Anaerobic Biodegradation of Long Chain Fatty Acids

Biomethanisation of biomass-associated LCFA as a challenge for the anaerobic treatment of effluents with high lipid/LCFA content

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Dissertação para Doutoramento em Engenharia Biológica e Química

na Universidade do Minho

Universidade do Minho

Escola de Engenharia

Departamento de Engenharia Biológica

2003

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Provas de Doutoramento realizadas em 24 de Outubro de 2003.

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AGRADECIMENTOS

Durante o decorrer deste trabalho, senti-me acompanhada por muitas pessoas tanto a nível científico como a nível pessoal. Quero aqui expressar os meus sinceros agradecimentos pela sua colaboração que foi, sem dúvida, preciosa para a concretização deste trabalho.

Refiro em primeiro lugar os meus orientadores Doutora Madalena Alves e Professor Manuel Mota que sempre me apoiaram e aconselharam e a quem devo um especial agradecimento pelas óptimas condições de trabalho proporcionadas, bem como pela valiosa ajuda a nivel da discussão crítica do trabalho. Mais do que uma excelente orientação, não posso ainda deixar de agradecer à Madalena Alves, o incentivo e encorajamento constantes, a partilha dos entusiasmos e dos desalentos, os valiosos ensinamentos e a preciosa amizade, com que me acompanhou, não só durante a realização deste trabalho, como durante os oito anos que temos de colaboração. A sua incondicional disponibilidade foi, sem dúvida, sempre uma importante fonte de energia e inspiração.

Gostaria ainda de agradecer ao colega e amigo Luis Amaral pela incansável disponibilidade demonstrada, bem como pela valiosa colaboração no trabalho de análise de imagem. Refiro ainda a importante colaboração do Doutor Eugénio Ferreira nesta parte do trabalho. Agradeço também aos colegas do Laboratório de Biotecnologia Ambiental, em especial à Olga Pires, Diana Sousa e Ana Julia Cavaleiro que colaboraram em alguns dos trabalhos descritos nesta tese, bem como à Lucia Neves e Pablo Kroff, pela boa camaradagem e apoio demonstrados.

Agradeço ainda o incansável apoio e sugestões do Doutor José Maria Oliveira durante as análises em cromatografia gasosa e ao "nosso" técnico Manuel Santos pela colaboração imprescindível na construção e montagem das instalações experimentais.

A todos os outros colegas e funcionarios do Departamento de Engenharia Biológica agradeço a camaradagem e as palavras amigas.

Parte deste trabalho foi efectuada no Laboratório de Microbiologia da Universidade de Wageningen, na Holanda, onde senti um carinhoso acolhimento e apoio por parte de todas as pessoas, e a quem expresso aqui os meus sinceros agradecimentos. *"Graag wil ik alle mensen van het Laboratorium voor Microbiologie bedanken voor hun gastvrijheid en de aangename werksfeer"*. I would like to express a special thank to Professor Antoon Akkermans and Professor Alfons Stams who made possible my stay at the Laboratory of Microbiology, and for the kind support and guidance in their areas of expertise. To Kees Roest, I gratefully acknowledge for teaching me all the details of the molecular techniques and sharing his scientific experience and knowledge, always in a prompt and enthusiastic way. Also his friendship and support were precious during my stay in Wageningen. Also to Erwin Zoetendal and Sergey Konstantinov, I am very thankful for their assistance in the application of the Molecular Analyst software to analyse DGGE profiles, as well as to Hans Heilig for the valuable technical assistance. I also thank to the other member of the Moleco's group: Cinzia, Reetta, Hauke, Elaine, Arjan, Kathrin, Mark, Nora, Maaike, Ineke, Wilma, Yao Wen, John, Andrea and other Moleco-students, for all the provided support and friendship. Fundação para a Ciência e Tecnologia and Fundação Calouste Gulbenkian are also acknowledged for the financial support provided for my stay in Wageningen.

Agradeço ainda aos familiares e amigos, pelo carinho e compreensão com que sempre me apoiaram e entenderam a minha frequente ausência, e em particular aos meus pais.... onde tudo começou.

Não posso deixar de referir o incentivo e apoio incondicional do Leon, bem como todo o carinho com que me acompanhou durante este trabalho. *"Lieve Leon, hartstikke bedankt voor alles"*. Thanks for "laugh with me" in the good moments and for "smile to me" in the less good ones.

