m^3_{CH4} \cdot t^{-1}$ for pure glycerol (Amon *et al*., 2006) but relatively close to the range of 217 - 308 $m^3_{CH4} \cdot t^{-1}$ reported for other glycerol fractions recovered by phosphate acidification or distillation processes (Siles *et al*., 2009). The presence of impurities (water, methyl ester, soap stock, methanol, etc.) and inorganic salts (sulphate, phosphate, soda, etc.) depends very much on the production process and the used raw materials, while is ultimately responsible for the decrease in methane yield as compared to pure glycerin.

7.3.2. Reactor performance

The reactor feeding strategy was implemented for the biomass adaptation from operation on PM alone to a complex mixture of PM, PP-ABP, and RG. This continuous experiment lasted 85 weeks and was divided in 3 stages based on feed composition and reactor control parameters. It consisted on starting feeding with pig manure in a first stage, progressively increasing the PP-ABP concentration in a second stage, adding then glycerin in the last stage, without fluctuations in the OLR.

The averaged values of operational and control parameters are shown in Table 7.2, classified per performance periods. The evolution of methane yield along the different periods for the applied hydraulic retention times and organic loading rates is represented

in Figure 7.2, while the comparison between the estimated methane flow, taking into account the composition and MPP of the wastes comprised in the influent, and the methane flow measured in the laboratory is shown in Figure 7.3. The differences between the estimated and experimental data were due to the introduction of different fresh pig manures with different composition, which MPP were estimated based on the MPP of the first pig manure used.
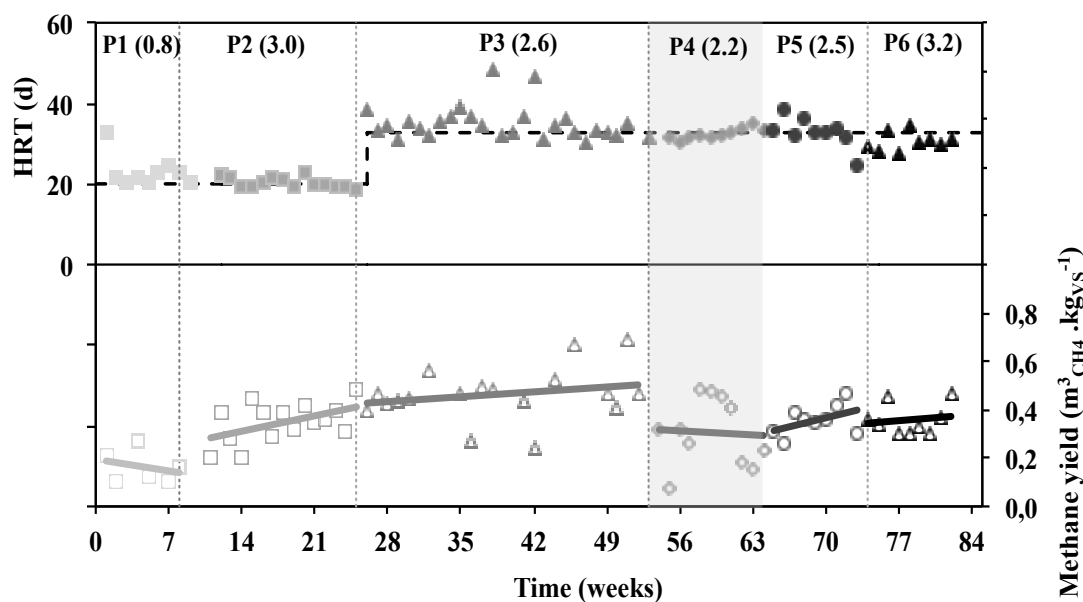
Figure 7.2. *Continuous operation: HRT and methane yield of every period (OLR is shown between brackets in $kg_{COD} \cdot m^{-3} \cdot d^{-1}$).*
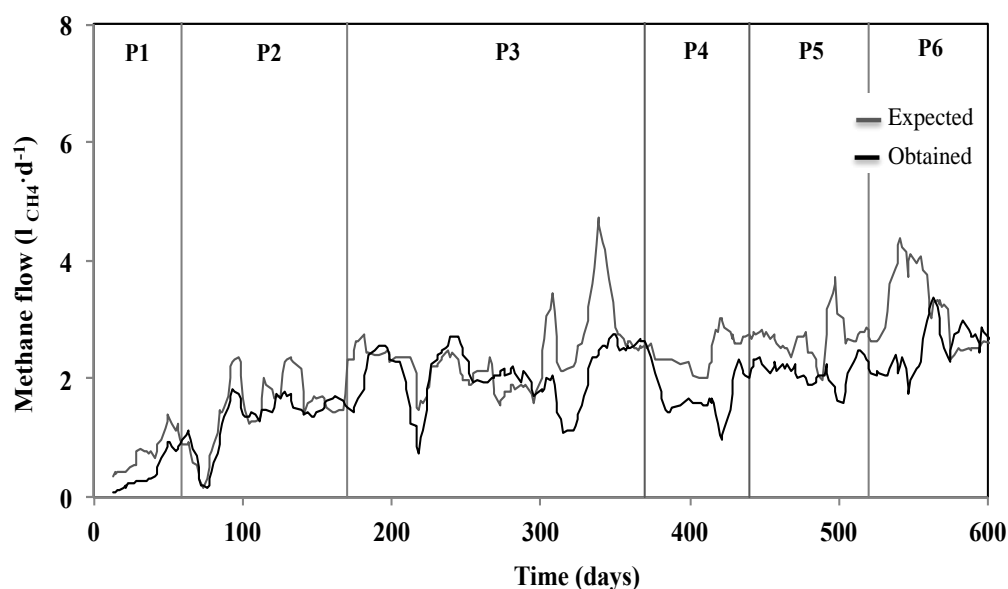
Figure 7.3. *Obtained and expected methane flow ($l_{CH4} \cdot d^{-1}$) during the operation of the CSTR along the different periods.*

Table 7.2. *Operation and control parameters during the continuous co-digestion with different feeding mixtures. Nomenclature: PM - pig manure, PP-ABP - pasteurized pig waste, RG - residual glycerin.*

Stage	1	2	2	2	3	3
Period	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Influent						
PM:PP-ABP:RG (% VS)	100:0:0	93:7:0	64:36:0	40:60:0	34:50:16	35:47:18
C/N (g·g^{-1})	6.3	6.1	5.9	4.0	8.0	10.3
N-NH$_4^+$ (g·kg^{-1})	2.69	3.08	2.62	1.69	1.61	2.14
Operation parameters						
HRT (d)	21	21	33	32	33	32
OLR (kg$_{COD}$·m^{-3}·d^{-1})	0.8	3.0	2.6	2.2	2.5	3.2
Control parameters						
COD removal (%)	30%	48%	44%	35%	51%	55%
CH$_4$ (% v·v^{-1})	65%	73%	73%	72%	71%	71%
m$^3_{CH4}$·m^{-3}·d^{-1}	0.22	0.47	0.39	0.28	0.48	0.64
m$^3_{CH4}$·kg$_{VSin}^{-1}$	0.15	0.35	0.43	0.31	0.38	0.38
m$^3_{CH4}$·t^{-1}	3.6	9.7	13.6	13.8	16.0	18.7
N-NH$_4^+$ (g·l^{-1})	1.81	2.95	3.28	2.68	2.30	2.42
N-NH$_3$ (g·l^{-1})	0.13	0.33	0.31	0.20	0.14	0.11
VFA (% effluent-COD)	1.5%	3.6%	2.8%	1.8%	4.2%	1.9%
pH	7.8	7.7	7.9	7.8	7.8	8.0
Propionic/acetic	0.17	0.32	0.21	0.24	0.82	0.88
Alkalinity ratio	0.19	0.19	0.22	0.27	0.22	0.24
Micro. samples (week)	8	23	55	67	73	86

The first period (P1) and also first stage, was the starting up of the reactor, feeding only with PM. The second stage consisted on supplementations with PP-ABP (expressed as percentage in relation to VS), which were added in three subsequent periods: 7% (P2), 36% (P3) and 60% (P4). The HRT in P2 was maintained at 21 days and the OLR increased until 3.0 kg$_{COD}$·m^{-3}·d^{-1}. The methane yield and production rate raised up to 9.7 m$^3_{CH4}$·t^{-1} and 0.47 m$^3_{CH4}$·m^{-3}·d^{-1}, representing an improvement of 168% and 114%, respectively, in relation to the previous phase in which only PM was applied (P1). The CH$_4$ biogas fraction also increased from 65 till 73% v·v^{-1}. Despite the higher concentration of ammonia and VFA measured in the effluent of P2 (0.33 g$_{NH3}$·l^{-1} and 3.6% COD) comparing to the effluent of P1, the system was considered stable because of the relatively high methane production rate.

It has been demonstrated that higher HRT facilitates the biomass adaptation in manure co-digesters (Viswanath and Nand, 1994; Salminen and Rintala, 2002; Murto *et al*., 2004) so in period P3, the HRT was increased from 21 till 33 days (Figure 7.2) in order to prevent potential problems due to the increase of the PP-ABP content from 7 to 36% VS in the feed. The COD removal efficiency and the methane yield experimented a slight decrease when compared to P2, despite the fact that there was neither VFA accumulation (2.8% COD) nor high ammonia concentration ($0.31 \text{g} \cdot \text{l}^{-1}$) in P3. As expected, with respect to P1, the biogas production was higher, achieving values of $0.43 \text{ m}^3_{CH4} \cdot \text{kg}_{VS}^{-1}$, which are slightly greater than the ranges of $0.27\text{-}0.35 \text{ m}^3_{CH4} \cdot \text{kg}_{VS}^{-1}$ previously reported for the co-digestion of slaughterhouse wastes with pig manures and fruit (Alvarez and Liden, 2008), or $0.38\text{-}0.43 \text{ m}^3_{CH4} \cdot \text{kg}_{SV}^{-1}$ with sewage sludge (Luste and Luostarinen, 2010).

The final increment in PP-ABP content, that reached the 60% of the fed VS, was performed in P4, which also resulted in a lower C/N ratio of 4 in the influent. However, the reactor behavior during this latter stage was unstable due to some mechanical problems such as obstruction in the feeding pump. As a consequence, progressive drop in methane production (Figure 7.2) and COD degradation (Table 7.2) was observed. However, an alkalinity ratio below the reference stability threshold of 0.4 (Callaghan *et al*., 2002) and a lower VFA concentration, in comparison to P3, indicated that methanogenesis was not significantly affected.

RG was introduced during the third and final stage, up to 16% and 18% of the fed VS for the periods P5 and P6, respectively. The difference between P5 and P6 was the OLR, which was increased from 2.5 to $3.2 \text{ kg}_{COD} \cdot \text{m}^{-3}\text{d}^{-1}$. In this period, glycerin was added as a mean for increasing C/N ratio of the influent from 6 till 10. An optimum C/N range of 20 to 30 has commonly been reported for an efficient use of nutrients and maximum methane yield (Viswanath *et al*., 1992; Wu *et al*., 2010). Yet, lower C/N ratios have also been suggested as optimal, particularly in the anaerobic digestion of swine manure. In an early study, Sievers and Brune (1978) revealed that the optimal C/N range for swine manure digestion was 15-19 in terms of maximum methane production. They also reported that, along with an increasing loading rate, the biogas production was stable in the digesters when the C/N was maintained between 6-16, when compared with digesters that were operated with a C/N of 20.

