PhD Thesis

UPC – Program on Environmental Engineering

STUDY OF METHODS FOR THE IMPROVEMENT OF THE ANAEROBIC DIGESTION OF LIPIDS AND LONG CHAIN FATTY ACIDS

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« *Il faut faire de la vie un rêve et faire d'un rêve une réalité* »

Pierre Curie

إلى أّمّي و أبي

لأنّه ما رضاء اللّه إلّا برضاء الوالدين

و لأنّ بريق السّعادة الذي ألمحه في عيناكما هو كنز لا يقدّر بثمن

على هذا الدرب سرت

Nizti,

Je t'aime et je t'aimerais pour toujours, non seulement pour ce que tu es mais pour ce que je suis quand nous sommes ensemble. Je n'oublierais jamais le chemin de notre vie qu'on avait dessiné ensemble sur un bout de papier. Ce rêve est maintenant une réalité, après tant de distance et de sacrifices. Je voudrais partager toute ma vie avec toi, respirer ton amour et entendre ta voix dans tous les bruits du monde. «Je t'aime»

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ABSTRACT

Anaerobic digestion is a versatile technology platform that transforms, in a biochemical process, diverse categories of biomass feedstock and organic waste to renewable energy, in the form of methane, and contributes to resources conservation and greenhouse gases emission mitigation. Lipid-rich waste and wastewaters have a high energy potential, however efficient methane recovery with conventional anaerobic digestion technology is not easy to achieve because of a wide assortment of operational problems mainly related to the accumulation of long chain fatty acids (LCFA), products of lipids hydrolysis, in the system. The objective of the present dissertation is to test and to evaluate new methodologies and strategies to improve the anaerobic digestion of high-strength lipid waste.

In a preliminary approach, the suitability and the attractiveness of high-strength lipid wastes and slaughterhouse wastes for biogas production was confirmed, suffice to control the applied organic load. The obtained results reinforced the existing knowledge describing the flotation and wash-out of substrate/biomass and the inhibition phenomena affecting the microbial population, as the main process drawbacks. The results of studies submitting anaerobic reactors to increasing concentrations of lipids/LCFA underlined the importance of the adsorption of LCFA onto the microbial cell membrane as limiting factor, guiding further research to found new technical approaches in order to control the biomass-LCFA adsorption dynamics.

The use of inorganic adsorbents to capture LCFA prior to the anaerobic digestion process or the application of sequential low-energy ultrasonic pulses in order to control the adsorption-desorption kinetics were tested with interesting results. However, the effectiveness of these strategies was limited by the proportion of inorganic adsorbent/LCFA and the cumulative damaging effect of ultrasonic treatment over biomass, respectively. Further studies are thus required to optimize the efficiency and the applicability of these strategies.

Efficient conversion of complex high-strength lipid waste to methane was proved to be possible in a novel reactor system configuration combining saponification pre-treatment and digested solids recirculation to the anaerobic digestion process, to increase solids retention time. A start-up step consisting on pulse-feeding cycles of the fatty waste prior to the semi-continuous process promoted an adapted microbial community for LCFA mineralization. The feasibility of this system configuration for solid slaughterhouse fatty waste was evidenced at lab scale reactors, reaching organic matter removal efficiencies higher than 90%. The comparison of this configuration with systems without saponification or without digested solids recirculation confirmed the synergistic effect of both strategies. The use of high throughput sequencing approach (454-pyrosequencing) to characterize the evolution of the biodiversity and the phylogenetic structure of the microbial community during the operation of the tested configurations concluded that a selection of a defined functional acidogenic population (β-oxidizers) was induced by substrate pretreatment. Contrary, the solids recirculation resulted in an enrichment of the methanogenic biodiversity, mainly of hydrogenotrophic archaea.

Based on the satisfactory results obtained with the strategies studied in the present dissertation, it is expected that lipid-rich waste valorization will be a real alternative to increase renewable energy production through anaerobic digestion process.

RESUMEN

La digestión anaerobia es un proceso bioquímico versátil que transforma diversas categorías de materias primas de biomasa y de residuos orgánicos en energía renovable en forma de metano, además de contribuir a la conservación de los recursos y la mitigación de emisiones de gases de efecto invernadero. Los residuos y las aguas residuales ricas en lípidos presentan un elevado potencial energético, sin embargo una eficiente recuperación del metano no es fácil de conseguir mediante las tecnologías convencionales de digestión debido a una gran variedad de problemas operativos relacionados, principalmente, con la acumulación en el sistema de ácidos grasos de cadena larga (LCFA), productos de la hidrólisis de los lípidos. El objetivo de la presente tesis es analizar y evaluar nuevas metodologías y estrategias de tratamiento para mejorar la digestión anaerobia de los residuos con alto contenido en lípidos.

En una primera aproximación, se confirmó el interés de los residuos ricos en lípidos y de los subproductos animales para la producción de biogás, mediante el control de la carga orgánica aplicada. Los resultados obtenidos refuerzan el conocimiento existente en la literatura especializada que describe la flotación, el lavado de sustrato y biomasa, y los fenómenos de inhibición por LCFA como los principales limitantes del proceso. Los resultados del estudio del comportamiento de reactores anaerobios sometidos a alimentación por pulsos de lípidos/LCFA señalaron la importancia de la adsorción de LCFA sobre la membrana celular como principal factor limitante, orientando la investigación hacia el desarrollo de nuevos enfoques técnicos con el fin de controlar la dinámica del proceso de adsorción de LCFA sobre la biomasa anaerobia.

En este sentido, el uso de adsorbentes inorgánicos para la captura de LCFA antes del proceso de digestión anaerobia o la aplicación secuencial de pulsos ultrasonidos de baja energía, con el fin de controlar la cinética de adsorción-desorción, aportaron resultados interesantes. Sin embargo, la eficiencia de estas estrategias fue limitada por la proporción adsorbente inorgánico/LCFA y por el efecto acumulativo contraproducente de los ultrasonidos sobre la biomasa, respectivamente. Otros estudios son por lo tanto necesarios para optimizar la eficiencia y la aplicabilidad de estas estrategias.

Una conversión eficiente de residuos complejos con alto contenido lipídico en metano se ha demostrado que era posible mediante una nueva configuración de sistema de reacción, combinando el pre-tratamiento de saponificación y la recirculación de sólidos al digestor anaerobio. Una etapa de puesta en marcha antes del proceso semi-continuo, que consistió en ciclos de alimentación por pulsos, promovió una comunidad microbiana adaptada para la mineralización de LCFA. La viabilidad de esta configuración para los residuos cárnicos sólidos se comprobó a escala de laboratorio, alcanzando un rendimiento de degradación de la materia orgánica superior al 90%. La comparación de esta configuración con sistemas sin saponificación o sin recirculación de los sólidos digeridos confirmó el efecto sinérgico de ambas estrategias. Paralelamente, el uso de técnicas avanzadas de biología molecular (454-pyrosequencación) para caracterizar la evolución de la biodiversidad y la estructura filogenética de la comunidad microbiana implicada durante la operación de las configuraciones estudiadas concluyó que el pre-tratamiento del sustrato favoreció la selección de una población acidogénica específica (β-oxidantes), mientras que la recirculación de sólidos promovió el enriquecimiento de la biodiversidad metanogénica, principalmente de arqueas hidrogenotróficas.

Basándose en los resultados satisfactorios obtenidos con las estrategias estudiadas en la presente tesis doctoral, se espera que la valorización de residuos ricos en lípidos represente una alternativa atractiva que permita aumentar la producción de energía renovable a través del proceso de digestión anaerobia.

RÉSUMÉ

La digestion anaérobie est un processus biochimique polyvalent qui transforme des diverses catégories de biomasse et de déchets organiques en énergie renouvelable, sous forme de méthane. Elle contribue également à la conservation des ressources et à la réduction des émissions de gaz à effet de serre. Les résidus et les eaux usées lipidiques présentent un potentiel énergétique élevé. Néanmoins une récupération efficace du méthane avec les technologies conventionnelles de la digestion anaérobie n'est pas facile à achever en raison d'une vaste gamme de problèmes opérationnels principalement liée à l'accumulation dans le système des acides gras à longue chaîne (LCFA), les produits de l'hydrolyse des lipides. L'objectif de la présente thèse est donc de tester et d'évaluer de nouvelles méthodes et stratégies pour améliorer la DA des déchets graisseux.

La pertinence et l'attractivité des déchets graisseux et des déchets d'abattoirs pour la production de biogaz ont été confirmé dans une première approche. En plus, les résultats obtenus ont renforcé les connaissances existantes décrivant la flottation et le lessivage du substrat et de la biomasse, et le phénomène d'inhibition qui affectent la population microbienne comme les principaux inconvénients du processus. Les résultats des études exposant des réacteurs anaérobies à des concentrations croissantes de lipides/LCFA ont souligné l'importance de l'adsorption des LCFA sur la membrane de la cellule microbienne comme le facteur limitant, dirigeant de nouvelles recherches pour apercevoir de nouvelles approches techniques afin de contrôler la dynamique de l'adsorption des LCFA sur la biomasse.

L'utilisation d'adsorbants minéraux pour capturer les LCFA avant le processus de digestion anaérobie ou l'application séquentielle d'ultrasons de faible énergie afin de contrôler la cinétique d'adsorption-désorption ont été testées avec des résultats intéressants. Cependant, l'efficacité de ces stratégies a été limitée par la proportion adsorbant inorganique/LCFA et par l'effet néfaste cumulatif du traitement par ultrasons sur la biomasse, respectivement. D'autres études s'avèrent donc nécessaires pour optimiser l'efficacité et l'applicabilité de ces stratégies.

Une conversion efficace des déchets complexes lipidiques au méthane a été prouvée d'être possible dans une nouvelle configuration de système de réacteur combinant le prétraitement de saponification et le recyclage des matières digérées avec le processus de digestion anaérobie, afin d'augmenter le temps de rétention des solides. Une étape de démarrage comprenant des cycles d'impulsion des déchets graisseux avant le procédé semi-continu a promu une communauté microbienne adaptée pour la minéralisation des LCFA. La faisabilité de cette configuration pour les déchets graisseux solides d'abattoirs a été démontrée dans des réacteurs à l'échelle de laboratoire, atteignant une efficacité d'élimination de matières organiques supérieure à 90%. La comparaison de cette configuration avec d'autres systèmes sans l'étape de saponification ou de recirculation des solides digérés a confirmé l'effet synergique de ces deux stratégies. L'utilisation de l'approche de séquençage à haut débit (454 pyroséquençage) pour caractériser l'évolution de la biodiversité et la structure phylogénétique de la communauté microbienne au cours de l'opération des configurations testées a conclu que le prétraitement du substrat a induit la sélection d'une population acidogène spécifique (β-oxydantes). Contrairement, la recirculation des solides a abouti à un enrichissement de la biodiversité méthanogène, principalement des archées hydrogénotrophe.

En se basant sur les résultats satisfaisants obtenus avec les stratégies étudiées dans cette thèse doctorale, il est attendu que la valorisation des déchets graisseux sera une véritable alternative pour augmenter la production d'énergie renouvelable à travers le processus de la digestion anaérobie.

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Chapter 1. Context, aim and thesis outline

In this chapter, the importance of renewable energy sources in the present concern about global climate change and energy crises and the importance of lipid-rich organic waste source valorization, especially through anaerobic digestion, are introduced as the subject of the present dissertation. The identified research needs and the key objectives of the thesis activities are exposed and, finally, the thesis outline is explained.

1.1. CONTEXT

One of the most important challenges that society is facing nowadays is the global energy crisis, which is a consequence of the foreseeable depletion of fossil fuels coupled with the unprecedented rising of crude oil prices. This critical situation imposes not only to focus on sustained economic use of the existing limited resources, but also to move efforts towards alternative technologies and renewable energy sources. Another important challenge that the humanity is facing is the climate change. Indeed, the global warming is due to the large scale anthropogenic emission of greenhouse gases (GHG), which are mainly caused by the generation of heat and power (Appels et al., 2011). In this scenario, renewable energies will hold a high level of importance in the future, in both developed and developing countries, because they have the potential to *(i)* cater for the increasing energy demand, *(ii)* to minimize environmental impact and *(iii)* to produce minimum secondary wastes, in addition to possessing other positive attributes such as being sustainable, globally available and easy to exploit (Abbasi et al., 2011; Panwar et al., 2011). In 2004, renewable energy accounted for over 15% of world primary energy supply (Sim et al., 2007), including biomass, hydropower, geothermal, solar, wind and marine energies (Panwar et al., 2011).

In this scenario, anaerobic digestion has been evaluated as one of the most-efficient and environmentally "friendly" technology for bioenergy production (Madsen et al., 2011). Anaerobic digestion is a robust biochemical process that combines waste management and energy production, in the form of biogas. Its application for the treatment of organic waste has been emerging spectacularly with an annual growth rate of 25% during recent years (Buffiere et al., 2008). Thousands of full-scale installations are in operation worldwide, treating mainly wastewater containing readily degradable organic pollutants such as volatile fatty acids and carbohydrates generated mainly from distilleries, pulp and paper, breweries and beverage industries (Alves et al., 2009). The biogas is a mixture of gases that is composed mainly of methane (CH_4) 40–70%, carbon dioxide (CO_2) 30–60%, and other gases 1–5%. In most cases, biogas, with a calorific value of about 16 – 20 MJ m^{-3} (Kurchania et al., 2010), is valorized energetically in a combined heat and power installation for the simultaneous generation of heat and electricity. These installations typically offer an electrical efficiency of 33% and a thermal efficiency of 45% (Appels et al., 2011). Recently, Tilche and Galatola (2008) computed

the potential contribution of anaerobic digestion to GHG for the 27 EU countries on the basis of their 2005 Kyoto declarations and using life cycle assessment data. This study shows that biogas may considerably contribute to reduce GHG emission, in particular if used as a biofuel, with a potential of covering almost 50% of the 2020 biofuels target and a 10% of all the automotive transport fuels.

Thus, the urgent issue in the future is to increase the valorization of organic waste with high theoretical biogas potential in order to increase the economic feasibility of the anaerobic digestion technology. Lipids constitute ideal substrates for methane production because of their high methane potential, i.e. 1.01 L_{CH4} g_{lipid}^{-1} vs. 0.37 L_{CH4} $g_{glucose}^{-1}$ (Kim et al., 2004), when compared with other organic substrate (protein or carbohydrates). Lipid-rich waste and wastewaters are mainly discharged from food processing industry, which include dairies, slaughterhouses, meat processing plants and olive oil mill processing facilities. Their amounts are significantly increasing as a consequence of the intensive livestock production, the increasing demand of meat products and the decline of the economic value of animal by-product after the bovine spongiform encephalopathy crisis. However, efficient methane recovery from high-strength waste and wastewater with conventional technology is not easily achieved because of a wide assortment of operational problems mainly related to long chain fatty acids, products of lipids hydrolysis.

The current status of scientific knowledge of the anaerobic treatment of lipid-rich wastes is focused on the physiological and ecological aspects of their syntrophic degradation on the description of the associated operational problems and on the study of the adsorption process as key phenomena related to microorganisms' inhibition. Considerable efforts were recently made to develop new solutions and systems to overcome all the limiting aspects of the anaerobic digestion of high-strength lipid waste/wastewater. The works constituting the present doctoral thesis have the aim to contribute to these efforts.

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1.2. OBJECTIVES

The main objective of this thesis is to test and to evaluate new methodologies and strategies to improve the anaerobic digestion of high-strength lipid wastes. In this context, several activities have been planned in order to achieve the following specific objectives.

To identify the process limitations when studying the degradation of synthetic and real lipid wastes.

- To study the toxic effect of LCFA, discriminating between the superficial adsorption and the metabolic inhibition.
- To evaluate the effect of additives addition (Bentonite, Serum Bovin Albumin) as strategies to enhance the bioavailability of lipids or to prevent the inhibition phenomena.
- To analyze and to contrast the hypotheses that the application of suitable controlled ultrasounds could influence the bio-augmentation of the biomass activity, the LCFA solubilisation or the adsorption-desorption dynamics.
- To propose and to evaluate different anaerobic reactors configuration adapted to the treatment of complex solid lipid-rich wastes.
- To assess the evolution of the microbial consortia (archaeal and bacterial) during the anaerobic digestion of complex solid lipid-rich waste and to identify potential important players in the process by means of high throughput sequencing technology (454-pyrosequencing).

1.3. RESEARCH CHRONOLOGY AND SCOPE

The activities of this dissertation were involved in the framework of OPA_LAP (Ref. ENE2007-65850, 2007-2010) and ADAMOX (Ref. CTM 2010-1812, 2010-2012) projects and financially supported by a research grant (Ref. BES-2008-008625, 2008-2012) of the Spanish Personal Research Training Program from the Spanish Ministry of Science and Innovation. The development of the research work was done at GIRO

Technological Center, in the context of the doctoral program on "*Environmental Engineering*" of the Universitat Politècnica de Catalunya –BarcelonaTECH (UPC), under the supervision of Prof. Xavier Flotats and Dr. Jordi Palatsi.

The two projects involved in this research had as specific scope the optimization of the anaerobic digestion of animal by-product (slaughterhouse wastes), characterized by high fat and protein content. Working lines of the present thesis were focused on testing advanced anaerobic treatment strategies for high lipid substrates in general, and solid slaughterhouse waste as specific case.

In the context of the present PhD thesis, I had also the privilege to collaborate with some scientists of praised reputation in this field of knowledge. In the context of the OPA_LAP project and thanks to funding provided by the Spanish Ministry of Science and Innovation (Ref. BES-2008-008625, 2008-2012), I gained an internship during three months in the *Laboratory of Biotechnology of University of Minho* (Braga, Portugal 10/2009 – 12/2009), under the supervision of Prof. Madalena Alves. This group of research is recognized by their studies about the LCFA inhibition phenomena and by the development of new concepts for the anaerobic digestion of lipids and LCFA. Serum Bovine Albumin addition was tested as a preventing strategy for LCFA adsorption during this stay, but unfortunately the results were not enough conclusive, reason why are not included in the present manuscript. I had also the possibility to collaborate with Dr. Audrey Battimelli at the *Laboratory of Environmental Biotechnology* (LBE-INRA, Narbonne France, 04/2011 – 10/2011), Centre that is known by the huge and original scientific production in the field of the environmental biotechnology. This stay lasted six months and was financially supported by the ADAMOX project and a grant from the Spanish Ministry of Science and Innovation (EEBB-2011-44023).

1.4. THESIS OUTLINE

Working lines of the present dissertation consist on the proposal and the testing of new strategies to optimize the anaerobic treatment of organic waste with high content in lipids and LCFA in general, and solid slaughterhouse waste as specific case. The improvement of the solubilisation and the bio-availability of lipids/LCFA has gained a special attention in the research working. New technical approaches to control the

biomass-LCFA adsorption dynamics and new reactor configurations have been also proposed as exhaustive strategies in this PhD thesis.

The subject of this thesis and its importance in the context of organic waste treatment through the anaerobic digestion technology is presented in the present **Chapter 1**. The main research objectives and an executive summary of the different activities performed in the framework of the thesis scope are also included.

Chapter 2 provides a detailed literature review of the current knowledge and state of the art on the anaerobic treatment of lipids and LCFA, focusing on biochemical and microbiological aspects. Common problems related to their degradation and the existing alternatives proposed to optimize the anaerobic digestion process were underlined. Main process limitation was ascribed to the adsorption of LCFA, products of lipids hydrolysis, onto the microbial surface cells, affecting the external nutrients transport and the protective functions of the microbial cells membrane, causing the sludge flotation and wash-out, in addition to the biological toxicity.

The first tasks of the present Thesis aimed to identify the problems, described in literature, of the anaerobic digestion of lipid-rich wastes, and to understand the pathway of LCFA inhibition and the importance of the adsorption process phenomenon. In the annexed information of **Annex.1**, category 2 and 3 sterilized solid slaughterhouse waste collected from a cattle and bovine rendering facility were co-digested with sewage sludge in batch and continuous conditions, testing different ratios of fatty substrate/sewage sludge. The working line of this study was focused on *(i)* the determination of the optimal sterilized slaughterhouse waste dosage for co-digestion with sewage sludge, by evaluating volatile solids removal efficiencies and CH_4 yields, and *(ii)* the evaluation of possible process inhibition related with free ammonia and lipids accumulation in the system. Results of fed-batch reactor experiment confirm the suitability and the attractiveness of sterilized slaughterhouse waste for biogas production. The optimal process conditions were established with 5% (w/w) of the fatty substrate corresponding to an OLR of 2.68 $kg_{VS}\ m^{-3}\ d^{-1}$. Those conditions allowed the improvement of CH_4 production by 5.7 times compared to sewage sludge mono-digestion. However, further higher fatty substrate addition caused process inhibition, initiated by NH_3 accumulation and finalizing with lipids-LCFA accumulation, foaming,

CH_4 methane yields reduction and overall process failure when the fatty substrate was added in the proportion of 10% (w/w). This research was produced during a collaboration with Mr. Peep Pitk, a PhD student from the Department of Chemistry of Tallinn University of Technology (Harjumaa, Estonia), who did a research stay at GIRO Technological Centre in the framework of ADAMOX project, reason why it is included in the annexed information. More specifically, in **Chapter 3**, a comprehensive picture on the operational problems that could be associated with the anaerobic digestion of lipid-rich wastes was provided by submitting two CSTR reactors (HRT = 30 days and OLR= 1 g_{COD} L^{-1} day^{-1}) to increasing pulses of glycerol trioleate, as lipid model, and oleic acid, as LCFA model. The results of this chapter confirmed that flotation, wash-out and the inhibition of anaerobic process were the main operational problems attributed to the anaerobic digestion of lipids/LCFA degradation. The flotation was found to be a rapid process that occurred even before the inhibition process. Moreover, the inhibitory effect of the fatty substrate over the anaerobic biomass was found to be concentration dependent and was directly related to the palmitic acid accumulated in the system. In parallel, a further study has evaluated the impact of the adsorption mechanisms on the process inhibition, by means of specific batch tests (monitoring LCFA evolution), by the additions of synthetic adsorbents (bentonite) and by molecular profiling of the predominant microorganisms and fluorescence staining microscopy imaging. A detailed description of this study was annexed in **Annex 2**. The results of bentonite addition highlight the importance of the adsorption process on the biological inhibition of LCFA, and confirm its positive effect on the improvement of the system robustness. Three strategies of adsorbents (bentonite) addition were compared, and the best scenario was to incubate the bentonite with LCFA prior to the anaerobic digestion process, in order to promote the exclusively LCFA-bentonite adsorption (LCFA capturing strategy) and thus to promote the protection of the biomass from LCFA adsorption-inhibition process. The second part of **Chapter 3** was thus to test and to evaluate this recommended strategy in anaerobic bioreactors (CSTR), in continuous operation conditions. Bentonite was added in the ratio 20% (w/w) and was fount to partly overcome the inhibitory effect of lipids pulses (glycerol trioleate). However it was not very reliable when the reactor was subjected to continuous load of oleic acid. The positive effect of bentonite addition was exceptionally noted with the lower applied oleate organic load (0.3 ± 0.009gCOD $L_{reactor}^{-1}$ d^{-1}), most likely due to the relatively low

bentonite/substrate ratio (20%) which was probably insufficient to capture all or at least the majority of the added substrate.

The hypothesis that the application of ultrasonic treatment with suitable controlled energy input could affect positively the bio-augmentation of the biomass activity, the LCFA (saturated and unsaturated) solubilization or the adsorption-desorption dynamics was studied in **Chapter 4**. These possible effects were studied simultaneously by means of batch tests, ultrasonicating the mixture of substrate-inoculum at different intervals of specific energy. The same experimental procedure was also applied to waste activated sludge and sewage sludge with the aim to obtain a comparison framework. The effect of ultrasounds on the organic matter solubilisation was determined by means of total and soluble COD measurement, while their impact on biomass activity and on the adsorption-desorption process was quantified by fitting the Gompertz equation to the methane production data and identifying the optimal parameter values. The results revealed that the ultarsonic treatment of the mixture inoculum-waste activated sludge or sewage sludge enhanced the methane production potential and the biomass activity as a result of the higher bio-available soluble substrate, till certain specific energy supply, for which the cells damage by sonication leads to an increase of the lag phase time in batch experiments. For LCFA, sonication at the tested specific energies does not modify the biodegradability of oleate and palmitate, while increases the biomass activity for oleate. In the case of palmitic acid, the ultrasonic treatment decreased significantly the lag time λ when the specific energy inputs was 1,640 $kJ \cdot kg_{TS}^{-1}$, increasing it for higher energy values. This finding open the discussion about possible effect of sonication over the adsorption-desorption kinetics. To confirm this, the ultrasonic treatment was applied in a sequentially scheme (every 3 days) to anaerobic digestion batch experiments with palmitate as substrate. The methane production rate was increased before the fifth dosage respect to the untreated sample, but decreasing later. Sequentially low energy dosages during the anaerobic digestion process of palmitate opens an interesting option to enhance its decomposition, but maximum acceptable cumulated energy value must be further studied.

In **Chapter 5**, novel reactor system configurations suitable for the anaerobic treatment of complex lipid-based waste were tested and validated. Solid slaughterhouse waste (fresh fat from cattle carcass) was selected as the model substrate since the specific

scope of the two projects in which the present dissertation is involved is the optimization of the anaerobic treatment of animal by-products. All limiting aspects on the anaerobic degradation of lipids, such as particulate substrate, slow hydrolysis rates, high suspended solids content, substrate-biomass flotation or wash-out, and possible process inhibition were considered in the experimental process design. The concept of these configurations were based on the combination of anaerobic digestion with/without waste saponification pretreatment (70ºC during 60 minutes) and with/without recirculation of the digestate solid fraction (ratio=20% w/w). Based on the positive stimulating effect of punctual pulses addition over the microbial community found in **Chapter 3**, an acclimatization period consisting on pulse-feeding cycles was applied to the anaerobic biomass prior to the semi-continuous loading of the solid fatty waste. The degradation of the raw substrate was shown to be the bottleneck of the whole process, obtaining the best performance and process yields in the reactor equipped with waste pretreatment and solids recirculation. Saponification promoted the emulsification and bioavailability of solid fatty residues, while recirculation of solids minimized the substrate/biomass wash-out and induced microbial adaptation to the treatment of fatty substrates.

The evolution of the microbial consortia (archaeal and bacterial) in each reactor configuration along the experimental time, studied in **Chapter 5**, and the identification of the potential important players in the process were assessed by means of high throughput sequencing technology (454-pyrosequencing) and presented in **Chapter 6**. Special attention has been given to the effects of lipid pre-treatment by alkaline hydrolysis and to the recirculation of solids from the reactor outflow.

Chapter 7 summarizes the main conclusions of the work done in this thesis and introduces some suggestions for further investigations in this research topic.

Chapter 2. Anaerobic digestion of lipid-rich waste

Waste and wastewater discharged from slaughterhouses facilities and food processing industry contains considerable amounts of fats and lipids. They constitute interesting substrates for the anaerobic digestion process due to their high theoretical methane potential. However, a wide assortment of operational challenges was associated with their treatment. This chapter presents the state of the art of the anaerobic treatment of high-strength lipid waste and wastewater. A brief characterization of this kind of wastes and a critical review of its anaerobic degradation and process limitations is reported in the following sections. Details of the biochemical mechanisms and the microbial communities involved in the anaerobic degradation of lipids and its intermediates, saturated and unsaturated long chain fatty acids (LCFA), are also discussed. A special focus was given to the problems caused by LCFA and to the existing alternatives, or strategies, proposed to optimize its anaerobic degradation.

2.1. LIPIDS AND LCFAs-RICH WASTE/WASTEWATERS CHARACTERISTICS

Fat, oil and grease (FOG) is a term commonly used to define the layer of lipid-rich materials from wastewater generated from the food processing industry, which include dairies, slaughterhouses, meat processing plants and olive oil mill processing facilities. These types of wastes are characterized by a high biological and chemical oxygen demand (Demirel et al., 2005) and by a high richness in biodegradable organic molecules, mainly proteins and fats (Vidal et al., 2000; Li et al., 2002; Cammarota and Freire, 2006). In general, the lipid concentration of those wastes is dependent on the specific industrial process. For example, the chemical characteristics of cooking wastes are dependent on the type of restaurant or food service establishment and on the type of the cooking treatment (Long et al., 2012). In dairy industry, the variability in the characteristics of the generated waste is also correlated with the season and the type of final product (milk, butter, yoghurt, ice-cream, cheese) (Demirel et al., 2005). Lipids concentrations in a dairy outflow were reported to vary between 900 and 3,000 mg L^{-1} (Kim et al., 2004a; Kuang et al., 2002). Also relatively high concentration of lipids, averaging from 6,600 mg L^{-1} (Beccari et al., 1998) to 25,000 mg L^{-1} (Becker et al., 1999) were quantified in olive oil mill effluents, while lower values, ranging from 200 to 1,300 mg L^{-1}, were detected in sunflower oil mill wastewater (Saatci et al., 2003).

Solid slaughterhouses wastes, or animal by-products (ABP), have gained a crucial position in the anaerobic digestion sector after the bovine spongiform encephalopathy disease crisis and the development of more stringent environmental legislation. The ABPs are considered as attractive substrate for methane production because of its high organic content, mainly composed by proteins and fats. Recently, Hejnfelt and Angelidaki (2009) and Palatsi et al. (2011) have characterized individual fractions of Danish and Spanish animal by-products, respectively, and determined their high methane potential yields. Palatsi et al. (2011) reported that the fat accounted for 68 – 82% of the total volatile solids in different mixtures of fresh pig and cattle slaughterhouse wastes. Relatively lower contents of lipids, in the range of 24 % - 30%, were reported by Hejnfelt and Angelidaki (2009) in mixtures of piggery slaughterhouse by-products, probably due to the presence of a higher proportion of meat fractions (as

meat and blood flour). In beef slaughterhouse wastewater about 40% of the total COD is composed by lipids (Saddoud and Sayadi, 2007).

Glycerids (triglycerides, phospholipids and glycolipids) are organic compounds recognized by their solubility in nonpolar organic solvents (e.g. ether, chloroform, acetone and benzene) and general insolubility in water. Lipids are glycerol bonded to long chain fatty acids (LCFA), alcohols and other groups by an ester or ether linkage. LCFA contain approximately 95% of the original lipid chemical oxygen demand. LCFA are carboxylic acids that contain normally even number of carbon atoms which are from 12 to 24. The carboxylic acid contains an aliphatic tail (chain), which is either saturated or unsaturated, depending on the absence or presence of double bounds. The qualitative composition of LCFA in fatty residues is variable and highly dependent on the origin of the lipid source. However, palmitic acid (hexadecanoic) and oleic acid (cis-9-octadecanoic) are, respectively, the most abundant saturated and unsaturated LCFA present in lipid-rich wastes.

2.2. BIOCHEMICAL FUNDAMENTALS OF THE ANAEROBIC DIGESTION OF LIPIDS AND LCFA RICH WASTE/WASTEWATERS.

Knowledge of the biochemistry and microbiology fundamentals, kinetics, and the technological aspects of anaerobic digestion is essential to ensure correct design and efficient anaerobic treatment. The anaerobic process proceeds through a series of parallel and sequential biological reactions involving several groups of microorganisms. The actual structured anaerobic digestion models describe the process with 4 different steps, in which the product of one step serves as a substrate for the next (Batstone et al., 2002). First, particulate organic matter (protein, carbohydrate and lipids) is disintegrated and hydrolyzed by hydrolytic-fermentative bacteria to their monomer or dimeric component (amino acids, sugar and LCFA). The hydrolysis products are further fermented, by acidogenic bacteria, into volatile fatty acids (VFA), alcohols and hydrogen. The latter are, in turn, oxidized in the acetogenesis step to acetate (Ac) and hydrogen (H_2). Finally Ac and H_2 are converted during the methanogenisis step into methane (CH_4) and carbon dioxide (CO_2) via acetoclastic and hydrogenotrophic archaea, respectively. Figure 2.1 displays a schematic representation of the anaerobic

digestion process and its main degradation steps. A special emphasis to the anaerobic degradation of lipids is given in the next section.

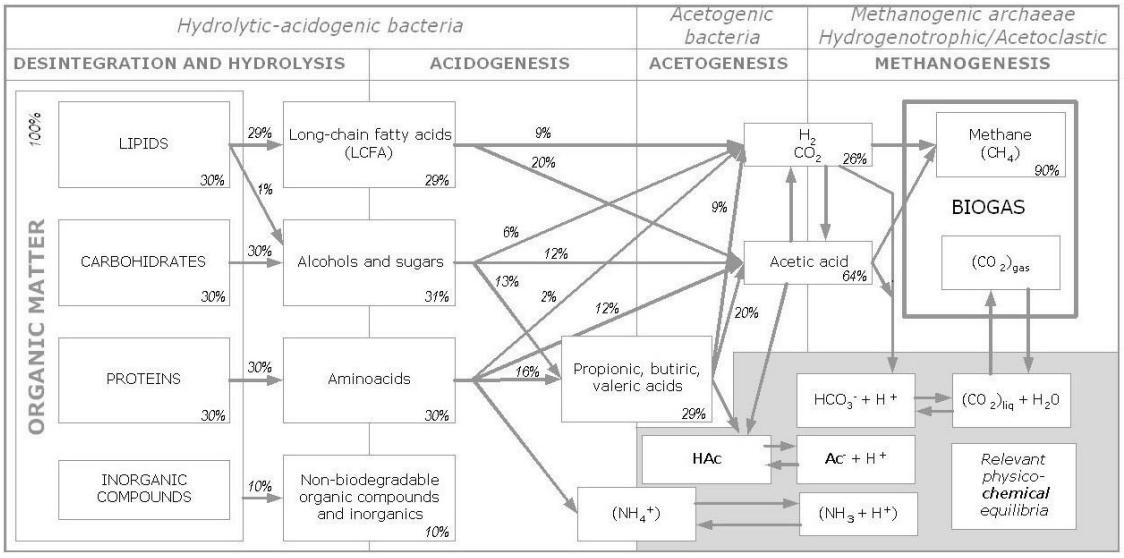

Figure 2.1. *Schematic representation of the anaerobic digestion process. Numbers indicate COD flux of materials, adapted from Bastone et al. (2002)*

2.2.1 Hydrolysis

Lipids, mainly present as triglycerides in waste and wastewaters, are hydrolyzed by extracellular enzymes to glycerol and long chain fatty acids (LCFA). This enzymatic reaction is executed by lipases at the lipid-water interface: the enzymes are firstly adsorbed at the lipid interface for their potential activation. The formation of the enzyme-substrate complex is followed by the hydrolysis reaction and finally the regeneration of the adsorbed enzyme (Jaeger et al., 1994; Gupta et al., 2004). It is evident that the hydrolysis rate is strongly dependent on the concentration of the hydrolytic biomass, on the substrate-biomass ratio and on the lumped effect of the type (triglycerides, phospholipids or sterols), physical state (solid or liquid) and the particle size of the lipid (Masse et al., 2002; Vavilin et al., 2008). Table 2.1 provides a review of the typical values of the first-order rate coefficients of fatty substrate, ranging between 0.005 day^{-1} and 0.76 day^{-1} (Vavilin et al., 2008). When the lipid-water interface is tiny, because of the large particle size (such as in solid slaughterhouse waste), the hydrolysis can be the rate-limiting step in the overall anaerobic lipid degradation process.

Few references are available about the microbial communities involved in the anaerobic hydrolysis of lipids and most of them are derived from studies on rumen bacteria. Main identified microorganisms were related to the genus *Clostridium* (Cirne et al., 2006a). Up to date, only 3 bacterial species have been isolated and identified as anaerobic hydrolizing bacteria. Dighe et al. (1998) isolated an obligatory anaerobic bacterium, named *Selenomonas lipolytica*, from a tropical anaerobic lagoon receiving wastewater from an edible oil mill factory. This bacterium was related to the genus *Selenomonas* and was able to hydrolyze lipids to glycerol and fatty acids. Later, *Thermosyntropha lipolytica* was isolated by Svetlitshnyi et al. (1996) and identified as thermophilic lipolytic alkali tolerant bacterium. Recently, a new strictly anaerobic mesophilic lipolytic bacterium, identified as *Clostridium lundense* was isolated from the bovine rumen by Cirne et al. (2006b). This *Clostridium* strain shows lipolytic activity, hydrolyzing olive, sesame and corn oils.

Table 2.1 *Kinetic coefficients of the first-order rate of lipid and high-strength lipid waste hydrolysis (adapted from Vavilin et al., 2008)*

Substrate	***k*** **(day^{-1})**	**T (ºC)**	**References**
Lipids	0.005 – 0.01	55	Christ et al. (2000)
Lipids	0.1 – 0.7		Garcia-Heras (2003)
Lipids	0.63	25	Masse et al. (2002)
Lipids	0.76		Shimizu et al. (1993)
Household waste	0.1	37	Vavilin and Angelidaki (2005)
Kitchen waste	0.34	35	Liebetrau et al. (2004)
Slaughterhouse waste	0.35	35	Lokshina et al. (2003)
Food waste	0.55	37	Vavilin et al. (2004)

2.2.2. Acidogenesis

The acidogesis is the first energy yielding step during the anaerobic digestion process and consists on the degradation of soluble substrates without the presence of an external electron acceptor. The acidogeneis takes place inside the cell of fermentative bacteria and yields acetate (Ac), other volatile fatty acids (VFA; propionate, butyrate, valerate...), hydrogen and other organic products, such as lactate and alcohols. In general, the acidogenic population represents about 90% of the total microbial community present in the anaerobic digester. These microorganisms have a short doubling time and therefore acidogenesis is usually considered as the fastest reaction in

the anaerobic digestion process (Zeikus, 1980; Mosey, 1983). The oxidation of LCFA requires an external electron acceptor and, therefore, is realized in the acetogenesis stage. However, the hydrogenation (see section 2.2.5.) of unsaturated LCFA occurs during the acidogenesis step (Mackie et al., 1991).

2.2.3. Acetogenesis

The organic products of the acidogenesis step and LCFA are further oxidized to Ac, hydrogen gas (H_2), and CO_2 by the action of obligate hydrogen-producing acetogens (OHPA). Propionate is also formed during the oxidation of odd-numbered LCFA. Fatty acids oxidation is coupled to the reduction of hydrogen ions or bicarbonate (functioning as external electron acceptors) to H_2 and formate, respectively. Under standard conditions, these oxidation reactions are thermodynamically unfavorable (positive Gibbs free energy values, $\Delta G^{o'}$). The complete conversion of the substrates only proceeds when hydrogen partial pressure in the medium is kept low to yield a negative $\Delta G^{o'}$ value. This is achieved by syntrophic association with hydrogen- (formate)-utilizing bacteria, usually hydrogenotrophic archaea, that act as hydrogen (formate) scavengers (Schink, 1997).

2.2.4. Methanogenesis

Methanogenesis is the last stage 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) and are more sensitive to environmental changes than hydrogenotrophic ones (Koster and Cramer, 1987; Alves et al., 2001; Lalman and Bagley, 2001). The generation times for methanogens range from 3 days at 35º C up to 50 days at 10º C (Gerardi, 2003), requiring therefore a high retention times (at least 12 days) in bioreactors to ensure the growth of a large methanogenic population, which make it the rate-limiting step of the whole anaerobic digestion process of not complex wastes (Huang et al., 2003).

2.2.5. Hydrogenation and β-oxidation of LCFA

LCFA degradation pathways have not been yet completely described in anaerobic environments. However, experiments with labeled ^{14}C pamitic acid (Nuck and Federle, 1996) and oleic acid (Weng & Jeris, 1976) pointed at β-oxidation as the principal mechanism involved in LCFA degradation under anaerobic conditions. β-oxidation is a multi-step process in which LCFAs are broken down to yield energy and acetyl-CoA as end product. Unsaturated and saturated LCFA are primarily adsorbed onto the cell surface and then transferred across the cytoplasmic membrane to bind to acyl-CoA synthetase which induces the activation of LCFAs. Transport and activation of LCFA (by acyl-CoA synthetase) are strongly coupled processes resulting in the production of fatty acyl-CoA (DiRusso et al., 1999). These molecules are then released into the microbial cells and the β-oxidation mechanisms starts. Cycles of β-oxidation reactions are finally carried which results in the production of one acetyl-CoA from each cycle of fatty acid β-oxidation. The remaining shortened LCFA acyl-CoA of one cycle serves as substrate in the next β-oxidation cycle, until the break-down of all carbons to acetyl-CoA and finally to acetic acid (Weng and Jeris, 1976, Sousa et al., 2009).

Saturated LCFA enter directly in the β-oxidation mechanisms. However discussion is opened about the exact mechanisms of unsaturated LCFA degradation. Most of the published studies suggest that unsaturated LCFA undergo to the β-oxidation process only after the hydrogenation of the double bond (Weng and Jeris, 1979; Lalman and Bagley, 2001; 2002). Contrary, some researchers have detected unsaturated LCFA during the anaerobic degradation of oleate and linoleate, hypothesizing the occurrence of a direct β-oxidation mechanism (Roy et al., 1986; Canovaz-Diaz et al., 1991; Lalman and Bagley, 2000). Nevertheless, the analysis of the free energy value (Thauer et al., 1977) of the two possible pathways of anaerobic degradation of unsaturated LCFA can provide some insights into which pathway is more feasible (Table 2.2). The negative values of $\Delta G^{o'}$ during the conversion of linoleate (C18:2), oleate (C18:1) and palmitoleate (C16:1) to oleate (C18:1), stearate (C18:0) and palmitate (C16:0), respectively, prove that the occurrence of the hydrogenation step prior to β-oxidation process is thermodynamically feasible. Also, in the case of linoleate (C18:2) degradation, a first hydrogenation step followed by a β-oxidation step, with the formation of palmitoleate (C16:1), was found to be thermodynamically feasible ($\Delta G^{o'} <$

0). Contrary, the direct oxidation of C18:1 to C16:1 can be considered thermodynamically unfavorable (Thauer et al., 1977).

2.3. MICROBIOLOGY of LCFA DEGRADATION

The β-oxidation of LCFA in anaerobic environment is thermodynamically unfavorable and requires the syntrophic cooperation of acetogenic bacteria (β-oxidizers) and acetoclastic-hydrogenotrophic archaea (McInerney et al., 2009). Up to date, 14 syntrophic fatty acid degrading bacterial species have been isolated and characterized. However, only 7 of these species were described as capable to degrade LCFAs with more than 12 carbon atoms. They all belong to the families *Syntrophomonadaceae* and *Syntrophaceae*, within the phyla *Firmicutes* and *Delta-Proteobacteria*, respectively (Sousa et al., 2009): *Syntrophomonas sapovorans* (Roy et al., 1986), *Syntrophomonas saponavida* (Lorowitz et al., 1989), *Syntrophomonas curvata* (Zhang et al., 2004), *Syntrophomonas zehnederi* (Sousa et al., 2007a), *Syntrophomonas palmitatica* (Hatamoto et al., 2007), *Thermosyntropha lipolytica* (Svetlitshnyi et al., 1996) and *syntrophus aciditrophicus* (Jacckson et al., 1999). The ability to degrade unsaturated LCFA is restricted to *S. sapovorans*, *S. curvata*, *S. zehnederi* and *T. lipolytica* (Sousa et al., 2009). The doubling times for fatty acid β-oxidizing bacteria ranged from 40h for *S. sapovorans* to 90h for *S. wolfei subsp. Wolfei* (Hansen et al., 1999).

