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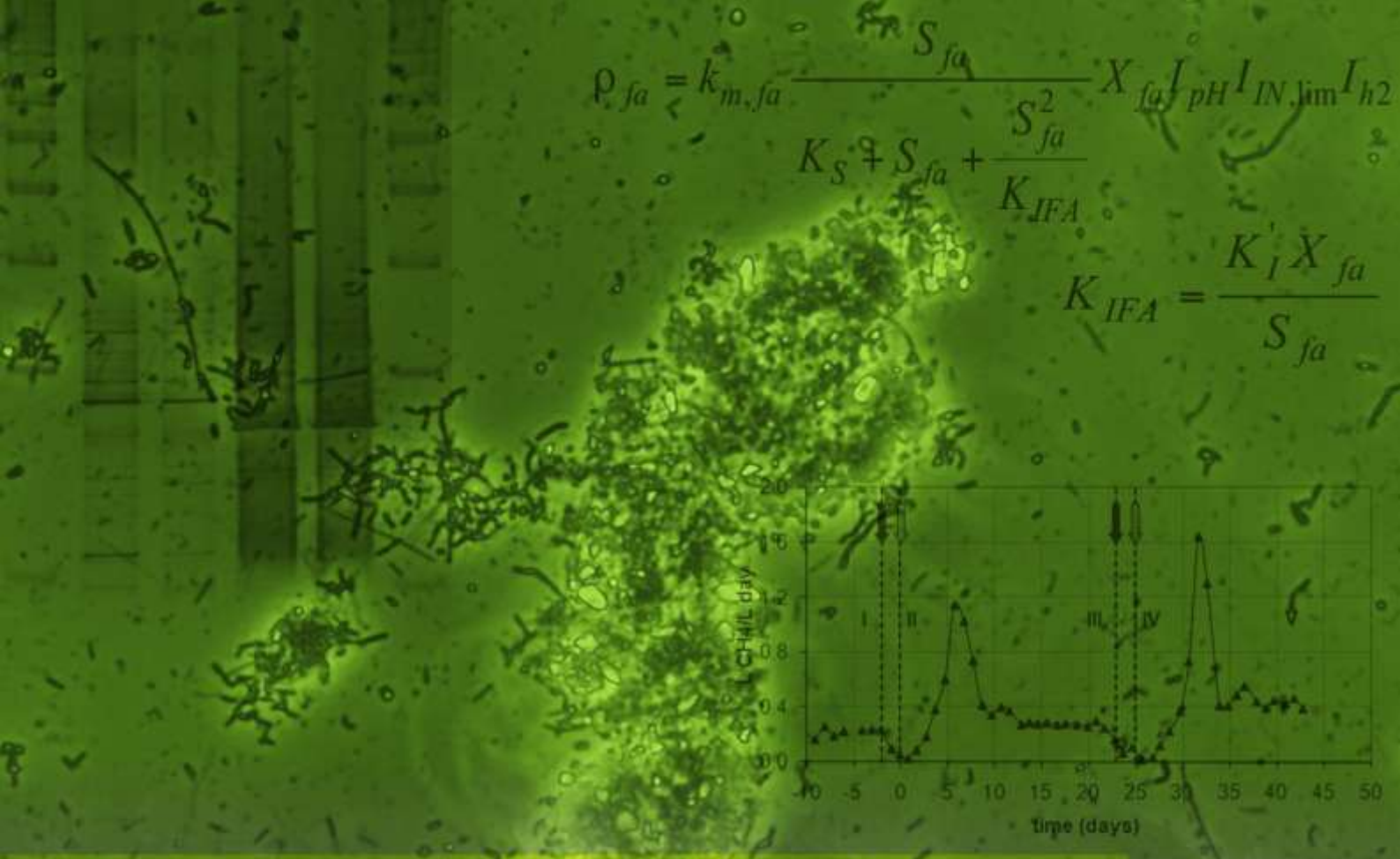
Anaerobic digestion of slaughterhouse waste. Impact of the LCFA inhibition

Jordi Palatsi Civit



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ANAEROBIC DIGESTION OF SLAUGHTERHOUSE WASTE: IMPACT OF LCFA INHIBITION

Doctoral Dissertation for PhD degree in Engineering

Supervisors of the thesis:

Dr. Xavier Flotats i Ripoll

Dra. Belén Fernández García

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Universitat de Lleida
Escola Tècnica Superior d'Enginyeria Agrària
Departament d'Enginyeria Agroforestal

ANAEROBIC DIGESTION OF SLAUGHTERHOUSE WASTE: IMPACT OF THE LCFA INHIBITION

***PhD* Thesis**

Supervised by Xavier Flotats (GIRO/UPC) and Belén Fernández (GIRO)

Jordi Palatsi Civit
Lleida – Novembre 2009

als meus pares,
Carmen i Manolo

a Sònia:

“....I don't care if Monday's black,
Tuesday, Wednesday, heart attack.
Thursday, never looking back.
It's Friday, I'm in love...:”
(Robert Smith)

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ABSTRACT Slaughterhouse wastes are interesting for the anaerobic digestion process regarding its high biogas production potential and because the current legal scenario promotes renewable energy production. The high lipid and protein content of those residues limit its treatment due to inhibitory processes, in particular the inhibition caused by long chain fatty acids (LCFA). The objective of the present disertation is to obtain a deeper insight on the LCFA inhibition process, the microorganism adaptation ability and the prevention/recovery of inhibitory phenomena.

In a preliminary approach, organic wastes generated in slaughterhouses are characterized, by studying the anaerobic biodegradability of waste mixtures containing diferents lipid/proteins concentrations. Anaerobic batch tests are performed at increasing substrate concentrations by sequential pulse feeding. From those experiments, the fast hydrolysis-acidogenesis of proteins is verified, being the lipids and LCFA degradation the main limiting step of the overall anaerobic process. Despite this limitation, the system is able to recover up to a higher methane production rate after each applied pulse.

In order to elucidate on the mechanisms of the recovery process, several strategies to recover LCFA inhibited reactors are tested. The increase of the biomass/LCFA ratio and the adition of bentonite to reduce the biodisponibility or the adsorption of LCFA over microbial cell walls, are found to be effective approaches in the operation of fullscale biogas plants. The obtained results reinforce the hypothesis of the adsorptive nature of the LCFA inhibition, and that the recovery process can be followed as an increase in the microbial activity.

The nature of the reported microbial activity improvement after subsequent sytem inhibition is characterized by three different techniques: 1) the study of specific microbial activities on different model substrates, 2) the application of molecular biology tools to monitor the microbial population structure and, 3) the development of kinetic expressions of the LCFA inhibition phenomena, based on the adsorption process, within the framework of ADM1 model of the International Water Association. The combined analysis of those confirmed that inhibition and adaptation phenomena are explained by a specific microbial growth, including adsorption in the metabolic LCFA inhibition process.

The adsorption-inhibition process is evaluated in detail by determining LCFA adsorption isotherms on granular sludge, LCFA toxicity test, and fluorescence microscopy techniques. This multidisciplinary approach results in the definition of an inhibition preventing strategy based on the introduction of competitive adsorbents, and on stating the importance of palmitate during ß-oxidation of LCFA.

This study contributes to the understanding of slaughterhouse wastes anaerobic treatment, the LCFA inhibition process, and the biomass adaptation phenomena. The physical adsorption process has been directly related with the LCFA metabolic inhibition, and a new mathematical kinetic expression is proposed. New strategies guiding the operation of anaerobic reactors are suggested in order to obtain high renewable energy yields from slaughterhouse wastes digestion.

RESUM. Els residus carnis, o subproductes animals, són interessants per al procés de digestió anaeròbia i producció de biogàs, donat el seu elevat potencial energètic i l'actual marc legislatiu que prima la producció d'energia renovable. Tot i així, l'elevat contingut en lípids i proteïnes d'aquests residus pot limitar el seu tractament en introduir fenòmens d'inhibició, dels quals el més important és el produït pels àcids grassos de cadena llarga (AGCL), resultants de la hidròlisi dels lípids. L'objectiu de la present tesis és aprofundir en el coneixement d'aquest procés d'inhibició, en la capacitat d'adaptació dels microorganismes i en la recuperació o prevenció dels fenòmens d'inhibició.

En una primera aproximació a la problemàtica, es caracteritzen residus orgànics d'escorxador, s'estudia la seva biodegradabilitat anaeròbia amb diferents relacions lípids/proteïnes i es realitzen assaigs discontinus seqüencials incrementant la concentració de substrat mitjançant pulsos consecutius. Es comprova que la hidròlisi i acidogènesi de proteïnes és molt ràpida i que la degradació dels lípids i AGCL limita la velocitat global del procés. Malgrat aquesta limitació, el sistema es recupera després dels pulsos aplicats, tot augmentant la taxa màxima de producció de metà.

Per tal d'estudiar el fenòmen de recuperació, s'estudien i desenvolupen diferents estratègies en reactors sotmesos a processos d'inhibició per AGCL. L'increment dels ratis biomassa/AGCL o l'adició d'additius com la bentonita, per tal de reduir la biodisponibilitat o l'adsorció dels AGCL sobre la biomassa activa, es mostren com estratègies funcionals d'utilitat en l'operació de plantes industrials. Els resultats obtinguts reforcen la hipòtesi de que la inhibició és deguda a adsorció d'AGCL sobre la membrana cel·lular i que la recuperació es pot mesurar mitjançant un augment de l'activitat dels microorganismes.

Per tal de dilucidar sobre la natura del augment de l'activitat en els processos de recuperació es caracteritza la inhibició-recuperació mitjançant tres tècniques: 1) estudi de les activitats dels microorganismes a diferents substrats 2) utilització de tècniques de biologia molecular per caracteritzar les poblacions, i 3) desenvolupant expressions cinètiques del procés d'inhibició, basades en l'adsorció, en el marc del model matemàtic ADM1 de la International Water Association. Mitjançant aquestes metodologies es comprova que els fenòmens d'inhibició i adaptació es poden explicar mitjançant un creixement poblacional específic i la inclusió dels fenòmens físic d'adsorció en el procés d'inhibició metabòlica.

Finalment, s'avalua de forma més detallada el procés d'adsorció-inhibició mitjançant la determinació de les isotermes d'adsorció i monitoritzant mitjançant assaigs amb biomassa granular i tècniques de microscòpia de fluorescència. Aquesta caracterització ha permès obtenir estratègies de prevenció de la inhibició per AGCL, mitjançant competència amb adsorbents sintètics, i concloure que l'àcid palmític és el limitant en el procés de β-oxidació dels AGCL.

Els resultats obtinguts constitueixen una base per al millor coneixement de les possibilitats de tractament anaerobi del residus carnis i dels processos d'inhibició per AGCL i adaptació de la biomassa. El procés físic d'adsorció ha estat directament relacionat amb el fenòmen d'inhibició metabòlica, obtenint-se una descripció matemàtica del mateix. Els resultats han permès plantejar estratègies operacionals, sent una eina a disposició d'operadors de plantes de biogàs per optimitzar la producció d'energia d'aquests residus mitjançant la seva digestió anaeròbia.

RESUMEN. Los residuos cárnicos, o subproductos animales, son interesantes para el proceso de digestión anaerobia y producción de biogás, dado su elevado potencial energético y el actual marco legal que prima la producción de energía renovable. A pesar de esto, el elevado contenido en lípidos y proteínas puede limitar su tratamiento, al introducir fenómenos de inhibición, de los cuales el más importante es el producido por ácidos grasos de cadena larga (AGCL), resultado de la hidrólisis de los lípidos. El objetivo de la presente tesis es profundizar en el conocimiento de este proceso de inhibición, en la capacidad de adaptación de los microorganismos t en la recuperación de sistemas inhibidos.

En una primera aproximación a la problemática, se caracterizan los residuos orgánicos de matadero, se estudia su biodegradabilidad anaerobia con diferentes relaciones lípido/proteína y se realizan ensayos discontinuos secuenciales incrementando la concentración de substrato mediante pulsos consecutivos. Se comprueba que la hidrólisis y acidogénesis de las proteínas es muy rápido y que la degradación de lípidos y AGCL limita la velocidad global del proceso. A pesar de esta limitación, el sistema se recupera después de los pulsos aplicados aumentando la tasa máxima de producción de metano.

A fin de estudiar el fenómeno de recuperación, se estudian y desarrollan diferentes estrategias en reactores inhibidos por AGCL. El incremento de los ratios biomasa/AGCL o la adición de aditivos como la bentonita, a fin de reducir la biodisponibilidad o la adsorción de los AGCL sobre la biomasa activa, se muestran estrategias funcionales de utilidad en la operación de plantas industriales. Los resultados obtenidos refuerzan la hipótesis de que la inhibición es debida a adsorción de AGCL sobre la membrana celular y que la recuperación se puede medir mediante un aumento de la actividad de los microorganismos.

A fin de dilucidar sobre la naturaleza del aumento de la actividad en los procesos de recuperación se caracteriza la inhibición mediante tres técnicas: 1) estudio de las actividades de los microorganismos a diferentes substratos, 2) utilización de técnicas de biología molecular para caracterizar las poblaciones, y 3) desarrollando expresiones cinéticas del proceso de inhibición, basado en la adsorbió, en el marco del modelo ADM1 de la International Water Association. Mediante estas metodologías se comprueba que los fenómenos de inhibición y adaptación se pueden explicar mediante un crecimiento poblacional específico y la inclusión de la adsorción en el proceso de inhibición metabólica.

Finalmente, se evalúa de forma detallada el proceso de adsorción-inhibición mediante la determinación de las isotermas de adsorción y monitorizando estos procesos mediante ensayos discontinuos con biomasa granular y técnicas de microscopia de fluorescencia. Esta caracterización ha permitido obtener estrategias de prevención de la inhibición por AGCL, mediante competencia con adsorbentes sintéticos, y concluir que el ácido palmítico es el limitante en el proceso de mutante β-oxidación de los AGCL.

Los resultados obtenidos constituyen una base para el mejor conocimiento de las posibilidades de tratamiento anaerobio de residuos cárnicos y de los procesos de inhibición por AGCL y adaptación de la biomasa. El proceso físico de adsorción se ha relacionado directamente con el fenómeno de inhibición metabólica, obteniéndose una descripción matemática del mismo. Los resultados han permitido plantear estrategias operacionales, siendo una herramienta a disposición de operadores de plantas de biogás para optimizar la producción de energía de estos residuos mediante su digestión anaerobia.

Background, introduction and objectives of this thesis

A brief introduction of the anaerobic digestion process and the treatment of slaughterhouse waste are presented in relation to the context and research objectives of this disertation.
A special focus was given to the implications of long chain fatty acids (LCFA) in relation to its inhibition potential towards the anaerobic biomass activity.
The results of this thesis were obtained from different bioreactor experiments (batch and continuous reactors, mesophilic and thermophilic operational regimes, suspended and granular sludge, adapted and un-adapted biomass). Consequently potential effects of those aspects are also introduced.
The adopted multidisciplinary methodological approach for the analysis of the experimental data is also described. This was based on the characterization of the microbial community dynamics by means of molecular biology techniques, and on the application of mathematical modelling tools to test hypothesis.
Finally, research objectives of this disertation are listed.

1.1. RESEARCH CHRONOLOGY AND SCOPE

The interest in biomass, as renewable energy source, has grown significantly due to the increasing concerns about the global warming issue and to the more stringent environmental legislation. In this scenario, anaerobic biogas production from organic waste plays an important role in contributing to the control of anthropogenic impacts by reducing the emissions of carbon dioxide (CO_2), *via* the substitution of fossil fuels, and by reducing methane (CH_4) emissions from organic waste storage and land application. Therefore, in the last decade, the research in this field has seen a renewed interest and the use of biogas has rapidly developed in many sectors.

The antecedents of the present dissertation coincided with my studies in Agricultural Engineering (1999-2002), time in which I started a collaboration with the **LEA-UdL-IRTA** (Laboratory of Environmental Engineering), funded by the University of Lleida and the Institute of Agrofood Research and Technology (IRTA, Catalonia). That research group was coordinated by Prof. **Xavier Flotats,** and with the partnership of **Elena Campos,** which has a significant experience on piggery and cattle manure treatment. My first tasks aimed with the monitoring of pig slurry anaerobic digesters. The economic viability of those anaerobic digestion plants depends, among other factors, on the specific production of methane per unit of treated residue. The high water content in pig slurry and the high ammonia concentration are the main causes of low methane yields. On the other hand, wastes from food industry, in particular lipid containing waste like effluents from slaughterhouses, are attractive for anaerobic digestion due to their high energetic potential, in terms of specific biogas production potential.

Solid slaughterhouse wastes, or animal by-products, were usually treated by rendering process (EC-IPPC2005). In previous decades, these by-products were commercialized as row materials for animal feedstuff, providing a valuable source of slaughterhouse income. In recent years, because of BSE (Bovine Spongiform Encephalopathy), the value of these materials has been reduced substantially, and in many cases, they have to be disposed as a waste (EC no 1771/2002). Further regulation by the European Parliament (EC no 92/2005) on the disposal and uses of animal by-products, allows biogas transformation if certain approved pre-treatments are applied, depending on its biohazard category.

Due to this new scenario and to the possibility of introducing high organic content waste into the anaerobic digestion process, the LEA-UdL-IRTA was involved in a project funded by the Spanish Ministry of Science and Education, which dealt with the mathematical modelling of the anaerobic digestion of complex waste (CAD/CRAI Ref. ENE2004-00724/ALT 2004-2007). In 2005, the research group was integrated into a new institution, the **GIRO** Technological Centre (Barcelona, Spain), and was involved into a specific project on the anaerobic digestion of animal by

products (OPA-LAP Ref. ENE2007-65850 2007-2010). Those two projects had a wide scope on process control, mathematical modelling, waste pre-treatment, reactor configuration, and complementary technologies for ammonia removal. In the framework of the cited projects, the present dissertation is focussed in one of the main difficulties in treating organic wastes with high lipid content, the inhibition caused by the accumulation of long chain fatty acids (LCFA).

During the development of the present PhD thesis (2004-2009), I had' the privilege to collaborate with some scientists and institutions of praised reputation within this field of knowledge. Prof. **Vasily Vavilin***,* from the *Water Problems Institute of the Russian Academy of Sciences* (Moscow, Russian Federation) introduced me to the mathematical modelling of biological processes. Within the framework of the CAD/CRAI project, I had the possibility to be a guest researcher (09/07-12/07) in the *Department of Environmental Engineering* of the *Technical University of Denmark* (Lyngby, Denmark), one of the reference research groups on anaerobic digestion, with the supervision of Prof. **Irini Angelidaki**. Also, within the context of the OPA-LAP project and thanks to funding provided by the Department of Universities, Research and Media Society of Catalonia Government (Grand BE-DGR 2008 BE1 00261) I had the possibility to collaborate (09/08-12/08) with Prof. **Madalena Alves** and the *Laboratory of Environmental Biotechnology* of *University of Minho* (Braga, Portugal), group that for the past 10 years has made important advances in the anaerobic digestion of lipid rich effluents.

1.2. INTRODUCTION TO THE ANAEROBIC DIGESTION OF SLAUGHTERHOUSE WASTE

1.2.1 The anaerobic digestion process

The anaerobic digestion process can be defined as a biological treatment in which the organic matter is decomposed by the action of microorganisms, in the absence of oxygen, producing a gas (biogas) composed mainly by methane and carbon dioxide, with a high energetic value. The process is performed trough a series of sequential biological reactions, involving different groups of microorganisms. In Figure 1.1, it is presented a schematic representation of the anaerobic digestion process and its main degradation steps. First, particulate organic matter is disintegrated into macro molecules. Proteins, carbohydrates and lipids are then degraded (hydrolysis) into sugars, amino acids and long chain fatty acids (LCFA). Sugars, amino acids and LCFA are further fermented (acidogenesis) into volatile fatty acids (VFA) and alcohols. Fermentation products are further oxidized (acetogenesis), with the production of acetate (Ac) and hydrogen (H_2). Finally, Ac and H_2 are converted into methane (CH_4) and carbon dioxide (CO_2) via acetoclastic and

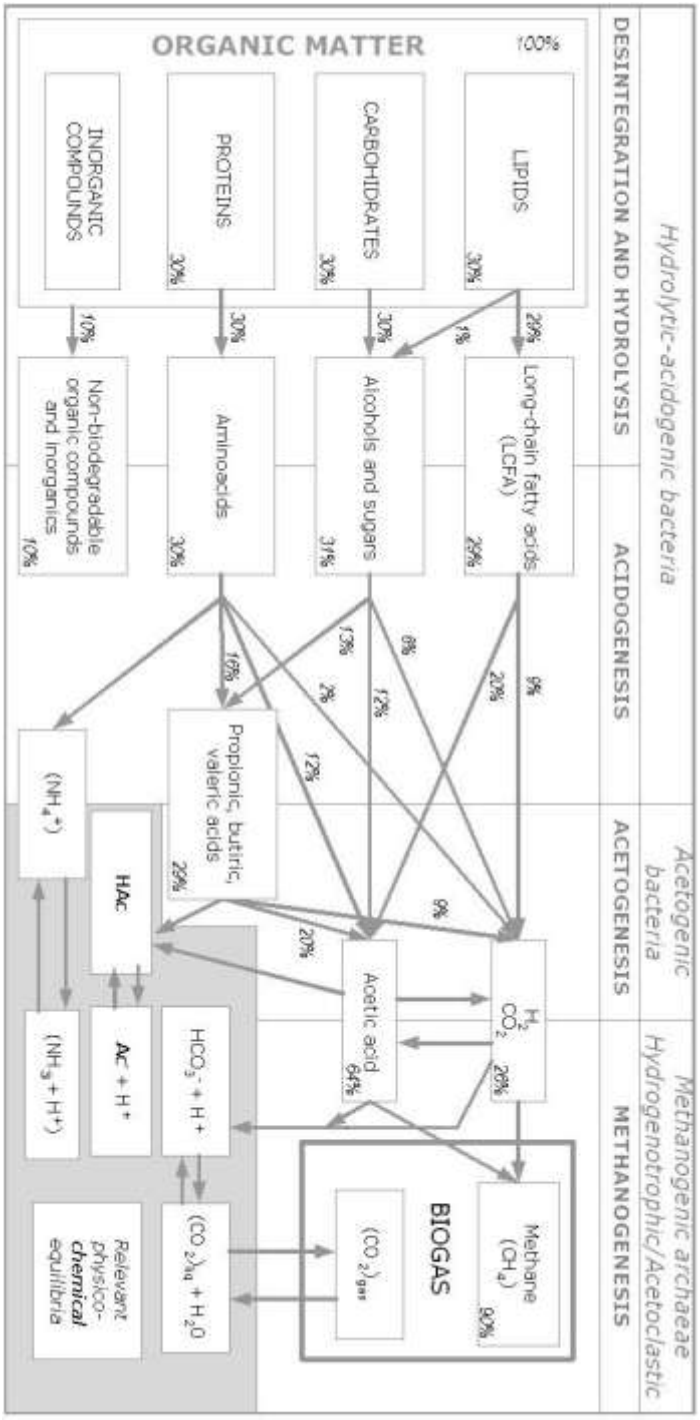

Figure 1.1*. Schematic representation of anaerobic digestion process. Numbers indicate COD flux of materials, adapted from Batstone et al. (2002).*

hydrogenotrophic archaeae (methanogenesis), respectively (Jeyaseelan, 1997; Angelidaki *et al.*, 1999; Batstone *et al.*, 2002). The main four process steps, disintegration/hydrolysis, acidogenesis, acetogenesis and methanogenesis are briefly described as follows.

Desintegration and Hydrolysis. Complex organic matter compounds are disintegrated into carbohydrates, proteins and lipids. Those macromolecules need to be hydrolyzed by extracellular enzymes (cellulases, proteases and lipases, respectively), excreted by fermentative bacteria, before being transported through the cell membrane.

This process is performed by facultative bacteria and, in the case of complex substrates, disintegration and hydrolysis can be the rate-limiting step in the whole anaerobic digestion process (Christ *et al.*, 2000; Vavilin *et al.*, 2008).

Acidogenesis. Sugars, amino acids, LCFA and glycerol formed during hydrolysis are converted into VFA and alcohols in the acidogenic step, without an external electron acceptor. Acetic acid is the main by-product formed during acidogenesis, but other intermediates like propionic, butyric and valeric acid are also reported to accumulated (Yu and Fang, 2002) and cause inhibition if the acetogenic and methanogenic populations do not efficiently degrade those intermediates (Batstone *et al.*, 2003; Pind *et al.*, 2003; Nielsen *et al*., 2007). The levels of accumulated VFA are reported to be indicators of a possible process unbalance.

Acidogenic population represents about *90%* of the total microbial population present in anaerobic digesters. They have a short doubling time and therefore acidogenesis is not normally considered a limiting step in the global anaerobic digestion process (Zeikus, 1980; Mosey, 1983)

Acetogenesis. The previously described reduced intermediates (VFA) are converted to Ac and H_2 in this step. Under standard conditions, these oxidative reactions are not energetically feasible, and they proceed only when the reaction products are removed from the system (by methanogens in syntrophic association). At low H_2 partial pressure, the reactions are thermodynamically favourable, and the energy variation is enough for the ATP synthesis and bacteria growth (Schink, 1997; Schink and Stams, 2002).

Acetate can also be synthesized by carbon dioxide reducing bacteria, usually referred as homoacetogenic bacteria but, thermodynamically, methane production from H_2/CO_2 is a more favourable pathway. Therefore, homoacetogenesis usually represents only a small percentage of the total Ac production during the anaerobic digestion (Batstone *et al.*, 2002).

Methanogenesis. Methanogenesis is the last step of the complete mineralization of organic matter and represents, in many cases, the rate-limiting conversion. The end products of the previous reactions, mainly H_2, CO_2 and Ac, are further converted into CH_4/CO_2 by methanogenic archaea. This process mainly occurs through two different metabolic pathways: hydrogenotrophic methanogenesis with the reduction of CO_2 and H_2, and acetoclastic methanogenesis with the degradation of Ac. Acetoclastic methanogens are responsible of about *70%* of the total CH_4 production in anaerobic bioreactors (Batstone *et al.*, 2002).

Methanogens occupy a crucial position in the whole degradation process, since the conversion of Ac and H_2/CO_2 to CH_4 affects the overall anaerobic degradation. Doubling time of these microorganisms is comparatively long, and it is reported to be the rate-limiting process in the whole anaerobic digestion of not complex wastes (Fang *et al.*, 1995; Huang *et al.*, 2003).

1.2.2 Slaughterhouse waste

The importance of the slaughterhouse waste, and animal by-products into our territorial context, is the consequence of the intensive livestock production and of the importance of the agro-food sector in Catalonia, in parallel with the development of a more stringent environmental legislation. The meat industry in Catalonia represents the main sector in the Spanish food industry (*20.3%*), and it is one of the most important in the European Union (EU). Every year, more than *10 million tons* of meat derived from healthy animals and not destined to direct human consumption, are produced in the EU. It is estimated that the total produced amount in Spain is over *2 Mtones/year* (EC 2005). Some of those materials are then transformed into a variety of products used in animal feed, cosmetic, pharmaceutical and in other industrial processes, but in some cases the unique alternative is their destruction, often by incineration.

Animal by-products are characterized by a high organic content, mainly composed by proteins and fats, but few references are available on the quantitative characterization and anaerobic potential of these products (Tritt and Schuchardt, 1992; Edström *et al*., 2003; Hejnfelt and Angelidaki, 2009). More experiences have been reported in the literature about the anaerobic treatment of slaughterhouse wastewaters, normally subjected to a primary treatment and, in some cases, to a secondary anaerobic digestion, usually based on upflow anaerobic sludge blanket (UASB) or expanded granular sludge bed (EGSB) reactors, due to the high organic loading rates (Torkian *et al.*, 2003; Mittal, 2006; Del Neri *et al.*, 2007).

The regulations adopted by the European Parliament and the Council introduces stringent conditions throughout the food and feed chains, requiring safe collection, transport, storage, handling, processing, uses and disposal of animal by-products (EC

no 1771/2002). Animal by-products are classified into three categories based on their potential BSE risk to animals, the public or to the environment, and sets out how each category must be disposed:

- *Category 1* materials (i.e. animal by-products presenting the highest biohazard such as BSE or scrapie, and residues with prohibited substances or environmental contaminants, e.g. hormones used for growth, dioxins, PCB) which must be completely disposed of as waste by incineration or landfilling after appropriate heat treatment.
- *Category 2* materials include animal by-products presenting a risk of contamination with other animal diseases (e.g. corpses of sick animals which died in the farm, or that are sacrificed in the context of farm disease control measures, or that present or at risk of containing residues of veterinary drugs). These residues may be recycled for uses other than feeding purposes after an appropriate treatment (e.g. biogas, composting, oleo-chemical products, etc).
- *Category 3* materials are by-products derived from healthy animals slaughtered for human consumption. These are the only materials that can be used in the production of feedstuff following an appropriate treatment in approved processing plants, and also for the anaerobic digestion and composting processes.

According to the risk category of the material, the new regulation (EC no 92/2005) has approved several pre-treatments, prior to the anaerobic digestion process, witch consist on: alkaline hydrolysis (*KOH, 150ºC, 4 bars, 3h*), high temperature and pressure treatment (*180ºC, 12 bars, 40min*), high pressure treatment (*220ºC and 25 bars* or rendering process) or a simple pasteurization process.

The effect of authorised pre-treatments on slaughterhouse waste characteristics remains controversial. Some positive effects, like reducing the particulate material size, and increasing the rates of solubilisation, hydrolysis and biodisponibility have been reported (Cammarota *et al.*, 2001; Cassini *et al.*, 2006). On the other side, some authors also reported the formation of recalcitrant toxic compounds which might hinder the subsequent biological treatment (Ajandouz *et al.*, 2008; Dwyer *et al.*, 2008). Masse *et al.* (2003) did not find a significant effect of thermal and enzymatic pre-treatment on the hydrolysis rate of slaughterhouse particulate fats. Henfelt and Angelidaki (2009) tested pasteurization (70ºC), sterilization (133ºC) and alkali hydrolysis (NaOH) on several animal by-products, but no effect was observed on methane yields compared to the fresh substrates. Assessing the benefits and limitations of all approved pre-treatments is out of the scope of the present

dissertation. Consequently, experimentation with slaughterhouse waste in the present dissertation has been restricted to *Category 3* material.

Because of the high fat and protein content of slaughterhouse waste and animal by-products, these substrates can be considered as adequate for anaerobic digestion plants, regarding the high potential methane yield. However, slow **hydrolysis** rate and **inhibitory** processes have been described as the limiting steps. In particulate and poorly hardly degradable materials like animal by-products, hydrolysis must be coupled to the growth of acidogenic bacteria, factor that can limit the overall process rate in case of unbalances (Vavilin *et al*., 2008). Furthermore, lipids can cause biomass flotation and wash-out. Also, during the hydrolysis by extracellular lipases, long chain fatty acids (LCFA) are produced. Those intermediates are well known as inhibitory species of the anaerobic digestion process (Angelidaki *et al*., 1990; Hwu *et al*., 1997). Ammonia is released during the degradation of protein under anaerobic conditions and its inhibitory effect on the anaerobic digestion process has also been reported (Hansen *et al*., 1996; Flotats *et al*., 2006). Consequently, most of the industrial experiences related to the anaerobic digestion of slaughterhouse waste are dealing with its co-digestion with other industrial, agricultural or domestic wastes as suitable substrates, particularly, in centralised biogas plants (Angelidaki and Ellegard, 2003; Resh *et al*., 2006).

1.2.3 Environmental and operational parameters

As in all biological mediated reactions, environmental and operational parameters affect the activity of the microbial community and influence the behaviour of the overall process. In particular: alkalinity, pH, presence of toxics or inhibitors, temperature and reactor configuration must be taken into consideration when analysing and comparing experimental results dealing with anaerobic slaughterhouse waste treatment.

pH, alkalinity and process stability. The previously described specific microorganisms responsible of the different anaerobic digestion steps (Figure 1.1) have different optimal pH values. The hydrolytic and acidogenic microorganisms have an optimal growth at pH above 6, while the activity of methanogens is reduced at a pH below 6.5 (Batstone *et al.*, 2002; Yu and Fang, 2002). Consequently, it is widely accepted that the optimal pH for the whole anaerobic digestion process can be established close to the neutrality (Clark and Speece, 1989).

Although the direct effect of pH on biomass activity, the monitoring of pH is not considered as a good parameter for process control, due to its logarithmic scale. An increase of one pH unit corresponds to a ten-fold variation on the proton concentration, and, consequently, relatively slight variations in pH might already be

the result of process imbalance and even process failure. Conversely, alkalinity (carbonate system equilibrium) or the system buffer capacity is more affected by the accumulation of intermediates (VFA and H_2) than the pH measurement, being a quick indicator of stress in digesters (see the physic-chemical equilibrium section in Figure 1.1).

Because of the different sensibility to pH and other environmental conditions, and to the diverse growth rates of acid forming and methane producing microorganisms, the imbalance between those groups can cause volatile fatty acids (VFA) accumulation, resulting in reactor instability (Chen *et al*., 2008). The VFA are the main intermediate species of the anaerobic digestion process. Consequently, the two parameters most frequently used to monitor digester stability are alkalinity and the direct measurement of VFA concentration (Ripley *et al.*, 1986; Hill *et al.*, 1987; Ahring *et al.*, 1995). The main anaerobic degradation intermediate and process indicator is acetate (Pind *et al.*, 2003), but propionate has also been proposed as a key control parameter (Nielsen *et al.*, 2007). In the anaerobic degradation of proteins, the monitoring of straight and branched chain butyrate and valerate is also important (Batstone *et al.*, 2003).

VFA accumulation can cause process inhibition as well. Propionic acid accumulation in the reactor has been described to cause inhibition on the acetogenesis (Fukuzaki *et al.*, 1990), and also on the acetoclastic methanogenesis (Barredo and Evison, 1991). Acetate accumulation has been reported to inhibit the acetogenesis from propionate (Fukuzaki *et al.*, 1990), the acetogenesis from butyrate (Ahring and Westermann, 1988), and also the methanogenesis step (Stafford, 1982; Ahring *et al.*, 1995), when present at high concentrations.

Hydrogen is also an important intermediate species and it is considered as an indicator parameter of the anaerobic process. H_2 accumulation can inhibit the acetogenesis, with the consequent VFA accumulation, being a potential inhibitor not only of the methanogenesis but also of the global anaerobic process (Fukuzaki *et al.*, 1990; Hill and Cobb, 1993).

Toxics and inhibitors. Besides the previously described substrate-product inhibitory compounds (VFA and H_2), the literature on anaerobic digestion illustrates a considerable disparity in the inhibition/toxicity levels of other substances, like ammonia, long chain fatty acids (LCFA), sulphide, light metal ions, heavy metals and other organic compounds (Chen *et al*., 2008). Due to the characteristics of slaughterhouse waste, ammonia and LCFA are considered the main potential inhibitory substances on the anaerobic digestion process of those wastes. These inhibitory phenomena are synthesized below.

Proteins are important components in organic wastes and they are often responsible for the high ammonium concentration during the anaerobic digestion, causing inhibition and process failure (Flotats *et al.*, 2006). The described inhibitory specie is the free **ammonia** (NH_3), since its inhibitory effect increases along with the pH and temperature values (Zeeman *et al.*, 1985). 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 microorganisms that are affected the most by ammonia inhibition are the methanogens (Koster and Lettinga, 1988; Robbins *et al.*, 1989), being acetoclastic methanogens more sensitive than hidrogenotrophic methanogens to high NH_3 concentration (Hansen *et al.*, 1998; Angelidaki and Ahring, 1993). A wide range of inhibiting ammonia nitrogen concentration values (NH_4^+-N), from *1.7* to *14 g* L^{-1}, have been reported in the literature to cause a *50%* reduction in methane production, as a function of pH, temperature and biomass adaptation (Chen *et al.*, 2008). Moreover, it has been suggested that the hydrolysis of proteins is affected by the ammonia content (Lü *et al.*, 2007).

Under anaerobic conditions, lipids are rapidly hydrolysed by extracellular lipases to long-chain fatty acids (**LCFA**) and glycerol. Hydrolysis of lipids is generally regarded as a fast process, while the overall conversion rate is limited either by further LCFA metabolic rates or by physical processes, as dissolution and adsorption of these acids (Cirne *et al.*, 2007). About *90%* of the COD originally contained in the lipids is conserved in the LCFA formed upon hydrolysis. Palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids are the most abundant saturated and unsaturated LCFA, respectively, present in organic waste and wastewater (Hwu *et al.,* 1998). LCFA are known to inhibit the methanogenic activity and its accumulation in anaerobic reactors is commonly reported as a major operational problem. The inhibitory effect was initially attributed to the permanent toxicity resulting from cell damage, and it is known to affect both syntrophic acetogens and methanogens (Rinzema *et al.*, 1994; Hwu *et al.*, 1998). Further studies have demonstrated that LCFA inhibition is reversible and that microorganisms, after a lag phase, are able to efficiently methanise the accumulated LCFA (Pereira *et al*., 2004). Adsorption of LCFA onto the microbial surface has been suggested as the mechanism of inhibition, affecting transport of nutrients through the cell membranes (Pereira *et al.*, 2005). LCFA inhibition is dependent on the type of microorganism, the specific surface area of the sludge, the carbon chain length and the saturation degree (number and position of the double carbon bonds) of LCFA (Hwu *et al.*, 1996; Salminen and Rintala, 2002). It has been reported that LCFA inhibit anaerobic microorganisms at very low concentrations, with IC_{50} values for C18:1 over *50-75 mg* L^{-1} (Alves *et al.*, 2001b; Hwu *et al.*, 1996), C16:0 over *1,100 mg* L^{-1} (Pereira *et al*., 2005) or C18:0 over

1,500 mg L^{-1} (Shin *et al.*, 2003) at mesophilic temperature range. Methanogens were reported to be more susceptible to LCFA inhibition compared to acidogens (Lalman and Bagley, 2002; Mykhaylovin *et al.*, 2005; Pereira *et al*., 2003).

However, inhibition caused by LCFA is a reversible process, and neither syntrophic acetogenic nor methanogenic activities are irreversibly damaged, and the rate of methane formation is able to recover the previous values and, in some cases, it can even be improved (possibly as the result of microbial adaptation), after the LCFA degradation had recommenced (Pereira *et al.*, 2003 and 2005).

Temperature. Traditionally, the anaerobic digestion of organic waste has been carried out at the mesophilic temperature range (*35-37ºC*), which satisfies a sufficient process stability and the low energy needs. When increasing the organic loading rates and sludge hygienization becomes an important requirement, thermophilic anaerobic digestion (*50-55ºC*) appears as an interesting alterative to the mesophilic digestion (Zábranská *et al*., 2000). As a biological process, the treatment capacity in anaerobic digestion depends on the microbial growth rate, which is defined as temperature dependent (Van Lier, 1995). Consequently, the growth rates of thermophilic bacteria are higher than those of mesophilic bacteria, and thermophilic anaerobic digestion can be more efficient in terms of organic matter removal and methane production rates than the mesophilic process (Zábranská *et al.*, 2000; Ahring *et al.*, 2002; Gavala *et al.,* 2003). In addition, digestion in the thermophilic range achieves a considerable pathogen reduction and higher resistance to foaming. On the other side, the thermophilic operational regime might result in a less stable process, more prone to VFA accumulation (Kim *et al.*, 2002; Palatsi *et al.*, 2009).

Another important aspect of the operational temperature regime is its effect on the solubility of gases, which decreases when increasing temperatures and, consequently, results in an increase of the liquid gas-transfer. That effect could be positive in the case of potentially toxic species (NH_3, H_2S and H_2) but also affects the CO_2 solubility and, consequently, the buffer system capacity and pH. The increase in temperature also affects the equilibrium of ionized-not and ionized forms, and results in a higher inhibitory effect of ammonia in thermophilic reactors (Van Lier, 1995). Although thermophiles are considered more susceptible to ammonia or LCFA toxicity compared to mesophiles, they can recover faster after inhibition due to their faster growth rates (Hwu and Lettinga, 1997).

Biomass and bioreactor configuration. The influence of the seed sludge used for inoculation (i.e suspended versus granular biomass), the reactor operation regime (batch, continuously operation, or feeding patterns), as well as biomass adaptation

processes must be considered as key parameters in the analysis and operation of anaerobic digesters dealing with slaughterhouse waste or lipid rich substrates.

Recent advances in the anaerobic digestion of lipid/LCFA based effluents have challenged the previously accepted theories on permanent LCFA inhibition/toxicity. The reduction on nutrients-metabolites transport rate through biological membranes due to the LCFA adsorption over cell walls has currently been accepted as the main the mechanism of inhibition (Pereira *et al*., 2005). Consequently, the LCFA inhibitory process may be considered as a function of the available biomass surface area. It has been reported that **suspended** and flocculent sludges, which have a higher surface area, suffered much greater LCFA inhibition than **granular** sludge (Hwu *et al.*, 1996). Nevertheless, the higher LCFA adsorption capacity and degradation of suspended sludge benefits the system recovery capacity (Pereira *et al.*, 2002a and 2002b).

The LCFA adsorption phenomenon is described as a fast process and a pre-requisite for the biodegradation of lipids while desorption is a consequence of biological activity and the rate limiting phenomenon (Hwu *et al*., 1998; Nadais *et al*., 2003). For this reasons intermittent operation (or **discontinuous feeding**) in anaerobic reactors dealing with lipids is defined as a promising process, due to its capacity to couple biological degradation with the adsorption phenomenon. Coelho *et al.* (2007) improved the efficiency of biological conversion and methanisation rates by introducing starving periods in UASB reactors treating daily wastewaters. Specific methanogenic tests performed by Nadais *et al.* (2006) showed a shift in the microbial population towards a better adapted species in intermittent feeding reactors. Discontinuous feeding system to promote the development of an active anaerobic community, for the efficient conversion of lipid-rich effluents, has also been suggested by Cavaleiro *et al.* (2008).

It has been reported that the reactor organic loading rate and the active microorganism concentration affect the ammonia inhibition (Angelidaki and Ahring, 1993). Van Velsen (1979) demonstrated that the **adaptation** of the methanogenic sludge allows the mesophilic digestion of piggery slurry to ammonia concentrations up to *3* $g_{N\text{-}NH4+}$ L^{-1}. Other authors have also proposed biomass adaptation processes related to high ammonia concentrations (Angelidaki and Ahring, 1993; Hansen *et al*., 1998). Experimental evidence has clearly demonstrated the possibility of obtaining stable digestion of manure with ammonia concentrations exceeding *5* $g_{N\text{-}NH4+}$ L^{-1}, after an initial adaptation period. Immobilizing the microorganisms with different types of inert material (clay, activated carbon, zeolite) has been demonstrated to reduce ammonia inhibition and allows a more stable process (Angelidaki *et al.*, 1990; Hanaki *et al.*, 1994; Hansen *et al.*, 1998). The positive effect of zeolite on the

anaerobic process could partially be attributed to the presence of cations such as Ca^{2+} and Na^{+} that counteract the inhibitory effect of ammonia (Borja *et al.*, 1996).

Despite the inhibitory effect of LCFA on anaerobic process, adaptation of the anaerobic biomass to relatively high LCFA concentrations has been reported. As mentioned previously, the discontinuous or pulse LCFA exposure, promoting the LCFA accumulation into the biomass prior to its degradation leads to increased tolerance towards LCFA (Nadais *et al.*, 2003 and 2006; Cavaleiro *et al.*, 2008). Digesters inoculated with an acclimated sludge exhibited higher methane yields than those inoculated with a non-acclimated sludge (Pereira *et al.*, 2002b). Other strategies, like the use of additives or co-substrates were demonstrated to achieve that objective. The inhibitory effect of LCFA could be reduced by adding calcium, because calcium can precipitate LCFA as calcium salt (Ahn *et al*., 2006). However, calcium addition cannot solve the problem of sludge flotation (Alves *et al*., 2001a and b). The addition of adsorbents (biofibers) in order to protect the biomass (Nielsen *et al*., 2007), or the addition of easily degradable substrates like glucose or cysteine (Kuang *et al*., 2006), have also been proposed. Other operational strategies, like the bioaugmenting of lipolytic bacterial strains, also led to a reduction in LCFA inhibition. However, it was not possible to confirm the survival of the bioaugmented lipolytic strains during experiments (Cirne *et al.*, 2007). The application of the electrochemical anodic conversion of lipids could be a promising technique to transfer the energetic potential of lipids into biogas, removing the potential toxic effect of LCFA (Gonçalves *et al.*, 2006). Two-stage anaerobic systems successfully overcame also the inhibition problems and showed a significant improvement in the process efficiency (Wang and Banks, 2003).