Finalmente, é devido um reconhecimento às instituições e organismos que contribuíram para a realização deste trabalho: à Universidade do Minho, e à Fundação para a Ciência e Tecnologia (FCT) e Fundo Social Europeu (FSE) pelo apoio financeiro concedido no âmbito do III Quadro Comunitário de Apoio (PRAXIS XXI/BD/20326/99).

ABSTRACT

This work was focused on the anaerobic biodegradation of Long Chain Fatty Acids, especially those that are associated to anaerobic sludge by mechanisms of adsorption, precipitation or entrapment. When continuously fed with oleic acid (EGSB reactors, influent concentrations between 2 and 8 g COD/l and HRT=1 day), suspended and granular anaerobic sludge accumulated palmitic acid. This LCFA was efficiently biomethanised in batch assays, but the addition of oleic acid inhibited this conversion. The application of stirring conditions (150 rpm) enhanced the biomethanisation rate, when compared to static conditions. The maximum plateau achieved in the methane production curve was considered an indirect measurement of the amount of accumulated LCFA and attained a value as high as 3272±877 mg COD-CH_4/gVSS, for the suspended sludge, which was mineralised at a rate of 243±9 mg COD-CH_4/(gVSS.day). The amount of accumulated palmitate and the rate of methane production were highly dependent on biomass structure, suspended sludge being more efficient than granular sludge for that purpose. The application of image analysis techniques gave important insights on relative importance of fragmentation and filament release during granular sludge deterioration, induced by the contact with oleic acid. The equivalent diameter of the aggregates larger than 1 mm increased with the increase on the amount of accumulated LCFA. After a threshold value of about 200 mg COD-CH_4/gVSS the aggregates were mainly located in a top floating layer. In the granular sludge the amount of accumulated LCFA exponentially increased with the total free filament length, but exponentially decreased with the increase on the percentage of aggregates smaller than 1 mm, which evidenced the importance of filamentous bacteria on the process of LCFA accumulation/degradation and the relative irrelevance of the surface area for the phenomenon. In granular and suspended sludge, the composition of the bacterial community, based on 16S rDNA sequence diversity, was more affected during the oleate loading process than the archaeal consortium. Archaeal consortium remained rather stable in the granular sludge, whereas in the suspended sludge the relative abundance of *Methanosaeta*-like organisms became gradually weaker. The combination of molecular-based sludge characterisation with physiological and morphological characterisation provided complementary information on the mechanisms of sludge disintegration, flotation and washout, in relation with the contact/accumulation of LCFA.

The specific methanogenic activity in the presence of individual substrates (acetate, propionate, butyrate, ethanol and H_2/CO_2) measured before and after the depletion of the accumulated LCFA (mainly palmitic) strongly contradicted the accepted theory of bactericidal effect of LCFA towards anaerobic consortia as well as their permanent toxic effect. Although the encapsulated sludge had only detectable methanogenic activity in H_2/CO_2 and ethanol (24±6 and 437±12 mg COD-CH_4/(gVSS.day), respectively), after allowing the mineralisation of the accumulated LCFA, all the measured activities increased significantly, attaining values of 533±95, 16±4, 222±71, 67±1 and 2709±38 mg COD-CH_4/(gVSS.day), for acetate, propionate, butyrate, ethanol and H_2/CO_2, respectively. Aceticlastic tolerance to LCFA toxicity and the capacity of LCFA biodegradation exhibited by the consortia were also improved after allowing the mineralisation of the biomass-associated LCFA. These results put in evidence the possible strong effect of diffusion limitations imposed by the LCFA layer that could hamper the access of the added substrates to the cells as well as the subsequent biogas release. This physical effect could easily mislead the data obtained in previous works that suggest severe conclusions about LCFA inhibitory effects. The methanisation kinetics of biomass-associated LCFA (mainly palmitic) was established in batch assays, according to an inhibition model based on Haldane's enzymatic inhibition kinetics. An optimal value of 1080 mg COD-CH_4/gVSS was determined for the amount of accumulated LCFA that could be mineralised at the maximal rate of 250 mg COD-CH_4/(gVSS.day). Near this optimal specific concentration of LCFA, the effect of adding VFA to the medium was studied, in order to evaluate their interactions with the methanisation of the biomass-associated LCFA. Different patterns were obtained for each individual substrate. Although acetate and butyrate were preferentially consumed by the consortium, in the case of propionate no evidence of a sequential consumption pattern could be withdrawn.