Most of the existing studies that characterize the phylogenetic affiliation and the dynamic of the microbial communities involved in the degradation of LCFA consist in continuous bioreactors experiments using oleate, stearate and/or palmitate as model substrate. These LCFA are the most abundant unsaturated and saturated LCFA encountered in lipid-rich wastes (Shigematsu et al., 2006; Sousa et al., 2007b; Palatsi et al., 2011; Salvador et al., 2012). Contrary, only few studies have been performed in reactors treating complex or "real" lipid-containing wastes, as agro-food industry wastewaters or slaughterhouse solid wastes (Rosa et al., 2009; Palatsi et al., 2011). Furthermore, despite the main role of *Syntrophomonadaceae* and Syntrophaceae in the anaerobic degradation of LCFA, only few numbers of these members were quantified in these studies (Shigmatsu et al., 2006; Palatsi et al., 2011). The limited biodiversity of these species, in association with the isolated phylogenetically diverse bacterial community, have opened the discussion about LCFA-degrading potential of other families (Hatamoto et al., 2007b).

Table 2.2 *Free energy values for selected reactions involving LCFAs (adapted from Lalman and Bagley, 2001)*

Parent	Product	Reaction	$\Delta G^{o'}$ (kJ mol^{-1})
Linoleic acid (C18:2)	Oleic acid	$C_{18}H_{31}O_2^- + H_2 \rightarrow C_{18}H_{33}O_2^-$	–78.6
	Palmitoleic acid	$C_{18}H_{31}O_2^- + 2H_2O \rightarrow C_{16}H_{29}O_2^- + C_2H_3O_2^- + H_2 + H^+$	–28.1
	Palmitic acid	$C_{18}H_{31}O_2^- + 2H_2O \rightarrow C_{16}H_{31}O_2^- + C_2H_3O_2^- + H^+$	–106.4
Oleic acid (C18:1)	Strearic acid	$C_{18}H_{33}O_2^- + H_2 \rightarrow C_{18}H_{35}O_2^-$	–78.6
	Palmitoleic acid	$C_{18}H_{33}O_2^- + 2H_2O \rightarrow C_{16}H_{29}O_2^- + C_2H_3O_2^- + 2H_2 + H^+$	50.5
	Palmitic acid	$C_{18}H_{33}O_2^- + 2H_{2O} \rightarrow C_{16}H_{31}O_2^- + C_2H_3O_2^- + H^+$	–27.8
Stearic acid (C18:0)	Palmitic acid	$C_{18}H_{35}O_2^- + 2H_2O \rightarrow C_{16}H_{31}O_2^- + C_2H_3O_2^- + 2H_2 + H^+$	50.8
Palmitoleic acid (C16:1)	Palmitic acid	$C_{16}H_{29}O_2^- + H_2 \rightarrow C_{16}H_{31}O_2^-$	–78.3
	Myristic acid	$C_{16}H_{29}O_2^- + 2H_2O \rightarrow C_{14}H_{27}O_2^- + C_2H_3O_2^- + H_2 + H^+$	–31.8
Palmitic acid (C16:0)	Myristic acid	$C_{16}H_{29}O_2^- + 2H_2O \rightarrow C_{14}H_{27}O_2^- + C_2H_3O_2^- + 2H_2 + H^+$	46.5
Myristic acid (C14:0)	Lauric acid	$C_{14}H_{27}O_2^- + 2H_2O \rightarrow C_{12}H_{23}O_2^- + C_2H_3O_2^- + 2H_2 + H^+$	48.6

Methanogenic archaea include mainly members of the phylum *Euryarchaeota*, assigned to at least 31 genera within five orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales* (Narinho et al., 2009). All isolated acetoclastic archaea belong to the order of *Methanosarcinales* comprising only two genera: *Methanosaeta* and *Methanosarcina* (Narinho et al., 2009). *Methanosaeta* methanogens have a high affinity for Ac and a relatively low growth rate, while *Methanosarcina* have faster growth rates and lower affinity for Ac, requiring minimum concentration of 1 mM (Liu and Whitman, 2008). Also, the hydrogenotrophic methanogens play an essential role in the overall LCFA degradation process by controlling the redox potential of the media, maintaining it at low level through metabolic H_2 consumption. Principally two groups of organisms closely affiliated to *Methanobacterium* (Sousa et al., 2007b; Salvador et al., 2012) and *Methanospirillium* (Shiegmatsu et al., 2006) genera were isolated and identified in anaerobic bioreactors fed with LCFA-based substrate (oleic and palmitic acid). *Methanospirillium* methanogens advantageously grows in systems with low H_2 concentration while *Methanobacterium* grows at high H_2 concentration.

2.4. OPERATIONAL CONCERNS OF THE ANAEROBIC DIGESTION OF LIPIDS AND LCFA-RICH WASTE

The anaerobic digestion of high-strength lipid wastes was markedly studied in the last decade due to their relatively high methane potential. A specific attention was paid to the identification and the evaluation of different operational problems caused by lipids, including the sludge flotation or wash-out and the inhibition of the microbial activity.

2.4.1. Sludge flotation and wash-out

Conventional high-rate anaerobic reactors, such as upflow anaerobic sludge blanket (UASB) reactor (Huw et al., 1998; Kim and Shin, 2010), expanded granular sludge bed (EGSB) reactor (Hwu et al., 1997; Pereira et al., 2002) and anaerobic filter (Alves et al., 2001) are usually used for the anaerobic treatment of lipid-rich wastewaters. Alves et al. (2009) have provided a detailed summary of the several reactors technologies applied to the anaerobic treatment of wastewater containing lipids and LCFA, the operational conditions and main process limitations in each case. Sludge flotation and wash-out, as a consequence of the adsorption process, is one of the most common operational

problem and lead in some cases to system failure. Rinzema et al. (1989; 1993) tested the treatment of LCFA-rich wastewaters in UASB and reported a complete wash-out when the system was overloaded (within 2-8 h). Recently, Jeganathan et al. (2006) treating complex oily wastewater from a food industry in three different UASB reactors at different operating conditions, detected operational problems at high organic loading rates. An increase in loading up to 5 kg_{COD} $m^{-3}d^{-1}$ caused lipids accumulation, sludge flotation and wash-out, with the consequent decrease in the degradation efficiency; up to values of 40-50%.All these studies highlight the detrimental impact of the sludge flotation on the overall system efficiency. Hwu et al. (1998a) concluded that the level of flotation was directly proportional to the LCFA loading rate and that the time required for the complete biomass flotation was shorter at higher loading rate. These researchers also found that the onset of flotation occurred at concentrations far below the LCFA toxicity limit, which might suggest that complete sludge bed wash-out is likely to be encountered prior to inhibition of methanogens. These results suggest that is possible that the process fails due to fluids dynamics problems before a complete inhibition of the microbial activities. In this way, Hwu et al. (1998b) demonstrated that the typical hydrodynamic parameters of EGSB reactors, a high superficial velocity (> 4 m/h) and short HRT (< 10h), are unfavorable for the anaerobic treatment of C18:1-based effluent, producing a severe wash out of sludge granules. Reactor operation with lower superficial velocity or longer HRT, and the addition of co-substrate (as glucose) was recommended for a successful anaerobic treatment (82 – 89% of COD removal efficiency).

2.4.2. LCFA toxicity

The inhibitory effect of LCFA was often studied in batch experiments conducted with single fatty acids or a mixture of LCFA. Research performed up to date concluded that LCFA inhibit the activity of both acetogenic and methanogenic activity, even at low concentration. However, the acetoclastic population is demonstrated more sensitive to the LCFA inhibition than the acetogenic and the hydrogenotrophic consortia (Koster and Cramer, 1987; Alves et al., 2001; Lalman and Bagley, 2001; 2000; 2002). As previously explained, the mechanism of LCFA inhibition has been usually attributed to their adsorption onto the cell membrane. However, only few studies have evaluated the importance of the adsorption process during the LCFA degradation (Hwu et al., 1998a; Pereira et al., 2005). Recently, Palatsi et al. (2012) and Zonta et al., (2013) gave a new

insight in the LCFA inhibition process, evidencing the clear impact of the physical phenomenon on the process inhibition.

The exact effect of the LCFA adsorption over the microbial cell was widely discussed. Initially, it was suggested that the adsorption process may alter the permeability, the protective functions (Demeyer and Henderickx, 1967; Galbraith and Miller, 1973a) and the energy metabolism (Galbraith and Miller, 1973b) of the cell membrane, causing irreversible changes that lead to cell damage and lysis. The toxic effect was, as consequence, reported to be a permanent phenomenon (Angelidaki and Ahring, 1992; Rinzema et al., 1994). These latters found that the acetoclatic methanogens did not adapt to LCFA neither upon repeated exposure to toxic concentration, nor after prolonged exposure to non-toxic concentration. These authors hypothesized that the system recovery was due to the growth of the minor quantity of the surviving microorganisms (at most 0.2%). More recent studies performed by Pereira et al. (2002a; 2003, 2004) have demonstrated that the permanent toxicity was incorrectly diagnosed. Results revealed that the anaerobic consortium remained active, even after extended contact with LCFA, and was able, after a lag phase, to efficiently methanise the accumulated LCFA. Pereira et al. (2004) proved the ability of the "floating" sludge to mineralize biomass-associated LCFA up to specific content of $5mg_{COD}$ VSS^{-1} and the enhancement of the sludge activity after its mineralization. In this way, Alves et al. (2001a) performed a gradual replacement of co-substrate by oleic acid in a continuous fixed bed reactor in order to prove the increase of the tolerance of acetoclastic bacteria to oleic acid toxicity. The reversibility of the inhibitory effect was related to a physical transport limitation effect of the adsorption process (Pereira et al., 2005). Recently, the inhibitory effect of LCFA has been also associated with the interference of the electron transport chain, impairment of the nutrient uptake and with the generation of toxic peroxidation and auto-oxidation product (Desbois and Smith, 2010).

Table 2.3 displays a literature review of the inhibitory concentrations (IC_{50}) exerted by various saturated and unsaturated long chain fatty acids over acetoclastic methanogens. It could be noted that the severity of LCFA toxicity on methanogenic consortia depend mainly on the sludge origin, the sludge surface area, the type of microorganisms, the carbon length and the saturation degree of LCFA:

- Sludge type: suspended and flocculent sludge, which have higher specific surface area, suffer much greater inhibition than did granular sludge (Hwu et al., 1996; Hwu and Lettinga, 1997; Pereira et al., 2002a).
- Sludge origin: the pre-exposure of the anaerobic biomass to fatty substrate (Alves et al., 2001a; Palatsi et al., 2010) and the use of acclimated biomass enhance the tolerance level of the microbial population toward LCFA (Pereira et al., 2001).
- The type of microorganisms: the acetoclastic methanogens are more sensitive to the LCFA inhibition (Lalaman and Bagley, 2000; 2001).
- The carbon length and the saturation degree of LCFA: unsaturated LCFA are generally considered more inhibitory than saturated LCFA (Lalman and Bagley, 2001; Shin et al., 2003, Kim et al., 2004). It could be related to an increase in the LCFA surface area by the multiple carbons bound, which in turn result in a greater surface being adsorbed to the microbial membrane cell. The accumulation of C16:0 in anaerobic systems can be explained by its low solubility. Novak and Kraus (1973) reported that the solubility property of some LCFA limits their degradation, as in the case of C18:0 and C16:0 acids, concluding that all unsaturated fatty acids are degraded about five times more rapidly than saturated acids.

Based in all these findings, Zonta et al., (2013) developed two new LCFA-inhibition models, state-compatible and easy to be integrated into the full ADM1 framework. New kinetics were considered to describe the bio-physics of the inhibitory process, including: i) adsorption of LCFA over granular biomass, ii) specific LCFA substrate (saturated/unsaturated) and LCFA degrading populations, and iii) a new variable that accounts directly for the damage of cell functionality.

Table 2.3. *Review of IC_{50} inhibitory concentration (mgCOD L-1) of the most common unsaturated and saturated LCFAs.*

	Reference	Sludge Type	Sludge Origin	Tº range	IC_{50} (mg_{COD} L^{-1})
Linoleic acid	Kim et al. (2004)	Granular	UASB/dairy wastewater	35º C	575
(C18:2)	Shin et al. (2003)	Granular	UASB/LCFA-glucose mixture	35º C	550 – 620
	Palatsi et al. (2012)	Granular	UASB/beer brewery	35º C	1500
	Kim et al. (2004)	Granular	UASB/dairy wastewater	35º C	2722
	Shin et al. (2003)	Granular	UASB/LCFA-glucose mixture	35º C	2700 - 2850
Oleic acid	Pereira et al. (2002a)	Granular	UASB/Brewery effluent	37º C	997 ± 75
(C18:1)	Hwu and Lettinga (1997)	Granular	UASB/potato processing	30º C	2064
	Hwu and Lettinga (1997)	Granular	UASB/potato processing	40º C	1994
	Koster and Kramer (1987)	Granular	UASB/potato processing	30º C	3820
	Hwu and Lettinga (1997)	Floculent	UASB/sucrose and VFA mixture	40º C	465
	Pereira et al. (2005)	Suspended	Municipal sludge digester	37º C	202 ± 29
	Pereira et al. (2002a)	Suspended	Laboratory digester/oleate	37º C	383 ± 46
	Cavaleiro et al. (2001)	Suspended	AF/Skim milk and oleate	37º C	58 – 621
	Alves et al. (2001b)	Suspended	AF/Skim milk and oleate	37º C	246 – 397
Stearic acid	Kim et al. (2004)	Granular	UASB/dairy wastewater	35º C	4782
(C18:0)	Shin et al. (2003)	Granular	UASB/LCFA-glucose mixture	35º C	3800 – 4480
Palmitic acid	Kim et al. (2004)	Granular	UASB/dairy wastewater	35º C	4530
(C16:0)	Shin et al. (2003)	Granular	UASB/LCFA-glucose mixture	35º C	3890 – 4400
	Pereira et al. (2005)	Suspended	Municipal sludge digester	37º C	3157 ± 143

2.5. NEW TECHNICAL APPROACHES FOR LIPIDS AND LCFA-RICH WASTE TREATMENT

The new reported findings about the lipids toxicity and degradation pathways have opened new research axed in technological development and treatment alternatives. Several researches have been focused in the identification and the study of the different operational concerns whereas other ones have attempted new alternatives and methodologies to prevent inhibition and to optimize the overall process. In the following section the main developed strategies are analyzed.

2.5.1. Improvement of lipids/LCFA bio-availability

As it is previously discussed, the values of the first-order constant of lipid hydrolysis present a large variation because it is highly dependent on the type (triglycerides, phospholipids or sterols), the physical state (solid or liquid), the particle size and the specific surface area of lipids-biomass. In the case of complex fatty waste, where high amounts of suspended solids are present, the liquefaction and the hydrolysis can be considered as the rate limiting step of the whole anaerobic process (Sayed et al., 1988, Vavilin et al., 2008). Several pretreatment techniques have been applied for the treatment of slaughterhouse, dairy and other food industry wastewaters in order to cope with their low bio-availability (Masse et al., 2001; 2003; Battimelli et al., 2009; 2010; Valladão et al., 2011; Leal et al., 2006; Mendes et al., 2006; Damasceno et al., 2008; Jeganathan et al., 2007; Cirne et al., 2006b).

Among these, the bio-enzymatic treatment that consists on the utilization of microorganisms and/or enzyme pools has gained great attention during the last years. Generally, low cost commercial lipases (Cirne et al., 2007; Jaganathan et al., 2007), preparations obtained from porcine pancreas (Masse et al., 2001; Masse et al., 2003, Mendes et al., 2006) or from the solid-state fermentation of the fungus *Penicillium restrictum* (Leal et al., 2006; Damasceno et al., 2008; Rosa et al., 2009; Valladão et al., 2011) are the most used. The possibility of bio-augmentation by the addition of anaerobic lypolytic bacteria (*Clostridium lundense*, DSM 17049^{T}) has been also investigated (Cirne et al., 2006b). Cammarota and Freire (2006) also reported the use of a "bio-additive" (that consists on various aerobic microorganisms; *i.e. Aerobacter aerogenes*, *Bacillus subtilis*, *Cellulomonas biazotea*, *Nitrobacter winogradskyi*) to deal with high fat content in wastewater. Mendes et al. (2005) recommended the use of a

mixture composed by emulsifiers, microorganisms (*Bacillus subtilis and Aspergillus niger*) and enzymes (protease, amylase, lipase, cellulose and pectinase) for enhancing the lipid removal from wastewaters. However, the effect of the bio-augmentation by aerobic microorganism is limited by the pretreatment requirement of oxygen supply and, thus, the increase of the cost of the process.

Alkaline pre-treatment (Masse et al., 2001) and saponification (Mouneimne et al., 2003; Battimelli et al., 2009; 2010) were also tested with the purpose to reduce the size of fat particles and to improve the emulsification of slaughterhouse wastes and wastewaters.

All these pretreatment strategies demonstrated an enhancement in the hydrolysis step, in the initial degradation rate and, accordingly, a reduction in the digestion time. However, these advantages on the overall degradation process could be limited due to the subsequent higher accumulation of LCFAs, causing potential process inhibition and failure (Cirne et al., 2006; Rosa et al., 2009; Battimelli et al., 2010).

2.5.2. Adsorbents and co-substrate addition.

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. Adsorbents as bentonite, a clay mineral known by its high bonding capacity, can outcompete with biomass in terms of sorptive capacity towards LCFA and, thus, its addition as a synthetic adsorbent in anaerobic digesters might influence the dynamics of LCFA-adsorption inhibition process (Palatsi et al., 2012). Indeed, Beccari et al. (1999) have verified the positive effect of bentonite addition (up to 15 g/l) when anaerobically treating olive mill effluents, both scarcely and strongly diluted. More recently, Mouneimne et al. (2004) proved that the use of bentonite, with a grease/bentonite ratio of 0.9, clearly enhanced the bioavailability and the biodegradation of domestic fatty residues treated in mesophilic continuously stirred tank reactor. Palatsi et al. (2009) also found that the addition of "natural adsorbents" like biofibers was a reliable recovery alternative for LCFA inhibited systems, reducing significantly the recovery time from 10 to 2 days. Digested fibers addition as a competitive tool, was also effective to reduce the oleate inhibition in anaerobic reactors digesting manure (Mladenovska et al., 2003; Nielson and Ahring, 2006).

The effectiveness of other co-substrates addition, like carbohydrates, amino acids and proteins, on the reduction of the LCFA inhibitory effect was also reported by several

researches (Hwu et al., 1998b; Kuang et al., 2002 and 2006). It was proven, at laboratory scale, that adding glucose or amino acid (cysteine), either singly or in combination, could be an alternative to decrease the toxicity of oleic acid in anaerobic conditions. This positive effect was attributed to the growth improvement of archaeal and bacterial cells and granular biomass formation. The presence of glucose could improve the growth of methanogens, acidogens and acetogens, while the addition of cysteine increased the number of bacteria cells (Kuang et al., 2006).

Based on these findings, co-digestion process could be also a promising method to enhance the anaerobic degradability of lipids.

2.5.3. Biomass adaptation

The improvement of the specific methanogenic activity after the batch depletion of the "floating" biomass-associated LCFA, reported by Pereira et al. (2004; 2005) suggests that the application of biomass acclimatization step can lead to an efficient treatment of LCFA-rich wastes. Consequently, several studies have recommended the use of a biomass previously exposed to LCFA-rich substrate to enhance the system performances and the resistance to shocks loads (Alves et al., 2001b; Pereira et al., 2001; Cavaleiro et al., 2008). The improved performances of reactors associated with the use of acclimated biomass suggest the application of a start-up step based on sequential feedings prior to continuous treatment of lipid-rich substrate. The application of repeated pulses feeding as a start-up strategy was widely investigated (Nielsen and Ahring, 2006; Cavaleiro et al., 2008; Palatsi et al., 2009). The positive effect is attributed to an increase in the tolerance level of both β-oxidizing bacteria and syntrophic methanogens toward LCFA (Nielsen and Ahring, 2006; Sousa et al., 2007b; Palatsi et al., 2010) and to a stimulating effect on the overall process (Nielsen and Ahring, 2006; Cavaleiro et al., 2008). Applying this strategy, Cavaleiro et al. (2009) were able to achieve a continuous treatment of lipids of up to 21 kg_{COD} m^{-3} day^{-1} with 99% of COD removal efficiency and 72% of methane yield. More recently, Cavaleiro et al. (2010) reported that the pulsed or discontinuous start-up step requires long reaction time and proposed the bio-augmentation of non-acclimated sludge with β-oxidizer bacteria, *Syntrophomonas zehnderi*, as a strategy to accelerate the reactor start-up phase and to promote the recovery of the inhibited process. The addition of this syntrophic bacteria, known by its ability to degrade both saturated and unsaturated fatty acids with

4-18 carbon atoms (Sousa et al., 2007a), resulted in a faster and higher oleate conversion to methane, reaching 89 ±5%.

2.5.4. New technological developments

Several attempts of conventional technology modification were developed with the aim to adapt them to the anaerobic treatment of high-fat-containing wastewaters.

The integration of a re-circulation unit to the anaerobic reactor was applied by many researches in order to recuperate the sludge-associated LCFA washed-out from the system (Hwu et al., 1997; Pereira et al., 2001; Erdirencelebi, 2011). This technique promoted the overall system stability by minimizing the biomass was-out and allowing larger accumulation and longer retention of LCFA-degraders in the reactor, giving rise to a lower inhibition. A periodic re-seeding of anaerobic reactors that suffered an important wash-out was also recommended (Hwu et al., 1998b; Jeganathan et al., 2006).

Some researcher also proposed two-phase system, composed of an acidogenic CSTR and a methanogenic UASB reactor (Kim et al., 2004b; Kim and Shin, 2010). Only partial saturation of the unsaturated LCFA is applied in the first phase of acidogenesis in order to allow the syntrophic cooporation of β-oxidizers with methanogens, necessary for the complete anaerobic degradation of LCFA, in the second step. An enhanced treatment performance, relatively to the single phase system, was found in the OLR threshold of 1.6 $_{kgCOD\text{-}LCFA}$ m^{-3} day^{-1} (Kim et al., 2004b). However, a high LCFA load resulted in a decrease in the methane yield (Kim et al., 2004b), associated with a longer phase time of methane production (Kim and Shin, 2010). Erdirencelebi (2011) proposed the use of three-stage configuration, consisting on a pre-acidification tank and two sequential UASB reactors in order to compensate the reduced performance of the first stage.

Other researches focused on developing novel reactor designs suitable for the treatment of lipid-rich wastes. Haridas et al. (2005) proposed the *Buoyant Filter Bioreactor* (BFBR) for the anaerobic treatment of lipid-rich complex wastewater. This system is able to decouple the biomass and insoluble COD retention time from the hydraulic retention time by means of a granular filter bed made of "buoyant polystyrene beads". Filter clogging is prevented by an automatic backwash driven by biogas release, which fluidizes the granular filter bed in a downward direction. This novel reactor design was tested at lab scale during 400 days using not pretreated dairy effluent. A higher COD removal efficiency in excess of 85% was achieved at a volumetric OLR of 10 Kg_{COD} m^{-}

^{3} day^{-1} (Haridas et al., 2005). Alves et al. (2007) developed a novel anaerobic reactor, the *Inverted Anaerobic Sludge Blanket* (IASB), for the removal of LCFA from fat-containing wastewater. Contrary to conventional anaerobic reactors, it avoids the need of good settling properties of sludge and exploits the problem of sludge flotation due to LCFA or biogas adsorption onto the sludge. Furthermore, it provides an increased specific sludge surface area for better LCFA degradation. It is fed from the top and is equipped with a separation step at the bottom. Reactor contents are thoroughly mixed by the novel combined action of a gas lift loop and a liquid recycle over the reactor. The reactor can be operated in continuous and sequential mode. Although it is specifically designed for lipid degradation, its application is not limited to this (Alves et al., 2007).

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Chapter 3. Response of the anaerobic digestion process to punctual and continuous loading of lipids and long chain fatty acids: The inhibition process and the use of bentonite as protective strategy

A comprehensive picture on the operational problems that could be associated with the anaerobic digestion of lipid-rich wastes was provided in the present study by submitting two CSTR reactors (HRT = 30 days, OLR= 1 g_{COD} L^{-1} day^{-1}) to punctual pulses of glycerol trioleate (lipid model, in R_{Lipid}) and its free fatty acid, oleate (LCFA model, in R_{LCFA}). A general disruption of the process stability was observed after the addition of the fatty substrate. Flotation and wash-out of the organic matter and inhibition of the microbial populations were identified as the main limiting aspects of lipids treatment. The flotation was proved to be a rapid process that occurred even before the inhibition process. In spite of the inhibition, the methanogenic activity was recovered along subsequent substrate contact, also confirmed by batch SMA tests. The incubation of the fatty substrate with 20% (w/w) of bentonite prior to its injection into the reactor was tested as alternative strategy to overcome the above mentioned problems. This protective strategy partly overcome the inhibitory effect of lipids pulses (in R_{Lipid}), by preventing the methane production decrease and the VFA accumulation in the system. In R_{LCFA}, the positive effect of bentonite addition was exceptionally noted when the oleic acid was added in an organic load of about 0.3 ± 0.009$g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}. However it was not very reliable when the reactor was subjected to increasing continuous loading of oleic acid. It could be due to the relatively low bentonite/substrate ratio (20%) which was probably insufficient to capture all or at least the majority of the added substrate.

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3.1. INTRODUCTION

The versatility of anaerobic digestion as an effective technology that combines the waste treatment and renewable energy production (in the form of biogas) has been extensively documented over the past decades. The energy yield of this process depends on the organic matter composition and it is especially elevated for high strength-lipid waste (Pereira et al., 2003). However, a wide assortment of operational challenges due to the application of uncontrolled lipids loads have been deeply reported (Palatsi et al., 2012; Pereira et al., 2003; Lalman and Bagley, 2001; Hwu et al., 1998b; Hwu et al., 1997; Koster and Cramer, 1987).

Under anaerobic conditions, lipids are hydrolyzed by extracellular lipases to long chain fatty acids (LCFA) and glycerol. Glycerol is further converted to short chain fatty acids (VFA) and alcohols. The LCFA are subsequently degraded via the β-oxidation mechanism to acetate (Ac) and hydrogen (H_2), which are finally converted to methane (CH_4) and carbon dioxide (CO_2), constituting the biogas mixture (Weng and Jeris, 1976). Angelidaki and Ahring (1992) initially reported a permanent toxic effect exerted by the accumulation of LCFA, resulting in cell membrane damage of both syntrophic acetogens and methanogens. Further studies have later contradicted this theory and demonstrated that LCFA inhibition is a reversible mechanism in which microorganisms, after a lag phase, are able to efficiently methanize the accumulated LCFA (Pereira et al., 2004). The adsorption of LCFAs onto the microbial cell surface is described as a pre-requisite for their biological degradation (Hwu et al., 1998a; Palatsi et al., 2012), and the mechanism of LCFA inhibition was ascribed to this adsorptive process, affecting the external nutrients transport and the protective functions of the microbial cells membrane (Zonta et al., 2012; Pereira et al., 2005). The degree of inhibition depends mainly on LCFA concentration, the specific surface area of the biomass, the carbon chain length and the saturation degree of LCFA (Koster and Cramer, 1987; Lalman and Bagley, 2001; Pereira et al., 2002; Palatsi et al., 2012).

Also, multiple studies conducted in anaerobic bioreactors, including upflow anaerobic sludge blanked (UASB) reactor (Koster and Cramet, 1987; Rinzema et al., 1993), expanded granular sludge bed (EGSB) reactor (Hwu et al., 1998b; Pereira et al., 2002) or anaerobic filter (Alves et al., 2001), experimented sludge flotation as a consequence

of LCFA adsorption to biomass (Hwu et al., 1998b). Flotation would definitively lead to sludge wash-out (Hwu et al., 1997) and in some cases to process failure (Hwu et al., 1998b). Sludge flotation depends more on the LCFA organic loading rate rather than on their concentration and it is likely to occur prior the inhibition of methanogens (Hwu et al., 1998a).

From all this reported experiences, it is evident that the key factor for a successful treatment of fatty substrates is to control the LCFA adsorption process. Although many researchers have studied the LCFA inhibition process in batch and continuous conditions, the exact behavior of anaerobic bioreactors submitted to inhibitory concentration of fatty substrate are not well known. The purpose of this work is to investigate the behavior of a completely stirred tank reactor (CSTR) submitted to increasing amounts of lipids or LCFA (C18:1), and to evaluate the effect of additives addition (bentonite) as a strategy to overcome the operational problems caused by the accumulation of LCFAs in the system and, thus, to improve the knowledge about the anaerobic digestion process of fatty residues.

3.2. MATERIALS AND METHODS

3.2.1. Substrate

Synthetic skim milk solution (SM) was used as basis substrate in the present study. It was prepared by diluting powder skim milk (Nestlé Sveltesse, La Lechera, Spain) with distilled water until a total chemical oxygen demand (COD) concentration of 30 $g_{COD}L^{-1}$. This substrate was buffered with sodium bicarbonate (3 g_{NaHCO3} L^{-1}) and periodically supplemented with macro- and micronutrients solutions following the composition described elsewhere (Ferrer et al., 2010).

Glycerol trioleate (Sigma-Aldrich; 65% of purity, USA) and sodium oleate powder salt (Riedel-de Haën/Sigma-Aldrich; 82% of purity) were selected as lipidic and LCFA substrate models, respectively. Triolein is a symmetrical triglyceride derived from glycerol bonded to three units of oleic acid, which is naturally present in oils and solid vegetable fats. Oleic acid, known by its high solubility, is the most abundant unsaturated LCFA present in fatty wastes (Valladão et al., 2011). Analytical grade powder bentonite ($Al_2O_34SiO_2H2O$, Sigma Aldrich) was used as the synthetic adsorbent.

3.2.1. Experimental set-up

The experiment of the current study was conducted in two laboratory scale continuously stirred-tank reactors (CSTR) with a working volume of 4.7 L. The reactors were inoculated with suspended biomass (22.5 g_{TS} L^{-1}) sampled from a mesophilic anaerobic digester of a municipal wastewater treatment plant located at La Llagosta (Barcelona, Spain). This biomass was previously maintained in an incubation chamber (35ºC) during 3 days in order to degrade the residual COD. Reactors were operated at mesophilic range (35ºC ±2) and under intermittent mixing conditions (150 rpm, 1 min on/4 min off). A start-up period was firstly monitored, in which both reactors were fed with the basis substrate (SM) thrice daily (156 ml per day), with 8-h intervals, using temporized peristaltic pump, resulting in an organic loading rate (OLR) of 1 g_{COD} $L_{reactor}^{-1}$ day^{-1}. Afterwards, the behavior of CSTR exposed to inhibitory concentrations of lipids and LCFA was investigated during Period I. The first reactor, named R_{Lipid}, was subjected to pulses of glycerol trioleate (GT) while the second one, identified as R_{LCFA}, received pulses of sodium oleate (C18:1). Period I lasted 203 days during which increasing concentration of substrates pulses were directly injected into the reactors. Tables 3.1 and 3.2 summarize the overall operating conditions of the different experimental steps for R_{Lipid} and R_{LCFA}, respectively. The purpose of the second stage of the experiment (Period II) was to evaluate the addition of bentonite as a strategy to overcome the different problems encountered in Period I. The fatty substrate (GT or C18:1) was captured by its incubation with bentonite (during 2 days under shaking) prior to its injection into reactors, in order to force its exclusively adsorption over bentonite. The quantity of the added bentonite represented 20% (w/w) of the fatty substrate weight. For R_{Lipid}, Period II lasted 85 days and comprised two additional pulses of a GT-bentonite mixture (1.58 g_{COD} L^{-1} and 3.05 g_{COD} L^{-1}). In R_{LCFA}, a continuous addition of oleate, previously mixed with 20% (w/w) of bentonite, was applied. The reactor was operated at the same conditions of the previous periods (HRT = 30 days, fed thrice daily with SM at OLR ≈ 1 g_{COD} L^{-1} day^{-1}), while the C18:1-bentonite mixture was manually injected once a day. Period II lasted 399 days in which three organic loads of C18:1- bentonite mixture (0.30, 0.58 and 0.70 $g_{COD\text{-}C18:1}$ L^{-1}) were daily applied in four different intervals, as detailed in Table 3.2. The continuous injection of the C18:1-bentonite mixture was interrupted three times, as shown in Table 3.2, to prevent the system failure.

The performance of the reactors was monitored by measuring the influent and effluent characteristics and biogas production. The total COD (CODt) of the influent was determined weekly while the effluent was characterized twice a week in terms of total and soluble COD (CODt, CODs). Volatile fatty acids (VFA), total Kjeldahl Nitrogen (TKN) and alkalinity were also determined in outflow samples. Samples were also collected from R_{Lipid} after each pulse or continuous addition of oleate for LCFA quantification. The biogas production, reactor temperature and pH were monitored daily, while biogas composition was determined three times per week by gas chromatography. The biogas production was measured in both reactors with an automated gas metering system, based on Mariotte flask (Veiga et al., 1990). A volumetric milligas counter (MGC-1 V3.1, Ritter ®, Germany) was also used in R_{Lipid} during the first 85 days of the experiment.

3.2.3. Specific methanogenic activity (SMA) assays

The SMA of reactor biomass, sampled at different operating time, was tested in triplicate in batch assays. A total of 5 SMA tests were performed with biomass samples from R_{LCFA}: 2 samples were collected in Period I at day 49 (1^{st} pulse) and day 86 (2^{nd} pulse), while 3 were withdrawn in Period II, during the continuous oleate addition at days 344, 393 and 645, respectively. The SMA of the biomass sampled from R_{Lipid} was assessed on day 32 (1^{st} pulse) and day 69 (2^{nd} pulse) of Period I.

The SMA was done in glass vials (120 mL), filled until a total volume of 50 mL, with 2.5 g_{VSS} L^{-1} of inoculum and using acetate (20 mM) and hydrogen (H_2/CO_2= 80/20 v/v) as substrate. Macro/micronutrients solution and 1 g_{NaHCO3} $g^{-1}_{CODadded}$ were added, as suggested by Soto et al. (1993) and Angelidaki et al. (2009). Strict anaerobic conditions were maintained by bubbling vials with a N_2/CO_2 gas mixture (80/20 v/v) and sealing them with rubber stoppers and aluminium crimps bungs. The substrates were then added and vials were incubated at 35ºC under continuous shaking (120 rpm). Control vials, without substrate addition were also performed to determine the residual CH_4 production of the inocula. The headspace of the vials was sampled periodically to monitor the CH_4 production by gas chromatography. SMA values were calculated as the initial slope of the net accumulated CH_4 production curve divided by the VSS content of each vial and expressed as $mg_{COD\text{-}CH4}$ g_{VSS}^{-1} day^{-1}.

3.2.4. Routine analysis

Total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), total and soluble chemical oxygen demand (CODt, CODs), total Kjeldahl Nitrogen (TKN) and pH were determined according to standard methods (APHA, 1995). Total and partial alkalinities (TA, PA) were analyzed following the protocol of Hill and Jenkins (1989). Volatile fatty acids (VFA) composition, i.e. acetic (Ac), propionic (Pro), iso-butyric and n-butyric (Bu), iso-valeric and n-valeric (Va) and caproic (Cp) acids were identified and quantified by a CP-3800 gas chromatograph (Varian, USA) fitted with a Tecknokroma TRB-FFAP capillary column (30 m × 0.32 mm × 0.25 μm) and FID detection (Campos et al., 2008). Saturated and unsaturated LCFA, from C12 to C24, were determined as fatty acids methyl esters (FAME) following the method described by Palatsi et al. (2009) based on direct methylation extraction procedure, using CP-3800 gas chromatograph (Varian, USA), fitted with CP7489: CP-Sil 88 FAME capillary column (50m × 0.25mm ×0.2μm) and FID detection. All determinations, except that of TA and PA, were done in duplicate.

Methane and carbon dioxide content in the biogas was determined by a CP-3800 gas chromatograph (Varian, USA) fitted with Haysep-Q 80-100 Mesh (2mx1/8" × 2.0mm) column (Varian, USA) and thermal conductivity detector (TCD). Helium was used as carrier gas (45 ml min^{-1}) and the temperature of the injection port, oven and detector were 50, 150 and 180ºC, respectively. Hydrogen content in the biogas was also analyzed by gas chromatography using a GC CP-3800 gas chromatograph (Varian, USA) fitted with Molecular Sieve SA 80-100 Mesh (2mx1/8" × 2.0mm) packed column (Varian, USA) and thermal conductivity detector (TCD), using Argon as carrier gas (45 ml min^{-1}).

3.3. RESULTS AND DISCUSSION

The operational conditions (Tº, pH, HRT, OLR) applied to R_{Lipid} and the efficiencies of the anaerobic process (average biogas and CH4 production, methane yields, VFA profile) were summarized in Figure 3.1 and Table 3.1. The whole performance data of R_{LCFA} (CH_4 production, LCFA uptake and VFA profile) were depicted in Figure 3.2, while the operational conditions and mean values of several process parameters were presented in Table 3.2.

3.3.1. Effect of punctual pulses on the process stability

The start-up of the experiment lasted 32 days and 49 days for R_{Lipid} and R_{LCFA}, respectively. Throughout this period, the two CSTRs reactors were operated under identical conditions (T°, HRT, OLR) and fed only with the SM solution. A significant fluctuation in CH_4 production was observed in both reactors at the beginning of the experiment, as shown in Figure 4.1 and Figure 4.2. This fluctuation could be related to the degradation of the residual COD, since the inoculum was kept during only 3 days in an incubation chamber (35ºC) and probably residual biomass was not completely removed. Far ahead, the COD removal efficiency was gradually improved and a steady state performance was consequently obtained in both reactors, as indicated by low VFA level and a stable CH_4 production. The methane production was about 305 ± 121 and 302 ± 51 L_{CH4} kg_{COD}^{-1} in R_{Lipid} and R_{LCFA} respectively, corresponding to a COD removal efficiency of 86%.

Period I with punctual pulses of GT in R_{Lipid} and its free fatty acid C18:1 in R_{LCFA} was then performed with the aim to evaluate operational problems of the anaerobic digestion of fatty substrate. In general, the addition of the fatty substrate disrupted the process stability of both reactors.

R_{Lipid} received a total of 5 pulses of GT with a concentration fluctuating between 2.53 and 14.45 g_{COD} L^{-1}. Although the addition of GT induced a gradual decrease in the methane production efficiency (Table 3.1), however differences between pulses were noted in the profile of the methane production (Figure 3.1). The first two pulses (almost the same concentration) resulted in an instantaneous drop in the methane production followed by a steep increase in VFA concentration, mainly acetate and propionate. Then, the CH_4 production increased progressively as well as the VFA were metabolized (P_1, P_2 in Figure 3.1). The acetate peak was lower in the second pulse (as was for the other acids) probably due to the adaptation of acetogenic and methanogenic microbial populations to the new substrate. This hypothesis will be discussed later, considering the results of SMA tests.

Table 3.1 *Operating conditions and performance data of R_{Lipid} reactor*

	time	**T°**	**pH**	**HRT**	**OLR**	**Lipidic Pulse**	**CH_4 production**	**CH_4 content**	**COD degradation**
	days	°C	-	days	$g_{COD}\ L_{reactor}^{-1}\ d^{-1}$	$g_{COD}\ L^{-1}$	$L_{CH4}\ Kg_{COD}^{-1}$	%	%
Start up	0 – 32	35.8 ± 0.3	7.8 ± 0.2	31.2 ± 3.9	0.97 ± 0.1	-	302 ± 51	70.4 ± 1.3	86.0
Period I: Punctual pulses of glycerol trioleate									
P_1	32 – 69	35.5 ± 0.3	7.8 ± 0.2	32.9 ± 4.6	0.91 ± 0.1	2.53	255 ± 65	68.7 ± 3.7	71.2
P_2	69 – 97	35.6 ± 0.5	7.7 ± 0.2	32.3 ± 4.5	0.97 ± 0.13	2.89	206 ± 88	72.8 ± 2.4	60.7
P_3	97 – 116	36.1 ± 0.1	7.8 ± 0.1	30.8 ± 2.8	0.96 ± 0.13	5.84	214 ± 100	73.3 ± 2.9	60.5
P_4	116 – 137	36.9 ± 0.3	7.8 ± 0.03	30.6 ± 2.5	1.02 ± 0.13	11.57	173 ± 91	70.7 ± 1.7	50.5
P_5	137 – 235	37.1 ± 0.2	7.9 ± 0.01	32.8 ± 3.6	0.96 ± 0.11	14.45	242 ± 91	70.0 ± 2.7	70.0
Period II: Punctual pulses of glycerol trioleate and bentonite									
P_6	235 – 258	37 ± 0.8	7.9 ± 0.05	33 ± 4.1	0.88 ± 0.11	1.58 *	264 ± 74	66.8 ± 2.5	70.4
P_7	258 – 320	36.8 ± 1.6	7.9 ± 0.1	32.1 ± 4.7	1.04 ± 0.31	3.05 *	288 ± 76	70.5 ± 1.9	76.4

* The quantity of bentonite represents 20% (w/w) of that of glycerol trioleate weight

Table 3.2 *Operating conditions and performance data of R_{LCFA} reactor*

	time	T°	pH	HRT	OLR of SM	C18:1 load	CH_4 production	CH_4 content	COD degradation
	days	° C	-	days	$g_{COD}\ L_{reactor}^{-1}\ d^{-1}$	$g_{COD}L^{-1}$ or $g_{COD}\ L^{-1}\ d^{-1}$ *	$L_{CH4}\ Kg_{COD}^{-1}$	%	%
Start up	0 – 49	36.8 ± 1.6	7.7 ± 0.3	31.1 ± 2	0.97 ± 0.09	-	305 ± 121	69.6 ± 2.6	85.8
Period I: Punctual pulses of oleate									
P_1	49 – 86	35.5 ± 0.3	7.9 ± 0.1	30.3 ± 4.6	0.97 ± 0.09	1.44	260 ± 85	69.4 ± 2.5	72.7
P_2	86 – 119	35.7 ± 0.6	7.7 ± 0.3	32.3 ± 2.6	1.03 ± 0.13	2.89	241 ± 190	67.5 ± 13	70.6
P_3	119 – 154	37.2 ± 0.9	7.7 ± 0.2	30.7 ± 2.5	1.02 ± 0.13	4.34	221 ± 161	68.8 ± 11	65.4
P_4	154 – 252	37.3 ± 0.6	n.d	31.7 ± 3.4	1.00 ± 0.08	5.79	217 ± 110	56.7 ± 9.8	62.9
Period II: Continuous addition of oleate and bentonite									
C_1	252 – 261	37.8 ± 0.3	n.d	29 ± 0.9	1.05 ± 0.07	0.70 ± 0.002 *	77 ± 45	41.1 ±. 4	21.9
SM feeding	261 – 338	37.3 ± 1.5	n.d	31.6 ± 3.5	1.02 ± 0.26	-	190 ± 143	63.7 ± 12.1	52.6
C_2	338 – 406	37.6 ± 1.2	7.6 ± 0.1	30.1 ± 3.3	1.08 ± 0.16	0.30 ± 0.009 *	176 ± 72	61.6 ± 7.9	52.7
C_3	406 – 491	35.2 ± 0.8	7.7 ± 0.1	31.0 ± 3.6	1.02 ± 0.16	0.58 ± 0.02 *	127 ± 31	56.7 ± 3.5	37.4
SM feeding	491 – 540	35.3 ± 0.5	n.d	34.6 ± 4.1	0.85 ± 0.14	-	457 ± 104	70.9 ± 5	127.2
C_4	540 – 567	35.1 ± 0.6	n.d	30.3 ± 4.5	1.03 ± 0.13	0.58 ± 0.002 *	85 ± 26	54.8 ± 8.7	24.1
SM feeding	567 - 651	35.3 ± 0.6	n.d	34.1 ± 3.1	0.86 ± 0.10	-	196 ± 31	55.6 ± 2.8	53.5

During period II, $OLR_{applied}$ represents the sum of the OLR of SM (skim milk) and OLR of C18:1
** The quantity of bentonite represents 20% (w/w) of C18:1 weight*

Conversely, an increase in the GT pulse concentration up to 14.45 g_{COD} L^{-1} (P_3, P_4 and P_5) induced an unexpected peak of methane production, mainly after the addition of P_5, as depicted in Figure 3.1a. Nevertheless, the methane production potential was very low (between 214 ± 100 and 242 ± 91 L_{CH4} Kg_{COD}^{-1}) relatively to the theoretical CH_4 potential and the corresponding COD removal efficiency, fluctuating between a minimum of 50.5% (during P_4, Table 3.1) and a maximum of 70% (during P_5, Table 3.1). In addition, acetate was accumulated in small amounts (<1,000 mg_{COD} L^{-1}), compared to the previous lower pulses, while propionate was accumulated only during the 5th pulse (Figure 1b). The other VFA intermediates (butyrate and valerate) were detected at low levels, not exceeding 300 mg_{COD} L^{-1}, as shown in Figure 1c. These results pointed to a possible substrate-biomass wash-out, instead of a system overloading or inhibition. In fact, onwards the 3rd pulse an oily layer floating on the top of R_{Lipid} was observed few hours after the pulse addition, which was then partially washed out from the reactor. The thickness of the floating layer was proportional to the pulses load as evidenced earlier by Hwu et al. (1998a). So, an important fraction of the pulse was lost by this way and the methane productivity corresponded to the remaining COD. These results proved that the flotation was a rapid process that occurred even before the inhibition process of the microbial consortia, which is consistent with the results reported by Hwu et al. (1998a). Biomass - adsorbed organic matter flotation and wash out were widely described as the main operational problem of the anaerobic treatment of fatty substrates, leading in some cases to eventual process failure (Rinzema et al., 1993, Hwu et al., 1997, Hwu et al., 1998a, Hwu et al., 1998b).