In summary, the toxicity of a given substance to anaerobic microorganisms can be reduced significantly by promoting biomass adaptation and by other operational strategies that ultimately result in a lower LCFA exposure. Currently, there is no clear evidence on whether the adaptation process is the result of a microbial population shift towards the enrichment of specific and better adapted degraders, or to the phenotypic adaptation of the existing microorganisms against high inhibitory concentrations (physiological acclimatation). The adaptation process frequently implies the reorganization of metabolic resources by the toxic substrate rather than a population change (Kugelman and Chin, 1971). The use of mathematical modeling and molecular biology techniques, have opened new insights in the investigation of those phenomena.

1.3. MATHEMATICAL MODELLING OF ANAEROBIC PROCESS

As in other biological treatment processes, anaerobic digestion models have become a valuable tool to increase the understanding of complex biodegradation

processes, to orientate experimental designs and to evaluate results, to test hypothesis, to reveal relations among variables, to predict the evolution of a system, to teach and to communicate using a common language, to optimize design plants and operating strategies, and for training operators and process engineers (Vanrolleghem and Keesman, 1996).

A join effort to create a unified language and to propose a general structured model has resulted in the anaerobic digestion model IWA-ADM1 (Batstone *et al*., 2002). IWA-ADM1 is a mechaniscistic model including disintegration, hydrolysis, acidogenesis, acetogenesis and methanogenesis. Substrate-based uptake Monod kinetics are used as basis for biochemical reactions. Inhibition functions included pH, H_2, and NH_3, mainly as reversible and non-competitiveinhibition functions. Also, physico-chemical processes are included, such as acid-base equilibrium and liquid-gas transfer (Batstone *et al*., 2002). Despite the general acceptance and use of ADM1 model, several processes were not yet included either because they were considered not relevant under more common applications or because of limited available research. From those non included processes, the disintegration/hydrolysis of complex substrates and LCFA inhibition can significantly affect the anaerobic digestion of slaughterhouse waste.

The cumulative effects of the different processes taking place during disintegration/hydrolysis have traditionally been simplified to simple first-order kinetics for the substrate biodegradation (Eastman and Ferguson, 1981; Batstone *et al*., 2002). For complex substrate or rate-limiting biomass to substrate ratios, the first-order kinetics should be modified in order to take into account the hardly degradable material (Batstone *et al.*, 2002; Vavilin *et al.*, 1996). It has been shown that models in which hydrolysis is coupled to the growth of hydrolytic bacteria also work well at high or at fluctuant organic loading rates (Vavilin *et al.*, 2008). Proteins are important components of many waste and often responsible for the high ammonia concentration during anaerobic digestion, causing inhibition of acetoclastic methanogens and possible process failure. In IWA-ADM1 model (Batstone *et al*., 2002) a free ammonia inhibition function is introduced only for acetoclastic methanogens, described as a non-competitive inhibition function. Besides, NH_3 inhibition, the possible effect of VFA in the hydrolysis of proteins has received a special attention. While Breure *et al.* (1986a), Yu and Fang (2003), and Flotats *et al.* (2006) concluded that VFA did not inhibit protein degradation, using gelatine as a model substrate, González *et al.* (2005) showed gelatine hydrolysis was severely inhibited by acetic acid, expressed as an inhibition constant in a non-competitive inhibition affecting a first order hydrolysis.

Despite the fact that LCFA inhibition is well documented and has a significant impact on the anaerobic digestion process, this phenomenon has not yet been

included in IWA-ADM1 Model because of the complexity of the inhibition phenomenon (Batstone *et al*., 2002). It has been proposed that inhibition is a result of LCFA adsorption over the cell surface. Therefore, factors such as cell surface area, pH, and possible adaptation have influence in the observed LCFA inhibition process.

In other developed models, LCFA inhibition is mainly modelled as a non-competitive process on the lipolytic, acetogenic or methanogenic activities. Angelidaki *et al.* (1999), studying manure codigestion with glycerol trioleate or bentonite bound oil degradation, considered a non-competitive LCFA inhibition on the lipolitic, acetogenic and methanogenic steps, and a Haldane's inhibition kinetics to the ß-oxidation process. Salminen *et al.* (2000) and Lokshina *et al.* (2003), using solid slaughterhouse waste, considered a non-competitive inhibition kinetics due to LCFA, affecting acetogenesis and methanogenesis.

However, the microbial aspects of the adsorption process and biomass adaptation to LCFA remain poorly characterized, and further modelling developments are required in order to link the results from physiological activity test and the microbial population dynamics throughout the whole adsorption-inhibition-adaptation process.

1.4. MOLECULAR BIOLOGY TECHNIQUES

The field of microbial ecology has evolved rapidly in the last decade due to the generalization of molecular biology tools for monitoring the microbial diversity in biological systems. The direct extraction of DNA from environmental samples, and the amplification and sequencing of specific genes containing usefull phylogenetic information has allowed the study of microbial communities in a culture independent-way, so that bias related to the selectivity of culture conditions is prevented. A number of methods based upon selective PCR amplification and molecular profiling and/or sequencing of environmental DNA extracts can be applied for the characterization of entire microbial communities. Thechniques such as clone library sequencing and molecular profiling by denaturing gradient gel electrophoresis (DGGE) can be used in the assessment of microbial diversity and community dynamics. Other methods, like fluorescence in situ hybridization (FISH) result in the visualization of specific taxa in relatively undisturved samples, so that the spatial distribution and quantification of specific microorganisms can be assessed.

The DGGE profiling of the small ribosomal subunit (16S rRNA) gene fragments has consolidated as a robust and relatively simple technique in the characterization of complex bacterial and archaeal communities. Separation of rRNA amplicons by **DGGE** is based on their sequence-specific denaturing position in a polyacrylamide

gel with a gradient of denaturing chemical (a mixture of urea and formamide). Complete denaturation of the DNA fragments is prevented by the addition of a GC-clamp at one amplicon end (30-50bp, added in the PCR amplification). Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing gel. This analysis generates an overall impression on the complexity of microbial communities, which can be easily compared to monitor population shifts (*Muyzer et al.*, 2008).

Calli *et al.* (2005) using (DGGE) cloning and DNA sequencing techniques, studied the diversity of methanogenic populations in anaerobic reactors subjected to extremely high ammonia levels, finding that members of the *Methanosarcina* genus were the predominant acetoclastic methanogens. Pereira *el al* (2002b) used DGGE, cloning and sequencing in samples from reactors fed with LCFA and found a high diversity of genotypes related to *Syntrophomonas spp*. LCFA-degrading bacteria have been found to be closely related to the *Syntrophomonadaceae* and *Clostridiaceae* families (Hatamoto *et al.*, 2007). Recently, a new anaerobe that only degrades LCFA in co-culture with *Methanobacterium formicium*, *Syntrophomonas zenderi* sp. nov, has been isolated (Sousa *et al*., 2008), reinforcing the hypothesis of the necessary presence of syntrophic methanogens for a reliable LCFA degradation.

As mentionned above, other molecular biology techniques are based on the hybridization of specific fluorescent molecular probes (FISH) on environmental samples, and on the microscopic observation for the direct monitoring of the microbial community structure. Menes and Travers (2006) developed a new FISH probe specific for the *Syntrophomonadaceae* family, and characterized its abundacy in wastewaters. Also, **Dye staining** can be used as an *in-situ* technique to study the spatial organization of substrates and microorganisms. The selected targets are labelled with stain fluorocromes that can be identified by confocal laser scanning microscopy (CLSM) or fluorescence light microscopy (LFM) techniques. In the literature, there are specific fluorocromes reported for total cells (*Syto 63*), dead cells (*Synox Blue*), proteins (*FITC*), lipids (*Nile Red*) and polisacarides (*Calcofluor White*), as described in Chen *et al.* (2007). The key to multiple fluorochrome experiments is to use highly specific dyes with minimum spectral peak interference (Murray, 2005). DAPI staining is a wildly applied method for biomass identification on anaerobic systems (Araujo *et al*., 2000; Solera *et al.*, 2007) and can be easily combined with *Nile Red* (lipids). Consequently the application of stain-dye and fluorescence light microscope introduces complementary information to other classic methodologies, like batch degradation or toxicity tests, providing new insights on the LCFA adsorption process (i.e. by monitoring the cells and lipids organization). Further research in more specific new dyes and more optimized procedures may establish methodologies of in situ and rapid LCFA quantification by

fluorescence intensity analysis, as it has been achieved in other biotechnology fields (Diaz *et al.*, 2008; Larsen *et al.*, 2008).

1.5. OBJECTIVES

The main difficulties during slaughterhouse waste anaerobic treatment are related to long chain fatty acids (LCFA) inhibition. Despite the fact that the most recent scientific literature strongly suggests that the inhibitory effect exerted by LCFA is due to their adsorption onto active biomass, the complete inhibition mechanisms and the resulting microbial interactions are not completely clear.

Therefore, the general objectives of this thesis are focussed on to obtaining a better understanding of the LCFA inhibition and the related biomass adsorption and adaptation phenomena. In order to achieve the general objective, this thesis works has been organized according to fit the following specific objectives:

- To characterize real and representative slaughterhouse waste and animal by-products, in order to determinate its potential methane yields and to identify the main difficulties for the anaerobic digestion process.
- To develop a fast and accurate analytical methodology to determinate free and adsorbed LCFA concentrations in biological samples.
- To test several methodologies to prevent or overcome LCFA inhibition in anaerobic reactors.
- To characterize the LCFA-biomass adsorption process and its influence over LCFA inhibition, by means of batch activity tests and microscopic observations.
- To identify and monitor the adaptation processes of biomass subjected to sequential LCFA inhibitory concentrations by means of culture independent molecular biology techniques.
- To develop a kinetic expression of the LCFA inhibition, according its physical nature, able to be used in a more general mathematical model, such us the IWA ADM1 model.

1.6. REFERENCES

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Thesis Outline

In the present chapter a summary of the main obtained results and a general discussion of this thesis are exposed. The specific difficulties on slaughterhouse waste anaerobic treatment, the inhibitory effect of long-chain fatty acids (LCFA), the research on possible strategies to prevent or to overcome inhibition, the study of the LCFA-biomass adsorption process and the mathematical modelling of LCFA inhibition and related physical process, are exhaustively presented in the subsequent chapters.

Slaughterhouse waste and animal by-products are one of the main residues produced by food industry. The new legal regulations and the possibility to apply new waste management regulated treatments, become an interesting opportunity for anaerobic digestion process and the production of renewable energy from this kind of wastes. Lipid-protein rich substrates are interesting for anaerobic digestion process due to its high expected methane yield, but also possible rate limiting steps and inhibitory processes have been reported, reducing the potential of a stable and controlled anaerobic process.

In **Chapter 1**, an introduction to the anaerobic digestion process, the possible difficulties on slaughterhouse waste treatment and the possible affecting environmental/operational parameters are briefly presented. The main difficulty is related to the anaerobic digestion of long chain fatty acids (LCFA), products of lipids hydrolysys. It has been reported that this inhibition is due to a physical adsorption of LCFA onto the microorganisms' membrane, witch decreases the external mass transport rate of substrates to the cell. Complementary techniques used in results discussion and the main research objectives of the present dissertation are presented also in this first Chapter.

The general objective of this Thesis is to obtain a better knowledge of the **LCFA inhibition** process and the related **adsorption** and **adaptation** phenomena. The subsequent paragraphs contains an executive summary of the works performed under this thesis scope

In **Chapter 3**, animal by-products, wastewaters and other organic waste produced in cattle-piggery slaughterhouse facilities were exhaustively characterized and representative mixtures, according to slaughterhouse waste flow rates, were produced. Those mixtures were defined as high organic content substrates (mainly by lipid and proteins), interesting for the anaerobic digestion process due to its theoretical high methane potential.

The different tested slaughterhouse waste mixtures, containing different proportions of lipid and protein (L/P), resulted on a different biodegradability indexes and methane yields. As expected, the obtained substrates methanogenic potential were high (*270-300* $L_{CH4}\ kg^{-1}_{CODin}$). Results showed that a low L/P ratio (an increase of protein concentration in the substrate) had a stimulating effect over the overall kinetics of slaughterhouse waste degradation, compared with substrates with a high L/P ratio, being the degradation of lipids and the LCFA the main rate limiting process steps in the overall substrate descomposition.

A simplified mathematical model was developed based on the results from biodegradability tests with similar slaughterhouse waste mixtures, as presented in the Annexed information, **Chapter 8.1**. Hydrolysis and acidogenesis were considered as a unique process steps in the simplified model. The degradation of proteins was

described by the classical first order kinetics, due to the relatively low hydrolysis rate of proteins compared to the uptake rate of amino acids during acidogenesis. In contrast, the comparatively low acidogenesis rate for LCFA required the combined process description for lipids (hydrolysis+acidogenesis), coupled to the growth of the specific biomass, being the Contois kinetics a suitable model for this purpose. Experimental results and model simulations showed the limiting effect of LCFA on slaughterhouse waste treatment, and the necessity of measuring the LCFA concentration (developed in Chapter4) for the mathematical modelling of hydrolysis of lipids and acidogenesis of LCFA as independent processes. A literature compilation of hydrolysis kinetic parameters of particulate substrates in anaerobic degradation, and a review of suitable kinetic expressions, is presented in the Annexed information of **Chapter 8.2**.

Organic concentrations in slaughterhouse waste mixtures of up to *15* g_{COD} L^{-1} exhibited a clear LCFA inhibitory effect, as observed in sequential batch tests where a long lag-phase in methane formation and accumulation of volatile fatty acids (mainly acetate) were monitored. The propionate accumulation profile also indicated a possible interaction of the lipids or LCFA on the degradation of proteins, since this acid is not a product of LCFA decomposition. The potential inhibitory effect of VFA accumulation on the protein hydrolysis process is also reported in the Annexed information of **Chapter 8.3**, where it is concluded that VFA are not inhibiting the hydrolysis step of proteins.

Despite the reported LCFA-inhibition in successive batch assays, the methanogenic activity was recovered and the slaughterhouse waste pulses were degraded, obtaining similar methane yields than those in non inhibited systems. The time course evolution of the degradation rates at increasing substrate concentration points to a biomass adaptation process. To determine whether or not this adaptation process is the result of a shift in the microbial community structure, culture independent techniques needs to be used in order to monitor the microbial dynamics throughout the batch experiments. These techniques will be introduced and tested in Chapter5.

From the results of Chapter 3, the main difficulties were arising from a probable LCFA inhibition process. Consequently, the aim of the subsequent thesis chapters was focussed on the study of the LCFA dynamics, the inhibition process produced by them and the microorganisms' adaptation process, as being the main identified limiting steps in the anaerobic digestion of slaughterhouse waste.

In **Chapter 4**, the inhibitory effect of LCFA was investigated in thermophilic (*55ºC*) anaerobic manure based reactors exposed to pulses of synthetic LCFA mixtures (oleate C18:1, stearate C18:0 and palmitate C16:0, considered as the main species in LCFA rich substrates). The assays were not designed as co-digestion assays

and manure was only selected as a basis substrate to confer system stability due to its high buffering capacity and nutrient concentration. The experimental set-up was designed to monitor the effect of successive LCFA inhibitory pulses and to test the effectiveness of different recovery strategies. The thermophilic range was selected for the depiction of a faster and clearer system response, that is, higher inhibition but fast biomass growth.

Initially, a fast and straight-forward methodology was developed for monitoring the soluble and adsorbed LCFA content on biological samples. The inhibitory effect of LCFA over anaerobic activity was assessed in toxicity batch tests. From those preliminary results, a LCFA concentration of $4\ g\ L^{-1}$ was considered sufficient to impose a clear and long lasting inhibition on the anaerobic biomass, and consequently, selected for the subsequent batch and semi-continuous assays.

After characterizing the LCFA inhibitory pulse, several operational strategies, such different reactor feeding patterns (manure feeding/no-feeding), dilution (with water/fresh manure/digested sludge) and addition of adsorbents (fibers/bentonite), were tested in order to accelerate the recovery of the inhibited biomass. Those experiments were performed in both batch and semi-continuous reactors, monitoring the CH_4 production, LCFA and VFA concentration profile.

The dilution with active inocula in order to increase the biomass/LCFA ratio, and the addition of adsorbents for reducing the bioavailable LCFA fraction, were found to be the best recovery strategies. In this way, the recovery time of the inhibited semi-continuously fed systems was reduced from *10* till *2 days*.

The use of adsorbents, like bentonite or digested fibers, is the most reliable simple, feasible, and cost-effective solution strategy for its scaling up in industrial facilities, where waste dilution is usually not feasible. This positive effect of adsorbents was related with the competition with biomass in adsorbing soluble LCFA and, thus, in reducing biomass exposure to LCFA. The introduction of microscopic observation and mathematical modelling tools in next Chapters will give more insights about this hypothesis. On the other hand, the generally accepted practice in real plants of stopping the feeding when an inhibition/imbalance of the process is detected is revealed to be the worst approach to overcome an LCFA inhibition episode in terms of recovery time and process stability.

The application of repeated LCFA pulses resulted in a faster recovery of the system, both in batch and semi-continuous reactors, and in an enhancement in methane production (from *0.04* to *0.16* $g_{COD\_CH4}\ g^{-1}_{VS}\ d^{-1}$) and degradation rates. That result suggests that the biomass present in the bioreactors is progressively adapted to tolerate high LCFA concentrations. The incubation time between subsequent pulses, or discontinuous LCFA pulses, seems to be a decisive process parameter to tackle LCFA inhibition.

In **Chapter 5**, the previously reported adaptation process to successive LCFA inhibitory pulses, expressed as higher capacity of the reactor system to recover its normal operational conditions, was further investigated by means of specific activity batch test, the characterization of the microbial populations by culture-independent molecular biology tools, and by the mathematical modelling of the involved biochemical and physical processes.

Specific activity test of biomass samples, taken during the continuous operation of the previously described reactors, evidenced the differentl sensitivities to LCFA of major microbial trophic groups. Increasing substrate utilization rates for ß-oxidizing bacteria (*163-219* $mg_{CODCH4}\ g^{-1}_{VS}\ d^{-1}$) and syntrophic methanogens (*68-91* $mg_{CODCH4}\ g^{-1}VS\ d^{-1}$) were obtained upon successive biomass exposition to LCFA inhibitory concentrations, which is in agreement with the previous reported capacity of the reactor system to recover its normal operational characteristics upon LCFA exposure.

A shift in bacterial and archaeal communities could explain the reported activity improvement, and that possibility, was studied by DGGE profiling of PCR-amplified 16S rDNA genes. DNA sequencing of DGGE predominant bands showed close phylogenetic affinity to ribotypes characteristic from specific ß-oxidation bacterial genera (*Syntrophomonas* and *Clostridium*), while the main syntrophic archaeae domain was related with the genus *Methanosarcina*. The population profiles of predominant eubacteria and archaea revealed that no significant shift on the microbial community composition took place upon biomass successive exposure to LCFA. Yet, the indigenous microbiota present in the daily manure reactor feeding might have attenuated any observable change on the eubacteria and archaeae population profile.

On the other hand, the hypothesis of LCFA adaptation as being the result of an enrichment of existing specific LCFA-tolerant populations was tested and confirmed by means of mathematical modelling tools. Despite the fact that LCFA inhibition is well documented and has a significant impact on the anaerobic digestion process, this phenomenon has still not been included in IWA ADM1 reference model. Consequently, using IWA ADM1 as a basis model, LCFA inhibition was introduced, firstly using classical inhibition model approximations (non-competitive and Haldane inhibition kinetics) and, finally, as a new kinetic function considering the relation between LCFA inhibitory substrate concentration and specific biomass content, due to the reported importance of adsorption process. The proposed Inhibition-Adsorption kinetics produced a better fit to the experimental results than the classical inhibition models, and provides a numerical expression of the physical adsorptive nature of the overall LCFA inhibition process. Consequently, modelling results also suggest that adsorption plays an important role in the overall LCFA

inhibition-adaptation process, and that there is a need to introduce modifications in IWA ADM1 model when dealing with the degradation of lipids.

The results of specific activity tests, together with the apparent stability of the microbial community structure, and the predicted increase in hydrogenotrophic methanogens and LCFA degrading populations along time by mathematical modelling results, strongly indicates that the observed LCFA adaptation process was the result of a physiological acclimation of existing populations or, at most, to the proliferation of specific, yet already existing, LCFA degrading bacteria and syntrophic methanogenic archaea.

In **Chapter 6**, an in-depth study of the adsorption process of LCFA over biomass and the possible competition with other adsorbents, as a preventing strategy to face with LCFA inhibition, was assessed by means of batch assays and microscopic observation techniques.

Two granular sludges from different origins were characterized in terms of methanogenic activity rate, LCFA toxicity and granule morphology. In relation to those experiments, an oleate concentration of $0.5\ g\ L^{-1}$ was considered enough to reduce the global activity of granular sludge, causing a clear and long-lasting inhibition, while no significant differences were reported in available surface area or syntrophic methanogenic activities between both sludges.

The adsorption isotherms of LCFA over bentonite and on inactivated anaerobic granular sludge were also assessed in batch experiments, being the adsorption capacity of these clay mineral materials clearly higher than that of granular sludge, according to the obtained *Freundlich* isotherms.

Batch test with un-adapted biomass showed a fast and non-limiting oleate partial ß-oxidation process, which was confirmed by the detection of palmitate as the main intermediate. The absence of inoculum adaptation to lipids or LCFA may have played a major role in the slow palmitate degradation or in the step-by-step LCFA overall degradation process. The discussion was focussed on the obtained LCFA degradation profiles, in particular on the different oleate and palmitate inhibitory behaviour due to their distinct adsorption properties, and on the biomass adaptation, especially in relation to the structure of the ß-oxidizing microbial community. The introduction of fluorescence staining and microscopy observation techniques confirmed the presence of palmitate adsorbed onto the anaerobic granular sludge, with the consequent implications on limitations to the external mass transport of substrate to the cell. Results obtained by the selected fluorescent dyes and the observation procedure were a qualitative approach to monitor the LCFA adsorption process on anaerobic granular sludge. The data provided by this innovative technique are complementary to the results from classical methodologies, like batch degradation or toxicity tests.

The addition of bentonite for the prevention of, instead of recovery, LCFA inhibition was also tested. Batch test results demonstrated that the use of competitive additives is a reliable strategy to improve the system performance, in terms of process stability, methane production delay or resistance to LCFA inhibitory concentrations.

This Thesis, therefore, constitutes a basis for better knowledge about slaughterhouse waste treatment and a better understanding of LCFA inhibition and adsorption/adaptation processes. Considering the obtained results, anaerobic digestion of lipid-rich wastes can be achieved if adequate LCFA/biomass ratios are condidered. The inhibition of the process can be prevented or recovered with competitive inorganic adsorbents and ensuring the growth/adaptation of the microorganisms. The inclusion of the proposed inhibition kinetics into the IWA ADM1 model can help to simulate the anaerobic digestion of high lipid-rich substrates, allowing to guide the desing and operation of reactors. Current results will help to obtain high removable energy rates from slaughterhouse wastes trough anaerobic digestion. The detailed conclusions and suggestions for further research are exposed in **Chapter 7**.

Anaerobic digestion of piggery and cattle slaughterhouse waste

ABSTRACT. Representative piggery and cattle slaughterhouse mixtures were characterized and its anaerobic biodegradability assessed by standardized batch tests.
The obtained methane potentials of slaughterhouse mixtures were high (*270-300* $L_{CH4}\ kg^{-1}_{COD}$) being interesting substrates for an anaerobic digestion process. However, the lipid content of those substrates has a limiting effect over the overall transformation process, resulting in a clear inhibitory phenomenon when lipid concentration reached values of *11.2* $g_{COD}\ L^{-1}$.
Although the severe inhibition process reported, monitored as a long lag-phase in methane production and a VFA accumulation, the system was able to recover activity and methane rates. That response was identified as an adaptation process to subsequent lipid exposition, making the pulse feeding method a reliable strategy

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3.1. INTRODUCTION

Solid slaughterhouse waste, or animal by-products, were usually treated by rendering process (EC-IPPC2005) in previous decades, providing a valuable source among the slaughterhouse incomes. In recent years, because of BSE (Bovine Spongiform Encephalopathy) the economical value of these materials has reduced substantially and in many cases have to be disposed off as a waste (EC no 1771/2002). Further modification of European Parliament and of the Council (EC no 92/2005) about disposal and uses of animal by-products, allows biogas transformation if approved pre-treatments depending on the by-product category (according to BSE risk) are applied (pasteurization, high pressure and temperature or alkaline hydrolysis).

Slaughterhouse wastes are characterized by its high organic content, mainly composed by proteins and fats. Few references are available on quantification, characteristics and anaerobic potential of animal by-products and waste from slaughterhouses. Tritt and Schuchardt (1992) and Edström *et al.* (2003) reported the first reviews of the material flows and possible treatment strategies in German and Swedish piggery and cattle slaughterhouses, respectively. Salminen and Rintala (2002) reported quantities and anaerobic biodegradabilities of waste produced in Finnish poultry slaughterhouses. Recently, Hejnfelt and Angelidaki (2009) have characterized individual fractions of Danish piggery animal by-products and determined its potential methane yields.

Because of their composition, high fat and protein content, slaughterhouse waste and animal by-products, can be considered a good substrate for anaerobic digestion plants, according to the high potential methane yield. However, slow hydrolysis rates and inhibitory process have been reported. In particulate hardly degradable materials, like animal by-products, the hydrolysis must be coupled with the growth of hydrolytic bacteria, and this factor can limit the overall process rate (Vavilin *et al.*, 2008). Furthermore, lipids can cause biomass flotation and wash-out, and during lipids hydrolysis by extracellular lipases, long–chain fatty acids (LCFA) are produced. Those intermediate are well described as inhibitory species (Angelidaki *et al.*, 1990; Hwu *et al.*, 1997). Also, ammonia is released during protein degradation and its inhibitory effects over anaerobic digestion (as NH_3 form) is reported elsewhere (Hansen *et al.*, 1996). For all those reasons, and due to the difficulties of its digestion as unique substrate, large experiences with anaerobic digestion of slaughterhouse by-products (mainly rumen, stomach or intestinal content and sludge from slaughterhouse wastewater treatment plants) consist on their co-digestion with other industrial, agricultural or domestic waste, as a suitable substrate for centralized biogas plants (Angelidaki and Ellegard, 2003; Resh *et al.*, 2006).

More experiences are reported in literature about the anaerobic treatment of slaughterhouse wastewaters. The increased atomisation of carcase dressing and incorporation of washing at every stage (scalding, bleeding, evisceration and tripe treatment) have increased water consumption in slaughterhouse facilities, and consequently the treatment requirements. Wastewaters from slaughterhouse are normally submitted to a primary treatment witch generally include the use of screens, settlers and fat separators (Martinez, *et al.*, 1995; EC-IPPC2005; Mittal, 2006). Some slaughterhouse wastewater treatment plants have a secondary anaerobic reactor, usually based on UASB or EGSB systems, due to the high organic content of these wastewaters. Large experiences at laboratory, pilot and industrial scale of those reactor configurations are reported in literature (Torkian *et al.*, 2003; Mittal, 2006; Del Neri *et al.*, 2007).

Although the reported difficulties in slaughterhouse waste treatment, such as, hardly degradable substrate, high organic content, ammonia and LCFA inhibitory processes or possible biomass wash-out, some strategies were remarkable. Most of them are based on adapting anaerobic biomass to efficiently degrade these substrates. Addition of adsorbents to protect biomass (Angelidaki *et al*., 1990), the application of feeding patterns like pulse feeding (Cavaleiro *et al*., 2008) or the addition of easily degradable substrates (Kuang *et al*., 2006) were demonstrated to achieve that objective.

The aim of this work is to study the anaerobic biodegradability of a real mixture of piggery-cattle slaughterhouse waste and to identify the main process difficulties.

3.2 MATERIAL AND METHODS

3.2.1 Analytical Methods

Total solids (TS), volatile solids (VS), suspended volatile solids (VSS), total Kjeldhal nitrogen (TKN), ammonia nitrogen (NH_4^+-N), chemical oxygen demand (COD) and pH were determined according to Standard Methods (APHA, AWA. WEF, 1995). According to the high organic content and lipids concentration of the sampled animal by-products, it was necessary to modify the COD close reflux titrimetric method (Standard Methods, 5220C), to force the reducing conditions, by increasing digestion temperature till 350ºC (2h) and sulphuric (H_2SO_4)/dichromate ($K_2Cr_2O_7$) reagents concentrations.

Fat content was determined by a Soxtec™ 2050 extraction equipment (Foss, Denmark) according to Standard Methods (APHA, AWA, WEF, 1995) and recommendations of n-hexane extractable material (HEM) for sludge, sediments, and solid samples (EPA 2005, Method 9071b).

Methane (CH_4) content in produced biogas was determined by a gas chromatograph CP-3800 (Varian,USA) fitted with Hayesep Q 80/100 Mesh (2mx1/8"x2.0mmSS) packed column (Varian, USA) and TCD detection, as described elsewhere (Angelidaki *et al.*, 2009).

VFA - acetate (Ac), propionate (Pro), iso and n-butyrate (Bu), and iso and n-valerate (Va) were determined with a CP-3800 gas chromatograph (Varian, USA), fitted with Tecknokroma TRB-FFAP capillary column (30m×0.32mm×0.25µm) and FID detection, according to Campos *et al.* (2008).

3.2.2 Slaughterhouse waste mixture

Solid slaughterhouse waste (animal by-products Category 2 and 3, according to the EC no 1774/2002) were collected from a piggery/cattle slaughterhouse facility (Huesca, Spain). The selected solid waste fractions were: *cattle/piggery meat* and *fatty waste*, *kidneys, lungs* and *livers*, *piggery stomach* and *intestinal mucus* and *cattle rumen content*. Other fractions like *hair*, *horns* or *toenails* were not considered due to their characteristics (low organic content and pumping operational problems). All sampled fractions were minced (*4mm*) and characterized according to Analytical Methods section. Protein content was estimated by the organic nitrogen content (difference between NTK and NH_4^+-N), applying a conversion factor of *6.25* $g_{PROTEIN}\ g^{-1}_{Norg}$. The difference between organic matter (as VS), fats (extracted by the soxtec method) and the previously estimated protein, was associated to carbohydrates. Theoretical mixture COD concentration was calculated applying the conversion factors for carbohydrates(C), proteins (P) and lipids (L) of; *1.06, 1.50* and *2.87* $g_{COD}\ g^{-1}$, respectively.

A preliminary mixture containing all those selected animal by-products, in a proportion analogous to the slaughterhouse by-products production streams, was produced (SW1). Due to the heterogeneity and high organic content of that mixture, and to better perform the sample characterization, minced SW1 mixture was freeze-dried (Telstar, Spain) previously to be analysed. The experimental measured COD value was further compared with the previously estimated theoretical COD concentration from the individual fractions.

The fraction *cattle meat* and *fatty waste* corresponded to a *40%* of the slaughterhouse solid waste production (SW1). Due to its high lipid content, this fraction provides an income to the slaughterhouse facility. Consequently, and to better study the effect of lipid and protein content over anaerobic digestion, a realistic mixture SW2, without that fraction, was also prepared and characterized.

Other waste streams were also produced in the slaughterhouse facility, like *waste blood*, wastes from slaughterhouse wastewater treatment plant and *wastewater*, which corresponds to an *85-90%* of total waste production. The

wastewater treatment facility of the present slaughterhouse consists on a primary treatment that includes several screens and a dissolved air flotation system (DAF). Consequently *screened waste* and *DAF sludge* were also considered as waste material flows.

All the previously reported slaughterhouse waste fractions (solid and liquid) were considered to perform two representative slaughterhouse mixtures (M1 and M2), which composition is a function of the contained fraction of animal by-products solid waste (SW1 and SW2, respectively)

3.2.2 Experimental set-up

The previously characterized slaughterhouse mixtures (M1 and M2) were used as substrates in anaerobic batch tests, to determinate the influence of different lipid/protein ratio and a possible process inhibition by increasing the organic content.

Initial, anaerobic biodegradability tests of the slaughterhouse mixtures were performed, following Soto *et al.* (1993) and Angelidaki *et al.* (2009). Substrates (M1 and M2) were introduced in glass flaks of *1,000 ml* (*500 mL* working volume) up to a final organic concentration of *5* g_{COD} L^{-1}, supplementing the media with macro and micronutrient solution. A bicarbonate solution was also added (*1* g_{NaHCO3} $g^{-1}_{CODadded}$) and the pH was adjusted to neutrality. Anaerobic suspended sludge, sampled from a mesophilic reactor of a large scale municipal wastewater treatment plant (Barcelona, Spain) was used as anaerobic seed, at a concentration of *5* g_{VSS} L^{-1}. The flasks were stirred and bubbled with N_2/CO_2 gas in order to remove O_2 before closing them with rubber stoppers. A reducing solution was finally added (*5 ml* of *10* g_{Na2S} L^{-1}). The flasks were incubated at *35ºC* under strict anaerobic conditions. Control vials, with only biomass and anaerobic media, were also run to obtain biogas production from residual organics at the inocula and to estimate the net biogas production from slaughterhouse mixtures.

Vials containing M2 substrate, were used to study the effect of sequential pulses of increasing COD concentration and to identify inhibitory process. After substrate exhaustion (methane plateau) in the biodegradability tests (1rst pulse), vials containing M2 mixture were opened and a higher concentration of that slaughterhouse mixture (*10* g_{COD} L^{-1}) was added (2nd pulse). The flasks were bubbled again with N_2/CO_2, closed and monitored till null methane production. Finally, a pulse of M2 substrate (3rd pulse) at *15* g_{COD} L^{-1}, was added, following the same procedure.

Each treatment was performed in triplicate, and CH_4 was monitored by gas headspace analysis, according to Analytical Methods section. Periodically, liquid samples (*2.5 mL*) were withdrawn to determine soluble VFA evolution. The obtained

methane production results were expressed as methanogenic conversion ($\%COD_{CH4}/COD_{in}$), methane yield ($L_{CH4}\ kg^{-1}_{CODin}$) and maximum methane production rate ($L_{CH4}\ kg^{-1}_{\ VSSin}$), determined by the maximum slope of the accumulated CH_4 production curve per unit of initial content of biomass (VSS).

3.3 RESULTS AND DISCUSSION

3.3.1 Slaughterhouse waste characterization

Characterization of individual solid animal by-products was summarized in Table 3.1. Representative minced mixtures containing the individual streams, according to Material and Methods sectionto obtain different lipid-protein (L/P) waste ratios (in SW1 and SW2), were also reported in Table 3.1.

The obtained mixtures can be defined as high organic content substrates (*870-1,350* $g_{COD}\ kg^{-1}$) composed mainly by lipids (*68-82% fats/VS*). Hejnfelt and Angelidaki (2009) reported mixtures of piggery slaughterhouse by-products (*blood, meat, fat, bones*, *pressed raw waste* and *bone flour*) but with a lower content of lipids (only *23.6% fats/VS*), probably due to the introduction of meal fractions and to the absence of cattle by-products. In the present mixtures, some slaughter fractions, like *meat tissues* or other small fraction from evisceration process like *confiscates*, are responsible of a remarkable protein content (*12-20% protein/VS*). Only the cattle rumen content fraction, which contained a significant amount of COD associated to carbohydrates (*42% of COD*), probable related to partially digested lignocellulosic material or crude fibre concentration of rummenal content (Triit and Schuchard, 1992), introduced a small amount of carbohydrates (*<10% carbohydrates/VS*) in the final SW mixtures (Table 3.1).

Characteristics of other slaughterhouse waste streams, produced by waste liquid and wastewater management, were summarized in Table 3.2. *Waste blood*, although being a by-product with a low solids concentration, is responsible of part of the protein content of the global slaughterhouse waste mixture (M). In literature there are reported values of organic nitrogen content in slaughterhouse blood of *25-40* $g_N\ L^{-1}$ (Tritt and Schuchardt, 1992; Lopez *et al.*, 2006; Hejnfelt and Angelidaki, 2009), similar to the ones summarized in Table 3.2. Solid waste from wastewater treatment process (*screening waste* and *DAF sludge*) also presented a remarkable organic content. Tritt and Schuchardt (1992) reported organic contents for those fractions of *30-40* $g_{COD}\ kg^{-1}$ and *95-400* $g_{COD}\ kg^{-1}$, respectively, in the range of the values shown in Table 3.2. Althougt being minor fractions, its interest have been reported by Loustarinen *et al.* (2009). In that work, the biogas production of sewage

Table 3.1*. Characterization of slaughterhouse fresh sampled fraction and representative solid waste mixtures according to materials flows (SW1 and SW2*

Parameter	Cattle meat and fatty	Piggery meat and fatty	Confiscates (kidneys, lungs and livers)	Piggery stomach and intestinal mucus	Cattle rumen content	SW1	SW2
TS (g kg^{-1})	885.58±4.81	564.54±19.18	244.89±2.86	182.89±7.88	116.86±5.04	**527.13**±7.47	**374.53**±7.50
VS (g kg^{-1})	854.14±4.80	557.33±20.79	219.67±3.57	179.72±7.88	108.68±4.35	**521.52**±7.70	**366.94**±7.23
TKN (g kg^{-1})	3.21±0.54	13.80±0.36	26.32±0.30	12.38±0.69	1.32±0.18	**10.75**±0.11	**11.69**±0.11
NH_4^+-N (mg kg^{-1})	148.51±2.06	389.70±64.67	1,512.37±19.53	1,645.81±15.00	60.83±2.05	**274.51**±77.64	**537.55**±195.10
$Protein_{estimated}$ *(g kg^{-1})*	*19.14*	*83.80*	*155.04*	*67.10*	*7.88*	***65.48***	***69.73***
Fat_{soxtec} (g kg^{-1})	762.79±2.39	467.30±14.90	46.60±1.50	86.64±0.01	18.42±1.18	**432.07**±4.80	**249.38**±2.45
COD (g kg^{-1})	n.d	n.d	n.d	n.d	n.d	**1,356.03**±21.66	**849.24**±77.99
$COD_{estimated}$ *(g kg^{-1})*	*2.294.47*	*1,473.46*	*385.40*	*376.85*	*152.01*	***1,363.66***	***871.01***

Table 3.2*. Characterization of slaughterhouse liquid streams and waste from wastewater treatment plant*

Parameter	Waste blood	Screening waste	DAF sludge	Wastewater
TS (g kg^{-1})	88.98±0.60	242.30±2.72	95.65±0.78	0.93±0.02
VS (g kg^{-1})	76.96±0.46	233.34±2.61	82.66±0.76	0.53±0.03
TKN (g kg^{-1})	11.76±0.11	12.40±0.32	5.87±1.52	0.15±0.01
NH_4^+-N (mg kg^{-1})	1,341.21±61.19	1,502.21±20.14	861.00±9.90	84.00±2.30
$Protein_{estimated}$ *(g kg^{-1})*	*65.12*	*68.12*	*31.29*	*0.41*
Fat_{soxtec} (g kg^{-1})	n.d	107.11±0.64	50.30±0.05	0.10±0.01
$COD_{estimated}$ *(g kg^{-1})*	*110.23*	*471.18*	*192.44*	*0.92*

sludge reactor was increased by the co-digestion with grease trap sludge from a meat processing plant.

Raw wastewater, from washing down and cleaning operations, presents a low organic content, mainly as proteins due to the usual blood content of those streams (del Pozo *et al.*, 2003). The main function of the *wastewater* stream (characterized in Table 3.2), in the present batch experiments, was to dilute the high organic content of animal by-products and other slaughterhouse fractions, allowing direct digestion of those substrates without the need of dilution.

Table 3.3 summarizes the estimated composition of the performed representatives slaughterhouse waste mixtures (M1 and M2), according to the previously described fractions and criteria explained in Material and Methods section. Those substrates present a solid content suitable to be loaded to an anaerobic reactor, with an estimated organic content of over *80-200* $g_{COD}\ L^{-1}$.

Table 3.3*. Estimated composition of the slaughterhouse wastemixtures tested in batch assays (M1 and M2)*

Estimated parameters	M1	M2
TS (g kg^{-1})	79.64	36.00
VS (g kg^{-1})	77.94	34.36
TKN (g kg^{-1})	1.89	1.67
NH_4^+-N (mg kg^{-1})	66.39	58.51
Protein (g kg^{-1})	11.38	10.08
Fat (g kg^{-1})	63.29	20.92
COD(g kg^{-1})	203.36	79.96

3.3.2 Batch anaerobic biodegradation of slaughterhouse waste.

First anaerobic batch tests were based on standardized biodegradability assays. The two slaughterhouse mixtures (M1 and M2) with different lipid/protein (L/P) ratio substrates (prepared from the adition of SW1 or SW2) were introduced inside buffered anaerobic media, at a concentration of *5* $g_{COD}\ L^{-1}$, in the presence of *5* $g_{VSS}\ L^{-1}$ of biomass, as described in Material and Methods section. Those conditions guaranteed an excess of biomass, avoiding overloading or inhibitory processes (Angelidaki *et al.*, 2009) and allowing to determinate methanogenesis and biodegradability indexes (related to *CODin*), according to Soto *et al*. (1993). With obtained results it was estimated the methane yield ($L_{CH4}\ kg^{-1}_{CODin}$), the maximum methane production rate ($L_{CH4}\ kg^{-1}_{VSS}$) and the substrate potential ($L_{CH4}\ kg^{-1}_{substrate}$). All those process indicators are summarised in Table 3.4. The time-profiles evolution of the accumulated net CH_4 production and VFA intermediates, both in COD units, are plotted in Figure 3.1.

From Table 3.4, it can be stated a high biodegradability index for both substrate mixtures (*94.9* and *86.1* % for M1 and M2, respectively), but obtaining lower values with protein rich mixture, and consequently also lower methane yield (*301.7* and

273.6 L_{CH4} kg^{-1}_{CODin} for M1 and M2, respectively). Those differences can be related with the content of hard-to-degrade proteins in animal by-products (matrix proteins, collagen and keratine), considered to be strongly resistant to proteinases because of their structural features (Suzuki *et al.*, 2006), remaining un-degraded at the end of the experimental time.