From a practical viewpoint, the results presented in this work suggest that, in order to enhance methane production, it should be advantageous to sequence cycles of adsorption/accumulation and degradation, when treating effluents with high lipid/LCFA content.

SUMÁRIO

Este trabalho incidiu sobre o estudo da degradação anaeróbia de Ácidos Gordos de Cadeia Longa (AGCL), especialmente os que se encontram associados à biomassa por mecanismos de adsorção, precipitação ou inclusão. Quando se alimentou biomassa suspensa e granular com ácido oleico, em contínuo (reactores EGSB, concentração da alimentação entre 2 e 8 g CQO/l e TRH=1 dia) verificou-se que em ambas as biomassas se acumulava ácido palmitico que era eficientemente convertido em metano, em ensaios realizados em reactor fechado. Verificou-se ainda que a adição de ácido oleico inibia esta conversão, e que a mesma era acelerada em condições de agitação. O patamar de cada curva permitiu estimar a quantidade de AGCL acumulada em cada situação, tendo-se observado um máximo de 3272±877 mg CQO-CH_4/gSSV na biomassa suspensa, que foi mineralizada a metano a uma taxa de 243±9 mg CQO-CH_4/gSSV.dia. Verificou-se ainda nesta experiência que a acumulação de AGCL na biomassa era altamente dependente da sua estrutura, sendo, neste caso, a biomassa suspensa significativamente mais eficiente do que a biomassa granular. A aplicação de técnicas de análise de imagem permitiu obter informação relevante sobre fenómenos de fragmentação e de libertação de filamentos durante o processo de deterioração granular, induzido pelo contacto com o ácido oleico. O diâmetro equivalente dos agregados maiores que 1mm presentes na base de RI aumentou com o aumento da quantidade de substrato acumulado e a partir duma certa quantidade (≈200 mg CQO-CH_4/gSSV) houve uma tendência para que estes agregados flutuassem. A quantidade de AGCL que acumulou na biomassa e a respectiva taxa de metanização aumentaram exponencialmente com o comprimento total de filamentos livres, mas diminuíram exponencialmente com o aumento da fracção de agregados menores que 1 mm, o que evidenciou a importância dos filamentos no processo de acumulação destes substratos e relativizou a importância do aumento da área superficial dos agregados para o mesmo fenómeno. A aplicação de técnicas moleculares para avaliar a diversidade microbiológica dos consórcios presentes em ambos os reactores, revelou que o domínio bactéria foi o mais afectado pelo contacto com o ácido oleico. Embora na biomassa granular o domínio arquea tivesse permanecido relativamente estável, na biomassa suspensa a abundância relativa de *Methanosaeta* diminuiu. A combinação de técnicas moleculares com dados fisiológicos e com os aspectos morfológicos quantificados por análise de imagem permitiram obter informação complementar sobre os mecanismos de fragmentação, flutuação e washout e sua relação com o contacto com o ácido oleico.

A actividade metanogénica na presença de substratos individuais (acetato, propionato, butirato, etanol e H_2/CO_2) foi medida antes e depois de mineralizados a metano os AGCL (essencialmente palmitato) acumulados na biomassa. Os resultados obtidos contrariaram fortemente a teoria estabelecida sobre o efeito bactericida e o efeito tóxico permanente destes ácidos gordos sobre o consórcio anaeróbio. Embora a biomassa encapsulada com AGCL apenas apresentasse uma actividade detectável em etanol, e H_2/CO_2, (24±6 and 437±12 mg CQO-CH_4/(gSSV.dia), respectivamente), após a mineralização deste substrato todas as actividades medidas aumentaram significativamente atingindo valores de 533±95, 16±4, 222±71, 67±1 e 2709±38 mg CQO-CH_4/(gSSV.dia), para acetato, propionato, butirato, etanol e H_2/CO_2, respectivamente. Estes resultados sugerem que os AGCL acumulados podem atrasar o acesso dos substratos adicionados para as células e também a libertação do biogás, provocando problemas de limitações difusionais, podendo este fenómeno físico estar na origem das conclusões demasiado severas obtidas por outros autores acerca dos efeitos inibitórios dos AGCL. A cinética de mineralização dos AGCL acumulados na biomassa foi estabelecida de acordo com um modelo de inibição baseado na cinética de inibição enzimática de Haldane. Obteve-se um valor óptimo de 1080 mg CQO-CH_4/gSSV para a quantidade de AGCL que poderia ser acumulado e posteriormente degradado a uma taxa máxima de 250 mg CQO-CH_4/(gSSV.dia). Estudou-se o efeito de adicionar ácidos gordos voláteis (AGV) a uma biomassa que possuía uma quantidade de AGCL perto do valor óptimo para avaliar as interacções destes substratos com a degradação do substrato já associado à biomassa. Verificou-se que, dependendo do AGV adicionado, se obtiveram diferentes perfis de consumo de AGV e AGCL. Acetato e butirato eram substratos preferenciais para o consorcio, mas quando em presença de propionato, não houve evidência dum consumo sequencial dos dois tipos de substratos.