The second reactor, R_{LCFA}, was exposed to 4 pulses of C18:1 during Period I (Table 3.2). Figure 3.2a shows that the addition of C18:1 pulses caused an instant drop in the CH_4 production. The intensity of this decline was proportional to C18:1 concentration and was a consequence of LCFA degradation process: oleic acid was completely depleted few days after its injection in the reactor (Figure 3.2b) and mainly metabolized to palmitic acid (C16:0) and to stearic acid (C18:0) in lower extend (Figure 3.2c). No significant amounts of other LCFA metabolites were detected. Also, the metabolism of LCFA resulted in a transient accumulation of VFA in the medium, particularly acetate and propionate. This accumulation lasted until the complete degradation of palmitic acid (Figure 3.2d).

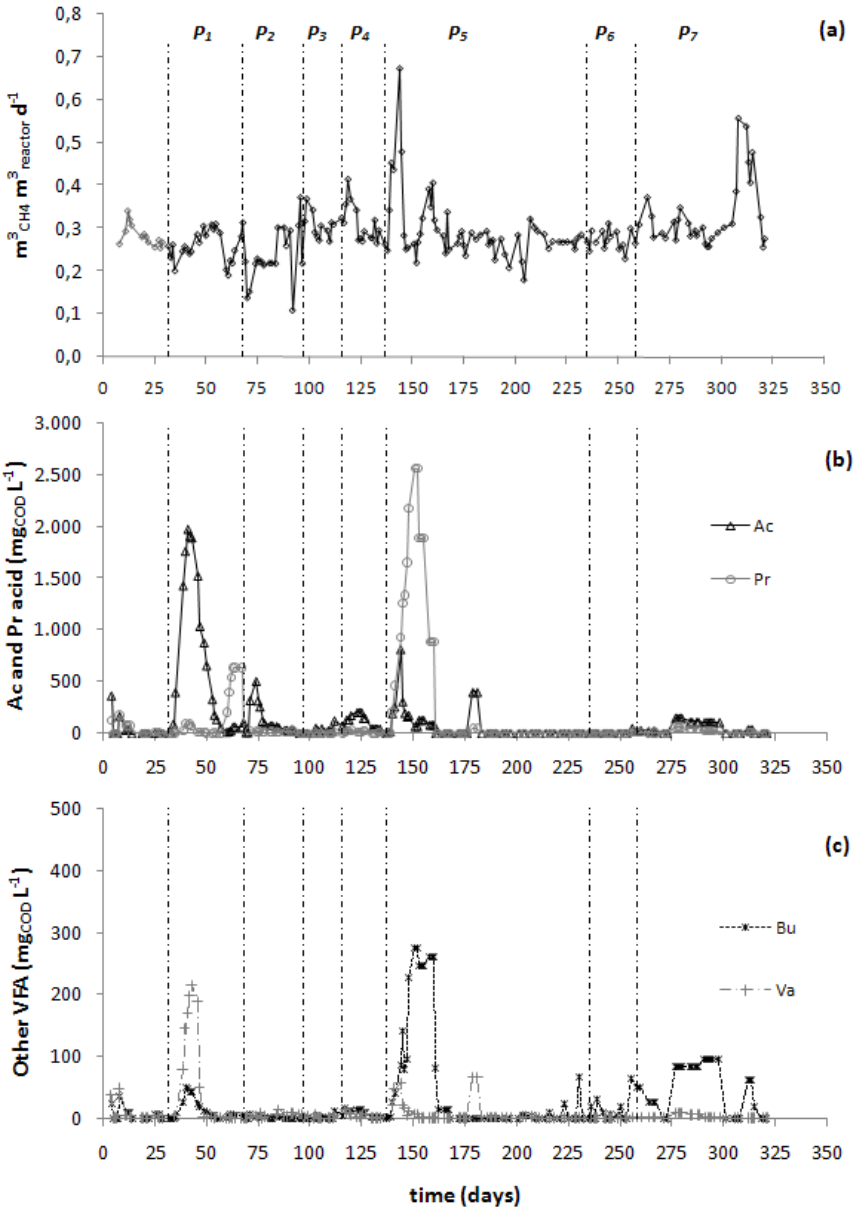

Figure 3.1 *Performances data of the R_{Lipid} reactor submitted to intermittent pulses of glycerol trioleate (GT), in terms of (a) methane production, expressed in $m^3_{CH4}\ ^{m\text{-}3}_{reactor}\ day^{-1}$ units, and (b) acetate, propionate and (c) other VFA intermediates (butyrate and valerate) profile expressed in COD equivalent concentration units ($mg_{COD}\ L^{-1}$). The vertical dashed lines indicate the timing of each pulse, characterized in Table 3.1.*

In spite of the produced inhibitory effect, the system was able to recover the activity, confirming the process reversibility (Pereira et al., 2002), and the CH_4 was increased concomitantly with the consumption of palmitate and VFA. The COD degradation efficiency was gradually decreased with the increase of oleic pulses concentration, as indicated in Table 3.2. Nevertheless, the pulse of 5.8 $g_{COD}\ L^{-1}$ (2 $g_{C18:1}\ L^{-1}$) severely

affected the system efficiency, obtaining a significant decline in all the process parameters (Table 3.2).

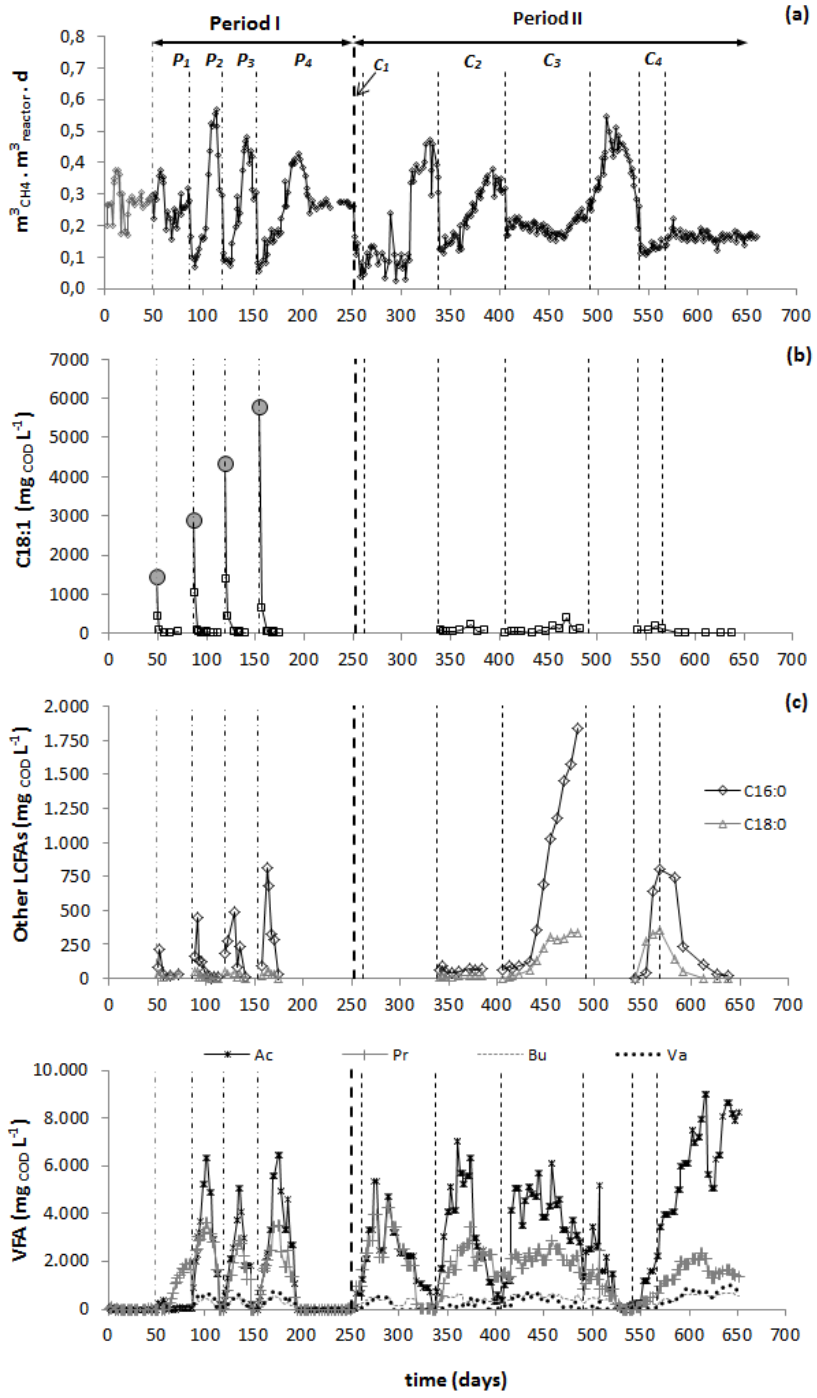

Figure 3.2 *Performances data of the R_{LCFA} reactor, in terms of (a) methane production, (b) oleic acid concentration, (c) other LCFA concentrations and (d) VFA intermediates profile during punctual pulses (period I) and continuous addition of C18:1 (period II). Methane productivity was expressed in m^3_{CH4} $m^{-3}_{reactor}$ $days^{-1}$ units and the other parameters were expressed in COD equivalent concentration units (mg_{COD} L^{-1}).The gray circles (in b) indicate*

the initial estimated concentration from the oleate pulses. The vertical dashed lines indicate the timing of each C18:1 addition, characterized in Table 3.2.

The same behavior of CH_4 production and VFA profile were also observed in the study of Nielson and Ahring (2006), when a CSTR reactor was submitted to pulses of C18:1 in the range of 0.5 – 2.0 $g_{C18:1}$ L^{-1}, reporting a severe inhibition following the pulse of 2.0 $g_{C18:1}$ L^{-1}. In contrast, Neves et al. (2009) revealed that controlled intermittent inputs of oil (effluent from canned fish processing industry), in the threshold of 12.0 g_{COD} L^{-1}, enhanced the methane production when co-digesting cow manure and food waste and, that only a pulse concentration of 18 g_{COD} L^{-1} promoted a persistent inhibition in the process. This higher tolerance, in comparison with the present study could be justified by the use of an acclimated inoculum (Pereira et al., 2001).

3.3.2. Effect of punctual pulses on the microbial activity

Besides the drawbacks in the process efficiency (inhibitory effect, flotation and wash out), pulses of GT and C18:1 had a stimulating effect on the microbial activities. Biomass samples withdrawn from both reactors just before the injection of the 1st and the 2nd pulses were characterized in terms of SMA in the presence of acetate and H_2/CO_2, and results were summarized in Table 3.3. At the end of the start-up period (day 49), the acetoclastic and the hydrogenotrophic activities of R_{LCFA} were 29.0 and 169.7 $mg_{COD\text{-}CH4}$ g_{VSS} day^{-1}, respectively. An improvement in both methanogenic activities was obtained following the first addition of oleate pulse (day 86, Table, 3), being 2.7 and 1.3 times higher for the acetoclastic and hydrogenotrophic activities, respectively. Likewise, the hydrogenotrophic activity of R_{Lipid} biomass exposed to the 1st pulse of trioleate (day 69) was 1.6 times higher than its activity at day 32.

Many studies have reported the beneficial effect of biomass exposure to fatty substrate on the microbial activity of acetogenic and methanogenic consortia (Nielson and Ahring, 2006; Cavaleiro et al., 2009; Neves et al, 2009; Palatsi et al., 2010). Recently, Cavaleiro et al. (2009) recommended a start-up period combining feeding phase and batch degradation phases in order to promote the development of an efficient anaerobic community for LCFA mineralization. However, contradictory results of the adaptation procedure were noted. Some authors found a high increase in the acetoclastic activity (Nielson and Ahring, 2006; Cavaleiro et al., 2009; the present study) while others reported the higher adaptation of the hydrogenotrophic population (Palatsi et al., 2010).

Difference in the initial composition of the microbial population of the inoculum could explain these different adaptation profiles.

Table 3.3 *Specific methanogenic activity ($mg_{COD\text{-}CH4}g_{VSS}$ day^{-1}) of biomass sampled from R_{Lipid} and R_{LCFA} at different operating time of the experiment.*

	Time (days)	Acetoclastic activity	Hydrogenotrophic activity
R_{Lipid}	32		
	69	n.d	88.9 ± 10.1
		16.2 ± 3.8	144.8 ± 8.8
R_{LCFA}			
Period I	49	29 ± 1.2	169.7 ± 3.6
	86	78.1 ± 1.3	225.2 ± 12.8
Period II	344	28.4 ± 6.6	109.1 ± 9.4
	393	64.5 ± 5.6	n.d
	645	37.9 ± 3.5	91.0 ± 7.7

n.d: not determined

3.3.3. Effects of bentonite addition

Lipid and LCFA adsorption onto the anaerobic biomass is the main factor determining its flotation and its biological toxicity. Therefore, the key for an efficient anaerobic digestion of lipid-rich wastes is to prevent or at least to minimize the adsorption process. A series of batch assays were performed, in parallel to the first stage of the current experiments and extensively detailed in the annexed information (Annex 2) and reported by Palatsi et al. (2012), in order to *i)* ensure the importance of the adsorption process during the LCFA degradation and *ii)* to test and to evaluate different effects of bentonite addition onto LCFA adsorption-inhibition process. The LCFA capturing, by incubation with bentonite forced the LCFA adsorption over bentonite, prior to vial inoculation with granular sludge, resulting as a reliable approach to improve the system robustness.

Based on these results, two additional pulses of GT previously incubated with 20% (w/w) of bentonite (2 days under shaking) were applied in R_{Lipid} (P_6 and P_7 in Figure 3.1 and Table 3.1). The protection of the biomass surface by the bentonite addition was found to partly overcome the inhibitory effect of GT pulses. As depicted in Figure 1 (in P_6 and P_7 section), neither the habitual methane production decrease nor the VFA accumulation were observed following the pulses injection. Moreover, the comparison

between the 7th pulse (P_7 = 3.05 g_{COD} L^{-1}) and the 2nd pulse (P_2 = 2.89 g_{COD} L^{-1}) revealed a significant improvement in the methane production efficiency (Table 3.1). Thus, it was decided to study the use of bentonite as a protective strategy in R_{LCFA} in continuous conditions.

This second stage of the experiment (period II) was started on day 252 in R_{LCFA} when C18:1-bentonite mixture was continuously added (manually once a day), together with the SM solution (with temporized pump thrice daily) and maintaining the same operating conditions as in period I. The applied OLR was modified as a consequence of the daily oleate addition (Table 3.2). The C18:1 was incubated with bentonite under shaking during 2 days prior to its injection into the reactor in order to force the exclusively LCFA adsorption over bentonite, as recommended by Palatsi et al. (2012). Relatively low bentonite/C18:1 ratio (20%, w/w) was used in the present experiment in comparison with that adapted by Palatsi et al. (2012) with the aim to avoid the risk of flocculent particles accumulation in the system. Firstly, C18:1 was added with an organic load of about 0.7 ± 0.002 $g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}, resulting in a total OLR (skim milk + C18:1) of 1.72 ± 0.11 g_{COD} $L_{reactor}^{-1}$ d^{-1}. The C18:1 addition lasted only 9 days (C_1, day 252 to day 261 in Figure 3.2) because all parameters of reactor efficiency were sharply dropped, recording the lowest values so far (Figure 3.2 and Table 3.2). Thus, the C18:1 injection was suppressed to prevent process failure, and the reactor was later fed with milk solution as sole carbon source during 77 days (261 – 338 day). As a result, accumulated VFA were gradually consumed and, concomitantly, the biogas production was recovered. LCFA quantification was not monitored during this step. Right after the stabilization of the CH_4 production, the oleate (bounded to bentonite) was again fed with skim milk solution in a moderate organic load (0.3 ± 0.009$g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}) during 68 days (C_2, day 338 – 406 in Figure 3.2). An immediate drop in the CH_4 production was registered simultaneously with VFA accumulation. However, VFA were rapidly biodegraded and the CH_4 production was gradually increased later. Interestingly, neither oleic acid nor palmitic acids were accumulated in the system during this period (C_2 in Figure 3.2). These results indicated that the continuous addition of C18:1 at an OLR of 0.3 ± 0.009$g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1} did not affect seriously the microbial consortia. The daily C18:1 load was thus doubled during period C_3 (0.58 ± 0.02 $g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}), resulting in a significant rise up of palmitic acid (> 1,750 mg_{COD} L^{-1}), stearic acid

and VFA in the system, associated with a remarkable fall in CH_4 production. The system suffered a severe inhibition and reactor performance parameters were not recovered throughout this stage that lasted 85 days (C_3 in Figure 3.1 and Table 3.1). The oleate addition was thus stopped and reactor was fed with SM solution as sole carbon source during 49 days until the recovery of reactor performance. A high COD degradation (127.2%), corresponding to CH_4 production of 457 ± 104 L_{CH4} Kg_{COD}^{-1}, was consequently achieved and related to the biodegradation of LCFA and VFA intermediates, accumulated during the previous C_3 stage. A final step of continuous C18:1-bentonite mixture addition was performed on day 540 applying the same organic load as the previous step (C_4 = 0.58 ± 0.002 $g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}). This step was interrupted after 27 days (on day 567) of reactor operation because the same behavior of system instability was detected. The system was not able to recover, and a persistent accumulation of VFA was observed, even after 84 days of feeding with SM as sole carbon.

The SMA test monitored during the Period II revealed a clear inhibition of both acetoclastic and hydrogenotrophic populations, which was correlated with LCFA accumulation in the system (SMA tests on day 344 and days 645, Table 3.3). Pereira et al. (2003) also reported a decrease of acetogenic, acetoclastic and hydrogenotrophic activities after an organic loading shock of LCFA. However this process was reversible and the methanogenic activity was improved after the complete degradation of LCFA (SMA test on day 393, Table 3.3). These results evidenced that the inhibitory effect is related with the adsorption of LCFA over the anaerobic biomass, which is in agreement with other studies (Palatsi et al., 2012; Hwu et al., 1998a). Zonta et al. (2013) developed a LCFA-inhibition mathematical model and explained how the adsorptive process can affect on the transport and protective functions of cell membranes, indicating the high sensitivity of acetoclastic population to LCFA. The SMA results of the present study also confirmed this conclusion. The beneficial effect of bentonite, used as a preventing or a protective strategy of the adsorption process, has been observed in different studies performed in batch (Palatsi et al., 2012; Mouneimne et al., 2004) and in continuous (Palatsi et al., 2009; Mouneimne et al., 2004) conditions. In the case of the present study, the effectiveness of the incubation of oleic acid with bentonite on LCFA degradation (no LCFA accumulation in the system) was exceptionally noted in C_2 stage

when C18:1 was added in an organic load of about 0.3 ± 0.009$g_{COD\text{-}C18:1}$ $L_{reactor}^{-1}$ d^{-1}. It could be related to the low ratio of bentonite/C18:1. It seems that the quantity of bentonite in relation to that of C18:1 was insufficient to capture all, or at least the majority of the added substrate. In this way, Mouneimne et al. (2004) proved that bentonite/grease ratio and mixing are the two essential factors that could influence on the performance of biodegradation of fatty substrate and recommended the use of a ratio of 0.9 in continuous assays (only 60 days of operation). Palatsi et al. (2012) also used a relatively high concentration of bentonite (5 $g_{Bentonite}$ L^{-1} / 0.5 $g_{C18:1}$ L^{-1}) to capture the LCFA. On the other hand, the application of a relatively high ratio for a long period of reactor operation, like the case of the present experiment (399 days), can lead to the formation of big flocculent particles. Indeed, Mouneimne et al. (2004) observed this phenomenon in batch assays when a bentonite/grease ratio beyond the optimal one was applied.

The quantification of LCFA during the oleic degradation helps to the understanding of the biochemical mechanism of unsaturated LCFA degradation. Although both saturated and unsaturated LCFA are degraded via β-oxidation, however discussion is opened about the exact pathway of unsaturated LCFA degradation (Sousa et al., 2009). The detection of palmitic acid as the main intermediate accumulating during oleic acid degradation in anaerobic bioreactors (the present study; Palatsi et al., 2012; Cavaleiro et al., 2009; Pereira et al., 2002) confirmed the occurrence of hydrogenation step prior to the β-oxidation mechanism. Stearic acid was also observed during the oleate conversion, although generally in low concentrations (the present study; Cavaleiro et al., 2009). Lalman and Bagley (2001) reported that the conversion of oleic acid to palmitic and stearic acid is energetically favorable.

3.4. CONCLUSION

The response of the anaerobic digestion process to punctual and continuous loading of lipids (Glycerol trioleate) and LCFA (oleate) was investigated in two CSTR reactors fed with skim milk solution as a basis substrate (HRT = 30 days and OLR= 1 g_{COD} L^{-1} day^{-1}). The results obtained in this study clearly underline the importance of the adsorption process on the biological toxicity and the physical flotation/wash-out of substrate/biomass. The incubation of the fatty substrate with an adsorbent agent

(bentonite) prior to its injection into the reactor was tested as a "protective" strategy that promotes the capture of the substrate by the bentonite and consequently prevents their adsorption into the biomass surface. This strategy was found to partly overcome the inhibitory effect of lipids pulses, by preventing the methane production decrease and the VFA accumulation in the system. However it was not very reliable when the reactor was subjected to increasing continuous load of oleic acid. Actually, the positive effect of bentonite addition was exceptionally noted with the lower applied oleate organic load ($0.3 \pm 0.009 g_{COD\text{-}C18:1}\ L_{reactor}^{-1}\ d^{-1}$). It could be due to the relatively low bentonite/substrate ratio (20%) which was probably insufficient to capture all or at least the majority of the added substrate.

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Chapter 4. Effect of ultrasonic treatment on the biomass activity and methane production potentials of sewage sludge, waste activated sludge and long chain fatty acids

The application of suitable controlled ultrasonic treatment as a strategy to enhance the biomass activity and the solubilisation of saturated and unsaturated LCFA was investigated in the present Chapter, together with sewage sludge for comparison purposes.

The main interesting results of this study were that the sonication at the tested specific energies had not affect the biodegradability of oleate and palmitate. Nevertheless, the slight increase of the biomass activity treating oleic acid, and the significant decrease of the lag phase time of methane production for the palmitic acid, both obtained at specific energy supply of 1600 $kJ \cdot kg_{TS}^{-1}$, highlights a slight effect of sonication treatment over the adsorption-desorption dynamics. The application of this energy input in a sequentially scheme (every 3-4 days) to anaerobic digestion batch experiments with palmitate as substrate demonstrated an improvement of the methane production rate until the fifth dosage respect to the untreated sample. Sequentially low energy dosages during the anaerobic digestion process of palmitate opens an interesting option, but maximum acceptable cumulated energy value must be further studied.

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4.1. INTRODUCTION

Low-frequency sonication (20 - 100 kHz) has gained a great attention over the last decades in many medical and industrial fields due to its high efficiency, low instrumental requirement and its economically viable performance (Rokhina et al., 2009). Sonication is a complex mechanism that combines physical effects and chemical reactions (Bougrier et al., 2005). When an ultrasound wave propagates into a liquid medium, it generates compression and rarefaction cycles that lead to micro-bubbles formation (cavitation bubbles). These bubbles grow in successive cycles until reaching an unstable diameter, collapsing violently and producing high pressure on the liquid – gas interface and an intense local heating, that can led to the decomposition of water into hydrogen atoms (H^+) and highly oxidative hydroxyl radicals (•OH) (Pilli et al., 2011). In the recent years, the ultrasonic application as a pre-treatment strategy to enhance the hydrolysis of sewage sludge prior to its digestion in anaerobic reactors has been largely investigated. Laboratory, pilot and full scale studies showed an improvement in sludge disintegration and substrate solubilisation, resulting in a significant increase of sludge biodegradability, total methane production rate and in the subsequent possible reduction in biomass retention time. In a recent publication, Pilli et al. (2011) detailed the positive effect of this technique, suggesting that sludge disintegration is expected to occur mainly by hydro-mechanical shear forces and the oxidizing effect of (•OH). Positive effects on sludge dewaterability (Huan et al., 2009), settleability (Pilli et al., 2011), volume-mass reduction (Na et al., 2007) and on heavy metals retention (Laurent et al., 2009) have been also reported. Consequently, the applicability of this pre-treatment in the anaerobic digestion field has been extended to different complex substrates, like sunflower oil cake (Fernández-Cegrí et al., 2012), high protein and lipid rich wastes (Luste et al., 2011; Li et al., 2012) or tannery wastewaters (Kamesware et al., 2011).

The propagation of ultrasonic waves at low frequency and low energy has been also described as beneficial for the metabolic performance of live systems by enhancing the membrane permeability and mass transfer within the microbial cells, promoting enzymes activity and biomass growth (Chisti, 2003; Xie et al., 2009; Zhang et al., 2008; Sclaäfer et al., 2000). The optimal ultrasonic conditions used for that purpose varied in each study, depending on the cells sensitivity to ultrasound and on the operational

conditions of each treatment (Chisti 2003). However, Zhang et al. (2008) proved that low frequency (25 kHz) was more effective than higher ones (80 kHz) to produce biomass stimulation, being the mechanical effects, instead of free radicals production, the main responsible of the process improvement. Bougrier et al. (2005) showed that the specific energy required to increasing biomass activity can be considered low, ranging from 1,000 to 3,000 kJ kg_{TS}^{-1}. Consequently, the optimal sonication energy exposure to be adopted must fit the requirement of increasing solubility and bioavailability of the substrate without damaging active cells, which could led to an increase on biomass activity.

The previously cited mechanisms and benefits of ultrasonic treatment pointed to the possibility of its usage to improve the anaerobic digestion of long chain fatty acids (LCFA). LCFA are the main intermediates of the anaerobic degradation of lipids, which are interesting for anaerobic digestion due to its high biogas potential (Hwu et al., 1998). Inhibition phenomena caused by LCFA onto anaerobic systems have been related to LCFA adsorption onto the biomass, affecting negatively the transport of nutrient and the protective functions of cell membrane, causing the inhibition of the anaerobic activity (Palatsi et al., 2012; Zonta et al., 2013). Moreover, the low solubility and the floatability of LCFA could limit its uptake by acidogenic microorganisms (Hwu et al., 1998), following the β-oxidation mechanism.

The aim of the present study was to analyze and to contrast the hypotheses that the application of suitable controlled ultrasounds could influence *(i)* the bioavailability of LCFA, *(ii*) the microbial activity of the biomass exposed to LCFA or *(iii)* the adsorption- desorption kinetics. In order to obtain a comparison framework, the same experimental procedure will be applied to waste activated sludge and sewage sludge, as complex substrates.

4.2. MATERIAL AND METHODS

4.2.1. Substrate and biomass characteristics

Waste activated sludge (WAS), and the mixed sewage sludge (SS), 60% primary sludge and 40% WAS, collected from a wastewater treatment plant (WWTP) located in Banyoles (Barcelona, Spain) were used in the present study. Anaerobically digested sludge (AS) from the mesophilic anaerobic digester of the same WWTP was used as

inoculums or anaerobic seed. Table 4.1 summarizes the chemical characteristics of SS, WAS and AS.

Sodium oleate (82% of purity, Riedel-de Haën/Sigma – Aldrich, USA) and sodium palmitate (98.5% of purity, Sigma-Aldrich, USA) powder salts were selected as saturated and unsaturated LCFA models, respectively.

Table 4.1 *Characterization (mean value ± standard deviation) of waste activated sludge (WAS), sewage sludge (SS) and the anaerobic inoculum (AS), used in the experiments.*

Parameters	Units	WAS	SS	AS
TS	g kg^{-1}	60.8 ± 1.1%	40.2 ± 0.4%	17.7 ± 0.9%
VS	g kg^{-1}	40.2 ± 1%	25.5 ± 0.8%	9.2 ± 1.4%
VS	%TS	66%	64%	52%
TSS	g kg^{-1}	n.d	n.d	16.7 ± 0.4%
VSS	g kg^{-1}	n.d	n.d	9.5± 0.1%
COD_t	g kg^{-1}	68.4 ± 2%	43.3 ± 3%	14.6 ± 1%

nd: Not determined

4.2.2. Ultrasonic treatment

The ultrasonic apparatus was a Sonifier® model S-250 D (Branson Sonic Power Co., USA), equipped with a titanium horn disruptor, working at a constant operational frequency (20 kHz) and variable power (max of 200W). The power amplitude was adjustable from 10 to 100%. The tip of the probe had a diameter of 13 mm and the apparatus was also equipped with a temperature probe.

Specific supplied energy (*sE*) was considered as the main variable parameter in the different experiments. The *sE*, expressed in $kJ \cdot kg_{TS}^{-1}$ units, was defined by Equation 1 (Bougrier et al., 2005; Fernández-Cegrí et al., 2012),

$$sE = \frac{P \cdot t}{V \cdot TS_0} \qquad \text{Eq. 1}$$

where P is the applied power (W), t is the exposure time (s), V is the treated sample volume (L) and TS_0 is the initial solids concentration ($g_{TS} \cdot L^{-1}$). Table 4.2 summarizes the different sonication conditions tested in the present study.

Table 4.2 *Ultrasonic treatment conditions.*

Substrate	Run	Nomenclature	***P*, Power Amplitude** (%)	***t*, Sonication Time** (s)	***sE*, Specific Energy** (kJ $kgTS^{-1}$)
WAS	R_1	$WAS_{1.4}$	30	480	79,560
	R_1	$WAS_{1.3}$	30	240	39,809
	R_1	$WAS_{1.2}$	30	30	4,975
	R_1	$WAS_{1.1}$	30	10	1,658
	R_1	$WAS_{1.0}$	0	0	0
SS	R_1	$SS_{1.2}$	30	60	9,657
	R_1	$SS_{1.1}$	30	10	1,610
	R_1	$SS_{1.0}$	0	0	0
	R_2	$SS_{2.2}$	30	5	745
	R_2	$SS_{2.1}$	15	5	381
	R_2	$SS_{2.0}$	0	0	0
LCFA	R_1	$Cx:N^*_{1.2}$	30	60	19,603
	R_1	$Cx:N^*_{1.1}$	30	10	3,264
	R_1	$Cx:N^*_{1.0}$	0	0	0
	R_2	$Cx:N^*_{2.2}$	30	5	1,640
	R_2	$Cx:N^*_{2.1}$	15	5	818
	R_2	$Cx:N^*_{2.0}$	0	0	0
AS	R_1&R_2	Inoculum	**	**	**

**LCFA nomenclature, Cx:N, where x is the number of carbons and N is the number of double bonds (C18:1 and C16:0, for oleic and palmitic, respectively)*

***Beakers and vials with inoculum alone (AS) where run for each tested P and t conditions (in WAS, SS and LCFA assays).*

4.2.3. Experimental set-up

4.2.3.1. Initial sonication assays (WAS, SS and LCFA).

The effect of ultrasonic treatment on organic matter solubility, substrate biodegradability and biomass activity were studied simultaneously by means of batch tests. A mixture of 1.5 g_{VSS} L^{-1} of inoculums (AS) and 5 g_{COD} L^{-1} of substrates (WAS or SS) were added to the basal anaerobic medium, with a total final volume of 50 mL, and placed in glass beakers. The basal anaerobic medium was composed of macronutrients and micronutrients solution diluted in unionized water, as described elsewhere (Angelidaki et al., 2009). The pH was then adjusted to neutrality, using HCl or NaOH, and the medium was buffered with 3 g_{NaHCO3} L^{-1}. In the case of LCFA experiments,

substrate concentration in the medium was reduced up to 2.5 g_{COD} L^{-1} because of the inhibitory proprieties of LCFAs over anaerobic biomass.

The previously described media was then subjected to ultrasonic treatment applying the corresponding selected *sE* (Table 4.2). Tested *sE* were in the range of *high* and *low* energy requirements to produce substrate hydrolysis-solubilisation or biomass activation, respectively. As shown in Table 4.2, two ranges of *sE* can be defined as: *sE* < 3,000 kJ kg_{TS}^{-1} and *sE* > 3000 kJ kg_{TS}^{-1}. Due to experimental limitations, the batches with SS and LCFA were performed in two different runs (R_1 and R_2), with an interval of time of 16 days. For the second run, SS was kept refrigerated at 4°C while the AS was stored in an incubation chamber at 35°C. Thus, a little decrease in the total COD was observed. This fact was considered in the set-up of R_2 experiments.

Each sonication experiment was performed in triplicate. One beaker per treatment was used for solubilisation (S_{COD}) determination. The S_{COD} represents the transfer of organic matter (COD) from the particulate fraction (substrate and inoculum) to the soluble fraction of the mixture. It was calculated using the difference between COD_s after sonication treatment and the initial soluble COD (COD_{s0}), versus the initial particulate COD (COD_{p0}), according to Equation 2 (Bougrier et al., 2005; Fernández-Cegrí et al., 2012),

$$S_{COD}(\%) = \frac{COD_S - COD_{S0}}{COD_{p0}} \cdot 100 \qquad \text{Eq. 2}$$

where the initial COD_{p0} was calculated as the difference between the initial total COD (COD_{t0}) and the initial soluble COD (COD_{s0}). The other two sonicated beakers were used for monitoring the biogas production along batch test, using 120 mL glass vials. Once media was introduced into vials, headspaces were bubbled with a N_2/CO_2 gas mixture (80/20 v/v) in order to remove O_2 before sealing them with rubber stoppers and aluminium crimps. A reducing solution was finally added (1 ml of 10 g_{Na2S} L^{-1}). Vials were placed in an incubation chamber at 35ºC under continuous shaking (120 rpm). Duplicates of vials with WAS, SS or LCFA, but without sonication treatment, were also incubated (controls). Same procedure was also applied to beakers and vials with only inoculum (AS), at the same *sE* inputs, in order to measure the residual biogas production by the inoculum (blank) and to estimate the net production by the substrate.

The time course of the biogas production was monitored by gas chromatography, sampling the vials head space periodically. The produced methane was calculated at standards conditions of pressure and temperature (0°C and 1 atm) and expressed as $mL_{CH4} \cdot g_{COD}^{-1}$. With the objective to quantify and to compare the effect of the different tested ultrasonic treatments, a Gompertz curve equation (Equation 3) was fitted to the methane production data (Palatsi et al., 2012),

$$G = G_{max} \cdot \exp\left[-\exp\left(\frac{r_m \cdot e}{G_{max}}(\lambda - t) + 1\right)\right] \qquad \text{Eq. 3}$$

where G is the accumulated methane production ($mL_{CH4} \cdot g_{COD}^{-1}$), G_{max} is the methane production potential ($mL_{CH4} \cdot g_{COD}^{-1}$), r_m is the maximum methane production rate ($mL_{CH4} \cdot g_{COD}^{-1} day^{-1}$), λ is the duration of the lag phase period on methane production (days) ant t is time (days).

4.2.3.2. Sequential sonication experiments with LCFA

The optimal sonication specific energy, found in the previous batch tests was selected as the sonication conditions for sequential batch tests. Batch assays were carried out in duplicate with the same initial inoculum (1.5 g_{VSS} L^{-1}) and substrate (2.5 g_{COD} L^{-1}) concentration as in the previous LCFA experiment. Now, a total of 7 ultrasonic pulses were applied at different operating time with an interval of 3 – 4 days. Propionic acid, in a concentration of 890 mg_{COD} L^{-1}, was added as tracing element after the first ultrasonic treatment, since it is not expected to be produced during palmitate degradation. Since it was not possible to apply the sonication treatment directly to the media when bottles were sealed, the vials were degassed, opened and sonicated in a beaker every time. Once the sonication was applied, the media was returned to their corresponding vial and bubbled with a N_2/CO_2 gas mixture. The volume of methane was corrected by sampling the headspace before and after each ultrasonic pulse.

4.2.4. Analytical methods

Total and volatile solids (TS, VS), total and volatile suspended solids (TSS, VSS), total and soluble chemical oxygen demand (COD_t, COD_s) where measured according to standard methods (APHA, 1995). Methane (CH_4) and carbon dioxide (CO_2) production

was determined by a CP-3800 (Varian, USA) gas chromatograph fitted with Hayesep Q 80/100 Mesh (2 m x 1/8" 2.0 m) packed column (Varian, USA) and thermal conductivity detector, as described elsewhere (Palatsi et al., 2009). Volatile fatty acids concentration (VFA; Acetate (Ac), Propionate (Pr), iso-Butyrate (iso-But), n-Butyrate (n-But), iso-Valerate (iso-Val), n-Valerate (n-Val) and Hexanoate (Hex) were measured also with GC and FID detection, following Palatsi et al. (2009).

4.3. RESULTS AND DISCUSSION

4.3.1. Effect of ultrasonic treatment on substrate solubilisation

The organic matter solubilisation, S_{COD}, for the different *sE* inputs and substrates tested (according to Table 4.2), is shown in Figure 4.1. From the obtained results it can be appreciated a general increase in S_{COD} when *sE* increases, for all the substrates (WAS, SS and LCFA). Lines in Figure 4.1 are the result of fitting data to a logarithmic curve, indicating that solubilisation increases fast at low *sE* ranges and slowly at higher values. Figure 1 also includes results of sonication experiences performed with inoculums alone, AS (blanks). The maximum applied *sE* to the inoculum beakers were 25,721 kJ kg_{TS}^{-1}, obtaining a maximum S_{COD} value of 8.6 % (Figure 4.1).

Regarding to the activated sludge experiments, and considering specific energies below 5,000 kJ kg_{TS}^{-1}, the obtained solubilisation values of the mixture of activated sludge-inoculum (WAS) were low, and in the same range of those obtained with the inoculum treated alone (Figure 4.1a). These results indicated that sonication did not affect solubilisation of WAS at this range of energy. For a higher supplied energy, the solubilisation risen up to reach a value of 26% at a *sE* of 79,560 kJ kg_{TS}^{-1}, while the extrapolated value for the inoculum, reached only a 10% of S_{COD}, approximately. Hence, subtracting the inoculum results, a maximum solubilisation of about 13–16 % could be obtained treating WAS at 39,809-79,560 kJ kg_{TS}^{-1}. These values are in the same order of magnitude of those obtained by Khanal et al (2006). Nevertheless, using a similar range of supplied energy (90,000 kJ Kg_{TS}^{-1}), Yan et al. (2010) was able to release 37% of the organic matter of WAS from the solid phase to the liquid phase. Bougrier et al. (2005 and 2006) reached 15% of solubilisation with *sE* ranging from 6,250 – 9,350 kJ $kg_{TS}^{-1}$and a maximum of 37% at *sE* of almost 15,000 kJ kg_{TS}^{-1}. In contrast, Salsabil et al. (2009) obtained a very low solubilisation (less than 10%) in

WAS experiments using a higher *sE*, around 108,000 kJ kg_{TS}^{-1}. Consequently it can be concluded that the comparison of WAS sonication experiences dealing with COD solubilisation is not always direct, and other factors such as sludge type, sludge concentration, chemical characteristics, ultrasounds frequency and density and treatment duration, must be considered (Salsabil et al., 2009; Haun et al., 2009; Groönross et al., 2005).

In the experiments with the mixed sewage sludge (SS) and the inoculum, the tested *sE* sonication values were reduced, and just a slightly solubilisation was obtained, as stated in Figure 4.1b. The maximal percentage of S_{COD} was almost 10% for the max tested *sE* of 9,657 kJ kg_{TS}^{-1}. Compared to the results of the corresponding inoculum, the curve profile was just smoothly above, indicating that the release of organic components to the liquid phase was poor. Similar results (S_{COD} of 10%) were obtained by Lehne et al. (2001) with a *sE* of 3,000 kJ kg_{TS}^{-1}. Recently, Benabdallah El-Hadj et al. (2007) achieved a SS solubilisation of 15% by pre-treating sludge at a *sE* of 11,000 kJ kg_{TS}^{-1}. Tiehm et al. (1997), using ultrasound at a frequency of 31 kHz and high acoustic intensities (3.6 kW), obtained higher COD solubilisation (30% after 96 seconds of treatment). Nevertheless, and according to the present results and related literature, it is widely accepted that the sonication of SS results in a less pronounced release of chemical oxygen demand compared to WAS treatment. The high percentage of primary sludge in the SS mixtures and the high VS/TS ratio of SS sludges could have influenced the lower efficiecy in organic matter release compared to WAS treatment (Groönross et al., 2005; Benabdallah El-Hadj et al., 2007).

The effect of ultrasounds on the mixtures of inoculum and LCFA (LCFA experiments) at different *sE* conditions is reported in Figure 4.1c. As it can be seen, the S_{COD} of the mixture of LCFA and biomass increased slightly with the increase of *sE* supply. However, independently of the sonication treatment, the solubility of biomass is reduced with LCFA addition (Figure 1c), specially in the case of sodium palmitate (C16:0). Probably the formation of a biomass-abosrbed LCFA "complex" have favoured this behaviour (Hwu et al., 1998). It is plausible then, that the required *sE* input to solubilize the new complex biomass-adsorbed LCFA would be higher than the tested

sE. The poor solubility of saturated LCFA (C16:0) could also interfere with the adsorption effect and on the organic matter release during the treatment.