Table 3.4. *Mean values and standard deviation of estimated biodegradability parameters*

Estimated parameters	M1	M2
L/P (COD/COD)	10.6	4.0
% Methanogenesis (% CODin)	86.2±0.3	78.2±2.2
% Biodegradability (% CODin)	94.9±0.3	86.1±2.4
Methane Yield (L CH_4 kg^{-1} CODin)	301.7±1.1	273.6±7.7
Max Methane Prod. Rate (L CH_4 kg^{-1} VSSin d^{-1})	19.9±0.1	28.2±0.2
Methane potential (L CH_4 kg^{-1} substrate)	102.1±0.4	33.2±0.9

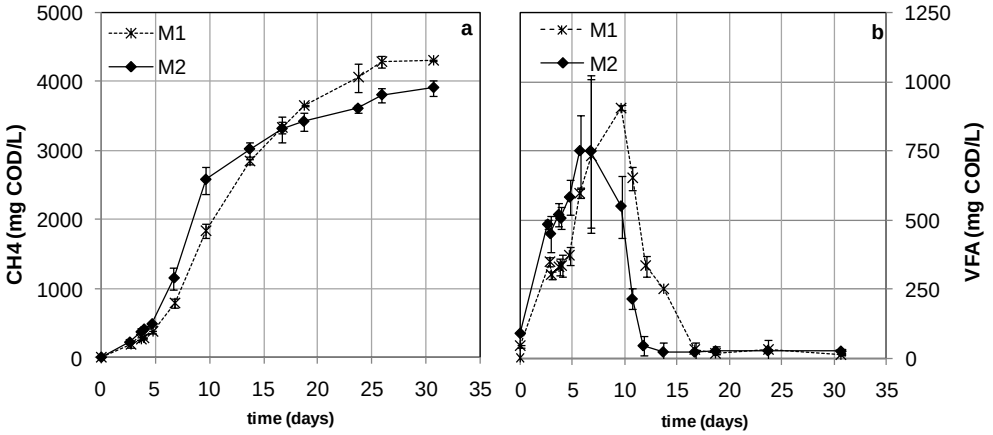

Figure 3.1*. CH_4 accumulated production (**a**) and intermediate VFA profile (**b**) during biodegradability assays of slaughterhouse mixtures M1 and M2. All parameters are expressed in COD equivalent concentration units (mg $_{COD}$ L^{-1}).*

Although the reported lower biodegradability index and methane yield in the protein rich substrate (M2), the global process kinetics seems to be faster than with the lipid rich substrate (M1). Higher maximum methane production rates were obtained when increasing the relative protein content (*19.9* and *28.2 L_{CH4} kg^{-1}_{VSS} d^{-1}*, for M1 and M2, respectively), according to Table 3.4. In lipid rich substrate (M1 vials) an initial delay in substrate degradation, monitored as a delay in the CH_4 formation (Figure 3.1a) and longer time accumulation of total VFA (Figure 3.1b), was observed. In literature, when treating higher concentration slaughterhouse lipidic wastes in anaerobic batch assays, it was reported even longer initial lag phases due to the limiting effect of LCFA (Hejnfelt and Angelidaki, 2009). Opposite, Kuang *et al*. (2006)

testing the addition of easily degradable proteins (cystein) to recover LCFA inhibited reactors, obtained a stimulating effect of proteins by increasing the number of bacteria cells, allowing faster lipid degradation. Consequently, these effects could explain the better results obtained with M2 mixture, compared with M1 substrate

Due to the higher COD content of lipids compared to proteins, the mixture with higher L/P ratio (M1) presented a higher methane potential (over *100* L_{CH4} $kg^{-1}_{substrate}$), but, as previously discussed, presented the possibility of higher process imbalance or delay. Consequently, mixture M2, containing a lower amount of fats over total COD, was selected as slaughterhouse substrate for further sequential batch tests, with increasing organic loads.

3.3.3 Sequential batch tests (*process inhibition*)

Anaerobic degradation of successive increasing concentrations of slaughterhouse substrate (M2) was also assessed in batch. After the previously described biodegradability assay, substrate concentration was increased up to *10* and *15* g_{COD} L^{-1}. Methane production and individual VFA concentration were monitored along experimental time (*120 days*), according to Material and Methods section and showed in Figure 3.2. Obtained results are summarized in Table 3.5.

Methane conversion of the *2nd* M2 pulse (*10* g_{COD} L^{-1}) reached a *74.9%* of the introduced COD, slightly lower value than the obtained in the *1rst* pulse or biodegradability test, while a similar methane yield value (*262.0* L_{CH4} kg^{-1}_{CODin}) was obtained, according to Table 3.5. From the methane production curve (Figure 3.5a) it was observed an earlier and faster methane formation, resulting in a higher maximum methane production rate, of *30.20* L_{CH4} kg^{-1}_{VSSin} d^{-1}, compared to the previous pulse or biodegradability assay (Table 3.5). Also the acetate (C2), propionate (C3) and butyrate (C4) consumption, after an initial accumulation, was faster (Figure 3.5b-3.5d).

Although in the *2nd* pulse the lipid and protein concentration have been increased up to values of *7.51* and *1.90* g_{COD} L^{-1}, respectively, not clear system inhibition was detected. Consequently, a *3rd* M2 pulse, of *15* g_{COD} L^{-1}, was applied following the same procedure. From the methane production curve (Figure 3.2a) it was observed a clear long lasting *lag phase* in methane formation of over *21 days*. Also, acetate (C2), propionate (C3) and butyrate (C4) were accumulated (Figure 3.2b-3.2d). Unfortunately only 2 vials resisted all the experimental time. This was the reason for the high results dispersions detected on *3rd* pulse (Figure 3).

Acetate (C2) is considered to be the main product of LCFA ß-oxidation process (Weng and Jeris, 1976), and it was found to be the main VFA accumulated in present batch test (Figure 3.2b). Since LCFA are not presented in nature with even carbon number, propionate (C3) accumulation must be related to protein degradation

(Figure 3.2c). Flotats *et al*. (2006) reported propionate as the longer time VFA accumulated during gelatine anaerobic degradation. Unfortunately, due to; the relative low protein content introduced in vials (max *2.7* $g_{CODprotein}$ L^{-1}in *3rd* pulse), the organic nitrogen contribution of biomass and due the low accuracy in N-NH_4^+ determinations, it was not possible to monitor protein degradation. However, Salminen *et al*. (2002) suggested that propionate degradation can be inhibited by LCFA accumulation. The reported lag-phase in CH_4 formation (Figure 3.1a) and the C2 and C4 accumulation (Figure 3.2b and d) in the *3rd* pulse, seems to be more related to lipids or LCFA inhibitory process than with ammonia inhibition, since ammonia concentration is much lower than values reported as inhibitory (Hansen *et al.*, 1996).

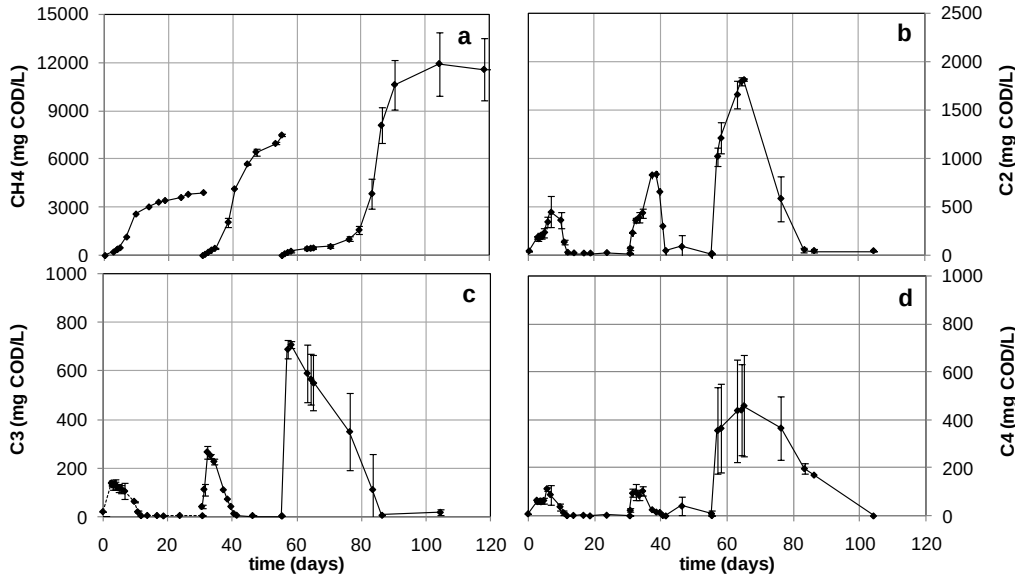

Figure 3.2. *CH_4 accumulated production and intermediate acetate (C2), propionate (C3), butirate (C4) and valerate (C5) profile during batch assay of slaughterhouse mixture M2 at different initial concentration (5, 10 and 15* g_{COD} L^{-1}*). All the others parameters are expressed in COD equivalent concentration units (mg* $_{COD}$ L^{-1}*).*

Table 3.5. *Mean values and standard deviation of estimated batch test parameters with M2 mixture*

Estimated parameters	1rst pulse	2nd pulse	3rd pulse
% Methanogenesis (% CODin)	78.2±2.2	74.9±0.8	77.8±3.4
Methane Yield (L CH4 kg-1 CODin)	273.6±7.7	262.0±2.8	272.2±11.8
Max Methane Rate (L CH4 kg-1 VSSin d-1)	**28.2±0.2**	**30.2±1.6**	**35.8±11.4**

LCFA inhibitory effect was initially been related to permanent toxic effect (Rinzema *et al.*, 1994). Further studies have demonstrated that the LCFA inhibitory effect is a reversible phenomenon, related to the physical adsorption of LCFA witch can hinder the transfer of substrate and metabolites through microbial cell walls (Pereira *et al.*, 2005). Consequently, LCFA inhibition is usually monitored as an initial delay in CH_4 production, or as a longer *lag phase* (Pereira *et al.*, 2005; Cavaleiro *et al.*, 2008; Hejnfelt and Angelidaki, 2009), according to the system response shown in Figure 3.2.

From present results, it can be stated an inhibitory process after the *3rd* pulse (corresponding to *11.2* $g_{CODlipid}\ L^{-1}$), probably related to LCFA inhibition. However, when system was recovered from inhibition (VFA intermediates consumed), and improvement on maximum methane production rate (*35.8* $L_{CH4}\ kg^{-1}_{VSSin}\ d^{-1}$) was achieved, obtaining a final methane yield (*272.2* $L_{CH4}\ kg^{-1}_{CODin}$) quite similar to the first pulse value or not inhibited system (Table 3.5). Those results could indicate a biomass adaptation phenomena or system improvement towards slaughterhouse waste degradation. As initial biomass in present experiments was not washed out from vials (only new substrate was added) the detected system "improvement" can be partially explained by biomass growth. Salminen *et al*. (2000) described the *waste-to-inocula ratio* as the main factor on solid poultry slaughterhouse waste treatment and possible process inhibition. In previous works about modelling the anaerobic degradation of similar slaughterhouse mixtures, there were obtained good model fittings considering a first order kinetics for protein degradation while it was necessary to use a Contois kinetics, that consider also the growth of hydrolytic-acidogenic bacteria population, to fit the initial delay in the hydrolysis/acidogenesis of lipids (Palatsi *et al.*, 2007).

Cavaleiro *et al.* (2008) evidenced the boundary of recurrent pulse feeding strategies to achieve higher acetotrophic methanogenic tolerance to LCFA and Neves *et al.* (2009) demonstrated that controlled intermittent inputs of oil can enhance methane production in co-digestion of cow manure and food waste. Consequently the recovery capacity should be partially related to specific biomass growth, but microbial biology techniques or accurate data modelling would be needed to determinate whether biomass growth is specifically related to lipid-LCFA degrading microorganisms.

The previous results suggest the need to study strategies to prevent or to recover reactors inhibition by LCFA, to study the influence of biomass/LCFA ratio and to study the inhibition-adaptation dynamics, in order to guide the design and operation of reactors loaded with high lipid contents, such as the slaughterhouse waste.

3.4 CONCLUSIONS

Animal by products, wastewaters and other organic waste produced in cattle-piggery slaughterhouse facilities were exhaustively characterized. Those substrates present a high organic content, mainly lipid and proteins, being interesting for an anaerobic digestion process.

Representative mixtures of slaughterhouse waste streams, with different lipid and protein content were performed, and anaerobic biodegradability assessed in standardized batch tests. As expected, the methane potential of lipid rich substrate was higher, but the protein content of the substrates seems to have an effect over the global anaerobic process kinetics, increasing the maximum methane rates when decreasing the lipid/protein ratio.

Increasing the concentration of slaughterhouse waste in sequential batch test, a clear inhibitory phenomenon was detected, monitored as a long lag-phase in methane formation and volatile fatty acids accumulation, probable associated to LCFA inhibition process. The propionate accumulation profile also indicated a possible interaction of proteins on lipids or LCFA degradation.

Although the severe reported inhibition, the system was able to recover the methanogenic activity and to degrade the slaughterhouse waste pulse, obtaining similar methane yields than in non inhibited systems or biodegradability assays. The reported system capacity to recover activity can be partially related with the growth of specific biomass. Consequently, pulse feeding strategies, for adapting microorganisms and efficiently degrade lipid rich substrates, were confirmed as a reliable strategy to face with slaughterhouse waste.

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Strategies for recovering inhibition caused by long-chain fatty acids on anaerobic thermophilic biogas reactors

ABSTRACT. Long chain fatty acids (LCFA) concentrations over *1.0 g L-1* were inhibiting manure thermophilic digestion, in batch and semi-continuous experiments, resulting in a temporary cease of the biogas production. The aim of the work was to test and evaluate several recovery actions, such as reactor feeding patterns, dilution and addition of adsorbents, in order to determine the most appropriate strategy for fast recovery of the reactor activity in manure based plants inhibited by LCFA. Dilution with active inoculum for increasing the biomass/LCFA ratio, or addition of adsorbents for adsorbing the LCFA and reducing the bioavailable LCFA concentration, were found to be the best recovery strategies, improving the recovery time from *10* to *2 days*, in semi-continuously fed systems. Moreover, acclimatisation was introduced by repeated inhibition and process recovery. The subsequent exposure of the anaerobic biomass to an inhibitory concentration of LCFA improved the recovery ability of the system, indicated as increasing degradation rates from *0.04* to *0.16* $g_{COD\_CH4}\ g^{-1}_{VS}\ d^{-1}$. The incubation time between subsequent pulses, or discontinuous LCFA pulses, seems to be a decisive process parameter to tackle LCFA inhibition in manure anaerobic codigestion.

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4.1. INTRODUCTION

Anaerobic digestion is a process widely applied for treatment of organic waste and residues, and in Denmark particularly for manure treatment. The economic viability of manure-based Danish centralized and farm-scale biogas plants depends on, among other factors, the specific production of methane per unit of treated waste material. The high water content, together with the high fraction of fibers in manure, is the main reasons for the low methane yield per weight. However, manure is excellent as a "matrix" to allow anaerobic digestion of concentrated industrial waste due to its high buffering capacity and its content of a wide variety of nutrients, necessary for optimal bacterial growth (Angelidaki and Ellegaard, 2003). On the other hand, waste from food industry, and especially lipid containing waste, have a high methane potential which can contribute to increase biogas production and consequently to improve the plant economy (Salminen and Rintala, 2002a).

In anaerobic treatment systems, lipids are rapidly hydrolysed by extracellular lipases to long-chain fatty acids (LCFA) and glycerol. LCFA are further degraded to acetate and hydrogen through β-oxidation process (Weng and Jeris, 1996). Exploitation of the biogas potential of lipids is difficult, because lipid containing waste often have low content of nutrients, low alkalinity (Angelidaki and Ahring, 1997a and 1997b) and, mainly, due to their toxicity towards the anaerobic digestion process (Hanaki *et al.*, 1981; Hwu *et al.*, 1996; Rinzema *et al.*, 1994). Moreover, problems with anaerobic treatment of lipids are caused by the adsorption of light lipid layer around biomass particles causing biomass flotation and wash-out (Hwu *et al.*, 1997).

Adsorption of LCFA onto the microbial surface has been suggested as the mechanism of inhibition, affecting transportation of nutrients to the cell (Alves *et al.*, 2001a; Alves *et al.*, 2001b; Hwu *et al.*, 1998). The LCFA inhibition is dependent on the type of microorganism, the specific surface area of the sludge, the carbon chain length and of the saturation (C=C) of LCFA (Hwu *et al*., 1996; Salminen and Rintala, 2002a). It has been reported that LCFA are inhibiting anaerobic microorganisms at very low concentrations, with IC_{50} values for oleate over *50* and 75 mg L^{-1} (Alves *et al.*, 2001b; Hwu *et al.*, 1996), palmitate over 1*,100 mg* L^{-1} (Pereira *et al.*, 2005) or stearate over *1,500 mg* L^{-1} (Shin *et al.*, 2003), at mesophilic temperature range. Although thermophiles are more susceptible to LCFA toxicity compared to mesophiles, they recover faster after LCFA-inhibition due to their faster growth rates (Hwu and Lettinga, 1997). Methanogens were reported to be more susceptible to LCFA inhibition compared to acidogens (Lalman and Bagley, 2002; Mykhaylovin *et al*., 2005; Pereira *et al*., 2003). Fortunately, inhibition caused by LCFA is a reversible process; neither syntrophic acetogenic nor methanogenic activities were irreversibly damaged, since the rate of methane formation increased dramatically within a short

time after the LCFA-biomass associated degradation had recommenced (Pereira *et al*., 2003 and 2005).

Inhibition by LCFA is often causing serious process problems in biogas plants. Therefore, methods to overcome inhibition would have a significant advantage for the safe and stable operation of codigestion plants. Although LCFA are inhibitory for the anaerobic biogas process at low concentrations, acclimatization of the anaerobic process to LCFA has been reported. Continuous or pulse exposure has lead to increased tolerance to LCFA (Alves *et al*., 2001a; Cavaleiro *et al*., 2008; Hwu *et al.*, 1997). Moreover, methods such as codigestion (Fernandez *et al*., 2005), addition of adsorbents (Angelidaki *et al.*, 1990) or addition of easily-degradable co-substrates, like glucose and cysteine (Kuang *et al.*, 2002 and 2006), have been used for overcoming LCFA inhibition. Discontinuous feeding of the system to promote development of an active anaerobic community, able to efficiently convert lipid-rich effluents, has been also suggested (Cavaleiro *et al.*, 2008; Nadais *et al.*, 2006).

Although many studies are dealing with LCFA inhibition, only limited attention has been paid to recovery strategies for an anaerobic process that has been inhibited by LCFA. In the present study we have tested and evaluated different strategies based on feeding patterns, dilution and absorption strategies, for fast recovery of LCFA inhibited anaerobic digestion of manure. The recovery strategies were investigated in batch and semi-continuously fed reactors. Moreover, the effect of process acclimatization was investigated by repeated inhibition by LCFA and subsequent process recovery.

4.2. MATERIALS AND METHODS

4.2.1 Analytical Methods

Total solids (TS), volatile solids (VS), total Kjeldhal nitrogen (TKN), ammonia nitrogen (NH_4^+-N) and pH were determined according to Standard Methods (APHA-AWA-WEF, 1995). Methane content (CH_4) and volatile fatty acids (VFA) in batch and semi-continuously fed reactors were measured with GC-TCD (MGC 82-12, Mikrolab a/s, Denmark) and GC-FID (GC 20100, Shimatzu, Japan), fitted with packed (¼" Molsieve+1/4"Cromosorb 102 and reference column: 1/8" Molsieve) and capillary (ZEBRON Phase ZB-FFAP) columns respectively, as described elsewhere (Angelidaki *et al.*, 1990)

For determination of LCFA in biological samples, some direct procedures based on direct methanolic-HCl solution were tested with good results (Neves *et al.*, 2009; Sönnichsen and Müller, 1999). In the present study, a new method using clorotrimethylsilane (CTMS) as fatty acids methyl esters (FAME) catalyst, without

prior extraction over lyophilized samples, was developed, based on Eras *et al.* (2004) methodology. This methodology can be used to determinate total fats and LCFA in solid, liquid or paste samples. Moreover the method allows small amount of sample to be used, reducing the reaction temperature and processing time, characteristics often needed on biological samples. Anaerobic reactor samples, from *0.5* to *1 mL*, were transferred together with Extraction Standard (ES), heptadecanoic acid (C17:0, 51610 Fluka puriss. >99.0%), to screwed pirex glass tubes (10 mL) and lyophilized overnight at -40ºC. For soluble LCFA ($LCFA_S$) determination, samples were previously centrifuged (*2x3,500 rpm*) and only soluble fraction was placed on the pirex tubes. Afterwards a magnetic stir bar was introduced together with *0.5 mL* of CTMS (CTMS GC Panreac 352776.0207) and *1 mL* of N_2 saturated methanol, under a hood fume, tighten the vials with teflon screw cup and shacked at vortex for *1 minute*. The tubes were introduced into aluminium block and maintained in stirring and heating (*90ºC*) for *1.5 h* reaction time. When the vials were at room temperature, were opened and *1* to *5 mL* of hexane was added (dilution in order to obtain the desired concentration of *0.5-600 mg* L^{-1}). Commercial powder $NaHCO_3$ was added till no reaction (effervescence) was detected, and finally *2 mL* of saturated solution of $NaHCO_3$ was added. The vials were shaken in vortex again and centrifuged (*10 min 3,500 rpm*) till phase separation. *900 µL* of the organic phase were directly transferred to GC vial, together with *100 µL* of methyl pentadecanoate (C15:0 FAME, Fluka 76560 puriss. p.a. standard for GC) as internal standard (IS).

FAME were identified and quantified by GC 3800 gas chromatograph (Varian, USA), fitted with CP7489:CP-Sil 88 FAME capillary column (50m0.25mm0.2µm, Varian, USA), flame ionization detector (FID) and equipped with auto sampler (CP 8400. Varian, USA). The FID was supplied with H_2 and synthetic air, while He was used as carrier and make-up gas with a flow rate of *2 mL* min^{-1}. Samples of *1µL* were injected in split mode. The oven initial temperature was *60ºC* during *1 min*, then increased to *100ºC* at *25ºC* min^{-1}, to *160ºC* at *10ºC* min^{-1}, to *240ºC* at *4ºC* min^{-1}, with a final isotherm step of *5 min*. Injector and detector temperature were set constant at *270ºC* and *300ºC* respectively. 36 different FAME from C6:0 to C24:1 were calibrated using FAME GC mixture (Supelco 18919-1AMP FAME Mix C4-C24) and IS, from *0.5-600 mg* L^{-1}, The recovery of LCFA, was determined by the ES (C17:0) recovered in blanks and real digested manure samples, and it was always over *87.5 %* in all determinations.

4.2.2 Substrates and Inoculum

Cow manure was used as basis substrate. The manure was diluted with distilled water in order to decrease the ammonia level and ensure that LCFA was the only

inhibitor in the experiments. The diluted manure used had an average concentration of *2.5% TS* and *2.0% VS* (Table 4.1).

Digested thermophilic effluent from a biogas pilot-scale plant (PP), digesting cow manure located at DTU (Kongens Lyngby, Denmark), with an average concentration of *3.0% TS* and *2.2% VS*, was used as initial inoculum for experiments. In the subsequent experiments, inoculum was provided from the effluent of the reactors used in the present experiments. Table 4.1 summarizes the characteristics of substrates, adsorbents and inoculum used in batch and semi-continuously fed reactors.

To impose LCFA inhibition to the biogas process, a LCFA mixture (LCFA), consisting of sodium oleate (C18:1), sodium stearate (C18:0) and sodium palmitate (C16:0) in a ratio of *40:10:50* (*w/w/w*) respectively (analytical grade, BDH Chemicals Ltd, Poole England), was used. This LCFA simulated the 3 major constituents in slaughterhouse wastewater sludge (Hwu *et al.*, 1998), which is considered to be one of co-substrates interesting in manure based biogas plants.

Commercial powder bentonite (Al_2O_3 $4SiO_2$ H_2O Prod 18609 Sigma-Aldrich, St. Louis USA) and fibers, obtained from filtered digested manure, were used as absorbents for the experiments testing adsorption strategies. Initially, fibers were obtained from a Danish manure centralized biogas facility, while in the subsequent experiments were manually obtained by filtration of digested manure from a pilot scale plant (Kongens Lyngby, Denmark). This caused some changes in composition (Table 4.1), however, the same VS amount of fibers were added to the reactors in all experiments.

Table 4.1. *Analysis of substrate, inoculum and adsorbents used in the experiments*

	Diluted Manure			**Inoculum**			**Fibers**		**Bentonite**
	BTA	**E1**	**E2**	**BTA**	**E1**	**E2**	**E1**	**E2**	**E1&E2**
TS (%w/w)	2,40 ±0.05	2.45 ±0.42	2.34 ±0.73	3.02 ±0.01	2.05 ±0.29	2.04 ±0.15	59.60 ±7.96	21.01 ±0.72	94.04
VS (%w/w)	2.0 ±0.05	1.98 ±0.38	1.93 ±0.65	2.25 ±0.01	1.47 ±0.20	1.44 ±0.18	34.80 ±4.87	18.80 ±0.70	5.09
TKN (g/kg)	-	1.31	1.41 ±0.25	-	-	1.32 ±0.06	-	5.17 ±0.20	0.28 ±0.21
NH_4^+-N (g/kg)	-	1.05	0.92 ±0.03	-	-	0.91 ±0.05	-	1.76 ±0.34	-
pH		7.52	7.49 ±0.24	-	-	7.68 ±0.19	-	8.21 ±0.01	~8 (10%H20)

4.2.3 LCFA toxicity assay (BTA)

A batch toxicity assay (BTA) was carried out to determine the toxicity level of LCFA, in manure based system, in order to estimate the amount to be added in reactors for achieving a clear long lasting inhibition of the anaerobic process.

120 ml vials were used in the BTA with a working volume of 40 ml. The assay included: blanks (*30 ml* of inoculum and *10 ml* of distilled water), controls (*30 mL* of inoculum and *10 mL* of diluted manure) and test vials with *30 ml* inoculum and *10 ml* of different dilutions of LCFA. The vials were inoculated under anaerobic conditions, while gassing with N_2 gas. Subsequently, the vials were closed with rubber stopper and aluminium crimps and were incubated at *55ºC* without agitation. The methane production in the head space of the vials was monitored by gas chromatography until biogas production ceased. Each LCFA concentration was conducted in triplicate. LCFA was added in the vials as a pulse, when the methane production from manure was increasing exponentially (at *day 5*). LCFA was added to a total concentration of *1.0*, *2.5*, *4.0* and *6.0 g* L^{-1} corresponding to *2.8*, *7.0*, *11.2* and *16.8* g_{COD} L^{-1}. Subsequently, vials were vigorously agitated until the LCFA was dissolved/emulsified. No LCFA was added in blanks and controls.

4.2.4 Reactors set-up, recovery strategies (E1 and E2)

To test the different recovery strategies eight reactors were used. Glass vials (*2.2 L* total volume; *1.0 L* working volume) closed with a rubber stopper were used as reactors. Through the rubber stoppers glass tubes with attached maprene tubes, were inserted for feeding and sampling (liquid/gas). Feeding was applied once a day (in semi-continuous experiments). The produced biogas, recovered in aluminium bags (PET/MET-ALU), was measured daily by water displacement system. The methane content of the gas was measured by GC analysis.

Recovery experiment 1 (E1) was aiming to test recovery strategies on un-adapted biomass (not pre-exposed to LCFA). All the reactors were run with manure until the process was stabilised (daily fed with fresh manure with a organic loading rate (OLR) of *1.0* g_{VS} L^{-1} day^{-1} and an hydraulic retention time (HRT) of *20 days*). This was done for achieving a stable methane production before inhibiting them with the LCFA (*4 g* L^{-1}). A control reactor ($R_{control}$), not inhibited and fed daily with fresh manure, was run during the whole experimental period. No feeding was applied to the reactors after inhibition (except for one case, see below). The recovery actions tested were:

- ***Feeding strategies***: a) No-feeding ($R_{no\text{-}feed}$) and b) continuous feeding (R_{feed}) with fresh manure, and HRT of *20 days* corresponding to an OLR of *1.0* g_{VS} L^{-1} day^{-1}.
- ***Dilution strategies***: Replacement of *40%* of the reactor content by: a) fresh manure (R_{manure}); b) digested manure or effluent from reactors before inhibition ($R_{inocula}$) and c) water (R_{water}).

- ***Adsorption strategies***: a) Addition of fibers (R_{fiber}), obtained from filtered digested manure and b) addition of bentonite powder ($R_{bentonite}$), both in the quantity of *5* g_{VS} L^{-1}.

E1 was repeated twice (RUN1 and RUN2), or two LCFA pulses were applied.

Recovery experiment 2 (E2) was aiming to test recovery strategies in the same reactors, pre-exposed to LCFA from E1. The reactors in E2 were daily fed with manure, and were subsequently exposed to inhibition by pulse addition of LCFA. The main difference between E1 and E2 was that in experiment E1 daily feeding with manure was ceased after LCFA was applied (except for R_{feed}), while in experiment E2 the daily feeding of the reactors with manure continued also after the initiation of the recovery strategy (except for $R_{no\text{-}feed}$). E2 was repeated twice (RUN 3 and RUN4), or two subsequent LCFA pulses were applied. Analysis of LCFA-FAME time course was only monitored in E2 by GC-FID.

The $R_{no\text{-}feed}$ was run only twice (one for E1 and other for E2 corresponding to RUN1 and RUN3), due to the long recovery time needed. For all the experiments, the recovery strategies tested were applied *48-72 hours* after inhibiting the system, in order to simulate full scale plant conditions, considering that some time would be necessary in an industrial facility to detect the inhibition problem and to apply the corrective strategy (at least *2 days* without biogas production). The reactors were kept inside *55ºC* incubators with continuous shaking during the whole experimental time. The experimental set up is summarised in Table 4.2.

Table 4.2. *Summary of the experimental set-up*

Exp	Reactor Config	Temp (ºC)	Agitation	LCFA (g/L)	RUN	Manure after recovery action
BTA	batch	55	no	1.0, 2.5, 4.0 and 7.0	-	-
E1	semi-continuous	55	shaker	4	RUN1&RUN2	no (except Rfeed)
E2	semi-continuous	55	shaker	4	RUN3&RUN4	yes (except Rno-feed)

To compare process performance in consecutive inhibited-recovered reactors, recovery time (days), the maximum methane production rate (g_{COD_CH4} g^{-1}_{VS} day^{-1}) and acetate maximum consumption rate (g_{COD_Ac} g^{-1}_{VS} day^{-1}) were calculated, per unit of initial measured VS (biomass). The recovery time was calculated as the time between the initiation of the recovery action and the time when the methane production rate exceeded the mean value of control reactor ($R_{control}$). The maximum methane production rate was calculated as the maximum slope of the methane yield curve, while the acetate consumption rate was calculated as the maximum

slope of the acetate consumption profile, when maximum methane production rate was achieved.

4.3. RESULTS AND DISCUSSION

4.3.1 LCFA toxicity assay

The methane production time course from the LCFA toxicity assay is shown in Figure 4.1. The methane production ceased after LCFA pulse, shown in Figure 4.1 as a decrease in the accumulated net methane production, because the methane production from control vials was subtracted (Control plotted in Figure 4.1). For all concentrations of LCFA over *1 g L^{-1}* tested, clear inhibition was detected. The methane production ceased and did not recover the control value for up to *12-17 days* for LCFA concentrations of *2.5-4.0 g L^{-1}*. For vials in witch *6.0 g L^{-1}* was added, more than *20 days* elapsed before methane production was recovered. From results, a concentration of *4.0 g L^{-1}* was chosen as the target LCFA concentration to impose inhibition on subsequent experiments E1 and E2, due to the clear and long lasting inhibition caused at this concentration.

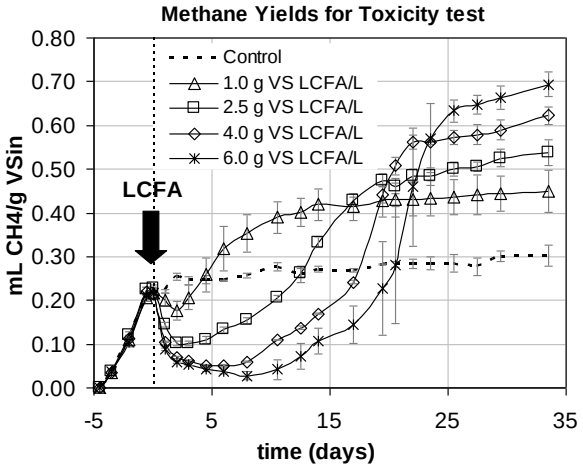

Figure 4.1. *Accumulated specific net methane production (mL CH4/g VSin) at different LCFA concentrations tested in batch experiment. Methane production of control vials was substracted from methane production of test vials with LCFA addition. Arrows indicate the LCFA time application.*

After the initial inhibition, the process self-recovered for all tested concentrations (Figure 4.1). This is in accordance with previous results, where the same pattern was observed, a temporary inhibition that was monitored as a *lag-*

phase. This phenomenon was reported to be adscribed to surface adsorption and transport sites (Cavaleiro *et al.*, 2008; Pereira *et al*., 2005).

4.3.2 E1: LCFA inhibition of un-adapted semi-continuous reactors and subsequent application of recovery strategies

As a part of the recovery strategy, the daily feeding with manure was ceased in all the reactors, after application of the LCFA pulse, except for the R_{feed} strategy and the $R_{control}$, which were fed daily with diluted fresh manure with an HRT of *20 days*. It was clear that the strategy of self-recover process ($R_{no\text{-}feed}$) was the strategy that resulted in the slowest recovery time, which was over *40 days*, compared to *9* or *7 days* in R*feed* for RUN1 and RUN2 respectively (Figure 4.2-Table 4.3). Additionally, VFA accumulation in $R_{no\text{-}feed}$ was significantly higher, *92.8 mM* compared to *47.2 mM* or *54.8 mM* in R*feed* for RUN1 and RUN2, respectively. The daily feeding of the reactor with manure (R*feed*), resulted in reduction of the inhibitory LCFA concentration, due to dilution by feeding. By calculating the expected methane production from the substrates introduced in R*no-feed* (methane production measured/theoretical production expected), it was found that over *90%* of the expected methane production was achieved. Oppositely, low methane recovery was obtained in R*feed*, indicating that part of the LCFA was washed undegraded out of the reactor, allowing the system to recover faster.

Table 4.3. *Process parameters obtained during E1 (RUN1 and RUN2).*

	Max Prod. Rate (L CH4/Lday)		Max VFA (mM)		Recovery time (days)	
RUN	**1**	**2**	**1**	**2**	**1**	**2**
Rcontrol	0.38	0.41	09.0	05.1		
Rno-feed	0.89		92.8		40	
Rfeed	1.04	1.15	47.2	54.8	9	7
Rinocula	0.81	0.50	28.3	47.9	3	3
Rmanure	1.17	1.34	39.7	43.3	4	3
Rwater	0.72	0.66	28.6	32.7	5	20
Rbentonite	0.99	1.29	50.1	56.3	7	17
Rfiber	1.39	1.68	39.2	83.7	5	6

The fastest recovery time was obtained, as expected, when the inhibited reactor was diluted with inoculum ($R_{inocula}$). *3 days* after the application of the recovery action, the process recovered and the lowest VFA accumulation was registered, *28.3 mM* (Figure 4.2 and Table 4.3). Dilution strategies, with the replacement of *40%* of reactor content, resulted in dilution of the initial LCFA concentration, estimated on

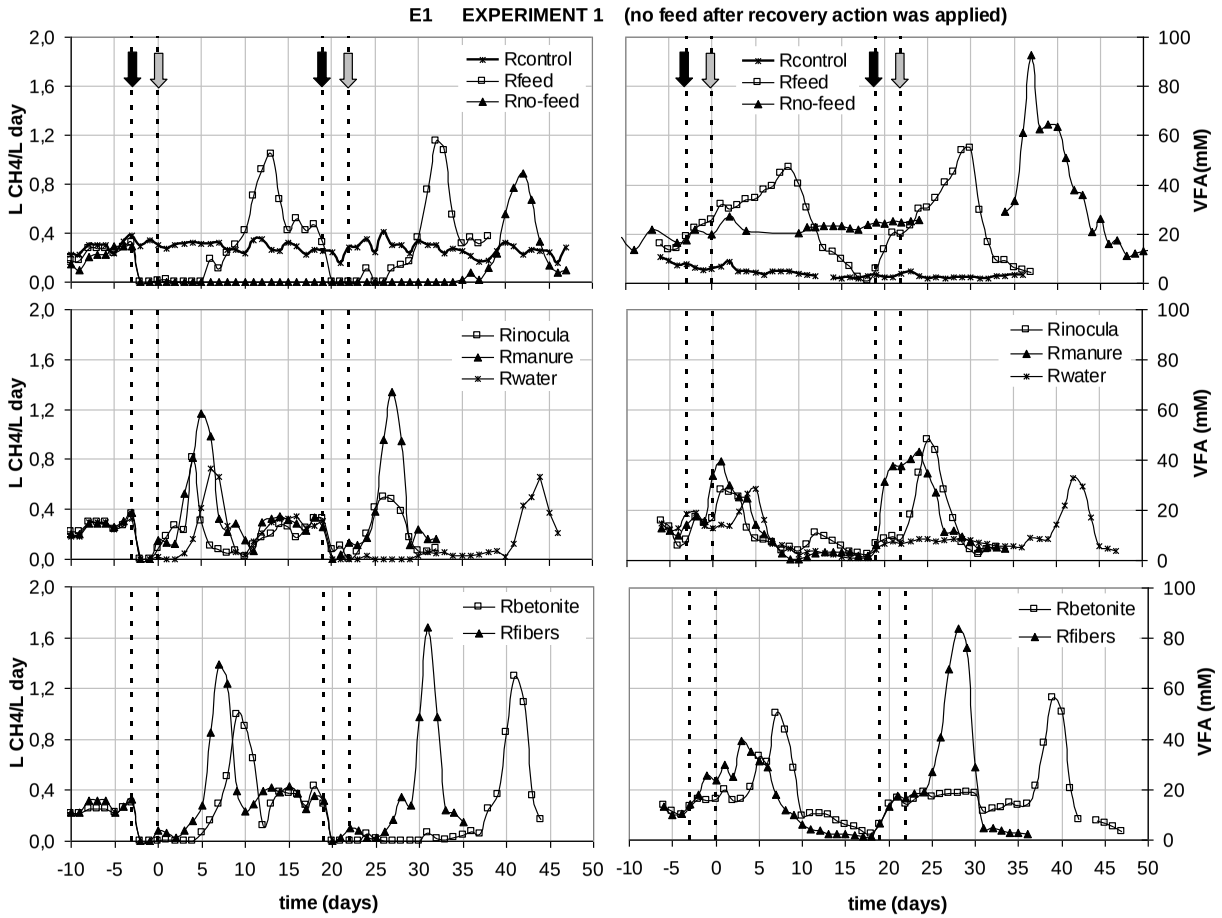

Figure 4.2. *Methane production (L CH4/L day) and VFA concentration (mM) during E1 (no feed after recovery action was applied). Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.*

2.4 g L^{-1} (*60%* compared to the initial concentration). The reactor diluted with manure (R_{manure}) also showed a fast recovery time (*4 days*), but the maximum methane production rate and the maximum VFA accumulated levels in R_{manure} were also higher, due to the extra organic material contained in the fresh manure compared to $R_{inocula}$ (Figure 4.2 and Table 4.3).However, in the second run (RUN2) those differences disappeared, with a very similar behaviour of $R_{inocula}$ and R_{manure}. The dilution introduced in R_{water} had a positive effect on the first run (RUN1) over inhibition, but the recovery time increased on the second run (RUN2), from *5* to *20 days* (Table 4.3), by the consecutive wash out of biomass and residual organic matter (2 consecutive dilutions by water introduced in only *21 days* without feeding the system). The longer recovery time in the R_{water} was attributed to the decrease also in the biomass content of the reactor which was not the case when dilution was made by inoculum ($R_{inocula}$) and fresh manure (R_{manure}). The content of biomass relative to LCFA concentration has been described as critical for the hydrolysis and acidification of lipids (Miron *et al.*, 2000; Salminen and Rintala, 2002b). The lipid-to-inoculum ratio has been previously shown to affect specific methanogenic activity during slaughterhouse waste digestion and LCFA inhibition (Salminen *et al.*, 2000). Similarly, we can conclude that the inhibitory effect of LCFA was not only depended on the LCFA concentration, as it was shown in batch toxicity assays (Figure 4.1), but also on the LCFA/biomass ratio, as it was shown by recovery time (Table 4.3) during discontinuous reactors operation when dilution with inoculum was applied.

The addition of adsorbents such as bentonite ($R_{bentonite}$) or fibers (R_{fibers}) had a positive effect on the recovery of the LCFA pulse, compared to the Rfeed (reduction of the recovery time from *9 days* in R_{feed} to *7* or *5 days* in $R_{bentonte}$ or R_{fiber} in RUN1 respectively), with similar or lower VFA levels in reactors where absorbent were added (Figure 4.2 and Table 4.3). Another advantage of using adsorbents as process recovery agents, compared to dilution strategies was the possibility of utilisation of the total biogas potential contained in the LCFA, as LCFA was retained in the reactor, contrary to the dilution strategies, where a significant part of the initial LCFA concentration (*40%*) was removed undegraded from thesystem. An exception of adsorption recovery actions behaviour was reported in E1, in the second run (RUN2), with an increase in recovery time (*6-17 days*). This was due to the lower amount of bentonite and fibers (*2.22* g_{VS} L^{-1}) that were used in RUN2 compared to the RUN1, as it was assumed that fibers and bentonite were still inside the reactors in significant amounts (reactors were not fed during E1, and only small amounts were retrieved for sampling analyses). This behaviour would be discussed later, together with E2 results.

4.3.3 E2: LCFA inhibition of pre-exposed biomass in semi-continuously fed reactors and subsequent application of recovery strategies.

This experiment was started approx. *2 months* after experiment E1 was finished. During those *2 months* the reactors were incubated at *55ºC* as batches. Thereafter, semi-continuous feeding of the reactors started with one daily feeding with diluted fresh manure at an HRT of *20 days* until constant production from diluted manure. Opposite to E1 feeding with manure was maintained during the entire experiment, except for $_{Rno\text{-}feed}$, to simulate full scale codigestion operation where feeding is rarely stopped.

As in E1, the $R_{no\text{-}feed}$ was the slowest to recover in experiment E2, although the recovery time was reduced to *10 days* compared to *40 days* in E1, and with lower accumulated VFA levels (Fig. 4.3 and Table 4.4). Daily feeding of the reactor with manure (Rfeed), improved the process performance, due to dilution and washing effect, in accordance with experiment E1. However, discontinuation of the feeding is the most common action, to recover inhibition in full scale biogas plants. It is broadly accepted that when a process is inhibited and stressed, continuing reactor loading would lead to further VFA accumulation and maybe acidification. However, in our study, where LCFA inhibition was the cause of imbalance, waiting for process self-recovery was the worse strategy.

The effect of the dilution strategies in experiment E2 was similar to experiment E1 (Figure 4.3 and Figure 4.2) and was confirmed by the total LCFA degradation profiles of $R_{inocula}$ and R_{water}. The concentration of total LCFA (C18:1, C18:0 and C16:0 in Figure 4.4) was reduced immediately after the dilution action with inoculum or water, to *60%*, of the original LCFA concentration. The main difference between $R_{inocula}$ and R_{water} was the higher content of microbial biomass in $R_{inocula}$, resulting in a faster LCFA degradation rate (slopes in Figure 4.4) and consequently in shorter recovery time and lower VFA accumulation levels compared to R_{water}, both in RUN3 and in RUN4 (Table 4.4). In Rwater dilution strategy in E2 a clear improvement compared to E1 was observed (Table 4.4 and Table 4.3), reducing the differences with the other dilution strategies (R_{water} compared to $R_{inocula}$ or R_{manure} in Table 4.4) by new biomass and organic matter introduced during daily feeding with manure. Dilution by manure still showed faster recovery compared to dilution with water (Table IV), which might be due to the higher biomass/LCFA ratio in Rmanure compared to Rwater. Similar results, where increasing the biomass/LCFA ratio by e.g. recirculation, could successfully recover LCFA inhibited process, have previously been reported (Hwu *et al.*, 1997; Mladenovska *et al.*, 2003; Salminen and Rintala, 2002b). In industrial facilities is not always easy to obtain new uninhibited inoculum, therefore, in such cases, dilution by fresh manure might be more practical.