Both methane yield and production rate in the digester improved: 23% and 11% in P5 with respect to P3. Amon *et al*. (2006) also used glycerin as carbon supplementation in

the digestion of pig manure and maize silage, with an increment of 19% in methane yield (from 0.57 to 0.68 $m^3_{CH4}\cdot kg_{VS}^{-1}$) after adding a 6% VS of glycerin in the total feeding, while Robra *et al*. (2010) found that maximum methane yields (0.52 $m^3_{CH4}\cdot kg_{VS}^{-1}$) were attained when 5-10% VS fed of glycerin was added to cattle slurry. There was an increase in VFA concentration (equivalent to 4.2% COD) in P5, but it decreased down to 1.9% COD in P6 due to a better COD removal efficiency, values that were in the range of the VFA concentration observed previously during P1. Related to the pig manure period, P1, the achieved methane yield was increased of 316% and 414% for P5 and P6, respectively, and the COD removal efficiency (51% and 55% COD removal in P5 and P6) also improved.

Although an increment in methane yield of 33% in P6 in relation to P5, addition of RG higher than 18% fed VS was discarded because of the imbalance propionic/acetic acid in the reactor. The inhibitory effect of propionic acid at concentrations of 1-6 $g\cdot l^{-1}$ on methanogenesis was already manifested with sporadically RG addition by Rétfalvi *et al*. (2011). Fountoulakis *et al*., (2010) concluded that crude glycerol addition at 1% v/v to sewage sludge co-digestion increased CH_4 production in the reactor above the expected theoretical value, as it was totally digested and furthermore enhanced the growth of active biomass in the system, but when glycerol in the feed exceeded 1% there was not a stable digestion process. They also observed that glycerol biodegradation takes place at a faster rate than that of propionate, and suggested that a glycerol overload in the reactor increases propionate concentration as well Angelidaki *et al*. (1998) assumed that glycerol biodegradation to propionate took place instantly, as an integral part of lipid hydrolysis.

In parallel with propionic acid accumulation, the highest values of pH and alkalinity ratio (8 and 0.24) were reached in P6, despite ammonia concentration in periods P5 and P6 was lower than in the previous periods (0.14 and 0.11 $g_{NH3}\cdot l^{-1}$ respectively), This result is consistent with other works that reported buffer capacity changes due to the accumulation of inorganic salts in the digester (Siles *et al*., 2009).

The question on whether a bioreactor remains stable over time is not easy to answer, as more than 140 different definitions of "stability" (properties and measure of stability) exist in ecology (Krakat *et al*., 2011; Fernández *et al*., 1999). So, the definition of ecosystem stability is referenced in many cases either to measurable parameters describing the function of the whole system or to the community composition (Fernández *et al*., 1999). For anaerobic digesters, stable performance implies steady-state production

and consumption of metabolites along the trophic chain. Selected metabolites that are generally monitored over time, as VFA/NH$_3$/H$_2$/CH$_4$, besides COD removal efficiency, were chosen as good functional stability indicators (Schoen *et al*., 2009). From this point of view, this co-digestion experience was operated along 595 days and functional stability in periods P1 to P3 and periods P5 and P6 was confirmed by a constant performance with respect to COD reduction and methane production, while an unstable period P4, between days 378 to 455, was observed.

7.3.3. Microbial community structure

Microbial community dynamics along the co-digestion experiment described previously have been characterized by DGGE profiling of PCR amplified eubacterial and archaeal 16S rRNA gene fragments (Figure 7.4).

The result was a pattern of bands indicating the predominant microbial species, defined as ribotypes, in the biomass taken along temporal series of influent and effluent samples. The depicted profiles showed that there were significant differences in the microbial community structure between influent and effluent samples and, to a lesser degree, during the different operational periods concerning the bioreactor biomass. This is particularly true for the observed eubacterial ribotypes, for which the DGGE profiles fluctuated significantly both in number and position of depicted bands, depending on the studied sample.

7.3.3.1 Eubacterial population

Up to twenty-two DGGE bands from the eubacteria were successfully excised and sequenced (Table 7.3). Predominant bacterial species primarily belonged to the phyla *Bacteroidetes* and *Firmicutes* (Table 7.3) but changes in community composition occurred over the complete co-digestion periods, being represented by six and five species, respectively. While the latter were observed mainly in the influent, the former were more abundant in the effluent samples.
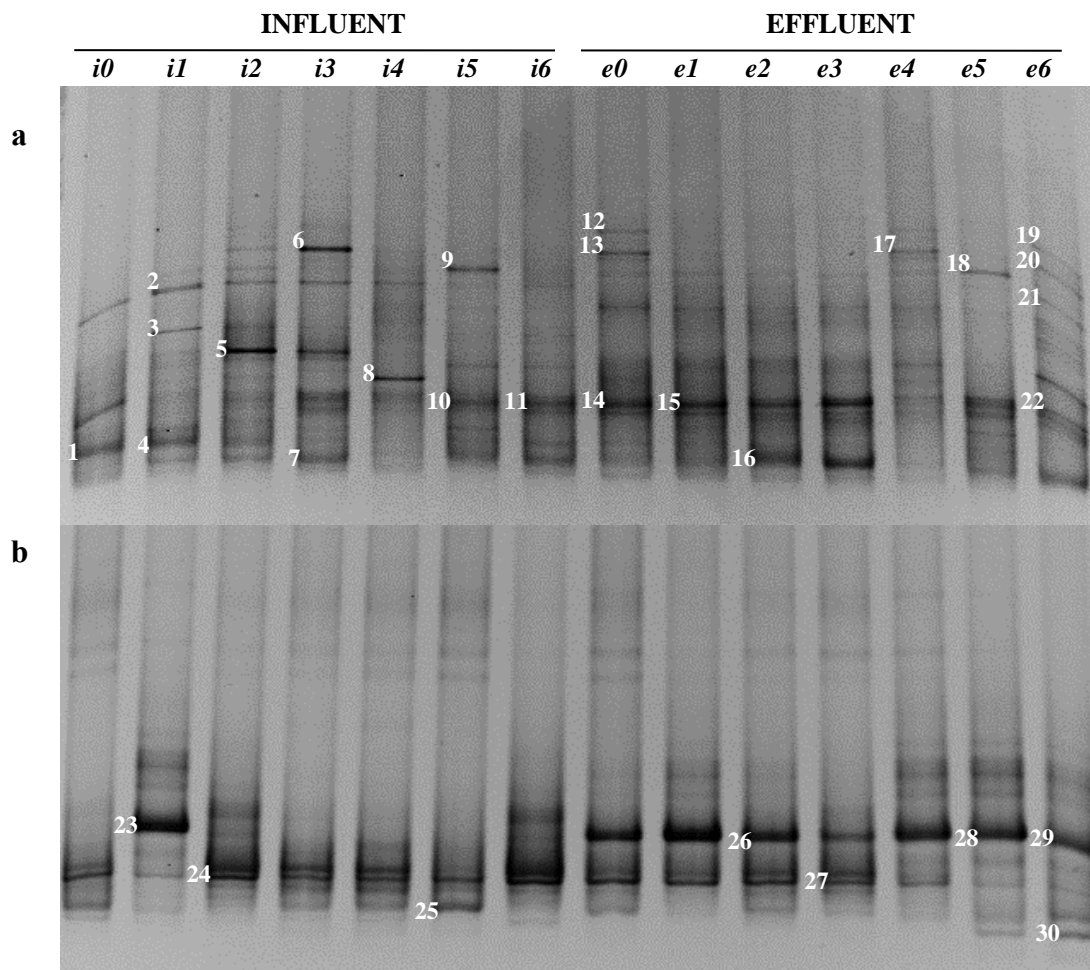
Figure 7.4. *DGGE profiles on 16S rRNA gene sequences from eubacteria (a) and archaeobacteria (b), amplified from total DNA extracts on influent and effluent samples taken during the co-digestion experiment. These samples corresponded to the different feed composition, as detailed in Table 7.2. Numbered bands were successfully excised and sequenced.*

This result is coherent with previous data, which pointed out the dominance of the phyla *Bacteroidetes* and *Firmicutes* in the anaerobic digestion process irrespective of the type of waste being treated (Chouari *et al*., 2005; Godon *et al*., 1997; Kampmann *et al*., 2012) because both have the ability to degrade a wide range of complex organic macromolecules, including proteins and carbohydrates (Bernardet and Bowman, 2005; Slepecky and Hemphill, 2005; Wiegel *et al*., 2005; Church, 2008; Scherr *et al*., 2012). Kampmann *et al*. (2012) also showed that *Bacteroidetes* and *Firmicutes,* were the most abundant bacterial groups in mesophilic reactors fed with a mixture of pig manure, maize silage, casein, starch and cream (33% fat), correlating the relative abundance of *Bacteroidetes* with acetate formation. Species from these two phyla are common inhabitants in the rumen (Kim *et al*., 2011) and in the human gut (Karlsson *et al*., 2011).

Identified members of *Bacteroidetes* from the digester biomass encompassed representatives of six different orders, especially after the addition of PP-ABP, being *S. canadensis* and *R. xylanolyticum* always detected, while *P. sulfuriphila* was found at an apparently lower abundance, understood as a fainter DGGE band. One single representative of the *Proteobacteria* phylum, belonging to the *Pseudomonadales* order, was observed, being *Pseudomonas pertucinogena* the closest phylogenetically defined specie (95% homology).

The *Firmicutes* group, on the other hand, contained representatives of five orders: *Clostridiales*, *Lactobacillales* and *Erysipelotrichales*. The phylum *Firmicutes* has often been associated to salt and acid tolerant anaerobic bacteria (Ludwig *et al.*, 2008). These bacteria are also able to produce enzymes involved in degradation of proteins and peptides (Schlüter *et al.*, 2008). The hydrolysis step is referenced to be conducted mainly by clostridia and bacilli (Schlüter *et al.*, 2008), but the former can perform secondary metabolism reactions involving the degradation of organic acids and amino acids to acetic acid, alcohols, CO_2 and H_2 (Shin *et al.*, 2011). The presence of lactobacilli order has been related to operational periods with a high VFA content, as observed here between periods P1 and P2 (Shin *et al.*, 2011).

There are a number of species that are not exclusive of the influent, since they have also been found in the effluent samples. Nevertheless, an increase in relative abundance points out the fact that some of these bacteria might play a key role in the methanogenic consortium. Despite the high diversity found in influent samples, the microbial community of effluent samples appeared more stable and less diverse. According to the theory of biological succession, the composition of biological communities is evolving in response to changes that occur in their environment. Although it is not possible to deduce whether the driving force of the community shift resides in one particular trophic level, it seems that, to achieve stability, a given arrangement among populations is more important than any one specific population (Fernández *et al.*, 1999). So, the observation of a relatively conserved DGGE microbial pattern along operation of a CSTR, where the inactive biomass is being washed out, is a strong indicator of stability.

The microbial composition of the influent varied along the continuous reactor experiments, which might primarily be attributed to the changing nature of the several fresh pig slurries among 595 days. One predominant ribotype identified in the initial

feeding sample *i0* (diluted pig slurry used in the starting up of the reactor) was closely related to the type strain of *Syntrophomonas sapovorans* (97%). The abundance of this species tended to decrease in subsequent influent samples, while the related *Syntrophomonas zehnderi* appears to be dominant in the *i1* sample. The genus *Syntrophomonas* is related to hydrolytic acidogenic and/or homoacetogenic bacteria (Archer and Kirsop, 1990; Melvin and Hobson, 1994). Both detected *Syntrophomonas* species are able of utilizing mono- and/or polyunsaturated LCFAs (Ahmad *et al.,* 2011). In particular, *S. sapovorans* is a proton reducing species that oxidizes saturated and unsaturated LCFA (Roy *et al.*, 1986).