It must be noted that during the sonication process, the temperature increased slightly for SS and LCFA experiments, with a maximum increment in temperature of 10ºC for the maximum tested *sE* level. Contrary, for WAS tests, the temperature increased up to values of 59.4±1.5ºC and 76.4±1ºC for sonication treatments at *sE* of 39,809 $kJ \cdot kg_{TS}^{-1}$ and 7 9,560 $kJ \cdot kg_{TS}^{-1}$, respectively. The different solids content of beakers, when media is prepared to reach the same total COD content with different substrates, could has also influenced this effect (Appels et al., 2012)

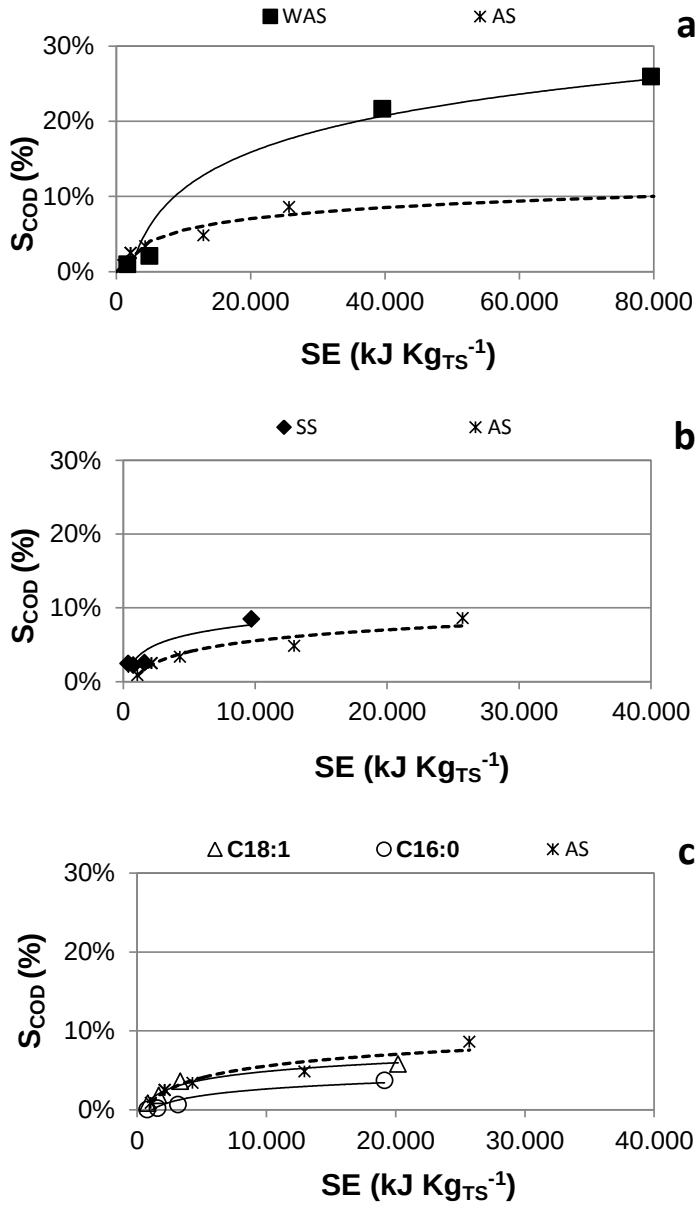

Figure 4.1 *Effect of specific energy on organic matter solubilisation, S_{COD} (%), for (a) WAS, (b) SS and (c) LCFAs* experiments.

4.3.2. Effect of ultrasonic treatment on substrate biodegradability and biomass activity

Different hypothesis can be formulated about the effect of the ultrasound treatment on substrate biodegradability and biomass activity. The solubilisation of organic matter and the possible enhancement of particles disintegration during the sonication treatment can lead to an improvement in the substrate biodegradability, measured by an increase of the

methane production potential (G_{max}). This fact could also improve the "bio-activity" of the biomass (as more "easily biodegradable matter" is "available") during the anaerobic digestion process, effect that can be measured by an increase in the methane production rate (r_m) and/or a decrease in the lag phase (λ) time (Zhang et al., 2008; Schläfer et al., 2000). Also, an increase on substrate biodegradability with a decrease on biomass activity, due to cells lysis by ultrasonication excess, will lead to an increase on G_{max}, a decrease on r_m and/or an increase of λ. Also possibe cells "bio-agumentation" by sonication could be monitored by variations in r_m and λ values (Li et al., 2013). All those possible effects and combinations are analyzed in detail in the following paragraphs. Table 4.3 shows these parameter mean values and 95% confidence intervals (95% CI) estimated by fitting Equation 3 to the accumulated methane production, for the different batch tests at the tested *sE* levels.

As stated in Figure 4.2, ultrasonic treatment at different *sE* inputs allowed an increase in the methane production of WAS, expressed in $mL_{CH4}\ g_{CODin}^{-1}$ units. For the untreated WAS ($WAS_{1.0}$ in Table 3), methane production potential was 125 $mL_{CH4}\ g_{CODin}^{-1}$, corresponding to a substrate biodegradability around a 36% (considering the theoretical value of 350 $mL_{CH4}\ g_{COD}^{-1}$, as the maximum value for complete anaerobic biodegradation). WAS biodegradability increased up to a 46.3%, when increasing the energy supply *sE* in the specific energy range of 0 – 39,809 kJ kg_{TS}^{-1} (Table 4.3 and Figure 4.2). This general trend confirms results obtained by many authors (Salsabil et al., 2009; Bougrier et al., 2006; Appels et al., 2012). Contrary, further increase in the applied *sE*, till 79,560 kJ kg_{TS}^{-1}, did not lead to an advantageous improvement of the methane production, which was in the same level of that obtained with an energy supply of 4,975 kJ kg_{TS}^{-1} (Table 4.3). These results means that although solubilisation could increase at high *sE* values, as shown in Figure 1a, at high energy dosages recalcitrant compounds could be formed, reducing the substrate biodegradability. Kim and Lee (2012) concluded that excess ultrasound irradiation could convert volatile solids into inert or inhibitory compounds to anaerobic digestion, as explanation of negative results from experiments with WAS at high energy dosages (around 140,000 kJ kg_{TS}^{-1}). This effect was also reported in other pre-treatments, as when a thermal pre-treatment is applied to substrates with high carbohydrate and protein contents (Rodriguez-Abalde et al., 2011). In fact, in the present experiments the thermal effect was also present since

WAS temperature reached 76ºC for the highest *sE* dosage. At those conditions, also some specific enzymes could be altered (such as some hydrolytic enzymes) impeding its function. The effect of ultrasonic treatment on four hydrolytic enzymes activities (proteasse, α-glucosidaes, acid phosphatase and alkaline phosphatase) on WAS were recently investigated by Yan et al. (2010), concluding that the sonication treatment had a positive effect on the activities of these enzymes, reaching the highest level at the specific energy of 30,000 kJ kg_{TS}^{-1} and decreasing significantly for higher values. The authors explained the increase on enzyme activities by the increase of soluble proteins and carbohydrates, while the activity decrease at high energy exposures was attributed to cells damage. Nevertheless, since maximum methane production rate (r_m) was not decreased and lag phase (λ) was not increased for the higher *sE* value ($WAS_{1.4}$ respect to $WAS_{1.2}$ or $WAS_{1.3}$ in Table 4.3), biomass activity seems to be not seriously affected or inhibited.

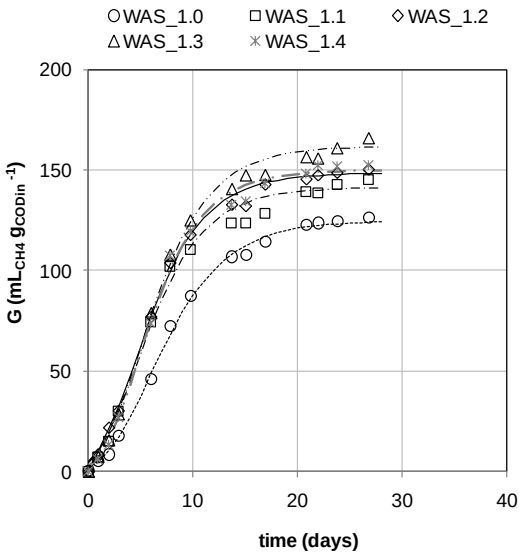

Figure 4.2 *Experimental data (point markers) and Gompertz fitting curves (lines) of accumulated methane production (mL_{CH4} g_{CODin}^{-1}), along experimental time for non-sonicated and sonicated WAS at different sE inputs (kJ kg_{TS}^{-1}).*

Table 4.3 *Effect of ultrasonic treatment on the anaerobic digestion process, quantified by the Gompertz equation parameters. Table summarizes mean parameters (G_{max}, rm and λ), confidence intervals (CI with α=0.05) and evaluations of the function fittings (r^2).*

Substrate	Nomenclature	*sE*, Specific Energy	*Gmax*, Max Methane Potential		*rm*, Methane Rate		*λ*, Lag Phase		r^2
		(kJ $kgTS^{-1}$)	(mL_{CH4} g_{COD}^{-1})	CI (95%)	(mL_{CH4} g_{COD}^{-1} day^{-1})	CI (95%)	(days)	CI (95%)	
WAS	$WAS_{1.4}$	79,560	150.18	2.67	16.14	1.52	1.29	0.45	0.996
	$WAS_{1.3}$	39,809	161.89	3.14	16.10	1.54	1.13	0.47	0.995
	$WAS_{1.2}$	4,975	148.29	2.28	15.06	1.21	0.82	0.37	0.997
	$WAS_{1.1}$	1,658	141.05	3.55	14.39	1.74	0.89	0.61	0.992
	$WAS_{1.0}$	0	125.01	2.53	11.21	0.87	1.62	0.40	0.998
SS	$SS_{1.2}$	9,657	193.96	4.67	18.47	1.98	6.84	0.59	0.996
	$SS_{1.1}$	1,610	180.47	3.30	16.57	1.47	3.65	0.50	0.997
	$SS_{1.0}$	0	166.94	2.03	15.75	0.90	1.60	0.31	0.998
	$SS_{2.2}$	745	160.75	3.45	18.80	2.55	2.76	0.58	0.995
	$SS_{2.1}$	381	142.96	3.00	21.25	3.57	2.71	0.61	0.994
	$SS_{2.0}$	0	146.01	1.48	19.64	1.46	2.05	0.29	0.999
C18:1	$C18:1_{1.2}$	19,603	348.49	2.91	36.74	1.38	14.93	0.20	1.000
	$C18:1_{1.1}$	3,264	355.17	4.73	31.92	1.57	13.95	0.27	0.999
	$C18:1_{1.0}$	0	351.58	5.14	30.23	1.55	14.48	0.33	0.999
	$C18:1_{2.2}$	1,640	335.65	4.89	24.74	1.83	10.38	0.58	0.999
	$C18:1_{2.1}$	818	324.18	5.76	30.68	3.50	11.05	0.71	0.998
	$C18:1_{2.0}$	0	346.63	8.65	27.36	3.50	11.35	0.92	0.997
C16:0	$C16:0_{1.2}$	19,603	343.84	14.04	28.12	4.29	15.55	0.96	0.994
	$C16:0_{1.1}$	3,264	355.31	6.34	36.79	2.82	12.92	0.37	0.998
	$C16:0_{1.0}$	0	345.96	5.09	36.04	2.27	13.58	0.31	0.999
	$C16:0_{2.2}$	1,640	319.69	16.44	24.15	4.31	15.44	1.27	0.995
	$C16:0_{2.1}$	818	316.52	9.42	24.41	2.89	18.79	0.80	0.997
	$C16:0_{2.0}$	0	328.45	9.72	28.25	3.73	19.72	0.81	0.996

Biodegradability or methane production potential obtained in SS experiments differs between tests performed during run R_1 and run R_2, and also between both un-treated samples ($SS_{1.0}$ and $SS_{2.0}$ in Table 4.3), being the G_{max} lower for the test done with the 16 days older inoculum ($SS_{2.0}$) (Figure 4.3). Although those differences, the general trend of methane production, and hence biodegradability, is to increase when *sE* increases (see Table 4.3 and Figure 4.3), with a significant maximum value of 194 mL_{CH4} g_{CODin}^{-1} (or a corresponding biodegradability of 55.4%) for a *sE* of 9,657 kJ kg_{TS}^{-1} ($SS_{1.2}$). Similar results were obtained by Benabdallah El-Hadj et al. (2007) with SS and *sE* values in the range 5,000 – 8,000 kJ Kg_{TS}^{-1}. Biomass activities, measured by r_m in Table 4.3, have also different range of values in the two runs, with a tendency to increase in run R_2 but without significant differences, taking into account the obtained CIs. The lag phase (λ) for run R_2 experiments is slightly higher than the values estimated for run R_1 (including blank vials or *sE*=0), which can be explained as a result of the 16 days inoculum storage time between the two runs. While the low *sE* levels have not affected the biomass lag phase duration on R_2 *runs*, the corresponding λ estimated parameter in R_1 has been significantly increased with higher energy dosages, reaching 6.84 days for a *sE* level of 9,657 kJ kg_{TS}^{-1} ($SS_{1.2}$ in Table 4.3), around 4 times the value estimated in blank vials during the same run ($SS_{1.0}$ in Table 4.3). Consequently, it seems that the complex SS-inoculum was more sensitive to sonication than WAS-inoculum, decreasing the concentration of viable microorganisms, but biomass activity could be maintained or slightly improved due to the increase of "bio-available" substrate concentration and biodegradability. This equilibrium between the increase in "bio-available" substrate and the decrease on "viable microorganisms" leads to different results depending of the relative solubilisation level of the substrate, explaining the different effects shown for WAS (higher solubilisation but not a significant effect on biomass activity) and SS (lower solubilisation but some effect on biomass activity at lower *sE* values). The media characteristics, and specifically the solids and organic matter content, could have influenced this behaviour.

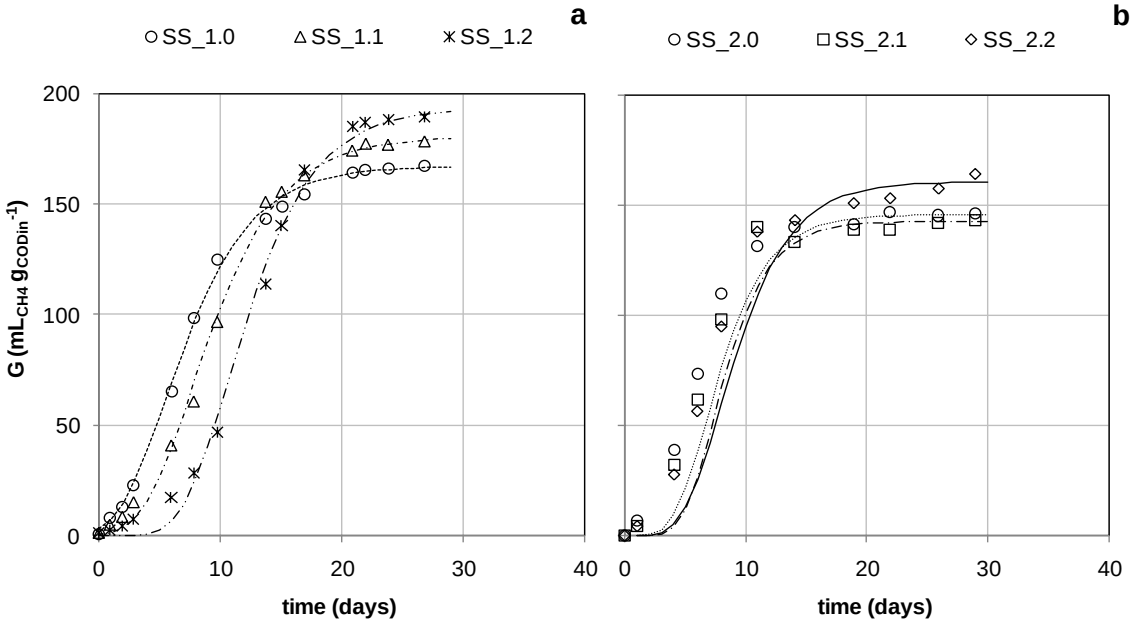

Figure 4.3 *Experimental data (point markers) and Gompertz fitting curves (lines) of accumulated methane production (mL_{CH4} g_{CODin}^{-1}), along experimental time for non-sonicated and sonicated SS at different sE inputs (kJ kg_{TS}^{-1}).* ***(a)*** *Run R_1 or experiment at high sE (> 3,000 kJ kg_{TS}^{-1}) and* ***(b)*** *Run R_2 or experiment at low sE (< 3,000 kJ kg_{TS}^{-1}).*

The effect of sonication on methane production from LCFA is illustrated in Figure 4.4, where the cumulative methane yields from oleate, C18:1 (Figure 4.4a and Figure 4.4b), and palmitate, C16:0 (Figure 4.4c and Figure 4.4d), is depicted as a function of *sE* dosage. The untreated LCFA samples ($LCFA_{1.0}$ or $LCFA_{2.0}$) were almost totally transformed to methane, with biodegradability close to 100%, after a long lag period (more than 10 days). In general, lag phase (λ) and maximum methane production rate (r_m) have been always higher for LCFA than for WAS or SS experiments (see Table 4.2).

In the case of C18:1, no significant difference in the maximum methane productions (G_{max}), methane production rate (r_m) and lag phase (λ) were observed between sonication dosage, *sE* (Figures 4.4a - 4.4b, and Table 4.3). Nevertheless, r_m values were higher for run R_1 than for R_2, probably due to the older inoculum used for R_2. Contrarily, λ values were significantly higher for R_1. Anyway the microorganism activity seems to not be damaged at the tested *sE*. The highest r_m value obtained at the highest energy dosage for oleic acid ($C18:1_{1.2}$ in Table 4.3) could be explained by

sonication enhancement of LCFA desorption, decreasing the surface mass transfer limitations and enhancing the biomass activity.

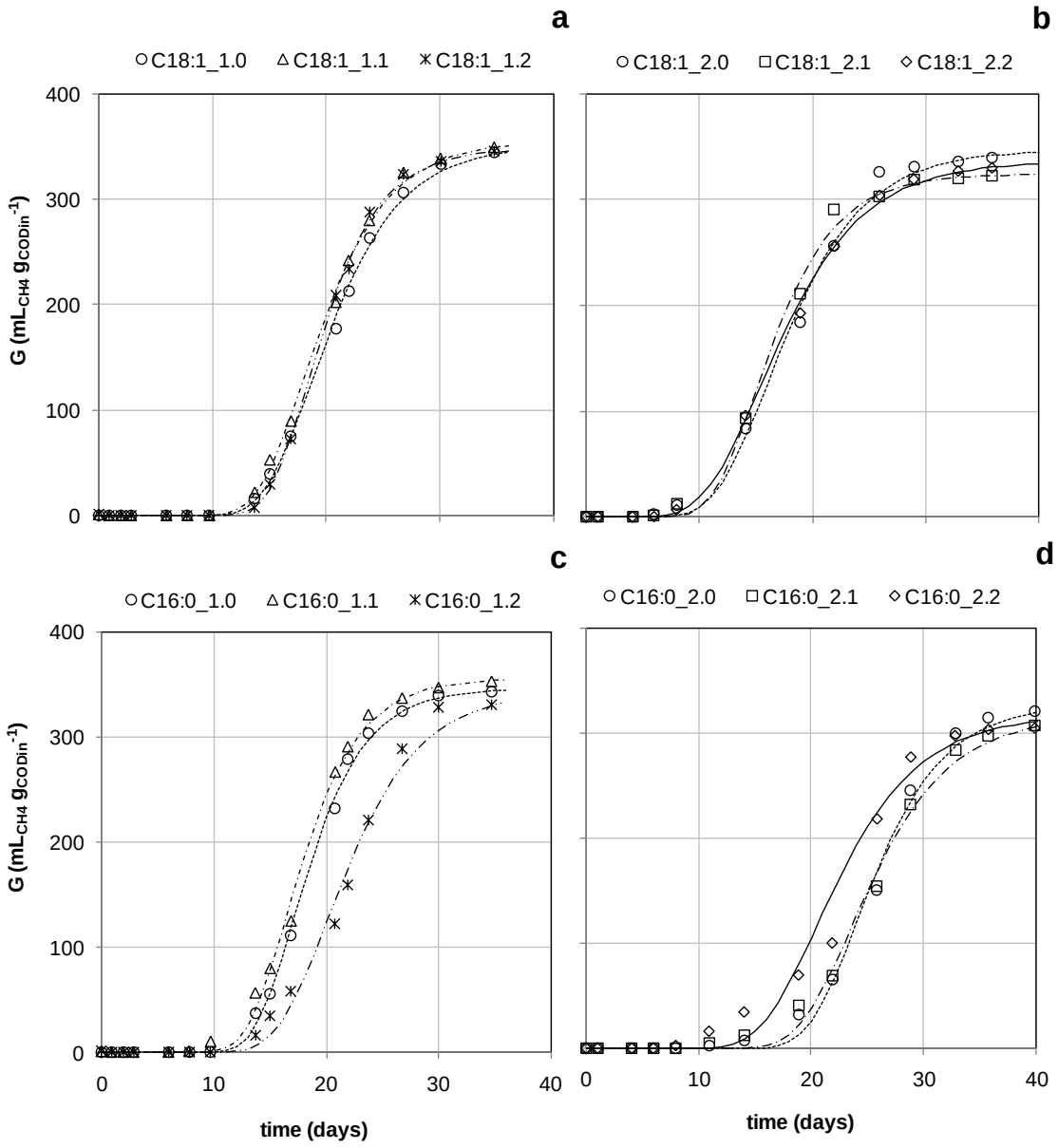

Figure 4.4 *Experimental data (point markers) and Gompertz fitting curves (lines) of accumulated methane production (mL_{CH4} g_{CODin}^{-1}), along experimental time for non-sonicated and sonicated LCFA at different sE inputs (kJ kg_{TS}^{-1}). (**a**) Run R_1 at high sE (> 3,000 kJ kg_{TS}^{-1}) with C18:1, (**b**) Run R_2 at low sE (< 3,000 kJ kg_{TS}^{-1}) with C18:1, (**c**) Run R_1 at high sE (> 3,000 kJ kg_{TS}^{-1}) with C16:0 and (**d**) Run R_2 at low sE (< 3,000 kJ kg_{TS}^{-1}) with C16:0.*

The effect of sonication treatment on the methane production profile from palmitate (C16:0) was also different depending on the experimental run (Figure 4.4c and Figure

4.4d). For R_1, with the higher tested *sE* values, the obtained G_{max} values were not significantly different between *sE* dosages. With the increase of *sE* in R_1, the r_m values decreased while the λ increased with energy dosage, *sE* (Table 4.2), indicating a possible cells damage. These results are consistent with literature experiences with a varied kind of complex lipid-rich wastes, like a FOG waste (composed by a mixture of waste frying oil, bacon grease and animal fat), synthetic kitchen waste and by-products from meat processing industry (Luste et al., 2011; Lueste et al., 2009; Li et a., 2013). However, authors explained these results by the fact that the pre-treatment enhanced significantly the lipid hydrolysis rate and induced, consequently, an accumulation of LCFA in the system which might have subsequently inhibited the biomass and hence increasing the lag phase time. Contrary, in the experiments with palmitate during R_2, the values of G_{max} and r_m were not significantly different when increasing the energy dosages (Table 4.3). In the case of the lag time λ, the values decreased when *sE* increased, with a significant lower value (15.4 days, around 4 days less than the control or $C16:0_{2.0}$, according to Table 4.3) for a *sE* of 1,640 $kJ \cdot kg_{TS}^{-1}$. This earlier biomass activation, without an increase of the methane production rate, could indicate a slight effect of sonication over the adsorption-desorption C16:0 kinetics. Improvements in biomass activity due to mass-transfer activation are consistent with results found by other authors (Bougrier et al., 2005; Lehne et al., 2001; Tiehm et al., 1997), who suggested that the optimal treatment conditions to activate biomass are for *sE* values in the range 1,000 – 3,000 kJ kg_{TS}^{-1}. Consequently, and according to the present results, a *sE* value of 1,640 kJ kg_{TS}^{-1} was selected for the sequential sonication experiments.

4.3.3. Sequential sonication during batch LCFA anaerobic digestion

In the case of our study, the beneficial effect of ultrasonic treatment on the anaerobic digestion of saturated LCFA (shorter lag phase time) could be related to an enhancement in the biomass activity. Because of the low solubility propriety of C16:0, only a part of the added substrate would be adsorbed onto the biomass, and perhaps the sound waves activated the "not yet adsorbed" biomass. Contrary, C18:1 dissolves rapidly in the liquid phase, and hence it could, in turn, be rapidly adsorbed onto the biomass and the effect of ultrasonic treatment is expected to be lower. Actually, ultrasonic treatment was widely used in transesterification of vegetable oil for biofuel production (Starvache et al., 2005) and in food industry emulsification fields

(Ramachandran et al., 2006; Cucheval and Chow, 2008), because of its positive effect on the enhancement of the lipase catalyzed reactions. The above-mentioned results open the discussion about the application of discontinuous controlled ultrasonic waves to C16:0 in order to promote the desorption-consumption steps. The mixtures of inoculum-C16:0 were subjected to 7 ultrasonic treatments at *sE* input of 1,640 kJ kg_{TS}^{-1}, applied at different digestion time with an interval of 3–4 days. Results of biodegradability batch tests are depicted in Figure 4.5.

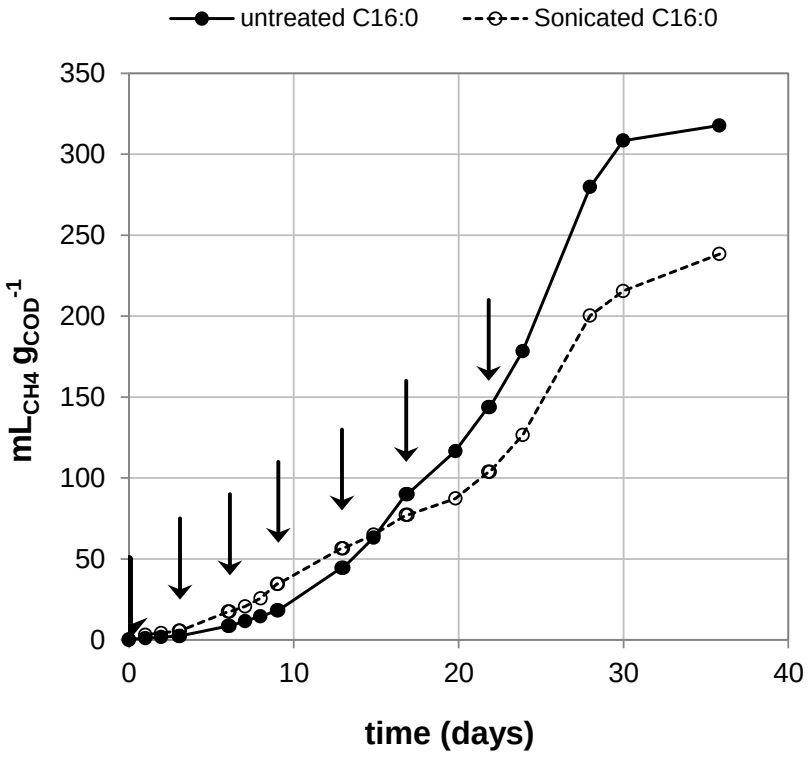

Figure 4.5 *Effect of repeated ultrasonic pulses with a specific energy input (sE= 1,640 kJ* kg_{TS}^{-1}*) on the methane production of C16:0 acid, applied at days indicated with the vertical arrow.*

After a lag phase time of about 3 days, 91% of the untreated palmitic acid was converted into methane in 36 days of digestion. The first ultrasonic treatment did not affect on the methane production which was similar to that produced by the untreated samples. Afterward, the subsequent ultrasonic treatment enhanced the methane production that was slightly higher until the 5th treatment as compared to the non-sonicated samples. Later, the rate of CH_4 production was decreased and C16:0 was

slowly degraded. The sonicated C16:0 was not completely digested, since only 79% of the organic matter was converted into CH_4, compared to control. Due to the experimental set-up (vials were opened and poured in each ultrasonic treatment), a loss of inoculum and likewise of the organic matter could be possible. Not absolute conclusion could be detected from the obtained results, however, it seem that subsequent discontinuous exposure of C16:0 to ultrasonic treatment promoted the adsorption-desorption cycles and hence reduced negative effects of LCFA onto the biomass at the beginning of the experiment, decreasing the effect later.

The profile of propionic (tracing element) and acetic acids degradation, depicted in Figure 4.6, could confirm this conclusion. The propionic acid added after the first ultrasonic pulse was rapidly depleted before the second pulse, in comparison with the nontreated vials, slowing afterwards its biomass uptake, indicating a possible damage of acetogenic microorganisms. The acetic acid evolution indicates a higher release rate till approximately day 7 for sonicated vials, suggesting an initial faster palmitate decomposition, but the later accumulation of acetic acid, over that of untreated vials, indicates a possible damage of acetoclastic methanogens.

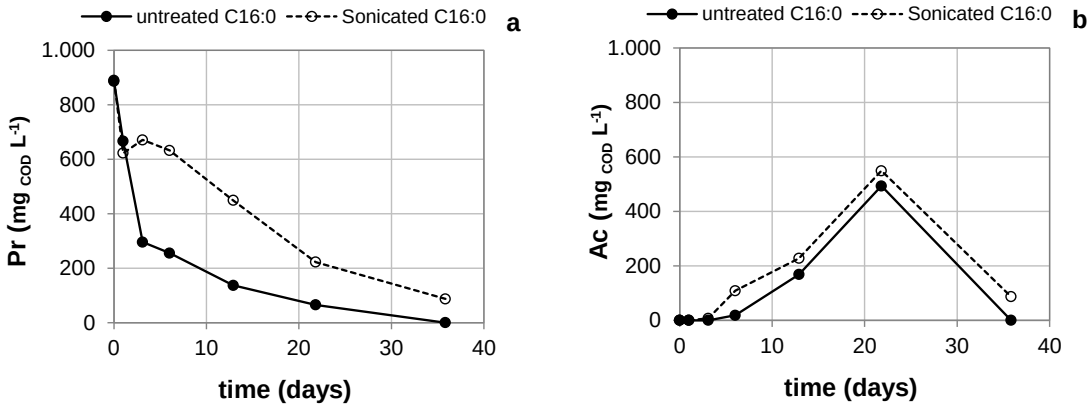

Figure 4.6 *Effect of repeated ultrasonic pulses with a specific energy input (sE= 1,640 kJ* kg_{TS}^{-1}*) on the propionic acid (a) and acetic acid (b) time evolution.*

The number of ultrasonic treatments should be controlled because excessive (or repetitive) treatment could lead to the breakdown of cells and consequently could affect the methane production rate. Complementary studies, like LCFA determination on the liquid and the solid phase and viable microbial community analysis, are required to well

understand the exact effect of repeated ultrasonic treatments on the anaerobic degradation of LCFA.

4.4. CONCLUSIONS

When treating by sonication mixtures of inoculum and waste activated sludge, or mixed sewage sludge, the methane production potential is enhanced while the biomass activity is increased as a result of the higher bio-avaliable soluble substrate, till certain specific energy supply, for which the cells damage by sonication leads to an increase of the lag phase time in batch experiments. This implies the existence of an optimal energy dosage for every mixture substrate – inoculum, with a positive effect on the above equilibrium.

For LCFA, sonication at the tested specific energies does not modify the biodegradability of oleate and palmitate, while increases slightly the biomass activity for oleate. With the same specific energy dosages, lag phase time was reduced when treating palmitate at an energy dosage of 1,640 kJ kg_{TS}^{-1}. Applying this pre-treatment in a sequentially scheme (every 3-4 days) to anaerobic digestion batch experiments with palmitate as substrate, the methane production rate was improved before the fifth dosage respect to the untreated sample, but decreasing later. Sequentially low energy dosages during the anaerobic digestion process of palmitate opens an interesting option to enhance its decomposition, but maximum acceptable cumulated energy value must be further studied.

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Chapter 5. Saponification pretreatment and solids recirculation as a new anaerobic process for the treatment of slaughterhouse waste

Different configurations of anaerobic process, adapted to the treatment of solid slaughterhouse fatty waste, were proposed and evaluated in this study. The tested configurations are based on the combination of anaerobic digestion with/without waste saponification pretreatment (70ºC during 60 minutes) and with/without recirculation of the digestate solid fraction (ratio=20% $_{w/w}$). After an acclimation period of substrate pulses-feeding cycles, the reactors were operated in a semi-continuous feeding mode, increasing organic loading rates along experimental time. The degradation of the raw substrate was shown to be the bottleneck of the whole process, obtaining the best performance and process yields in the reactor equipped with waste pretreatment and solids recirculation. Saponification promoted the emulsification and bioavailability of solid fatty residues, while recirculation of solids minimized the substrate/biomass wash-out and induced microbial adaptation to the treatment of fatty substrates.

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5.1. INTRODUCTION

Besides proper waste management practices, the concept of "waste to energy" is currently being promoted as an opportunity to face the fossil fuel crisis, the global warming and the consequent stringent environmental legislation. In this context, the anaerobic digestion of organic waste plays a crucial role as a technology that combines waste treatment and renewable energy production, in the form of biogas. High strength lipid containing wastes are interesting substrates for the anaerobic digestion process due to its high theoretical methane potential. Considerable amounts of this kind of substrates are generated from food processing industry, from the production of vegetable and animal oils, and from slaughterhouses facilities (Appels et al., 2011). Recently, Hejnfelt and Angelidaki (2009) and Palatsi et al. (2011) have characterized individual fractions of Danish and Spanish animal by-products, respectively, and determined its high potential methane yields.

Under anaerobic conditions, lipids are hydrolyzed by extracellular lipases to long chain fatty acids (LCFA) and glycerol. The LCFA are subsequently degraded via the β-oxidation mechanism to acetate and hydrogen, which are further converted to a methane-carbon dioxide mixture, known as biogas (Weng and Jeris, 1976). The rate of lipid hydrolysis depends, among other factors, on the specific type of lipid (triglycerides, phospholipids or sterols), the lipid particles size and the biomass specific surface area (Massé et al., 2003). In the case of particulate fatty substrates, where high amounts of suspended solids (SS) are present, the hydrolysis can be considered as the rate limiting step of the whole anaerobic process (Sayed et al., 1988, Vavilin et al., 2008). Also, released LCFAs have to be adsorbed onto biomass to be degraded (Hwu et al., 1998) and this phenomenon can affect the transport (Pereira et al., 2005) and/or protective functions (Galbaraith and Miller, 1973) of cell membranes, inhibiting the anaerobic activity (Lalman and Bagley, 2001; Palatsi et al., 2012). Lipid and LCFA adsorption onto biomass have also been reported to cause sludge flotation and wash-out, process that can take place at concentrations below the LCFA toxicity limit (Hwu et al., 1997).

Several pretreatment techniques have been investigated to reduce the particle size and to promote the solubilization of lipids. Among these, saponification (Battimelli et al., 2009 and 2010), enzymatic hydrolysis (Masse et al., 2003) and enzymatic bio-augmentation

(Cirne et al., 2006) have been claimed to enhance the hydrolysis rates. Furthermore, new treatment strategies developed to prevent problems related to LCFA inhibition have been proposed. The use of acclimated biomass (Cavaleiro et al., 2008), the addition of adsorbents as biofibers or bentonite (Palatsi et al., 2009) and the application of feeding strategies, based on sequential LCFA accumulation-degradation steps (Cavaleiro et al., 2009) have been suggested as possible alternatives.

Conventional high-rate anaerobic reactors, such as upflow anaerobic sludge blanked (UASB) reactor (Kim and Shin, 2010), expanded granular sludge bed (EGSB) reactor (Pereira et al., 2002) or anaerobic filter (Alves et al., 2001), have been used for the anaerobic treatment of lipid-rich inflows. Also, novel reactor designs as the *Buoyant Filter Bioreactor* (BFBR) and the *Inverted Anaerobic Sludge Blanket* (IASB) reactor, have been proposed for the treatment of lipid-rich wastes and slaughterhouse wastewaters, respectively (Haridas et al., 2005; Alves et al, 2007). However, the treatment efficiency of these technologies is limited by the content of solids in the substrate, opposite to plug flow (PF) and continuously stirred tank reactors (CSTR) that allows higher solids content. Consequently, further process optimization and new technological developments are still required for the treatment of complex lipid-rich wastewater and, specifically, for that containing solid fatty waste substrates. Different modifications in the design of conventional anaerobic reactors have been proposed as well in order to overcome the problems related to sludge flotation and wash-out. Biomass recirculation (Hwu et al., 1997; Pereira et al., 2001) and partial phase separation (Kim and Shin, 2010) were assayed for that purpose.

The objective of the present study is to test and validate a new reactor system configurations that are suitable for the treatment of complex lipid-based solid waste. All limiting aspects on the anaerobic degradation of lipids, such as particulate substrate, slow hydrolysis rates, high suspended solids content, substrate-biomass flotation or wash-out, and possible process inhibition have been considered in the process design. The concept of this system combines *(i)* the waste pretreatment through saponification, *(ii)* the anaerobic digestion in a completely mixed anaerobic reactor and *(iii)* the inclusion of a solids recirculation stage.

5.2. MATERIAL AND METHODS

5.2.1. Slaughterhouse fatty waste

The solid waste used as substrate in the present experiments was flesh fat from cattle carcass collected in a slaughterhouse located in Narbonne (France). This substrate was homogenized, sieved at 5 mm to remove the fat-containing membranes and stored at -20°C. Fresh substrate was characterized in terms of total solids (TS), volatile solids (VS), total chemical oxygen demand (COD) and long chain fatty acids content (LCFA), according to Analytical Methods section.

5.2.2. Saponification pre-treatment conditions

Saponification consists on the reaction between a lipid and an alkali, resulting in the production of LCFA salts (soap) and glycerol release. The saponification pretreatment was conducted in batch-mode inside an enclosed glass reactor coupled with a condensates recovery system. The reaction was performed in alkali conditions (sodium hydroxide, NaOH, at 32 % w/w concentration) under continuous mixing (1,000 rpm) at 70ºC during 60 minutes. NaOH was added in stoichiometric excess, adopting an equivalent ratio of 0.04 $mol_{NaOH}.g^{-1}_{\ CODsubstrate}$, as proposed by Battimelli et al. (2009). The excess of alkali was checked during the pretreatment by monitoring the pH, which was maintained around 12.

Soaps were prepared at four different organic matter concentrations of fatty substrate (5 %, 10 %, 15 % and 30 % of VS). The soap concentration was selected taking into account the homogeneity and fluidity at room temperature. The efficiency of the saponification process was assessed by total and free LCFA determination, according to Analytical Methods section.

5.2.3. Experimental set-up

Experiments were conducted in three anaerobic continuously stirred tank reactors (CSTR) of 5 L, maintained at mesophilic conditions (35ºC). All reactors were inoculated with mesophilic granular sludge sampled from an UASB reactor of a sugar facility (Marseille, France). Sampled granular seed sludge was broken by high shear stress agitation and added into the CSTR reactors in a high concentration (32.9 ± 0.6 $g_{VSS}\ L^{-1}$), in order to obtain a

high initial biomass content. Three different operational strategies were investigated, summarized as follows:

- In the first reactor, or **configuration R1**, saponification pre-treatment was applied to the substrate, with the aim to enhance the emulsification and bioavailability of lipids. A recirculation step of the solid fraction of reactor outflow was also coupled to the anaerobic digestion process in order to overcome problems related with biomass/substrate flotation and wash-out. Effluent was manually withdrawn from reactors before each feeding cycle, centrifuged (6,500 rpm, 20 minutes at 20ºC) and the solid fraction reintroduced into the reactor, with a volumetric recirculation ratio around 20% of the outflow (w/w).
- In the second reactor, or **configuration R2**, the saponified substrate (soap) was anaerobically digested, but in this case the recirculation step was not applied in order to evaluate the influence of the solids recirculation, comparing the system performance and efficiency respect to configuration R1.
- In the third reactor, or **configuration R3**, raw waste (not saponified) was directly digested in the anaerobic reactor, including the effluent solid fraction recirculation step, with the aim of quantifying the influence of the saponification pre-treatment on process efficiency, comparing the system performance and efficiency respect to configuration R1.

The same operational procedure was followed in all tested configurations or reactors:

- Initial sludge starvation (**period 0**). In order to remove the residual biodegradable organic matter contained in the inoculum, CSTRs were maintained in batch conditions without feeding until residual biogas production. After that, the anaerobic activity of the sludge was evaluated by a series of easily biodegradable compound (ethanol) injections. In total, 9 ethanol pulses were performed and 3 increasing concentrations were tested: 0.9 g_{COD} L^{-1} (4x), 2.3 g_{COD} L^{-1} (3x) and 4.5 g_{COD} L^{-1} (2x). At the end of this period, the inoculum activity ($g_{COD\text{-}CH4}$ g_{VSS}^{-1} d^{-1}) and the minimal biogas production rate ($mL_{biogas}min^{-1}$), representing the endogenous biogas production, were determined for each reactor as described by Battimelli et al. (2009).

- Acclimation period (**period I**). The 3 different reactor configurations (R1, R2, and R3) were tested in pulses-feeding conditions. A new cycle (feeding of fatty waste) was started when the system presented a stable response to the tested inflow pulse (plateau in biogas production). A total of 12 pulses-feeding cycles were carried out, testing two different substrate concentrations: 1 g_{COD} L^{-1} from cycle 1 to 7 (period I.1) and 2 g_{COD} L^{-1} from cycle 8 to 12 (period I.2). As previously explained, saponification pretreatment (in R1 and R2) and solids recirculation (in R1 and R3) were applied when necessary.
- Semi-continuous feeding period (**period II**). In the semi-continuous mode, the reactors were fed 3 times per week with an equivalent hydraulic retention time (HRT) of 33 days. Two organic loading rates (OLR) were tested: 0.95 g_{COD} L^{-1} d^{-1} (period II.1) and 2.2 g_{COD} L^{-1} d^{-1} (period II.2), increasing the inflow organic concentration (g_{COD} L^{-1}) and maintaining the HRT. Saponification pretreatment and solids recirculation were also applied when necessary.
- Final starvation (**period III**). After the last substrate feeding (day 112), reactors were maintained in batch conditions in order to estimate the final degradation rates as well as the amount of substrate accumulated in the reactor along experimental time (up to day 156), in relation to the saponification and recirculation effects after long term operation of reactors.

During all these periods the reactors inflows (raw or saponified) were supplemented with macronutrients solution, as described in Alves et al. (2001). Biogas production and pH were continuously measured by a MilliGas counter flow meter (MGC-1, V3.1, Ritter ®, Germany) and a pH probe (pH InPro 4800/120/PT1000, Mettler Toledo, France), respectively. Gas flow rate and pH data were acquired by a PC acquisition system. Biogas volumes were calculated under standard conditions of temperature and pressure (STP). Once per week reactor outflows were analyzed in terms of total and volatile suspended solids (TSS/VSS), soluble chemical oxygen demand (CODs) and volatile fatty acid concentration (VFA). LCFA were also analyzed at the beginning of the starvation period (period III). All measurements were done according to Analytical Methods section.