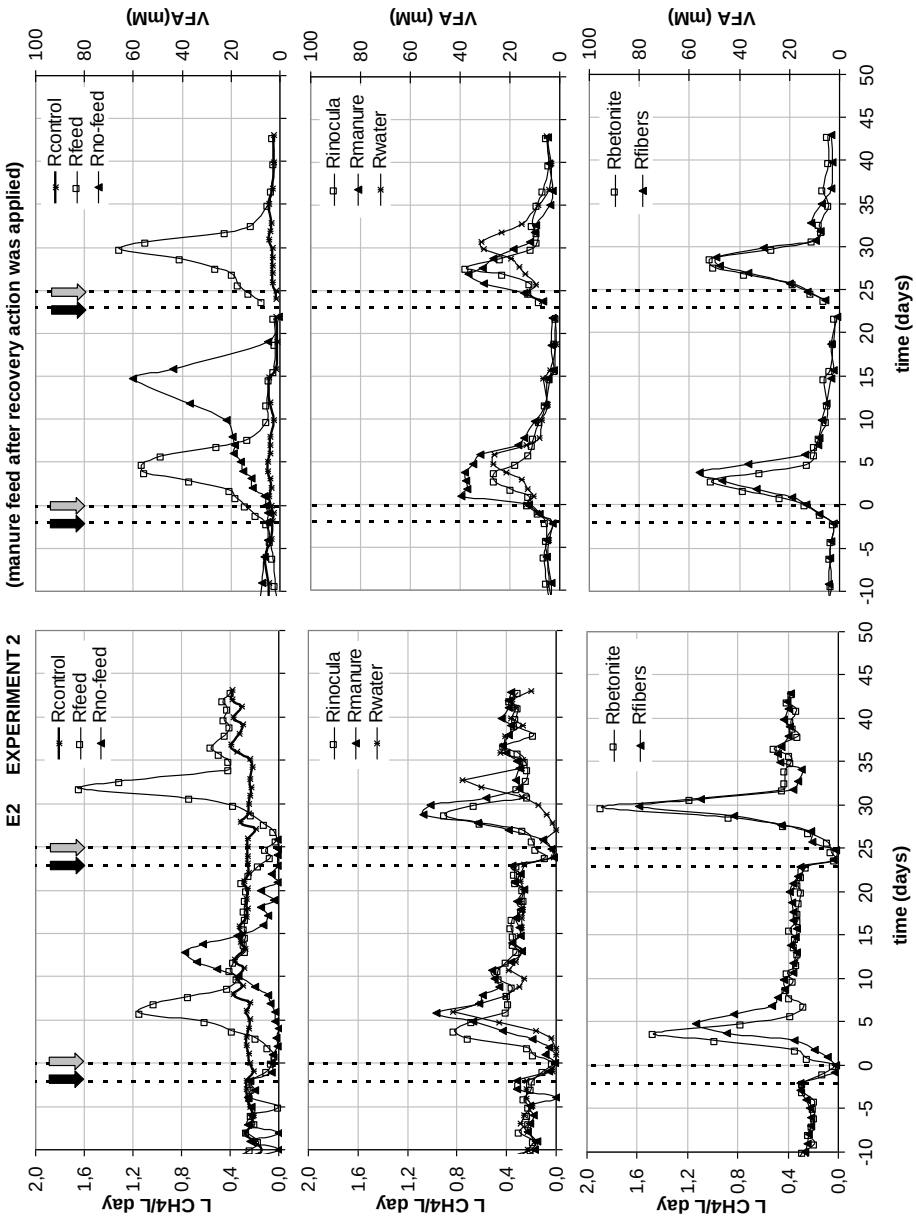

Figure 4.3. *Methane production (L CH4/L day) and VFA concentration (mM) during E2 (semi-continuous feeding with manure after recovery action was applied). Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.*

Table 4.4. *Process parameters obtained during E2 (RUN3 and RUN4)*

	Max Prod. Rate (L CH4/Lday)		Max VFA (mM)		Recovery time (days)	
RUN	**3**	**4**	**3**	**4**	**3**	**4**
Rcontrol	**0.37**	**0.39**	**04.8**	**04.3**		
Rno-feed	**0.77**		**60.4**		**10**	
Rfeed	**1.14**	**1.64**	**56.3**	**65.3**	**4**	**5**
Rinocula	**0.82**	**0.90**	**26.4**	**37.7**	**3**	**3**
Rmanure	**0.96**	**1.07**	**39.3**	**36.6**	**4**	**2**
Rwater	**0.83**	**0.76**	**26.6**	**31.4**	**5**	**7**
Rbentonite	**1.47**	**1.88**	**51.2**	**41.0**	**2**	**3**
Rfiber	**1.13**	**1.58**	**56.1**	**49.3**	**3**	**3**

Addition of adsorbents ($R_{bentonite}$ and R_{fiber}) as recovery strategy in experiments E2 improved the recovery time compared to R_{feed}, from *4-5 days* to *2-3 days*, and showed a higher utilisation of LCFA (Figure 4.3 and Table 4.4), which was in accordance to the observations in E1. Beccari *et al.* (1999) observed positive effect of bentonite addition during anaerobic degradation of olive oil mill wastewaters, while Nielsen *et al.* (2006), reduced oleate inhibition by adding biofibers (digested fibers) to continuously fed reactors digesting manure. Those reports proposed that adsorbents were able to bind the lipids or LCFA on their surface, lowering the adsorption to the microbial cells, and thus stimulating methane production. Adsorption is considered as a rapid physico-chemical mediated phenomenon, while desorption is biologically mediated (Hwu *et al.*, 1998; Nadais *et al.*, 2003; Ning *et al.*, 1996). Bentonite and fibers were added to the reactors 2 days after the LCFA pulse, and consequently a significant part of LCFA may have already been adsorbed to the biomass. This previous absorption to biomass might have been the reason for the absence of clear effect in Figure 4.4, where the concentration of total LCFA just after the application of recovery strategy in R_{fiber} or $R_{bentonite}$ was quite similar to R_{feed}. By measuring the soluble fraction of LCFA ($LCFA_S$), i.e. the fraction non associated to particles, in RUN4, the day after the application of the recovery strategy, a lower concentration of $LCFA_S$ was found in $R_{bentonite}$ (*81.4* $mg_{C18:1}\ L^{-1}$ or *110.7* $mg_{C16:0}\ L^{-1}$) compared to R_{feed} (*179.4* $mg_{C18:1}\ L^{-1}$ or *270.7* $mg_{C16:0}\ L^{-1}$). This was consistent with the assumption that absorbents such as bentonite can result in recovery of the process, by binding LCFA and thus removing the cause of inhibition.

In E1 a reduced (R_{fiber}) or negative ($R_{bentonite}$) effect of recovery action in the RUN2 was observed (RUN1 compared to RUN2 in Figure 4.2 and Table 4.3). This could be explained with the assumption that the residual absorbents from RUN1 may not possess the same absorbent capacity as "*un-used*" adsorbents. Active adsorption sites of remaining adsorbents might have been occupied by biomass or remaining organic matter. Adsorbents, like bentonite, have been described as

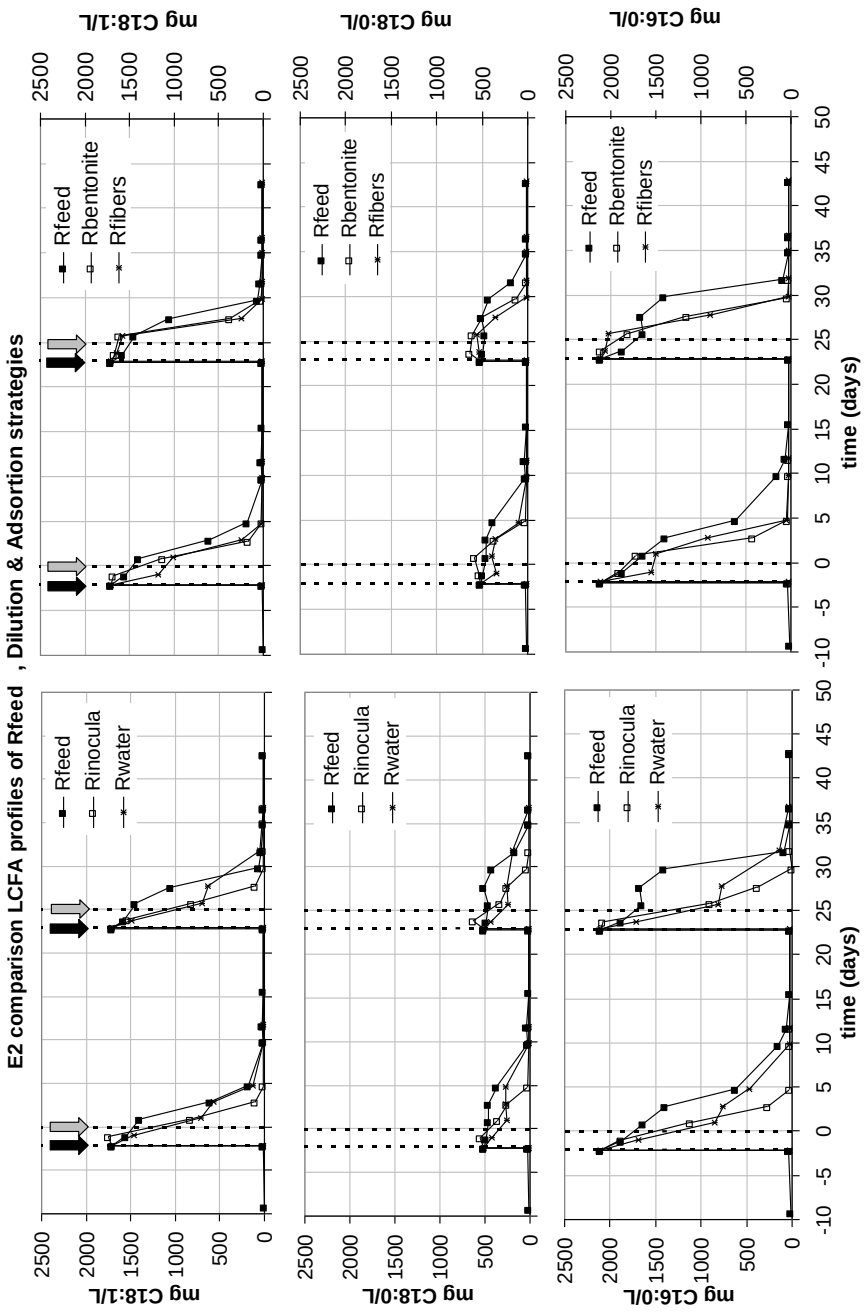

Figure 4.4. *LCFA degradation profiles during E2 (semi-continuous feeding with manure after recovery action was applied) for control (Rfeed), dilution and adsorption strategies. Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.*

support matrices for immobilization of anaerobic consortia, due to their adsorption capacity overmicroorganism (Chauhan and Ogram, 2005; She *et al.*, 2006). During

the E2, in both runs of adsorption strategies (RUN3 and RUN4), the same quantity of adsorbents was used (*5* g_{VS} L^{-1}), resulting in a very similar behaviour of the system for both runs (Figure 4.3 and Table 4.4).

From the present results, it seems that LCFA inhibition is related with binding of LCFA to the microbial surface causing physical hindrance of the transport of nutrients through the cell membrane, and thus causing inhibition of cell function. Other possible mechanisms of resistance, such as flocculation, aggregation or complex structures formation (adsorbent-cell-LCFA) have also been reported (Hulshoff *et al.*, 2004; Kuang *et al.*, 2002 and 2006). In any case, addition of organic or inorganic material, such as fibers from digested manure or cheap clay minerals like bentonite as remediation medium for lipid inhibited processes, could with advantage be introduced in industrial plants.

4.3.4 Adaptation of the system to LCFA pulses

The system was adapted to repeated exposure of the biomass LCFA in both E1 and E2 experiments. Direct comparison between E1 and E2 is not possible as different feeding patterns were applied. However, in 2 of the reactors the exact same strategies and feeding procedure were applied for all the runs; namely in $R_{no\text{-}feed}$ and R_{feed}.

From the $R_{no\text{-}feed}$, the process adaptation after the repeated LCFA pulses can be clearly seen as a reduction of the recovery time from *40* to *10 days* and as a lower VFA accumulation, *92.8 mM* compared to *60.4 mM* for the RUN1 and RUN3 respectively (Figure 4.2-4.3, and Tables 4.3-4.4). The observed adaptation is in agreement with previously reported by Cavaleiro *et al.* (2008), Nadais *et al.* (2006), and Sousa *et al.* (2007), where is it proposed that discontinuous treatment of LCFA, or LCFA pulses, would promote the development of an active anaerobic community, able to efficiently degrade LCFA. It is important to mention that, during the time between experiment E1 and E2, the reactors have been incubated without feeding, as batches, for a period of 2 months. In the literature, periods of non-feeding have been related with an improvement of the capacity for degradation of fatty waste in terms of production, adsorption capacity and system stability (Coelho *et al.*, 2006).

The other strategy that had identical set-up for all the runs and can easily be used for elucidation of any adaptation of the process was the strategy applied in R_{feed}. In Figure 4.5 all R_{feed} experiments (E1 and E2) are shown together, with overlapping time axis, in order to be able to visually compare the time needed for process recovery (days), the maximum specific methane production rate (g_{COD_CH4} g^{-1}_{VS} day^{-1}) and acetate maximum consumption rate (g_{COD_Ac} g^{-1}_{VS} day^{-1}) as process parameters. The process seemed to adapt to the LCFA, with subsequent LCFA pulses. Only in RUN4 similar recovery time was achieved but, for all subsequent

runs, higher maximum methane production or acetate maximum degradation rates were observed (Figure 4.5). Nielsen *et al.* (2006) have similarly shown that a system submitted to previous oleate pulses, induced an increase in the tolerance level of acetoclastic methanogens towards oleate. The adaptation or increased resistance to LCFA detected in R_{feed} and $R_{no\text{-}feed}$, can possibly be attributed to an increase in microbial biomass (higher biomass/LCFA ratio), or to changes in the microbial populations (selection of more LCFA resistant species), or changes in population structure (aggregate formation or more resistant structures).

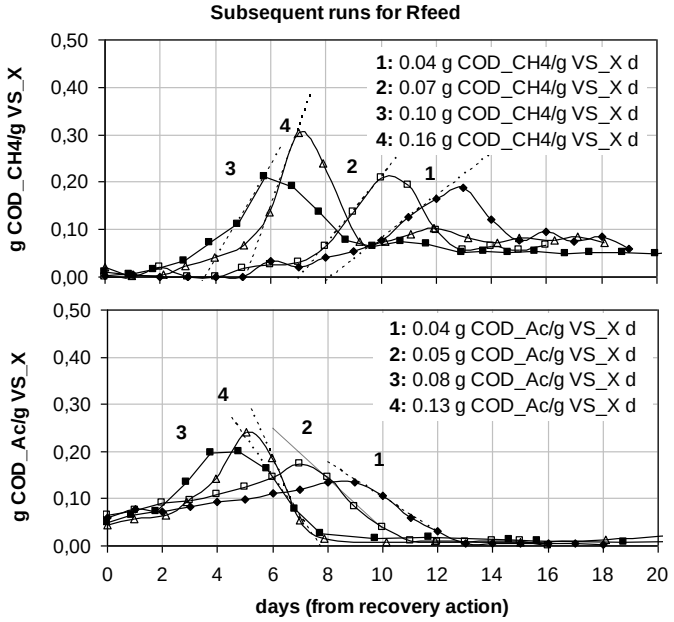

Figure 4.5. *Methane production rate (g COD_CH4/g VS day) (A), and acetate consumption rate (g COD_Ac/g VS day) (B), for all semi-continuous-feeding runs (Rfeed). Numbers indicate the subsequent runs. Discontinuous-lines are the calculated maximum slopes of methane and acetate rates.*

4.4. CONCLUSIONS

Among the seven recovery strategies tested and evaluated, dilution of the reactors content with inoculum, thus increasing the biomass/LCFA ratio, or the addition of adsorbents, were found to be the best strategies to recover thermophilic manure reactors submitted to LCFA inhibition. The use of adsorbents seems to be the most reliable strategy for application on industrial facilities, where it is not easy to introduce dilution, emerging as a simple, feasible and cost-effective solution. The effect of adsorbents was related with competition with biomass in adsorbing LCFA,

thus reducing their inhibitory effect, mainly due to the surface adsorption and transport sites saturation. On the other hand, broadly accepted practice, in real plants, to stop the feeding when an inhibition/imbalance of the process is detected revealed to be the worst approach to face LCFA inhibition in terms of recovery time and process stability.

Repeated subsequent LCFA pulses on biogas reactors, resulted in faster recovery of the system, both in batch and semi-continuous reactors, and in an enhancement in methane production and acetate consumption rates, suggesting an increase or adaptation/tolerance process.

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Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: Batch tests, microbial community structure and mathematical modelling

ABSTRACT. Biomass samples taken during the continuous operation of thermophilic anaerobic digestors fed with manure and exposed to successive inhibitory pulses of long-chain fatty acids (LCFA) were characterized in terms of specific metabolic activities and 16S rDNA DGGE profiling of the microbial community structure. Improvement of hydrogenotrophic and acidogenic (ß-oxidation) activity rates was detected upon successive LCFA pulses, while different inhibition effects over specific anaerobic trophic groups were observed. Bioreactor recovery capacity and biomass adaptation to LCFA inhibition were verified. Population profiles of eubacterial and archaeal 16S rDNA genes revealed that no significant shift on microbial community composition took place upon biomass exposure to LCFA. 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 archaeae was related with the genus *Methanosarcina*. The hypothesis that biomass adaptation was fundamentally of physiological nature was tested using mathematical modeling, taking the IWA ADM1 as general model. New kinetics considering the relation between LCFA inhibitory substrate concentration and specific biomass content, as an approximation to the adsorption process, improved the model fiting and provided a better insight on the physical nature of the LCFA inhibition process.

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5.1. INTRODUCTION

Lipid containing waste are interesting substrates for biogas production because of their high methane yield potential. Lipids are initially hydrolyzed to glycerol and long chain fatty acids (LCFA), which are further converted by syntrophic acetogenic bacteria to hydrogen (H_2) and acetate (Ac), and finally to methane (CH_4) by methanogenic archaea. The degradation of LCFA takes place through the ß-oxidation pathway, which has been reported as the rate-limiting step of the whole anaerobic digestion process (Lalman and Bagley, 2002). LCFA are known to inhibit the methanogenic activity. The inhibitory effect was initially attributed to permanent toxicity resulting from cell damage and it is known to affect both syntrophic acetogens and methanogens (Hwu *et al.*, 1998). Further studies have demonstrated that LCFA inhibition is reversible and that microorganisms, after a lag phase, are able to efficiently methanise the accumulated LCFA (Pereira *et al*., 2004). Adsorption of LCFA onto the microbial surface has been suggested as the mechanism of inhibition, affecting the transport of nutrients into the cell (Pereira *et al.*, 2005).

Recent advances in molecular microbial ecology have brought new insights on the specific microorganisms that are involved in the ß-oxidation process. LCFA-degrading bacteria have been found to be closely related to the *Syntrophomonadaceae* and *Clostridiaceae* families (Hatamoto *et al.*, 2007; Sousa *et al.*, 2007). These microorganisms are commonly proton-reducing acetogenic bacteria that require the syntrophic interaction with H_2-utilizing methanogens and acetoclastic methanogens (Sousa *et al*., 2007). Biomass adaptation to inhibitory levels of LCFA has recently been reported in several studies (Nielsen and Ahring, 2006; Cavalaleiro *et al.*, 2009; Palatsi *et al.*, 2009). Currently, it is not clear whether this adaptation process is the result of a microbial population shift towards the enrichment of specific and better adapted LCFA-degraders (population adaptation), or to the phenotypic adaptation of the existing microrganisms towards high LCFA concentrations (physiological acclimatation).

Despite the fact that LCFA inhibition is well documented and has a significant impact on the anaerobic digestion process, this phenomenon has still not been included in IWA ADM1 reference model (Batstone *et al*., 2002). In other developed models, LCFA inhibition is mainly modeled as a non-competitive process on the lipolytic, acetogenic or methanogenic activities (Angelidaki *et al*., 1999; Salminen *et al*., 2000; Lokshina *et al*., 2003). However, LCFA adsorption phenomena or the microbial aspects of the LCFA inhibition/adaptation process remain poorly characterized. Further modelling developments are required in order to relate the results from physiological activity tests and the characterization of microbial population dynamics throughout the whole LCFA inhibition/adaptation process.

The aim of the present study is to gain a deeper insight on the LCFA inhibition and adaptation process of the anaerobic consortium. Specific physiological activity rates and the microbial structure composition in biomass samples obtained from reactors exposed to LCFA pulses were compared. These samples were characterized by means of culture-independent molecular profiling of dominant eubacterial and archaeobacterial populations, respectively. The obtained results were used in the implementation and testing of a new LCFA inhibition kinetics expression, in the framework of the IWA ADM1 model (Batstone *et al.,* 2002).

5.2. MATERIAL AND METHODS

5.2.1 Analytical Methods

Total solids (TS), volatile solids (VS), total Kjeldhal nitrogen (TKN), ammonia nitrogen (NH_4^+-N) and pH were determined according to Standard Methods (APHA, AWA, WEF, 1995). Methane content in the biogas (%CH_4) and volatile fatty acids concentration in the liquid media (VFA), corresponding to acetate (Ac), propionate (Pr), *iso*- and *n*-butyrate (Bu), *iso*- and *n*-valerate (Va) and hexanoate (Hex), were measured in a gas chromatograph fitted with a flame ionization detection (GC-FID 20100, Shimatzu, Japan). Two different capillary columns, Porapak 60/80 Molsieve (6ft 3mm) and ZEBRON Phase ZB-FFAP (30mx0.53mmx1.00 µm), were used for CH_4 and VFA determination, respectively (Angelidaki *et al.*, 2009).

5.2.2 Biomass and specific batch test

Samples from the outflow of semi-continuous thermophilic (*55ºC*) laboratory completely stirred reactors, fed with manure and exposed to two successive LCFA pulses (*4 g* L^{-1}), were used in subsequent anaerobic batch activity assays and in the molecular characterization of the microbial community structure. The LCFA pulse was composed by a mixture of sodium oleate (C18:1), sodium stearate (C18:0) and sodium palmitate (C16:0) in a ratio *40:10:50* (*w/w/w*) respectively (analytical grade, BDH Chemicals Ltd, Poole England), since these are the main constituents in lipid-rich wastewaters (Hwu *et al.*, 1998). Manure was fed in the influent as the basic substrate at hydraulic retention time (HRT) of *20 days*, and a corresponding organic loading rate (OLR) of *1.0 g VS* L^{-1} d^{-1}. Fresh manure was diluted with distilled water prior to its use, in order to decrease the ammonia level (*1.41±0.25 g TNK* L^{-1}*; 0.92±0.03 g* NH_4^+*-N* L^{-1}) and ensure that the pulse of LCFA was the only inhibitory cause throughout the experiments. Samples were withdrawn from reactors at different stages; before each LCFA pulse (samples I and III), when the process was clearly inhibited (samples II and IV), and when it recovered and reached a new

steady state (sample III and V). The sampling program is shown in Table 5.1. The time between sampled biomass I and III was *25 days*, and between samples III and V was 24 days. So, in all cases, more than one HRT had elapsed before it was assumed that a new state was established. The concentration of LCFA in the reactors at sampling times II and IV, were approximately *4 g* L^{-1}, while LCFA were not detected at samples III and V. A detailed description on the experimental set-up and operation of the sampled reactors can be found in Palatsi *et al.* (2009).

Specific batch activity tests of non-inhibited (samples I, II and V) and LCFA inhibited biomass (samples II and IV) were performed in anaerobic batch assays with specific substrates, according to Table 5.1. Glass bottles (*118 mL* total volume) were inoculated with *2.5 g VS* L^{-1} from bioreactor sampled biomass, resuspended in basic anaerobic medium (Angelidaki *et al.*, 2007), previously amended with *31mM* $NaHCO_3$. A reducing solution of sodium sulfide (*3.20 mM* Na_2SO_3) was also added up to a final liquid total volume of *50 mL* and the pH was adjusted to neutrality. The flasks were stirred and bubbled with N_2 gas in order to remove O_2 before sealing them with rubber stoppers and aluminum crimps. In order to measure the aceticlastic methanogenesis and acetogenetic activity rates, the bottles were supplemented with *20mM* and *10mM* of acetate (Ac) and butyrate (Bu), respectively, while the hydrogenotrophic methanogenesis was assayed by injecting *70 mL* H_2 and *40 mL* CO_2 in the headspace (*1atm, 20ºC*), as described by Angelidaki *et al*. (2009). Additional batches with inhibited and non-inhibited biomass were included as controls, without the addition of any substrate, to determine the methane production derived from the depletion of the LCFA adsorbed onto the biomass (for samples II and IV) and from the utilization of residual organic matter (for samples I, III and V). Activity tests were conducted in quadruplicate (3 vials for CH_4 analysis and 1 vial for VFA determination). Methane and VFA were monitored in the head space and in the liquid medium, respectively. Batch tests set-up and monitored variables are presented in Table 5.1.

The specific biomass activity rate was determined by linear regression on the initial slope of the accumulated methane production curve, and was expressed as *mg* COD_{CH4} *g* VS^{-1} d^{-1}. For substrates that are not directly converted into methane, like butyrate or LCFA, the methane production rate is only a valid measure of syntrophic activity, when the aceticlastic and hydrogenotrophic steps are not the rate limiting process (Dolfing and Bloemen, 1985). Consequently, the maximum specific substrate utilization rate in the assays with butyrate was also calculated from the steepest linear decline in substrate concentration (*mg* COD_{Bu} *g* VS^{-1} d^{-1}), as described by Nielsen and Ahring (2006). In control vials with inhibited biomass (*Control+LCFA* in II and IV samples, according to Table 5.1), the LCFA maximum

Table 5.1. *Summary of batch tests set-up and monitored variables in experimental assays*

Sample	LCFA inhibition	Days from LCFA pulse	Added substrate (*k*)	Initial substrate concentration in vials (kg COD m^{-3})	Monitored variables (*j*)
I	**NO**	-1	H_2/CO_2 (A)	$Sg_{h2}(0)$=0.04/0.04/0.04	Sg_{CH4}
III		+24 (-1)	Ac	$S_{ac}(0)$=1.49/1.50/1.31	S_{ac}, Sg_{CH4}
V		+48 (+23)	Bu	$S_{bu}(0)$=1.76/1.67/1.54	S_{bu}, S_{ac}, Sg_{CH4}
			Control	-	S_{ac}, Sg_{CH4}
II	**YES**	+2	H_2/CO_2 (A)(+LCFA)	$Sg_{h2}(0)$=0.04/0.04(+$S_{fa}(0)$=2.23/2.64)	S_{ac}, Sg_{CH4}
IV		+27 (+3)	Ac(+LCFA)	$S_{ac}(0)$=2.00/1.35(+$S_{fa}(0)$=2.23/2.64)	S_{ac}, Sg_{CH4}
			Bu(+LCFA)	$S_{bu}(0)$=2.00/1.67(+$S_{fa}(0)$=2.23/2.64)	S_{bu}, S_{ac}, Sg_{CH4}
			Control(+LCFA)	(+$S_{fa}(0)$=2.23/2.64)	S_{ac}, Sg_{CH4}

Note: *Roman numbers indicate biomass samplings from reactors. LCFA pulses were introduced in reactors on day 0 and day 25. Days in parenthesis indicates time from the second LCFA pulse. (A) Gas substrate units kmol m^{-3}. Sfa(0) is LCFA remaining concentration from reactors pulse (4 g L^{-1}) adsorbed onto biomass introduced in vials. Different values for initial substrate concentration in vials, correspond to different sampled biomass or different batch test (I to V).*

specific utilization rate was estimated from initial maximum slope of Ac production (*mg* COD_{Ac} *g* VS^{-1} d^{-1}), assuming that Ac was the main product from LCFA β-oxidation (Batstone *et al.*, 2002).

5.2.3 Molecular analysis of microbial community

The effect of LCFA pulses on the anaerobic microbial community composition of both eubacterial and archaeal domains was analyzed at beginning and at the end of reactor operation (samples I and V, according to Table 5.1). Reactor samples of *2 mL* were fixated in *1 mL* of guanidine thyocyanate (4M-Tris-Cl pH 7.5:0.1M, autoclaved) and *0.5 mL* of N-lauroyl sarcosine (10% N-LS autoclaved) and stored at *-20ºC* until further processing. The total DNA was extracted by using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., USA), according to the instructions of the manufacturer. The V3-V5 variable regions of the eubacterial 16S rDNA gene was amplified by the polymerase chain reaction (PCR) using the F341 and R907 primers (Yu and Morrison, 2004). A nested approach was applied to amplify archaeal 16S rDNA by using the primer pairs ARCH0025F-RCH151R and F344-R915 for the first and the nested PCR reactions, respectively (Raskin *et al*. 1994). The forward primer used in the generation of the DGGE amplicons included a GC clamp at the 5' in order to stabilize the melting behavious of the DNA fragments during DGGE. All PCR reactions were performed in a Gradient Mastercycler (Eppendorff, Germany).

Approximately *300 ng* of purified PCR product was loaded onto a *8%* (w/v) polyacrylamide gel (*0.75 mm*), with a denaturing chemical gradient ranging from *30* to *70%* (100% denaturant stock solution contained 7M urea and 40% formamide). DGGE was performed in 1×TAE buffer (40 mM tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a DGGE-4001 System (CBS Scientific, USA) at *100 V* and *60°C* for *16 h*. DGGE gels were stained for *45 min* in 1×TAE buffer containing SybrGold (Molecular Probes, USA) and then scanned under blue light by means of a blue converter plate and a transilluminator (GeneFlash Synoptics Ltd., USA).

Relevant DGGE bands were excised with a sterile filter tip, resuspended in *50 µl* sterilized Milli-Q water, and stored at *4°C* overnight. These extracts were subsequently reamplified by PCR and sequenced. Sequencing was accomplished using the ABI prism BigDye Terminator v. 3.1 cycle sequencing kit (Perkin-Elmer Applied Biosystems, USA) and an ABI 3700 DNA sequencer (Perkin-Elmer Applied Biosystems, USA), according to instructions of manufacturer. Sequences were edited using the BioEdit software package v. 7.0.9 (Ibis Biosciences, USA) and compared against the NCBI genomic database with the BLAST search alignment tool (NCBI, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotides sequences obtained in the present study have been deposited in the GenBank database under accession numbers GQ468297 to GQ468308.

5.2.4 Mathematical modeling and parameter estimation

Processes related to monitored variables (Table 5.1) were modeled with IWA ADM1 as basic model implemented in MatLab (The Mathworks, USA), applying the same structure, nomenclature and units (Batstone *et al*., 2002). Data obtained from activity batch test and molecular microbiology analysis, were used to estimate several unknown parameters and the initial biomass concentrations. The default values for kinetic parameters and stochiometric coefficients suggested by Batstone *et al*. (2002) for thermophilic operation were adopted, with the following exceptions: *a)* the value of LCFA inhibition constant on hydrogenotrophic methanogenesis ($K_{I,h2\ fa}$), which is not given for thermophilic range in the ADM1 model, was assumed to be the same as for mesophilic, $K_{I,h2\ fa}=5\ 10^{-6}$ *kg COD* m^{-3}; *b)* the adopted value for the liquid-gas mass transfer coefficient was $k_La= 45\ d^{-1}$; and *c)* the pH was assumed to be constant, since a buffering solution was added to each vial and no significant pH change was detected. In all simulations, the initial value for inorganic nitrogen was $S_{in}(0)=10^{-2}$ *kmol N* m^{-3}. The initial specific substrate concentration in each vial, used as model initial vector, are summarized in Table 5.1.

The time course of the variables monitored in vials with non-inhibited biomass (samples I, III and V), with H_2/CO_2, Ac and Bu as substrates (Table 5.1), were used to estimate the initial concentration of H_2, Ac and Bu degrading microbial populations, $X_i(0)$ (*kg COD_X* m^{-3}), by a sequential estimation procedure (step-by-step, where the found values were then used as fixed parameters in next step), using ADM1 and its default biochemical parameters values (Batstone *et al*., 2002), as indicated in Table 5.2.

Different approaches were considered concerning the modelling of the inhibition phenomena observed on the activity tests with inhibited biomass (samples II and IV, according to Table 5.2).

The first assumption (A1) consisted on a direct application of the *IWA ADM1 Model* using the suggested biochemical parameters (Batsone *et al.*, 2002) and the calculated initial biomass content ($X_{h2}(0)$, $X_{ac}(0)$, *and* $X_{bu}(0)$), for assays I and III. This initial biomass content was considered to be equal to subsequent sampled inhibited biomass, samples II and IV respectively, since the time delay between the sampling of non-inhibited and inhibited biomass was only *2-3 days* (Table 5.1). With those assumptions, the initial amount of LCFA degrading microorganisms, $X_{fa}(0)$ (*kgCOD_X* m^{-3}), and the maximum LCFA uptake rate, $k_{m,fa}$ (*kg COD_S kg* COD_X^{-1} d^{-1}), were estimated by a multiple parameter optimization procedure, using the time evolution data of all the monitored variables during activity tests of inhibited biomass (samples II and IV), according to Table 5.2.

Table 5.2. *Process rates modifications used in different model approaches*

Batch	Model Approach	Process rate (ρ_j, kg COD m^{-3} d^{-1})	Estimated parameters
I,III,V	IWA ADM1	IWA ADM1	$X_{h2}(0), X_{ac}(0), X_{bu}(0)$
II,IV	(A1) IWA ADM1	IWA ADM1	$X_{fa}(0), k_{m,fa}, K_I$
	(A2) Inhibition Model	*Haldane kinetics for LCFA uptake rate* $\rho_7 = k_{m,fa}\frac{S_{fa}}{K_S + S_{fa} + \frac{S_{fa}^2}{K_I}} X_{fa} I_{pH} I_{IN,lim} I_{h2}$ *Non-competitive term for Ac uptake rate* $\rho_{11} = k_{m,ac}\frac{S_{ac}}{K_S + S_{ac}} X_{ac} I_{pH} I_{IN,lim} I_{NH3,Xac} \frac{K_I}{K_I + S_{fa}}$ *Non-competitive term for H_2 uptake rate* $\rho_{12} = k_{m,h2}\frac{S_{h2}}{K_S + S_{h2}} X_{h2} I_{pH} I_{IN,lim} \frac{K_I}{K_I + S_{fa}}$	$X_{fa}(0), k_{m,fa}, K_I$
	(A3) Inhibition-adsorption Model	replace K_I by K_{IRA} in ρ_7, ρ_{11} and ρ_{12} with $K_{IRA} = \frac{K_I' X_{fa}}{S_{fa}}$	$X_{fa}(0), k_{m,fa}, K_I'$

Note: Nomenclature and units were maintained from IWA ADM1 (Batstone et al., 2002)

The second approach (A2), named as *Inhibition Model*, considered the uptake of LCFA to be described by the Haldane's inhibition kinetics and both methanogenic processes (uptake of acetate and hydrogen) to be affected by a non-competitive term with a common LCFA inhibition constant, K_I (kgCOD_S_I m^{-3}), as shown in Table 5.2. Such inhibition kinetics has already been proposed by other authors. Angelidaki *et al*. (1999), studying manure codigestion with glycerol trioleate or bentonite bound oil degradation, considered a non-competitive LCFA inhibition on the lipolitic, acetogenic and methanogenic steps, and the *Haldane* inhibition kinetics on the ß-oxidation process. Salminen *et al*. (2000) and Lokshina *et al*. (2003), using solid slaughterhouse waste, considered a non-competitive inhibition kinetics due to LCFA, affecting acetogenesis and methanogenesis. With those assumptions, new initial values for $X_{fa}(0)$, $k_{m,fa}$ and K_I, were estimated by multiple parameter optimization (Table 5.2).

The last approach (A3), was named as *Inhibition-Adsorption Model*, and included a simple mathematical expression for the description of the physical adsorption process of LCFA onto the biomass, as an inhibition mechanism. Adsorption is considered as a rapid physico-chemical phenomenon, while desorption (degradation) is a biologically mediated process by LCFA-degraders (Hwu *et al*., 1998). Pereira *et al*. (2004) proposed a modification of the *Haldane* equation for the LCFA inhibition process, which considers the adsorbed substrate per VS unit, S_{ba} ($M_{substrate}\ M_{biomass}^{-1}$), instead of total substrate concentration (S_{fa}). Consequently, by adopting this concept, the proposed *Inhibition-Adsorption Model* assumes the following hypothesis: *a)* the inhibition of LCFA uptake process can be expressed by the Haldane kinetics; *b)* a non-competitive reversible inhibition term can be used on acetogenesis and methanogenesis; *c)* in the previous inhibition processes, the inhibitory constant (K_I) is replaced by a new inhibitory term, $K_{IFA}=K_I'\cdot X_{fa}/S_{fa}$, proportional to the specific ratio between the LCFA degrading population and the substrate (X_{fa}/S_{fa}), being higher (less inhibition) when this ratio value increases (Table 5.2).

The objective function was minimized, in the sequential parameter estimation procedure, for each step or specific substrate *k*, according to Eq 5.1,

$$fobj_k = \sum_j w_{kj}\left(\sum_{i=1}^{n_{kj}}(y_{kji}^{*}-y_{kji})^2\right) \qquad \text{Eq.5.1}$$

where, y_{kji}^{*} represents the measured value of variable *j*, in vial *k*, at time *i*, and y_{kji} is the corresponding simulated value. Variable *j* from vials *k* has n_{kj} measured values at successive different times *i*. The weight factor, w_{kj}, used in optimization was defined as Eq 5.2,

$$w_{kj} = ((n_{kj}(\max(y_{kj}^{*}) - \min(y_{kj}^{*}))^2)^{-1} \qquad \text{Eq.5.2}$$

with $max(y^*_{kji})$ and $min(y^*_{kji})$, being the maximum and minimum measured value of variable *j* in vial (step) *k*. The objective function used in the multiparameter estimation procedure, with datasets II and IV, was calculated according to Eq.5.3, and the optimization routine followed the downhill simplex method as implemented in the MatLab package.

$$fobj = \sum_{k} fobj_k \qquad \text{Eq.5.3}$$

Model data fitting accuracy was measured by the coefficients of determination R2 defined in Eq.5.4,

$$R^2_{kj} = 1 - \frac{\sum_{i=1}^{nkj} (y^*_{kji} - y_{kji})^2}{\sum_{i=1}^{nkj} (y^*_{kji} - \bar{y}^*_{kji})^2} \qquad \text{Eq.5.4}$$

where $\bar{y}^*_{kji}$ is the mean of n_{kj} measured values of variable *j* from vial *k.*

5.3. RESULTS AND DISCUSSION

5.3.1 Specific batch tests

The first set of analyzed batch tests were those with biomass taken from the reactors, just before the application of LCFA pulses (samples I and III, in Table 5.1), and when the system had recovered from a previous inhibition stage (sample V, in Table 5.1). Results of activity batch tests on specific substrates; H_2/CO_2, Ac and Bu, respectively, as model substrates for the main trophic groups, are summarized in Table 5.3. Mean separation was performed on the calculated rates by Multiple Range Test (MRT) with a significance level α= 0.05 (Sheskin, 2000).

Table 5.3*. Substrate utilization rates of non-inhibited biomass (I, III and V).*

Substrate	Unit	I	III	V
H2/CO2	mg CODCH4/g VS-1 d-1	91.1±5.9 a	131.7±6.6 b	147.2±3.7 c
Ac	mg CODCH4/g VS-1 d-1	127.7±6.5 a	122.9±8.2 a	135.0±10.7 a
Bu	mg CODCH4/g VS-1 d-1	183.4±18.8 a	181.8±2.6 a	183.9±37.4 a
	mg CODBu/g VS-1 d-1	-263.8	-285.8	-230.9

Note: Different letters in rows indicate significant differences between rates (α=0.05).

A significant increase on the hidrogenotrophic methanogenic activity rate was observed after subsequent inhibitory stages (Table 5.3), in samples I to V (from *91.1* to *147.2 mg* COD_{CH4} *g* VS^{-1} d^{-1}), while the net acetoclastic methanogenic activity remained at a relatively similar level along time (*127.7*, *122.9* and *135.0 mg* COD_{CH4} *g*

VS^{-1} d^{-1}, for samples I, III and V). These results are in agreement with previous findings on suspended sludge and fixed bed reactors subjected to LCFA inhibition, which concluded that hydrogenotrophic methanogens appeared to be more resistant to LCFA inhibition than acetoclastic methanogens (Templer *et al.*, 2006).
Concerning the acetogenic activity, the *n*-butyrate (Bu) uptake rate remained fairly constant (*263.8*, *285.8* and *230.9 mg* COD_{Bu} gVS^{-1} d^{-1} respectively for samples I, III and V) and no significant statistical differences were found in terms of methane production rate (in COD_{CH4} units, according to Table 5.3). Similary, Nielsen and Ahring (2006) found that the maximum substrate utilization rate for Ac and Bu by biomass from thermophilic anaerobic reactors, fed with a mixture of cattle and pig manure and subjected to oleate pulses (*2 g* L^{-1}), decreased or remained constant, while the methanogenic activity rate from H_2/CO_2, but also from formate and Ac, experienced an increase.

To analyze the inhibitory effect of LCFA pulses on specific activities of representative trophic groups, a second set of batch tests were run with biomass, sampled *2-3 days* after each LCFA pulse, when biogas production in the reactor evidenced a clear inhibition (biomass samples II and IV, according to Table 5.1). Tests were performed with H_2/CO_2, Ac, and Bu as methanogenic and acetogenic model substrates, respectively. Samples II and IV had remaining LCFA adsorbed onto the biomass. Additionally, one set of vials were included as controls, *Control(+LCFA),* incubated without any substrate supplementation in order to monitor the ß-oxidation process. The specific activities of inhibited biomass, II and IV, are summarized in Table 5.4.

Table 5.4. *Substrate utilization rates of LCFA inhibited biomass (II and IV).*

Substrate	Uniits	II	IV
H2/CO2(+LCFA)	mg CODCH4/g VS-1 d-1	67.6±7.9 a	90.8±2.7 b
Ac(+LCFA)	mg CODCH4/g VS-1 d-1	44.6±1.3 a	56.7±4.4 a
Bu(+LCFA)	mg CODCH4/g VS-1 d-1	183.9±3.8 a	174.0±15.4 a
	mg CODBu/g VS-1 d-1	-183.2	-161.8
Control(+LCFA)	mg CODCH4/g VS-1 d-1	163.3±8.7 a	218.8±16.1 b
	mg CODAc/g VS-1 d-1	104.9	153.6

Note: Different letters in rows indicate significant differences between rates (α=0.05).

In general, a clear reduction in all monitored metabolic activities was observed upon the application of each LCFA pulse (Table 5.4 compared to Table 5.3). During batch activity tests on LCFA inhibited biomass, the remaining LCFA content (from the reactor pulse and adsorbed onto the biomass) was completely consumed and the methane production reached a maximum plateau close to the expected theoretical value. These results confirm that LCFA inhibition is a reversible phenomenom, since neither syntrophic acetogenic nor methanogenic activities were irreversibly

damaged, which is in accordance to what has previously been reported (Pereira *et al*., 2004). Yet, acetoclastic methanogenesis was the most affected activity by LCFA (*44.6-56.7 mg* COD_{CH4} gVS^{-1} d^{-1}, compared to *127.7-122.9 mg* COD_{CH4} $g\ VS^{-1}$ d^{-1} for the LCFA-inhibited and uninhibited biomass, as shown in Tables 5.4 and 5.3, respectively). Those vials exhibited not only lower methane production rates but also a longer lag-phase, compared to the activities before the LCFA pulse. The hydrogenotrophic methanogenesis was the metabolic activity affected the least by LCFA inhibitory pulses, with rate values up to *90.8 mg* COD_{CH4} gVS^{-1} d^{-1} (Table 5.4), very similar to the system hydrogenotrophic activity prior to the LCFA inhibitory pulse (*91.1 mg* COD_{CH4} $g\ VS^{-1}$ d^{-1}, Table 5.3).

The results of the present study are in agreement with the hypothesis of LCFA-induced transport limitation (Pereira *et al.*, 2005). Those authors found that hydrogen, the smallest methanogenic substrate molecule, was the first to be transformed into methane in LCFA inhibited systems, in relation to other substrates of higher molecular weight, due to its higher diffusivity through the LCFA adsorbed layer.

It has also been described in the literature that methanogens are more susceptible to LCFA inhibition than acidogens (Lalman and Bagley, 2002; Mykhaylovin *et al.*, 2005), which is also in agreement with the lower differences in acetogenic activities detected on Bu vials, before and after LCFA inhibition (I-II on Table 5.3 and III-IV on Table 5.4).