A second dominant band was found along all influent samples, which was related to an uncultured bacterium from an anaerobic digester treating pig slurry, being *Erysipelothrix tonsillarum* the closest phylogentically defined match (91% of sequence homology). The genus *Erysipelothrix* has commonly been associated with erysipelas disease in swine, chickens and other animals, being faeces the possible carriers (Pal *et al.*, 2010). The sequence from band 5 (observed in *i1*, *i2* and *i3*) was identical the type strain of *Bacteroides coprosuis*. This specie has previously been isolated from a swine-manure storage pit by Whitehead *et al*. (2005), who also found out that the end products of the glucose metabolism were acetate, succinate and propionate, while gelatine was not hydrolyzed. The more ubiquitous band 3 (samples *i1* to *i4*) was highly homologues to an uncultured microorganism from a digester treating pig slurry, but it was poorly related (86% sequence homology) to *Parapedobacter soli* as the closest known species. Kim *et al*., (2008) observed that *P. soli* is a gram-negative, non-spore-forming, rod-shaped, non-motile bacterium, isolated previously from soil from a ginseng field in Korea able to utilizing mono- and/or polyunsaturated LCFAs, such as *S. zehnderi* (Ahmad *et al*., 2011).

A ribotype identical to *Ruminofilibacter xylanolyticum*, a rumen bacterium, was observed in *i1*, *i2* and *i5*. This species is involved in digestion of xylan and was detected in a full-scale biogas plant fed with maize silage, green rye and liquid manure (Kröber *et al*., 2009). It is also present in energy crops, manure and in grass silage fibers immobilized on zeolite (Weiß *et al.,* 2011), and showed a pronounced hydrolytic xylanase activity that might catalyze the degradation of fibers in pig slurries. Finally, a new ribotype was observed among the dominant populations in samples *i5* and *i6*, which was highly homologous to an uncultured microbe from an aerobic reactor treating pig slurry. Yet, the

closest phylogenetically defined match, with just 85% sequence homology, was the species *Solitalea canadensis*.

The most abundant ribotype within this category has a 99% sequence homology to the species cluster formed by *Trichococcus flocculiformis*, *T. palustris* and *T. pasteurii*. They have been characterized as fermentative, aero-tolerant and gram-positive filamentous bacteria, which have been isolated from bulking sludge and mainly degrade monomeric and dimeric carbon sources (Scheff *et al.*, 1984). These species are able to fermentate glucose by producing lactate, formate, acetate and ethanol as the organic end products (Liu *et al.*, 2002). Jiang *et al.* (2010) observed that *T. pasteurii* was one of the dominant methanol-using acetogens in soil samples, while Tandukar *et al.* (2009) suggested that this species is capable of carrying out dinitrification as well as reduction of heavy metals.

Other ribotypes that were present in both influent and effluent samples were associated to *Petrimonas sulfuriphila* (92% in sequence homology), *Clostridium disporicum* (98% homologous) and *Pseudomonas pertucinogena* (95% homologous). *Petrimonas sulfuriphila* is a mesophilic, strictly anaerobic, fermentative bacterium that was isolated previously from a producing well of a biodegraded oil reservoir (Grabowski *et al.*, 2005). This species are known to ferment carbohydrates and some organic acids, producing acetate, H_2 and CO_2. Elemental sulphur and nitrate can be used as electron acceptors, being reduced to sulphide and ammonium respectively (Grabowski *et al.*, 2005). *Clostridium disporicum* is a starch hydrolyzing bacteria that ferments sugars to acids (Horn, 1987) and it has been found in swine hind gut, biosolids of pork manure and swine slurry (Cook *et al.*, 2010). This species of *Clostridium* is known to be especially resistant to environmental stress (Peu *et al.*, 2006).

There was a main cluster of five species that were detected in effluent samples from periods P1, P2 and P3 (Table 7.3). It was constituted by *Ruminofilibacter xylanolyticum*, *Petrimonas sulfuriphila, Clostridium disporicum, Trichoccocus* spp. and *Pseudomonas pertucinogena*. The presence of these species might be attributed to the background population of the pig manure from the feeding. When comparing *e1*, *e2* and *e3* with the unstable period *e4*, they mainly differed by the presence of *Petrimonas sulfuriphila* that could be related to the increase in the PP-ABP and indirectly the sulphur concentration.

Period P5 was characterized by the addition of glycerin as a second co-substrate, and the relatively high PP-ABP content in the feeding (50% of the total COD) prompted the

increase in the propionic/acetic ratio in the effluent (Table 7.2). Under these new operational conditions two species were determined to be specific in *e5*, *Syntrophomonas zehnderi* that has already been described previously as a specific syntrophic LCFA degrading bacterium (Sousa *et al.,* 2008*)* and *Ruminofilibacter xylanolyticum*, which was also detected in influent samples.

Syntrophomonas species have a very restricted substrate range (fatty acids with 4-18 carbon atoms) and coexist in consortia with methanogens or sulphate reducing bacteria, which use the H_2 released from fatty acid degradation as energy source as well as removing the inhibitory effect of H_2 on *Syntrophomonas* species (McInerney *et al*., 1981). In this case, the specific propionic accumulation detected in this period may be the explanation why *Syntrophomonas* was not observed in any other effluent sample different from *e5*. Propionate is oxidized to acetate and carbon dioxide in anaerobic environments, being a reaction energetically favourable when archaea remove reducing equivalents as H_2 or formate. Syntrophic propionate degradation has been demonstrated for several *Syntrophobacter* species via different routes such as the non-randomizing pathway via butyrate (de Bock *et al*., 2001) where part of the propionate is carboxylated to butyrate and then degraded to acetate. Cocultures of *Smithella propionica* or *Syntrophobacter* strains in syntrophy with hydrogen- and formate-utilizing methanogen produce less CH_4 and more acetate (de Bock *et al*., 2001).

In the effluent *e6*, the highest RG content in the feed increased the sulphate content inside the reactor (Table 7.2), being the eubacterial profile different from all other periods, including *e5*. For example, two new species were observed: *Parapedobacter soli* (it was already observed in *i1-i5*), also *Bacteroidetes propionicifaciens*. This species is a strictly anaerobic bacterium, isolated previously from rice-straw residue from a methanogenic reactor treating waste from cattle farms (Ueki *et al*., 2008). Most *Bacteroidetes* species produce acetate and succinate as major products from glucose and species that produce propionate as a main end product are less common, but *B. propionicifaciens* with cobalamin and haemin can produce abundant amounts of propionate (Ueki *et al*., 2008). This species can use different compounds (arabinose, fructose, galactose, glucose, mannose, cellobiose, maltose, glycogen, starch, dextrin, amygdalin, lactate and pyruvate) as growth substrate and produces acetate and propionate (Ueki *et al*., 2008). In comparison to *e5,* two additional species were detected: a H_2S producer bacterium close to *Solitalea Canadensis* (85% homology) and a sulphur reducing bacterium affiliated to

Petrimonas sulfuriphila (92% homology) also present in *e0* and *e4* (the adaptation and the unstable period respectively).

7.3.3.2 Archaeal population

DGGE profiles from the archaeal populations (Figure 7.4b) yielded a total of eight ribotypes, which were successfully excised and sequenced (Table 7.4). It is noteworthy that the observed biodiversity is appreciably lower than that in the eubacteria, and its structure is also more conserved over time being less affected by the operational changes. The obtained phylogenetic assignments were similar to other works in that *Methanosarcinales* and *Methanomicrobiales* were dominant in swine manure biogas reactors (Schmidt *et al*., 2000; Mladenovska *et al*., 2003; Karakashev *et al.,* 2005; Hansen *et al.*, 1999). *Methanosarcinales* is divided into two families, *Methanosarcinaceae* and *Methanosaetaceae*. *Methanosarcinaceae* are able to utilize CO_2, methylated compounds as well as acetate and they play a crucial role in methane formation during anaerobic degradation in biogas fermenters (Mladenovska *et al*., 2006, Narihiro *et al*., 2009). *Methanosaetaceae* is a strict acetotrophic group of methanogens that have often been detected in biogas reactors working under a mesophilic temperature regime (McHugh *et al*., 2003; Laloui-Carpentier *et al*., 2006).

Methanomicrobiales are hydrogenotrophic methanogens that may serve as the hydrogen-utilizing partners of syntrophic fatty acid β-oxidizing (SAO) bacteria in the digester (Hansen *et al*., 1999). They can also growth in co-culture with species of the family *Syntrophobacter* (Siggins *et al*., 2012) such as *Syntrophomonas sapovorans* that degrade linear saturated fatty acids with 4–18 carbon atoms in co-culture with *Methanospirillum hungatei* (Alves *et al*., 2009; Hatamoto *et al*., 2007).

Three species were detected in the influents: *Methanosaeta concilii* (*i1*), *Methanosarcina barkeri* (*i0, i2-i6*) and *Methanosarcina mazei* (*i3-i5*). The first one is a strict aceticlastic methanogen (Plugge *et al*., 2010) observed only in the influent from period P1, constituted for pig slurry, as a predominant band. This species uses acetate as electron donor with an optimum growth temperature of 35 - 40°C and it has a low *Ks* value compared to other acetate-utilizing methanogens (Rastogi *et al*., 2008).

Methanosarcina barkeri is a hydrogenotrophic or acetoclastic methanogen, highly specialized and versatile, able to produce methane from molecular hydrogen, carbon dioxide, carbon monoxide, acetate, or methanol as a methanogenic substrate (Hippe *et*

al., 1979; Lessner *et al*., 2006; Bryant and Boone 1987). Under adverse conditions methane will mainly be produced by *M. barkeri*, which has a fast doubling time (about 1.5 days), and grows well at near neutral pH, but is a poor scavenger with a low affinity for acetate (Barber and Stuckey, 1998).

Methanosarcina mazei also uses several carbon sources, but its growth and CH_4 production readily occur at the expense of H_2-CO_2, methanol, or trimethylamine. Liu *et al.* (1985) observed that acetate was used very slowly, and formate was not utilized, its growth was most rapid when H_2-CO_2 was provided in combination with trimethylamine or methanol. Both species are tolerant to changes in pH, with optimum pH levels of 5–7 and 5.5–8 for *M. barkeri* and *M. mazei,* respectively (Liu *et al*., 1985; Van Leerdam *et al*., 2008).

The presence of *M. mazei* in the influents of periods P3 to P5, a methylotrophic methanogen (Fernández *et al*., 1999) which appears to be controlled by environmental conditions such as cation concentration (Raskin *et al*., 1999), was attributed to a change in the manure management and/or storage time in the centralized treatment facility where it came from. These results are similar to other studies that have shown that hydrogenotrophic methanogens dominated the archaeal community of pig manure slurries (Whitehead and Cotta, 1999; Hori *et al*., 2006; Mladenovska *et al*., 2006). The only available substrate for methanogens inside the rumen is H_2/CO_2, since formed VFA are absorbed by the rumen epithelium and converted to animal proteins, being not available for utilization as C source by acetoclastic methanogens, thus hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*) can multiply easily (Rastogi *et al*., 2008). So, the dominance of hydrogenotrophic methanogens (in manure samples) might be an indicator of a good manure management since the presence of VFA may be an indicator of biological degradation during long storage periods.