5.2.4. Analytical Methods

Analytical measurements, i.e. total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), and chemical oxygen demand (COD) were determined according to Standard Methods (APHA, 1995). Due to the high lipid content of substrates, the COD analytical procedure was modified according to Noguerol et al. (2012). Biogas composition (CH_4 and CO_2) was determined using a Clarus 580 gas chromatograph (Perkin Elmer, USA) equipped with two capillary columns Rt Q-Bond (30mx0.32mmx10μm) and a Molsieve 5A (30mx0.32mmx30μm) and thermal conductivity detector (TCD). Volatile fatty acids (VFA) composition in the liquid phase was determined using a Clarus 580 gas chromatograph (Perkin Elmer, USA) equipped with an Elite-FFAP capillary column (15m x 0.53mm) and flame ionization detection (FID).

Total LCFA concentration, $LCFA_t$ (sum of glycerides and "free" LCFA, $LCFA_f$), from C12 to C24, were determined as fatty acids methyl esters (FAME) based on direct in situ trans-esterification protocol, as described by Palatsi et al. (2009). FAME were identified and quantified by GC CP-3800 gas chromatograph (Varian, USA), equipped with CP-Sil 88 FAME capillary column (50m0.25mm 0.2μm,) and FID detection. The quantification method of LCFA not forming part of glycerides, $LCFA_f$, was based on a multiple extraction-concentration procedure, as described elsewhere (Alosta et al., 2004). Briefly, 3x consecutive extractions were performed, including a Extraction Standard addition, (ES=Heptadecanoic acid, C17, Fluka puriss >99.0%) with acidified Hexane:Ether (1:1) mixture. Recovered organic phase was transferred to a GC vial, together with Internal Standard (IS=Tridecanoic acid, C13, Sigma puriss >99.0%). Due to the chromatographic method conditions, only $LCFA_f$ were separated, identified and quantified by GC CP-3800 gas chromatograph (Varian, USA), fitted with TRB-FFAP capillary column (30m×0.32mm×0.25μm) and FID detector. A mixture solution of individual unsaturated fatty acids standards (containing C10, C12, C14:0, C16:0, C18:0 and C20:0 standards, UN10-1KT, Supelco) was used for calibration.

5.3. RESULTS AND DISCUSSION

5.3.1. Substrates and biomass characterization

Slaughterhouse fatty wastes (raw and saponified) characteristics are summarized in Table 5.1. Raw slaughterhouse fatty waste had a high organic content (99.9% VS/TS and 2.6 g_{COD} g^{-1}_{waste}), mainly due to its high lipid content (Table 5.1). Saturated and mono-unsaturated fatty acids were the major identified component, representing 66.1% and 32.1% of lipid content, respectively. These values are in the range of what has been previously reported in slaughterhouse fractions (Palatsi et al., 2011; Valaldaõ et al., 2011). In particular, palmitic (C16:0), stearic (C18:0) and oleic acids (C18:1) are found to be the most important LCFA, representing 88.3% of the total lipid content, with respective individual fractions of 24.4%, 32.4% and 31.5%.

Similar results were previously reported by Battimelli et al. (2010), analyzing a similar slaughterhouse fatty waste. Difference between $LCFA_t$ and $LCFA_f$ measurements indicated that 94.7% of the lipidic matter of the flesh fat from cattle carcass was composed of glycerides and only 5.3% was present in free fatty acid form (Table 5.1). The maximum methane potential of the raw waste was estimated considering the COD of the individual $LCFA_t$ composition present in substrate.

To easily feed the saponified waste into anaerobic reactors, it was necessary to have a liquid media (soap). Only the soap prepared with a 5% of organic matter (%VS) was homogeneous and fluid at ambient temperature. The obtained substrate was composed by 89.1% of $LCFA_f$ and, only a 10.9% remained in glyceride form (difference between $LCFA_t$ and $LCFA_f$ values in Table 5.1). As a result, it can be concluded that the saponification pretreatment presented a lipid hydrolysis efficiency of 89%. Furthermore, the $LCFA_f$ composition in the saponified substrate was equivalent to the one quantified in the raw waste (24.6 % of C16:0, 34.0 % of C18:0 and 32.4 % of C18:1), confirming that the saponification at moderate temperature does not alter the LCFA composition (Kallel et al., 1994; Battimelli et al., 2010).

The initial biomass concentration inoculated in each reactor (period 0) was 32.9 ± 0.6 g_{VSS} L^{-1}. Biomass activity was determined through the successive ethanol injections in each

reactor. After successive increasing ethanol loads, a steady methane production was rapidly achieved with values of 327 ± 5, 340 ± 8 and 345 ± 5 L_{CH4} kg_{COD}^{-1} for R1, R2, and R3, respectively. The final obtained methane production represented a mean value > 95% of the theoretical methane potential. Consequently, the methanogenic activity in all reactors (0.27 ± 0.06, 0.23 ± 0.05 and 0.17± 0.02 $g_{COD\text{-}CH4}$ g_{VSS}^{-1} d^{-1} in R1, R2 and R3, respectively) was considered high enough to start substrate feeding. At the end of this period, the endogenous biogas production was also estimated in each reactor, with a mean value for all reactors of 0.14 ± 0.03 mL_{biogas} min^{-1}. This value was considered in further assays to calculate net biogas production and to estimate COD balance.

Table 5.1 *Raw and saponified substrate characterization*

Parameter	**Raw waste**	**Saponified waste**
TS ($g\ kg^{-1}$)	979.1	ND
VS ($g\ kg^{-1}$)	978.6	ND
Total COD ($g_{COD}\ g^{-1}_{waste}$)	2.60	ND
$LCFA_t$ ($g_{COD}\ g^{-1}_{waste}$)	2.51	2.38
C12:0	0.001	0.001
C14:0	0.073	0.067
C16:0	0.634	0.590
C18:0	0.843	0.817
C18:1	0.820	0.778
other LCFA	0.139	0.128
$LCFA_f$ ($g_{COD}\ g^{-1}_{waste}$)	0.129	2.12
C12:0	-	-
C14:0	0.001	0.054
C16:0	0.019	0.507
C18:0	0.039	0.744
C18:1	0.068	0.704
other LCFA	< 0.001	0.101
% $LCFA_f$ (COD-$LCFA_f$/COD-$LCFA_t$)	5.3 %	89.1%
% Glycerides (COD-$LCFA_t$/COD)	96.4 %	nm*

**nm-. Not measured*

5.3.2. Reactors performance

The instant biogas production, expressed as L_{biogas} $hour^{-1}$, during the acclimation (period I) and the semi-continuous feeding periods (period II) is represented in Figure 5.1 and Figure 5.2, respectively. A summary of the operational conditions, efficiency (in organic matter degradation) and methane yields for each reactor or strategy are presented in Table 5.2.

With the aim to adapt the biomass to degrade fatty substrates, low feeding patterns were applied during period I. Pulses were repeated with a low frequency, giving enough time for efficient substrate degradation. Initially, weak and slow substrate degradation was observed in all reactors. Furthermore, the biogas potentials in R2 and R3 during the first 2 cycles were as low as the endogenous biogas production. Thus, those cycles were considered as the start-up of the experiment (grey curves, in Figure 5.1), and were not included in parameters calculation of Table 5.2.

After this initial start-up, the biogas production was progressively improved in all the reactors during period I.1, along the successive feeding pulses (indicated by vertical dashed lines in Figure 5.1). The "equivalent" organic loading rate during period I.1 was estimated in 0.22 g_{COD} $L^{-1}day^{-1}$ (Table 5.2). The reactors treating saponified waste (R1 and R2) displayed very similar biogas production, reaching a maximum value of 0.17 L_{biogas} $hour^{-1}$. In these reactors, the substrate (in terms of COD) was efficiently converted into CH_4 (98% and 91% in R1 and R2, respectively, according to Table 5.2). The lowest system performance was obtained in the reactor treating raw waste (R3), with a low organic matter degradation and low methane yield (59% COD and 206 L_{CH4} kg_{COD}^{-1} respectively, according to Table 5.2). Even after the long period of low frequency pulses (end of period I.1) the maximum instantaneous biogas production in R3 was still very low, around 0.13 L_{biogas} $hour^{-1}$ (Figure 5.1).

During period I.2, the pulse-feeding frequency was increased up to an "equivalent" OLR of 0.9 g_{COD} L^{-1} day^{-1}, introducing a clear instability or inhibitory effects over biogas production, according to the reported values of Table 5.2. The COD degradation rate was notably decreased, down to 62% and 58% respectively in R1 and R2, showing that the applied OLR or feeding frequency was in the limiting levels of biomass tolerance.

The COD degradation rate was also slightly decreased in the reactor treating raw waste, R3, (from 59% to 54%) during this period. At the end of this period (period II.2) it was reported in all the reactors outflows the highest soluble COD values (0.30-0.38 g_{COD} L^{-1}), with a maximum in R2 rector (0.38±0.15 g_{COD} L^{-1}).

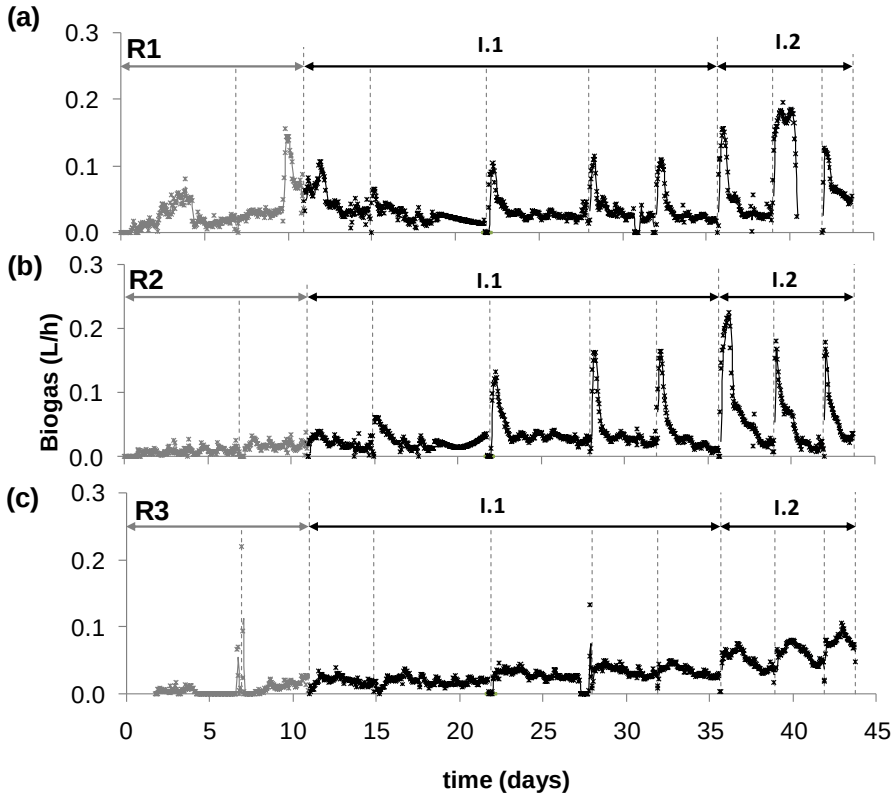

Figure 5.1 *Instant biogas production (L_{biogas} $hour^{-1}$) during the pulses-feeding period (period I) for reactor configurations (**a**) R1, (**b**) R2 and (**c**) R3. Periods I.1 and I.2 have an estimated OLR of 0.2 and 0.88 g_{COD} L^{-1} d^{-1}, respectively. The vertical dashed lines indicate timing of each feeding cycle, while gray part of the curves represent the start-up period, not considered for parameter estimation.*

Table 5.2 *Summary of anaerobic system set-ups and obtained efficiencies, for each reactor configuration (R1, R2 and R3) and periods (I.1, I.2, II.1 and II.2). Results of the system start-up of each period (first 2 feedings) were not included in mean parameters estimation.*

	HRT (days)	**OLR** (g_{COD} L^{-1} day^{-1})	**Recirculation** (% w/w)	***CH_4*** **production** (L_{CH4} kg^{-1} $_{COD}$)	**CH_4 content** (%)	**$COD_{degradation}$** (%)	**Maximum CH_4 rate** (L_{CH4} L^{-1} $_{reactor}$.day^{-1})
Period I.1							
R1	163.7 ± 49	0.221 ± 0.06	20.7 ± 0.03	344 ± 75	76 ± 0.02	98	0.37
R2	164.3 ± 50	0.222 ± 0.06	-	318 ± 133	75 ± 0.03	91	0.41
R3	164.5 ± 50	0.233 ± 0.05	20.9 ± 0.01	206 ± 76	69 ± 0.08	59	0.15
Period I.2							
R1	85.6 ± 20	0.877 ± 0.23	21 ± 0.004	218 ± 71	75 ± 0.004	62	0.50
R2	86.2 ± 20	0.873 ± 0.29	-	203 ± 95	75 ± 0.004	58	0.42
R3	86.7± 20	0.892 ± 0.34	21.7 ± 0.01	190 ± 23	71 ± 0.01	54	0.28
Period II.1							
R1	33.19 ± 0.17	0.949 ± 0.01	19.4 ± 0.01	325 ± 49	74 ± 0.01	93	0.71
R2	33.09 ± 0.14	0.948 ± 0.01	-	302 ± 53	74 ± 0.01	86	0.70
R3	33.14 ± 0.17	0.941 ± 0.02	20.3 ± 0.01	324 ± 50	72 ± 0.01	93	0.46
Period II.2							
R1	33.18 ± 0.31	2.130 ± 0.06	19 ± 0.01	341 ± 98	75 ± 0.02	97	1.71
R2	33.08 ± 0.30	2.151 ± 0.03	-	315 ± 92	77 ± 0.02	92	1.32
R3	33.20 ± 0.30	2.164 ± 0.01	20 ± 0.01	302 ± 75	74 ± 0.02	86	1.12

In this reactor it was also detected the maximum acetate concentration (0.41±0.04 g_{COD} L^{-1}), while values in R1 and R3 were always below 0.20 g_{COD} L^{-1}(data not shown). However, the maximum methane rate (L_{CH4} $L^{-1}_{reactor}$ day^{-1} in Table 5.2) was clearly increased in reactors that included solids recirculation (R1 and R3). Not absolute conclusions can be obtained from this period, because part of COD that had not been degraded in the previous pulses could interfere on the system response, and consequently on the corresponding parameters estimation. Nevertheless, during the whole pulse-feeding period (period I), the main differences between reactors performance seems to depend more on the type of the substrate (raw or saponified waste) than on the solids recirculation step, as expected, due to the low re-circulation rate.

The two first feedings cycles during the semi-continuous period, period II (gray curve, in Figure 5.2), were also not considered for parameter estimation (Table 5.2), as they were considered as a transition period, necessary to fix the reactors HRT. During the period II.1, the HRT was stable at 33 days, and the OLR was fixed close to 1 g_{COD} L^{-1} day^{-1}. During this period, clear differences were observed between R1 and R3 in relation to the kinetic of biogas production (Figure 5.2 and maximum methane rates of Table 5.2). However, no great difference was detected in the global COD degradation efficiency, with a mean value for both reactors of 93% (Table 5.2). Probably, the long HRT and the low tested OLR (0.94-0.95 g_{COD} L^{-1} day^{-1}) were suited enough to efficiently degrade both LCFA (in R1) and glycerides (in R3). Also, clear differences between reactors treating saponified waste, with or without the solids recirculation step (R1 and R2), were detected throughout this period (period II.1), evidencing the beneficial effect of solids recirculation.

Maintaining the HRT stable at 33 days, the OLR was increased up to values close to 2 g_{COD} L^{-1} day^{-1}, during period II.2. In general, the performance of all the tested strategies or reactors was improved during this period. At the end of this period, the instantaneous biogas production reached maximum values of 0.6, 0.4 and 0.31 L_{biogas} $hour^{-1}$ in R1, R2 and R3, respectively (Figure 5.2). Several explanations to these results are plausible: *i)* the recovery of washed-out LCFA by recirculation and *ii)* the increase of LCFA-adapted biomass inside reactor, reducing the ratio inhibitor-substrate/microorganisms. Pereira et al. (2001) reported the benefits of LCFA-associated biomass recirculation in anaerobic filters.

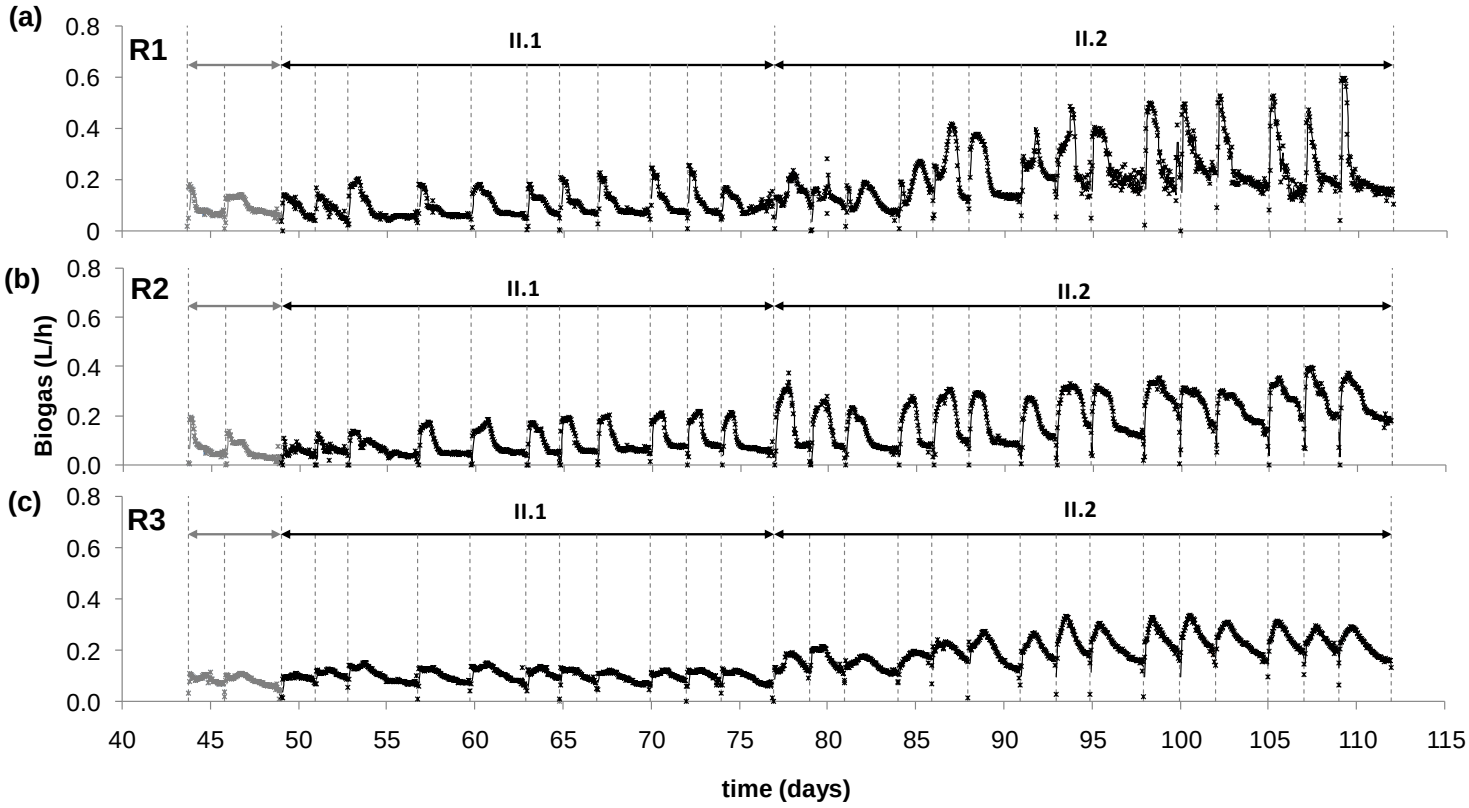

Figure 5.2 *Instant biogas production (L_{biogas} $hour^{-1}$) during the semi-continuosus feeding period (period II) for reactors configuration (**a**) R1, (**b**) R2 and (**c**) R3. Periods II.1 and II.2 have an estimated OLR of 0.94 and 2.0 g_{COD} L^{-1} d^{-1}, respectively. The vertical dashed lines indicate timing of each feeding cycle, while gray part of the curves represent the start-up period, not considered for parameter estimation.*

The effect of washed-out biomass recirculation over methane production in EGSB reactors has been also previously reported by Hwu et al (1997). These authors concluded that the recirculation of solids allowed the development and retention of highly concentrated LCFA-oxidizers per unit of volatile suspended solids. The specific effect of the studied reactor configurations over substrate wash-out prevention or over biomass enrichment-adaptation is further discussed in the next section, considering the results of the starvation period (period III), the COD balance and a specific analysis of the evolution of methane rates.

5.3.3. Solids recirculation and biomass adaptation

To study the effect of the reactor configuration (R1, R2 and R3) on the possible biomass/substrate wash-out, weekly samples of reactors content were collected during the experimental time to monitor the VSS evolution (Figure 5.3).

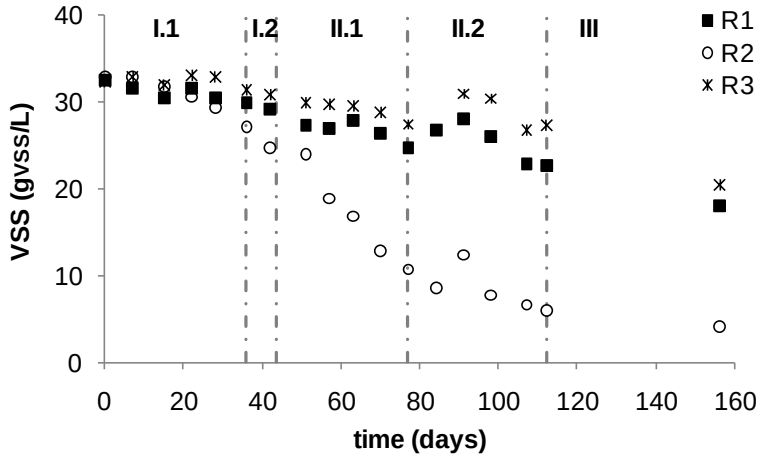

Figure 5.3 *Suspended solids concentration, VSS (g_{VSS} L^{-1}), along experimental time, for each reactors configuration: R1 (■), R2(○) and R3(*). The vertical dashed lines indicate the different periods I.1, I.2, II.1 and II.2.*

As shown in Figure 5.3, the VSS concentration decreased in all tested reactor configurations and differences between reactors were mainly related to the applicability of solids recirculation step. As expected, the highest VSS content was maintained in reactors configured with the solids recirculation step (R1 and R3). Its VSS content presented a slow tendency of decrease along sampling time, in comparison with the reactor without solids recirculation step (R2), where the total VSS content was

drastically reduced up to values of 5.89 g_{VSS} L^{-1}. Throughout starvation period (period III in Figure 5.3), all reactors suffered a VSS reduction due to the expected residual COD consumption and biomass decay in batch conditions.

To elucidate about which VSS reduction during the starvation period corresponds to previously not degraded substrate and/or to biomass decay, the accumulated methane produced during starvation period, expressed as $g_{CH4\text{-}COD}.L^{-1}{}_{reactor}$, was monitored (Figure 5.4). Also, reactor samples were collected at the beginning of the starvation period and $LCFA_t$ concentrations were quantified to reveal a possible LCFA accumulation and to estimate the COD balance during the starvation period (Table 5.3).

Table 5.3 *Organic matter balance (COD) during starvation period (period III) for reactor configurations R1, R2 and R3*

Parameter (g_{COD} L^{-1})	**estimation**	**R1**	**R2**	**R3**
LCFAt detected before starvation (day 112)	A	4.95	3.50	11.76
COD added in last feed (day 112)	B	4.70	4.70	4.70
Total available COD during starvation period	C = (A+B)	9.65	8.20	16.46
Accumulated methane production (day 156)	D	12.14	10.4	25.17
Produced methane, not from substrate (day 156)	E = (C-D)	2.49	2.20	8.71

The effluent sampled from R1 at the end of feeding period (period II.2) accumulated an amount of 4.95 $g_{LCFAt\text{-}COD}$ L^{-1} (Table 5.3). One hour after taking this sample, R1 was submitted to the last feeding pulse (day 112) of 4.70 g_{COD} L^{-1}(Table 5.3) and was maintained at batch conditions (starvation) during 44 days. The total estimated COD content (9.65 g_{COD} L^{-1}) was subsequently degraded during the starvation period, producing 12.14 $g_{COD\text{-}CH4}$ L^{-1} (Table 5.3). Consequently, all the introduced COD was successfully degraded (in less than 10 days, Figure 5.4) and 2.49 $g_{COD\text{-}CH4}$ L^{-1} of the produced methane was assumed to come from decayed biomass (estimated in 1.75 g_{VSS} L^{-1}), according to Table 5.3. When the solids recirculation step was not applied, in R2, the VSS concentration declined dramatically (as seen in Figure 5.3). Along experimental time, we noted that the effluent collected from R2 was more diluted,

compared with those withdrawn from reactor with biomass recirculation (R1 and R3) and floating particles were observed in the reactor outflow. $LCFA_t$ analysis indicated that this reactor accumulated the lesser amount of $LCFA_t$ (3.50 $g_{COD\text{-}LCFA}$ L^{-1} in Table 5.3). At the end of the starvation period, R2 presented the lowest biogas production, 10.4 $g_{COD\text{-}CH4}$ L^{-1} (in Figure 5.4 and Table 5.3). These results evidenced the substrate and biomass wash-out, that leaves the reactor with the effluent. If it is assumed that all the remaining COD was successfully degraded, 2.20 $g_{COD\text{-}CH4}$ L^{-1} of the produced methane was not from substrate but due to biomass decay (estimated in 1.55 g VSS/L), according to Table 5.3. The reactor treating raw waste (R3) accumulated the highest amount of $LCFA_t$ (11.76 $g_{COD\text{-}LCFA}$ L^{-1}, Table 5.3), that was later degraded in the starvation period. Therefore, R3 registered the higher and slowest methane production, 25.17 $g_{COD\text{-}CH4}$ L^{-1} (in Figure 5.4 and Table 5.3), during starvation period and, also, the higher residual methane production (8.71 g_{COD} L^{-1}, according to Table 5.3). It must be noted that in the estimation of this high residual COD value, could have interfere some floating glycerides, not sampled in the LCFA determination (day 112) but finally degraded (day 156) producing methane. Anyway, considering that both R1 and R3 configurations include a recirculation step, the only reason for a higher residual production in R3 should be related with a higher biomass content. According to the substrate characteristics (raw waste) in this reactor it is necessary the presence not only of B-oxidizing/syntrophic methanogens community but also of hydrolytic population.

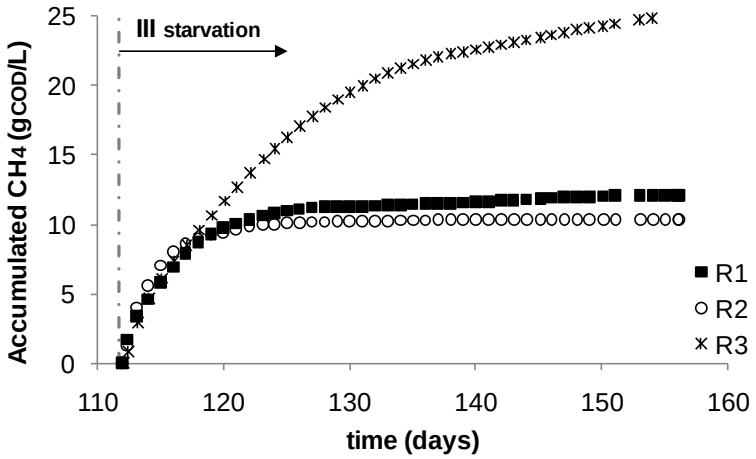

Figure 5.4 *Specific methane production ($g_{CH4\text{-}COD}$ L^{-1}), during the starvation period (period III), for each reactor configuration: R1 (■), R2(○) and R3(*).*

According to the obtained results, the solids recirculation step appears as a promising technique in the anaerobic digestion of fatty residues since it prevents the wash-out of biomass-substrate and improves system efficiency. To elucidate about the hypothesis of an improvement on process efficiency related with a specific biomass growth or adaptation phenomena, the methane rates (calculated as initial slopes of methane production and expressed in L_{CH4} $L^{-1}_{reactor}$ day^{-1} units) obtained after each substrate feeding cycle were estimated for each reactor configuration (R1, R2 and R3). Those methane rates, plotted along experimental time, are fitted to sigmoid curves, as a function shape that can be related with biomass growth (in Figure 5.5). The maximum methane rates observed in each operational period (period I and II) have been previously summarized in Table 5.2.

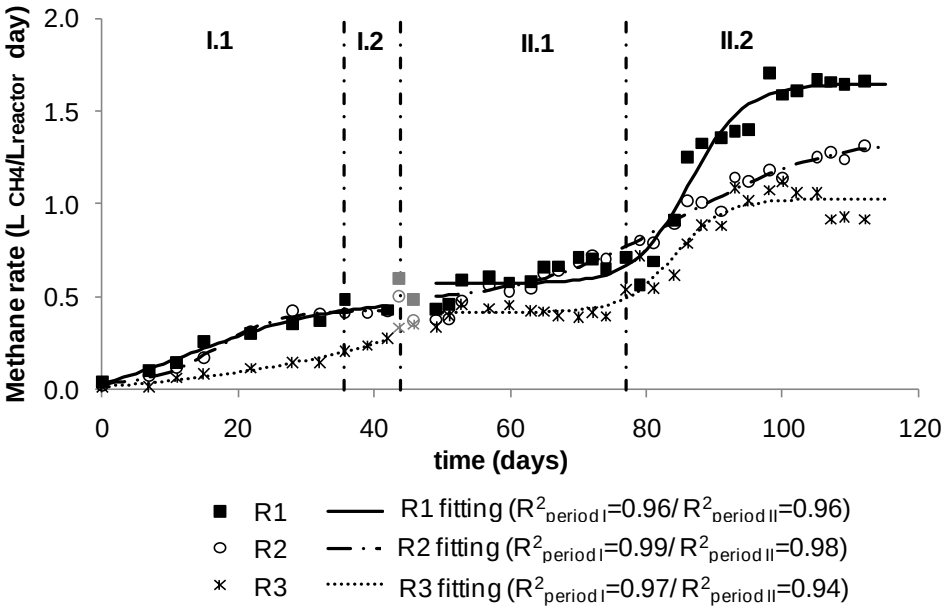

Figure 5.5 *Experimental data (point markers) and sigmoidal fitting curves (lines) of methane rate (L_{CH4} $L_{reactor}$ day^{-1}), along experimental time, for each reactor configuration: R1 (■), R2(○) and R3(*). Determination coefficients (R^2) of curve fittings for each reactor and operational period (Period I or II) are also indicated.*

In general, all the reactors showed an enhancement of methane rates along time. During pulses feeding operating period (period I), reactors treating saponified waste, with or without solids recirculation (R1 or R2), exhibited a similar evolution of the methane rates along time. In fact, it is expected that the recirculation at low frequency does not

significantly affect the biomass concentration in the system (due to the low recirculation rate) and, thereby, reactors R1 and R2 might present similar evolution during this period. The main differences were again reported in the reactor treating raw waste (R3), where the lower methane rates pointed to a possible limitation in the hydrolysis process. It have been previously reported that in systems treating complex wastes the hydrolysis step could be an important bottleneck of the overall anaerobic process (Masse et al., 2003). Petruy and Lettinga (1997) attributed the poor biodegradation of milk-fat emulsion to the limiting rate of liquefaction, as evidenced by the low hydrolysis constant, k_{hyd} of 0.01 day^{-1}. Similar results were also found by Sayed et al. (1988) treating slaughterhouse wastewaters with high amounts of suspended solids. On the other hand, difference in methane rates evolution between R1 and R2 were revealed when OLR was increased (Period II, in Figure 5.5). During this period, reactors designed with solids recirculation (R1 and R3) were able to rapidly adapt to an increase on organic loading rate (transition from 1 to 2 g_{COD} L^{-1} day^{-1} between period II.1 and II.2 in Figure 5) whereas the reactor without biomass recirculation (R2) exhibited a slow transition. Consequently, the benefits of recirculation process over biomass enrichment or adaptation were clearly stated. Also the long term operation of reactors in contact with LCFA or glycerides could have influenced the system response. Cavaleiro et al. (2009) recommended the application of a step-feeding start up procedure based on sequencing feeding-batch reaction phases, with a stepwise increased feeding rate, in order to produce a specialized and efficient anaerobic community for LCFA mineralization. Nielsen and Ahring (2006) and Palatsi et al. (2010) also improved the biomass adaptation through successive biomass contact to lipids/LCFA. Further complementary studies, based on high throughput sequencing techniques (massively parallel pyrosequencing), are currently underway with reactors biomass samples in order to check these hypothesis and to analyze the population dynamics dependence on reactor operation and configuration.

5.4. CONCLUSIONS

A novel system configuration concept to improve the anaerobic treatment of solid fatty slaughterhouse waste was tested and evaluated. The system is based on the application of saponification pretreatment prior to anaerobic digestion process, and on the inclusion

of a recirculation of the solid fraction of the effluent. An enhancement on methane rates and on COD degradation efficiency was achieved. Saponification is a promising pretreatment procedure for enhancing the emulsification and bioavailability of solid fatty residues, while solids recirculation minimizes the substrate/biomass wash-out and induces the microbial community adaptation to the treatment of lipid/LCFA based substrates.

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Chapter 6. Microbiome dynamics during the anaerobic treatment of slaughterhouse wastes

This chapter outlines the study performed using high throughput sequencing technique (454-pyrosequencing) to investigate the microbiome dynamics during the anaerobic treatment of solid slaughterhouse fatty waste. A total of 18,027 and 12,571 filtered-qualities DNA reads of bacterial and archaeal domains, respectively, were retrieved from 4 reactors biomass samples and were analysed to evaluate the microbial biodiversity and to identify the potential important players in the process. Especial attention has been given to the effect of lipid pre-treatment by saponification and to the recirculation of solids from the reactor outflow. Feeding with fatty substrate leaded to drastic reductions in the bacterial diversity, pointing to an enrichment process towards more specific and adapted bacterial population that was highly dependent on the substrate characteristics (saponified or raw waste). The anaerobic treatment of fatty substrate was linked to a strong development of representatives from *Syntrophomonadaceae* and *Syntrophaceae* families. However, the predominance of *Syntrophus spp.* above *Syntrophomonas* in the biomass treating raw waste points to the fact that it plays a crucial role in the anaerobic degradation of more complex substrates. Contrary, the archaeal diversity was increased along reactor operation, reflecting an enrichment of a more complex microbial community. This enrichment process was more impacted by solids recirculation than by the substrate pre-treatment (saponification). A clear shift from the obligate acetotrophic Methanosaeta genus to hydrogenotrophic archaeobacteria was observed in all the reactors, particularly to *Methanospirillum ssp.*

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6.1. INTRODUCTION

In the recent years, the optimization of anaerobic digestion (AD) efficiency when treating different wastes, in particular those containing significant amounts of lipids, has received a great deal of attention. These substrates are characterized by a high potential biogas yields but lipids are also one of the most problematic components in AD systems, causing sludge flotation and biomass/substrate wash-out, as well as inhibition of the microbial activity (Hwu et al., 1998). In order to overcome these process limitations, several approaches have been tested in AD reactors treating lipid-rich substrates, such as the implementation of thermal, alkali or enzymatic hydrolytic pre-treatments (Battimelli et al., 2010; Mendes et al., 2006), the application of feeding strategies based on accumulation-degradation stages (Cavaleiro et al., 2009), the use of acclimated biomass (Cavaleiro et al., 2010) or the development of high solids retention systems (Alves et al., 2007), as well as some of these strategies combined (Affes et al., 2013).

In the anaerobic digestion process, the hydrolysis of lipids yields long chain fatty acids (LCFA) and glycerol. Glycerol is further fermented to volatile fatty acids (VFA) and alcohols while LCFA are further converted to acetate (Ac) and hydrogen (H_2) via the β-oxidation process and, finally, to methane (CH_4) and carbon dioxide (CO_2), gas mixture known as biogas, formed during the so-called methanogenesis. Hydrolysis is generally considered as a relatively fast process, but some authors have demonstrated that it could be the rate limiting step in the case of particulate substrates, including lipids (Mendes et al., 2006). Once lipids have been hydrolyzed to LCFA, these latter compounds need to be adsorbed onto the anaerobic biomass for further degradation (Hwu et al., 1998). This adsorptive process can affect the external transport of nutrients and the protective function of the cell membrane, thus causing microbial inhibition (Zonta et al., 2013). This inhibitory process is generally considered to be a reversible process, depending on the LCFA type (saturated/un-saturated) and the microbial community structure of the associated degrading biomass (Sousa et al., 2009; Zonta et al., 2013). Also, the β-oxidation process is not thermodynamically feasible without syntrophic concurrence of acetogenic bacteria (β-oxidizers) and acetoclastic-hydrogenotrophic archaea (McInerney et al., 2009).

The understanding of the microbial interactions involved in the overall degradation process is thus essential to optimize the AD of lipid-rich wastes. Up to date, 14 syntrophic fatty acid degrading bacterial species have been isolated and characterized but only 7 of these species were described as capable of degrading LCFA with more than 12 carbon atoms. They all belong to the families *Syntrophomonadaceae* and *Syntrophaceae*, within the phyla *Firmicutes* and delta-*Proteobacteria*, respectively, while the two main syntrophic partners were closely affiliated to the genera *Methanobacterium* and *Methanospirillium* (Sousa et al., 2009). Despite this general knowledge, only few studies have been performed in reactors treating complex or "real" lipid-containing wastes, such as agro-food industry wastewaters or slaughterhouse solid waste (Rosa et al., 2009; Palatsi et al., 2011). The microbial community has typically been analyzed by culture-independent molecular approaches that are based on the extraction of total DNA and PCR amplification and sequencing of 16S rDNA genes, e.g. clone library sequencing or molecular profiling by Denaturing Gradient Gel Electrophesis (DGGE). In some studies, RNA-based stable isotope-probing (SIP), fluorescence in situ hybridization (FISH) and quantitative PCR (qPCR) have been also used (Hatamoto et al., 2007; Shigematsu et al., 2006). The inconvenient of all these techniques is the relatively limited number of ribotypes that can be sequenced, thus providing a very limited depiction on the complexity of the whole microbial community. The development of the 454-pyrosequencing, a second-generation massive sequencing technology based on the sequencing-by-synthesis principle, has opened new possibilities of high-throughput and cost-effective exploration of complex microbiomes, such as those present in anaerobic digesters (Kröber et al., 2009; Schlüter et al., 2008; Krause et al., 2008; García et al., 2011). Yet, to our knowledge, no specific studies have been performed on the microbial communities from anaerobic digesters involved in lipid degradation.

The aim of this study was to investigate the microbial community structure of the biomass enriched in anaerobic reactors for treating solid slaughterhouse fatty waste. Especial attention has been given to the effects of lipid pre-treatment by alkaline hydrolysis (saponification) and to the recirculation of solids from the reactor outflow. A high throughput sequencing strategy was implemented for the in-depth 16S rRNA microbiome analysis of both eubacteria and archaeobacteria domains.

6.2. MATERIAL AND METHODS

6.2.1. Bioreactors operation and biomass sampling

Different strategies to optimize the biodegradation and energy recovery from slaughterhouse solid fatty waste (fresh fat from cattle carcass) were evaluated in three laboratory-scale anaerobic digesters (R_1, R_2 and R_3) of 5L each, operated in parallel under mesophilic conditions. These strategies consisted on system feeding with saponified (R_1 and R_2) or fresh (R_3) fatty substrate, and with the recirculation of solids (R_1 and R_3) versus a non-recirculation operation mode (R_2). Substrate characteristics, saponification and recirculation conditions, as well as the detailed description of the experimental set-up, are extensively reported in Affes et al. (2013) (**Chapter 5**)

Biomass samples were withdrawn at the beginning and at the end of each reactor operation, and kept at -22ºC until further processing. All reactors were inoculated with mesophilic granular sludge sampled from an UASB reactor of a sugar industry facility (Marseille, France). Before feeding with slaughterhouse waste, the reactors were subjected to a starvation stage in order to remove the residual organic matter from the inoculum (period P_0). At the end of this period (set as t=0), all the collected samples were pooled into one single sample, codified as S_0, since the operational conditions during this preliminary phase were virtually identical in all the reactors. Operation with slaughterhouse waste (fresh or saponified) was initiated in a pulse-feeding regime (P_{PF}), consisting in the application of discontinuous feeding cycles during 41 days. A total of 12 pulses-feeding cycles were carried out, testing two substrate concentrations (1 and 2 $g_{COD}\ L^{-1}$). Subsequently, reactors were operated in a semi-continuous feeding mode (P_{SC}) in which hydraulic retention time (HRT) was set at 30 days (including solids recirculation when necessary, as in R_1 and R3). During this period the reactors were fed three times per week during 73 days and two organic loading rates (OLR) were tested, 0.95 and 2.15 $g_{COD}\ L^{-1}\ d^{-1}$. The experiments were finished after 114 days of operation, when biomass samples from each reactor were collected and codified as S_1, S_2 and S_3, respectively.

6.2.2. Molecular processing conditions

Microbiome analysis using 16S rRNA sequences was performed on both eubacterial and archaeobacterial microbial domains. Genomic DNA from each biomass sample (S_0,

S_1, S_2 and S_3) was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The quantity and quality of the DNA was verified by electrophoresis on a 0.7% (w/v) agarose gel and visualized with ultra violet light upon staining with ethidium bromide. Tag-encoded FLX-amplicon pyrosequencing (TEFAP) of bacterial and archaeal 16S rRNA genes was performed on a Genome Sequencer FLX instrument using Titanium protocols and reagents (Roche Applied Sciences, Indianapolis, USA). Concerning the PCR conditions, one single amplification step with a total of 30 cycles was carried out. The reaction solution contained a mixture of Hot Start and HotStar high-fidelity Taq polymerases. Concerning the primers targeting 16S rRNA genes, the primer pairs Gray28F *5′ GAGTTTGATCNTGGCTCAG* and Gray519r *5′ GTNTTACNGCGGCKGCTG* were used for targeting the eubacterial domain, while the archaeobacteria were amplified with the ARCH 349F *5′ GYGCASCAGKCGMGAAW* and the ARCH 806R *5′ GGACTACVSGGGTATCTAAT* primer set. A detailed description of PCR amplification and pyrosequencing procedures are reported in Dowd et al. (2008). Obtained sequences were submitted to Short Read Archive (SRA) of NCBI with accession number SRA067024.

6.2.3. Bioinformatics and data analysis

The processing of the obtained DNA reads was mainly conducted with the software MOTHUR v1.24, based on the pipelines described by Schloss et al. (2009). Raw reads were screened and denoised, removing low-quality sequences. These filtered reads were then aligned, and screened to reduce sequencing noise, by a pre-clustering step. Chimeric sequences were also removed using UChime tool. Sequences were assigned to operational taxonomic units (OTUs) using the average neighbour method implemented in MOTHUR, considering a cut-off value of 3% (sequence similarity of 97%). The defined OTUs were used to calculate the microbial richness (S_{obs} and *Chao*1) and diversity (*Shannon*, *invSimpson* and sampling *coverage*) estimators. Rarefaction analyses were also performed using the same software package. The CANOCO software package for multivariate analysis (Wageningen Center for Biometry, The Netherlands) was used for performing a Correspondence Analysis (CA) on the OTUs abundance matrix of both *Eubacteria* and *Archaeobacteria*. The obtained samples and

OTUs scores were depicted in a 2D biplot, which represented the phylogenetic assignment of the predominant OTUs (relative abundance >0.5%). The processed sequences were also phylogenetically assigned by bootstrap analysis (cut-off score of 50%) on Greengenes (GG) taxonomy data set to represent community composition of each individual biomass sample. The predominant identified microorganisms were also compared to the NCBI genomic database with the BLAST search alignment tool (Altschul et al., 1990).