In relation to the control vials, LCFA batch *Control(+LCFA)*, a clear improvement on the ß-oxidation process along time was observed (from *163.3* to *218.8 mg* COD_{CH4} $g\ VS^{-1}$ d^{-1} or from *104.9* to *153.6 mg* COD_{Ac} $g\ VS^{-1}$ d^{-1} in terms of substrate production rate, for the tests II and IV, respectively). Mladenovska *et al*. (2003) described that the biomass of digested manure and lipids was more active and had higher initial rates of methane production than the biomass of only digested manure (not exposed to lipids). These results were related to the importance of the interaction microorganism-substrate-particle size and, in particular, to the effect of lipids on cell density and aggregation. Pereira *et al*. (2004) reported an enhancement on the microbial activity upon depletion of adsorbed LCFA, by favouring specific degrading populations, while Nielsen and Ahring (2006) also reported an increasing oleate tolerance (from *0.3* to *0.7 g* L^{-1}) in manure thermophilic systems exposed to oleate pulses. Different explanations for this behavior were hipotesized, like the induction of higher hydrolysis rates, an increase on biomass concentration or changes in the microbial composition.

The observed differential LCFA inhibition effect on distinct trophic groups might, in principle, be related to an enrichment of specific populations involved on LCFA degradation process. Therefore, a shift in bacterial and archaeal communities can

not be excluded and was studied further by means of molecular biology techniques and mathematical modelling tools, as described in the subsequent paragraphs.

5.3.2 Microbial community structure

DGGE molecular profiling of PCR amplified eubacterial and archaeal 16S rDNA ribotypes was performed on biomass taken at the beginning (sample I) and at the end (sample V) of reactor operation (Table 5.1). Despite the fact that both sampling events were separated in time by more than *40 days* (equivalent to two HRT intervals), and that the biomass suffered two inhibitory LCFA pulses and subsequent recoveries stages during this period, no significant differences were observed in the microbial community structure of eubacterial and archaeal populations (Figure 5.1).
Up to 12 DGGE bands were successfully excised, reamplified and sequenced. BLAST sequence comparison against NCBI genomic database resulted in close maches with several uncultured ribotypes from the *Clostridiaceae,Syntrophomonadaceaae, Bacillaceae* and *Synergites*, all families that belong to the *Firmicutes* eubacterial phylum (Figure 5.2).

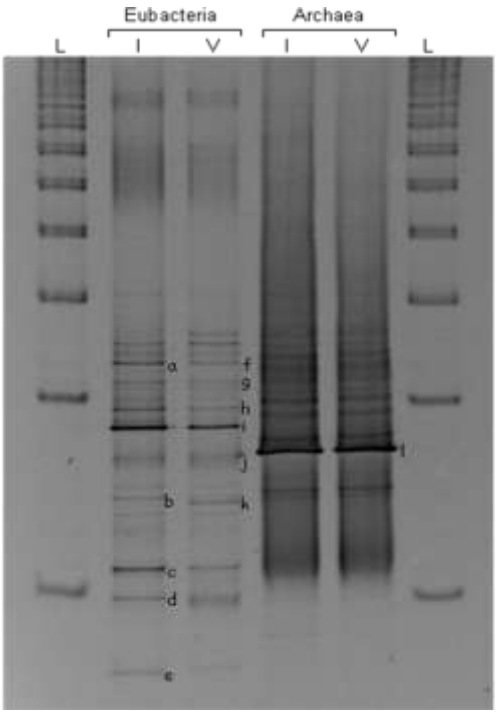

Figure 5.1*. DGGE profiles on eubacterial and archaeal 16S rDNA amplified from samples I and V. A standard ladder (L) has been used at both gel ends in order to check the DNA migration homogeneity. Successfully excised and sequenced bands have been named with lower-case letters*

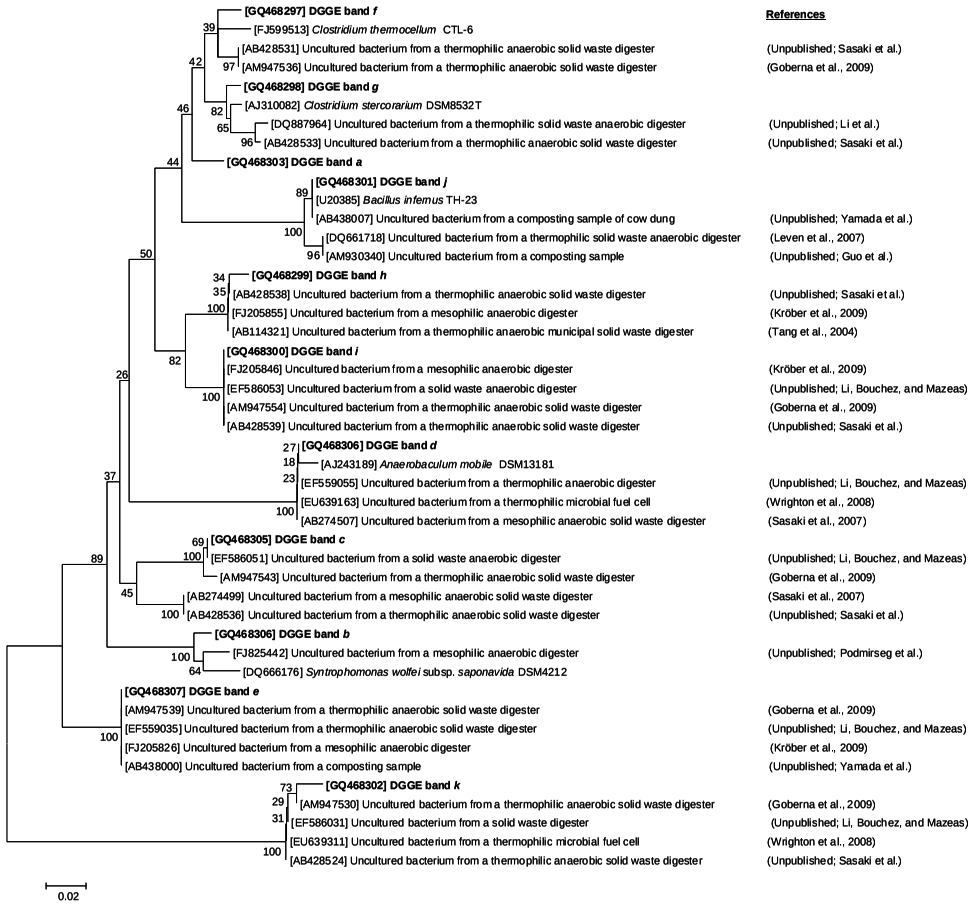

Figure 5.2. *Phylogenic tree on eubacterial16S rDNA from DGGE excised bands (Figure 5.1) and from homologous sequences deposited at the GenBank database (accession numbers are given between box brackets). The tree was generated using the Neighbour-joining algorithm and the Kimura 2-parameter correction, and was bootstrapped 500 times. Values beside the nodes represent the percentage of branch support given by bootstrap analysis.*

The *Clostridiaceae* appears to be one of the most represented bacterial families in the microbial community of anaerobic digesters. In our study, the DGGE bands *f* and *g* are related to uncultured bacteria previously found in different solid waste-thermophilic anaerobic bioreactors (*95*-97% of sequence homology), and to the type strains of *Clostridium thermocellum* (*93%*) and *Clostridium stercolarium* (*95%*), respectively, as the closest phylogenetically defined matches. The sequence from band *a* also clustered with the *Clostridiaceae* family, but its poor homology (*88%*) with database sequences indicates that it might belong to a yet undescribed taxon.

The sequence from band *b* was relatively homologous (*95%*) to an uncultured bacterium from an anaerobic digester and, more distantly (*93%*), to the type strain of *Syntrophomonas wolfei* subsp. *saponavida*. The *Syntrophomonas* genus has been described previously as specific syntrophic LCFA degrading bacteria (Sousa *et al.,* 2008).

Band *j* sequence was identical to that of the type strain of *Bacillus infernus*, the only strictly anaerobic species in the genus *Bacillus* (Boone *et al.*, 1995). This halotolerant and thermophilic bacterium is characteristic from deep terrestrial subsurface areas. Yet, very similar uncultured ribotypes (*98-99%* sequence homology) were obtained during the composting of hyperthermophilically pre-treated cow dung and from a thermophilic anaerobic digester of solid waste (Leven *et al.*, 2007).

The sequence from band *d* was identical to a number of uncultured ribotypes obtained from solid waste anaerobic digesters, and closely related to that of the species *Anaerobacterium mobile* (*98%* sequence homology). This is a novel anaerobic, thermophilic, and sliglhy halotolerant bacterium able to ferment organic acids and some carbohydrates into acetate, hydrogen, and CO_2 (Menes and Muxi, 2002).

No reference strains were found to be sufficiently related to the sequences from bands *h*, *i*, *k* and *e* for its phylogenetic assignment, but they were highly homologous, or ever identical, to a number of uncultured ribotypes obtained predominantly from thermophilic anaerobic reactors degrading organic solid waste (Goberna *et al*., 2009; Kröber *et al*., 2009; Tang *et al*., 2004; Wrighton *et al*., 2008). Interestingly, the number of coincident, or highly related, ribotypes found in this work in relation to the previously cited studies is remarkable (Figure 5.2). These results suggest that the environmental conditions present in the thermophilic anaerobic digestion of solid waste promote de formation of relatively stable microbial consortia.

In relation to the archaeal domain, a single predominant band was observed in the DGGE profiles (band *l*). The associated sequence was *97%* homologous to that of the *Methanosarcina thermophila* type strain. This thermophilic archeon is a

methanogen that has been found in a wide variety of thermophilic anaerobic digesters treating organic waste. Sequence homology of band *I* was higher in relation to another strain of the same species that was enriched in a thermophilic anaerobic digester operated at high concentration of volatile fatty acids (Hori *et al*., 2006). Mladenovska *et al*. (2003) compared the digestion of cattle manure at mesophilic conditions to the digestion of a mixture of manure with glycerol trioleate (2% w/w). Despite different reactor performance no differences were found in the diversity of archaea, being the vast majority of the detected ribotypes phylogenetically close to *Methanosarcina siliciae.* Karakashev *et al*. (2005) studied the influence of environmental conditions and feeding on methanogenic populations in a real scale biogas plants, reporting a dominance of *Methanosarcinaceae* members on manure digesters. Kaparaju *et al.* (2009) also reported the predominance of *Methanosarcinaceae* on the pilot plant (Kogens-Lyngby, Denmark), which was used as source of inoculum for semi-continuous reactors sampled in the present study (Palatsi *et al*., 2009). Hence, the origin of the inoculum, the daily manure feeding and the thermophilic regime might have exherted a strong influence on the enrichement of specific methanogenic populations.

5.3.3 Mathematical modeling and parameter estimation

Data from batch activity assays were used to test the three model approaches (A1-A3) as summarized in Table 5.2. The main aim was to determine whether the observed biomass adaptation process to LCFA can be explained by an increase of specific degrading populations (X_i), and/or a modification of the adsorption-inhibition process, once a species composition shift has been excluded as the reason for the observed adaptation.

In order to estimate the initial biomass content of specific trophic groups, $X_i(0)$, the experimental data from batch activity tests, with H_2/CO_2, Ac and Bu as substrates and not inhibited biomass (batch with samples I, III and V), was used in a direct implementation of *IWA ADM1 Model* and a sequential parameter estimation procedure (as described in Material and Methods section). Estimates on the initial biomass content of specific trophic groups, $X_i(0)$, are summarized in Table 5.5a. Goodness-of-fit coefficient R^2 of modeled results ranged from *0.78* to *0.99* (data not shown). As an example, the simulations and experimental data for Bu batch activity tests (witch included the previously initial estimated concentrations for hidrogenotrophic and acetoclastic methanogens), and the corresponding R^2 coefficients, are depicted in Figure 5.3. When the initial population concentration, $X_i(0)$ and the maximum uptake rate, $k_{m,i}$ were simultaneously estimated at each step, the obtained $k_{m,i}$ values were relatively close to those suggested by Batstone *et al*.

Table 5.5a. *Estimated parameter values for non inhibited batch tests data sets I, III and V.*

Model Approach	Estimated parameter	Results		
		I	III	V
IWA ADM1	$X_{h2}(0)$	$5.89\ 10^{-4}$	$5.08\ 10^{-4}$	$2.33\ 10^{-3}$
	$X_{ac}(0)$	$1.30\ 10^{-2}$	$1.26\ 10^{-2}$	$1.70\ 10^{-2}$
	$X_{bu}(0)$	$5.53\ 10^{-4}$	$1.52\ 10^{-3}$	$1.68\ 10^{-3}$

Units; Xi (kg COD m^{-3})

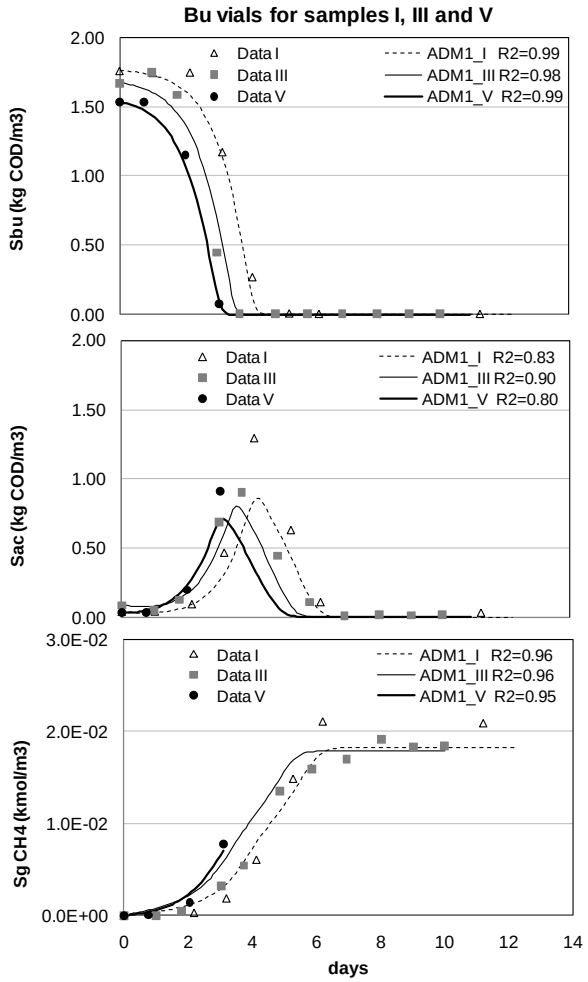

Figure 5.3. *Experimental data (nkj point markers) and IWA ADM1Model results (lines) for activities (k) to Bu for non-inhibited biomass I, III and V. Coefficients of determination (R2) for model fitting are indicated in every graphic.*

(2002) and no significant differences in the coefficients of determination were found. Moreover, at the tested initial substrate concentrations (in activity assays,$S_i(0)>>>Ks_i$), the sensitivity of the system to variations on the half saturation constants (Ks_i) was extremely low, as expected (Dochain and Vanrolleghem, 2001), and this constant was not possible to be identified. For this reason, the sequential parameter estimation of initial X_i values by adopting the suggested biochemical parameters by IWA ADM1 (Batstone *et al*., 2002), was considered adecuate. Due to technical difficulties on the measurement of the methane production in batch V, caused by an operational problem on the GC-FID, model fitting in this particular batch was based mainly on the VFA production-degradation profile (Figure 5.3).

Based on the estimated initial biomass content of specific microorganisms, $X_i(0)$, an initial acetoclastic methanogenic population stability can be outlined (Table 5.5a). However, the initial hydrogenotrophic methanogenic population, $X_{h2}(0)$, increased along sampling time, which could explain the observed improvement of the hydrogenotrophic activity (Table 5.3). From the microbial community analysis, it was not possible to differentiate between methanogenic populations, because the most abundant isolated archaeae was affiliated to the genus *Methanosarcina* (Figure 5.2).

In the analysis of batch reactors with inhibited biomass (data-sets II and IV), the initial amount of hydrogenotrophic methanogens $X_{h2}(0)$, aceticlastic methanogens $X_{ac}(0)$, and butyrate acetogens $X_{bu}(0)$, was assumed to be the same as in tests with non-inhibited biomass (samples I and III), as explained in the Material and Methods section (Table 5.5a). The initial content of LCFA in batch tests II and IV ($S_{fa}(0)$, *2.23* and *2.64 kg COD* m^{-3}) was identical to that from the previous LCFA pulse in the reactor, adsorbed on the biomass. As general procedure, in each tested approach with inhibited batch tests data, a multiple parameter estimation ($X_{fa}(0)$, $k_{m,fa}$ and K_I) was performed for batch test II and the obtained kinetic parameter values were then used in the estimation of the initial LCFA degrading population, $X_{fa}(0)$, as the sole parameter optimized in batch test IV (Table 5.5b).

The first approach (A1) to estimate $X_{fa}(0)$ and $k_{m,fa}$ parameters was the *IWA ADM1 Model* (Table 5.2). Figure 5.4 shows, as example, the experimental and predicted values for the inhibited sample IV. Although the predicted methane production curve and S_{ac} or S_{bu} evolution values are acceptable in some cases, it was not possible to find an unique set of parameters ($X_{fa}(0)$ and $k_{m,fa}$) able to fit all experimental data together, with sufficiently high coefficients of determination (Figure 5.4). Hence, the need to introduce modifications in *IWA ADM1* model, in order to express adequately the LCFA inhibition process is justified.

The second tested approach (A2)*, Inhibition Model*, introduced the *Haldane* inhibition kinetics for the ß-oxidation and the reversible non-competitive inhibition kinetics for acetate or hydrogen methanogenesis (Table 5.2), as previously reported

(Angelidaki *et al*., 1999; Salminen *et al*., 2000; Lokshina *et al*., 2003). The estimated parameter values for batch test II are shown in Table 5.5b. An increase in the initial LCFA degrading population, $X_{fa}(0)$, from $2.40 \cdot 10^{-3}$ to $4.45 \cdot 10^{-2}$ *kgCOD_X* m^{-3}, in batch test IV was detected, maintaining a maximum degradation rate and inhibition constant of $k_{m,fa}$=21.69 *kg COD_S kg COD_X*$^{-1}$ d^{-1} and K_I=3.35 *kg COD* m^{-3}, respectively. Coefficients of determination and model fitting for sample IV are shown in Figure 5.4.

The last approach (A3), *Inhibition-Adsorption Model*, replaced the constant inhibitory factor, K_I, by a term K_{IFA} proportional to the ratio X_{fa}/S_{fa} (Table 5.2) to model the adsorption effect of LCFA on the cell walls. Estimated parameter values for test II were presented in Table 5.5b. An increase in the initial LCFA degrading population $X_{fa}(0)$, from $9.89 \cdot 10^{-4}$ to $1.30 \cdot 10^{-3}$ *kg COD* m^{-3}, was also detected in sample IV (Table 5.5b), while initial $K_{IFA}(0)$ value remained around *1.15 kg COD* m^{-3}. An example of the obtained coefficients of determination and model fittings for sample IV are shown in Figure 5.4.

Table 5.5b. *Estimated parameters values for inhibited batch tests data sets II and IV.*

Model Approach	Estimated parameter	Results	
		II	IV
(A1) IWA ADM1	X_{fa}	3.00 10^{-4}	3.70 10^{-3}
	$k_{m,fa}$	22.37	22.37
(A2) Inhibition Model	X_{fa}	2.40 10^{-3}	4.45 10^{-2}
	$k_{m,fa}$	21.69	21.69
	K_I	3.35	3.35
(A3) Inhibition-adsorption Model	X_{fa}	9.89 10^{-4}	1.30 10^{-3}
	$k_{m,fa}$	124.33	124.33
	K_I	2.37 10^{3}	2.37 10^{3}

Units; Xi (kg COD m-3); Km,fa (kg COD_S kg COD_X-1 d-1); KI and KI' (kg COD m-3)

The best model fittings were obtained with the *Inhibition-Adsorption Model*, which was able to reproduce not only the lower production rates when the system was inhibited but also the longer lag-phase during system inhibition. Although the obtained parameter set is probably not unique, these results could be considered as a first approach to express the importance of the LCFA/biomass ratio in the adsorption-inhibition process.

Modelling results suggest that adsorption plays an important role in the overall LCFA inhibition-adaptation process, and that there is a need to introduce

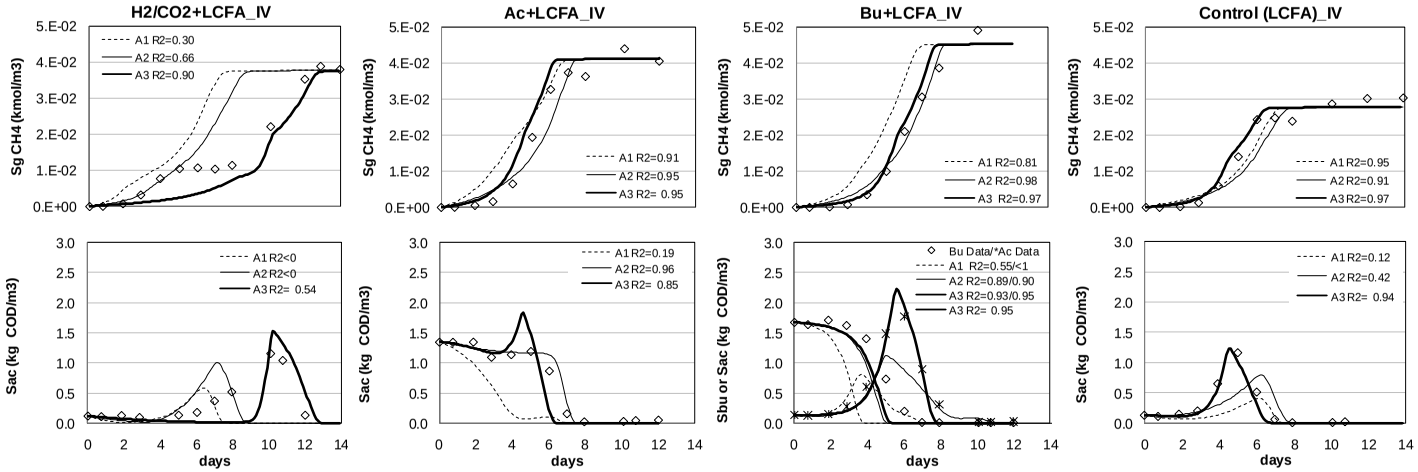

Figure 5.4. *Experimental data (point markers) and different model assumptions (A1-A3) results (lines) for activities to H_2/CO_2, Ac, Bu and LCFA (Controls) in terms of CH_4 (kmol CH_4 m^{-3}) cumulative production and substrate degradation (kg COD m^{-3}) for inhibited biomass IV. Coefficients of determination (R^2) for model fitting areindicated in every plot*

modifications in *IWA ADM1* model when dealing with the degradation of lipids. Although the proposed *Inhibition-Adsorption Model* produces a satisfactory fitting of experimental results and provides a better representation of the physical nature of the overall LCFA inhibition process, additional experimental data specifically designed to study biosorption phenomena is needed to mathematically express the adsorption-inhibition process. It is important to notice that for all tested modelling approaches, an increase in the initial hydrogenotrophic methanogens and LCFA degrading population occurred along time. The obtained batch experimental data and modeling results, together with the apparent stability of the microbial community structure, might explain the observed LCFA adaptation process as the result of a physiological acclimatation of existing populations or, at most, to the proliferation of specific, yet already existing, LCFA degrading bacteria and syntrophic methanogenic archaea.

5.4. CONCLUSIONS

Activity assays of anaerobic biomass exposed to successive LCFA inhibitory pulses evidenced the recovery capacity of ß-oxidizing bacteria and syntrophic methanogens, while no significant microbial community shift occurred. A new LCFA-inhibition kinetics was proposed within the IWA ADM1 model framework, which resulted in better fits to the experimental results and provided a numerical expression of the process, in accordance to the adsorptive nature of the inhibition.

The predicted increase in hydrogenotrophic methanogens and LCFA-degrading populations along time, together with the observed stability of the microbial community, indicate that the observed adaptation process is of physiological nature.

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Long-chain fatty adsorption and inhibition: Use of adsorption competitive additives as a preventing strategy on anaerobic granular sludge

ABSTRACT. To study long chain fatty acids (LCFA) adsorption and inhibitory process over anaerobic biomass, two different granular sludges were characterized in terms of methanogenic activity rate, LCFA toxicity, and granular morphology. The possibility of introduce competitive additives (bentonite), as a preventing strategy to face with LCFA inhibition, was also tested, and the respectively adsorption isotherms estimated.
A clear inhibitory effect of oleate (C18:1) concentration of 5 g C18:1 L-1was found for both sludges. Palmitate (C16:0) was confirmed to be the main intermediate of C18:1 degradation in not adapted systems, and C16:0 accumulation, mainly adsorbed onto biomass, was confirmed by fluorescence staining and microscopy observation techniques. Although the C18:1 tested concentration inhibited the anaerobic digestion process, the inhibition was reversible and the system was able to recover after the consumption of adsorbed LCFA. The introduction of bentonite as a competitive adsorbent was demonstrated to be a reliable strategy to improve the system resistance to LCFA, affecting the kinetics of the LCFA adsorption-inhibition process, due to its higher adsorption capacity compared to granular biomass.

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6.1. INTRODUCTION

Lipids are interesting substrates for anaerobic digestion process due to its high methane yields. Lipids are initially hydrolyzed to glycerol and to long chain fatty acids (LCFA), which are further converted (ß-oxidation process) by syntrophic acetogenic bacteria to hydrogen (H_2) and acetate (Ac), and finally to methane (CH_4), by methanogenic archaeae (Weng and Jeris, 1976). LCFA are the main intermediates of lipid hydrolysis process, and several problems, related with sludge flotation, washout and toxic effect, have been reported (Rinzema *et al*., 1994; Hwu *et al*., 1998). Further studies have demonstrated that the LCFA inhibitory effect is a reversible phenomenon, related to the physical adsorption of LCFA and their accumulation on the cell walls, hindering the transfer of substrates and metabolites (Pereira *et al.*, 2005; Palatsi *et al.*, 2009b).

Oleate (C18:1) and palmitate (C16:0) were considered to be the major constituents in lipid-rich wastewaters (Hwu *et al.*, 1998). C16:0 has been proposed to be the main intermediate, and key inhibitory specie, during oleate degradation via ß-oxidation (Lalman and Bagley, 2001; Pereira *et al*., 2002). The reported C16:0 accumulation in anaerobic systems could be related to its low solubility, saturation degree, adsorption properties (Kanicky and Shah, 2007; Pereira *et al*., 2005), and also to the presence or absence of a specific microbial community (Sousa *et al.*, 2007). The LCFA adsorption over granular sludge has been reported to be a rapid surface physical phenomenon if compared to biological degradation (Hwu *et al*., 1998).

Several methods to prevent, overcome or recover LCFA inhibited anaerobic systems have been reported. The use of inoculum already acclimatized to LCFA treatment (Alves *et al*., 2001), feeding procedures based on sequential accumulation and degradation steps (Cavaleiro *et al*, 2009), the addition of easily degradable co-substrates (Kuang *et al*., 2006) or the introduction of adsorbents as a recovery agents (Nielsen and Ahring, 2006; Palatsi *et al*., 2009a), have been discussed as a possible strategies to limit LCFA inhibitory effects.

The aim of the present study is to gain a deeper insight on the adsorption and inhibition process of LCFA onto anaerobic granular biomass, by means of granular sludge characterization, activity-toxicity anaerobic batch tests and fluorescence staining microscopy techniques. The possibility of introduce competitive additives (adsorbents), as a preventing strategy to face with LCFA inhibition, was also tested

6.2. MATERIAL AND METHODS

6.2.1. Analytical Methods

Total solids (TS), volatile solids (VS), suspended volatile solids (VSS) and pH were determined according to Standard Methods (APHA, AWA, WEF, 1995).

Biogas and methane production was monitored by pressure transducer and gas chromatography techniques (FID and TCD), as described elsewhere (Angelidaki *et al*., 2009).

Volatile fatty acids (VFA) - acetate (Ac), propionate (Pro), *iso* and *n*-butyrate (Bu), and *iso* and *n*-valerate (Va) were determined with a CP-3800 gas chromatograph (Varian, USA), fitted with Tecknokroma TRB-FFAP capillary column (30m×0.32mm×0.25µm) and FID detection, after sample acidification and extraction, as described by Campos *et al*. (2008).

Long chain fatty acids (LCFA) – laurate (C12:0), myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0) and oleate (C18:1) - were determined as fatty acids methyl esters (FAME) by CP-3800 (Varian, USA) gas chromatograph and FID detection. Samples were previously centrifuged at *4,500 rpm* to analyze solid or liquid phase LCFA content ($LCFA_S$ or $LCFA_L$) by two different etherification and detection procedures, according to Neves *et al*. (2009) and Palatsi *et al.* (2009). Methanolic-HCl or CTMS (cholotrimethylsilane) solutions as FAME catalyst, and capillary columns Teknokroma CP-Sil 52 CB TR-Wax (30mx0.32 mmx0.25µm) or Varian CP-Sil 88 FAME (50mx0.25mmx0.2µm) for detection, were used, respectively. A proficiency testing exercise between both methodologies was performed, and differences in results were lower than *10%*, allowing further results comparison.

6.2.2. Experimental set-up

Biomass characterization

Two different anaerobic granular sludges were sampled from industrial beverage wastewater treatment plants: (A) from a beer brewery, in A Coruña (Spain), and (B) from a fruit juice processing industry, in Lleida (Spain). Both seed sludge were characterized in terms of VSS content ($g_{VSS}\ L^{-1}$), methanogenic activity rate ($mL_{CH4}\ g^{-1}_{VSS}\ d^{-1}$) and granule morphology.

Classical methanogenic activity test in acetate (Ac) and hydrogen (H_2/CO_2) were performed in batch as described elsewhere (Angelidaki *et al.*, 2009). Granule morphology characterization was performed in *20 samples* containing more than *1,200 granules* (*>0.2* $g_{biomass}$ per sample) by image analysis of digitised images (*768×574* pixel size, *256* grey levels) using *Analyze Particle Tool* of ImageJ package software (National Institutes of Health, USA). Images were binarized and particles

sizes were evaluated by equivalent diameter and specific surface ($cm^2\ g^{-1}_{VSS}$), calculated from particle projected area, based on Pereira *et al*. (2003).

LCFA inhibition batch test

Several LCFA concentrations were tested in methanogenic activity assays performed with both granular biomas to select the LCFA concentration that causes a *clear* and *long-lasting* inhibition for use in further experiments. Oleate (C18:1) was selected as LCFA substrate model, considering that C18:1was one of the major constituents in lipid-rich wastewaters and according to its high solubility (Hwu *et al*., 1998). C18:1, used in all assays, was introduced from a stock solution ($5\ g_{C18:1}\ L^{-1}$) of *purum sodium oleate powder salt* (Riedel-de Haën/Sigma-Aldrich; *82%* C18:1/LCFA). Concentrations of C18:1 from *0.1* to *2.0* $g_{C18:1}\ L^{-1}$ were tested in *120 mL* glass vials (*50 mL* working volume; $5\ g_{VSS}\ L^{-1}$ of granular sludge and $1\ g_{NaHCO3}\ g^{-1}_{CODadded}$), under strict mesophilic (*35ºC*) anaerobic conditions (vials bubbled with N_2 and closed with rubber butyl stoppers and aluminium crimps) and continuous shaking (*150 rpm*). Methane production obtained at each tested oleate concentration was expressed as a global LCFA activity rate, determined by the initial slope of the accumulated CH_4 production curve per VSS content of biomass ($mL_{CH4}\ g_{VSS}^{-1}\ d^{-1}$).

LCFA adsorption isotherms

Adsorption isotherms for oleate over bentonite and over anaerobic granular sludge (A) were assessed in batch. Glass vials of *1 L*, with *500 mL* of final media working volume, were used. Media was composed by demineralised water, sodium bicarbonate ($1\ g_{NaHCO3}\ g^{-1}_{CODadded}$) and bentonite ($0.4\ g_{BENTONITE}\ L^{-1}$) or inactivated anaerobic granular sludge ($5\ g_{VSS}\ L^{-1}$) as sorbents. Bentonite was introduced as *analytical grade* ($4SiO_2\ H_2O$, Prod 285234, Sigma-Aldrich) reagent. Biomass was inactivated to differentiate adsorption from biological desorption or LCFA degradation (Hwu *et al*, 1998). Chemically inactivation, incubating biomass at *4ºC* with formaldehyde solution (*4%* v/v) during *2 hours* , was selected to prevent possible cell wall damage caused by other inactivation treatments, like autoclaving (Ning *et al*., 1996). Oleate concentrations from *0.5* to *5.5* $g_{C18:1}\ L^{-1}$ were introduced as LCFA sorbate model, from the previously described stock solution.

Bottles were maintained in anaerobic conditions and under agitation with a continuous shaker (*150 rpm*) inside incubators set at mesophilic conditions (*35ºC*). Liquid phase samples were withdrawn periodically from vials to monitor soluble oleate concentration (C; $mg_{C18:1soluble}\ L^{-1}$). Obtained experimental values were fitted to an asymptotical exponential decay curve in time ($C=C_e+\alpha e^{-\beta t}$), to determine the equilibrium oleate concentration in the liquid phase (C_e; $mg_{C18:1soluble}\ L^{-1}$). Obtained C_e values were used to calculate the LCFA adsorbed concentration per unit of

adsorbent (bentonite or inactivated biomass) and to fit results to a *Freundlich* isotherm model ($C_{ad}=KC_e^{1/n}$), where C_{ad} is the equilibrium amount of sorbate on sorbent (C_{ad}; $mg_{C18:1adsorbed}\ g^{-1}_{TSadsorbent}$), and *K* and *1/n* are the *Freundlich* parameters. Sorbent concentrations were expressed in TS units to allow further comparison between bentonite and biomass adsorption isotherms.

Addition of bentonite as a strategy to prevent LCFA inhibition

Addition of bentonite as a strategy to prevent LCFA inhibition over anaerobic granular sludge was tested in batch (*120 mL* vials with a final working volume of *50 mL*). Granular biomass (A or B) was previously exhausted, under mesophilic (*35ºC*) anaerobic conditions, till residual methane production (*2 days*). As biomass from the brewery reactor (sludge A) was sampled more than *2 months* before the present assay, an acetate pulse (*30 mM*) was added to those vials to activate the inoculum, and to better identify further LCFA inhibition process. LCFA inhibition is usually monitored as an initial CH_4 production delay or as a longer lag phase (Hwu *et al.,* 1998; Pereira *et al.*, 2005; Cavaleiro *et al.*, 2008). If biomass is not previously activated, results can lead in some cases in doubts between a *real* inhibition or *simple* delay due to substrate complexity. As biomass from fruit juice industry (sludge B) was sampled immediately before the present assay, the described activation step was omitted.

Different strategies were tested to force C18:1 adsorption over bentonite and to prevent biomass inhibition. Table 6.1 summarizes the batch tests set-up for addition of bentonite as a strategy to prevent LCFA inhibition.

Table 6.1*. Experimental set-up of adsorption-inhibition batch experiments*

Sludge A	**Day -2** (sludge exhaustion)	**Day 0** (activation)	**Day +1** (inhibition)
T_A	0.5 g $_{BENTONITE}$ L^{-1} + 5 g $_{VSS(A)}$ L^{-1}+3 g $_{NaHCO3}$ L^{-1}	+30 mM $_{Ac}$	+0.5 g $_{C18:1}$ L^{-1}
C_A	5 g $_{VSS(A)}$ L^{-1}+3 g $_{NaHCO3}$ L^{-1}	+30 mM $_{Ac}$	+0.5 g $_{C18:1}$ L^{-1}
BL_A	5 g $_{VSS(A)}$ L^{-1}+3 g $_{NaHCO3}$ L^{-1}	+30 mM $_{Ac}$	-
Sludge B	**Day -4** (LCFA-bentonite adsorption)	**Day-2** (exhaustion)	**Day 0** (inoculation)
T_B	5.0 g $_{BENTONITE}$ L^{-1} + 0.5 g $_{C18:1}$ L^{-1}+3 g $_{NaHCO3}$ L^{-1}	0.5 L $_{sludge\ B}$ under anaerobic conditions	+5 g $_{VSS(B)}$ L^{-1}
C_B	0.5 g $_{C18:1}$ L^{-1}+3 g $_{NaHCO3}$ L^{-1}		+5 g $_{VSS(B)}$ L^{-1}
BL_B	3 g $_{NaHCO3}$ L^{-1}		+5 g $_{VSS(B)}$ L^{-1}

For the sludge A, the preventing strategy consisted in the addition of bentonite into buffered vials together with biomass, before the C18:1 pulse (T_A vials, in Table 6.1). Concentrations of $0.5\ g_{BENTONITE}\ L^{-1}$ and $5\ g_{VSS}\ L^{-1}$, respectively, were selected from previous assays as competitive adsorbent and biomass concentration,

respectively. After the previously described activation step (*30 mM_{Ac}*), vials were inhibited with an oleate pulse of *0.5 $g_{C18:1}$ L^{-1}*.

Based on the results obtained with sludge A, some changes were introduced in the experimental set-up performed with sludge B. A higher concentration of bentonite (*5 $g_{BENTONITE}$ L^{-1}*) was mixed with oleate (again *0.5 $g_{C18:1}$ L^{-1}*) in the buffered media (vials T_B), during *4 days* to force bentonite adsorption, prior to vials inoculation with sludge B (*day 0*).

Control vials (C), with LCFA and biomass but without bentonite, and blank vials (BL), with only biomass, were also run for sludges A and B. All vials were maintained at *35ºC* under continuous shaking (*150 rpm*). Each treatment was performed in triplicate for biogas analysis (CH_4 and H_2), and *10 vials* per treatment were withdrawn periodically to determinate liquid and solid LCFA concentrations ($LCFA_L$ and $LCFA_s$) and soluble VFA profile.

6.2.3. Microscopy observation techniques

Granules, free and submitted to LCFA pulse, from the first set of adsorption-inhibition batch experiments (vials C_A and BL_A, according to Table 6.1), were stained and examined under fluorescence light microscopy (FLM) using a BX51 (Olimpus, Japan) microscope. To better observe the LCFA adsorption process, sampled granules were also sectioned using a cryostat CM 1900 (Leica, Germany) before observation. All obtained images were analysed using ImageJ (National Institutes of Health, USA) package software.

Before sectioning and staining, granular sludge samples were settle at *4ºC* and the supernatant was carefully removed. Afterwards, granules were washed and re-suspended in phosphate buffered saline (PBS) media. Cells were fixed adding *3 volumes* of formaldehyde (*4%* v/v in PBS) to *1 volume* of pellet cells. Samples were incubated overnight, washed, re-suspended in PBS and finally stored at *4ºC* for further observation. For cutting granules in cryomicrotome, granules were placed in a base of OCT mold (Optimum Cutting Temperature media, Sakura Finetek, USA) as described in Batstone *et al*. (2004). Frozen blocks were sectioned on cryostat (sections of *10 µm*) with a knife temperature of *-20ºC*, a cabinet temperature of *-18ºC*, and mounted onto microscopic slides for staining and observation.

Stain was performed with multiple fluorochrome dyes. DAPI (4',6-diamidino-2-phenylindole, Sigma, Spain) was used as probe for biomass or total cells (DNA) while Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, Sigma, Spain) was selected as probe for hidrophobicity sites (lipids). Dyes stock-working solutions of *10 µg mL^{-1}* were prepared and used over sectioned fixed granules directly staining the microscopic slides, for *15 min* in darkness at room temperature, based on Diaz *et al*. (2008) and Chen *et al*. (2007). A further washing step with PBS was implemented to

remove the excess of dye. The FLM settings were *Ex365-370/B 400/LP 421* and *Ex 530-550/B 570/LP 591* for blue (DAPI) and red (Nile Red) channels respectively.

6.3. RESULTS AND DISCUSSION

6.3.1. Biomass characterization

Table 6.2 summarizes main results of granular sludges characterization, according to Material and Methods section.

Table 6.2. *Granular biomass characterization*

Parameter	sludge A	sludge B
Sludge biomass content ($g_{VSS}\ L^{-1}$)	8.81±0.13	8.87±0.02
H_2 activity rate ($mL_{CH4}\ g^{-1}_{VSS}\ d^{-1}$)	72.4±4.4	32.8±1.6
Ac activity rate ($mL_{CH4}\ g^{-1}_{VSS}\ d^{-1}$)	34.4±5.0	45.0±2.5
Mean size as equivalent diameter (mm)	1.96±0.72	2.04±0.93
Specific surface area ($cm^2\ g^{-1}_{VSS}$)	620.50±41.57	540.13±58.65

From results of batch activity test (Table 6.2), it can be stated that both sludge had a similar activity to acetate as methanogenic substrate model, while the hidrogenotrophic activity rate of sludge A was higher than for sludge B. Acetate is considered to be the main product of the ß-oxidation process (Weng and Jeris, 1976) and most of LCFA-degrading microorganisms are known to be proton reducing bacteria, which require syntrophic interaction with H_2-utilizing and acetoclastic methanogens (Schink, 1997; Lalman and Bagley, 2001). Consequently, the balance between acidogenic bacteria and archaeae communities plays a central role in the LCFA degradation process, and the quality of inocula, in terms of methanogenic activity, can influence the overall LCFA degradation process (Pereira *et al*., 2002). For those reasons, dealing with LCFA inhibition, it is important to consider the reported methanogenic activity rates in further discussion of adsorption-inhibition batch tests (Table 6.1).

LCFA inhibitory effect has been related to surface phenomenon (Pereira *et al*., 2005; Nielsen and Ahring, 2006; Palatsi *et al*, 2009). Therefore, dealing with LCFA degradation, it is important to consider the biomass available surface for LCFA adsorption. Mean values of granule size, calculated as equivalent diameter, do not give enough information about sludge morphology due to its high dispersion or standard deviation (Table 6.2). It was necessary to analyze granule size distribution in sampled biomass (data not shown) to estimate equivalent surface area. Although the detected differences in particle size distribution, the obtained value of the

specific surface area (*620.50* and *540.13* $cm^2\ g^{-1}_{VSS}$, respectively) was quite similar for both sludges (Table 6.2).

6.3.2. LCFA inhibition batch test

Global LCFA degradation rates exhibited by sludge A and B.for each tested oleate concentration were calculated as initial slope of specific methane production ($mL_{CH4}\ g^{-1}_{VSS}\ d^{-1}$), according to Material and Methods section. A clear response (exponential decay curve fitting) to increasing initial C18:1 concentration was obtained for both sludges, as plotted on Figure 6.1.

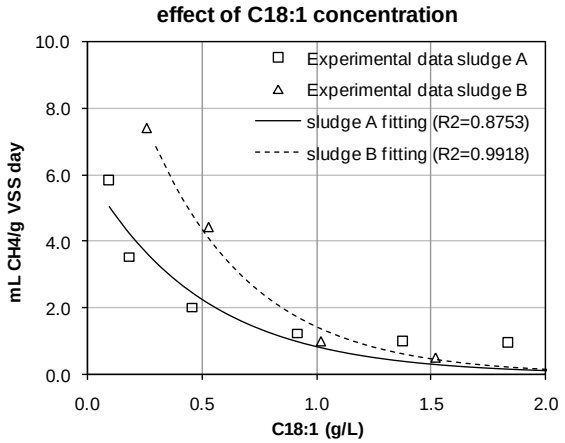

Figure 6.1. *Effect of tested oleate concentration (gC18:1 L-1) on the initial specific methane production rate (mLCH4 g-1VSS d-1) of granular sludge A and B. Markers represent experimental values while lines represent the fitting to an exponential decay curve. Coefficients of determination for curve fitting (R2) are indicated in the figure.*

From Figure 6.1, it can be stated a major resistance of sludge B, than sludge A, to oleate concentrations up to *1.0* $g_{C18:1}\ L^{-1}$, while those differences disappear at higher initial oleate concentrations. Those differences between both sludges response could be explained by differential methanogenic activity of archaeae communities (syntrophic interaction with LCFA degraders), differential available granule surface area (LCFA inhibition as surface related phenomenon) or differential acidogenic or ß-oxidizing bacterial microbial structure (biomass adaptation). From the results of biomass characterization (Table 6.2), the slightly lower methanogenic activity rate and the estimated specific surface area of sludge B, can not explain the higher reported resistance of that biomass to a given LCFA concentration. Part of the higher sensitivity of sludge A might be attributed to the absence of previous biomass exposition to lipids or LCFA adaptation (biomass from brewery industry, no lipid

containing). Contrary, biomass B, obtained from fruit juice wastewater treatment plant, has been probably in contact to higher lipid content, as the contained in fruit peels press liquor waste (Galí *et al*., 2009). Pereira *et al*. (2005) and Palatsi *et al*. (2009a and 2009b) observed a considerable increase of sludge activity, or resistance to LCFA, after batch depletion of the accumulated LCFA or after subsequent LCFA contact. Sousa *et al*. (2007) reported the promotion of specific ß-oxidizers populations, dominated by members of *Clostridiaceae* and *Syntrophomonadaceae* families in lipids acclimated cultures. The hypothesis of a higher concentration of ß-oxidizing bacteria on sludge B, might be confirmed by molecular biology tools or other microbiology techniques.