In case of the effluents, *Methanosaeta concilii* and *Methanosarcina barkeri* were identified in all samples (*e0-e6*) and one species from *Methanomicrobiales*: *Methanospirillum hungatei* was observed in *e5* and *e6*, when glycerin was added. *M. hungatei* produces methane only from H_2–CO_2 or formate, but not from acetate or ethanol and methanol, being a strictly hydrogenotrophic methanogen (Ferry *et al*., 1974). The occurrence of *Methanosarcina* and *Methanosaeta* could be due to the acetic and ammonia concentrations in the reactor (Table 7.2) were always under the threshold level for growth both families (Westermann *et al*., 1989; Jetten *et al*., 1990; Ahring, 1995).

7.3.3.3. Statistical multivariate analysis

Direct gradient analysis on the relationship between microbial community dynamics in response to changes on selected physicochemical parameters was performed by canonical correspondence analysis (CCA). A species abundance matrix was generated upon the relative intensity of the depicted DGGE bands, while average values for each operational period on VFA, AR, NH_3 and the ratio propionic/acetic and C/N were used as the environmental matrix (Figure 7.5).
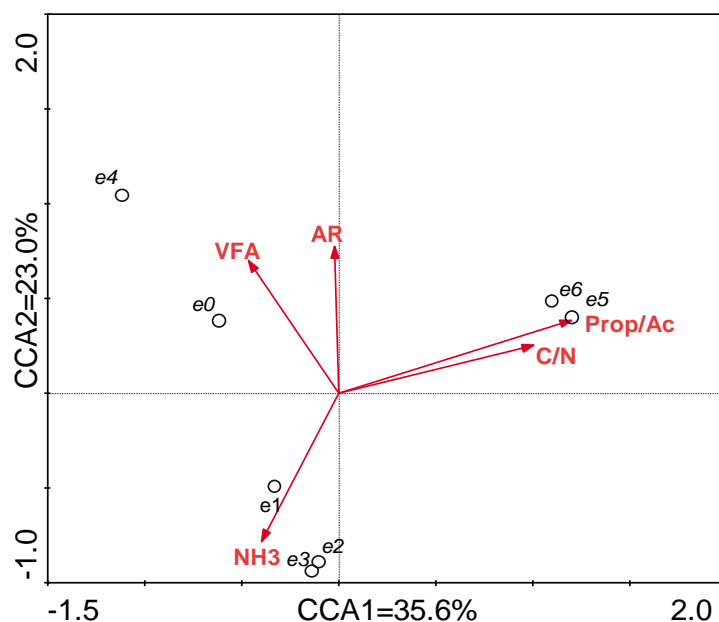
Figure 7.5. *CCA biplot on the microbial community structure (based on DGGE profiles) and selectedphysico-chemical parameters (VFA, AR, propionic/acetic ratio, C/N ratio and NH₃), in reactor effluent samples taken at the end of the different operational phases.*

The CCA sample scores and environmental parameter vectors were depicted in a biplot, which encompassed up to 58.6% of the total species data variance, and a 76.7% of the species-environment relation. The ordination pointed towards the existence of two gradients; the first followed the trend of propionic/acetic and C/N ratio, which correlated significantly with the microbial community structure in periods P5 and P6 when glycerin was added ($p \leq 0.05$). Samples from the periods P1 to P3 were arranged through a second gradient of decreasing VFA and AR, and increasing NH_3 content. With this respect, the instability from period P4 was reflected by a relatively high VFA content. On the other hand, supplementation of ABP in the feeding was related to an increase in NH_3 concentration. As expected, AR is correlated directly with VFA and inversely with NH_3. Nevertheless, in this case, the correlation between these environmental parameters and

the resulting microbial community structure was not significant. This is consequent with the relatively similar eubacterial DGGE profiles in samples *e1* to *e4*.

7.4. CONCLUSIONS

Co-digestion of pasteurized animal by-product with pig manure gave good results, and it was further improved by the addition of glycerin as carbon source. The best results related to methane production potential were obtained with the highest C/N, in periods P5 and P6, upon glycerin addition. The highest methane yield value was obtained in period P5 (18.7 $m^3_{CH4} \cdot t^{-1}$), which represented an increment of 414%, 246% and 139% with respect to the period P1 (3.6 $m^3_{CH4} \cdot t^{-1}$ with pig manure alone), P2 and P3, with a binary mixture of manure and slaughterhouse waste respectively. Significant differences in the methane production rates ($m^3_{CH4} \cdot m^{-3} \cdot d^{-1}$), comparing PM alone and co-digestion of PM:PP-ABP:RG have been found. High ammonium values were obtained in period P2 and P3, due to high PM ammonia value but inhibition processes were not observed.

PCR-DGGE has proved to be a useful tool for analyzing and investigating the dynamics of the microbial community in the anaerobic co-digestion of pasteurized slaughterhouse waste with pig slurry and glycerin. The microbial community of the domain *Eubacteria* was more sensitive to operational changes than the domain *Archaea*.

The CCA analysis showed correlation between the eubacterial communities in the effluent samples of every period and the physic-chemical parameters. Glycerin addition resulted in a significant change in the enriched microbial populations.

Table 7.3. *DGGE bands of eubacteria (Figure 7.4a): designations and accession numbers for the band sequences and levels of similarity to related organisms. Note: T Type strain.*

Band	Sample	Phylum/ Family	Reference species, strain or uncultivated microorganism (environmental source)	Accession	H
1	i0	*Firmicutes/ Clostridiaceae*	Uncultured (anaerobic reactor treating pig slurry) *Syntrophomonas sapovorans DSM3441^T*	HQ155840 NR_028684	98 97
2	i0-i6	*Firmicutes/ Erysipelotrichaeae*	Uncultured (anaerobic reactor treating pig slurry) *Erysipelothrix tonsillarum ATCC43339^T*	HQ156132 NR_040871	95 91
3	i1-i4; e6	*Bacteroidetes/ Sphingobacteriaceae*	Uncultured (anaerobic reactor treating pig slurry) *Parapedobacter soli DCY14^T*	GQ139189 NR_044119	98 86
4	i0-i3, i5, i6; e5	*Firmicutes/ Clostridiaceae*	Uncultured (anaerobic reactor treating pig slurry) *Syntrophomonas zehnderi OL-4^T*	GQ133946 NR_044008	99 94
5	i1-i3	*Bacteroidetes/ Bacteroidaceae*	*Bacteroides coprosuis JCM13475^T*	AB510699	100
6, 12, 13, 17, 19, 20	i2, i3; e0, e4, e6	*Bacteroidetes/ Porphyromonadaceae*	Uncultured (anaerobic reactor treating pig slurry) *Petrimonas sulfuriphila BN3^T*	GQ137794 NR_042987	99 92
7, 16	i1-i3, i6; e0-e3, e5, e6	*Firmicutes/ Carnobacteriaceae*	*Trichococcus flocculiformis DSM2094^T* *Trichococcus palustris DSM9172^T* *Trichococcus pasteurii DSM 2381^T*	NR_042060 NR_025435 NR_036793	99 99 99
8	i2-i4; e0,e4	*Proteobacteria/ Pseudomonadaceae*	Uncultured (aerobic reactor treating pig slurry) *Pseudomonas pertucinogena IFO 1416^T*	HM069956 NR_040799	99 95
9, 18	i1,i2,i5; e0-e5	*Bacteroidetes/ Rikenellaceae*	*Ruminofilibacter xylanolyticum S1^T*	DQ141183	100
10, 11, 14, 15	i2-i6; e0-e6	*Firmicutes/ Clostridiaceae*	Unidentified (swine feces) *Clostridium disporicum DS1^T*	FJ753830 NR_026491	98 98
21	e6	*Bacteroidetes/ Bacteroidaceae*	Uncultured (aerobic reactor treating pig slurry) *Bacteroides propionicifaciens JCM14649^T*	GQ137107 AB510706	95 91
22	i5, i6; e1-e4, e6	*Bacteroidetes/ Sphingobacteriaceae*	Uncultured (aerobic reactor treating pig slurry) *Solitalea canadensis DSM3403^T*	GQ134100 NR_040906	98 85

Table 7.4. *DGGE bands of archaeobacteria (Figure 7.4b): designations and accession numbers for the band sequences and levels of similarity to related organisms. Note: T Type strain.*

Band	Sample	Phylum/Order	Reference species, strain or uncultivated microorganism (environmental source)	Accession number	H
23, 26, 28, 29	i1, e0-e6	Euryarchaeota / Methanosarcinales	Uncultured (activated sludge) *Methanosaeta concilii DSM2139^T*	AB489236 NR_028242	100 99
24, 27	i0, i2-i6, e0-e6	Euryarchaeota / Methanosarcinales	Uncultured (anaerobic reactor treating pig slurry) *Methanosarcina barkeri DSM800^T*	JN173201 AJ012094	100 98
25	i3, i4, i5	Euryarchaeota / Methanosarcinales	*Methanosarcina mazei DSM2053^T*	NR_041956	99
30	e5, e6	Euryarchaeota / Methanomicrobiales	Uncultured (anaerobic reactor treating MWS) *Methanospirillum hungatei NBRC100397^T*	CU917418 AB517987	99 96

7.5. ACKNOWLEDGEMENTS

7.6. REFERENCES

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Chapter 8. Final conclusions and suggestions for further research

This Chapter reports the main conclusions obtained. Suggestions for further research related to ABP anaerobic digestion are also presented.

8.1. FINAL CONCLUSIONS

In this thesis, anaerobic digestion was proposed as a useful and suitable technology for treating and valorizing ABP, that are produced in high amounts in Europe and Spain. This process offers several advantages such as energy production. The studies reported in this dissertation focused in the feasibility of solid slaughterhouse for anaerobic digestion, with emphasis in previous pre-treatments, studying their effect on the chemical composition of organic matter, besides its disintegration kinetics together with its methane yield and production rate. Also a continuous co-digestion process was carried out with thermally pre-treated ABP, pig manure and glycerin, where the microbiological communities involved in anaerobic digestion was monitored. From the overall results, the following major conclusions can be obtained:

In **Chapter 3**, the anaerobic digestion of poultry and piggery ABP at mesophilic range showed that both substrates were suitable for this biological process, but when thermal pre-treatments included in the European legal framework were applied, different results were obtained depending on the initial composition of fats, proteins and carbohydrates. The pre-treatments solubilized both ABPs but in the case of piggery ABP, with high concentration of fat and little amount of carbohydrates, the pasteurization and sterilization improved considerably the methane production rate and yield. In the case of poultry ABP, the pasteurization did not improve the yield and also produced a decrease in the methane production rate. The theory that some recalcitrant compounds created during the Maillard reactions between carbohydrates and proteins was postulated.

In **Chapter 4,** using TGA-MS and FTIR techniques to carry out a deep characterization of the organic material before and after pasteurization of the poultry and piggery ABPs the theory proposed in Chapter 3 was confirmed. These techniques demonstrated different changes in the organic material after the thermal pre-treatment and in poultry ABP was possible to observe the formation of new complex nitrogen related compounds after the pasteurization.

In **Chapter 5**, a novel high pressure pre-treatment showed to be suitable for increasing effectively the solubilization of the pasteurized piggery ABP after all the selected conditions (200, 400 and 600 MPa at environmental conditions for 15 minutes) and for improving protein decomposition during the anaerobic process. Although it did not

enhance the methane production and rate notably, no recalcitrant or more complex compounds were formed.