6.3. RESULTS

6.3.1. Bioreactors performance and sampled biomass.

The operational conditions and main process efficiencies obtained in the three assayed system configurations (R_1, R_2 and R_3) are summarized in Table 6.1. A more detailed description of the experimental results can be found in Affes et al (2013) (**Chapter 5**). To summarize, the best performance in terms of substrate degradation, biogas yields and methane production rates were achieved when both saponification pre-treatment and solids recirculation were applied, in system R_1. It has been suggested that both strategies act synergically. Saponification improved the bioavailability of LCFA while diminishing the hydrophobicity of lipids. On the other hand, the recirculation of solids resulted in the substrate dilution which also minimizes the substrate and biomass wash-out, thus ultimately prompting the enrichment and adaptation of active biomass (Affes et al., 2013).

Pyrosequencing analysis on the depicted reactors samples yielded 18,027 DNA eubacterial reads. The length of these sequences ranged from 150 to 555 bp, with an average value of 429 bp. The 24.6% of these reads were removed upon quality filtering and data processing (remaining 13,598 reads with an average length of 168 bp). Concerning the archaeal domain, pyrosequencing yielded 12,571 filtered-quality reads (mean length of 298 bp) for bio-informatics post-processing. Both bacterial and archaeal data were used in subsequent biodiversity and community structure analysis. In the following sections is described how the reactors operation along time (from initial, P_0, to final, P_{SC}) with the different system configuration (R_1, R_2 and R_3) induced specific changes in the microbial community of sampled biomass (S_0, S_1, S_2 and S_3).

Table 6.1 *Summary of the different system configurations, operating conditions and obtained anaerobic process efficiencies.*

System	Period	HRT (days)	*OLR* ($g_{COD}\ L^{-1}day^{-1}$)	*Saponification*	*r* Recirculation (%w/w)	*B* Biogas ($L_{biogas}\ kg^{-1}_{COD}$)	D, Degradation (%COD)	(v_{max}) maxCH_4 rate ($L_{CH4}\ L^{-1}_{reactor}.day^{-1}$)
R_1	P_0	-	-	-	-	438.27 ± 8.50	91	-
	P_{PF_1}	163.7 ± 49*	0.22 ± 0.06*	*yes*	20.7 ± 0.03	371.50 ± 102.11	98	0.37
	P_{PF_2}	85.6 ± 20*	0.88 ± 0.23*	*yes*	21.0 ± 0.01	290.84 ± 94.83	62	0.50
	P_{SC_1}	33.2 ± 0.2	0.95 ± 0.01	*yes*	19.4 ± 0.01	444.36 ± 66.87	93	0.71
	P_{SC_2}	33.2 ± 0.3	2.13 ± 0.06	*yes*	19.0 ± 0.01	448.80 ± 131.33	97	1.71
R_2	P_0	-	-	-	-	456.27 ± 17.8	96	-
	P_{PF_1}	164.3 ± 50*	0.22 ± 0.06*	*Yes*	*No*	323.77± 177.52	91	0.41
	P_{PF_2}	86.2 ± 20*	0.87 ± 0.29*	*Yes*	*No*	271.87 ± 127.69	58	0.42
	P_{SC_1}	33.1 ± 0.1	0.95 ± 0.01	*Yes*	*No*	374.80 ± 112.69	86	0.70
	P_{SC_2}	33.1 ± 0.3	2.15 ± 0.03	*Yes*	*No*	409.54 ± 120.14	92	1.32
R_3	P_0	-	-	-	-	464.37 ± 26.4	99	-
	P_{PF_1}	164.5 ± 50*	0.23 ± 0.05*	*No*	20.9 ± 0.01	216.24 ± 110.59	59	0.15
	P_{PF_2}	86.7 ± 20*	0.89 ± 0.34*	*No*	21.7 ± 0.01	267.12 ± 34.10	54	0.28
	P_{SC_1}	33.1 ± 0.2	0.94 ± 0.02	*No*	20.3 ± 0.01	432.10 ± 68.88	93	0.46
	P_{SC_2}	33.2 ± 0.3	2.16 ± 0.01	*No*	20 ± 0.01	407.95 ± 101.73	86	1.12

** Estimated*

Table 6.2 *Estimates of richness and diversity values for bacterial operational taxonomic units (OTUs), defined with 97% of similarity, for the different reactor samples and their corresponding confidence intervals (c.i.) with α=0.05.*

Sample	nseqs	sobs	Chao		Shannon		1/Simpson		Coverage
		OTUs	mean	(c.i.)	mean	(c.i.)	mean	(c.i.)	(%)
S_0	1,792	473	948.3	814.8 –1,134.1	5.0	5.0 – 5.1	51.1	44.8–59.4	84.5
S_1	4,690	189	377.3	299.4 – 510.2	1.5	1.4 – 1.5	1.7	1.6 – 1.7	97.8
S_2	5,731	188	386.7	304.3 – 527.6	1.4	1.4 – 1.5	1.7	1.7 – 1.8	98.1
S_3	1,385	163	300.3	239.8 – 408.5	31	3.0 – 3.2	6.8	6.1 – 7.6	93.9

Table 6.3 *Estimates of richness and diversity values for archaeal operational taxonomic units (OTUs), defined with 97% of similarity, for the different reactor samples and their corresponding confidence intervals (c.i.) with α=0.05.*

Sample	*nseqs*	s_{obs}	*Chao*		*Shannon*		*1/Simpson*		*Coverage*
		(OTU_S)	mean	(c.i.)	mean	(c.i.)	mean	(c.i.)	(%)
S_0	1,388	18	19.2	18.1-28.4	1.2	1.2-1.3	2.8	2.5-2.8	99.7
S_1	5,478	58	77.2	64.9-112.0	1.7	1.7-1.8	4.0	3.9-4.0	99.6
S_2	4,339	28	33.1	29.1-51.4	1.3	1.3-1.4	2.9	2.8-3.0	99.8
S_3	1,366	29	40.0	31.7-73.1	1.7	1.7-1.8	4.3	4.1-4.5	99.2

6.3.2. Microbial community richness and biodiversity

From bacterial reads processing, a total of 1,172 OTUs were observed from sampled biomass, at a sequence cut-off value of 3%, being the different statistical estimates of richness and diversity summarized in Table 6.2. These results were complemented with a rarefaction analyses performed on each biomass sample (Figure 6.1a). Estimators of bacterial richness (S_{obs} and *Chao*1) indicated that the number of different OTUs was significantly highest in the initial sample (S_0) when compared to the biomass samples taken at the end of each reactor operation (S_1, S_2 or S_3), according to Table 6.2. Biodiversity indices (*Shannon* and *1/Simpson*) also confirmed the trend towards the selection of a more restricted microbial community along reactors operation (Table 6.2). Specifically, the highest bacterial biodiversity values were registered in the reactor treating raw waste (sample S_3, Table 6.2), indicating that saponification pre-treatment prompted a comparatively less diverse bacterial population (samples S_1 and S_2). These results were also supported by the rarefaction curves, which also showed a clear reduction in the bacterial biodiversity along the three different bioreactor experiments (Figure 6.1a).

Concerning the biodiversity of the archaeal domain, the obtained reads accounted for 84 OTUs (with 3% similarity cut-off). Richness and diversity statistics as well as rarefaction curves are also represented in Table 6.3 and Figure 6.1b, respectively. Contrary to the bacterial domain, the initial sample (S_0) showed the lowest richness and diversity indices (Table 6.3).

The estimated number of OTUs, as indicated by the *Chao*1 index, increased along operational time in all the systems, reflecting an enrichment of a more complex microbial community (values in Table 6.3). The biodiversity indices (*Shannon* and *1/Simpson*) increased slightly during operation in the reactors configured with solids retention by recirculation (samples S_1 and S_3) compared to the reactor without this step (sample S_2), as shown in Table 6.3. Consequently, and contrary to the eubacteria, the archaeal diversity seems to be more impacted by the higher solids retention time, induced by recirculation, than by the substrate pre-treatment (saponification).

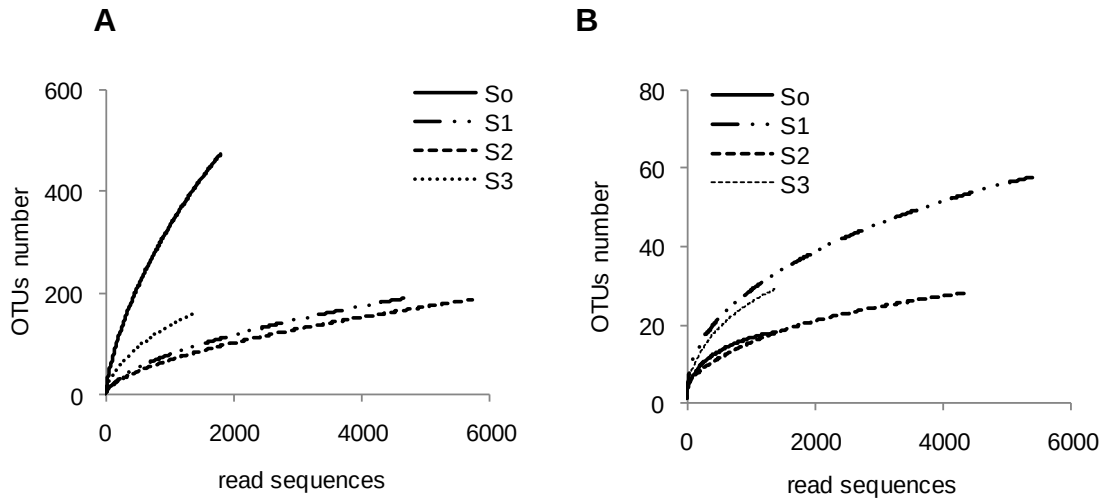

Figure 6.1 *Rarefaction curves depicting the effect of (**A**) bacterial and (**B**) archaeal sequences number on the operational taxonomic units (OTUs) identified at the end of the different treatment systems operation (S_1, S_2, and S_3) in relation to the initially inoculated biomass (S_0). Sequences were grouped by 97% of similarity using MOTHUR software.*

6.3.3. Multivariate analysis

A Correspondence Analysis (CA) was performed on the OTUs abundance matrix for *Eubacteria* and *Archaeobacteria*, and the obtained samples and OTUs scores have been represented in a biplot along the two first ordination axes (Figure 6.2). These results showed that up to the 95.4% and 86.8% of the data total variance, respectively for the eubacterial and archaeobacterial domains, have been represented. In relation to the bacteria (Figure 6.2a), it can be clearly observed that an enrichment process took place in all reactors, since the community structure at the end of the experiments was very specific and less diverse in relation to that of the initial biomass. Interestingly, sample scores on the reactors fed with saponified waste (S_1 and S_2) were relatively similar and significantly unrelated to the reactor fed with raw waste (sample S_3). Phylogenetic assignment on the most dominant OTUs revealed that anaerobic digestion on the assayed lipid-rich raw substrate was linked to a strong development of representatives from the *Syntrophus* genus (sample S_3 in Figure 6.2a). Instead, substrate pre-treatment by saponification resulted in the predominance of OTUs that were associated to the *Syntrophomonas* genus, regardless of the implementation of a solids recirculation regime (samples S_1 and S_2 in Figure 6.2a).

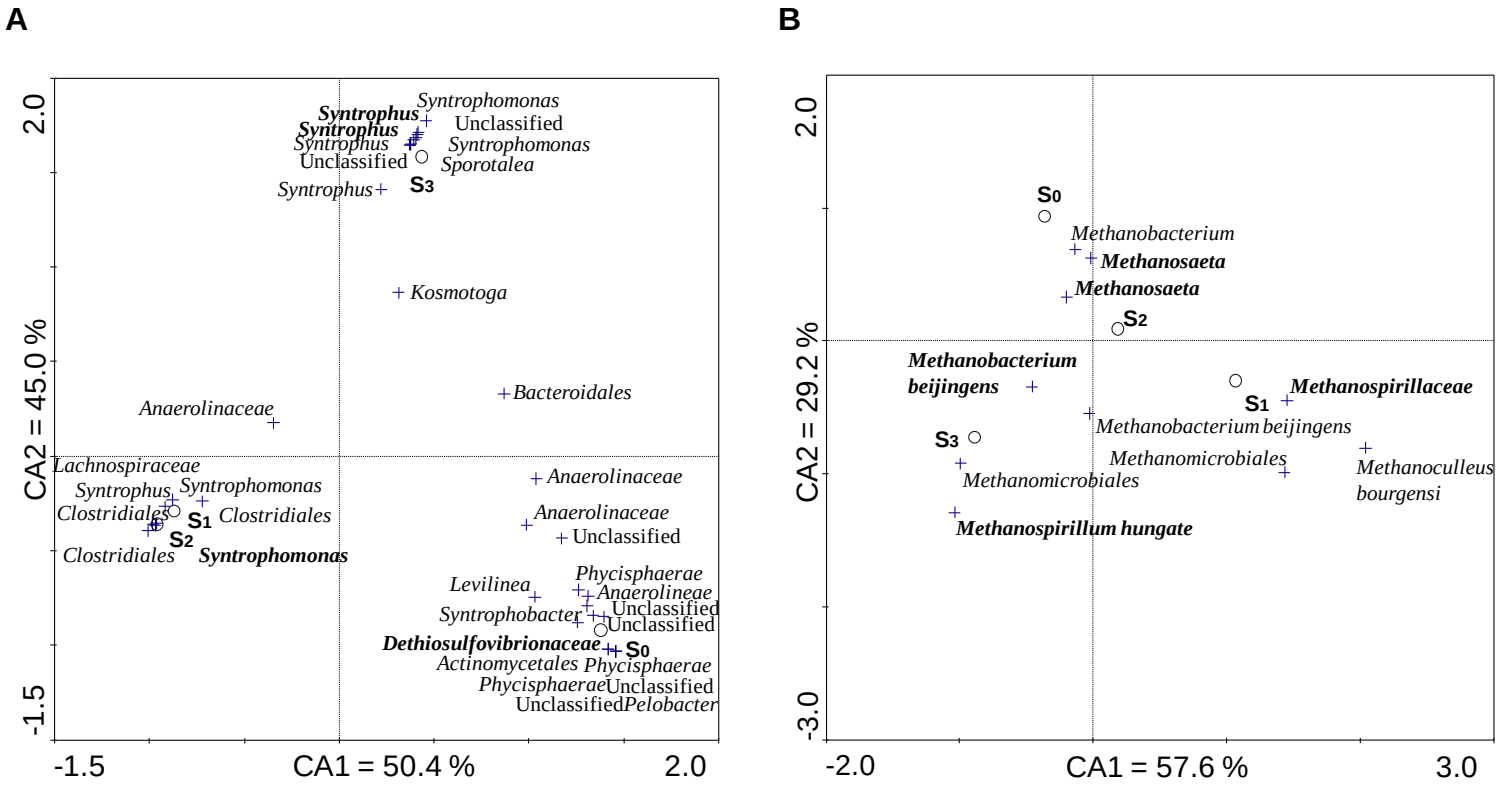

Figure 6.2 *Correspondence Analysis (CA) ordination biplot on sample scores (circles) and OTUs as species scores (crosses) corresponding to the (**A**) bacterial and (**B**) archaeal communities obtained at the end of the different treatment systems (S_1, S_2, and S_3) in relation to the initially inoculated biomass (S_0). Only those OTUs with a relative abundance higher than 1% have been depicted and phylogenetically assigned (bootstrap support > 95%). OTUs with a relative abundance higher than 10% have been labeled in bold characters. The percentage of variance of species data explained by the first two axes is indicated in the labels.*

Microbial community dynamics on the archaeal domain followed a different pattern in relation to the eubacterial domain, as shown by the CA results (Figure 6.2b). Initially the methanogenic population was dominated by *Methanosaetaceae* members (sample S_0 in Figure 6.2b).

Those OTU_S were partially conserved in the reactor treating the saponified waste without solids recirculation (sample S_2 in Figure 6.2b). Contrary, when this step was not omitted (recirculation included) biomass resulted in the enrichment of specific OTUs that were phylogenetically assigned to *Methanobacteriales* and *Methanomicrobiales* orders (both strict hydrogenotrophic methanogens) depending on the inflow characteristics, saponified (sample S_1) or raw waste (sample S_3), respectively (according to Figure 6.2b).

6.3.4. Phylogenetic analysis and population structure

The taxonomic classification of all bacterial and archaeal reads in each sample (S_0, S_1, S_2 and S_3) has been represented in Figure 6.3 and Figure 6.4, respectively.

The initial biomass sample (S_0) presented the highest percentage of unclassified bacterial sequences (27.3%, 31.4%, 39.9% and 55.8% at *phylum*, *class*, *order* and *family* level, respectively). Nevertheless, 76.1% of the classified reads were assigned to only 5 phyla: *Chloroflexi*, *Synergistetes*, *Proteobacteria*, *Bacteriodetes* and *Firmicutes* (Figure 6.3). *Anaerolineales* (*Chloroflexi*) and *Synergisteales* (*Synergistetes*) were the most abundant orders (21.4 and 13.3%, respectively, data not shown). A total of 53 bacterial families were identified, of which only 2 families were predominant: *Anaerolineaceae* (12.2%) and *Dethiosulfovibrionaceae* (11.9%), respectively, while the other 50 families were present in a low relative abundance ≤ 4% (data not shown). This initial microbial community (sample S_0) was subjected to long-lasting feeding with saponified waste and to solids retention during R_1 operation (sample S_1). Compared to S_0, the relative abundance of *Synergistetes* and *Bacteroidetes* phylums in S_1 were reduced while *Proteobacteria* and *Cloroflexi* were maintained (Figure 6.3). Furthermore, *Firmicutes* phylum was drastically increased (from 4.2 to 85.0%), and specially the family *Syntrophomonadaceae* that was selectively enriched, dominating the microbiota (79.6% according to Figure 6.3).

Syntrophomonadaceae family has been extensively described and isolated as a syntrophic LCFA degrader (Sousa et al., 2009; Hatamoto et al., 2007; Shigematsu et al., 2006). It must be noted that this family was identified as non-abundant taxa (only 1.10 %) in the original inoculum (S_0 in Figure 6.3). All the *Syntrophomonadaceae* sequences were classified as *Syntrophomonas* at genus level, but not assigned at specie level with sufficient confidence considering GG database.

These unique sequences were also compared with NCBI genomic database, based on BLAST similarity search. Sequences matched with *S. curvata* and *S. sapovorans* (Hansen et al., 1999) species, but with low similarity indices (max of 96% and 94%, respectively). A similar evolution of bacterial communities was obtained in the reactor treating saponified waste without solids recirculation step, R_2 (sample S_2 in Figure 6.3). *Firmicute* phylum was clearly enriched (92.9%) being *Syntrophomonadaceae* also the dominant family (79.2%), according to Figure 6.3. Contrary, a different shift in the bacterial community was obtained in the reactor treating raw waste with solids recirculation step, R_3 (sample S_3 in Figure 6.3). In this case *Syntrophomonadaceae* (*Firmicutes*) and *Syntrophaceae* (*Proteobacteria*) were the highest enriched families, up to 15.0% (8.2%) and 59.1% (56.1%) respectively (Figure 6.3). In this case, the *Syntrophaceae* members were the dominant family. Local BLAST search of theses sequences matched with *Smithella* (max 94% *S. propionica*) and *Syntrophus* (max 91% *S. buswelly* and S *gentianae*) species, previously described by Liu et al. (1995) and Wallrabenstein et al. (1995).

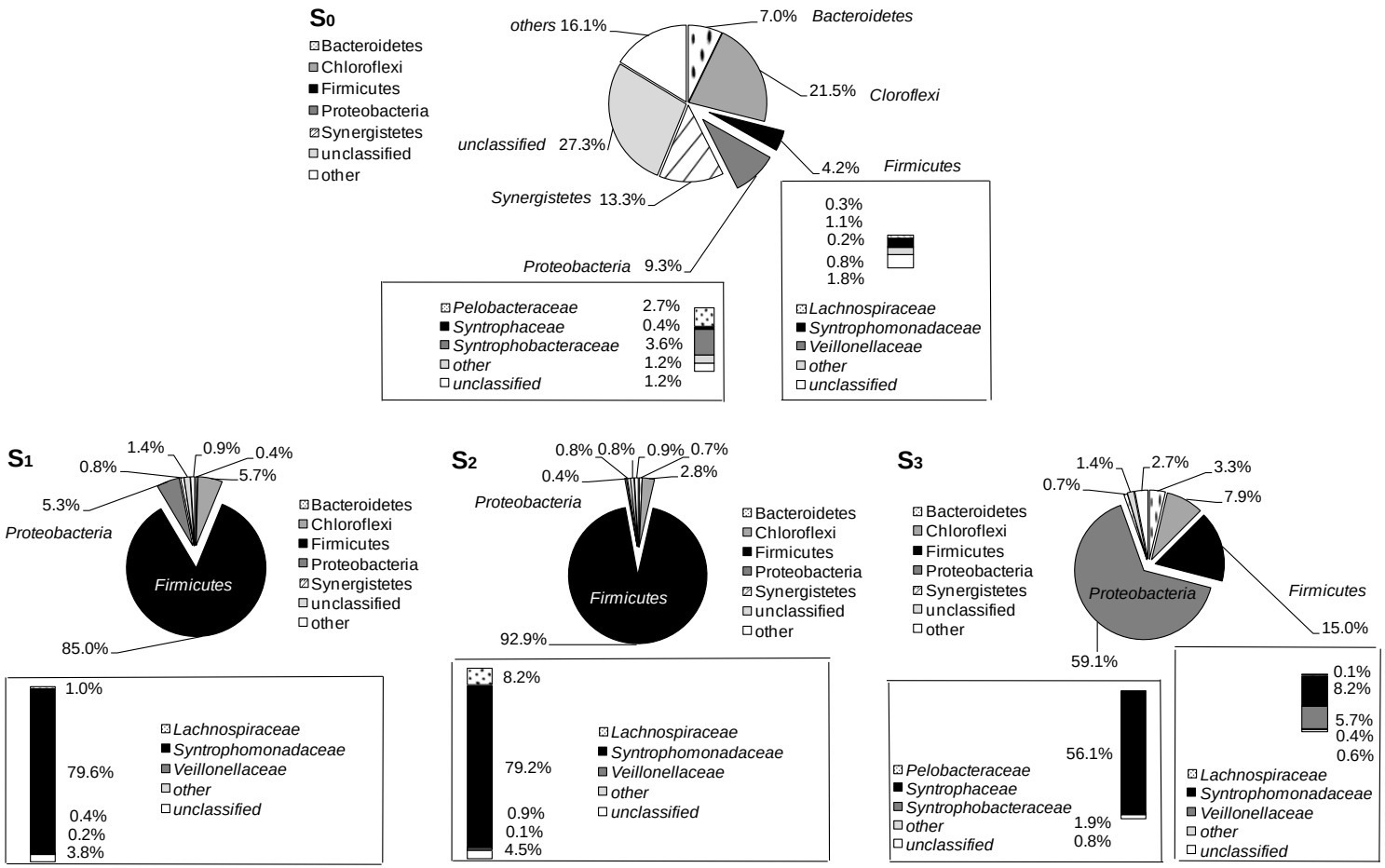

Figure 6.3 *Taxonomic classifications at phylum and family level of the obtained pyrosequencing reads of bacterial community at the end of the different treatment systems operation (S_1, S_2, and S_3) in relation to the initially inoculated biomass (S_0). Relative abundance was defined as the number of sequences affiliated to each category divided by the total number of sequence per sample. Sequences with relative abundance lower than 0.1% were categorized as "others", while sequences that were not classified at the defined level were assigned to "unclassified" group.*

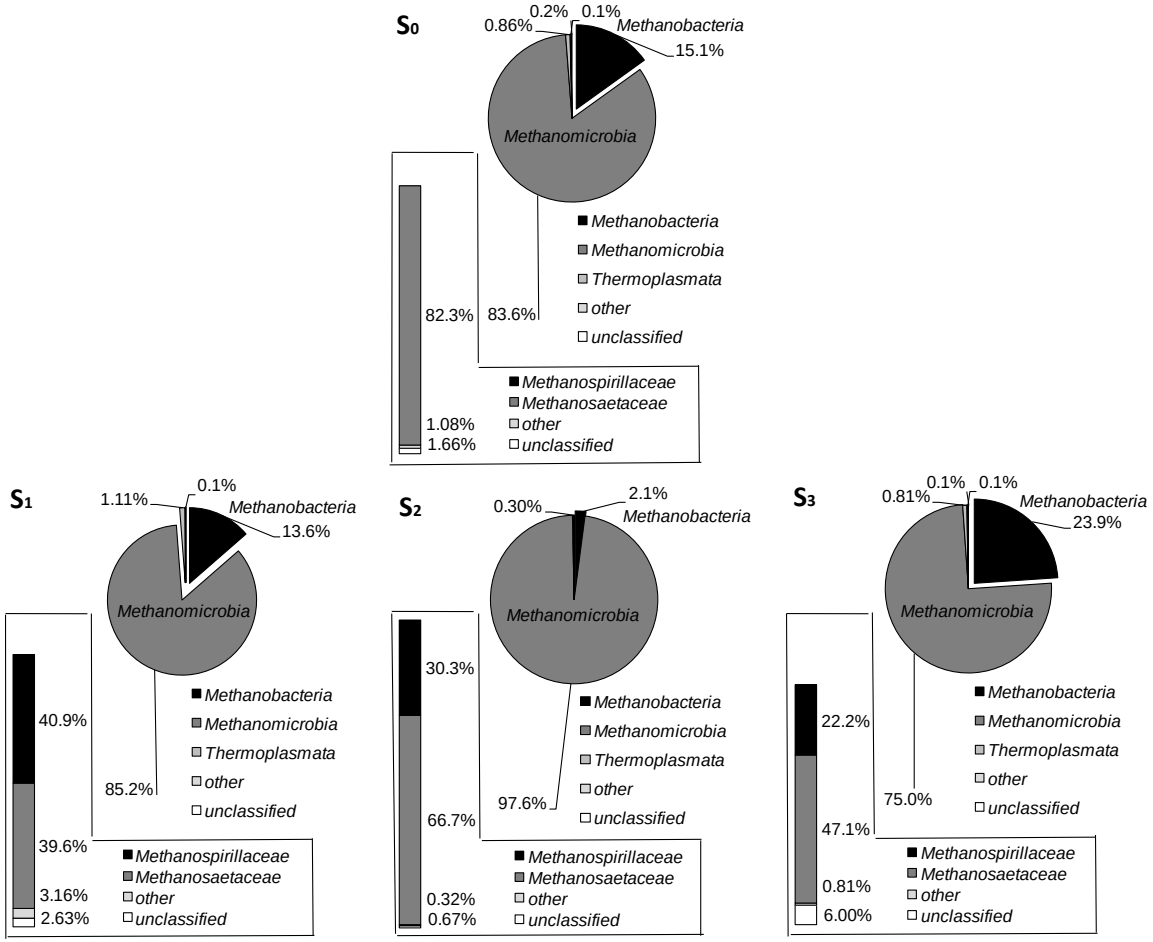

Figure 6.4 *Taxonomic classifications at class and family level of the obtained pyrosequencing reads of archaeal community at the end of the different treatment systems operation (S_1, S_2, and S_3) in relation to the initially inoculated biomass (S_0). Relative abundance was defined as the number of sequences affiliated to each category divided by the total number of sequence per sample. Sequences with relative abundance lower than 0.1% were categorized as "others", while sequences that were not classified at the defined level were assigned to "unclassified" group.*

Regarding to the Archaeal domain, all the classified sequences belong to *Euryarchaeota* plylum. The dominant class and families for each biomass sample are presented graphically in Figure 6.4. Initially, the original archaea samples in all reactors (S_0) was dominated by 2 families; 82.3% of *Methanosaetaceae* (*Methanomicrobia* class) and 14.9% of *Methanobacteriaceae* (*Methanobacteria* class), according to Figure 6.4. Sequences related to these identified families showed a high similarity with *Methanosaeata concilii* (max of 99%) and *Methanobacterium beijingens* (max of 99%) species, according to BLAST search, previously described by Eggen et al., 1990 and Ma et al., 2005. A clear shift in the archaeal community was observed along reactors operation, promoting the appearance of a second *Methanomicrobia* family, the *Methanospirillaceae* members (40.9%, 30.3% and 22.2% in S_1, S_2, and S_3, respectively, according to Figure 6.4), initially not detected in the initial biomass (S_0). BLAST local search of these affiliations resulted in a max 97% of similarity to *Methanospirillum hungatei* (Wright and Pimmic, 2003). Also *Methanobacteriacea* and *Methanosaetaceae* families class suffered different variations, function of treatment strategy. More specifically, in the reactor treating saponified waste with solids retention (sample S_1), the proportion of pyrosequencing reads that were related to *Methanosaetaceae* family was greatly decreased (from 82.3% to 39.6%) while *Methaobacteriaceae* members were maintained (from 15.1% to 13.6%), according to Figure 6.4. In R_2 (digesting saponified waste without solids retention) although the relative abundance of *Methanosaetaceae* was also decreased (from 82.3% to 66.7%), it still remains as the dominant, while the *Methanobacteriaceae* mainly disappeared (2.1%), according to Figure 6.4. Contrary, the anaerobic treatment of raw wastes with solids retention (sample S_3) promoted the maintenance of *Methanobacteriaceae* members (increasing up to 23.9%) and the reduction of Methanosaetaceae (up to 47.1%), according to Figure 6.4.

6.4. DISCUSSION

We have previously demonstrated, in **Chapter 5**, that substrate pre-treatment by saponification and solids retention by outflow recirculation significantly improves the anaerobic digestion of fatty slaughterhouse waste (Affes et al., 2013). This phenomenon was then attributed to the combined action of enhanced emulsification and bioavailability of solid fatty residues with the minimization of substrate/biomass wash-

out. These operational strategies exert quite specific selective pressures towards the microbial community of methanogenic biomass, as revealed here by a high throughput DNA sequencing approach.

When looking at general biodiversity indicators (Table 6.2 and Figure 6.3), it can be clearly seen that the number of expected species (regarded as OTUs with a 3% sequence similarity cut-off) in the domain *Eubacteria* is about one order of magnitude larger than that on the *Archaeobacteria*. Such differences between the two microbial domains have been outlined through conventional clone library sequencing of samples from different anaerobic digesters (Rivière et al., 2009), and can be attributed to the convergence of several hydrolytic and fermentative pathways that are predominantly encompassed in the *Eubacteria*, towards methanogenesis as the top trophic level, being this metabolic process a very specialized and exclusive trait in the *Archaeobacteria*. Hence, this observation is in agreement with general ecology rules on the interplay between biodiversity and trophic complexity.

Considering that the inoculum arose from an industrial digester treating a complex wastewater rich in carbohydrates, and thus unrelated to fatty wastes, it is not surprising that feeding with lipids leaded to drastic reductions in the expected number of species, particularly in the *Eubacteria*, as depicted by rarefaction curves (Figure 6.3). Similar decreases in biodiversity have previously been observed in anaerobic reactors, upon adaptation to specific operation parameters or substrates which might select for more specialized microbial populations (Garcia et al., 2011). In relation to this, the functional biomass enriched upon feeding with raw lipids (S_3) should have the physiological capacity to emulsify and hydrolyze the substrate prior to the degradation of the produced LCFAs and, consequently, an equilibrium between hydrolytic and ß-oxidizing microbial populations must be reached for the process to be feasible. Yet, only β-oxidizers might be required when applying saponified lipids (R_1 and R_2) as this pre-treatment had a hydrolysis efficiency as high as 90% (Affes et al., 2013). Rarefaction on OTU incidence (Figure 6.3) points to a further reduction in bacterial biodiversity when feeding with saponified lipids (samples S_1 and S_2), in relation to the reactor treating raw substrate (sample S_3). However, the non-parametric species richness estimate Chao1 showed no significant differences between S_1, S_2 and S_3 (Table 6.2). It is important to consider though that the Chao1 estimate has been reported to underestimate the true

richness at relatively low sample sizes (Hughes et al. 2001), which is the case of S_3 in relation to S_1 and S_2. Biodiversity synthetic indices (Shannon and Simpson) further support the observed reduction in microbial complexity upon saponification (Table 6.2). The selective effects of exposure to saponified and non-saponified lipids on the eubacterial component have also been evidenced in terms of microbial community composition and dynamics (Figure 6.2). From a more diverse microbial population in the original biomass, which appears to be formed by a mix of bacterial taxa capable of sulphate–reduction (*Dethiosulfovibrionaeae, Syntrophobacter, Phycisphaerae)* with other that are related to the fermentation of carbohydrates (*Levilinea, Pelobacter*). Such microbial population profile is in agreement with the origin of the methanogenic biomass from a bioreactor treating wastewater from a fruit processing industry. In parallel with a reduction in biodiversity, lipid exposure resulted in the enrichment of members of the *Syntrophaceae* and *Syntrophomonadaceae* (Figures 2 and 3). The detection of representatives from these families has been reported in methanogenic biomass subjected to LCFA-rich effluents (Hansen et al., 1999; Hatamoto et al., 2007; Sousa et al., 2009) and in reactors treating complex fatty wastes, such as slaughterhouse solid waste and wastewaters (Palatsi et al., 2011; Rosa et al., 2009). It is interesting to contemplate though the distinct dominance pattern of *Syntrophus* spp. (*Syntrophaceae*) and *Syntrophomonas* spp. (*Syntrophomonadaceae*) when treating raw (S_3) and saponified fats (S_1 and S_2), respectively (Figure 6.2). Both genera have been described in connection to the metabolism of LCFA in co-cultures with methanogens (Jackson et al. 1999; Yoochatchaval et al. 2011). Hence, the predominance of *Syntrophus* spp. above *Syntrophomonas* in S_3 points to the fact that it plays a crucial role in the anaerobic degradation of more complex substrates, such as lipids (Figure 6.3). Conversely, saponification of fats, which resulted in a more LCFA-rich substrate, lead to the enrichment of *Syntrophomonas* well over *Syntrophus*. Besides this major taxa, metabolism of lipids and LCFA has also been related to certain species in *Bacteroidetes*, *Spirocaetes*, *Clostridiales* and *Coprothermobacters* (Shigematsu et al., 2006; Hatamoto et al., 2007), which were also found in lower amounts in this study.

Contravening the above described biodiversity decreasing trend in the *Eubacteria*, feeding with fatty wastes caused the opposite effect concerning the *Archaeobacteria*. Microbial dynamics between hydrogenotrophic and acetoclastic methanogens were

closely related to the lipids/LCFA feeding regime and to the retention of solids, as depicted by multivariate analysis on OTU relative abundance (Figure 6.2). A clear shift from the obligate acetotrophic *Methanosaeta* genus to hydrogenotrophic archaeobacteria was observed in all the reactors, particularly to *Methanospirillum* ssp. (Figure 6.4). This genus encompasses strictly hydrogenotrophic methanogens that thrive as hydrogen scavengers in LCFA degrading cultures (Hatamoto et al., 2007; Roy et al., 1986; Lorowitz et al., 1989), and it has been identified in samples collected from an anaerobic reactor treating synthetic wastewater containing oleic and palmitic acids (Shigematsu et al., 2006). *Methanospirillum* has also been used in syntrophic co-cultures with different *Syntrophus* spp. Dominance of *Methanospirillum* spp. appears to be prompted by biomass recirculation (S_1 and S_3), rather than by saponification pre-treatment. In relation to the original biomass (S_0), the relative abundance of representatives from the *Methanobacteriaceae* family was similar in the bioreactor fed with saponified lipids (S_1) and increased when feeding with raw lipids (S_3), along reactor operation with biomass recirculation, but experienced a significant drop when the recirculation of solids was non-operational (S_2; Figure 6.4). Biomass wash out from the reactor, which occurs when the dilution rate exceeds microbial growth rates, appears to be the main selection mechanism for these archaea. These results are in agreement with the previous detection of *Methanobacterium* in high rate biomass retained anaerobic digesters (EGSB) feed with oleic acid (Pereira et al. 2002) and its tolerance towards relatively high LCFA loads (Salvador et al. 2012; Sousa et al., 2009). Considering the assayed reactor experimental conditions (OLR < 2 g_{COD} $L^{-1}_{reactor}.day^{-1}$; HRT = 30 days; solid retention time ~ 60 days), the differences in the predominance of hydrogenotrophic methanogens belonging to the *Methanospirillaceae* and *Methanobacteriaceae* families might be driven by their affinity for H_2, as the latest grows at relatively high H_2 concentrations.

6.5. CONCLUSION

The biodiversity and the phylogenetic structure of the microbial community residing in 3 different reactors configured with/without solids recirculation and fed with saponified/raw solid slaughterhouse wastes were analyzed using high throughput sequencing approach. These operational strategies exerted quite specific selective

pressures towards the microbial community of anaerobic biomass that was phylogenetically different in relation to that of the initial biomass. The bacterial biodiversity was highly dependent on the substrate characteristics (raw/saponified), while the archaeal one was more impacted by solids retention induced by the recirculation step. Changes in the bacterial diversity from a more diverse microbial population in the original biomass (formed by a mix of bacterial taxa capable of sulphate–reduction and carbohydrate fermentation) to a less diverse community, predominated by representatives from *Syntrophomonadaceae and Syntrophaceae* families (known by their ability to anaerobically degrade lipids and LCFA), explain the gradual improvement in the methane production rate observed along reactor operation. The predominance of *Syntrophus* spp. above *Syntrophomonas*, in the biomass treating raw waste, points to the fact that it plays a crucial role in the anaerobic degradation of more complex substrates. Contrary to the *Eubacteria* domain, biomass exposure to fatty substrate induced an increase in the archaeal biodiversity. A clear shift from the obligate acetotrophic *Methanosaeta* genus to hydrogenotrophic archaeobacteria *(Methanospirillaceae* and *Methanobacteriaceae* families) was observed in all the reactors, particularly to *Methanospirillum* ssp. Dominance of *Methanospirillum* spp. appears to be promoted by biomass recirculation, rather than by saponification pre-treatment.

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Chapter 7. General conclusions and suggestions for further research

Based on the proposed objectives, this section gives an overview of the main conclusions of the work constituting this dissertation. Suggestions for further research and perspectives of slaughterhouse waste treatment are also presented.

7.1. GENERAL CONCLUSIONS

Anaerobic digestion has been evaluated as an integral part of the solution for the actual concerns about climate change, global energy crisis and waste management. Results of **Annex 1** highlight the suitability and the attractiveness of slaughterhouse waste, characterized by their high content on protein and lipids, for biogas production. However, long chain fatty acids (LCFA) and ammonia (NH_3), released during lipids hydrolysis and acidogenesis of amino acids, respectively, act as inhibitors at high concentrations and, therefore, they limit the application of high organic loading rate anaerobic systems. This finding urges to understand the pathway of the inhibition process in order to found alternatives and methodologies to make the process more profitable, being the main objective of this dissertation with a special emphasis on lipids and LCFA valorization. Flotation and wash-out of the organic matter and inhibition of the microbial populations were identified as the main limiting aspects of lipids-rich wastes treatment, caused principally by their physical adsorption onto the membrane, mechanism required for their degradation (**Chapter 3** and **Annex 2**).

Consequently, the key for an efficient anaerobic digestion of lipid-rich wastes is to prevent or at least to minimize the adsorption process. The LCFA capturing, by incubation with bentonite to force the exclusively LCFA adsorption over bentonite prior to the anaerobic digestion process (LCFA capturing strategy) tested in batch conditions, resulted a reliable approach to improve the system robustness. However, the application of this strategy in anaerobic bioreactor subjected to increasing continuous loading of oleic acid was not found to be very promising, probably due to the relatively low bentonite/substrate ratio which could be insufficient to capture all or at least the majority of the fatty substrate (**Chapter 3** and **Annex 2**).

Application of low-energy ultrasonic treatment, in a sequentially scheme (every 3-4 days) could be an interesting strategy to control the adsorption-desorption dynamics and to improve the activity of the biomass exposed to saturated LCFA. However, the cumulative nature effect of ultrasonic treatment must be taken into account in order to achieve high process performances without damaging the microbial cells (**Chapter 4**).

Efficient conversion of complex high-strength lipid waste to methane is possible in a novel reactor system configuration that integrates waste pre-treatment (saponification) and digested solids recirculation to the anaerobic digestion process. A start-up step,

consisting on pulses-feeding cycles of the fatty waste prior to the semi-continuous process, is also recommended in order to promote an adapted microbial community for LCFA mineralization. The applicability of this system configuration for solid slaughterhouse fatty waste as a model of "real" lipidic waste was proved at lab scale reactors. Results suggest that both strategies act synergically: saponification promoted the emulsification and the bioavailability of solid fatty residues, while the recirculation of solids resulted in substrate dilution which also minimizes the substrate and biomass wash-out, thus ultimately prompting the enrichment and adaptation of active biomass (**Chapter 5**).

The biodiversity and the phylogenetic structure of the microbial community of the biomass enriched during the operation of this reactor system configuration was analyzed using high throughput sequencing approach, being the first specific investigation that depicts the whole physiology of lipids degraders in anaerobic digesters (**Chapter 6**). Biomass exposure to fatty substrate induces a drastic change on the bacterial biodiversity, from highly diverse microbial population in the original biomass to a less diverse community, predominated by members of *Syntrophomonadaceae and Syntrophaceae* families, previously characterised by their ability to anaerobically degrade lipids and LCFA. The predominance of *Syntrophus* spp. over *Syntrophomonas*, in biomass treating raw waste, points to the fact that this specie would play a crucial role in the anaerobic degradation of more complex substrates (emulsifications/hydrolysis and β-oxidation). Contrarily, the biomass retention time increase, obtained by solids recirculation, induced an increase in the archaeal biodiversity, with a clear shift from the obligate acetotrophic *Methanosaeta* genus to hydrogenotrophic archaeobacteria *(Methanospirillaceae* and *Methanobacteriaceae* families), particularly to *Methanospirillum* ssp.

All the strategies proposed in the present dissertation, i.e. the addition of bentonite, the application of ultrasonic treatment, the application of an acclimation period based on pulses-feeding cycles were proved to be reliable strategies to guide further research on methane production from lipid-rich wastes/wastewaters. However, the novel system configuration with combination of saponification pre-treatment and digested solids recirculation arises as the best option, opening a clear alternative to be "easily "applied at conventional full scale continuous stirred reactors. It is expected that the results of the

present thesis will be the basis for new developments and innovations of anaerobic bioreactors operation in order to achieve a higher renewable energy production from lipids-rich waste.