Despite the reported differences in the C18:1 inhibitory effect over sludge A and B, a concentration of *0.5* $g_{C18:1}$ L^{-1} was considered enough to reduce the global biomass activity, causing a *clear* and *long-lasting* inhibition phenomena (Figure 6.1). Consequently, *0.5* $g_{C18:1}$ L^{-1} was selected as the LCFA inhibitory concentration to be tested in further adsorption-inhibition batch experiments (Table 6.1).

6.3.3. LCFA adsorption isotherms

Adsorption batch experiments, of oleate over granular biomass (sludge A) and over bentonite, were performed according to Material and Methods section. Figure 6.2 shows the evolution of $C18:1_{soluble}$ concentration in the vials with bentonite or granular inactivated biomass as sorbents (Figure 6.2a and 6.2c, respectively), and the corresponding estimated *Freundlich* isotherms (Figure 6.2b and 6.2d, respectively). Other models, like *Langmouir,* were also tested, obtaining similar fittings coefficients (data not shown).

The higher adsorption capacity of bentonite compared with the tested anaerobic granular sludge (A), in terms of equilibrium amount of sorbate on sorbent (C_{ad}), emerges clearly from the experimental results (Figure 6.2). The obtained values of *Freundlich* parameters for chemically inactivated granular sludge (*K=19; 1/n= 0.442*) were similar to ones reported by Hwu *et al* (1998) with oleic acid and thermal inactivated granular sludge (*K=12 and 1/n= 0.521*, also plotted in Figure 6.2d). No references were found in literature for adsorption of LCFA over bentonite. Nevertheless, if it is assumed that adsorption can be described by a physical theory, surface and concentration dependent (Ning *et al*, 1996; Hwu *et al*, 1998), the higher specific surface area of bentonite (Raposo *et al*, 2004) compared to anaerobic granular sludge (Table 6.2), may result in a higher adsorption capacity per unit of sorbant, as it was shown in Figure 6.2b, with a corresponding set of parameters for a *Freundlich* isotherm fitting of *K=2.5* and *1/n=1.042*.

From the obtained results, a concentration of *0.5* $g_{BENTONITE}$ L^{-1} was considered enough for a fast and complete adsorption of the selected LCFA inhibitory concentration (*0.5* $g_{C18:1}$ L^{-1}) in the further experiments of bentonite adition as a strategy to prevent inhibition (Table 6.1). According to the calculated specific surface areas of sludge B (Table 6.2), its adsorption isotherm is considered to be similar to sludge A.

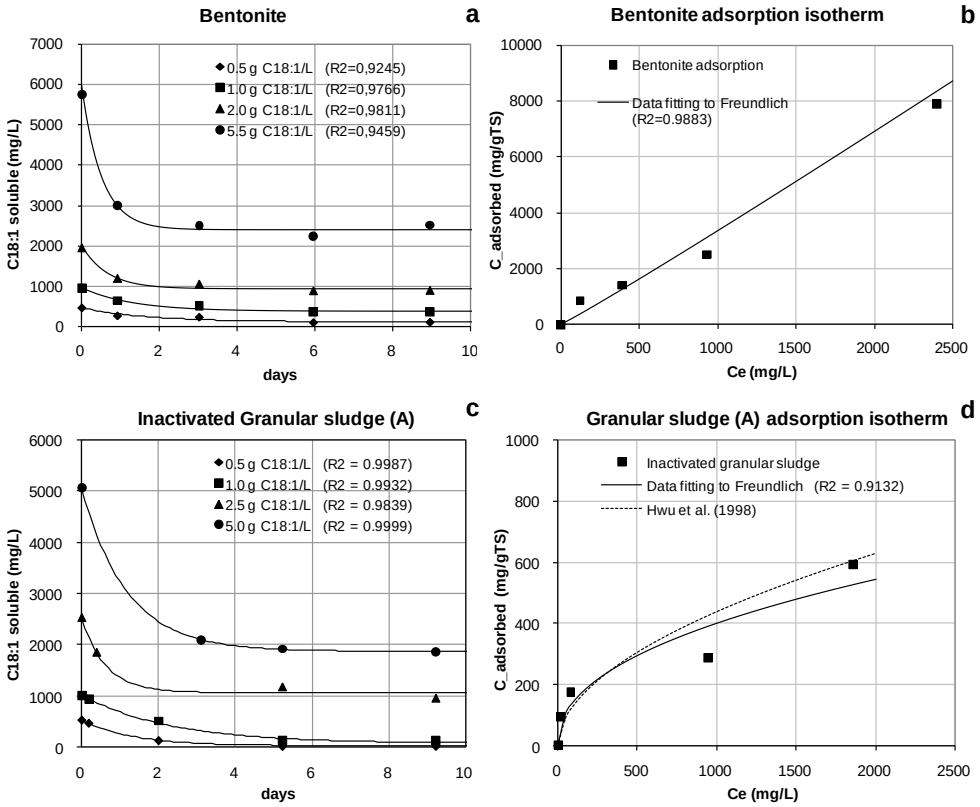

Figure 6.2*. Evolution of oleate concentration in liquid phase,* $C18:1_{soluble}$*, in the batch adsorption assay performed with bentonite (**a**) and with inactivated anaerobic granular sludge A (**c**), to calculate the corresponding equilibrium concentration (Ce, mg* L^{-1}*). Results were fitted to a Freundlich isotherm model (—) for bentonite (**b**) and sludge A inactivated biomass (**d**) and compared with available literature values (--).*

6.3.4. Addition of bentonite as a strategy to prevent LCFA inhibition (sludge A)

Based on the previous results (biomass characterization, LCFA inhibition batch tests and adsorption batch experiments) an *adsorption-inhibition* batch test, with sludge A, was designed (Table 6.1) to study the addition of a bentonite as a strategy

to prevent LCFA inhibition. Figure 6.3 shows the time course of main detected LCFA, oleate and palmitate, in the solid and liquid phase ($LCFA_S$ and $LCFA_L$) VFA accumulation, and CH_4 production in the bentonite addition treatment (T_A), controls (C_A) and blank (BL_A) vials. All monitored parameters were expressed in equivalent chemical oxygen demand units (COD) to facilitate comparison.

As LCFA pulse was introduced from a solubilised stock solution (see Material and Methods section), it was considered that, initially (*day 1*), LCFA were completely contained on liquid phase (Figure 6.3b and 6.3d). Notice that initial palmitate concentration (*day 1* in Figure 6.3d) was due to the synthesis grade of sodium oleate salt reagent (see Material and Methods part) and not to the beginning of a C18:1 degradation process. The disappearance of C18:1 from the liquid media was very fast (in less than *5 days*), including the control vials (C_A), where no competitive adsorbent (bentonite) was added (Figure 6.3b). C18:1 disappearance from solid phase was attributed to a partial degradation to palmitate, as shown in Figure 6.3c. C16:0 was mainly present accumulated on solid phase and no significant concentrations of C16:0 were detected on liquid phase samples after initial adsorption process (Figure 6.3d). Palmitate has been proposed as the main intermediate in oleate degradation via ß-oxidation (Lalman and Bagley, 2001; Pereira *et al*., 2002). No palmitoleate (C16:1) or other intermediates were detected in liquid or solid phase samples (data not shown) during C18:0 consumption, consistently with the hypothesis of hydrogenization of unsaturated LCFA prior to ß-oxidation process (Weng and Jeris, 1976; Lalman and Bagley, 2001). The maximum levels of C16:0 in the solid phase were reached at *day 7-10* (Figure 6.3c) with few differences between treatments.

Some reports suggest a specific microbial community able to degrade saturated or unsaturated LCFA, explaining the fact of palmitate accumulation by the absence of specific microbial species (Sousa *et al*., 2007). In other reports, where it is investigated the effect of degree, type and position of LCFA unsaturation in the formation of lipid monolayer, it is suggested that the C16:0 intermolecular distance in monolayer is lower compared to C18:0 (Kanicky and Shah, 2007). Those factors could increase the limiting effect of the C16:0 accumulation over the nutrient transport through the cell walls (Pereira *et al*., 2005), producing a process inhibition as the reported on Figure 6.3.

The degradation of LCFA, via ß-oxidation, produced an accumulation of acetate in the medium (Figure 6.3e), that is maintained till complete degradation of palmitate. No significant concentrations of other intermediates were detected, in accordance with other studies (Weng and Jeris, 1976; Angelidaki and Ahring, 1995; Cavaleiro *et al*., 2008 and 2009).

The inhibition caused by the LCFA pulse resulted in an immediate stop in methane production (except for BL_A vials, with only Ac, according to Figure 6.3f). Methanogenesis was reported to be more susceptible to LCFA inhibition compared to acidogenesis (Lalman and Bagley, 2001; Mykhaylovin *et al*, 2005). Nevertheless LCFA inhibition was a reversible process as methane formation was able to recover after *30-35 days*. No significant differences were also reported in CH_4 production between bentonite addition treatment (T_A) and control vials (C_A).

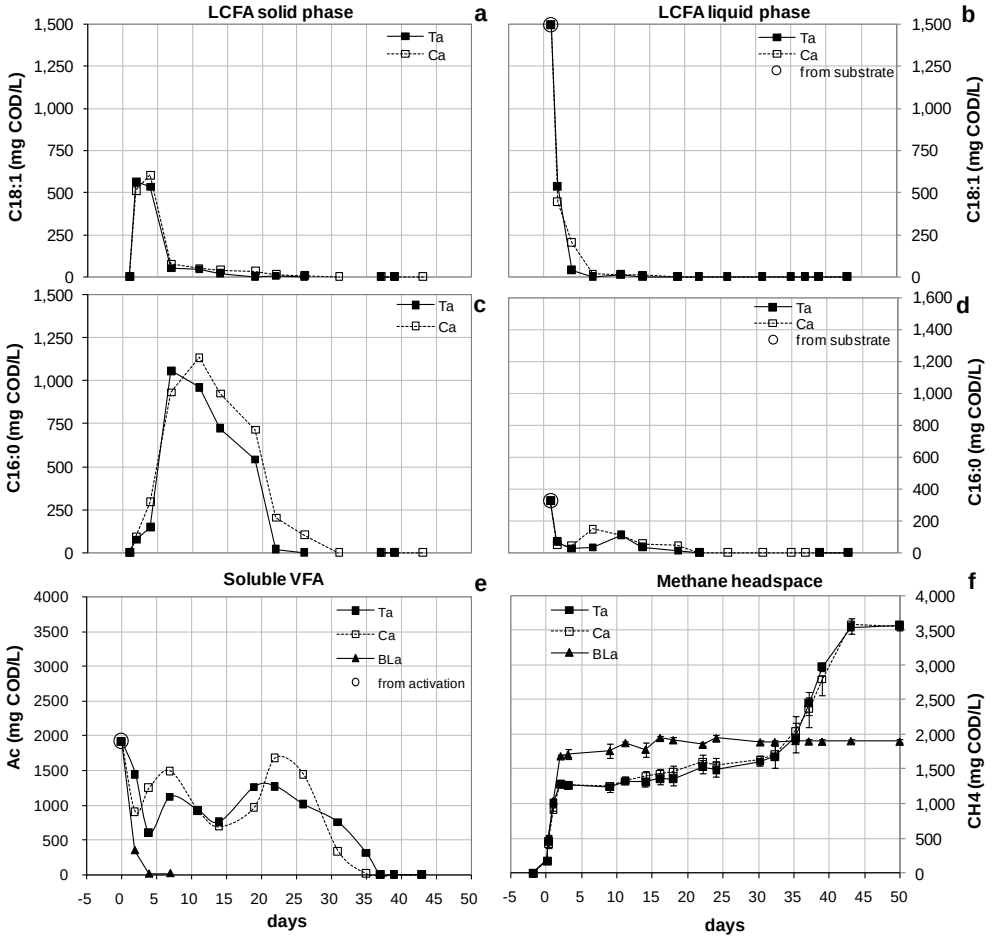

Figure 6.3. *Comparison of LCFA concentration in the solid phase (**a** and **c**) and in the liquid phase (**b** and **d**), acetate profile (**e**) and methane formation (**f**), for bentonite treatment (T_A), control (C_A) and blank (BL_A) vials. All parameters are expressed in COD equivalent concentration units (mg $_{COD}$ L^{-1}). The circles indicate the initial estimated concentration from LCFA pulse introduced in the vials.*

Present results showed a fast and not limiting step for the oleate partial ß-oxidation process and confirmed the palmitate as the main intermediate and key inhibitory specie. The high LCFA inhibitory effect, reported in the previous inhibition batch test of sludge A (Figure 6.1), together with the previous discussed absence of inoculum adaptation to lipids or LCFA content, may had played a main role in the *slow* palmitate degradation or in the *step-by-step* LCFA overall degradation process, clearly presented in Figure 6.3.

6.3.5. Microscopic examination of granules (sludge A)

Intermediates of LCFA degradation, like palmitate, have been proposed to be encapsulated (entrapped), precipitated or adsorbed over sludge, as function of the LCFA (saturated or unsaturated) and biomass (suspended or granular) source (Pereira *et al*., 2005). To confirm that C16:0 was mainly adsorbed onto the biomass cell walls, causing biomass inhibition, and not precipitated in media, granules from previous batch test were sectioned using a cryostat and examined by dye staining and FLM microscopic observation, according to Material and Methods section. Samples from C_A and BL_A vials where taken from adsorption-inhibition batch experiments at *day 10*, when mainly all C18:1 was consumed and maximum C16:0 levels in solid phase were detected (Figure 6.3c). Figure 6.4 shows an example of the appearance under FLM of BL_A (Figure 6.4 a-c) and T_A (Figure 6.4 d-f) stained sections.

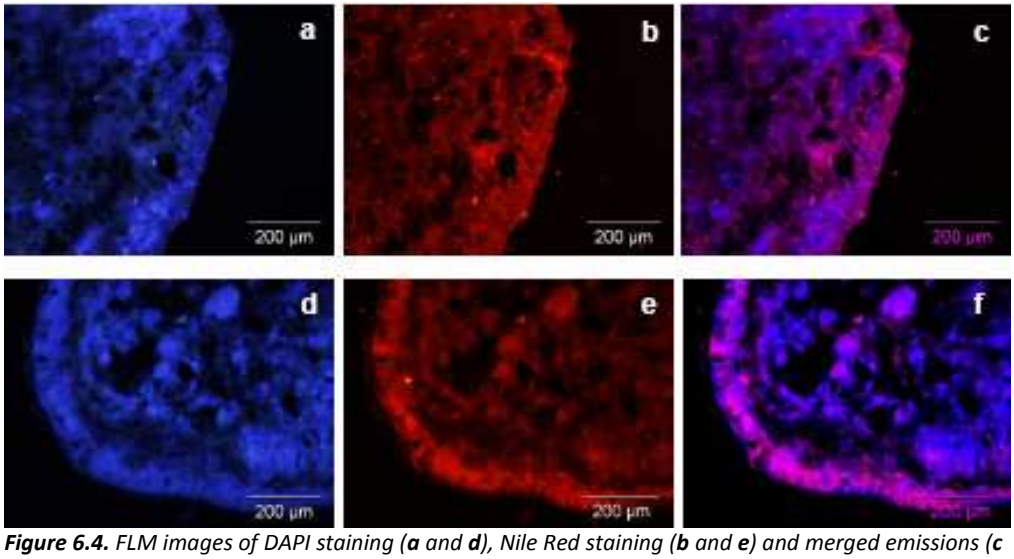

Figure 6.4. *FLM images of DAPI staining (**a** and **d**), Nile Red staining (**b** and **e**) and merged emissions (**c** and **f**), of BL_A and C_A sectioned granules, respectively*

Unfortunately, Nile red was not able to differentiate between phospholipids and LCFA adsorbed onto cell walls. Diaz *et al*. (2008) reported a shift of the Nile Red emission spectrum from red to yellow, with oleyl cholesteryl ester, triolein and oleic acid, but not between oleic acid and phospholipids. Consequently the expected response for palmitate adsorption must be in terms of signal intensity, not in terms of Nile Red signal presence or absence. According to those statements, from all obtained images, and as example in Figure 4, it can be stated a higher Nile Red signal in the outer layer of C_A granules, compared with BL_A granules, where no LCFA pulse was introduced, confirming that C16:0 intermediate was adsorbed onto granule surface.

Results of the selected fluorescent dyes and observation procedure gave a qualitative approach to monitor the process of LCFA adsorption over anaerobic granular sludge, as complementary information to classic methodologies, like batch degradation or toxicity tests. Further research, newly more specific dyes and more optimized procedures, may establish methodologies of *in situ* and rapid LCFA *quantification* by fluorescence intensity analysis, as it has been achieved in other biotechnology fields (Diaz *et al*, 2008; Larsen *et al*, 2008).

6.3.6. Addition of bentonite as a strategy to prevent LCFA inhibition (sludge B)

In the adsorption-inhibition batch experiments performed with sludge A, the expected preventing effect of bentonite addition, over LCFA inhibitory process, was not detected (Figure 6.3). The quantity of competitive adsorbent selected in T_A vials (Table 6.1) was estimated from the bentonite adsorption isotherms (Figure 6.2), as the bentonite concentration that allows the fast and complete adsorption of LCFA pulse. In that way, and from the adsorption experiment set-up, it was considered exclusively a LCFA-bentonite system. The possible competition between granular sludge and bentonite for adsorption of the introduced LCFA pulse was not taken into account. Works with higher ratio of concentrations of sorbents, used as additives after a LCFA inhibition, obtained clearer effects (Nielsen and Ahring, 2006; Palatsi *et al.*, 2009). Furthermore, spatial distribution and probability of the bentonite-LCFA or biomass-LCFA adsorption occurrence, function of its concentration and particle density, might had play an important role in the obtained results. For that reasons, a new experimental set-up was designed for sludge B. In T_B vials, higher bentonite concentration (*5* $g_{BENTONITE}\ L^{-1}$) was incubated with LCFA in the experimental vials during *4 days*, prior to inoculate vials with sludge B, in order to force LCFA adsorption only over bentonite and better prevent biomass inhibition, as summarized on Table 6.1.

Figure 6.5 shows the time course of $LCFA_S$ and $LCFA_L$ evolution, VFA accumulation and CH_4 production in the bentonite addition treatment (T_B), controls

(C_B) and blank (BL_B) vials. All monitored parameters were expressed in equivalent chemical oxygen demand units (COD) to facilitate comparison, while time scale was the same as in Figure 6.3, to better compare results.

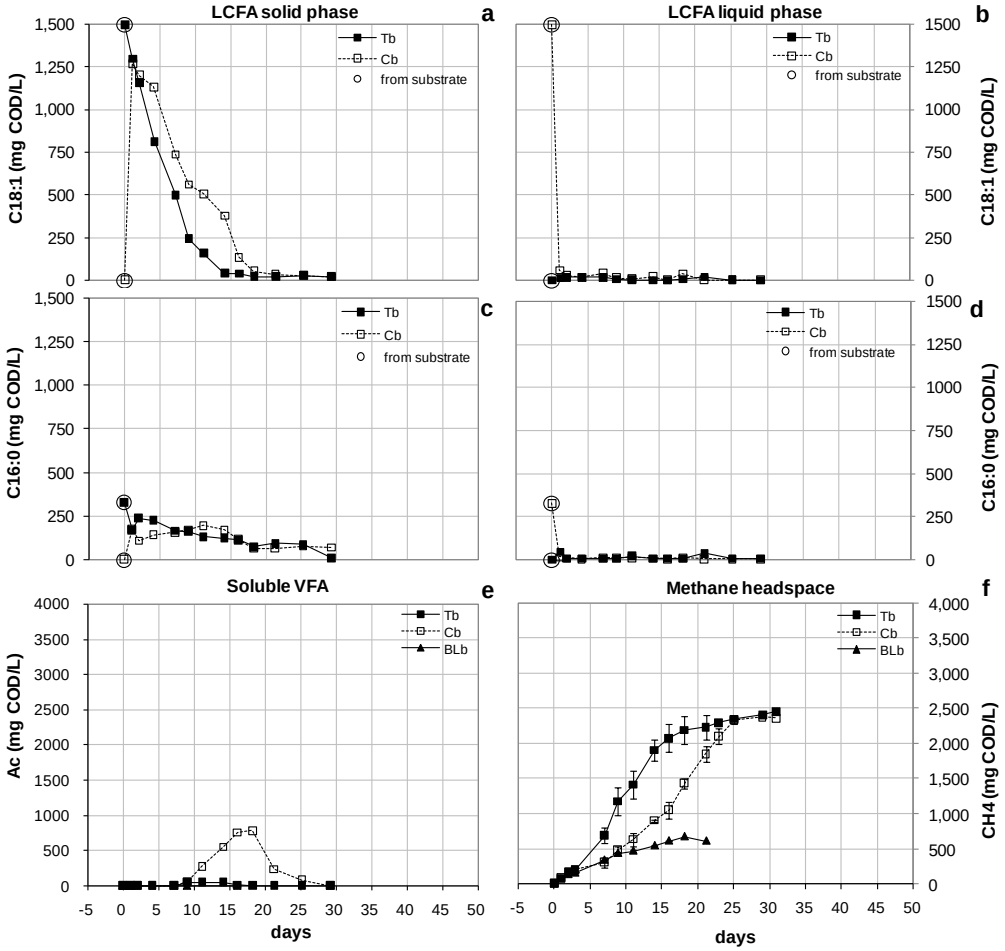

Figure 6.5. *Comparison of LCFA concentration in solid phase (**a** and **c**) and liquid phase (**b** and **d**), acetate profile (**e**) and methane formation (**f**), for bentonite treatment (T_B), control (C_B) and blank (BL_B) vials. All parameters are expressed in COD equivalent concentration units (mg $_{COD}$ L^{-1}). The circles indicate the initial estimated concentration from LCFA pulse introduced in the vials.*

As LCFA was incubated during *4 days* in the buffered media, in the presence or absence of bentonite (Table 6.1), it was considered that at *time 0* the LCFA were completely solubilised in the C_B vials, while in T_B vials the LCFA were completely adsorbed over bentonite (Figure 6.5a-6.5d). Notice that initial C16:0 concentrations

(*day 0* in Figure 6.5c and 6.5d) was due to the synthesis grade of sodium oleate reagent. In control vials (C_B), where bentonite was not present, initial soluble oleate concentration (Figure 6.5b) was rapidly detected in solid phase or adsorbed over biomass (Figure 6.5a). In the present experiment, and from the C18:1 degradation evolution (Figure 6.5a) it was possible to observe differences between treatments, being the degradation rate higher for the treatment where bentonite was added (T_B, or preventing strategy). Furthermore, C18:0 degradation did not produce the expected C16:0 peak, or intermediate accumulation on sampled solid phase (Figure 6.5c), neither in T_B or C_B vials.

The higher reported resistance of sludge B to LCFA toxicity (Figure 6.1), together with the differential inoculum origin and the suggested major adaptation of sludge B to lipid treatment, might have influenced on the different LCFA degradation profile presented in Figure 6.5. Cavaleiro *et al*. (2009) detected accumulations of C16:0 in reactors treating oleate based influents only during the first contact with C18:1 or LCFA pulses. After *2-3 cycles* of LCFA feeding, C16:0 was not accumulated on the system, in agreement with the present results.

As in the previous adsorption-inhibition batch experiments, no palmitoleate (C16:1), myristate (C14:0) nor other LCFA ß-oxidation intermediates, were detected in liquid or solid phase samples and only an acetate accumulation was detected. The reported differences between T_B and C_B vials, in terms of C18:1 consumption, were now also clearly detected by the VFA evolution profile, with clear Ac accumulation in C_B vials (Figure 6.5e). Contrary, no significant acetate concentrations was detected on bentonite vials (T_B) and methane production rate was higher than for control (C_B) vials (Figure 6.5f).

The results of the present adsorption-inhibition batch tests demonstrate that it was possible to prevent LCFA inhibition by addition of competitive adsorbents like clay mineral bentonite. Those additives can compete with biomass for LCFA adsorption and, consequently, influence the kinetics of the LCFA adsorption and inhibition process.

6.4. CONCLUSIONS

Comparison of LCFA adsorption over anaerobic granular sludge and over clay mineral bentonite, in terms of Freundlich adsorption isotherms, evidenced a clear and higher adsorption capacity of bentonite.

Batch test with not adapted biomass showed a fast and not limiting step for oleate partial ß-oxidation and confirmed palmitate as the main intermediate or the key inhibitory specie. Obtained LCFA degradation profiles focus the discussion on the differential oleate and palmitate inhibitory behaviour due to differentiated

adsorption properties, biomass adaptation or ß-oxidizing microbial community structure. The tested fluorescence staining and microscopy observation techniques evidenced the presence of palmitate adsorbed onto anaerobic granular sludge, with the consequent potential implications over membrane transport and LCFA inhibitory effect.

Further batch test results, forcing the occurrence of LCFA-bentonite adsorption, demonstrated the use of competitive additives to be a reliable strategy to improve system performance, in terms of process stability, methane production delay or resistance to LCFA inhibitory concentrations.

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6.5. References

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General Conclusions and suggestions for further research

Finally, according to the proposed objectives, the present chapter summarizes the main conclusions of this dissertation. Suggestions for further research and perspectives of slaughterhouse waste treatment are also presented.

7.1. GENERAL CONCLUSIONS

From the results of slaughterhouse waste characterization and biodegradability tests it was stated the high interest of those substrates for the anaerobic digestion process due to the high potential methane yields. However, the diverse characteristics of tested wastes and the different obtained biogas production profiles, indicate specific kinetics for protein and lipids degradation. In relation to the later, the present study was focussed on the study of the limiting inhibitory effect of long chain fatty acids (LCFA) on the degradation process. Although severe LCFA inhibition was monitored under laboratory conditions, the system capacity to recover the activity was confirmed and the possible occurrence of biomass adaptation was also identified (**Chapter 3** and **Annexed Information**).

Consequently, in order to adapt and optimize the anaerobic treatment to lipid rich substrates, several strategies to recover systems subjected to LCFA inhibition were tested in thermophilic batch and continuous reactors (**Chapter 4**). The dilution of the reactor contents with an active inoculum in order to increase the biomass/LCFA ratio, and the addition of adsorbents, were found to be the best strategies to recover LCFA inhibited reactors.

The development of a fast and accurate methodology to measure LCFA in biological samples pointed to the importance of measuring not only liquid but also solid samples, due to the magnitude of LCFA adsorption on the biomass.

The effect of introducing adsorbents to recover the activity of inhibited reactors was related with the competition with the biomass in adsorbing LCFA, indicating the physical nature (surface adsorption and transport sites saturation) of LCFA inhibition.

Repeated LCFA pulses on thermophilic biogas reactors, resulted in a faster recovery of the activity after each applied pulse, and in an enhancement in process rates. This point again (as mentioned in Chapter 3) the occurrence of adaptation/tolerance process in the biomass.

In order to identify the nature of the reported adaptation/tolerance process (Chapter 3 and Chapter 4), biomass subjected to successive inhibition LCFA pulses was studied in a multidisciplinary approach by means of specific activity batch tests, the characterization of the microbial populations by culture-independent techniques, and by the mathematical modeling of the involved biochemical and physical processes (**Chapter 5**).

The community of eubacteria and archaeae in sampled biomass was studied by molecular biology tools (PCR amplification, DGGE profiling, and sequencing of DNA), and many of the identified microorganisms were closely related to species found previously in anaerobic digesters, where relatively high concentrations of LCFA are likely to occur. Different sensitivities to LCFA of major microbial trophic groups was

evidenced (by activity tests) and the adaptation process upon exposition to successive LCFA inhibitory pulses was related to the proliferation of ß-oxidazing bacteria and syntrophic methanogens, rather than to a specific shift in the microbial community structure.

A new LCFA-inhibition kinetics was proposed within the IWA ADM1 model framework, which resulted in better fits to the experimental results and provided a numerical expression of the process, in accordance to the adsorptive nature of the inhibition. The predicted increase in hydrogenotrophic methanogens and LCFA-degrading populations along time, together with the observed stability of the microbial community, indicate that the observed adaptation process is of physiological nature.

In accordance with the reported importance of the adaptation (Chapter 3 and 5) and adsorption processess (Chapter 4 and 5), a comprehensive study on those processes was undertaken with granular biomass. Parameters that were considered to affect the adsorption/inhibition/adaptation phenomena were: LCFA concentration, methanogenic syntrophic activity, microbial community structure, granule surface area, and ratio LCFA/active biomass (**Chapter 6**).

Batch test with non-adapted biomass showed a fast and not limiting step for the oleate partial ß-oxidation process and pointed the palmitate as the main intermediate or the key inhibitory specie during oleate degradation. The introduction of fluorescence staining and microscopy observation techniques evidenced the presence of palmitate adsorbed onto anaerobic granular sludge (not precipitated), with the consequent implications on membrane transport and LCFA inhibitory effect.

When adsorbents were introduced as a LCFA-inhibition preventing strategy, it was demonstrated that competitive additives were a reliable mean to improve the treatment of lipid rich substrates, in terms of process stability, shorter methane production delay, or biomass tolerance of LCFA inhibitory concentrations.

In summary, the general conclusions of this thesis are:

- The LCFA inhibition phenomena are directly related to the adsorption of LCFA onto the active biomass.
- The main intermediate product of oleate ß-oxidation, in non-adapted systems, is palmitate, which is responsible for the process inhibition.
- The use of inorganic adsorbents, such as bentonite, can be used to prevent the inhibition of microorganisms and, also, to recover reactors inhibited by LCFA.
- The adaptation process of microorganisms to succesive LCFA pulses is of physiological nature. That is, the growth of the specific microbial

populations that allows the adaptation to higher LCFA concentrations, instead of a change in the population structure, is the main mechanism.

- The kinetics of the inhibition process is related to the ratio LCFA/biomass, and the developed kinetics clearly shows its capacity to fit and top predict experimental data, in the framework of the IWA ADM1 model.

Considering the conclusions, the anaerobic digestion of lipid-rich wastes can be achieved if adequate LCFA/biomass ratios are applied. The inhibition of the process can be prevented or recovered with competitive inorganic adsorbents and ensuring the growth/adaptation of the microorganisms. The inclusion of the proposed inhibition kinetics into the IWA ADM1 model can help to simulate the anaerobic digestion of high lipid-rich substrates, allowing to guide the desing and operation of reactors.

Eventually, the obtained results will help to obtain high renewable energy rates from slaughterhouse wastes trough anaerobic digestion.

7.2. SUGGESTIONS FOR FURTHER RESEARCH AND PERSPECTIVES

Althought the results described in this thesis contributes to the general know-how on the anaerobic digestion of lipid waste and LCFA inhibition, some aspects have to be taken into account with respect to future studies on this subject.

Further studies are needed to improve knowledge about different degradation patterns of saturated or unsaturated LCFA (palmitate/oleate). Quantitative molecular biology tools, like real time PCR and new microscopy observation techniques, might shed new insights on these diverse behaviours. Also, new model developments, considering different microbial groups or different adsorption behaviour of saturated/unsaturated LCFA could orientate future research opportunities.

The use of adsorbents, like bentonite, has been demonstrated to be a reliable strategy to prevent or to overcome LCFA inhibition. However, bentonite is an inert material and do not contribute to biogas formation. Further research, using additives or co-substrates, which could contribute to LCFA solubilisation and transport trough the cell walls, like glycerol and albumin, could improve the system efficiency and the methane yield.

New reactor designs, that allow the coupling of a fast adsorption with a rate limiting the degradation of LCFA by means of system recirculation or sequential feeding-reaction process, and could improve the scale-up of industrial applications in slaughterhouse facilities.

Annexed Information

This chapter contains annexed scientific output related with the Thesis scope

8.1. **Palatsi, J**., Fernández, B., Vavilin, V.A., Flotats, X. (2007). Anaerobic biodegradability of fresh slaughterhouse waste: interpretation of results by a simplified model. In: *11th World Congress on Anaerobic Digestion (AD11). Bioenergy for our future*. Brisbane (Australia), 23-27 septiembre 2007.

8.2. Vavilin, V.A., Fernandez, B., **Palatsi, J.**, Flotats, X. (2008). Hydrolysis kinetics in anaerobic degradation of particulate organic material: an overview. *Waste Management,* **28**(6): 939-951.

8.3. Flotats, X., **Palatsi, J**., Ahring, B.K., Angelidaki, I. (2006). Identifiability study of the proteins degradation model, based on ADM1, using simultaneous batch experiments. *Water Science and Technology*, **54**(4): 31-39.

Anaerobic biodegradability of fresh slaughterhouse waste. Interpretation of results by a simplified model

J. Palatsi[1], B. Fernández[1], V.A. Vavilin[2] and X. Flotats[1]

[1] GIRO Technological Centre. Rambla Pompeu Fabra 1, E-08100 Mollet del Vallès, Barcelona, Spain (E-mail: xavier.flotats@giroct.irta.es)
[2] Water Problems Institute of the Russian Academy of Sciences. Gubkina 3, Moscow, 119991, Russia

Abstract
Anaerobic digestion of slaughterhouse waste is a complex process for which mathematical models can serve to understand this complexity and to predict failure situations. A simplified model of slaughterhouse waste anaerobic digestion was developed to study the effect of different initial lipids to proteins ratios. Experimental data on the production of methane and volatile fatty acids (VFA) were used for parameter identification. The model fitted the experimental data relatively well. Results showed that methanogenesis developed relatively fast due to the methanogenic bacteria already presented in the inoculum. The hydrolysis/acidogenesis of proteins and lipids was described by the first-order and the Contois kinetics, respectively. Two peak values of acetate concentration were measured. According to the model, the second peak in acetate concentration occurred because the combined process hydrolysis/acidogenesis of lipids was dependent of the growth rate of the related hydrolytic/acidogenic bacteria.

Keywords
Anaerobic digestion; Slaughterhouse waste; Hydrolysis, Lipids, Proteins, First-order and Contois kinetics

INTRODUCTION

The enzymatic hydrolysis of biopolymers like carbohydrates, proteins, and lipids, which are the main components of the organic waste, results in the production of monosaccharides, amino acids, glycerol and long chain fatty acids (LCFA), respectively. In the subsequent acidogenesis stage, these products are transformed into volatile fatty acids (VFA), mainly acetate, and hydrogen, which are precursors for methane formation. In general, acidogenesis is usually considered much faster than hydrolysis, being the latter stage the limiting step for the overall process. Traditionally, the first-order kinetics has been used to describe the hydrolysis of

carbohydrates, proteins and lipids in a complex waste (Christ *et al*., 2000; Salminen *et al*., 2000; Batstone *et al*., 2002; Lokshina *et al.*, 2003). However, in a number of papers it was shown that at high, or fluctuating, organic loading rates, models describing particulate hydrolysis, coupled to the growth of hydrolytic bacteria, provide better results (Vavilin *et al.,* 2004; Vavilin *et al.,* 2007).

Slaughterhouse waste are characterized by different amounts of proteins and lipids, which are the main components, and with low carbohydrates content. Its anaerobic digestion can be conceptually described by all the biological and physico-chemical reactions included in the ADM1 model (Batstone *et al.,* 2002), adding inhibition of several microbiological steps by LCFA (Rinzema *et al*., 1994). A primary tool to characterize a substrate is the biodegradability assay. Assays with different contents in the major macromolecules can result into different methanization rates and biodegradability values. Standardized methods for the biodegradability study are designed to perform the assay in such a condition where microbial biomass is not a limiting factor. In this situation, hydrolysis process should be expressed by a first order kinetics respect to substrate. A limited content of a given hydrolytic specific biomass can produce biodegradability test results difficult to understand or to explain.

When hydrolysis is the rate limiting step of the overall anaerobic digestion process, kinetic expressions for this step could be enough for obtaining simplified models to predict methane production. When hydrolytic/acidogenic biomass concentration is not limiting the process, a first order expression can led to satisfactory simplified models. When the biomass is limiting the hydrolytic/acidogenesis processes, two-phase surface-related models has shown to be useful to predict the sigmoid type curve obtained for methane production in this situation, which conceptually describes the complex multi-step hydrolysis process (Vavilin *et al*., 1996). The Contois kinetics of hydrolysis uses a single parameter to represent saturation of both substrate and biomass, but is as good at describing the hydrolysis experimental data as the two-phase surface-related model (Vavilin *et al*., 2007).

The presence of different pools of organic matter in the substrate and different concentration levels of microbial groups in the inoculum, during the biodegradability study of a complex waste, can result into different responses and, hence, into experimental results difficult to interpret. The objective of the present study is to analyse results of anaerobic biodegradability assays of fresh slaughterhouse waste with different proteins to lipids ratio, using a simplified model based on the hydrolysis/acidogenesis processes as the rate limiting steps of its anaerobic decomposition.

MATERIALS AND METHODS

Experimental design

Samples of Category 2 and 3 animal by-products (EU Regulation CE 1774/2002) were collected from a piggery and cattle slaughterhouse located at Binefar (Spain). The selected fractions were: waste fat and meat tissues of pork and cows, lungs, livers and kidneys from pork, cattle digestive tract content and pork blood. Samples from primary sludge and the wastewater from the slaughterhouse wastewater treatment plant were also taken. A mixture containing all the fractions in a proportion analogous to that generated in the slaughterhouse facilities was prepared (M3). Two additional mixtures (M1 and M2) containing a lower fat content, in relation to the protein, were also prepared (see Table 1).

***Table 1**. Composition of the mixtures M1, M2 and M3 of slaughterhouse waste used in the T1, T2 and T3 assays, respectively.*

	M1		M2		M3	
TS (g/kg)	36.00	±0.75	54.99	±0.42	80.84	±1.40
VS (g/kg)	34.36	±0.14	53.21	±0,41	79.12	±1.38
VS (% TS)	84.76%		85.21%		85.73%	
N_{TK} (mg/kg)	1680.38	±18.66	1853.44	±7.35	1903.25	±26.96
NH_4^+-N (mg/kg)	58.44	±0.15	65.19	±0.48	67.30	±1.04
Protein (g/kg)	10.14	±0.12	11.17	±0.04	11.47	±0.16
Fat_{Soxlet} (g/kg)	20.87	±0.05	38.74	±0.35	64.34	±1.17
COD (g/kg)	79.88	±0.26	133.83	±1.10	206.53	±3.64

These mixtures were used in batch anaerobic biodegradability tests based on Soto *et al*. (1993). Three tests in triplicate were performed, named T1, T2 and T3 respectively for each mixture (M1, M2 and M3). Glass flaks of *1000 ml* were filled with *500 g* of the selected mixture, up to a final concentration of *5 g COD/l*, and were supplemented with macro and micronutrient solution. An alkaline solution was also added (*1 g $NaHCO_3$/g COD*) and the pH was adjusted to neutrality. Digested sewage sludge from Lleida (Spain) wastewater treatment plant was used as inoculum, at a constant concentration of *5 g VSS/l* for all the experiments. The flasks were stirred and bubbled with N_2/CO_2 gas in order to remove O_2 before closing them with rubber stoppers. A reducing solution was finally added (*5 ml of 10 g Na_2S/l*). The flasks were incubated at *35ºC* for *31 days*. The time course of methane production and VFA concentrations were followed by gas chromatography (TCD and FID) using the methods described in Campos *et al*. (2007). Analytical determinations for Total solids (TS), volatile solids (VS), volatile suspended solids (VSS), chemical oxygen demand (COD), total Kjeldhal nitrogen (N_{TK}), ammonia nitrogen (NH_4^+-N), pH and fat content (Fat_{Soxlet}) were adapted from Standard Methods (APHA, 1995). Protein concentration was estimated from organic nitrogen content.

Simplified model of anaerobic digestion of slaughterhouse waste

A scheme of the simplified anaerobic digestion model used is presented in Fig.1, based on the following assumptions:

1. Only proteins (X_{PR}) and lipids (X_{LI}) were considered, since carbohydrates content in the waste was less than 7*%.*
2. Hydrolysis/acidogenesis of proteins was described as a first-order reaction, while the hydrolysis of lipids was described according the Contois kinetics.
3. Three groups of substrate specific microorganisms where considered: (i) lipid hydrolytic/acidogenic –$X_{ha\text{-}LI}$, (ii) acetogenic -X_{pro}, and (iii) acetoclastic methanogenic -X_{ac}.
4. Acetate (S_{ac}) and propionate (S_{pro}) were considered as representatives of VFA because of the low concentration of the other acids measured (*iso* or *n-butyrate* and *iso* or *n-valerate*).
5. Hydrogen was not included in the model, assuming that hydrogen is converted very quickly to methane
6. Inhibition was not taken into account because the highest measured VFA concentration was less than 600 mg/l and NH_4^+-N concentration was less than 600 mg/l at the end of experiments.

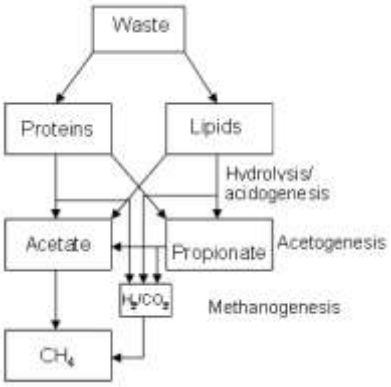

Fig 1. *Scheme of the simplified model*

The chemical reactions considered are described as follows. Hydrolysis/acidogenesis processes:

Proteins:

$$C_{16}H_{30}O_8N_4 + 8H_2O \rightarrow 6C_2H_4O_2 + C_3H_6O_2 + 2H_2 + CO_2 + NH_3 \quad (1)$$

Lipids:

$$C_{47}H_{96}O_9 + 27H_2O \rightarrow 7C_2H_4O_2 + 11C_3H_6O_2 + 28H_2 \quad (2)$$

Acetoclastic methanogenesis:

$$C_2H_4O_2 \rightarrow CH_4 + CO_2 \quad (3)$$

Hydrogenotrophic methanogenesis:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (4)$$

Anaerobic oxidation of propionate:

$$C_3H_6O_2 + 2H_2O \rightarrow C_2H_4O_2 + 3H_2 + CO_2 \quad (5)$$

Hydrogenotrophic methanogenesis process was not included, in order to simplify the number of microbial populations, and an immediate methane formation resulting from the reactions (1), (2), and (5), according to the reaction (4), was considered. Alkalinity was not considered as a limiting factor. The model consists on 7 processes and 8 components. Expressed in matrix form, its biochemical rate coefficients and kinetic rate equations are shown in Table 2. For comparison purposes, the first-order kinetics of lipid hydrolysis was also tested, without considering a specific microbial population for the hydrolysis/acidogenesis step.