Do ponto de vista prático, os resultados apresentados nesta Tese sugerem que deverá ser vantajoso aplicar um processo sequencial de adsorção/acumulação e posterior degradação, no tratamento de efluentes com elevados teores de lípidos/AGCL.

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AD – Anaerobic digestion.

bp - Base pair.

COD - Chemical oxygen demand.

$\Delta G^{o'}$ - Standard free energy change

DGGE - Denaturing gradient gel electrophoresis.

EGSB - Expanded granular sludge bed.

EDS - Energy dispersion spectroscopy.

GC - Gas chromatography.

HRT - Hydraulic retention time.

IC_{50} – Toxic concentration that reduces microbial activity in 50%.

LCFA - Long chain fatty acids.

minFD - minimum Feret diameter.

PCR - Polymerase chain reaction.

SD - Standard deviation.

STP - Standard temperature and pressure conditions.

UASB - Upflow anaerobic sludge blanket.

VFA - Volatile fatty acids.

vol - Volume.

VSS - Volatile suspended solids.

Vup - Upflow superficial velocity.

wt - Weight.

1 GENERAL INTRODUCTION

Abstract
This Chapter reviews current knowledge on the anaerobic biodegradation of lipid/LCFA based effluents. With a special focus on these compounds, a general overview of microbial and biochemical aspects of the Anaerobic Digestion process is presented. Therein, some methodologies for AD monitoring are reviewed and some reported problems associated to the AD treatment of lipids/LCFA containing wastewaters are discussed. Finally, the outline of the thesis is described.

1.1 SCOPE OF THIS THESIS

Anaerobic Digestion (AD) presents a great potential in the production of energy from renewable sources and has become increasingly recognized as a sustainable and environmentally friendly technology for the treatment and disposal of waste and wastewater. Its application to industrial wastewater was enhanced in the last years due to the development of new high-rate treatment systems. However, there are still some points that need further investigation in order to obtain a higher overall AD efficiency. Digestion of lipids is certainly one of the most important and less known topics in this area, and research is necessary to enhance the application of anaerobic digestion to a range of difficult effluents, such as slaughterhouse, edible oil, dairy or olive oil effluents.

Due to the increasing interest in high-rate AD processes, anaerobic consortia (especially those with a granular organization) have been deeply studied in the last years. A renewed knowledge of the role and metabolic interactions of anaerobic microorganisms has emerged, as well as some doubts on some established assumptions about their extreme sensitivity to toxicants and to general adverse conditions. In this context, the severe conclusions about Long Chain Fatty Acid toxicity that were reported in the literature in the early nineties, represented a challenge for the research group from the Environmental Biotechnology Laboratory, University of Minho, to develop research in this field. Previous results obtained in this laboratory, evidencing an efficient biomethanisation of high amounts of LCFA accumulated onto anaerobic sludge, were the driving force for this work.

1.2 OVERVIEW OF ANAEROBIC DIGESTION

1.2.1 BIOCHEMICAL AND MICROBIOL FUNDAMENTALS

Anaerobic digestion is the biological degradation of organic mater by a complex microbial ecosystem, in the absence of oxygen, through multiple sequences of metabolic pathways, involving many different kinds of interacting trophic groups. Methane and Carbon Dioxide are the main final products. The general simplified flow sheet of anaerobic degradation of organic mater, disregarding the possible contribution of sulfate reducers, is reported in Figure 1.1. Basically, the AD process can be divided in 4 main degradation steps: hydrolysis, fermentation or acidogenesis, syntrophic acetogenesis and methanogenesis. Given the diversity of anaerobes and the complex biochemistry involved, only a brief description of the main characteristics of importance will be considered in each degradation step. Special emphasis will be given on the biodegradation of lipid compounds.