7.2. SUGGESTIONS FOR FURTHER RESEARCH AND PERSPECTIVES

The results described in this dissertation highlight the effectiveness of the anaerobic digestion of lipid-rich wastewaters when certain alternatives or requirements are accomplished. Further research can be focused into three main axes:

- The use of developed molecular biology tools, such as 454-pyrosequencing technique, for the characterization of the microbial consortia developed in anaerobic bioreactors treating complex lipid-rich waste can give important insights of metabolic pathways, and thus new ideas for an efficient process. The bio-augmentation approach can be a promising solution in the future.
- Most studies that deal with the inhibition of lipids and LCFA use synthetic substrates, which in most cases contain specific LCFAs, or a mixture of different LCFA as sole carbon source. However, in real situations, lipid-rich waste/wastewaters discharged from slaughterhouse, dairies or food processing industries contain also other components (carbohydrates and proteins) that could interfere in LCFA inhibitory process or in the microbial activity. It will be interesting to adapt the novel system configuration, proposed in this thesis, to the anaerobic treatment of lipid-protein rich substrates, taking into account the inhibitory effect of both LCFA and ammonia. Inhibition by ammonia affects mainly to acetoclastic methanogens, but in lesser extend syntrophic acetate oxidation mircroorganisms (SAO) or the hydrogenotrophic methanogens. However, SAO have a generation time higher than 40 days. Consequently, system operation promoting SAO growth, by high biomass retention times, could be a possible solution for the future and synergic with practices for reducing inhibition by LCFA.
- Finally, it is necessary to test the reactor system configuration (saponification and solids retention time increase) proposed in this dissertation at pilot and full scales, and to develop new operational methods for the existing full scale plants, in order to increase their energy production and economical profitability.

Annexed Information

Annex 1. Co-digestion of sewage sludge and sterilized solid slaughterhouse waste: methane production efficiency and process limitations

Pitk, P., Kaparaju, P., Palatsi, J., Affes, R., Vilu, R.

Bioresource Technology (in press) *http://dx.doi.org/10.1016/j.biortech.2013.02.029*

Annex 2. Influence of adsorption and anaerobic granular sludge characteristics on long chain fatty acids inhibition process

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Water Research 46, 16 (2012) 5268 – 5278.

Annex 1. Co-digestion of sewage sludge and sterilized solid slaughterhouse waste: methane production efficiency and process limitations

The rendering product of Category 2 and 3 Animal By- Products is known as sterilized mass (SM) and it is mainly composed of fat and proteins, making it interesting substrate for anaerobic digestion. Batch and semi-continuous laboratory experiments were carried out to investigate the effect of SM addition in co-digestion with sewage sludge on methane production and possible process limitations. Results showed that SM addition in the feed mixture up to 5% (w/w), corresponding to 68.1% of the organic loading, increased methane production 5.7 times, without any indication of process inhibition. Further increase of SM addition at 7.5% (w/w) caused methane production decrease and volatile solids removal reduction, that was mainly related to remarkably increased free

Pitk, P., Kaparaju, P., Palatsi, J., Affes, R., Vilu, R.

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1. INTRODUCTION

Increasing demand for meat products has led to a concurrent increase in solid slaughterhouse wastes production worldwide. In the European Union, solid slaughterhouse waste treatment and utilization have been regulated by the Animal By-Product (ABP) Regulation EC No 142/2011 (European Parliament and the Council, 2011) in order to protect public and animal health (e.g. epidemics of bovine spongiform encephalopathy). There are two thermal treatment strategies for ABP. Pasteurisation (70 °C, for 60 minutes) for Category 3 and sterilization (133 °C, 3 bars for 20 minutes) for Category 2 ABP, before it could be used as substrate in the biogas plants. Mixture of Category 2 and 3 ABP has to be sterilized before it can be fed into biogas plants.

Anaerobic digestion (AD) of sewage sludge (SS) has been largely applied at industrial and municipal wastewater treatment plants (WWTP) for decades. It is a well- known, efficient and environmentally sustainable technology which enables simultaneous energy recovery as biogas, as well as stabilisation and volume reduction of sludge (Luostarinen et al., 2009). AD has also been considered as one of the best alternatives for energy recovery from ABP and slaughterhouse wastes (Hejnfelt and Angelidaki, 2009; Martinez- Sosa, et al., 2009; Palatsi et al, 2011). According to the ABP Regulation EC No 142/2011 the product of the rendering process, sterilized mass (SM), can be used as substrate for AD. SM is considered as an attractive substrate for AD due to its high organic content (mainly in the form of proteins and fats) and due to its high methane potential (Pitk et al., 2012). However, slow hydrolysis rates, operational problems and possible process inhibition have been reported when treating ABP. One of the major problems associated with the anaerobic treatment of ABP is the high fat and lipid content, that can cause sludge flotation and biomass washout, as well as the potential inhibition of microbial activity due to the produced-accumulated long chain fatty acids (LCFA) (Alves et al., 2009; Edström et al., 2003). Floating LCFA could affect substrate bioavailability and biomass activity, thus causing common plant operation obstacles such as fouling-scum overflow and process inhibition (Salminen and Rintala, 2002). In addition, ammonia is also produced during the anaerobic degradation of nitrogen containing compounds, such as proteins contained in ABP (Resch et al., 2011). Free (un-ionised) ammonia (NH_3) has been suggested to be the main cause of inhibition in high nitrogen loaded AD processes (Chen et al., 2008). The

hydrophobic ammonia molecule may diffuse passively into the cell, causing proton imbalance, and/or potassium deficiency in microorganisms, particularly in methanogens (Salminen and Rintala, 2002). Free NH_3 concentration increases with increase in pH and temperature. Thus, the inhibitory effect of ammonia nitrogen on AD process depends on ammonium concentration, pH and temperature (Angelidaki and Ahring, 1994). A wide range of inhibiting ammonia concentrations have been reported in the literature. The total ammonia nitrogen (TAN, NH_4^+ and NH_3) concentrations that can cause a 50% reduction in methane production range have been reported to be from 1.7 to 14 g L^{-1} (Chen et al., 2008; Nakashimada et al., 2008).

Only few studies have focused on the use of ABP rendering products as co-substrates for biogas production (Bayr et al., 2012; Hejnfelt and Angelidaki, 2009; Pitk et al., 2012). On the other hand, several studies have successfully demonstrated the use of dissolved air flotation sludge and grease trap sludge also as feedstock for biogas production in co-digestion processes (Creamer et al., 2010; Davidson et al., 2009; Luostarinen et al., 2009; Silvestre et al., 2011). The present study was therefore focused on evaluating the process performance and methane production during anaerobic co-digestion of SS with SM. The effect of organic loading rate, with respect to the dosage of SM in the feed, on maximum methane production was also determined. In addition, potential process limitations due to free NH_3 concentration, organic overloads (volatile fatty acids (VFA) accumulation) and lipids (or LCFA) inhibition were also evaluated.

2. MATERIAL AND METHODS

2.1 Substrates and inoculum

Sewage sludge (SS) was collected from a full scale WWTP facility (Tallin, Estonia). SS used as substrate in the present experiments, was obtained from a primary clarifier and it mainly contains primary sludge and small proportion of waste activated sludge, that is recycled to the primary clarifier. To consider the variations in sludge characteristics in the sampled WWTP facility, up to five SS sampling campaigns were performed (SS_1-SS_5). Inoculum (I), used as anaerobic seed sludge in batch test and lab reactors, was also obtained from the full-scale anaerobic digester of the WWTP, treating a mixture of primary and secondary sludge.

Category 2 and 3 sterilized ABP was produced in a cattle and bovine rendering facility (Rakvere, Estonia). As a first step of rendering process, Category 2 and 3 materials were disaggregated (particle size < 50 mm) in a crusher and transported to the cookers, where it were sterilized at 133^0 C, 3 bar for 20 minutes. The final product is known as sterilized mass (SM) and it was used as co-substrate in the present experiments. Un-fractioned SM contained bone particles (< 50 mm). Bone particles were manually removed from the sampled SM to avoid feeding problems in the lab-scale reactors.

2.2 Batch experiments

Biomethane potential (BMP) tests were carried out with OxiTop-C (WTW, Weilheim, Germany) respirometric system in accordance with the protocol proposed by Angelidaki et al., (2009). BMP experiments were performed with SM and SS_1 samples to determine substrates methane potential and assess biodegradability. Details of the batch experimental set-up are described elsewhere (Pitk et al. 2012). Briefly, to each assay, substrate (SS or SM) and inoculum (I) were added in a volatile solids (VS) ratio ($VS_{substrate}/VS_{inoculum}$) of 0.50 and 0.25 for SS/I and SM/I, respectively. Vials (in duplicate for each assay) were flushed with N_2 gas, sealed immediately with airtight stoppers and incubated at 37.5 °C under continuous mixing condition. Methane production from I vials (controls) was subtracted from the SM and SS tests to determine net methane potentials.

2.3 Reactor experiments

Process performance and CH_4 yields during the anaerobic co-digestion of SS with SM was conducted in 2 plexiglas lab reactors (5 L total capacity) with a working volume of 4.5 L. Reactors were operated at 37 ± 1 °C, by hot water circulating through the reactors waterjacket, and under intermittent mixing conditions (160 rpm, 15 min on/off) by magnetic stirrer (MAG MS7, IKA, Germany). Substrate feeding was performed once per day (including weekends) during the whole experimental period (220 days). SS was fed using a sterile plastic 100 ml syringe, while SM was manually fed through an inlet pipe at the top of the reactor.

Reactor 1 (R1) was inoculated with 4.5 L of inoculum and maintained in batch conditions (day 1 to 15) for initial inoculum starvation (removal of residual organic

matter). After this initial step, reactor was exclusively fed with SS (from day 15 to 37) with a loading rate of 225 mL_{SS1} day^{-1}, resulting in an hydraulic retention time (HRT) of 20 days. Thereafter, co-digestion of SM with SS was initiated (day 37 onwards). HRT was fixed in 22.5 days (for feeding convinience), while the proportion of SM in the feed was gradually increased from 0 to 10 % (w/w), in 2.5 % steps along the experimental period.

Reactor 2 (R2) was considered as the control reactor and it was fed only with SS. Start-up of R2 was similar to that of R1 and feeding was started on day 122, when a 7.5% of SM was being added in R1. Thereafter, both R2 and R1 were operated with the same SS throughout the experimental period (days 122-220). R2 was also operated with a HRT of 22.5 days.

Biogas production in reactors was continuously measured with a on-line milligascounter (MGC-1 V3, Ritter®, Germany). Reactors temperature and pH were monitored daily, while methane content in produced biogas (%CH_4) was measured weekly. Effluent characteristics were analysed at the end of each loading period, according to Analytical methods section.

2.4 Analytical methods and calculations

Total (TS) and volatile (VS) solids, total chemical oxygen demand (COD_{tot}), total nitrogen (TN), total phosphorous (TP), and ammonium (TAN) were analysed depending on the process conditions and OLR regime changes. Analyses were conducted by accredited laboratories Estonian Environmental Research Centre and Agricultural Research Centre. pH, TN, TP, total organic carbon (TOC), sulphur, potassium, magnesium, raw fat and raw protein in SM were determined according to EVS-EN ISO and ISO standard methods. HACH-LANGE spectrophotometer DR 2800 and cuvette tests were used for the determination of COD_{tot}, TP, and NH_4^+ in both SS and Inoculum. TS and VS contents were determined according to Standard Methods (APHA, 1998).

Methane content in the biogas was analysed with gas chromatograph (Model 3700, with thermal conductivity detector (TCD) and PorapakQ column 1.8 m x 3.17 mm) and N_2 was used as carrier gas. VFA analysis was performed using GC 2014 ATF/SPL (Shimadzu, Japan) gas chromatograph equipped with a Zebron ZB–WAXplus capillary column (35m x 0.25 mm x0.25 μm) and flame ionization (FID) detector. Total LCFA

from C12 to C24), including LCFA forming part of glycerides, were determined according to the method described by Palatsi et al. (2009), based on direct methylation-extraction procedure. LCFA were identified and quantified by GC CP-3800 gas chromatograph (Varian, USA), fitted with CP7489:CP-Sil 88 FAME capillary column (50m0.25mm 0.2µm) and FID detection.

Theoretical CH_4 potential at standard conditions (STP, 0ºC and 1bar) was estimated according to the following equation (Eq. 1), based on the protein, lipid and carbohydrate contents of the subtrates, as suggested by Angelidaki and Sanders (2004):

$$CH_4 \text{ yield } (dm^3_{CH4}\ kg^{-1}_{VS}) = 496*X + 1{,}014*Y + 415*Z \qquad \text{Eq. 1}$$

where *X* =% of proteins *Y* = % of lipids and *Z*= % of carbohydrates.

The unionised fraction of the ammonia nitrogen (NH_3) was calculated according the equation (Eq. 2), as described by Körner et al. (2001):

$$NH_3\ (\%\ \text{of TAN}) = 100/(1+10^{(pKa-pH)}) \qquad \text{Eq. 2}$$

$$pKa = 0.09108+272.92/(273.2+T) \qquad \text{Eq. 3}$$

where *pKa* is the dissociation constant dependent of temperature and *T*, the temperature in Celsius degrees (°C).

3. RESULTS AND DISCUSSION

3.1 Substrates characterization

Fresh SS from WWTP was sampled five times (SS_1-SS_5) during the experimental period and the variations in samples composition are shown in Table 1. TS content fluctuated around 3.3 ± 0.4 while VS content remained more or less stable (65.6 ± 1.8% of TS). The variations in SS characteristics were related to sampling time (seasonal variation) and to the settling efficiency in primary settler (daily basis variation). On the other hand, variation in COD, TN and TP concentrations among the five SS samples were lower than 30% (Table 1). Chemical composition of SM is also presented in Table 1. After the dry-rendering process, SM contained only 4 % water. The organic matter present in SM was mainly composed by fats and proteins (55.9% and 31.1% of the VS content, respectively) reaching a high TOC value of 553 ± 13.5 $g_{TOC}\ kg^{-1}_{TS}$ and a C/N ratio of 9.3 ± 0.7 (Table 1).

Table 4.1 *Characterization of sewage sludge (SS) and sterilized mass (SM) used in the study.*

			SS					**SM**
Parameter	SS1	SS2	SS3	SS4	SS5	**SS_{MEAN}**	**Parameter**	
TS (%)	3.1	4.1	3.2	3.1	3.1	3.3 ± 0.4	TS (%)	96 ± 1.3
VS (%)	65.9	68.2	65.9	63.3	65.8	65.6 ± 1.8	VS (%)	87 ± 1.4
pH	N.D.	5.9	n.d	6.8	n.d	6.36 ± 0.6	C:N	9.3 ± 0.7
COD (g L^{-1})	41.7	43.8	30.3	32.1	35.6	36.7 ± 5.9	TOC (g kg^{-1} TS)	553 ± 13.5
TN (mg L^{-1})	1526	1346	1165	1449	1259	1,349 ± 144	TN (g kg^{-1} TS)	59.8 ± 6.1
NH_4^+ (mg L^{-1})	184	224	139	197	235	195.8 ± 37.8	TP (g kg^{-1} TS)	22.3 ± 0.1
TP (mg L^{-1})	568	666	587	708	543	614.4 ± 69.7	Ca (g kg^{-1} TS)	34.6 ± 1.4
TOC (g L^{-1})	n.d	n.d	9.3	n.d	8.1	8.7 ± 0.9	Mg (g kg^{-1} TS)	1.1 ± 0.1

n.d-not determined

3.2. Batch experiments

BMP experiments were conducted with only one of the five SS samples. This sample (SS_1) had a 3.1% of TS and a 65.8% of VS/TS. The methane potential of SS_1 estimated by the BMP test was 239.9 m^3_{CH4} t^{-1}_{VS} (or 5.0 m^3_{CH4} t^{-1}). As expected, due to the high organic content, SM had a higher methane yield of 719.25 m^3_{CH4} t^{-1}_{VS} (or 590.5 m^3_{CH4} t^{-1}). The obtained methane yield correspond to >99% of the theoretically estimated SM methane potential (721.1 m^3_{CH4} t^{-1}_{VS}, according to Equation 1), indicating a high biodegradability and energetic potential of the SM.

The experimental methane yield obtained for SM in the BMP test was a 14% lower than the previously reported values by Pitk et al. (2012) with samples obtained in the same rendering facility. This is expected due to the daily variations of the ratio of slaughtered cattle/pigs in the facility, and consequently characteristics of the SM differ in time. If different facilities are sampled, this heterogeneity could be higher. For example, Bayr et al. (2012) reported a significantly lower methane yields (515 m^3_{CH4} t^{-1}_{VS} or 343 m^3_{CH4} t^{-1}) for similar substrates.

3.3. Reactor experiment

3.3.1 Process performance

Reactors operational conditions (HRT, OLR and nitrogen loading rate (NLR)) and efficiencies ($VS_{removal}$, methane production and yields) are summarized in Fig.1 and Table 2. After the described reactor start-up (first 15 days), R1 was fed with SS alone with a HRT of 20 days and at an equivalent OLR of 1.4 kg_{VS} m^{-3} d^{-1}, obtaining a stable methane production of 1.5 ± 0.2 L_{CH4} day^{-1} (Fig.1). Methane yield during the mono-digestion of SS_1 (days 15-37) was 233.9 ± 37.4 m^3_{CH4} $t^{-1}_{VSadded}$ (6.59 ± 1.1 m^3_{CH4} t^{-1}). This value is quite similar to the obtained value in the BMP test. Mean VS removal efficiency during this period was 46.8% (Table 2 and Fig.1).

Co-digestion of SS with SM was initiated on day 38 and HRT was regulated to 22.5 days. Initially, SM was introduced to feeding mixture in a proportion of 2.5%. This small fraction represents an important organic content of the reactors input, representing a 42.9% in terms of VS, increasing the reactor OLR up to value of 2.13 kg VS m^{-3} day^{-1}. Methane production was rapidly increased up to values of 3.8 ± 0.3 L_{CH4} day^{-1} (Fig.1), corresponding to a mean methane yield of 396.1 ± 29.3 m^3_{CH4} t^{-1}_{VS}. On day 63, the SM

proportion was increased up to a 5% of the input, increasing reactor OLR and methane yield up to 2.68 kg_{VS} m^{-3} day^{-1} and 618.9 ± 70.7 m^3_{CH4} t^{-1}_{VS}, respectively, without any sign of process imbalance. Contrary, when the SM was added at an amount of 7.5% (OLR of 3.55 kg_{VS} m^{-3} d^{-1}), the process efficiency started to decrease. Process limitations were detected by a gradual decrease in methane production and by the stabilization of the VS removal rates (Table 2). Reactor operation at 7.5% of SM was divided into two separate periods of 43 days (P_1 and P_2). During P_1 (days 116–159), methane yield was 644.8 ± 63.6 m^3_{CH4} t^{-1}_{V} with a VS removal efficiency similar to the one obtained with the addition of a 5% of SM (Table 2). This indicated process imbalance, as ratio of highly biodegradable SM in input mixture had increased. Definite implications of process imbalance appeared during P_2 (days 160-202), in which methane production started to decrease in conjunction with a reduction in VS removal rates (Table 2). Finally, increase in SM amount in the feed ratio up to 10% w/w (representing 81.92% in terms of VS daily load) resulted in a clear decrease in methane production (Fig.1). After two weeks of operation at 10% of SM addition, process started to show symptoms of failure with intensive foaming and clogging of gas pipes that led to termination of the experiment.

R2 was used as a control reactor and was started on day 122 of the experiment, at the same time when 7.5% SM loading was initiated in R1. R2 was also operated with a HRT of 22.5 days and with OLR of 0.9 ± 0.04 kg_{VS} m^{-3} d^{-1}, producing 92.9±17.0 m^3_{CH4} t^{-1}_{VS} (1.9 ± 0.4 m^3_{CH4} t^{-1}) with a VS removal efficiency of 41.6. These values are lower than the ones reported during the SS_1 mono- digestion in R1, due to the lower COD content of SS_3-SS_5 and probably to a lower biomass activity of R2 compared with R1. Still, those values can be considered "normal" for anaerobic digestion of SS (Davidsson et al., 2008; Luostarinen et al., 2009).

Effluent composition was analysed at the end of each loading regime and the results are presented in Table 3. Results showed that organic concentrations increased in accordance with the increasing OLR, nitrogen load and influent TS content increase. To conclude, the addition of 2.5% and 5% of SM increased CH_4 production by 2.9 and 5.7 times (respective to R1 SS mono-digestion) without detecting any process instability. First instability was detected with SM addition at 7,5% (representing an ORL of 3.55 kg_{VS} m^{-3} day^{-1}, and a nitrogen loading rate of 0.25 kg_{TN} m^{-3} day^{-1}). Further SM addition

of 10% resulted in nitrogen load increase up to maximum load of 0.32 kg N $m^{-3}day^{-1}$.

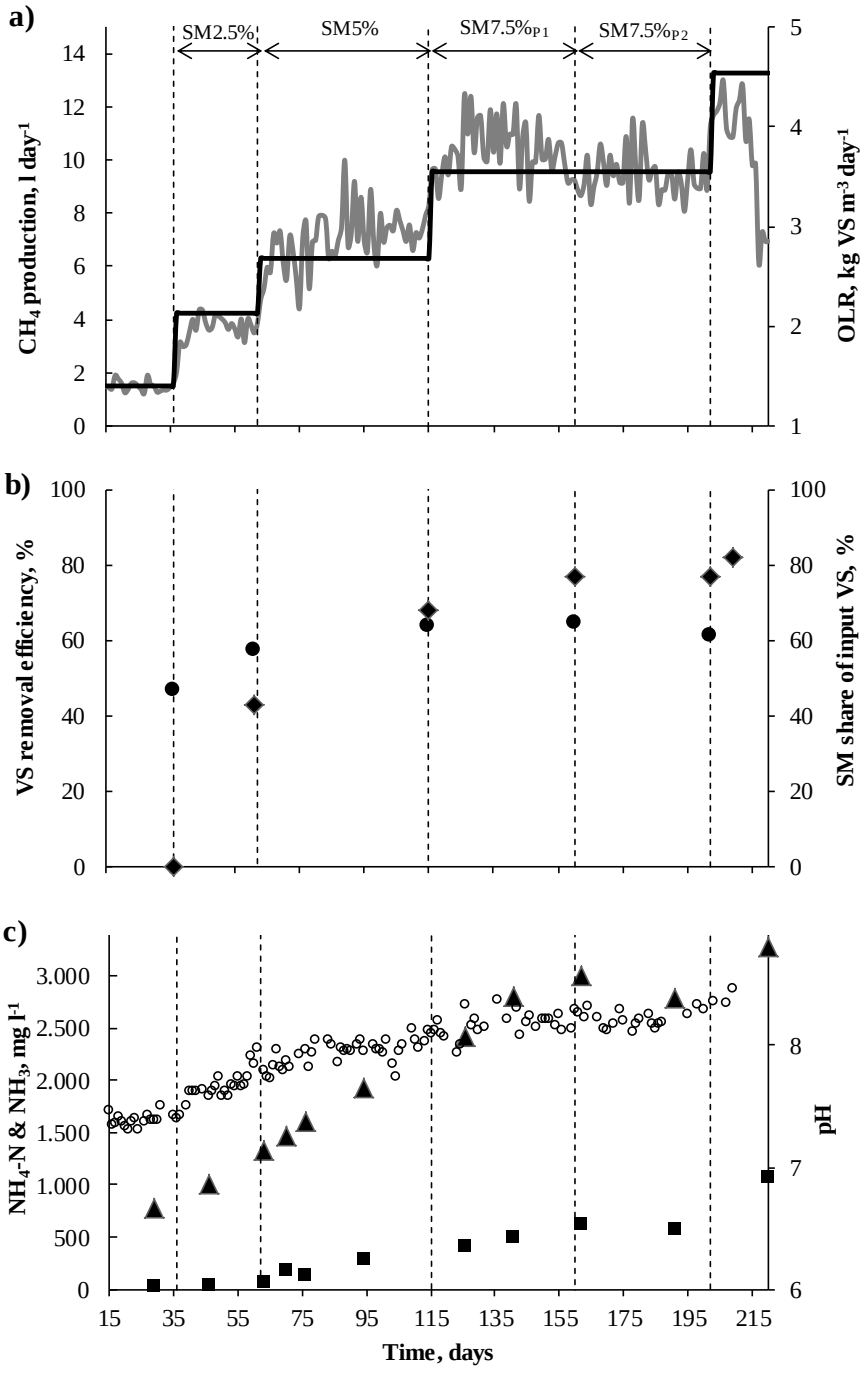

Fig.1. *Reactor1 (R1) process parameters: 1a) cumulative methane production, mL day^{-1} (–), organic loading rate, kg_{VS} m^3 day^{-1} (–); 1b) SM share of input VS, % (♦), VS removal efficiency (●), %; 1c) pH (o), ammonium, mg L^{-1} (▲) and free ammonia, mg L^{-1} (■) concentration patterns. Dashed vertical lines indicate the different feeding periods of sterilized mass co-digestion.*

Table 3 *Mean values of reactors (R1 and R2) outflow characteristics at the different operational periods.*

				R1			R2
Parameter	SS	SM2.5%	SM5%	SM7.5%$_{P1}$	SM7.5%$_{P2}$	SM10%	SS
pH	7.3 ± 0.1	7.5 ± 0.1	7.9 ± 0.1	8.1 ± 0.1	8.2 ± 0.1	8.4 ± 0.1	7.4 ± 0.1
COD_{tot} (mg L^{-1})	19.9	30.9	34.6	48.4	47.5	52.9	20.2±1.5
NH_4^+ (mg L^{-1})	783	1326	1931	2795	2993	3700	585±51.1
Free NH_3 (mg L^{-1})	28 ± 4.2	85.5 ± 6.4	246.5 ± 34.6	548 ± 33.9	645 ± 41	1035.5 ± 115.3	21 ± 6.3
P_{tot} (mg $L^{-1)}$	621	1227	1396	1946	2000	2443	572.5 ± 55.9
LCFA ($mg_{COD\text{-}LCFA}$ g^{-1})	n.d	n.d	n.d	1170 ± 6.4	1330 ± 63.4	(2001 ± 309.2/ 9172 ± 701.2)*	744 ± 198.2

*n. d- not determined; *sample from bottom of R2/ sample from foamy upper part of R2; (P1) and (P2)- different periods of SM7.5% loading*

This increase in N was 5.3 times higher compared to initial SS mono-digestion value causing a clear TAN accumulation in the reactor over the optimal values required for stable reactor operation.

In the present study, optimal OLR and methane production obtained during 5% SM addition (2.68 kg_{VS} m^{-3} day^{-1} and 618.9 ± 70.7 m^3_{CH4} $t^{-1}_{VS,}$ respectively) were higher than those reported in the literature. Salminen and Rintala (2002) have reported methane yields of 520–550 m^3 $_{CH4}$ t^{-1}_{VS} under mesophilic conditions for solid slaughterhouse waste operated at considerably lower OLR of 0.8 kg_{VS} m^{-3} day^{-1} with 50 days HRT. Hejnfelt and Angelidaki (2009) reported only 40% higher methane production during co-digestion of 5% pork by-products mixed with pig manure at 37 °C compared to digestion of manure alone. On the other hand, Bayr et al. (2012) reported higher methane yields of 720 m^3 $_{CH4}$ t^{-1} at OLR of 1.0 and 1.5 kg_{VS} m^{-3} day^{-1} in mesophilic CSTR experiments. However, the above authors used only energy rich rendering and slaughterhouse wastes for co-digestion.

3.2.2 Process limitations

To elucidate the possible causes of the detected process imbalance in R1, when the SM addition was >5%, the reactor content composition was analysed in the end of the different loading periods. TAN and NH_3 concentration evolution in conjunction with OLR and pH are shown in Fig.1c and summarized in Table 3. TAN concentration during mono-digestion of SS_5 in R2 was 585 ± 51.1 mg_{NH4+} L^{-1}. At 5% of SM loading in R1, TAN concentration was increased up to values of 1,931 mg $_{NH4+}$ L^{-1}, although the process remained stable (Fig.1). These NH_4^+ values were similar to the values of 2.1–3.1 g $_{NH4+}$ L^{-1}, reported to be non-inhibitory for anaerobic digestion (Procházka et al., 2012). Ammonium accumulation increased with the SM loading at 7.5%. Moreover, the estimated free NH_3 concentration reached values of 596.5 ± 68.6 g $_{NH3}$ L^{-1} during the 7.5% of SM addition (days 116-202), according to Fig.2 and Table 3. This NH_3 level detected in R1 were within the inhibitory range reported in literature. Gallert and Winter (1997) indicated that NH_3 concentrations of 560 – 680 mg $_{NH3}$ L^{-1}caused a 50% inhibition of methanogenesis at pH of 7.6 under thermophilic condition. Similarly, Angelidaki and Ahring (1994) reported a decrease in biogas yield during anaerobic digestion of manure when NH_3 levels reached a concentration of 700 mg $_{NH3}$ L^{-1}. The

over 2-fold increase in NH_3 concentrations at SM7.5% (596.5 ± 68.6 mg_{NH3} L^{-1}) period compared to SM5% loading (246.5 ± 34.6 mg_{NH3} L^{-1}) was produced due to the high NLR and the high pH values. The direct relation between the free ammonia concentration (causing process inhibition) and OLR to methane yield is graphically reported in Fig.2. As most of the organic loading during co-digestion came from nitrogen rich SM, then methane yield relation to OLR had similar pattern to ammonia inhibition curve. Methane yield increased concurrently with NH_3 concentration up to value of 548 ± 33.9 mg_{NH3} L^{-1} (period P1 of 7.5% SM addition). Higher NH_3 concentrations (during period P2 of 7.5% SM addition) caused process imbalance and methane yields decrease (Fig.2). Finally, NLR in R1 continued increasing along SM addition (up to SM10%) causing a clear NH_3 accumulation (1,035.5±115.3 mg_{NH3} L^{-1}) in the reactor over the optimal required values for stable operation, concluding in an inhibitory process.

VFA monitoring along experimental time did not indicate an organic overload or other process imbalance in R1. As an example, in Table 4 are summarized the VFA production and consumption after a feeding pulse during $SM7.5\%_{P2}$ addition period, when the VS removal efficiency and methane production started to decrease. After feeding, VFA concentration reached a maximum concentration of 505 $mg_{COD\text{-}VFA}$ L^{-1}, within few hours, and then started to decrease. Acetate and iso-butyrate were the dominant identified VFAs, while the propionate concentration remained stable throughout feeding cycle between 12 and 22 $mg_{COD\text{-}VFA}$ L^{-1}. At the end of the daily feeding cycle (24 h), total VFA concentration reached the similar level of 108 $mg_{COD\text{-}VFA}$ L^{-1} as before the feeding cycle. Nakakubo et al (2008) studying process indicators of ammonia inhibition during thermophilic animal manure digestion, concluded that acetate and propionate were not always accumulated in ammonia inhibited systems, and consequently not always used as process indicators.

Therefore, some LCFA measurements were carried out in the reactor effluents in order to assess the possible inhibition due to fats or LCFA accumulation. One LCFA sample was taken from R2, as control, while three samples from R1, during SM7.5% and SM10% loading regimes were selected (Table 3). Total LCFA concentration in R2 was 744.4 ± 198.24 $mg_{COD\text{-}LCFA}$ g^{-1}_{sample}. On the other hand, total LCFA concentration in R1 was between 1,170 ± 6 and 1,330 ± 63 $mg_{COD\text{-}LCFA}$ g^{-1}_{sample} during SM7.5% loading.

Palatsi et al. (2009) reported that LCFA concentrations over 2.8 $g_{COD\text{-}LCFA}$ g^{-1}_{sample} were necessary to inhibit thermophilic digestion of manure in batch and semi-continuous experiments (resulting in a temporary cease of the methane production). Thus, the imbalance or inhibition detected in R1 during SM7.5% addition seems to be more related with ammonia inhibition than with organic overloads or LCFA inhibitory effect. Contrary, when R1 was submitted to SM10% loads, reactor resulted in intense foaming and clogging of gas outlets and thus the experiment had to be terminated. Two samples were collected for LCFA analyses, one from the top foamy layer and the other one from the lower part of the reactor. LCFA concentration in the top layer was as high as 9,172 ± 701.2 $mg_{COD\text{-}LCFA}$ g^{-1}_{sample}, mainly formed by palmitic and oleic acids, while the detected value in the lower part of the reactor was of only 2,001 ± 309.2 $mg_{COD\text{-}LCFA}$ g^{-1}_{sample}. Thus, complete process failure at SM10% loading was apparently due to an accumulation of LCFA. Lü et al (2007) demonstrated that high ammonia concentrations could also affect the lipid hydrolysis rates and the LCFA accumulation-inhibitory process.

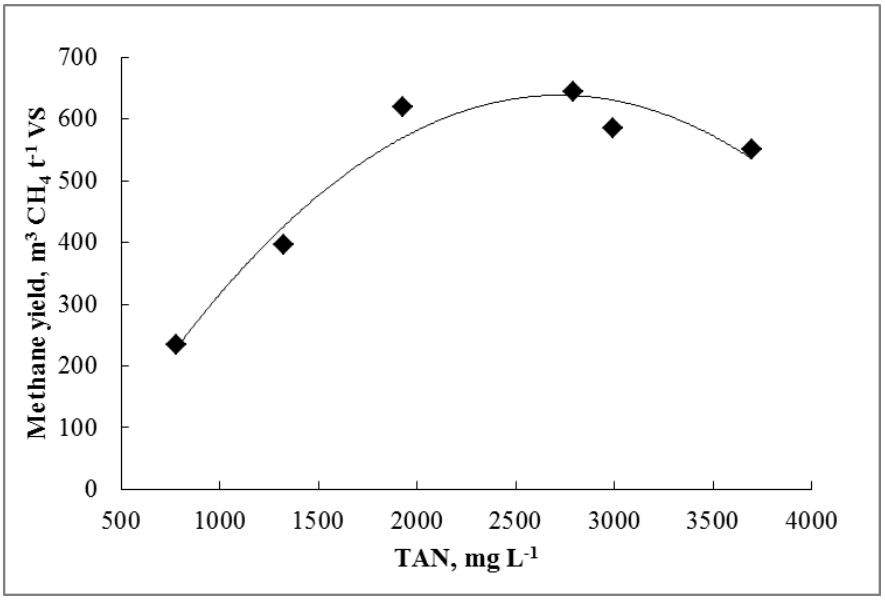

Fig.2. Relation between TAN (■) concentration and OLR (o) to methane yield at stable process conditions of different sterilized mass loading periods.

Table 3 *Mean values of reactors (R1 and R2) outflow characteristics at the different operational periods.*

				R1			R2
Parameter	SS	SM2.5%	SM5%	SM7.5%$_{P1}$	SM7.5%$_{P2}$	SM10%	SS
pH	7.3 ± 0.1	7.5 ± 0.1	7.9 ± 0.1	8.1 ± 0.1	8.2 ± 0.1	8.4 ± 0.1	7.4 ± 0.1
COD_{tot} (mg L^{-1})	19.9	30.9	34.6	48.4	47.5	52.9	20.2±1.5
NH_4^+ (mg L^{-1})	783	1326	1931	2795	2993	3700	585±51.1
Free NH_3 (mg L^{-1})	28 ± 4.2	85.5 ± 6.4	246.5 ± 34.6	548 ± 33.9	645 ± 41	1035.5 ± 115.3	21 ± 6.3
P_{tot} (mg $L^{-1)}$	621	1227	1396	1946	2000	2443	572.5 ± 55.9
LCFA ($mg_{COD\text{-}LCFA}$ g^{-1})	n.d	n.d	n.d	1170 ± 6.4	1330 ± 63.4	(2001 ± 309.2/ 9172 ± 701.2)*	744 ± 198.2

*n. d- not determined; *sample from bottom of R2/ sample from foamy upper part of R2; (P1) and (P2)- different periods of SM7.5% loading*

4. Conclusion

Results of semi-continuous reactor experiments confirmed the suitability and attractiveness of sewage sludge co-digestion with SM. Based on the obtained data, optimal process conditions were established at loading rate of 5% of SM, with an equivalent OLR below 2.68 kg_{VS} m^{-3} d^{-1}. Those conditions enhanced methane production by 5.7 times compared to SS mono-digestion. During this period NH_3 concentration remained below inhibitory levels (<500 mg_{NH3} L^{-1}). Higher SM addition (up to 10% w/w) caused process imbalance, initiated by NH_3 accumulation and finalizing with lipids-LCFA accumulation, foaming, methane yield reduction and termination of the experiment.

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Annex 2. Influence of adsorption and anaerobic granular sludge characteristics on long chain fatty acids inhibition process

The impact of LCFA adsorption on the methanogenic activity was evaluated in batch assays for two anaerobic granular sludges in the presence and absence of bentonite as synthetic adsorbent. A clear inhibitory effect at an oleate (C18:1) concentration of 0.5 gC18:1 L-1 was observed for both sludges. Palmitate (C16:0) was confirmed to be the main intermediate of C18:1 degradation in not adapted sludge and its accumulation was further evidenced by fluorescence staining and microscopy techniques. LCFA inhibition could be decreased by the addition of bentonite, reducing the lag-phase and accelerating the kinetics of LCFA degradation, concluding in the importance of the adsorptive nature of the LCFA inhibitory process. Granule morphology and molecular profiling of predominant microorganisms revealed that biomass adaptation to LCFA could modify the intermediates accumulation profiles and process rates.

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1. INTRODUCTION

Anaerobic digestion is a highly sustainable waste treatment process because it combines organic matter removal with energy production in the form of biogas. The energy yield depends, among other factors, on the organic matter composition (generally defined as the ratio between lipids-proteins-carbohydrates). In particular, lipids are interesting substrates for the anaerobic digestion process due to the high potential methane yield. Under anaerobic conditions, lipids are initially hydrolyzed to glycerol and long chain fatty acids (LCFA), which are further converted by acetogenic bacteria (ß-oxidation process) to hydrogen (H_2) and acetate (Ac), and finally to methane (CH_4) by methanogenic archaea.

LCFAs are the main intermediate by-product of the lipid degradation process, and their accumulation in anaerobic digesters has been related with problems of sludge flotation, biomass washout and inhibition of the microbial activity (Rinzema *et al*., 1994). These LCFA inhibitory effects have been associated to the interference with the electron transport chain, impairment of the nutrient uptake, inhibition of specific enzyme activities, or to the generation of toxic peroxidation and autooxidation products (Desbois and Smith, 2010). It has long been stated that adsorption of LCFA onto the cell membrane is the main factor determining its biological toxicity (Galbraith and Miller, 1973). More recently, Pereira *et al*. (2005) evidenced the importance of LCFA on transport limitations at the level of the cell membrane, and on how this affects the overall anaerobic digestion process. The fact that LCFA inhibition is reversible suggests that it is partially driven by LCFA adsorption onto the biomass and that the methanogenic activity might thus be resumed once the LCFA that have been accumulated onto the biomass are progressively metabolized.

Oleate (C18:1), stearate (C18:0) and palmitate (C16:0) are the major constituents in lipid-rich wastes and wastewaters (Batimelli *et al*, 2010; Valladão *et al*., 2011). The inhibitory effect of a specific LCFA has been defined as concentration dependent, but it is also related to the LCFA chain length and degree of saturation (Lalman and Bagley, 2001). Differences on cell membrane properties between bacteria and archaea also play an important role on LCFA toxicity (Zheng *et al.*, 2005). C16:0 has been proposed to be the main intermediate and the key inhibitory specie during the anaerobic degradation of C18:1 via the ß-oxidation process (Pereira *et al*., 2002). Recently, several studies have

published valuable information dealing with the diversity of saturated/unsaturated LCFA degraders and syntrophic methanogens interactions (Sousa *et al*., 2007).

Different strategies have been studied to prevent or to recover lipid/LCFA inhibited systems. The solubilization of lipid waste via saponification (Battimelli *et al*., 2010) or enzymatic pre-treatments (Valladão *et al*., 2011), the application of feeding procedures based on sequential LCFA accumulation-degradation steps (Cavaleiro *et al*., 2009), the addition of easily degradable co-substrates (Kuang *et al*., 2006) or the addition of adsorbents as recovery agents (Palatsi *et al*., 2009) have been proposed as possible strategies to limit the inhibitory effects.

Despite the extensive literature references about LCFA inhibition, only few studies focus on how important the adsorption process during the LCFA degradation is (Hwu *et al*., 1998; Pereira *et al*., 2005). The aim of the present study is to monitor the LCFA adsorption over granular sludge and to evaluate its impact on process inhibition, by means of: specific batch tests (monitoring LCFA evolution), addition of synthetic adsorbents (bentonite), molecular profiling of the predominant microorganisms and fluorescence staining microscopy imaging.

2. MATERIAL and METHODS

2.1. Physicochemical and biological characterization of biomass

Two different anaerobic granular sludges were used in present experiments, sampled from industrial beverage wastewater UASB reactors: sludge-A from a beer brewery (A Coruña, Spain) and sludge-B from a fruit juice processing industry (Lleida, Spain). Both sludges were characterized in terms of granular morphology, methanogenic activity and bacterial community structure.

The characterization of the granular morphology of both sludges (20 samples, containing more than 1,200 granules per sample or >0.2 g per sample) was performed analyzing digitalized images (768×574 pixel size, 256 grey levels) with the 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 the particles projected area, according to Pereira *et al*. (2003).

Methanogenic activity tests (SMA) of both sludges on acetate (Ac) and hydrogen (H_2/CO_2) were performed in batch anaerobic vials (120mL total volume vials with 50 mL of media working volume) at mesophilic temperature (35ºC), as described by Angelidaki *et al*. (2009).

The original microbial community structure was depicted by molecular profiling. The total DNA of biomass samples of both sludges was extracted and bacterial 16S rDNA gene fragments were amplified by polymerase chain reaction procedure (PCR). Amplicons were subsequently resolved by denaturing gradient gel electrophoresis (DGGE) analysis. Relevant DGGE bands were excised, reamplified by PCR and sequenced as described in Palatsi *et al*. (2010). Sequences were edited using the BioEdit software package v.7.0.9 (Ibis Biosciences, USA), compared against the NCBI genomic database with the BLAST search alignment tool (Altschul *et al*., 1990), and related to phylogenetic groups by using the RDP Naive Bayesian Classifier (Wang *et al*., 2007). Obtained nucleotide sequences have been deposited in the GenBank database under accession numbers HM100129 to HM100135.

2.2. LCFA adsorption isotherms on bentonite and granular sludge

The adsorption isotherms for oleate on bentonite and on anaerobic granular biomass (sludge-A) were determined in batch experiments (1 L vials with 500 mL of media working volume). Media was composed by demineralized water, sodium bicarbonate (1 $g_{NaHCO3}\ g^{-1}_{CODadded}$) and bentonite or inactivated anaerobic granular sludge as sorbents. In order to differentiate LCFA adsorption from biological degradation, biomass was previously inactivated by incubation (2h at 4ºC) with formaldehyde solution (4% v/v). This protocol was selected to minimize cell wall damage produced by other protocols (Ning *et al*., 1996). Sodium oleate at a concentration range from 0.5 to 5.5 $g_{C18:1}\ L^{-1}$ was added as LCFA sorbate model. Vials were maintained at mesophilic (35ºC) anaerobic conditions under continuous shaking (150 rpm).

Liquid samples were withdrawn periodically from the vials to monitor the soluble C18:1 concentration (*C*; $mg_{C18:1soluble}\ L^{-1}$). These measurements were fitted to a time course asymptotical exponential decay curve ($C=Ce+\alpha e^{-\beta t}$) to determine the equilibrium C18:1 concentration in the liquid phase (*Ce*; $mg_{C18:1soluble}\ L^{-1}$). Obtained *Ce* values, for each tested concentration, were used to fit a *Langmuir* isotherm model (Eq. 1):

$$C_{ad} = \frac{C_{max} K C_e}{1 + K C_e} \qquad \textit{Eq. 1}$$

where C_{ad} is the equilibrium amount of sorbate per unit of sorbent ($mg_{C18:1adsorbed}$ $g^{-1}_{TSadsorbent}$), C_{max} is the maximum sorptivity of C18:1 on sorbent ($mg_{C18:1adsorbed}$ $g^{-1}_{TSadsorbent}$) and K is the *Langmuir* equilibrium constant (L $mg^{-1}_{C18:1soluble}$). Sorbent concentrations were expressed in total solids (TS) units to compare biomass and bentonite adsorption capacity.