Table 2*. Biochemical coefficients and kinetic rate equations of the simplified model studied.*

Component i → / Process *j* ↓	X_{PR}	X_{LI}	X_{ha-LI}	X_{pro}	X_{ac}	S_{pro}	S_{ac}	S_{CH4}	*Rate (ρ_j, kg COD·m^{-3}·d^{-1})*
Hyd/acid. PR	*-1*					*0.21*	*0.73*	*0.06*	$k_{hyd.PR} X_{PR}$
Hyd/acid. LI		*-1*	Y_1			*(1-Y_1)0.58*	*(1-Y_1)0.21*	*(1-Y_1)0.21*	$k_{m.LI} \frac{X_{LI}}{K_{LI} X_{ha-LI} + X_{LI}} X_{ha-LI}$
Uptake pro				Y_2		*-1*	*(1-Y_2)0.57*	*(1-Y_2)0.43*	$k_{m.pro} \frac{S_{pro}}{K_{pro} + S_{pro}} X_{pro}$
Uptake ac					Y_3		*-1*	*(1-Y_3)*	$k_{m.ac} \frac{S_{ac}}{K_{ac} + S_{ac}} X_{ac}$
Decay X_{ha-LI}			*-1*						$k_{dec.Xha-LI} X_{ha-LI}$
Decay X_{pro}				*-1*					$k_{dec.Xpro} X_{pro}$
Decay X_{ac}					*-1*				$k_{dec.Xac} X_{ac}$

RESULTS AND DISCUSSION

Results of the anaerobic biodegradability test are shown in Table 3. These results indicate a quasi complete anaerobic biodegradability of these waste, presenting T2 the lower biodegradability values. This treatment is characterized by the higher concentration of carbohydrates (6.8 % COD), estimated as the difference between total COD and the COD of proteins and lipids.

Time evolution of propionate, acetate and accumulated methane for the three biodegradability tests are shown in Fig. 2 Best fittings of the simplified model considering Contois kinetics for lipids hydrolysis/acidogenesis are also shown in Fig. 2, while Fig. 3 shows the best fitting for the model considering first-order kinetics respect to lipids for the same coupled degradation step. In this latter case, the fitting

of the model was less accurate and the two characteristic measured peaks of acetate concentration were not possible to be simulated.

Table 3. *Results of the biodegradability tests. Average values of three replicates.*

Treatment	**T1**	**T2**	**T3**
Initial lipids/proteins ratio (COD/COD)	4.5	6	11.6
Methanogenesis (% COD/COD)	92.30	89.39	93.02
Biodegradability (% COD/COD)	97.30	96.80	99.60
l CH_4 kg^{-1} SV_{in}	751.19	779.92	849.84
l CH_4 kg^{-1} DQO_{in}	323.05	312.85	325.57
Nm^3 CH_4 t^{-1} substrate	25.8	41.5	67.24

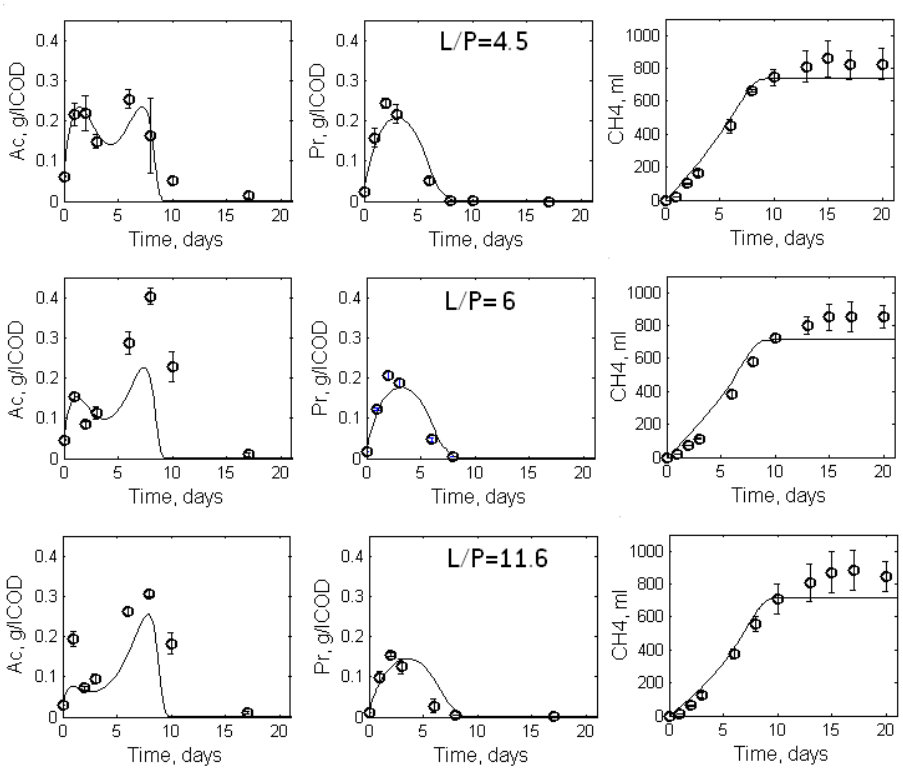

Fig 2. *Time-course of propionate, acetate and accumulated methane for T1 (L/P=4.5), T2 (L/P=6) and T3 (L/P=11.6) biodegradability tests of slaughterhouse waste. L/P ratio units: COD·COD^{-1}. Lines: model prediction with hydrolysis/acidogenesis of lipids expressed by Contois kinetics.*

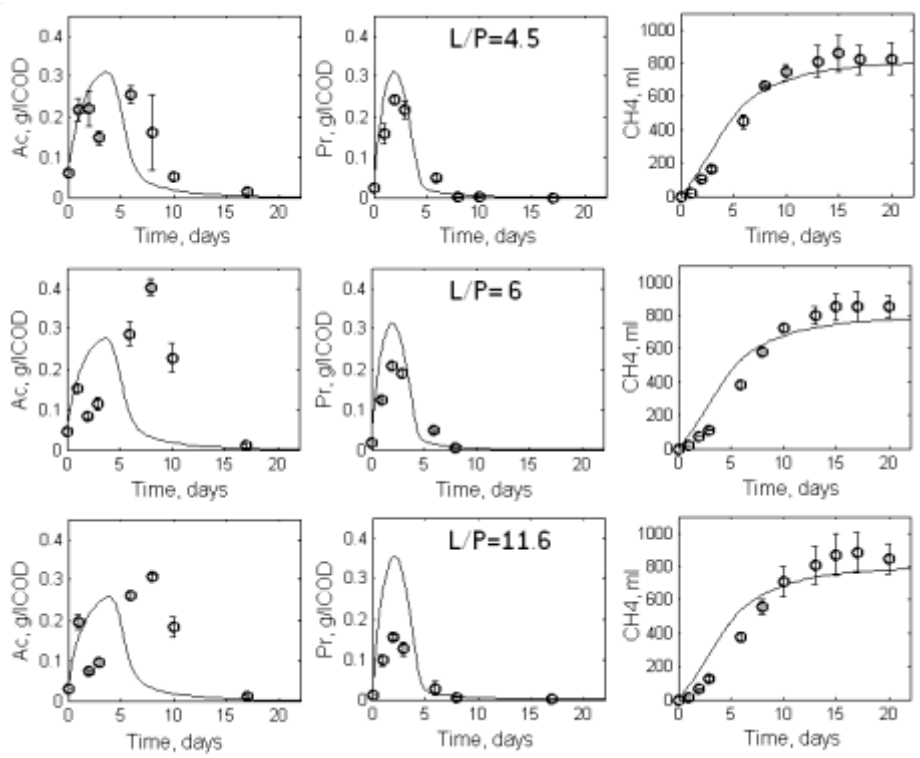

Fig 3. *Time-course of propionate, acetate and accumulated methane for T1, T2 and T3 biodegradability tests of slaughterhouse waste. Lines: model prediction with hydrolysis/acidogenesis of lipids expressed by first-order kinetics.*

Because the hydrogenotrophic methanogenesis was taken into account only indirectly (we did not consider a growth of hydrogenotrophic bacteria), predicted methane production in all experiments began something earlier. There was no big difference in methane production curves corresponding to the different initial lipids/proteins (L/P) ratios. The methanogenesis process developed rather quickly because of high initial concentration of acetoclastic methanogens. Two peaks in acetate concentration can be observed from experimental data. According to the model, immediately after the start, acetate and propionate concentrations increase quickly due to hydrolysis/acidogenesis of proteins, and decreasing later due to its uptake. However, hydrolysis rate of lipids increases in time because the growth of hydrolytic/acidogenic bacteria population. Thus, soon after the acetate concentration decrease, there is a second peak due the increasing rate of lipids decomposition and consequent acetate release. When lipids were exhausted,

propionate and acetate concentrations decreased quickly due to acetogenesis and methanogenesis with rather sharp decrease of methane production rate.

It has been shown (Vavilin *et al*., 2007) that hydrolysis can be described by the Contois kinetics when the rate of this process is controlled by the hydrolytic biomass concentration. That is, when the microbial biomass to particulate substrate ratio is extremely low. Results of the present study show that Contois kinetics can be used also when the combined hydrolysis/acidogenesis must be considered together in a simplified model for treating limited experimental data, and when the process is characterized also by low specific biomass concentration. This could be the situation of the current experiments, where inoculum was adapted to a relatively high protein concentration but not to lipids. Contrast of present assumptions must be done performing similar experiments with same substrates and with different biomass to substrate ratios.

The relatively low hydrolysis rate for proteins compared to the acidogenesis uptake rate for amino acids (Batstone *at al*., 2002; Flotats *et al.*, 2006) allows the simplification assumption of considering the combination of the two consecutive process expressed by the reaction rate representing the hydrolysis step (usually first order if hydrolytic biomass is not rate-limiting). In contrast, the comparatively lower acidogenesis rate for LCFA makes necessary to describe the combined process for lipids coupled to the growth of the specific biomass, for which Contois kinetics has shown to be a suitable model.

CONCLUSIONS

Contrary to the hydrolysis/acidogenesis of proteins, which can accurately be described by the first-order kinetics, the hydrolysis/acidogenesis of lipids has to be coupled to the growth of the specific activity biomass. The Contois kinetics has shown to provide an accurate description of this combined process. The simplified model developed was successful to explain the propionate and acetate profiles obtained from three biodegradability tests applied to slaughterhouse waste with different lipids to proteins ratios. The high anaerobic biodegradability values obtained make anaerobic digestion of slaughterhouse waste an interesting process for energy recovering.

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Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview

V.A. Vavilin [a], B. Fernandez [b], J. Palatsi [b], X. Flotats [b,*]

[a] *Water Problems Institute of the Russian Academy of Sciences, Moscow 119991, Russian Federation*
[b] *GIRO Technological Centre, Rambla Pompeu Fabra 1, E-08100 Mollet del Vallès (Barcelona), Spain*

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Abstract

The applicability of different kinetics to the hydrolysis of particulate organic material in anaerobic digestion is discussed. Hydrolysis has traditionally been modelled according to the first-order kinetics. For complex substrate, the first-order kinetics should be modified in order to take into account hardly degradable material. It has been shown that models in which hydrolysis is coupled to the growth of hydrolytic bacteria work well at high or at fluctuant organic loading. In particular, the surface-related two-phase and the Contois models showed good fits to experimental data from a wide range of organic waste. Both models tend to the first-order kinetics at a high biomass-to-waste ratio and, for this reason, they can be considered as more general models. Examples on different inhibition processes that might affect the degradation of solid waste are reported. Acetogenesis or methanogenesis might be the rate-limiting stages in complex waste. In such cases, stimulation of hydrolysis (mechanically, chemically or biologically) may lead to a further inhibition of these stages, which ultimately affects hydrolysis as well. Since the hydrolysis process is characterized by surface and transport phenomena, new developments in spatially distributed models are considered fundamental to provide new insights in this complex process.

1. Introduction

Exhaustive studies have recently been performed in order to improve the efficiency of the anaerobic digestion of solid wastes (Mata-Alvarez, 2003; Hartmann and Ahring, 2006). Anaerobic degradation of complex organic material has been described as a sequential process that involves the steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al., 2002). Although, the hydrolysis of particulate organic material has been considered the rate-limiting step in anaerobic digestion (Pavlostathis and Giraldo-Gomez, 1991), some authors have emphasised that the hydrolytic process still remains as the least well defined step (Miron et al., 2000; Gavala et al., 2003). The cumulative effects of the different processes taking place during hydrolysis have traditionally been simplified to a single first-order kinetics for the substrate biodegradation (Eastman and Ferguson, 1981). However, relatively high hydrolysis rates were reached in anaerobic biodegradability tests with a high inoculum-to-substrate ratio (Fernandez et al., 2001), showing some degree of dependence of hydrolysis to biomass concentration or activity. Consequently, the first order kinetics appears to be not applicable in all circumstances indicating that an in-depth understanding of the different processes involved is needed to accurately describe hydrolysis.

The objective of this paper is to compile and review the information available in the scientific literature relative to the kinetics of the hydrolysis process, highlighting the models in which hydrolysis is coupled to growth of hydrolytic bacteria, as well as to substrate heterogeneity. The prediction goodness of the reviewed models was compared against available experimental data. The concepts of rate-limiting step in anaerobic digestion and inhibition of hydrolysis at high loads of particulate substrates are also discussed.

* Corresponding author. Tel.: +34 93 5796780; fax: +34 93 5796785.
E-mail address: xavier.flotats@giroct.irta.es (X. Flotats).

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2. Disintegration, solubilisation and enzymatic hydrolysis

The concepts of disintegration, solubilization and enzymatic hydrolysis are usually expressed by the general kinetic term of hydrolysis in most of the practical cases presented in the literature (Batstone et al., 2002). Particulate carbohydrates, proteins and lipids, as well as particulate and soluble inert material are the products of the disintegration of composite material. Monosaccharides, amino acids, long chain fatty acids and glycerol are the products of the enzymatic degradation of particulate carbohydrates, proteins and lipids, respectively, and microorganisms benefit from the soluble products and produce the corresponding hydrolytic enzymes.

Hydrolysis of organic polymers is carried out by extra cellular enzymes (hydrolases). The parallel enzymatic steps with cellulases, proteinases and lipases account for the difference in hydrolysis rate of the particulate carbohydrates, proteins and lipids, respectively (Stryer, 1988). During degradation of monosaccharides, amino acids and long chain fatty acids, hydrolysis products, volatile fatty acids (acetate, butyrate, propionate, lactate, etc.) and hydrogen are formed, being precursors for methane production.

The stage of acidogenesis, following the hydrolysis stage, is usually the quickest step during the anaerobic digestion of complex organic material. For efficient methane production it is important to have a balance between the reaction rates of the different steps involved in the anaerobic digestion of complex organic material. When a process is composed of a sequence of reactions, the overall rate is determined by the slowest reaction, named the rate-limiting step (Hill, 1977). The rate-limiting step in anaerobic digestion with suspended organic matter is normally considered to be the hydrolysis of solids (Pavlostathis and Giraldo-Gomez, 1991). According to Batstone et al. (2002), hydrolysis can be represented by two conceptual models:

(a) The organisms secrete enzymes to the bulk liquid where they are adsorbed onto a particle or react with a soluble substrate (Jain et al., 1992).
(b) The organisms attach to a particle, produce enzymes in its vicinity and benefit from soluble products released by the enzymatic reaction (Vavilin et al., 1996).

The Michaelis–Menten kinetics may be applied for the hydrolysis of a soluble substrate, and is expressed as:

$$\frac{dS}{dt} = k \cdot E \frac{S}{K_m + S} = V_m \frac{S}{K_m + S}, \quad (1)$$

where S, E are the substrate and enzyme concentrations, $V_m = kE$ is the maximum hydrolysis rate, k is the maximum hydrolysis rate constant, and K_m is the half-saturation rate coefficient. According to Goel et al. (1998), the hydrolysis of soluble starch follows the model (1) where the enzyme concentration is proportional to the sludge (biomass) concentration.

3. The first-order kinetics of carbohydrate, lipid and protein degradation

The following system of differential equations, describing hydrolysis as the first-order reaction not directly coupled to the bacterial growth, is considered:

$$\frac{dS}{dt} = -kS, \qquad \frac{dP}{dt} = \alpha kS, \quad (2)$$

where S is the volatile solids (VS) concentration, P is the product concentration, k is the first-order rate coefficient, and α is the conversion coefficient of VS to product. After integration the product concentration is expressed as:

$$P = P_0 + \alpha S_0 (1 - e^{-kt}), \quad (3)$$

where P_0 and S_0 are the initial product and substrate concentrations, respectively. A non-linear regression may be used to estimate the values of coefficients k and α and their standard deviations.

Fig. 1 shows the first-order kinetics of hydrolysis/acidogenesis for a complex substrate (cattle manure) at thermophilic conditions (55 °C) and at different initial waste concentrations. Acetate was the most significant product with concentration of ten times higher than the other volatile fatty acids (VFA). The acetate data corresponding to the initial waste concentration of 62 g VS l^{-1} was used for the calibration and the other VFAs (100 and 31 g VS l^{-1}) were used for validation of the first-order kinetics. Results from the first-order kinetics fitted the experimental data reasonably well.

During protein degradation nitrogen is released in form of ammonia. Fig. 2 shows the first-order kinetics for gela-

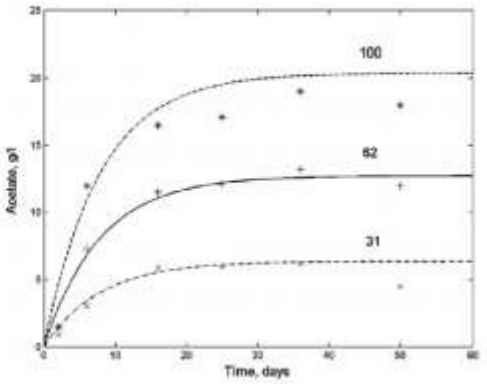

Fig. 1. Time profiles of the acetate concentration during cattle manure thermophilic anaerobic degradation at different initial waste concentrations (in g VS l^{-1}). Symbols refer to the experimental data and lines to the model (2) predictions with $k = 0.128 \pm 0.0206\ d^{-1}$, $\alpha = 0.206 \pm 0.0084$ g acetate g VS^{-1}. Data were taken from Montane (2001).

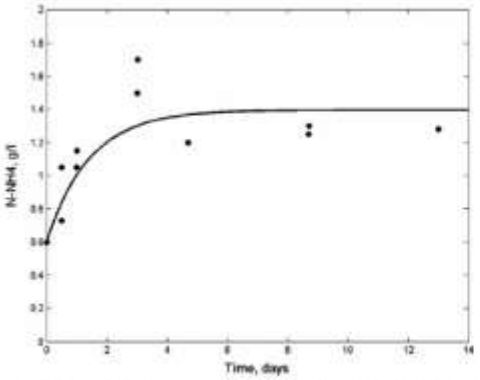

Fig. 2. Time profile of the ammonia concentration during hydrolysis of gelatine. Symbols refer to the experimental data and lines to the model predictions with $k = 0.7\,d^{-1}$, $x = 0.14\,g\,N\text{-}NH_4\,g^{-1}$ VS. Initial concentration of gelatine was 5.7 g COD/l. Data were taken from Flotats et al. (2006).

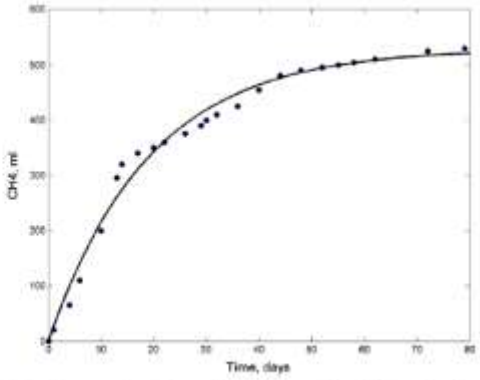

Fig. 3. Time profile of the methane volume released during pig slurry (80% w/w) and pear waste (20% w/w) degradation under thermophilic conditions (55 °C). Symbols refer to the experimental data and lines to the model predictions with $k = 0.052 \pm 0.003\,d^{-1}$ and $x\,S_0 = 530 \pm 11$ ml. Data were taken from Campos (2001).

tine degradation under thermophilic conditions (55 °C), estimated from ammonia released during the fermentation of amino acids. In these experiments, after parameter value analysis, it was concluded that hydrolysis was the rate limiting step, making possible the current simplifying assumption. Interestingly, the hydrolysis rate constant obtained by regression at Fig. 2 is very similar to that previously determined by Flotats et al. (2006) using a more complex model and time-course data of VFA, methane and ammonia from four different experiments with gelatine.

The system (2) with methane as the final product may also be used if hydrolysis becomes the slowest step in comparison to the other steps of solids conversion to methane (acidogenesis, acetogenesis and methanogenesis). This concept is illustrated in Fig. 3, where methane volume released from a complex waste was used to estimate the values of hydrolysis coefficients.

Table 1 summarizes the typical values of rate coefficients for different substrates that can be found in the literature. A wide range of values of the first-order rate coefficient can be seen for composite and simpler organic materials including carbohydrates, lipids and proteins sources. This wide range of values can be explained by different experimental conditions, different hydrolytic biomass to substrate ratios and the lumped effect of disintegration and hydrolysis. In order to distinguish between these effects, the model ADM1 (Batstone et al., 2002) proposes to consider disintegration as a separate hydrolysis processes. If the disintegration rate of composite material is much less than the rates of carbohydrate, protein and lipid hydrolysis, the influence of hydrolysis rates may be neglected (Rusdi et al., 2005; Feng et al., 2006).

Veeken and Hamelers (1999) studied the temperature dependence of the first-order hydrolysis rate of six solid

Table 1
Kinetic coefficients of the first-order rate of hydrolysis

Substrate	k (day^{-1})	T (°C)	References
Carbohydrates	0.025–0.2	55	Christ et al. (2000)
Proteins	0.015–0.075	55	Christ et al. (2000)
Lipids	0.005–0.010	55	Christ et al. (2000)
Carbohydrates	0.5–2.0		Garcia-Heras (2003)
Lipids	0.1–0.7		Garcia-Heras (2003)
Proteins	0.25–0.8		Garcia-Heras (2003)
Lipids	0.76		Shimizu et al. (1993)
Lipids	0.63	25	Masse et al. (2002)
Cellulose	0.04–0.13		Gujer and Zender (1983)
Cellulose	0.066	35	Liebetrau et al. (2004)
Kitchen waste	0.34	35	Liebetrau et al. (2004)
Biowaste	0.12	35	Liebetrau et al. (2004)
Cattle manure	0.13	55	Present study
Pig manure	0.1	28	Vavilin et al. (1997)
Proteins (gelatine)	0.65	55	Flotats et al. (2006)
Municipal solid waste	0.1	15	Bolzonella et al. (2005)
Office paper	0.036	35	Vavilin et al. (2004)
Cardboard	0.046	35	Vavilin et al. (2004)
Newsprint	0.057	35	Vavilin et al. (2004)
Food waste	0.55	37	Vavilin et al. (2004)
Forest soil	0.54	30	Lokshina and Vavilin (1999)
Forest soil	0.09–0.31	20	Lokshina and Vavilin (1999)
Slaughterhouse waste	0.35	35	Lokshina et al. (2003)
Household solid waste	0.1	37	Vavilin and Angelidaki (2005)
Primary sludge	0.4–1.2	35	O'Rourke (1968)
Primary sludge	0.99	35	Ristow et al. (2006)
Secondary sludge	0.17–0.60	35	Ghosh (1981)
Crops and crop residues	0.009–0.094	35	Lehtomaki et al. (2005)

organic waste components and estimated the average activation energy using the Arrhenius equation ($64 \pm 14\ kJ\ mol^{-1}$). They concluded that the hydrolysis rate is controlled by enzyme kinetics if the hydrolytic enzyme concentration exceeds the available amount of adsorption sites of the particulate substrate. In this case, hydrolysis can be described well by first-order kinetics (South et al., 1995).

4. Biodegradability of complex substrates

First-order kinetics can only be applied when the rate-limiting factor is the surface of the particulate substrate, and bioavailability or biodegradability related phenomena do not interfere (Sanders et al., 2003). The biodegradability and hence the biogas potential of a complex substrate depends on the content of biodegradable carbohydrates (including cellulose, hemicellulose and lignin fractions), proteins and lipids (Angelidaki and Sanders, 2004). Cellulose is a main polymer in lignocellulosic biomass and in many organic wastes. The biodegradability of a particulate substrate is dependent on the lignin content (Chandler et al., 1980) and on the structure of the lignocellulosic complex (Tong et al., 1990), while the rate of cellulose degradation depends on the enzymatic activity, as well as on the physical–chemical conditions of the cellulose polymers (Klesov and Rabinovich, 1978). South et al. (1995) developed an enzyme-adsorption based kinetic model showing that the rate of hydrolysis increases with the concentration of hydrolytic enzymes and with availability of adsorption sites.

The complete biodegradability of a given substrate can be determined by long-term batch digestion studies (Kayhanian, 1995), but the reaction period should be specified in these cases. Eq. (4) shows the applicability of the first-order kinetics corrected by the non-degradable fraction of a complex substrate:

$$\frac{dS}{dt} = -k(S - \beta S_0), \tag{4}$$

where S_0 is the initial substrate concentration, and β is the non-degradable fraction of the substrate. This latter parameter can greatly fluctuate depending on the organic substrate. Kayhanian et al. (1991) and Kayhanian and Tchobanoglous (1992) reported that the biodegradable fraction of various substrates, expressed in VS units, are: 82% (food waste), 72% (yard waste), 82% (office paper), 67% (mixed paper), and 22% (newsprint). Municipal solid waste (MSW) should be divided into easily degradable and recalcitrant fractions to model its degradation (Vavilin et al., 2006). The profusely used Activated Sludge Model No. 2, which considers enzymatic hydrolysis under anaerobic, anoxic and aerobic conditions, divides these substances into inert, rapidly and slowly hydrolysable (Henze et al., 1995).

Instead of model (4), different types of kinetics including *n*-order reaction may be applied to describe complex substrate hydrolysis. As an example, Fig. 4 shows the applicability of the following equation:

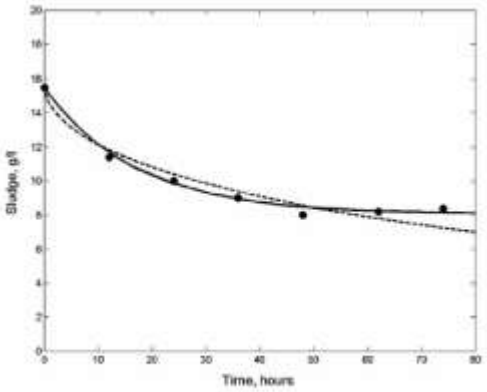

Fig. 4. Time profile of the sewage sludge concentration during disintegration and hydrolysis. Symbols refer to the experimental data and lines to the model predictions with $k = 0.06\ h^{-1}$, $\beta = 0.52$ (model (4), solid line) and $K = 2.5\ g\ l^{-1}\ h^{-1}$, $K_S = 50$ (model (5), dashed line). Data were taken from Park et al. (2005).

$$\frac{dS}{dt} = -\frac{KS}{\hat{K}_S(S - S_0) + S}, \tag{5}$$

where K is the maximum hydrolysis rate which in turn depends on hydrolytic biomass or enzyme concentration, and K_S is an additional model coefficient.

Eq. (4) can be changed to introduce a biomass concentration X:

$$\frac{dS}{dt} = -\hat{k}X^n(S - \beta S_0), \tag{6}$$

where $\hat{k}$ is a rate constant and n is a power index. Fig. 5 shows gelatine degradation at different initial sludge concentrations, using model (6).

A negative correlation between the hydrolysis rate constant and the methane yield was observed by Neves et al. (2006), suggesting that hydrolysis was not the limiting factor for the studied wastes. One important finding is that the substrate hydrolysis rate depends strongly on the origin and the previous acclimation of the anaerobic culture (Gavala et al., 1999). The biochemical pathways of different organic materials may be mutually affected. According to Breure et al. (1986), a complete degradation of protein in the presence of carbohydrates often cannot be achieved in anaerobic wastewater treatment.

5. Surface-related kinetics and two-phase model of hydrolysis of particulate substrate

Hills and Nakano (1984) demonstrated a linear relationship between the gas production rate and the inverse of the particle diameter for tomato solid waste, with average particle diameters from 0.13 to 2.0 cm. Similar results were obtained by Sharma et al. (1988) with agricultural and for-

est residues. Sanders (2001) confirmed this relationship for starch particles with much smaller characteristic sizes. A decrease of particle radius R was then described as a linear function with time:

$$R = R_0 - \frac{kt}{\rho}, \tag{7}$$

where R_0 is the initial average radius, ρ is the substrate density, k is the surface based hydrolysis constant and t is time. Valentini et al. (1997) used an exponential relationship between the rate constant of cellulose hydrolysis and the average particle diameter:

$$k = k_0 e^{-(d/d_0)}, \tag{8}$$

where d is the particle diameter, and k_0 and d_0 are constants.

Vavilin et al. (1996) developed the following rate function of hydrolysis for different shape particles:

$$r = kS_T^{1-n}S^n, \tag{9}$$

where S and S_T are the current and initial substrate concentrations, respectively; and n is the degree index that equals to 2/3, 1/2 and 0 for spherical, cylinder and plate-form particles, respectively. The last case is equivalent to zero-order kinetics. The hydrolytic constant is a function of the ratio between the characteristic sizes of bacteria and particles hydrolyzed:

$$k = 6r_m \frac{\rho_X}{\rho_S}\frac{\delta}{d_S} \quad \text{(spherical particles)}, \tag{10}$$

$$k = 4r_m \frac{\rho_X}{\rho_S}\frac{\delta}{d_S} \quad \text{(cylinder particles)}, \tag{11}$$

where r_m is the maximum specific hydrolysis rate; ρ_X and ρ_S are the bacterial and particle densities, respectively; δ denotes the depth of the bacterial layer and d_S is the current

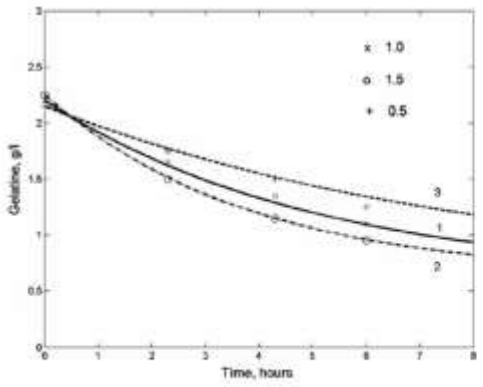

Fig. 5. Time profile of gelatine concentration at several initial relative sludge concentrations (g l^{-1}). Symbols refer to the experimental data and lines to the model (6) predictions with $kX^{0.5} = 0.2\ h^{-1}$, $\beta = 0.28$. Data were taken from Sanders (2001).

diameter of particles. Thus, for different particle shapes, a rate constant value is inversely proportional to the characteristic size of the particle.

The complete enzymatic hydrolysis stage is a complex multi-step process for carbohydrates, proteins and lipids, which may include multiple enzyme production, diffusion, adsorption, reaction and enzyme deactivation steps. In the simplest first-order kinetics, the rate of hydrolysis does not depend on hydrolytic biomass concentration and, hence, it can not be described with a typical sigmoid-type curve when the hydrolysis rate increases in time. However, during hydrolysis the particulate substrates contact the hydrolytic microbial cells and the released enzymes, so that two main phases might be taken into account for a description the hydrolysis kinetics. The first phase is a bacterial colonization in which the hydrolytic bacteria cover the surface of solids. Bacteria on or near the particle surface release enzymes and produce the monomers that are utilized by the hydrolytic bacteria. The daughter cells fall off into the liquid phase and then they try to attach to some new place on a particle surface. Thus, a direct enzymatic reaction as the intermediate step of the total two-phase process may be rather quick in comparison with the stages of bacterial colonization and surface degradation. When an available surface is covered with bacteria, the surface will be degraded at a constant depth per unit of time (second phase).

The surface-related hydrolysis kinetics model that takes into account the colonization of waste particles by hydrolytic bacteria has been developed by Vavilin et al. (1996):

$$\rho = \hat{\rho}_m \frac{\beta X}{1+\beta X}\frac{S}{K_S+S}, \tag{12}$$

where ρ and $\hat{\rho}_m$ are the current and maximum hydrolysis rates, respectively; S is the volatile solid waste concentration, X is the concentration of hydrolytic (acidogenic) biomass, β is the equilibrium constant equal to the ratio between the adsorption and desorption rate constants in the Langmuir function; and K_S is the half-saturation coefficient for the volatile solid waste concentration S.

Microorganisms attached to a particle produce enzymes in the vicinity of this particle and benefit from soluble products released by the enzymatic reaction. The Contois model that uses a single parameter to represent saturation of both substrate and biomass is as good at fitting the data as the two-phase model:

$$\rho = \rho_m X \frac{S}{K_X X + S} = \rho_m X \frac{S/X}{K_X + S/X}, \tag{13}$$

where ρ_m is the maximum specific hydrolysis rate; and K_X is the half-saturation coefficient for the ratio S/X. The surface-related (12) and Contois (13) models have the same limiting cases:

(i) exponential biomass growth (surface-related model: $\beta X \ll 1$, $S \gg K_S$; Contois model: $S/X \gg K_X$):

$$\rho \approx \hat{\rho}_m \beta X = \rho_m X, \tag{14}$$

and (ii) first-order kinetics (surface-related model: $\beta X \gg 1$, $S \ll K_S$; Contois model: $S/X \ll K_X$):

$$\rho \approx \frac{\hat{\rho}_m}{K_S} S = \frac{\rho_m}{K_X} S = kS. \quad (15)$$

By introducing the hydrolytic biomass concentration the following system of differential equations describing hydrolysis coupled to the bacterial growth is written instead of the model (2):

$$\begin{aligned} \frac{dS}{dt} &= -\rho(S,X), \\ \frac{dX}{dt} &= Y\rho(S,X), \\ \frac{dP}{dt} &= \alpha\rho(S,X), \end{aligned} \quad (16)$$

where X is the biomass concentration, Y is the biomass yield coefficient, and $\rho(S,X)$ is the rate function depended on substrate and biomass concentration. In the system (16) the biomass decay process was neglected. In such a case, the system (16) can be integrated and a non-linear regression gives the coefficient values and their standard deviations (Lokshina and Vavilin, 1999; Vavilin et al., 2004; Lehtomaki et al., 2005). Figs. 6 and 7 show an applicability of the two-phase and the Contois equations during starch and brewery-spent grains degradation. In general, it can be concluded that when the evolution of the hydrolysis products follows a sigmoid-type curve, the hydrolysis rate is dependent on the biomass content or on the enzyme concentration.

The models (2)–(16) were written assuming that hydrolysis was the rate-limiting stage in anaerobic digestion. An example in which hydrolysis is described as the rate-limiting step by the Contois kinetics for the degradation of waste and residual organic material in the inoculum is presented in Fig. 8. Reasonably good modeling results were obtained for calibration using only two sets of experimental data corresponding to 28.8 g VS l^{-1} and only inoculum. In some systems involving degradation of solids, the stages of acetogenesis and methanogenesis could be the rate-limiting steps instead of hydrolysis. In such cases, the rate of final product formation P is not strictly dependent on the

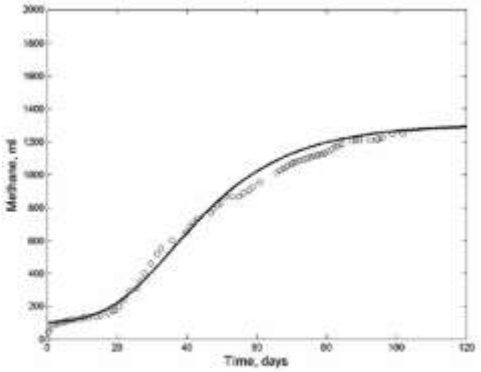

Fig. 7. Time profiles of the methane volume released during degradation of brewery-spent grains at the mesophilic conditions (37 °C). Symbols refer to the experimental data and lines to the model (16) predictions with the Contois kinetics at $\rho_m = 1.5\ d^{-1}$, $K_X = 30\ g\ g^{-1}$, $\alpha = 115\ ml\ CH_4\ g^{-1}$ VS, $X_0 = 0.05\ g\ l^{-1}$. It is assumed that 100 ml of CH_4 formed quickly from soluble organic material presented initially. Data were taken from Fernandez et al. (2001).

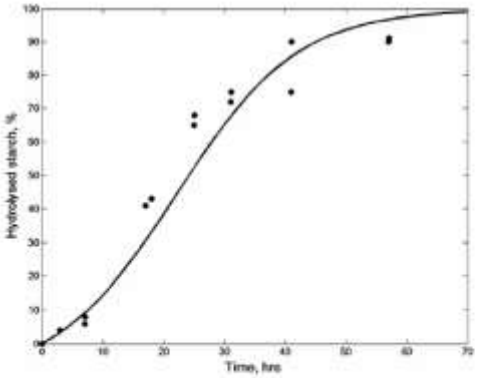

Fig. 6. Time profiles of the efficiency of starch hydrolysis. Symbols refer to the experimental data and lines to the two-phase model predictions assuming the first-order kinetics of surface degradation with $S \ll K_S$ and $k = \frac{\hat{\rho}_m}{K_S} = 0.15\ h^{-1}$, $\beta = 20\ l\ g^{-1}$ VS, $X_0 = 0.1\ g\ l^{-1}$. Data were taken from Sanders (2001).

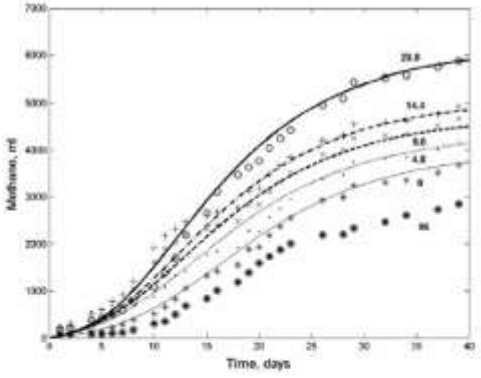

Fig. 8. Methane accumulation curves during household solid waste anaerobic digestion in batch reactors for different volatile solids concentrations ($g\ l^{-1}$). Symbols: experimental data (Angelidaki et al., 2006); curves: batch model prediction with the Contois kinetics of hydrolysis according to Lokshina et al. (2005): waste: $\rho_m = 1.25\ d^{-1}$, $K_X = 12\ g\ g^{-1}$; residual organic material in inoculum: $\rho_m = 2.5\ d^{-1}$, $K_X = 22.5\ g\ g^{-1}$; $Y = 0.1\ g\ g^{-1}$, $X_0 = 0.175\ g\ l^{-1}$.

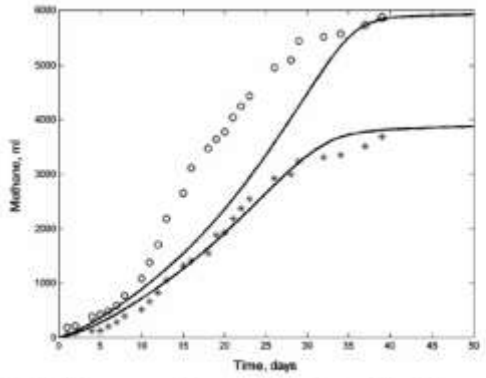

Fig. 9. Methane accumulation curves during household solid waste anaerobic digestion in batch reactors with 0 (inoculum alone; asterisk) and 28.8 g/l VS substrate (circle). Symbols: experimental data (Angelidaki et al., 2006); curves: batch model prediction with the first-order kinetics of hydrolysis (waste: $k = 0.1\ d^{-1}$; residual organic material in inoculum: $k = 0.3\ d^{-1}$) and Monod kinetics for acetoclastic methanogenesis ($\rho_{Ac} = 1.0\ d^{-1}$, $K_S = 5\ g\ l^{-1}$, $Y = 0.05\ g\ g^{-1}$).

hydrolysis rate. Sigmoid-type methane accumulation curves presented in Fig. 8 can be simulated by the Monod model (Fig. 9) if acetoclastic methanogenesis is assumed to be the rate-limiting step. However, such an assumption was not valid for all data sets obtained with different initial waste concentrations (compare Figs. 8 and 9).

The models described in Fig. 8 cannot explain experimental data obtained with initial concentrations of 96 g VS l^{-1}, which is in the range of causing inhibition by a high organic solids to inoculum ratio. In general, a balance between hydrolysis and methanogenesis during anaerobic digestion is very important as some of the intermediates are known to be inhibitors. In these cases, models must be adapted in order to account for inhibitory phenomena.

Palatsi et al. (unpublished data) described the hydrolysis/acidogenesis in complex mixtures of proteins and lipids from slaughterhouse waste by the first-order and the Contois kinetics, respectively. The resulting model was capable of explaining the dynamics of acetate accumulation obtained in different batch experiments, which was characterized by two peaks of acetate concentration, the result of different hydrolysis rates for fats and proteins. According to this model, the second peak could be explained because the hydrolysis rate of lipids increased in time due to the hydrolytic/acidogenic biomass growth.

6. Mechanical pre-treatment

To enhance anaerobic biodegradation, several processes, from comminution to cell disintegration, have been tested (Palmowski and Muller, 2003; Delgenes et al., 2003). Particle size reduction to increase the available specific surface represents an option for accelerating the digestion process. Kayhanian and Hardy (1994) indicated that the methane production rate was inversely proportional to the feedstock particle size. Wen et al. (2004) showed that decreasing the particle size from 840–590 to 590–350 μm enhanced glucose yield by 29% after 96 h-treatment of animal manure, but further decrease in particle size had no effect. Mechanical size reduction was found to be efficient for enhancing the biogas potential production from fibre-rich materials like maple leaves and hay stems, which were difficult to digest (Palmowski and Muller, 2000) or wastes like manure (Hartmann et al., 2000). However, Masse et al. (2003) showed that there was no significant particle size effect on pork fat hydrolysis.

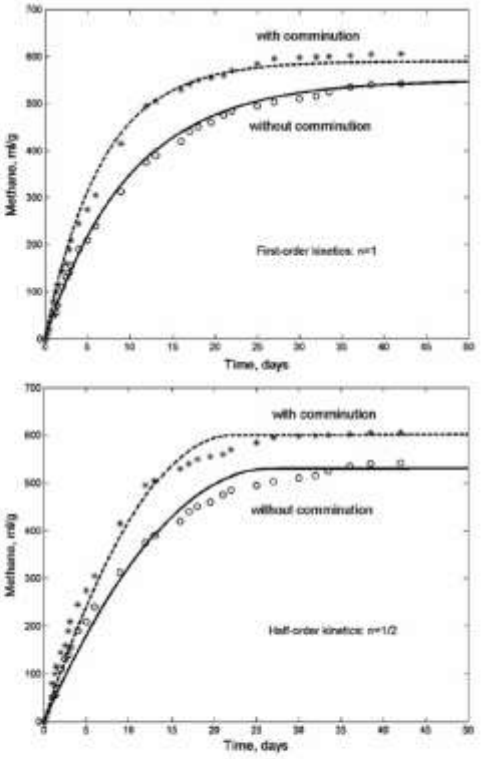

Fig. 10. Time profiles of the specific methane volume released during degradation of hay at the mesophilic conditions (35 °C) with and without comminution. Symbols refer to the experimental data (Palmowski et al., 2001) and lines to the model predictions: First-order kinetics without comminution ($k = 0.1\ d^{-1}$, $\alpha = 550\ ml\ g^{-1}$ VS), first-order kinetics with comminution ($k = 0.15\ d^{-1}$, $\alpha = 590\ ml\ g^{-1}$ VS), half-order kinetics without comminution ($k = 0.075\ d^{-1}$, $\alpha = 530\ ml\ g^{-1}$ VS), half-order kinetics with comminution ($k = 0.090\ d^{-1}$, $\alpha = 600\ ml\ g^{-1}$ VS).

Fig. 10a and b shows the comminution effect on anaerobic digestion of hay using the first-order and half-order kinetics of hydrolysis. The traditional first-order kinetics showed a better fit to experimental data. In spite of the significant reduction of characteristic size of particles (from higher than 1.6 mm to less than 0.2 mm), the first-order and half-order kinetic constants increased only by 1.5 and 1.2 times, respectively. This corresponds to an increase in ratio of surface areas of only 1.3 (Palmowski et al., 2001), due to the cylindrical shape of these particles.