In **Chapter 6** the disintegration kinetics for raw and pre-treated ABPs are shown. The results confirmed the previous chapter's data for piggery ABP and the increment in the disintegration and the hydrolysis rate, observed after thermal pre-treatment (pasteurization), was characterized by an observed coefficient k'_{dis}. A methodological procedure was defined and used with different solid substrates. Applying a modified BMP test with higher inoculum concentration, which allowed obtaining a logistic-type curve, to the particulate fraction of a solid waste, the correlation between the model and the experimental results was satisfactory.

Regarding **Chapter 7,** the co-digestion of ABP with pig manure and glycerin resulted in a feasible method of stabilization and valorization of the ABPs, where no inhibitions were observed in spite of the high LCFA and ammonia concentrations in the feeding. The approach of adding glycerin to the anaerobic reactor to improve the C/N ratio was a suitable option but until a maximum limit and after that, some imbalance between acetic and propionic appeared. The microbial community involved in the co-digestion process, studied by means of DGGE, was changing during the experimental time, especially in *Eubacteria* domain while *Archaea* domain remained quite stable.

As general conclusions, all the studied ABPs were highly bio-degradable and suitable for anaerobic digestion although their attainable degradability and methane yield relayed on the substrate composition and the applied pre-treatment. It was demonstrated that pasteurization can affect the disintegration kinetics, improving them in the piggery case. The strategy of the co-digestion was proved to be reliable to optimize the anaerobic digestion of ABP. These data might be directly utilized in practical implementation of anaerobic digestion of such materials; the obtained results will help to obtain high renewable energy rates from this industrial by-product through anaerobic digestion.

8.2. SUGGESTIONS FOR FURTHER RESEARCH

Although this thesis described the anaerobic digestion of ABP as an effective treatment method, there are still some lacks in the knowledge or improvements that could be done. As for example:

- A better understanding of thermal pre-treatment effect on protein, fat and carbohydrate fractions of the ABP, to distinguish between biological and physical effects, is needed.

- To apply high pressures (200, 400 and 600 MPa) to poultry ABP to check if Maillard compounds and/or other recalcitrant materials were formed and also to study if any of these pre-treatments could be useful to sterilize the organic material.

- A development of a new methodology for an accurate COD fractioning for disintegration parameters determination would be useful.

- To determine quantitatively the microbial community structure and changes by means of q-PCR with specific probes can give valuable information.

- To carry out experiments with different kind of continuous reactors, where more co-digestion experiments with different C/N ratios could be done to determine its effects on the anaerobic digestion process.

Annex 1. Determination of COD in heterogeneous solid or semisolid samples using a novel method combining solid dilutions as preparation step followed by optimized closed reflux and colorimetric measurement

This paper reports the development of an innovative sample preparation method for the determination of the chemical oxygen demand (COD) in heterogeneous solid or semi-solid samples, with high suspended solids and COD concentrations, using an optimized closed reflux colorimetric method. The novel method, named solid dilution (SD), is based on a different technique of sample preparation, diluting the sample with magnesium sulfate ($MgSO_4$) previously to COD determination. With this, it is possible to obtain a solid homogeneous mixture much more easily analyzable. Besides, a modification of concentration and ratio of reagents was optimized to make the closed reflux colorimetric method suitable for complex substrates with COD levels ranging from 5 to 2,500 $g_{O2} \cdot kg_{TS}^{-1}$. The optimized method has been tested with potassium hydrogen phthalate (KHP) as primary solid standard, and using different solid or semi-liquid substrates like pig slaughterhouse waste and sewage sludge, among others. Finally, the optimized method (SD/SM-CRC) was intensively tested in comparison to the standard titrimetric method (SM-ORT) using different certified reference materials (CRM). The developed method was found to give higher accuracy, 1.4% relative standard deviation (RSD) vs. 10.4%, and bias of 2.8% vs. 8.0%, in comparison to the standard open reflux titrimetric method.

1. INTRODUCTION

Chemical oxygen demand (COD) is defined as the amount of oxygen in the form of a strong oxidizing agent consumed in the oxidation of organic compounds, and it is used as one of the main water quality parameters at wastewater treatment facilities. Mathematical models such as the ASM for activated sludge processes (Hence *et al*., 2000) and the ADM1 for the anaerobic digestion processes (Batstone *et al*., 2002) use COD as a key measure for organic components variables.

The increasing interest on anaerobic digestion process for treatment of a widely range of organic waste types has lead to the necessity to obtain reliable measures of the organic matter content of the waste substrates and its transformed products, in order to characterize the process efficiency. COD is the best unit to express organic matter, since it is conservative along the biological processes and, therefore, its measure is a limiting factor for having good substrate and process characterizations. While the COD determination for waters is currently performed without significant problems, these appear when this measure is applied to solid or semi-solid heterogeneous waste with high fat contents, such as manure or slaughterhouse solid waste, obtaining results with large confidence intervals. The reliable measure of COD of solid substrates has become an issue of concern for many laboratories dealing with solid organic matter characterization, but there is a lack of references about the adequate procedures (Angelidaki *et al*., 2002; Yadvika *et al*., 2006; Raposo *et al*., 2008).

Recently, some researchers have applied the APHA–AWWA–WPCF (2005), Standard Methods for the Examination of Water and Wastewater, for COD determination of solid samples (Veeken and Hamelers, 1999; Neves *et al*., 2004) or high suspended solid content samples, such as poultry and cattle manure (Güngör-Demirci and Demirer, 2004), pig manure (Montalvo *et al*., 2005), olive mill wastewater (Raposo *et al*., 2004) or municipal waste sludge (De la Rubia *et al*., 2006), among others.

Since the APHA–AWWA–WPCF method of COD determination has been mainly used for water and wastewater samples (values below $1 g_{O2} \cdot l^{-1}$), it seems to be inappropriate for solid or semi-liquid samples having high percentage of suspended solids, due to the heterogeneity of the substrate. To solve this problem, several fold dilutions of the original sample were used, with the corresponding loss of accuracy and repeatability in the COD values of replicates, as reported by Liao and Lo (1985) and Yadvika *et al*. (2006). On the

other hand, Raposo *et al*. (2008) demonstrated that their modified and optimized method for determining COD based on the DIN 38414-S9 (1986) was applicable to solid substrates and solutions with high suspended solid content. Recently, in 2011, similar studies were carried out by Zupančič and Roš with similar results and drawbacks (Zupančič and Ro*š, 2012).*

In September 2008, twenty-six labs, from sixteen countries, participated in an interlaboratory study (PT) of COD measurements using solid substrates and liquid samples with high suspended solid content (Raposo *et al*., 2009). Results from laboratories performance, expressed as z-score (Crosby *et al*., 1995), explained in supporting information section, indicated that only 36% of the results were satisfactory. This suggests that the majority of participating laboratories obtained inappropriate COD results. In September 2009, a second PT of COD determination was carried out. The results obtained were improved by around 30% compared with the results of the first PT scheme. This result demonstrates that analytical performance of COD in difficult samples can be improved by regular participation in PT (Raposo *et al*., 2010). Although this is an improvement in COD determination in complex substrates, it is obvious that analytical methods for COD analysis of solid samples and liquid samples with high concentrations of suspended solids should be additionally optimized.

In order to improve the COD measure of heterogeneous solid and semi-solid waste, the present study was carried out to develop a suitable method of COD determination, based on optimization of Standard method 5220 D-closed reflux colorimetric (APHA–AWWA–WPCF, 2005) and the application of previous solid dilution of samples with COD values ranged between 2,000-3,000 $g_{O2} \cdot kg_{TS}^{-1}$, which have been impossible to be analyzed with precision and accuracy until now.

2. MATERIAL AND METHODS

2.1. Materials

Digestion of samples was carried out using standard materials as the APHA–AWWA–WPCF method of COD determination explains (see details in supporting information). Samples were also analyzed using available commercially Kit LCK914 COD, range of measure 5-60 $g_{O2} \cdot l^{-1}$, from Hach Lange.

2.2. Apparatus

Samples of pig slaughterhouse waste were lyophilizated before analysis using a laboratory freeze-dryer Telstar Cryodos 50 (Spain) and grinded to obtain meat flour.

2.3. Reagents

Siliceous earth purified and calcined extra pure USP NF and magnesium and sodium sulfate extra pure Scharlau brand were used for solid dilutions of samples.

2.4. Samples

Three different types of samples were used to confirm the effectiveness of this new COD determination method: pig slaughterhouse wastes (PSW) thermally pre-treated, used as co-substrate for an anaerobic digestion reactor, as example of solid substrate with high COD values difficult to analyze; other different heterogeneous solid or semi-liquid wastes (SSW) as example of complex samples not lyophilizated; and two different certified reference materials (CRM) used for final assessment of precision and accuracy. More details of samples are shown in supporting information section.

2.5. Procedure

APHA–AWWA–WPCF standard method 5220 B-open reflux titrimetric (SM-ORT)

The raw or lyophilizated sample (10–50 mg) was weighted directly inside the glass digestion vessels and 50 ml of distilled water were added. Then, 25 ml of the digestion reagent and 75 ml of sulfuric acid reagent were added slowly and carefully to the mixture while mixing thoroughly. Then, procedure and calculation was carried out following standard method 5220 B-open reflux titrimetric indications (see supporting information).

Kit LCK914 COD Method (LCK)

200 mg of the raw or lyophilizated sample was weighted directly inside the screw-cap test tube of the kit. Taking into account that the total weight always must be approximately 200 mg, distilled water should be added when the maximum weight of sample is not reached. Procedure and calculation was carried out following supplier instructions detailed in supporting information.

Optimized APHA–AWWA–WPCF standard method 5220 D-closed reflux colorimetric (SM-CRC)

10-200 mg of the raw or lyophilizated sample was weighted directly inside the screw-cap test tube. Different concentration and volumes of digestion and sulfuric acid reagents (see Table S-1 in supporting information) were tested by varying $K_2Cr_2O_7$ concentration from 0.5 to 1 N, and digestion reagent and sulfuric acid reagent ratio from 1:1 to 1:2 simultaneously, in order to increase the method applicability range, due to the high COD levels that presented the different substrates analyzed. Then, the rest of process was identical to the previous LCK method.

Solid Dilution Method (SD)

Approximately 200 mg of the raw or lyophilizated sample was thoroughly mixed and grinded with 1-2 g of silicious earth, or sodium or magnesium sulphate as diluting agent, then 50-200 mg (100 mg as default value) of mixture was weighted directly inside the glass digestion vessels or inside the screw-cap test tube, depending on SM-ORT or optimized SM-CRC method used to analyze samples after solid dilution. Following, diluted solid or semi-liquid substrates were analyzed using either method previously explained.

2.6. Calculations

The calculation of the COD-1$_{theo}$ was assessed using the empirical equation detailed in the ISO 10707 (1994). The calculation of the COD-2$_{theo}$ was estimated by the sum of the COD values coming from the protein and fat fractions, principal constituents of the pig slaughterhouse waste (see supporting information).