2.3. Selection of LCFA inhibitory concentration

The inhibitory effect of LCFA over anaerobic granular sludges (sludge-A and sludge-B) was assessed in batch assays. Increasing concentrations of C18:1 from 0.1 to 1.5 $g_{C18:1}$ L^{-1} were tested in 120 mL glass vials (50 mL of working volume) containing 5 g_{VSS} L^{-1} of granular sludge and 1 g_{NaHCO3} $g^{-1}_{CODadded}$, under strict mesophilic (35ºC) anaerobic conditions and continuous shaking (150 rpm). Those tests were carried out to determinate the amount of C18:1 that causes a clear and long lasting inhibition on biogas production over the selected inoculum. Specific methane rates were determined by the initial slope of the accumulated CH_4 production curve per biomass content unit (mL_{CH4} g^{-1}_{VSS} d^{-1}), to better compare different sludges responses.

2.4. Influence of the adsorptive process over LCFA inhibition

Three different experiments with bentonite addition were tested in batch assays in order to evaluate and quantify the effect of the LCFA-adsorption over LCFA inhibitory process (Table 1).

The first experiment (E1) was performed with sludge-A (*Ta_1* vials in Table 1) and consisted in the *prevention* of granular sludge inhibition by the addition of bentonite (day -2). After a biomass activation step with acetate (day 0), vials were inhibited with an oleate pulse (day +1). The second experiment (E2) was also tested with sludge-A (*Ta_2* vials in Table 1) but was focused on the study of the possible *recovery* of the degradation capacity of granular sludge upon oleate exposure or inhibition (day+1) by means of bentonite addition (day +2). Vials were also previously activated with acetate

(day 0). The third experiment (E3) was performed with sludge-B (*Tb* vials in Table 1) and was intended to *capture* LCFAs by incubating a higher concentration of bentonite with oleate (day-4), to force exclusively the LCFA adsorption over bentonite, prior to the inoculation with granular sludge (day 0). Since sludge-B arises from a steady state large-scale reactor, and was sampled immediately before the present assay, the sludge was previously exhausted (day-2) and the activation step with acetate was omitted.

In all experiments (Table 1), vials were previously buffered (3 g_{NaHCO3} L^{-1}) and the initial biomass and oleate concentrations were set constants (5 g_{VSS} $L^{-1}$and 0.5 $g_{C18:1}$ L^{-1}, respectively). Control vials (*Ca* and *Cb*), with LCFA and biomass but without bentonite, and blank vials (*BLa* and *BLb*) with only biomass, were also run for all the experiments. Incubations were carried out at 35ºC under shaking (150 rpm) and anaerobic conditions. Each treatment was performed in triplicate for biogas analysis (CH_4), and 12 vials per treatment were withdrawn during incubations in order to determine long chain fatty acids present in the solid and liquid phase ($LCFA_S$ and $LCFA_L$, respectively), and the volatile fatty acids (VFA) profile. All parameters were expressed in COD units to better follow the mass balance.

To quantify the adsoprtion effect of bentonita addition, a *Gompertz* equation (Eq. 2) was fitted to the methane production profiles in E2 experiments:

$$P = P_{\max} \cdot \exp\left[-\exp\left(-\frac{r_m \cdot e}{P_{\max}}(t-\lambda)+1 \right) \right] \qquad \textit{Eq. 2}$$

where P is the accumulated methane production (mg COD_{CH4} L^{-1}), which is expressed as a time t function (day), P_{max} is the methane production potential (mg COD_{CH4} L^{-1}), r_m is the maximum methane production rate (mg COD_{CH4} L^{-1} day^{-1}) and λ is the lag phase period on biogas production(day).

Table 1 *Experimental set-up of batch experiments to evaluate LCFA adsorption effect.*

Experiment	**Day -2**	**Day 0**	**Day +1**	**Day+2**
E1 and E2	**(sludge exhaustion)**	**(activation)**	**(inhibition)**	**(recovery)**
Ta_1 (E1)	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}	-
Ta_2 (E2)	5 $g_{VSS(A)}$ L^{-1}+3 g_{NaHCO3} L^{-1}	+30 mM_{Ac}	+0.5 $g_{C18:1}$ L^{-1}	+0.5 $g_{Bentonite}$ L^{-1}
Ca	5 $g_{VSS(A)}$ L^{-1}+3 g_{NaHCO3} L^{-1}	+30 mM_{Ac}	+0.5 $g_{C18:1}$ L^{-1}	-
BLa	5 $g_{VSS(A)}$ L^{-1}+3 g_{NaHCO3} L^{-1}	+30 mM_{Ac}	-	-
Experiment	**Day -4**	**Day -2**	**Day 0**	
E3	**(LCFA-bentonite adsorption)**	**(sludge exhaustion)**	**(inoculation)**	
Tb (E3)	5.0 $g_{Bentonite}$ L^{-1} + 0.5 $g_{C18:1}$ L^{-1}+3 g_{NaHCO3} L^{-1}		+5 $g_{VSS(B)}$ L^{-1}	
Cb	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}	
BLb	3 g_{NaHCO3} L^{-1}		+5 $g_{VSS(B)}$ L^{-1}	

2.5. Microscopy observations

For microscopy observation, intermediate (day +10) samples of granular sludge-A vials (Table 1), free (*BLa*) and submitted to LCFA pulse (*Ca*), were fixated (4% v/v formaldehyde in PBS) and embedded in Tissue-Tek OCT (optimal cutting temperature) compound (Sakura Finetek), as described in Batstone *et al.* (2004). Granule sections of 10 μm were obtained with a cryostat (CM 1900 Leica, Germany), operated with a knife temperature of -20ºC and a cabinet temperature of -18ºC. Slides were stained with multiple fluorochrome dyes. DAPI (4,6-diamidino-2-phenylindole, Sigma) was used as probe for biomass or total cells, while Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, Sigma) was selected as probe for hidrophobicity sites (lipids), based on Diaz *et al.* (2008). Images were obtained with a BX51 (Olympus, Japan) fluorescence light microscope (FLM). Observation settings were Ex365-370/B400/LP421 and Ex530-550/B570/LP591 for blue (DAPI) and red (Nile Red) channels, respectively. Merged images were obtained using ImageJ (National Institutes of Health, USA) package software.

2.6. Analytical Methods

Sodium oleate powder salt (Riedel-de Haën/Sigma-Aldrich; 82% C18:1/LCFA) was selected as LCFA substrate model due to its high solubility and its major presence on fatty wastewaters (Hwu *et al*., 1998). Analytical grade powder bentonite (Al_2O_3 $4SiO_2$ H_2O, Sigma-Aldrich) was selected as the synthetic adsorbent.

Total solids (TS), volatile solids (VS), volatile suspended solids (VSS) and pH were determined according to Standard Methods (APHA, 1995). Biogas and methane (CH_4) 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 after sample acidification with a CP-3800 gas chromatograph (Varian, USA), fitted with Tecknokroma TRB-FFAP capillary column (30m×0.32mm×0.25μm) and FID detection (Palatsi *et al*., 2009). 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), based on direct LCFA methilation-extraction procedure, with a CP-3800 gas

chromatograph (Varian, USA), fitted with the capillary column Varian CP-Sil 88 (50mx0.25mmx0.2µm) and a FID detector, according to Palatsi *et al*. (2009). Obtained experimental samples were centrifuged (4,500 rpm 10 min), to differentiate between solid and liquid phase LCFA content ($LCFA_S$ or $LCFA_L$, respectively).

3. RESULTS and DISCUSSION

3.1. LCFA adsorption isotherms on bentonite and granular sludge

Bentonite has been considered a good adsorbent for organic species because of its high specific surface area, high porosity and surface activity. Scanning electron microscopy (SEM) and FT-IR spectrometric analysis evidenced the porous structure of the clay mineral and the possible interaction of LCFA molecules with the silanol (SiO_2) groups of bentonite (Demirbas *et al*. 2006). A *Langmuir* model was fitted against the experimental data and the obtained parameters were compared with the literature (Figure 1). The obtained oleate maximum sorptivity value (C_{max}) on sludge-A was 373.78 $mg_{C18:1adsorbed}\ g^{-1}_{\ TSadsorbent}$ and the equilibrium constant (K) was $1.7 \cdot 10^{-3}$ L $mg^{-1}_{C18:1soluble}$. These isotherm parameters were lower than those previously reported by Hwu *et al*. (1998), using a *Freundlich* isotherm model and thermally inactivated granular sludge. With respect to bentonite, the estimated C_{max} and K values were 901.76 $mg_{C18:1adsorbed}\ g^{-1}_{\ TSadsorbent}$ and $1.23 \cdot 10^{-2}$ L $mg^{-1}_{C18:1soluble}$, respectively, which evidenced the relatively high adsorption capacity of the clay based mineral sorbent compared with biomass (sludge-A). These values were comparable to those obtained by Mouneime *et al.* (2004) with bentonite suspensions and solid fatty wastes.

Assuming that adsorption can be described as a physical phenomenon dependent on the sorbent surface and sorbate concentration (Ning *et al*., 1996; Hwu *et al*., 1998), the higher specific surface area of clay minerals like bentonite (Raposo *et al*., 2004) in relation to the anaerobic granular sludge (further reported), explains the higher adsorption capacity of the former. These results show that bentonite outcompetes granular biomass in terms of sorptive capacity towards LCFA and, thus, its addition as a synthetic adsorbent in anaerobic digesters might influence dynamics of LCFA-adsoption- inhibition process.

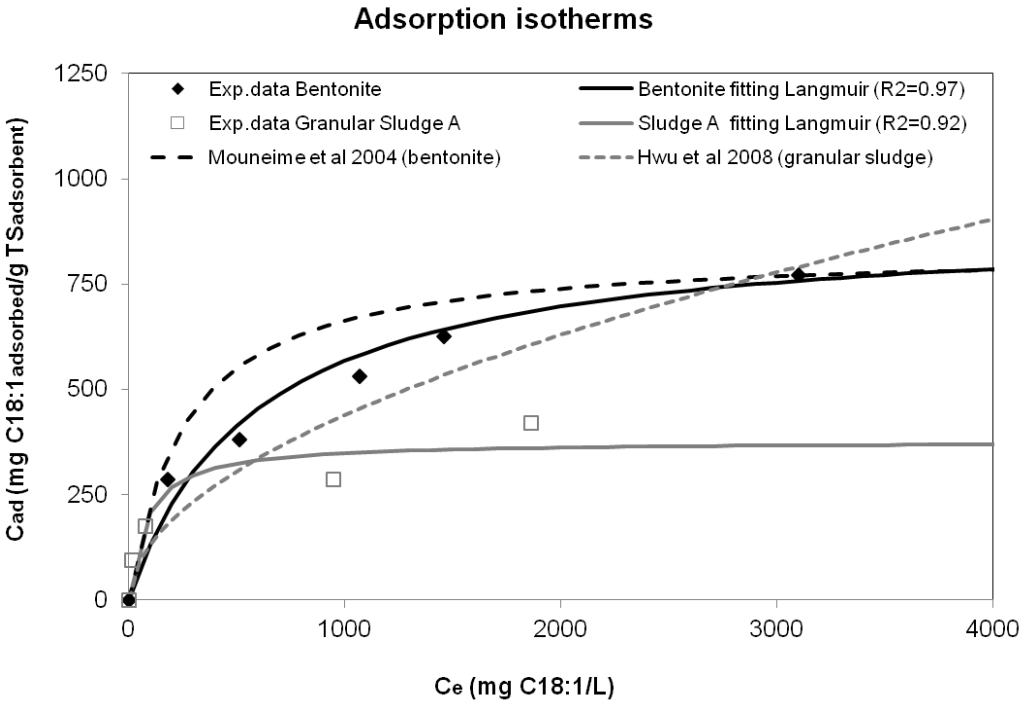

Figure 1 *Estimated adsorption isotherms, using a Lagmuir isotherm model, for bentonite and inactivated sludge-A, and comparison with available literature values. Markers represent experimental data while lines represent data fitting to isotherm models. Coefficients of determination for data fitting (R2) are also indicated in the figure legend.*

3.2. LCFA inhibitory concentration

LCFA degradation rates by sludge-A and sludge-B were estimated, according to Material and Methods section, using the initial slope of the specific methane production rate at different initial LCFA concentrations. Exponential decay curves were fitted to the obtained degradation rates (Figure 2).

A concentration of 0.5 $g_{C18:1}$ L^{-1} (aprox. 1.5 g_{COD} L^{-1}) caused a decrease on the methane production rate higher than 50% in both sludges (sludge-A and sludge-B), according to Figure 2. This value was selected as the reference oleate concentration in subsequent batch experiments (Table 1). It is interesting to observe that sludge-B displayed a higher degradation capacity of oleate in relation to sludge-A, at least up to concentrations of 1 $g_{C18:1}$ L^{-1} (Figure 2). The different resistances of sludge-A and sludge-B to C18:1 inhibition will be discussed later, considering the results of biomass characterization (granules morphology, methanogenic activity and molecular profiling).

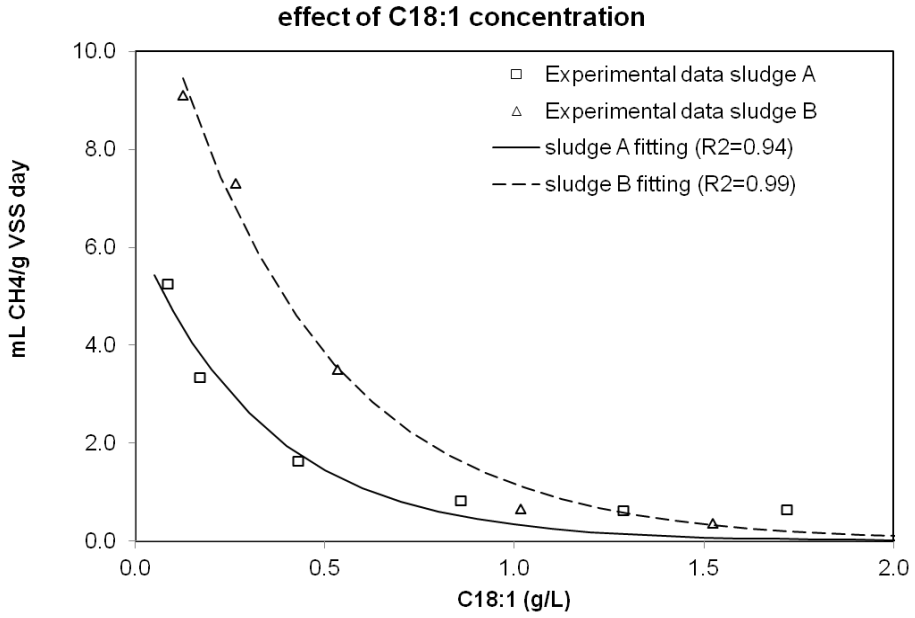

Figure 2 *Effect of tested initial LCFA (C18:1) concentration (expressed as* $g_{C18:1}\ L^{-1}$*) on the initial specific methane production rate (*$mL_{CH4}\ g^{-1}_{VSS}\ d^{-1}$*) of granular sludge-A and sludge-B. Markers represent experimental values while lines represent the fitting to an exponential decay curve. Coefficients of determination for data fitting (*R^2*) are also indicated in the figure legend.*

3.3. Influence of the adsorptive process on LCFA inhibition

Based on the previous results (adsorption isotherms and LCFA inhibitory concentration determination) different experiments were tested to study the effect of adsorptive process on LCFA inhibition and to quantify the LCFA adsorption-inhibitory effect by applying bentonite additions, as described previously in the Materials and Methods section (Table 1).

3.3.1. Addition of bentonite prior or after LCFA inhibition (E1 and E2)

The time course evolution of the $LCFA_S$ and $LCFA_L$ uptake, acetate transient accumulation, and methane production were monitored in E1 and E2, and showed in Figure 3.

Considering that the oleate pulse was added from an aqueous stock solution, it was assumed that this LCFA was exclusively contained in the liquid phase (Figure 3b and 3d). The adsorption of LCFA from the liquid media to solid phase was quite fast in all treatments, including the control vials (*Ca*) with no bentonite (Figure 3a and 3c).

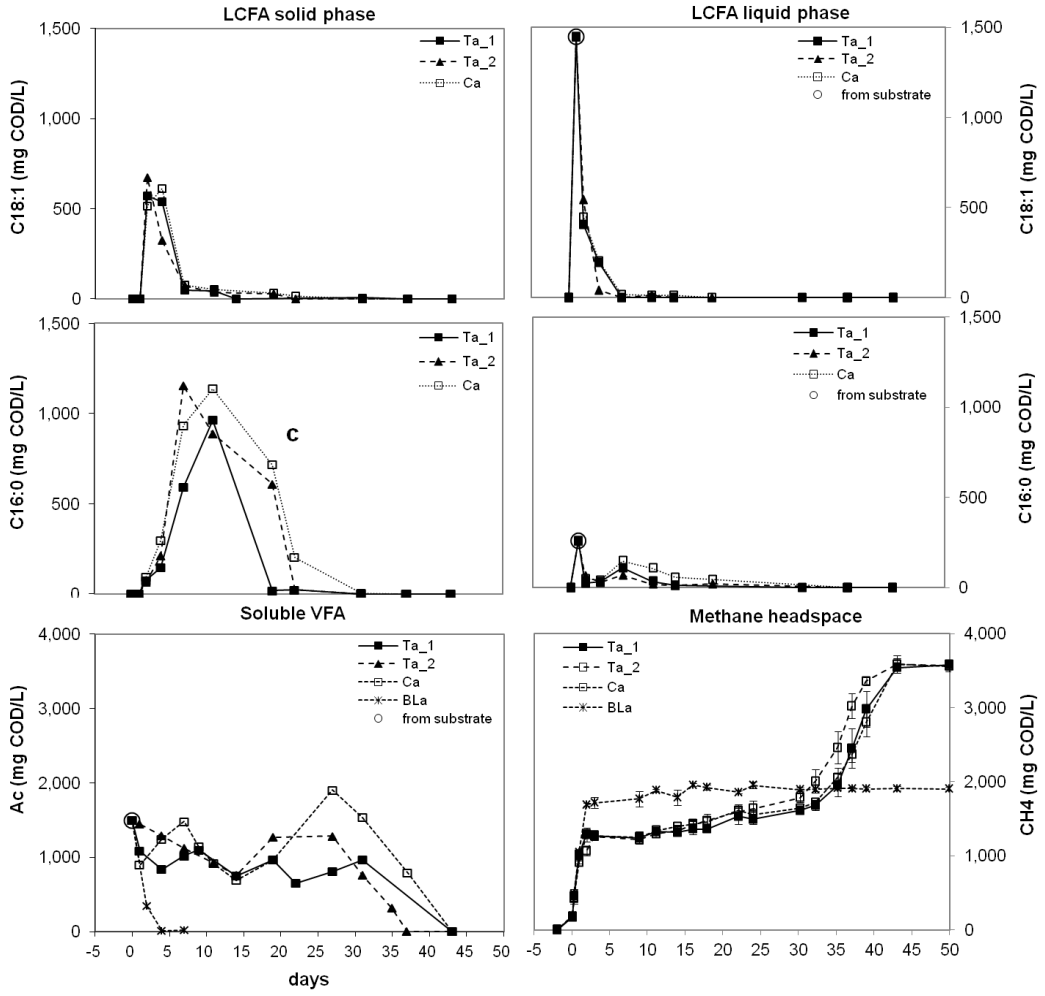

Figure 3 *Comparison between E1 and E2 batch tests in terms of LCFA concentration in the solid phase (a and c) and in the liquid phase (b and d), acetate profile (e) and methane formation (f), for bentonite (T_{A_1} or T_{A_2}), control (C_A) and blank (BL_A) vials. All parameters are expressed in COD equivalent concentration units (mg $_{COD}$ L^{-1}). The circles indicate the initial estimated concentration from LCFA pulse added into the vials.*

Oleate uptake from the solid phase was followed by a clear accumulation of palmitate, primarily on the solid phase as well, as shown in Figure 3c. No significant amounts of stearate nor palmitoleate were detected in liquid or solid phase samples (data not shown), which is consistent with the hypothesis of hydrogenization of unsaturated LCFA prior to the ß-oxidation process (Lalman and Bagley, 2001).

The metabolism of LCFA resulted in a transient accumulation of acetate in the medium (Figure 3e), which lasted until the complete degradation of palmitic acid. No significant amounts of other VFA intermediates (valerate, butyrate or propionate) were detected (data not shown), which is in agreement with others studies (Cavaleiro *et al.*, 2009). The inhibition caused by the LCFA pulse also resulted in an immediate halt on methane

production. However, this process was reversible as methane formation resumed after 30-35 days (Figure 3f), when the remaining LCFA were completely depleted (Figure 3a and 3c).

No significant differences were observed in LCFA degradation, accumulation of acetate, and methane production, between bentonite (*Ta_1*) and control (*Ca*) vials in experiments E1 (Figure 3), indicating that bentonite had little effect on preventing or modifying LCFA inhibition. A possible explanation for this unexpected phenomenon in E1 is the potential interaction between bentonite and biomass prior to the oleate pulse (4 days contact, Table 1). Clay minerals, like bentonite, have been largely used as support materials for the growth of anaerobic biofilms, due to their biomass adsorption capacity (Arnaiz *et al.* 2006). Furthermore, the spatial distribution and probability of *bentonite-LCFA* or *biomass-LCFA* adsorption occurrence, which is a function of LCFA concentration and particle density (0.5 $g_{Bentonite}$ L^{-1} vs 5 $g_{VSSbiomass}$ L^{-1}), might have played an important role in the results. Mouneimne *et al*. (2004) considered this factor in the calculation of the adsorption isotherms for multiple species systems (*biomass-clay mineral-LCFA*). Consequently, if biomass was attached to bentonite particles prior to LCFA pulse, the preventing effect expected in experiment E1 might have been severely hampered as the free adsorption sites available for LCFA in bentonite surface might have experienced a strong reduction (see scheme *a* in Figure 4).

On the other hand, relatively few differences were observed in the LCFA degradation profile and methane production rates, between bentonite (*Ta_2*) and control vials (*Ca*) in the experiment E2, which resulted in a slightly faster acetate consumption and methane production rate (Figure 3). If it is assumed that LCFA β-oxidation process was performed over the granule cell wall surface (only Ac and H_2 delivered to the media, but not other LCFA intermediates), the addition of bentonite would affect only the initially (day +2, Table 1) added LCFA concentration not yet adsorbed onto biomass (low according to Figure 3). Consequently, the *recovering* effect of E2 would be poor (see scheme *b* in Figure 4).

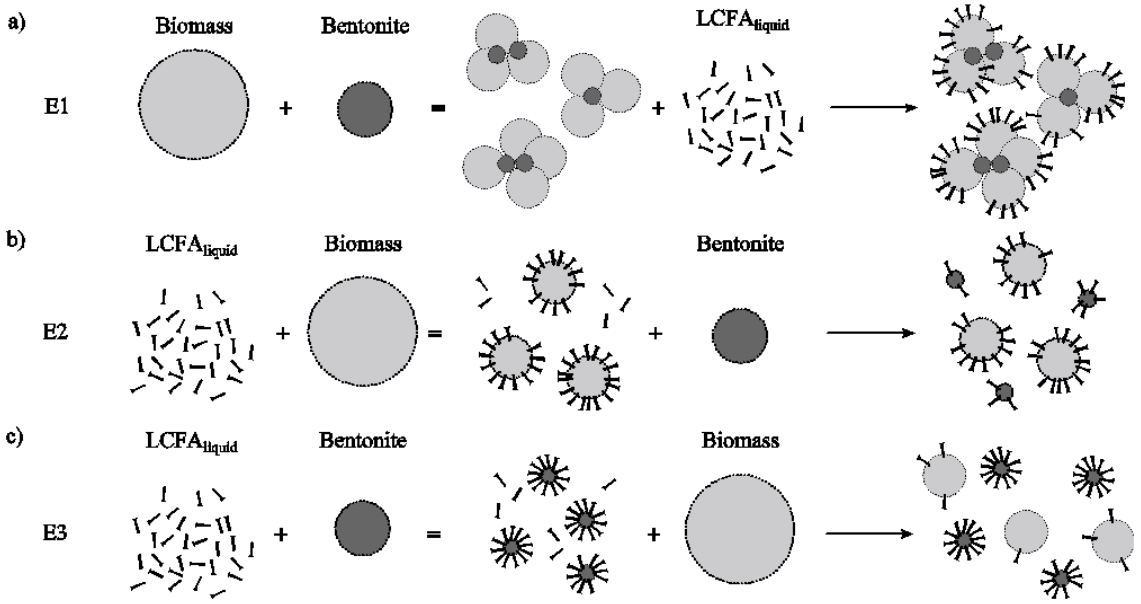

Figure 4 *Scheme of the bentonite addition effect onto LCFA adsorption-inhibition process in E1, E2 and E3 experiments*

In order to confirm that the measured accumulation of palmitic acid on the solid phase (LCFAs) is effectively adsorbed onto the granule surface (no β-oxidation intermediates delivered to the media) causing biomass inhibition, rather than just precipitating in the bulk media, granules from the control (*Ca*) and blank vials (*BLa*) were taken at day *+10*, when most of the oleate had already been consumed and maximum palmitate levels were detected in the solid phase (Figure 3c). Granules from *Ca* (incubations with biomass and oleate) and *BLa* (only biomass for endogenous activity), vials were sectioned, stained with DAPI-Nile Red, and examined under fluorescent light microscopy (FLM). Figure 5 shows an example of the appearance under FLM of *BLa* (Figure 5 a-c) and *Ca* (Figure 5 d-f) stained sections.

Nile Red is a hydrophobicity indicator but with this stain it is not possible to differentiate between the natural phospholipids of the cell membrane and the adsorbed LCFA (Diaz *et al*., 2008). Yet, the more intense Nile Red signal observed in the outer layer of the *Ca* granules (white arrow in Figure 5f) in relation to the *BLa* further supports the fact that palmitate formed upon the metabolism of oleate is adsorbed on the granule surface, with the subsequent potential implications in membrane transport limitations or in other LCFA inhibitory effect.

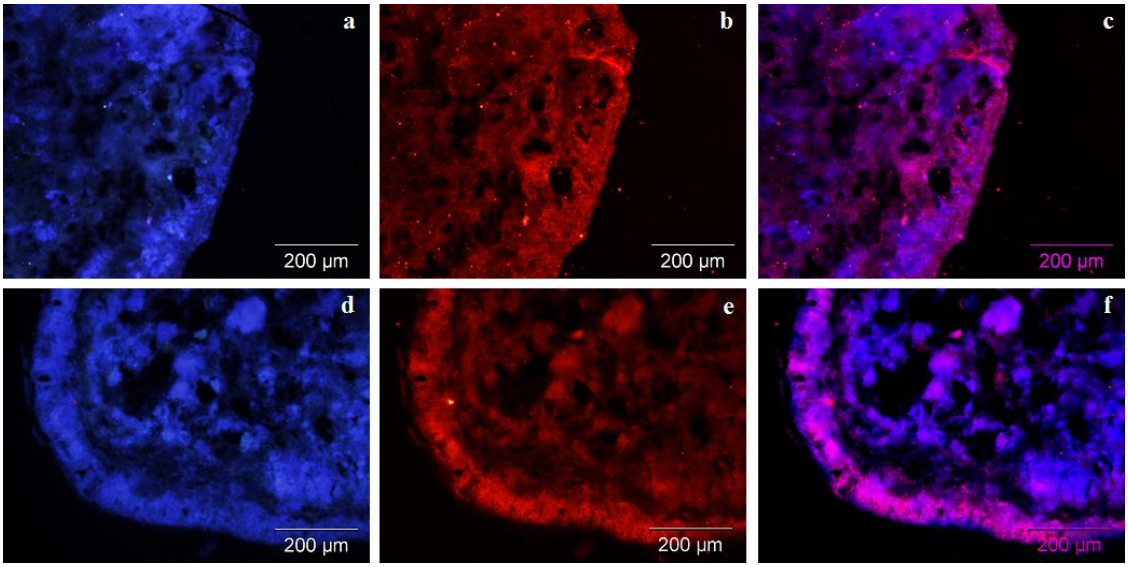

Figure 5 *FLM images of DAPI staining (a and d), Nile Red staining (b and e) and merged emissions (c and f), of BL_A and C_A sectioned granules, respectively.*

3.3.2. *Addition of bentonite for LCFA capturing (E3)*

To confirm the implications of LCFA adsorption over process inhibition, a different new experiment (E3) was designed with sludge-B. In this case, bentonite was also incubated with oleate during 4 days prior to biomass addition (Table 1), in order to promote LCFA-bentonite adsorption or LCFA *capturing* for the enhanced protection of biomass from LCFA adsorption-inhibition. A higher content of adsorbent (5 $g_{bentonite}$ L^{-1}) was added to have a more even biomass/bentonite particle distribution ratio, as previously reported by Mouneimne *et al*. (2004).The time course evolution of the $LCFA_S$ and $LCFA_L$ uptake, acetate transient accumulation, and methane production were monitored in E3, according to Figure 6. The plot scales were maintained to that of the previous experiments (Figure 3), in order to facilitate the direct comparison of the obtained results.

Since in E3 oleate was incubated during 4 days in a buffered medium with and without bentonite (Table 1), at the time of biomass addition (day 0) it was considered that all oleate was solubilized in *Cb* vials and adsorbed on *Tb* vials (Figure 6a and 6b). From the evolution of the oleate biodegradation pattern, clear differences were observed among treatments. Oleate biodegradation rates were higher when bentonite was added (Figure 6a). A similar positive effect has been described on sepiolite addition during anaerobic degradation of oleate (Cavaleiro *et al*. 2010). This phenomenon was then

claimed to be the result of a decrease in the LCFA inhibitory effect because of the physical adsorption, but also to an improved metabolite transfer between acetogens and methanogens due to the higher proximity between those groups promoted by biofilm formation. This effect was not observed in the experiment E1, where biomass-bentonite adsorption was promoted (Figure 3). Furthermore, in the experiment E3, oleate degradation did not produce the expected accumulation of palmitate in the solid phase, neither in the treatment vials nor in the controls (Figure 6c), which points to the fact that sludge-B was probably more adapted to the metabolism of LCFA, as already indicated by the comparatively higher oleate biodegradation rate (Figure 2). Cavaleiro *et al* (2009) reported transient palmitate accumulation in reactors fed with oleate-based influents but, because of biomass adaptation, palmitate was not longer detected after two or three cycles of LCFA feeding. To clarify these aspects further biomass (sludge-A and sludge-B) characterization (granule morphology, methanogenic activity and bacterial community structure) was performed and further discussed in the next paragraphs, to explain their different LCFA biodegradation patterns. It must be noticed as well that sludge-B consisted of fresh granules collected from an operative UASB reactor from a fruit juice processing industry and, despite the preliminary starvation step, it still conserved a relatively high residual organic matter when compared to sludge-A (*BLb* vs *BLa*, methane production in Figures 3 and 6). The presence of easily biodegradable co-substrates, such as glucose and cysteine, which are likely to be present in higher amounts on sludge-B, has been found to enhance the LCFA degradation rates (Kuang *et al* 2006). Despite the intrinsic differences on the assayed sludges, the LCFA adsorptive action of bentonite in experiment E3, *capturing* LCFA, confirmed the implications of the LCFA-adsorptive process onto biomass, and confirms the adsorbent addition to be a reliable approach to improve the process robustness, by intervening on the kinetics of the LCFA adsorption and biological inhibition process (Figure 4c). In particular, the acetate accumulation was prevented (Figure 6e) and methane production was improved (Figure 6f) upon bentonite addition. A *Gompertz* equation (Eq. 2) was fitted to the methane production profile on both, treatment (*Tb*) and control (*Cb*) vials. as described in Figure 6f. For an equivalent estimated P_{max} in *Cb* and *Tb* vials (2,569.4±106.6 and 2,445.6±32.9 mg COD_{CH4} L^{-1}, respectively), the lag phase for bentonite vials (*Tb)* was significantly decreased from 6.2±0.8 to 3.0±0.7 days (51.3%)

whilst the maximum methane production rate was improved, from 130.5±12.2 to 187.1±10.3 mg COD_{CH4} L^{-1} day^{-1} (43.3%). Thus, it can be concluded that the LCFA-adsorptive process partially explain the LCFA inhibition process (nutrient transport limitation) with a clear effect on the overall degradation process, but there are still some initial inhibitory or toxic effect (cell or membrane damage) that must be also considered. Further complementary studies, which include mathematical modeling of the experimental data, are underway and will help to elucidate the relevance of specific factors that are associated to membrane damage.

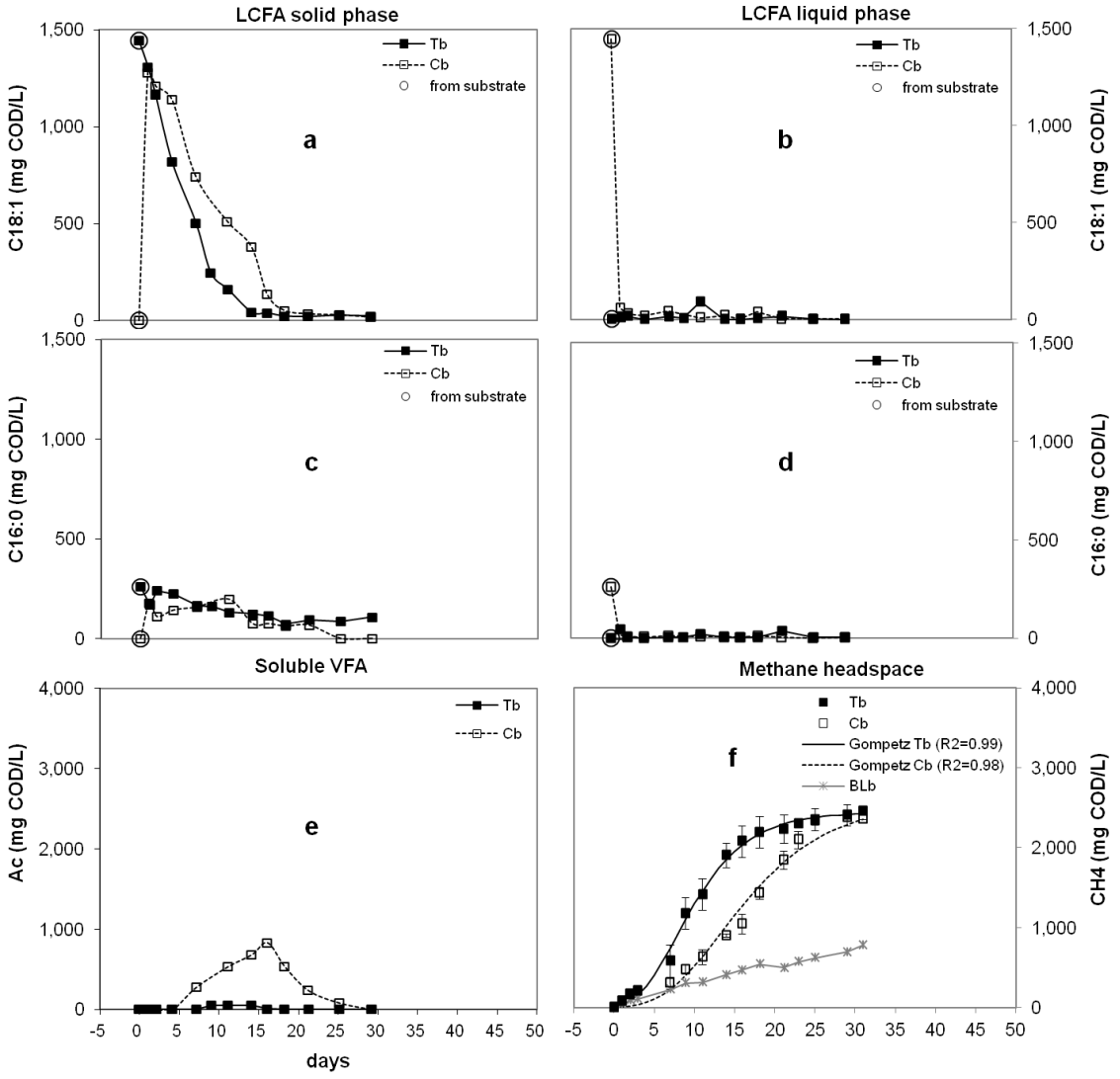

Figure 6 *E3 batch tests in terms of LCFA concentration in the solid phase (a and c) and in the liquid phase (b and d), acetate profile (e) and methane formation (f), for bentonite (T_B), control (C_B) and blank (BL_B) vials. All parameters are expressed in COD equivalent concentration units (mg $_{COD}$ L^{-1}).The circles indicate the initial estimated concentration from LCFA pulse added into the vials.*

3.4. Influence of sludge characteristics on LCFA inhibition

Considering that adsorption is a surface-related phenomenon, sludge specific area might be related to the LCFA inhibitory effect as well, as earlier proposed (Hwu *et al*., 1998). Therefore, the specific surface areas (cm^2 g^{-1}VSS) of both granular sludges were estimated by image analysis (Table 2). The obtained values on the specific area of sludges-A and sludge-B were quite similar and did not explain the differences on the LCFA inhibitory effect. On the other hand, LCFA-degrading microorganisms are known to be proton reducing bacteria, which interact syntrophically with H_2-utilizing and acetoclastic methanogens (Schink, 1997; Lalman and Bagley, 2001). Consequently, the balance between acetogenic bacteria and archaeal communities plays a central role in the LCFA metabolism, influencing the overall process rate (Pereira *et al*., 2002). Nevertheless, since sludge-B had a lower methanogenic activity (both acetoclastic and hydrogenotrophic activity) than sludge-A, it is not possible to conclude that differences concerning LCFA biomass tolerance are directly related to the methanogenic activity (Table 2).

Table 2 *Summary of granular biomass characterization (mean values and standard deviation)*

Parameter	Sludge-A	Sludge-B
Sludge biomass content (g_{VSS} L^{-1})	8.81±0.13	8.87±0.02
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
Hydrogenotrophic SMA (mL_{CH4} g^{-1}_{VSS} d^{-1})	179.66±1.66	88.26±9.09
Acetoclastic SMA (mL_{CH4} g^{-1}_{VSS} d^{-1})	128.93±14.81	69.69±1.97
C18:1 initial content (µg g^{-1} $TS_{biomass*)}$	<d.l	10.5±0.7
C16:0 initial content (µg g^{-1} $TS_{biomass*}$)	16.5±2.1	105.0±2.8

Acetogenic and/or ß-oxidizing bacteria can also influence sludge behavior in relation to LCFA tolerance and metabolism. A considerable increase in the ß-oxidative activity of anaerobic sludge has been observed upon LCFA pulses (Pereira *et al*., 2005; Palatsi *et al*., 2010). Significant differences in the microbial community structure of bacterial populations from initial biomass samples (sludge-A and sludge-B) were observed upon DGGE profiling of PCR amplified bacterial 16S rDNA ribotypes (Figure 7).

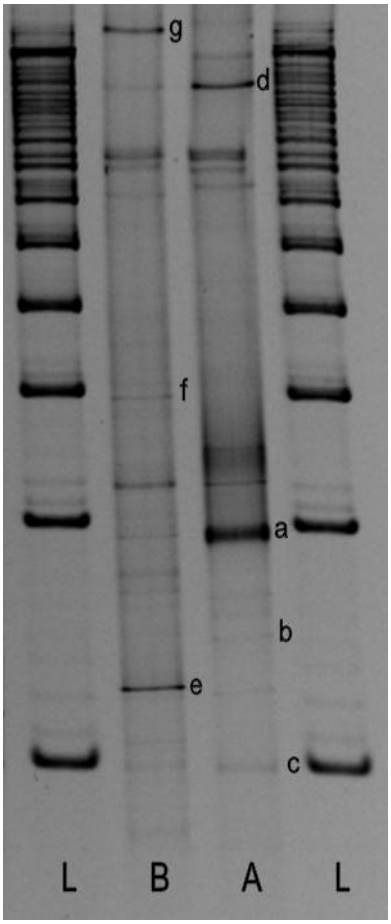

Figure 7 *DGGE profiles of eubacterial 16S rDNA amplified from sludge-A and sludge-B. A standard ladder (L) has been used at both gel ends in order to check the DNA migration homogeneity. Successfully excised and sequenced bands have been named with lower-case letters.*

Up to 7 bands from the obtained profiles were successfully excised, reamplified and subsequently sequenced (Table 3). BLAST sequence comparisons against the NCBI genomic database resulted in close matches with uncultured ribotypes in most of cases, previously described in anaerobic granular reactors (Narihiro *et al*., 2009; Riviere *et al*., 2009). Several phylogenetic groups were identified, mainly related with fermenting bacteria and acetate or propionate oxidative microorganisms, but none of them was directly related to specific LCFA degrading bacteria. Considering the origin of the tested sludges, from industrial plants where lipids might only be present in residual amounts, the absence of bacterial species related to LCFA metabolism among the dominant fraction of the bacterial community is thus not surprising. Consequently, the initial number of bacteria responsible for LCFA degradation may not be high enough for yielding visible bands in a DGGE profile. Other molecular high throughput

sequencing techniques, such as pyrosequencing, might give a deeper insight in relation to the presence and biodiversity of ß-oxidizing bacteria. Nevertheless, the obtained results clearly reveal that the origin of the biomass is relevant to the LCFA metabolism. Sludge-A was obtained from a brewing industry, where its wastewaters are expected to be free of lipids. Conversely, sludge-B was obtained from a fruit juice facility, and the wastewater could have been in contact with higher lipid content from fruit peels or press liquor wastes. This fact would be in agreement with the analysis of the initial LCFA profile performed on both sludges (Table 2), in which a significantly higher amount of LCFA was found in sludge-B compared to sludge-A. Further complementary studies, which include mathematical modeling of the experimental data, are underway and will help to elucidate the relevance of the specific factors that are associated to the biomass.

4. CONCLUSIONS

Batch incubations of methanogenic biomass from two different origins have clearly shown the inhibitory effect of oleate at concentrations above 0.5 $g_{C18:1}\,L^{-1}$. Nevertheless, this process was reversible and methanogenic activity recovered, showing that partial ß-oxidation was a non-limiting step. Palmitate was confirmed as the main intermediate in less adapted biomass and the main LCFA inhibitory species. Solid and liquid LCFA characterization and fluorescence staining microscopy inspection of the biomass, performed once all oleate was consumed from the liquid phase, demonstrated that palmitate was mostly adsorbed on granules surface. Oleate adsorption over anaerobic granular sludge and bentonite was characterized by means of Langmuir isotherms, which revealed the higher adsorption capacity of bentonite. Batch experiments designed to quantify the importance of the LCFA adsorption process on biological inhibition, by means of bentonite addition, proved that the protection of granule surface from LCFA-rich streams is a reliable approach to improve the system robustness, by affecting LCFA-biomass adsorption dynamics. Nevertheless, there is part of the inhibitory effect that still must be related with a LCFA toxic effect. Yet, biomass adaptation to LCFA is equally important for the anaerobic treatment of lipids.

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