Fig. 11a and b shows the comminution effect, with same characteristic size reduction as in previous experiments, on the anaerobic digestion of rice grains modelized by using Contois and two-phase kinetics for hydrolysis, considering

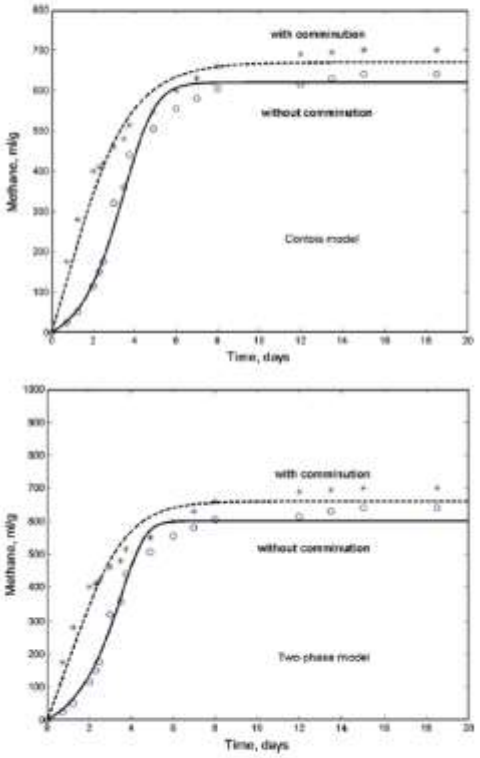

Fig. 11. Time profiles of the specific methane volume released during degradation of rice grains at the mesophilic conditions (35 °C) with and without comminution. Symbols refer to the experimental data (Palmowski et al., 2001) and lines to the model predictions: Contois kinetics (without comminution: $\rho_m = 8\ d^{-1}$, $K_X = 5\ g\ g^{-1}$, $\alpha = 620\ ml\ CH_4\ g^{-1}$ VS, $X_0 = 0.45\ g\ l^{-1}$; with comminution: $\rho_m = 12\ d^{-1}$, $K_X = 20\ g\ g^{-1}$, $\alpha = 670\ ml\ CH_4\ g^{-1}$ VS, $X_0 = 0.45\ g\ l^{-1}$), two-phase kinetics (without comminution: $\hat{\rho}_m = 9\ ml\ d^{-1}$, $K_S = 30\ g\ l^{-1}$, $\beta = 41\ g^{-1}$, $\alpha = 600\ ml\ CH_4\ g^{-1}$ VS, $X_0 = 0.9\ g\ l^{-1}$; with comminution: $\hat{\rho}_m = 13\ ml\ d^{-1}$, $K_S = 220\ g\ l^{-1}$, $\beta = 41\ g^{-1}$, $\alpha = 660\ ml\ CH_4\ g^{-1}$ VS, $X_0 = 6.0\ g\ l^{-1}$).

spherical particles. Both models showed a similar fit to the experimental data. A significantly higher initial hydrolytic biomass content (from 0.45 to 2.5 g/l for the Contois kinetics and from 0.9 to 6.0 g/l for the two-phase kinetics), as well as the half-saturation coefficients (K_X and K_S), were obtained in the comminution experiments. This means that both models were equivalent to the first-order model of hydrolysis when comminution was applied. In this case, the increase in ratio of surface areas extends to 2.9 (Palmowski et al., 2001).

The other way to promote hydrolysis is through the biological and physico-chemical pre-treatment of the substrate to break down the polymer chains into soluble components (Mace et al., 2001; Delgenes et al., 2003; Park et al., 2005). However, during anaerobic digestion of pork fat in slaughterhouse wastewater, long chain fatty acids (LCFA) oxidation was the rate-limiting step (Masse et al., 2002). Thus, mechanical or enzymatic hydrolysis pre-treatments of fat-containing wastewaters should not substantially accelerate anaerobic treatment.

In actual digester conditions, it is not possible to know the distribution of particles with different shapes, and the proportion that are degraded from the inner and the outer surfaces (Hobson and Wheatley, 1992). Thus, it may be assumed that the first-order model of hydrolysis is the simplest approximation of the actual processes.

7. Steady state models

Assuming a first-order kinetic expression for the hydrolysis process, and that this process is the rate-limiting step in anaerobic digestion, the following equations may be used for substrate (suspended solids) and specific methane production for a completely-mixing stirred-tank reactor (CSTR) operating at steady state:

$$S_e = \frac{S_{in}}{1 + k_h T}, \tag{17}$$

$$B = \frac{\alpha(S_{in} - S_e)}{S_{in}} = \alpha \frac{k_h T}{1 + k_h T} = B_0 \frac{k_h T}{1 + k_h T}, \tag{18}$$

where S_{in}, S_e are the influent and effluent solids concentration, B is the specific methane production and T is the solids retention time. The conversion coefficient α of VS to product P for the maximum specific methane production is written as B_0. Ristow et al. (2006) showed that for a fluctuating influent primary sludge concentration, the standard deviation corresponding to the first-order kinetic coefficient was relatively large, $k = 0.992 \pm 0.492\ d^{-1}$. Eastman and Ferguson (1981) were the first who used Eq. (17) for the estimation of particulate sludge reduction measured in COD units, introducing an additional refractory coefficient for the non-degradable fraction of the particulate substrate.

Chen and Hashimoto (1978) developed the following equations for the solid substrate concentration and specific methane production for a CSTR digester operating at steady state:

$$S_e = \frac{KS_{in}}{Y\rho_m T - (1-K)} = \frac{KS_{in}}{\mu_m T - (1-K)}, \quad (19)$$

$$B = \frac{x(S_{in} - S_e)}{S_{in}} = B_0\left\{1 - \frac{K}{\mu_m T - (1-K)}\right\}, \quad (20)$$

where K is the model coefficient, Y is the biomass yield coefficient, ρ_m is the maximum specific substrate removal rate and $\mu_m = Y\rho_m$ is the maximum specific growth rate of biomass. In fact, Eqs. (19) and (20) are based on Contois kinetics (13) taking into account that the biomass value is dependent on the difference between the initial and current substrate concentrations, $X = Y(S_0 - S)$, and that the coefficient K is presented as $K = K_X Y$. The applicability of the Contois kinetics for the description of continuous anaerobic digestion processes of particulate organic matter was demonstrated in a number of papers (e.g. Chen and Hashimoto, 1980; Domenech and Flotats, 1997). Vavilin et al. (2001) reported that Contois kinetics is preferable to the traditional first-order kinetics when considering the optimal design of a two-phase anaerobic digestion system treating complex solid wastes.

Both the Chen and Hashimoto, and the Contois models describe a wash out phenomenon of the hydrolytic biomass in CSTR reactor if:

$$T \leq T_{cr} = \frac{1}{\mu_m}. \quad (21)$$

Introducing the refractory coefficient R for a non-degradable fraction of particulate substrate into (19), Chen and Hashimoto (1978) obtained the following equation:

$$S_e = S_{in}\left\{R + \frac{(1-R)K}{Y\rho_m T - (1-K)}\right\}. \quad (22)$$

A number of authors used the Chen and Hashimoto model (Hill, 1982; Samson and Leduy, 1986; Lema et al., 1987; Maraval and Vermande, 1990; Flotats, 1993; He et al., 2006). However, it was found that the waste type, temperature and influent solids concentration affected the values of α,ρ_m and K (Hashimoto et al., 1981). At $K \gg 1$, the Chen and Hashimoto model transforms into the first-order model with the rate coefficient $k = \mu_m/K$. Chen and Hashimoto (1978) and Hill (1982) determined experimentally that the value of K increased over 1 when S_{in} increased and, hence, when biomass concentration increased, at steady state. This observed increase of K with biomass concentration confirms that the first-order kinetics can be used when the amount of biomass is not limiting the hydrolysis process.

8. Inhibition of hydrolysis

Different types of inhibition caused by high concentrations of LCFA, VFA, H_2 and NH_3 as well as by acidic or alkaline pH, have been observed in anaerobic digesters (Batstone et al., 2002; Lokshina et al., 2003). Inhibitory studies have mainly been focused on acetoclastic methanogens and acetogens, while less attention has been paid to the inhibition of hydrolysis. Hydrolysis can be inhibited by the accumulation of amino acids and sugars (Sanders, 2001; Kadam et al., 2004). During cellulose degradation, cellobiose as the intermediate product may be a stronger inhibitor than glucose (Duff and Murray, 1996). Non-ionized VFA are other possible inhibitors (De Baere et al., 1985; ten Brummeler et al., 1991). Some controversy can be found in the literature about the inhibitory effect of VFA.

Llabres-Luengo and Mata-Alvarez (1988) proposed a kinetic model for MSW degradation where VFA acted as inhibitors, but they did not consider the effect of pH. With the same kind of substrate, Veeken and Hamelers (2000) used a Contois type of kinetics affected by a non-competitive inhibition term due to VFA, with an inhibition constant of 30 g VFA l^{-1}, with satisfactory results. Veeken et al. (2000) designed a set of experiments to elucidate the mechanisms of VFA inhibition, concluding that no inhibition by VFA or by non-ionized VFA can be measured at pH values between 5 and 7, and that acidic pH was the inhibitor factor. They proposed a linear function of pH inhibition in the interval between 5.0 and 7.0:

$$k = 0.048\,pH - 0.172, \quad (23)$$

where k is the first-order hydrolysis coefficient in day^{-1}. Functions of this form have a limited applicability, and the non-competitive inhibition function operating as a multiplier of the rate coefficient is more widely used. For manure digestion, Angelidaki et al. (1993, 1999) proposed a structured model where hydrolysis of particulate matter was represented by a first-order reaction affected by a non-competitive reversible inhibition due to VFA. This model has been used by other authors with satisfactory results (Keshtkar et al., 2003). A generalization of the non-competitive inhibition function, multiplying the rate coefficient, has been used by Vavilin and Angelidaki (2005) for expressing inhibition of hydrolysis by VFA:

$$f(I) = \frac{1}{1 + (I/K_I)^n}, \quad (24)$$

where I is the inhibitor concentration, K_I is the inhibition constant and n is a degree index.

The effects of pH and acetate on the hydrolysis of carbohydrate differed from those on the hydrolysis of protein (He et al., 2006). Specifically for the hydrolysis of proteins, the study of the possible effect of VFA has received especial attention. While Breure et al. (1986) and Yu and Fang (2003) concluded that VFA do not inhibit protein degradation, using gelatine as substrate, Gonzales et al. (2005) clearly showed that acetic acid reduced the gelatine hydrolysis rate in a mesophilic saline environment, with 0.229 g COD-Ac l^{-1} as the inhibition constant for a non-competitive inhibition affecting a first-order hydrolysis. In contrast, Flotats et al. (2006) showed that no inhibition by VFA occurred during gelatine hydrolysis.

Low pH and high lipid concentration can also affect the hydrolysis (Palenzuela-Rollon, 1999). It has been stated

that lipid hydrolysis hardly occurs without methanogenic bacteria that keep pH at non-acidic levels and VFA at non-toxic concentrations. Lu et al. (2004) studied enzymatic activity during the start-up of dry anaerobic mesophilic and thermophilic digestion of the organic fraction of MSW. It was shown that the low hydrolysing protease activity during the first 2–3 weeks was due to inhibition by low pH.

Vavilin et al. (2006) and Jonsson et al. (2006) modelled MSW decomposition in 1001 landfill simulation reactors and concluded that inhibition of the hydrolytic and methanogenic processes occurred during the acidogenic phase. The degradation of the readily degradable waste fraction generated conditions that inhibited the degradation of recalcitrant waste at a pH of 5.6. The addition of methanogenic inoculum activated the decomposition of solids, decreased VFA concentration and initiated the biodegradation of phthalate esters. It was shown that the hydrolysis of phthalate diesters must be described coupled with the growth of hydrolytic biomass; with a biomass yield Y corresponding to alcohol as the intermediate product (Vavilin et al., 2005).

Lokshina et al. (2003) showed a temporary inhibition of the hydrolysis of household waste (HSW) at acidic pH and of the slaughterhouse solid waste (SSW) degradation at neutral and alkaline pH. Simulations showed that it was most likely that hydrolysis was inhibited by high VFA concentrations in the case of HSW, lowering the pH, and by high LCFA concentrations in the case of SSW. These compounds increased during hydrolysis and acidogenesis of the easily degradable fractions. Both systems recovered after an increase of the methanogenic biomass.

It is difficult to distinguish the inhibitory effects caused by pH or VFA. Previous works indicate that VFA accumulation induces a pH decrease, lowering the hydrolysis rate and making pH the effective inhibitor factor. Gradients of pH around the hydrolysable particles, which might not be measurable in the bulk liquid, could explain the different results found in the literature. Hence, the level of homogenization or mixing, and the concentration of methanogenic biomass lowering VFA, even at microniche level, would both be factors affecting the hydrolysis rate.

The influence of different ratios of lipids (from 5% to 47%) on the hydrolysis of artificial waste was studied by Cirne et al. (2007). By testing the effect of lipase addition during the hydrolysis of lipids, it was shown that this amendment enhanced hydrolysis but also produced intermediates (LCFA) that caused inhibition of the subsequent steps of anaerobic digestion, which ultimately also affected the hydrolysis. This work demonstrated that a stimulation of hydrolysis may have a negative effect if all stages are not in balance.

9. Distributed models

Hydrolysis of particulate organic matter is characterized by surface phenomena (colonization by enzymes or biomass and degradation reactions) and transport phenomena (enzymes from the bulk liquid or reaction products from the surface). As previously mentioned, reaction products such as VFA can accumulate in the biomass boundary layer and induce local pH values that inhibit the process, although this effect would not be detected in samples from the bulk liquid. Therefore, transport rate by diffusion or convection around particles can control the process. Models developed for describing the process in a completely mixed reactor can present a lack of reliability for explaining hydrolysis and the whole anaerobic digestion process of solid organic matter. When mass transfer is crucial for explaining solid waste degradation, spatially distributed models offer the required tool for integrating and explaining the experimental knowledge, both in engineered landfills (White et al., 2004), in landfill simulation reactors (Vavilin et al., 2006; Jonsson et al., 2006) or in bioreactors (Vavilin et al., 2002; Vavilin et al., 2003a; Vavilin et al., 2003b; Vavilin et al., 2004).

At present, 2D distributed models have been applied to batch anaerobic co-digestion of MSW and digested manure (Vavilin and Angelidaki, 2005), and 2D and 3D distributed models to a continuous-flow reactor with non-uniform influent concentration distribution (Vavilin et al., 2007). These models successfully predicted experimental results considering the effect of mixing intensity and the waste-to-biomass ratio. In the previous 3D distributed model, hydrolysis of MSW was described by the Contois kinetics (13) and the hydrolysis of the residual organic material in inoculum (digested manure) was described by the first-order reaction (2), both affected by a VFA inhibition function (24).

In general, results in this area show that hydrolysis efficiency in anaerobic digestion must be viewed in the context of the complex interaction between the different biomass species and intermediate products, and their relative spatial distribution. It is considered that new theoretical and experimental developments with space distributed models will help in the comprehension and modeling of the complex hydrolysis process.

10. Conclusions

The first-order kinetics had traditionally been used to describe the hydrolysis process in anaerobic digestion, but it may be inaccurate to describe the hydrolysis of certain complex substrates. In such cases, first-order kinetics should be corrected by taking into account the hardly biodegradable material. As improvements of the previous model, two models have been developed: the Contois kinetics that considers growth of hydrolytic/acidogenic biomass, and the two-phase kinetics considering surface colonization and biodegradation separately. In general, these models show a better fit to experimental data at a high or fluctuant organic loading rate. The first-order kinetics model is a particular case of these two models at a high biomass to substrate ratio and, therefore, they

can be considered as more general models. At high organic loading inhibition of hydrolysis should be considered when an analysis of the efficiency of anaerobic digestion of complex substrates is carried out. In such cases, methanogenesis or acetogenesis can be the rate-limiting steps in anaerobic digestion. To describe these phenomena numerically, a more complex structured model should be used. However, at present, it is still not possible to adopt a general model applicable under all circumstances. The new spatially distributed models that consider mass transfer processes, which are still in the developing stage, will provide new insights into the complex hydrolysis process.

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Identifiability study of the proteins degradation model, based on ADM1, using simultaneous batch experiments

X. Flotats*, J. Palatsi*, B.K. Ahring and I. Angelidaki*****

*Laboratory of Environmental Engineering, Centre UdL-IRTA, University of Lleida, Avda Rovira Roure 191, E-25198 Lleida, Spain (E-mail: *xavier.flotats@irta.es*)

**Department of Biotechnology, Technical University of Denmark, B-227, 2800 Lyngby, Denmark

***Department of Environment and Resources, Technical University of Denmark, B-115, 2800 Lyngby, Denmark

Abstract The objective of the present study is to analyse kinetic and stoichiometric parameter values of gelatine anaerobic degradation at thermophilic range, based on an experiment designed to elucidate if volatile fatty acids (VFA) are inhibitors of the hydrolysis process. Results showed that VFA are not inhibiting the hydrolysis process. The ADM1 model adequately expressed the consecutive steps of hydrolysis and acidogenesis, with estimated kinetic values corresponding to a fast acidogenesis and slower hydrolysis. The hydrolysis was found to be the rate limiting step of anaerobic degradation. Estimation of yield coefficients based on the relative initial slopes of VFA profiles obtained in a simple batch experiment produced satisfactory results. From the identification study, it was concluded that it is possible to determine univocally the related kinetic parameter values for protein degradation if the evolution of amino acids is measured in simultaneous batch experiments, with different initial protein and amino acids concentrations.

Keywords Acidogenesis; amino acids; anaerobic digestion; gelatine; hydrolysis; proteins

Introduction

Proteins are important components of many wastes and are often responsible of the high ammonia concentration during anaerobic digestion, causing inhibition of acetoclastic methanogens and possible process failure. As soluble proteins hydrolyse very fast, the subsequent release of volatile fatty acids (VFA) and ammonia can produce overloading and inhibition problems if an increase on the organic loading rate related to proteins overcomes the capacity of the system. A calibrated and validated mathematical model, such as the ADM1, helps to predict these situations and to plan successful operation methods. In the ADM1 (Batstone *et al.*, 2002), protein anaerobic decomposition is represented with the following steps: hydrolysis of soluble proteins to amino acids (AA); acidogenesis from AA to VFA, H_2 and inorganic nitrogen ($N\text{-}NH_4^+$); acetogenesis from VFA to acetate; and methanogenesis from acetate and H_2 to methane.

Hydrolysis of soluble protein is modeled using a first-order process without any inhibition function (Batstone *et al.*, 2002). Inhibition of hydrolysis at low pH levels is generally accepted, but some controversy on a possible inhibition by VFA is found in the literature for solid waste. Veeken *et al.* (2000) designed a set of experiments to elucidate if this inhibition effect takes place, concluding that no inhibition by VFA occurred at pH values between 5 and 7.

Specifically for the hydrolysis of proteins, the study of the possible effect of VFA has received special attention. While Breure *et al.* (1986a) and Yu and Fang (2003) concluded that VFA did not inhibit protein degradation, using gelatine as substrate, González

et al. (2005) clearly showed that gelatine hydrolysis was severely inhibited by acetic acid at pH 7, with 0.229 g COD-acetate/l as the inhibition constant for a non-competitive inhibition affecting a first-order hydrolysis. Gelatine is a heterogeneous mixture of water-soluble proteins of high average molecular weight; it is not found in nature and is derived from collagen. Collagen is the fibrous protein constituent of skin, cartilage, bone, and other connective tissue, making gelatine a good protein model for basic studies aiming at slaughterhouse waste biological treatment.

A simple experimental method for estimating parameters, although with limitations, is the use of simultaneous batch experiments (SBE). This method is based on the measure of the evolution of some components in a set of batches characterized by different initial conditions for some known components and equal initial values for others (Flotats *et al.*, 2003). As in other systems, the optimal experimental design is influenced by the parameter values (Vanrolleghem *et al.*, 1995), which are not previously known. Therefore, information from previous experiments is required in order to have a first estimation of the parameter values, and to choose the appropriate different initial concentrations.

Kinetic parameters for hydrolysis of proteins and for the acidogenesis of amino acids present a wide range of values in the literature (see Appendix A in Batstone *et al.*, 2002). Consequently, some level of uncertainty appears for choosing the appropriate SBE initial conditions, if a given protein degradation must be characterised. The objective of the present study is to orientate further SBE for the calibration of a protein (gelatine) decomposition model, using batch experiments previously done to elucidate if VFA act effectively as inhibitors of gelatine hydrolysis.

Materials and methods

Experimental design

Degradation of gelatine was tested in 116 ml vials, with four initial VFA concentration levels (0, 2.2, 5.2 and 11.2 g COD/l corresponding to 0, 30, 70 and 150 mM VFA, with a molar distribution of acetate-*Ac*, propionate-*Pro*, butyrate-*Bu* and valerate-*Val* of 85:10:3:2 respectively) and with 5.7 g COD/l of gelatine in all vials, placed in an incubator at 55 °C. Experimental conditions and analytical methods were the same as in Flotats *et al.* (2003). Initial concentration of ammonia was 0.6 g $N\text{-}NH_4^+$-N/l. Experiments were performed in triplicate and the duration was 27 days. VFA and methane were sampled frequently at the start (5 samples during first 24 hours) and less frequently later, with 18 total samples per vial. Ammonia nitrogen were analysed by standard methods, with 7 samples per vial until the 13th day. pH was measured at the end of the experiment.

Derivation of stoichiometry

Application of Ramsay algorithm. Table 1 shows the calculation method derived from Stickland reactions proposed by Ramsay (1997) and Ramsay and Pullammanappallil (2001), applied to gelatine with the average amino acids composition indicated by Merck (2001). Aromatic products represent 4.9% of COD and are cresol and phenylacetate. It was assumed that the aromatic compounds are consumed by acidogenic population with a yield value of 0.0592 C-mole biomass (B)/C-mole AA (Ramsay, 1997), producing Ac and Pro from cresol and Ac from phenylacetate. It is important to note that H_2 is not a product of gelatine anaerobic degradation based on Stickland reactions. Biomass yield value was calculated from the ATP moles produced (10 g B/mole ATP). The above-mentioned assumptions produced two sets of stoichiometric coefficients to be tested (Table 2). Based on amino acid contents, the derived gelatine formula is $CH_{2.0461}O_{0.5719}N_{0.3170}S_{0.0022}$.

Table 1 Determination of stoichiometric coefficients for gelatine degradation, based on Ramsay (1997) algorithm

Amino acid (AA)	Content		mole/mole AA								
	% w/w	mole AA/C-mole gelatine	C2-Ac	C3-Pro	C4-Bu	C5-Val	H_2	N-NH_3	CO_2	Aromatic acids	ATP
Glycine	26.25	0.0970	1				−1	1			
Alanine	9.06	0.0282	1				2	1	1		1
Valine	2.58	0.0061			1		2	1	1		1
Leucine	3.37	0.0071				1	2	1	1		1
Isoleucine	1.54	0.0032				1	2	1	1		1
Cysteine	0.15	0.0003	1				0.5	1	1		1
Methionine	0.79	0.0015		1			1	1	1		1
Phenylalanine	2.38	0.0040					2	1	1	1[1]	1
Proline	16.05	0.0387	0.5	0.5		0.5	−1	1			
Serine	3.47	0.0091	1				1	1	1		1
Threonine	2.03	0.0047	1		0.5		−1	1			1
Tyrosyne	0.79	0.0012	1				1	1	1	1[2]	1
Aspartic acid	6.09	0.0127	1				2	1	2		2
Glutamic acid	10.85	0.0204	1		0.5			1	1		2
Arginine	8.87	0.0141	0.5	0.5		0.5	−1	4	1		1
Lysine	4.95	0.0094	1		1			2			1
Histidine	0.79	0.0014	1		0.05			3	1		2
Tryptophan	0.00	0.0000					2	1	1	1[3]	1
Hydroxyproline	0.00	0.0000	0.5	0.5		0.5	−1	1			
TOTAL	mole/C-mole gelatine		0.2110	0.0279	0.0288	0.0368	−0.0197	0.3139	0.1222	0.0052	0.1582

[1,2,3]These aromatic acids are phenylacetate (C8), cresol (C7) and indole acetate (C10), with a final result of 0.004, 0.0012 and 0.0 mole/C-mole of gelatine, respectively

Table 2 Stoichiometric coefficients tested in the present study

Source	Y g COD B/g COD AA	Ac g COD Ac/g COD AA	Pro g COD Pro/g COD AA	Bu g COD Bu/g COD AA	Val g COD Val/g COD AA	H_2 g COD H_2/g COD AA	IN g N/g COD AA
Table 1	0.071	0.427	0.099	0.146	0.242	−0.032	0.134
Table 1[1]	0.074	0.458	0.103	0.146	0.242	−0.023	0.134
Experiment[2]	0.086	0.595	0.102	0.114	0.086	–	0.130
Experiment[3]	0.074	0.604	0.103	0.116	0.088	–	0.134

1: Considering degradation of aromatic acids; 2: Considering yield coefficient from Angelidaki *et al.* (1999) and from VFA relative production from initial slopes obtained in present experiments; 3: same of experiment[2], but considering yield coefficient obtained from Table 1[1]

Initial VFA slopes. Based on the relative initial slope of VFA profiles, measured at the present experiment for the batch of 0 mM VFA initial concentration, two biomass yields were tested (Table 2): the value used by Angelidaki *et al.* (1999) at thermophilic range, that is 0.086 g COD B/g COD AA (close to the value proposed by ADM1) and the value obtained by the Ramsay algorithm.

Problem definition and simplification assumptions

The unknown kinetic parameters are related to the hydrolysis of proteins and the uptake of amino acids processes (processes 3 and 6 in Table 3.1 of Batstone *et al.*, 2002). Since the initial concentration of NH_4^+-N was high enough, the term $I_{IN,lim}$ was neglected. pH was assumed not to vary significantly along the experiment. Since H_2 was not expected to be produced from gelatine (see Table 1) and H_2 partial pressure was not measured, uptake of H_2 process has been lumped together with the VFA producing it, applying stoichiometry and kinetic parameter values for *Ac*, *Pro* and *Val* from Flotats *et al.* (2003), and stoichiometric values for *Bu* from Angelidaki *et al.* (1999).

Mathematical and computing methods

The output function was defined as the vector whose components are the measured state variables evolution: *Ac*, *Pro*, *Bu*, *Val*, NH_4^+-N and methane. Parameter identifiability was studied by the expansion in Taylor series of the output function around the initial time. The successive derivatives were expressed as a function of the unknowns, trying to obtain a determined system of equations for calculating these parameters univocally (Dochain and Vanrolleghem, 2001)

Table 3 Estimated parameter values and its statistical analysis for the best global fitting

Correlation matrix	Hydrolysis		Acidogenesis of AA			Uptake of VFA	
	$k_{hyd,pr}$ (d^{-1})	$\mu_{max,aa}$ (d^{-1})	$K_{S,aa}$ (g COD /l)	$(X_{aa})_0$ (g COD/l)	$(X_{ac})_0$ (g COD/l)	$(X_{pro})_0$ (g COD/l)	$(X_{c4})_0$ (g COD/l)
$k_{hyd,pr}$	1.00						
$\mu_{max,aa}$	−0.07	1.00					
$K_{S,aa}$	0.91	0.05	1.00				
$(X_{aa})_0$	0.78	−0.52	0.81	1.00			
$(X_{ac})_0$	−0.14	−0.48	−0.30	0.02	1.00		
$(X_{pro})_0$	−0.06	−0.17	−0.09	0.01	−0.40	1.00	
$(X_{c4})_0$	−0.01	−0.34	−0.16	0.05	−0.12	0.39	1.00
Parameter value	0.649	15.987	1.815	0.105	$8.8 \cdot 10^{-2}$	$3.7 \cdot 10^{-4}$	$7.7 \cdot 10^{-2}$
Standard dev.	0.066	27.66	4.97	0.401	$2.5 \cdot 10^{-3}$	$3.6 \cdot 10^{-5}$	$5.2 \cdot 10^{-3}$
CI (95%)	±0.13	±54.27	±9.75	±0.78	$\pm 4.9 \cdot 10^{-3}$	$\pm 7.1 \cdot 10^{-5}$	$\pm 1.0 \cdot 10^{-2}$
t-test value	9.82	0.58	0.36	0.26	34.93	10.32	14.87
t-test prob.(%)	100	71.83	64.25	60.38	100	100	100

For fitting the model to the SBE results, and to estimate unknown parameter values, the objective function to be minimized was the sum of the reciprocals of the multiple determination coefficients ($\Sigma[1 - r^2]$) for the four SBE and for the six measured components together, using a combination of a direct search method and a conjugate gradient method (Flotats *et al.*, 2003). The sub-model for proteins degradation from ADM1 and the kinetic values which appear in appendix A of Batstone *et al.* (2002) were tested. A possible non-competitive inhibition by VFA has been evaluated.

Results and discussion

Based on Taylor series expansion up to the 3rd derivative, it is concluded that kinetic parameters for hydrolysis and acidogenesis are not identifiable with the present experimental setup. With a null initial concentration of AA, only the group $k_{hyd,pr} \cdot k_{m,aa} \cdot (X_{aa})_0 / K_{S,aa}$ is identifiable. For separating these parameters, different initial concentrations of gelatine and AA, and the measurement of the time evolution of AA, are required. With the present experimental design, the structural identifiability study concludes that the initial biomass concentrations for VFA degraders are identifiable.

Based on the time course of ammonia release (Figure 1), we concluded that gelatine hydrolysis is not significantly inhibited by VFA, at the tested VFA concentrations. Separation of ranges only appears at the 13th day, with lower values for the highest initial VFA concentration (150 mM). Experimental values for the six measured components and for the four SBE are shown in Figures 2–5, where some outlier measurements have been rejected.

As expected from the identifiability study, the practical identification process cannot provide a unique set of kinetic parameters, the system being undetermined with the present experimental design. Since VFA production/consumption can be predicted, the obtained data will help to provide orientations about the approximate kinetic values.

The Ramsay algorithm predicts an *Ac* yield value consistent with those reported by Yu and Fang (2003) and Breure *et al.* (1986a). While Yu and Fang (2003) obtained lower *Ac* production at 55 °C that at 35 °C, the opposite was measured by Fang and Chung (1999), which is in accordance with our results. The yield value for *Pro*, obtained by present experiments, is similar to that predicted by the Ramsay algorithm, and lower than those obtained by Yu and Fang (2003) and Breure *et al.* (1986a). While the Ramsay algorithm predicts higher yields for *Val* than *Bu*, consistent with results from Breure and van Andel (1984), the opposite was found in our experiments, in Breure *et al.* (1985) and in Yu and Fang (2003). Breure *et al.* (1986a) obtained the two different *Bu*–*Val* relative productions depending on the experimental conditions (dilution rate and pH). In addition

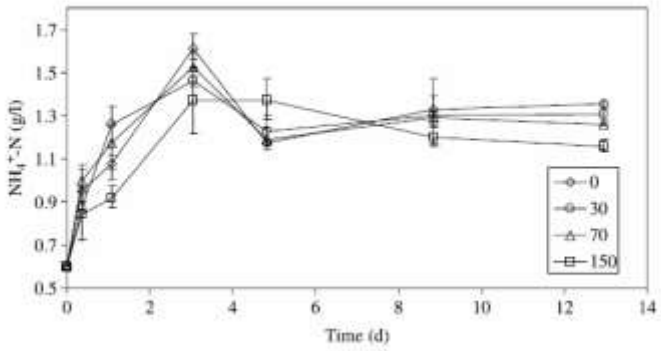

Figure 1 Concentration of NH_4^+-N (g/l) for the four SBE, at the indicated initial values of VFA (mM)

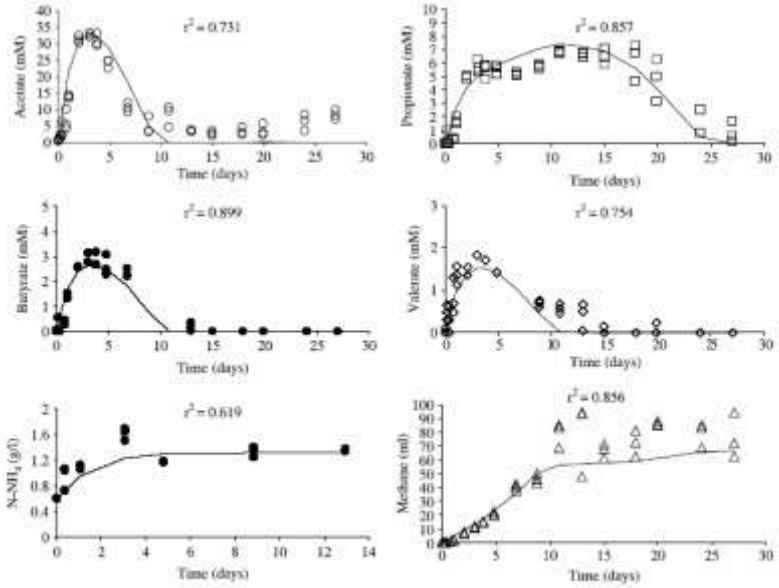

Figure 2 Experimental and predicted values for the 0 mM VFA initial concentration

to pH (Yu and Fang, 2003), other factors that can modify the protein degradation profile are the presence of another carbon source (Breure *et al.*, 1986a), the adaptation of micro-organisms (Breure *et al.*, 1986b) and the bacterial culture used (Ollivier *et al.*, 1986). It is difficult to draw clear conclusions about the exact VFA distribution from a given protein.

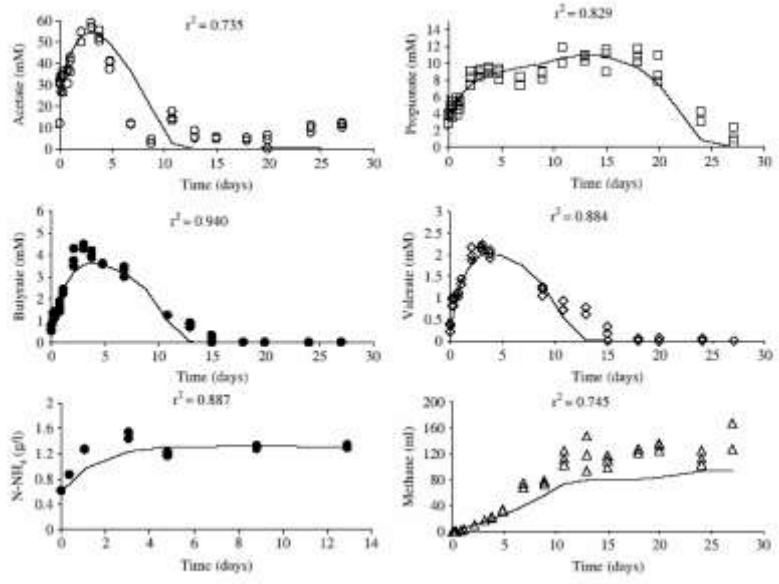

Figure 3 Experimental and predicted values for the 30 mM VFA initial concentration

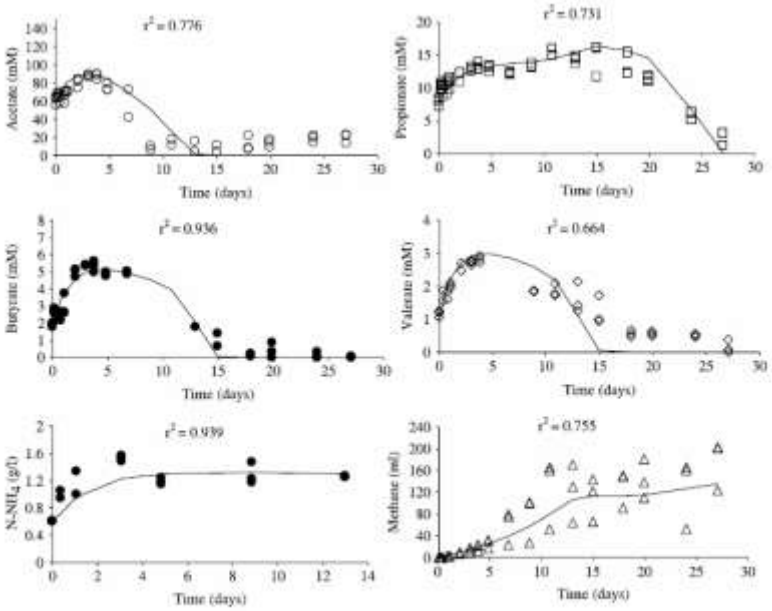

Figure 4 Experimental and predicted values for the 70 mM VFA initial concentration

The negative yield for H_2, obtained from Table 1, is consistent with results from Fang and Yu (2002), where partial pressure of hydrogen decreased as gelatine concentration increased in the influent, with measurements at steady-state operation. Although H_2 has not been measured in the present study, the profiles of VFA evolution have been very well

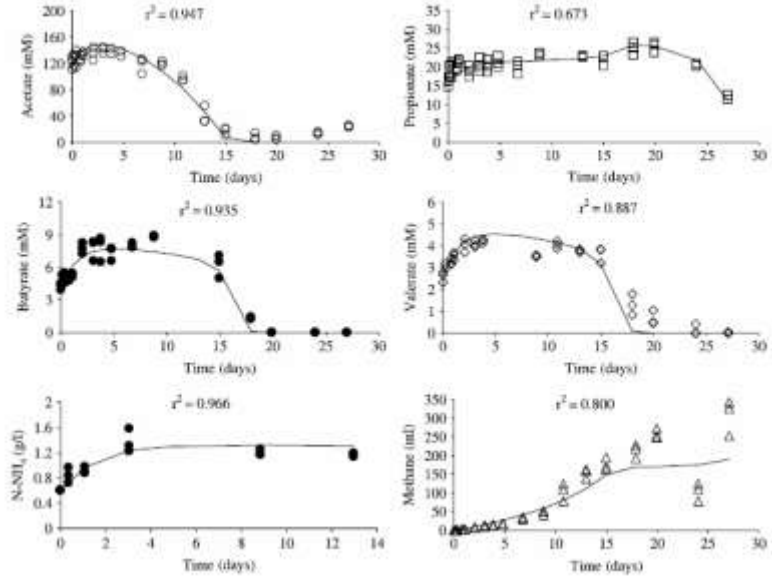

Figure 5 Experimental and predicted values for the 150 mM VFA initial concentration

predicted without considering inhibition by H_2, indicating that the prediction about the H_2 yield sign at Table 1 could be adequate for gelatine.

All kinetic values related to hydrolysis of proteins and to acidogenesis of AA indicated in Appendix A from Batstone *et al.* (2002), including values from González *et al.* (2005), have been used as a first guess for the practical optimization process. Many different combinations of values resulted in good fittings for the experiment[2,3] coefficients. However, coefficients produced applying the Ramsay algorithm never resulted in good experimental fit. The best global fitting (shown in Figures 2–5) has been obtained with values indicated at Table 3, using experiment[2] coefficients from Table 2.

Estimated initial biomass concentrations of VFA degraders present a high statistical significance and narrow confidence intervals (CI), indicating that these values can be univocally approximated with the present experimental design, as previously derived from the structural identifiability study. Estimated kinetic values express a fast acidogenesis and slower hydrolysis, as suggested by many authors (Breure and van Andel, 1984; Nagase and Matsuo, 1982), hydrolysis being the rate limiting step for gelatine degradation. Nevertheless, based on the statistical analysis of Table 3, it is only possible to conclude about the hydrolysis process, with an estimated constant presenting a low standard deviation and a high statistical significance (based on the Student's *t*-test). Parameters related to acidogenesis present a high uncertainty level.

The obtained hydrolysis constant value is very close to $0.6\,d^{-1}$, obtained by Gavala *et al.* (2003) from experimental data of Nagase and Matsuo (1982) for gelatine. Estimated $\mu_{max,ac}$ value is very close to that obtained by Siegrist *et al.* (2002) at thermophilic range. The result obtained considering inhibition of hydrolysis by VFA provides an inhibition constant high enough (328 g COD VFA/l, statistical analysis not shown) for considering this inhibition not significant at the tested VFA levels, as shown in Figure 1, clearly in opposition with the results of González *et al.* (2005).

Although the narrow confidence interval obtained for $k_{hyd,pr}$, in contrast to the acidogenesis parameters, the correlation matrix indicates that present SBE experiments cannot provide enough information for a complete separation between parameters from hydrolysis and from acidogenesis, as predicted. Further SBE experiments must be designed for separating the two process kinetics and for avoiding the present uncertainty for acidogenesis parameters. To measure AA evolution along the batch experiments using different initial concentrations for proteins and AA, ensuring values into the range of the CI of $K_{S,aa}$, and using the same initial inoculum concentration, should provide appropriate data for an adequate parameter identification work.

Figures 2–5 show that NH_4^+-N is released before VFA production. This fact can be explained by an acidogenesis process constituted by several consecutive steps, with ammonia release at the first step and VFA production at the final step, as described by Stickland reactions. This means that if ammonia is used as unique indicator in batch experiments, higher reaction rates than those obtained using VFA as indicators could be obtained. Repeating the practical optimization process without considering ammonia data, reaction rates obtained for acidogenesis are slightly lower than those of Table 3, with better statistical significance. It is concluded that VFA are better indicators of acidogenesis process.

Conclusions

The Ramsay algorithm cannot always predict the correct VFA production profile, but can orientate values for biomass yield and the H_2 sign (production or consumption). A simple batch for estimating the relative initial slopes of VFA profiles can help to approximate yield coefficients for VFA.

The obtained results indicate that VFA are not statistically significant inhibitors of gelatine hydrolysis at the tested levels of VFA. The estimated kinetic parameters indicate that hydrolysis is the rate limiting step of the degradation of gelatine, the acidogenesis of amino acids being a fast process. Nevertheless, the estimated parameters related to acidogenesis have been characterized by wide confidence intervals. Our results will orientate further identification works conducted in order to overcome the uncertainty found for parameters related to the amino acids uptake process.

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ABSTRACT Slaughterhouse wastes are interesting for the anaerobic digestion process regarding its high biogas production potential and because the current legal scenario promotes renewable energy production. The high lipid and protein content of those residues limit its treatment due to inhibitory processes, in particular the inhibition caused by long chain fatty acids (LCFA). The objective of the present disertation is to obtain a deeper insight on the LCFA inhibition process, the microorganism adaptation ability and the prevention/recovery of inhibitory phenomena.

In a preliminary approach, organic wastes generated in slaughterhouses are characterized, by studying the anaerobic biodegradability of waste mixtures containing diferents lipid/proteins concentrations. Anaerobic batch tests are performed at increasing substrate concentrations by sequential pulse feeding. From those experiments, the fast hydrolysis-acidogenesis of proteins is verified, being the lipids and LCFA degradation the main limiting step of the overall anaerobic process. Despite this limitation, the system is able to recover up to a higher methane production rate after each applied pulse.

In order to elucidate on the mechanisms of the recovery process, several strategies to recover LCFA inhibited reactors are tested. The increase of the biomass/LCFA ratio and the adition of bentonite to reduce the biodisponibility or the adsorption of LCFA over microbial cell walls, are found to be effective approaches in the operation of fullscale biogas plants. The obtained results reinforce the hypothesis of the adsorptive nature of the LCFA inhibition, and that the recovery process can be followed as an increase in the microbial activity.

The nature of the reported microbial activity improvement after subsequent sytem inhibition is characterized by three different techniques: 1) the study of specific microbial activities on different model substrates, 2) the application of molecular biology tools to monitor the microbial population structure and, 3) the development of kinetic expressions of the LCFA inhibition phenomena, based on the adsorption process, within the framework of ADM1 model of the International Water Association. The combined analysis of those confirmed that inhibition and adaptation phenomena are explained by a specific microbial growth, including adsorption in the metabolic LCFA inhibition process.

The adsorption-inhibition process is evaluated in detail by determining LCFA adsorption isotherms on granular sludge, LCFA toxicity test, and fluorescence microscopy techniques. This multidisciplinary approach results in the definition of an inhibition preventing strategy based on the introduction of competitive adsorbents, and on stating the importance of palmitate during ß-oxidation of LCFA.

This study contributes to the understanding of slaughterhouse wastes anaerobic treatment, the LCFA inhibition process, and the biomass adaptation phenomena. The physical adsorption process has been directly related with the LCFA metabolic inhibition, and a new mathematical kinetic expression is proposed. New strategies guiding the operation of anaerobic reactors are suggested in order to obtain high renewable energy yields from slaughterhouse wastes digestion.

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