3. RESULTS AND DISCUSSION

3.1. Optimized Colorimetric Method (SM-CRC)

The main objective of this study was to apply the APHA–AWWA–WPCF colorimetric method (SM-CRC) to solid or semi-liquid heterogeneous substrates with COD levels ranging from 5 to 2,500 $g_{O2} \cdot kg_{TS}^{-1}$. Therefore, it was necessary to optimize the reagents ratio and $K_2Cr_2O_7$ concentration, because of using the concentrations recommended by SM-CRC standard method (concentration of $K_2Cr_2O_7$ around 0.20 N and ratio of

digestion and sulphuric acid reagents around 1:2), the upper linear range of COD determination (lower than 15 $g_{O2} \cdot kg_{TS}^{-1}$) was too low for high organic content samples. Accordingly, in order to increase the linear range of the analytical method, and thus its applicability to substrates with high COD levels, higher concentrations of $K_2Cr_2O_7$ in the digestion reagent (from 0.5 to 1.0 N), and different ratio of digestion and sulphuric acid reagents (from 1:2 to 1:1) were tested. The criteria selected to choose the optimized conditions were the slope of the calibration curve, and the upper linear range. The solubility of $K_2Cr_2O_7$ decreases when the ratio of sulphuric acid reagent increases in the reaction media. As can be seen in Table S-1 (see supporting information), in some cases the solubility of $K_2Cr_2O_7$ was exceeded, especially in blank and in low COD concentration standards when a precipitate of the excess of unreacted $K_2Cr_2O_7$ appeared. As a result, the optimized conditions chosen were 0.5 N for the concentration of $K_2Cr_2O_7$ and a ratio of digestion and sulphuric acid reagents 1:1, because maximum slope was achieved and upper liner range reached was 65 $g_{O2} \cdot kg_{TS}^{-1}$, without precipitation problems.

If samples are homogeneous enough, linear range of optimized SM-CRC method can be easily increased by reducing the amount of sample taken. Since total sample weight must be always approximately 200 mg, distilled water should be added, when the maximum weight of sample is not reached. In those conditions, it was possible, for solid or semi-liquid substrates, the COD determination in a range from 4.5 to 1,300 $g_{O2} \cdot kg_{TS}^{-1}$. Table S-2 of supporting information shows the amount of sample necessary, and the lower and the upper linear range values of the optimized SM-CRC method.

Although this range is enough for most substrates analyzed, in case of heterogeneous samples, or samples with COD levels higher than 1,300 $g_{O2} \cdot kg_{TS}^{-1}$, like lyophilizated pig slaughterhouse waste (PSW), which COD values frequently reaches 2,000-3,000 $g_{O2} \cdot kg_{TS}^{-1}$, it is required a previous sample dilution step before COD determination.

3.2. Solid Dilution Method (SD)

Solid Diluting Agent (SD/SM-ORT method)

Since aqueous dilution was impossible even with hot water, due to the high fat content of the lyophilizated pig slaughterhouse samples, a different strategy was tested, consisting

of solid dilution of the samples. Thus, different solid substances were tested to check which could yield better results as diluting agent.

Preliminary tests were carried out with siliceous earth, which initially seemed to be a good candidate for diluting agent, due its inert character. Therefore, one sample of lyophilizated pig slaughterhouse substrate PSW-1 was analyzed in triplicate with and without solid dilution, SM-ORT and SD/SM-ORT methods, respectively. As Table 1 shows, not remarkable improvement was obtained with this reagent, since it was not possible to achieve a homogeneous solid dilution. Instead, much better results were obtained using $MgSO_4$ as dilution agent. Due to the poor repeatability of the standard analytical method for difficult samples, some COD values, obtained by SM-ORT and SD/SM-ORT method diluted with siliceous earth, appear to differ unreasonably from the others in the set and could be considered as outliers. In order to verify whether these suspect values should be rejected, a two-sided Grubbs´ test, recommended by ISO norm in preference to Dixon´s test (*Q*-test), was applied (Miller and Miller, 2000). As the critical value of G was 1.155 (P=0.05), slightly higher than the calculated values of G (see Table 1), the suspect values were not rejected.

The coefficient of variation (CV) of SD/SM-ORT method using $MgSO_4$ as dilution agent was found to be less than 4%, much lower than 22% without solid dilution or than 12% when diluted with siliceous earth, reflecting much better precision with this substance. Furthermore, one-sided *F*-test (Miller and Miller, 2000) was applied to confirm whether the new SD/SM-ORT method was more precise than the standard SM-ORT method. The calculated value of F was 2.6 for siliceous earth and 39 for $MgSO_4$. Since the critical value of $F_{2,2}$ was 19 (P=0.05), only SD/SM-ORT method using $MgSO_4$ as dilution agent demonstrated to be more precise than the standard method.

Magnesium sulfate monohydrate and anhydrous sodium sulfate are habitually used to dry sludge, soil and sediment in environmental samples (EPA Method 9071B, 1998). Therefore, using these reagents it is possible to dilute and to dry the samples simultaneously, obtaining a fine powder more homogeneous than the original sample. Besides, solid dilution of the sample previous to the COD determination allows taking more amount of sample, thus the sample can be more representative in case of heterogeneous samples.

Table 1. *Comparison of COD values obtained in preliminary studies using different diluting agents to carry out solid dilutions (SD: standard deviation, CV: coefficient of variation).*

Sample	Method	Dilution	Diluting Agent	COD ($g_{O2} \cdot kg_{TS}^{-1}$)	Mean	SD	CV (%)	G-Test	F-test
PSW-1	SM-ORT	No	None	1,579* 2,306 2,446	2,110	465	22	1.142	
PSW-1	SD/SM-ORT	6.5	Siliceous Earth	2,417 2,632 2,060*	2,370	289	12	1.073	2.6
PSW-1	SD/SM-ORT	10.5	MgSO$_4$	2,167 2,115 2,020	2,101	75	3.5		39

* Values considered suspect of being outliers.

In order to confirm the improvement obtained using MgSO$_4$ as diluting agent, SD/SM-ORT method was tested using two different types of substrates, lyophilizated pig slaughterhouse waste (PSW-2 and PSW-3) and municipal sewage sludge (SSW-1). Table 2 shows the comparison of COD values obtained with and without solid dilution from different substrates. For SD/SM-ORT method, dilution factor from 5 to 9 was adjusted to fit into the working range of the analytical method, considering the COD value obtained previously using SM-ORT method. Therefore, since the total solid percentage of SSW-1 ($\approx$12%) was lower than PSW-2 and PSW-3 ($\approx$55%), and since COD value in $g_{O2} \cdot kg_{TS}^{-1}$ was lower than for PSW-2 and PSW-3, the dilution should be lower for this sample. Again, the COD values suspected to be outliers were tested applying a two-sided Grubbs´ test. Since the critical value of G=1.155 (P=0.05) exceeds the calculated values of G (see Table 2), the suspect values were retained. As can be seen in Table 2, a remarkable improvement in precision can be achieved using solid dilution previously to COD determination using SD/SM-ORT method instead of using direct analysis, SM-ORT method, with CV average values being 8% and 27%, respectively. Nevertheless, applying one-sided F-test to the results obtained, since the critical value of $F_{2,2}$ was 19 (P=0.05), only the first sample PSW-2 demonstrated that the precision of SD/SM-ORT method was significantly better than the standard SM-ORT method. As a consequence, the new method should be additionally optimized applying solid dilution to a closed reflux colorimetric method for COD determination (SD/SM-CRC method).

Table 2. *Comparison of COD values obtained from different substrates without solid dilution (SM-ORT method), and diluting the sample with MgSO4 before the analysis (SD/SM-ORT method).*

Sample	Method	Dilution	Diluting Agent	COD ($g_{O2} \cdot kg_{TS}^{-1}$)	Mean	SD	CV (%)	G-test	F-test
PSW-2	SM-ORT	No	None	915* 1,205 1,514	1,211	299	25	0.990	
	SD/SM-ORT	9.3	MgSO$_4$	1,485 1,468 1,457	1,470	14	0.9		478
PSW-3	SM-ORT	No	None	1,082* 1,450 1,704	1,412	313	22	1.055	
	SD/SM-ORT	9.3	MgSO$_4$	1,756 1,553 1,486	1,599	141	8.8		5
SSW-1	SM-ORT	No	None	2,347* 1,421 1,253	1,674	589	35	1.143	
	SD/SM-ORT	5.0	MgSO$_4$	1,354 1,295 1,657	1,435	194	14		9

* Values considered suspect of being outliers

SD/SM-CRC method

First step was the confirmation of the applicability of SD/SM-CRC method to solid samples using standard pure solid substrates, such as KHP standard for COD determination, internationally accepted (APHA–AWWA–WPCF (2005). Table 3 gives the comparison between theoretical and experimental COD values, obtained by direct analysis or different solid dilutions of KHP and $MgSO_4$ or Na_2SO_4 ranging from 14 to 30-fold. In this case, good results were obtained with direct analysis because, unlike difficult or complex samples, KHP is a homogeneous substrate and its COD concentration fits into the working range of the analytical method SM-CRC (90-1,300 $g_{O2} \cdot kg_{TS}^{-1}$). In general, for solid dilutions good correlations between theoretical and experimental values were obtained, with bias less than 4%, except for 30-fold dilutions using $MgSO_4$ or Na_2SO_4. This can be explained because for solid dilutions, like for liquid dilutions, dilution error increases when dilution factor rises. Therefore, 20-fold dilutions should not be exceeded for this method. In order to decide whether the difference between the experimental value obtained with SD/SM-CRC method and the known value is significant, a t-Test was applied (Miller and Miller, 2000). Since the calculated t values were less than the critical value of $t_2 = 4.3$ (*P*=0.05), for most cases, the null hypothesis was retained, that is, there is not evidence of systematic error, except for 30-fold dilution using $MgSO_4$.

A similar approach was applied for real samples, using two different pig lyophilized substrates (PSW-4 and PSW-5). First, an elemental analysis was carried out to obtain the elemental composition of the substrates analyzed. The elemental analysis was carried out in triplicate. These data were used to estimate the molecular formula (see Table S-3 of supporting information). After setting up the general molecular formula of substrates used, the COD-1$_{theo}$ values were estimated. On the other hand, fat and organic nitrogen analyses of samples were carried out in order to calculate COD-2$_{theo}$ (see Table S-4 in supporting information section). All analytical determinations of fat and nitrogen were carried out in triplicates. Table 4 shows the theoretical results obtained for COD calculated using two different ways (COD-1$_{theo}$ and COD-2$_{theo}$), and also the experimental values of COD obtained by the SD/SM-CRC method. Table 4 indicates that acceptable correlations between theoretical and experimental COD values were obtained, especially considering the complexity of substrates analyzed. The bias were less than 4%, similar than for standard pure solid substrates, such as KHP.

Table 3. *Theoretical vs experimental COD values obtained for different artificial samples prepared with KHP using SD/SM-CRC optimized method.*

Diluting Agent	Dilution	KHP (mg)	Diluting agent weight (mg)	KHP or Solid dil. weight (mg)	COD_{theo} $(g_{O2} \cdot kg_{TS}^{-1})$	COD_{exp} $(g_{O2} \cdot kg_{TS}^{-1})$	Mean COD_{exp} $(g_{O2} \cdot kg_{TS}^{-1})$	SD	Bias (%)	t-test
None	No	9.4 10.0 10.2	0.0	9.4 10.0 10.2	1,176	1,177 1,174 1,175	1,175	1.3	-0.1	0.9
$MgSO_4$	14.2	75.7	1,000.5	106.8 102.4 101.4	1,176	1,157 1,164 1,146	1,155	9.1	-1.8	3.9
$MgSO_4$	20.7	50.9	1,001.9	153.8 155.1 158.0	1,176	1,163 1,171 1,196	1,176	17	0.0	0.0
$MgSO_4$	30.0	35.3	1,022.0	207.2 201.1 209.6	1,176	1,230 1,229 1,225	1,228	2.5	4.4	36
Na_2SO_4	14.0	77.3	1,000.4	32.0 38.8 24.9	1,176	1,152 1,145 1,206	1,168	33	-0.7	0.4
Na_2SO_4	21.1	50.3	1,011.6	109.5 115.2 106.8	1,176	1,185 1,180 1,159	1,175	13	-0.1	0.2
Na_2SO_4	29.5	35.3	1,007.3	92.7 109.6 115.3	1,176	1,281 1,217 1,248	1,249	32	6.2	4.0

Comparison between the experimental values obtained with SD/SM-CRC method using real samples (PSW-4 and PSW-5), and calculated COD-1$_{theo}$ and COD-2$_{theo}$ values were carried out by applying t-Test. Since the calculated values of t were always lower than the critical value of $t_2 = 4.3$ (P=0.05), the null hypothesis was retained again, meaning that no evidence of systematic error analyzing real samples with the new method was found in comparison with the calculated values.

Another experiment was carried out to check the homogeneity and stability of solid dilutions prepared. Three different aliquots of the same sample (PSW-5) were used to prepare three different solid dilutions (A, B, C). Then, each solid dilution was analyzed in triplicate using SD/SM-CRC method. Table 5 shows that the results obtained in each aliquot were quite similar. In order to check whether the mean values of the three different aliquots differ significantly, an analysis of variance (ANOVA) (Miller and Miller, 2000) was applied. Since the calculated value of *F* (2.20) is lower than the critical value of *F* (5.14 *P*=0.05), the null hypothesis was accepted; that is, the aliquots means do not differ significantly. Furthermore, aliquot A was analyzed one week later and the COD value was almost the same. Again, a comparison of both experimental means was carried out, by applying *t-test*. The observed value of t = 0.15 is less than the critical value of $t_4 =$ 2.78 (P=0.05) for 4 degrees of freedom. Therefore the null hypothesis was retained, no evidence that the solid dilution was unstable along time.

Table 4. *Comparison between theoretical and experimental COD values obtained for two different lyophilizated pig waste substrates using SD/SM-CRC optimized method.*

Sample	COD exp	Mean COD$_{exp}$ $(g_{O2} \cdot kg_{TS}^{-1})$	SD	COD-1$_{theo}$ $(g_{O2} \cdot kg_{TS}^{-1})$	Bias-1 (%)	*t*-test COD-1$_{theo}$	COD-2$_{theo}$ $(g_{O2} \cdot kg_{TS}^{-1})$	Bias-2 (%)	*t*-test COD-2$_{theo}$
PSW-4	2,448 2,499 2,532	2,493	42	2,515	-0.9	0.9	2,499	-0.2	0.2
PSW-5	2,596 2,541 2,538	2,559	33	2,484	3.0	4.0	2,533	0.7	0.9

Following, the optimized method was additionally tested after a series of COD experiments carried out using more lyophilizated pig slaughterhouse samples (PSW-6 and PSW-7). Then, the samples were tested for COD analysis using SM-ORT method as well as optimized SD/SM-CRC method for comparing real samples. The experimental

COD values obtained using both methods are presented in Table 6. The suspected COD values were again checked applying a two-sided Grubbs´ test. Since the critical value of G=1.155 (P=0.05) exceeds the calculated values of G (see Table 6), once more the suspect values were retained.

It is evident, from the results that for this kind of complex substrates the SM-ORT method failed to produce precise results. Probably, this could be due to the small amount of sample analyzed (typically 10-20 mg) that cannot be representative, especially for heterogeneous samples. Another problem was the high fat content of the samples that lead to the formation of two phases in the glass digestion vessels during refluxing which remained undigested due to a bad contact of the solid sample with oxidizing reagents, resulting again in poor repeatability of results. All these problems were overcome by diluting the samples with MgSO4 before COD determination. This resulted in marked improvement in the precision of results, as can be seen in Table 6; average CV of SD/SM-CRC method was less than 2%, much better than 31% obtained with SM-ORT method.

Table 5. *Comparison of COD values obtained from different solid dilutions with MgSO$_4$ of three different aliquots of the same sample, using SD/SM-CRC optimized method.*

Sample	COD ($g_{O2}.kg_{TS}^{-1}$)	Mean	SD	CV (%)	t-test
PSW-5 (A)	2,546 2,615 2,840	2,667	153	6	
PSW-5 (B)	2,596 2,541 2,538	2,559	33	1	
PSW-5 (C)	2,535 2,544 2,423	2,500	68	3	
PSW-5 (A)*	2,632 2,691 2,637	2,653	32	1	0.15

Table 6. *Comparison of COD values obtained from different pig lyophilizated pig waste samples using SM-ORT method, and diluting the sample with MgSO4 before the analysis using SD/SM-CRC optimized method.*

Sample	Method	Dilution	Diluents	COD $(g_{O2} \cdot kg_{TS}^{-1})$	Mean	SD	CV (%)	COD$_{theo}$	Bias	*G*-test	*F*-test
PSW-6	SM-ORT	No	None	2,299 1,983 3,035*	2,439	540	22	2,297	6.2%	1.104	
	SD/SM-CRC	11.4	MgSO$_4$	2,342 2,321 2,388	2,350	34	1.5		2.3%		248
PSW-7	SM-ORT	No	None	2,914 1,275* 2,050	2,080	820	39	2,494	-16.6%	0.981	
	SD/SM-CRC	15.7	MgSO$_4$	2,415 2,398 2,458	2,424	31	1.3		-2.8%		706

Values considered suspect of being outlier

Besides, one-sided F-test was applied to confirm whether the new SD/SM-CRC method was more precise than the standard SM-ORT method. The calculated value of F was 248 and 706 for PSW-6 and PSW-7 respectively. Since the critical value of $F_{2,2}$ was 19 (*P*=0.05), SD/SM-CRC method using $MgSO_4$ as dilution agent demonstrated to be much more precise than the standard method. Moreover, much better accuracy was also obtained with SD/SM-CRC method; average bias was 2.6%, much lower than 11.4% obtained with SM-ORT method.

Next step was to check the applicability of SD/SM-CRC method to different complex solid or semi-liquid substrates not lyophilizated (SSW). Five different complex raw samples, indicated in Table S-5 of supporting information, were analyzed. Dilution factor was adjusted from approximately 3 to 11 in order to fit into the working range of SD/SM-ORT method, considering the COD value expected or obtained previously using SM-ORT method. As Table S-5 shows, the average CV obtained for this kind of samples was around 3%, similar than for standard pure solid substrates or for lyophilizated samples. Consequently, difficult or complex substrates also can be analyzed by the optimized method with repeatable results.

Finally, two different certified reference materials, CRM-1 and CRM-2, were used for final assessment of precision and bias. Both solid samples were analyzed for COD determination (9 replicates were carried out in each case) by two different methods: standard APHA–AWWA–WPCF open reflux titrimetric method (SM-ORT) and, after solid dilution of the samples with $MgSO_4$, the novel optimized closed reflux colorimetric method (SD/SM-CRC). The COD_{theo} was considered as the assigned COD value for each sample. The assigned values for samples CRM-1 and CRM-2 were 988 and 956 $g_{O2} \cdot kg_{TS}^{-1}$, respectively (Raposo *et al*., 2009; Raposo *et al*., 2010). Table 7 gives the experimental and theoretical COD values for both samples, using the standard and optimized analytical methods.

Annex 1

***Table 7.** Comparison of COD values obtained using CRM solid substrates using optimized SD/SM-CRC and standard SM-ORT methods.*

Sample	Method	Dilution	Sample Weight (mg)	COD_{exp} ($g_{O2} \cdot kg_{TS}^{-1}$)	Mean	SD	CV (%)	COD_{theo} ($g_{O2} \cdot kg_{TS}^{-1}$)	Bias (%)	*G*-test	*F*-test
CRM-1	SM-ORT	No	22	818							
			38	853							
			41	864							
			30	980							
			29	973	900	81	8.9		-8.9		
			34	900							
			24	800							
			33	873							
			26	1,039*				988		1.723	
	SD/SM-CRC	11.9	106	928							
			100	955							
			100	976							
			101	934							
			115	956	953	15	1.5		-3.5		31
			116	951							
			106	960							
			104	958							
			111	965							
CRM-2	SM-ORT	No	32	851							
			31	1,034*						1.387	
			44	866							
			44	880							
			41	856	888	105	11.8		-7.1		
			34	836							
			32	794							
			31	1,093*						1.944	
			27	784				956			
	SD/SM-CRC	11.0	82	925							
			77	926							
			70	928							
			70	924							
			78	943	935	12	1.3		-2.2		71
			71	938							
			73	951							
			76	956							
			74	922							

* Values considered suspect of being outliers

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The COD values of the SM-ORT method suspect of being outliers were checked again applying a two-sided Grubbs´ test. Since the critical value of G, for a sample size of nine, was 2.215 (P=0.05), and for all cases exceeds the calculated values of G (see Table 7), every one of the suspect values were retained. The precision and bias was assessed for each analytical method and for both samples. As is shown in Table 7, the coefficient of variation is found to be less than 2% for optimized colorimetric method with solid dilution, SD/SM-CRC, which is much lower than the CV value for SM-ORT method (around 10%). On the other hand, the average bias for SD/SM-CRC method was less than 3%, while for SM-ORT method it was significantly higher, around 8%. Moreover, one-sided F-test was once more applied to confirm whether the new SD/SM-CRC method was more precise than the standard SM-ORT method using CRM samples. The calculated value of F was 31 and 71 for CRM-1 and CRM-2, respectively. Since the critical value of $F_{8,8}$ was 3.438 (P=0.05), SD/SM-CRC method using $MgSO_4$ as dilution agent demonstrated again to be much more precise than the standard method.

4. CONCLUSIONS

This work proposes a new method based on modification in the APHA–AWWA–WPCF open reflux titrimetric or closed reflux colorimetric methods, SM-ORT or SM-CRC respectively, for estimation of COD in heterogeneous solid or semi-liquid samples, consisting of a novel preparation method based on solid dilutions (SD), using $MgSO_4$ as diluting agent. In this way, it is possible to take more amounts of sample, increasing its representativeness and improving precision and accuracy in results. An extensive experimental study was carried out using lyophilizated pig slaughterhouse waste samples, proving that the modified method is useful and very appropriate to measure the organic content of solid or semi-liquid substrates with values ranging from 5 to 2,500 $g_{O2} \cdot kg_{TS}^{-1}$. The applicability of the optimized method was intensively tested for organic pure standards, like KHP, using $MgSO_4$ and Na_2SO_4 as diluting agents, and for different heterogeneous substrates with high organic contents, also with satisfactory results, with CV values around 4%. Intensive study using two certificated reference materials (CRM) confirmed that the modified method gave better precision as well as accuracy in results as compared to the SM-ORT method (1.4% RSD vs. 10.4%, respectively) and bias (2.8% vs. 8.0%, respectively). Additionally, a 93% reduction in the volume of chemicals consumed (from 100 ml of SM-ORT method to 7.2 ml of SD/SM-CRC method, as can be seen in procedure and in Table S-1) and toxic waste generated was achieved using SD/SM-CRC method, providing an economical an environmental benefit to laboratories dealing with COD determination of high organic strength waste.

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Supporting information accompanying the Annex 1

Table of content of SI

Supporting Material and Methods

Materials

Digestion of samples were carried out in 40 mm × 300 mm glass digestion vessels and 635 mm jacket condensers with ground-glass joint, or in 13 mm x 100 mm borosilicate culture tubs, with TFE-lined screw caps, for open or closed reflux method, respectively. Erlenmeyer flasks of 100 ml nominal capacity and burettes of 10 ml nominal capacity were used for titrimetric method.

Apparatus

Digestion of samples were carried out at 150±2 ℃ in a Bloc Digest-20 from J.P. Selecta (Spain) or LT 200 thermostat from Hach Lange (Germany), for open or closed reflux method, respectively.

COD determinations using colorimetric method were carried out using a DR 2800 Spectrophotometer from Hach Lange.

For theoretical COD determination, elemental analysis was carried out using a Truespec CHNS from Leco (USA). Lipids content was determined by an automatic soxhlet extraction system using a Soxtec 2050 from Foss (Denmark). Nitrogen NH_4-N determination was carried out by automatic distillation Büchi B-324 unit (Switzerland), and automatic titration using a 702 SM Titrino from Metrohm (Switzerland).

Reagents

Potassium hydrogen phthalate (KHP): primary standard grade Panreac brand, standard for COD determination internationally accepted. Digestion reagent containing potassium dichromate ($K_2Cr_2O_7$) 0.24 or 0.5 N primary standard grade, mercuric sulfate ($HgSO_4$), and sulfuric acid 95-97%; and also sulfuric acid reagent containing 1% of silver sulfate (Ag_2SO_4) in sulfuric acid 95-97% reagent grade Scharlau brand, were prepared following indications of Standard method 5220 B-open reflux (AOAC Official Method 991.20, 2006). Ferroin solution 0.025 M redox indicator and ferrous ammonium sulfate (FAS) solution 0.25 N, used for titrimetric method were prepared following the same indications.

Sodium hydroxide solution 40% W/V extra pure, boric acid solution 4% W/V and hydrochloric acid solution 0.1 Mol/l (0.1 N) Scharlau brand were used for NH_4-N determination. EDTA calibration sample LECO corporation brand was used for calibration of Truespec CHNS from LECO used for elemental analysis. n-Hexane 96% reagent grade, ACS, Reag. Ph Eur Scharlau brand was used for lipids determination. All these reagents were used for theoretical COD determination.

Samples

Pig slaughterhouse waste (PSW) is a heterogeneous substrate characterized by high COD due to its high concentration of lipids and proteins. With the aim of increasing homogeneity, the samples were previously lyophilized, removing water content by sublimation. Then, samples were grinded by a small mincer in order to obtain a more homogeneous consistency.

Other different solid or semi-liquid wastes (SSW) were also studied, such as solid fraction of sewage sludge from a municipal wastewater treatment plant, effluent from an anaerobic digestion reactor treating different organic waste, waste from an enzymatic hydrolysis process of animal by-product and two different mixtures of animal by-products, meat and bone meal, pig manure and sewage sludge. In all these cases the substrates were not lyophilizated.

Certified reference materials (CRM) used for final assessment of precision and accuracy were:

- Sample CRM-1 was sewage sludge provided by LGC Promochem as certified reference material (CRM 055-050), produced and certified by Resource Technology Corporation (USA and UK).

- Sample CRM-2 was also a sewage sludge produced by Resource Technology Corporation (USA and UK) and provided for characterization as a new certified reference material (CRM SeWS).

Procedure

APHA−AWWA−WPCF standard method 5220 B-open reflux titrimetric (SM-ORT)

After joining the condenser in the digestion vessels and placing the vessels in the block heater fixed at 150 ºC for 2 h, the tubes were removed and cooled at room temperature. Finally, 25 ml of the digestion vessel were transferred to 100 ml Erlenmeyer flask, 25 ml of distilled water and one or two drops of ferroin indicator solution were added. Then, the excess of potassium dichromate was titrated with ferrous ammonium sulfate (FAS) 0.25 N until the color changes from blue-green to reddish-brown. A blank containing 50 ml of distilled water was run simultaneously for each group of samples.

Calculation: the chemical oxygen demand of a dry solid sample was calculated using the following equation:

$$COD \ (g \ O_2 \ Kg^{-1}) = \frac{(FAS_{BL} - FAS_{SAMPLE}) \ x \ N_{FAS} \ x \ 8}{W_{SAMPLE}}$$

where FAS_{BL} is the volume of FAS used in the titration of blank sample (ml); FAS_{SAMPLE} is the volume of FAS used in the titration of the solid sample (ml); N_{FAS} is the concentration of reducing reagent (N); and W_{SAMPLE} is the weight of solid sample (g). Further raw sample was used to determine the substrate moisture and the dry sample weight.

Kit LCK914 COD Method (LCK)

The tubes were heated at 150 ºC for 2 h in a thermostat. After allowing the tubes to cool at room temperature, COD were determined by measuring the absorbance of the digested assay solution at 605 nm on a spectrophotometer, and interpolating in a standard curve (5–65 $g_{O2} \cdot kg^{-1}$), with KHP as the chemical standard reference and correlated to the COD level taking into account that the theoretical oxygen demand of substrate is 1.176 $mg_{O2} \cdot mg^{-1}$. If samples, standards and blanks were run under same conditions, COD level in sample was calculated as follows:

$$COD\ (g\ O_2\ Kg^{-1}) = \frac{mg\ O_2\ in\ final\ volume\ x\ 200}{W_{SAMPLE}}$$

where W_{SAMPLE}: weight of solid sample (g).

Calculations

Interlaboratory study (PT) of COD

The performance of each laboratory in an interlaboratory study (PT) of COD measurements was assessed by the internationally accepted z-score calculated by the following equation:

$$Z = \left| \frac{X_i - A}{SD} \right|$$

where Xi is the experimental value, A is the assigned or "true" value and SD is the standard deviation.

Theoretical oxygen demand (COD$_{theo}$)

The calculation of the COD-1$_{theo}$ was determined, defining it as the stoichiometric amount of oxygen required to oxidize a compound to end products, mainly CO_2 and H_2O, although other additional products should be produced when the organic compound contains heteroatom in the molecule. The chemical composition of KHP and two different lyophilizated pig substrates were determined by means of elemental analysis using a Leco Truespec CHNS. The data of chemical compositions was determined by three replicates samples of each substrate. Then, COD-1$_{theo}$ was calculated using the empirical equation detailed in the ISO 10707 (1994).

The calculation of the COD-2$_{theo}$ was also determined, where COD-2$_{theo}$ was estimated by the sum of the COD values coming from the protein and fat fractions, main constituents of the pig slaughterhouse waste used for this paper. Fat determination was carried out by an automatic soxhlet extraction system using a Soxtec 2050, by EPA method 9071b n-Hexane extractable material (HEM) for sludge, sediment, and solid samples (1998).

Protein concentration was estimated from the organic nitrogen content using a factor of 6.38 $g_{protein} \cdot g_{Norg}^{-1}$, as suggested by AOAC (Official Method 991.20, 2006). Organic nitrogen was estimated from elementary nitrogen analysis result determined using a Leco Truespec CHNS (AOAC Official Method 992.15, 2006) and subtracting NH_4-N. NH_4-N was analyzed using a B-324 distillation unit from Büchi, by automatic titration using a 702 SM Titrino from Metrohm, by Standard Methods 4500-NH_3B Preliminary Distillation Step and 4500-NH_3 C Titrimetric method (APHA–AWWA–WPCF, 2005).

The COD values were obtained using different indexes obtained from the oxidations of a standard protein ($C_5H_7NO_2$) and glycerol trioleate as standard fat ($C_{57}H_{104}O_6$) (Angelidaki *et al*., 2002 and 2004), that is, 1 g of protein is considered equivalent to 1.42 g COD and 1 g of fat equivalent to 2.90 g COD.

SUPPORTING REFERENCES

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4500-NH3 B Preliminary Distillation Step and 4500-NH3 C Titrimetric method. APHA–AWWA–WPCF, Standard Methods for the Examination of Water and Wastewater, 21st edition, Washington, DC, 2005.

Table S-1. *Effect of $Cr_2O_7K_2$ concentration in the digestion reagent and of the digestion reagent / sulfuric acid reagent ratio on the slope and linear range of calibration curve for the SM-CRC method.*

$Cr_2O_7K_2$ (N)	$V_{digestion}/V_{sulfuric}$ (ml)	Slope	Upper Linear Range ($g_{O2}\cdot kg^{-1}$)	Observations
1.0		$1.5 \ 10^{-5}$	< 85	
0.75	2.2 / 5	$1.6 \ 10^{-5}$	< 65	
0.5		$1.7 \ 10^{-5}$	< 45	
0.25*		$1.8 \ 10{-5}$	< 15	
1.0		$2.0 \ 10^{-5}$	< 85	$Cr_2O_7K_2$Precipitation
0.75	3.6 / 3.6	$2.6 \ 10^{-5}$	< 75	$Cr_2O_7K_2$Precipitation
0.5		$2.7 \ 10^{-5}$	< 65	
Kit LCK 914	(**)	$3.6 \ 10^{-5}$	< 60	

(*) Concentration of reagents recommended in SM-CRC standard method (**) Unknown

Table S-2. *Lower and upper linear range (LR) of the optimized SM-CRC method, depending on the amount of weighted sample used.*

Sample Weight (mg)	Lower LR ($g_{O2}\cdot kg^{-1}$)	Upper LR ($g_{O2}\cdot kg^{-1}$)
200	4.5	65
100	9.0	130
50	18	260
20	45	650
10	90	1,300

Table S-3. *TS, VS and elemental composition of two different substrates used for COD-1_{theo} assessment.*

Sample	TS (%)	VS (%)	Elemental composition (%)					Estimated formula
			C	H	N	O	S	
PSW-4	98.36	97.33	64.36	11.47	2.67	18.54	0.29	$C_{536}H_{1138}N_{19}O_{116}S_1$
PSW-5	98.08	96.46	65.74	10.55	4.68	15.15	0.34	$C_{547}H_{1047}N_{33}O_{95}S_1$

Table S-4. *Lipid and protein composition of two different substrates used for COD-2$_{theo}$ assessment*

Sample	Lipids (g·kg$_{TS}^{-1}$)	CODlipids (g$_{O2}$·kg$_{TS}^{-1}$)	Ntotal (g·kg$_{TS}^{-1}$)	NH4-N (g·kg$_{TS}^{-1}$)	Norg. (g·kg$_{TS}^{-1}$)	Protein (g·kg$_{TS}^{-1}$)	CODprot. (g$_{O2}$·kg$_{TS}^{-1}$)
PSW-4	786	2,278	26.7	2.3	24.4	156	221
PSW-5	750	2,176	43.1	2.8	40.3	257	365

Table S-5. *Experimental COD values obtained from different substrates using SD/SM-CRC optimized method.*

Sample	Sample Type	Dilution	COD (g$_{O2}$·kg$_{TS}^{-1}$)	Mean	SD	CV (%)
SSW-2	Solid fraction from a municipal sewage treatment plant	6.1	2,356 2,423 2,316	2,365	54	2.3
SSW-3	Wasted enzymatically hydrolyzed animal by-products	10.7	2,142 2,248 2,126	2,172	66	3.0
SSW-4	Effluent of anaerobic reactor	2.6	1,417 1,281 1,324	1,341	70	5.2
SSW-5	Mixture of animal by-products and meat and bone meal	2.9	1,606 1,568 1,638	1,604	35	2.2
SSW-6	Mixture of pig manure, sewage sludge and meat and bone meal	3.1	1,927 1,877 1,967	1,923	45	2.3