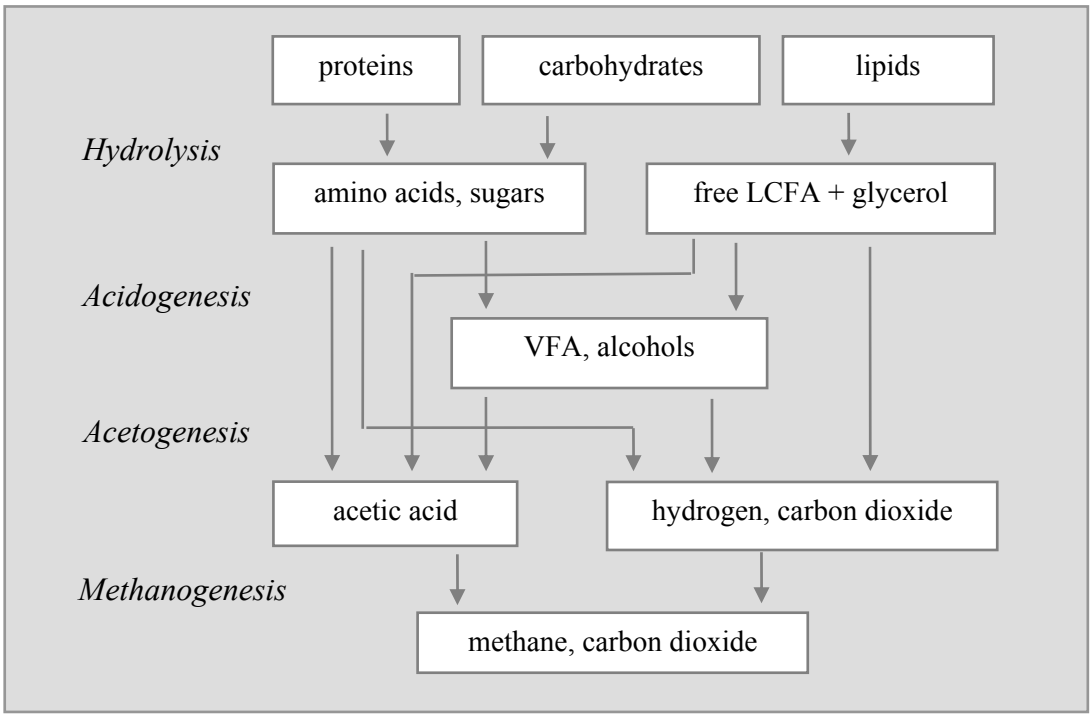

Figure 1.1 Simplified representation of the anaerobic digestion process (adapted from Gujer and Zehnder, 1983).

1.2.1.1 HYDROLYSIS

In the hydrolysis step, proteins, carbohydrates and lipids in the form of complex suspended compounds and colloidal matter are converted into their monomer or dimeric components, such as amino acids, single sugars and long chain fatty acids (LCFA). Organisms cannot take up non-soluble and particulate substrates that are too large to pass through the cell membrane and, therefore, extra-cellular enzymes (cellulases, amylases, proteases, lipases) are released to cleave polymers into smaller substrate molecules. This is normally a slow process, which can, in the case of complex substrates, be the rate-limiting step in the whole degradation process (Parkin and Owen, 1986).

Hydrolysis of lipids

Lipids are glycerol bonded to LCFA, alcohols, and other groups by an ester or ether linkage. Triacylglycerols, also called neutral fats, are the most abundant family of lipids and are hydrolysed by extracellular lipases to yield glycerol and LCFA. Glycerol is further degraded via acidogenesis while LCFA are degraded to acetate, H_2 and CO_2 through the β-oxidation process (Syntrophic acetogenesis) (Stryer, 1995). LCFA vary in chain length and number of inter-carbon double bonds. Those with no double bound are saturated, with single double bonds are mono-unsaturated, and with two or more are poly-unsaturated. Due to their amphiphilic structure, composed of a hydrophobic aliphatic tail and a hydrophilic carboxylic head, free LCFA will prefer the lipid-water interface instead of moving into the bulk solution. If further conversion of these free LCFA is slow, they can form a physical barrier that shields lipid surface from lipases action, or can alter the surface tension (Rietsch *et al*., 1977; Verger, 1980) thus affecting hydrolysis rate. Rather than the enzymatic activity, the slower hydrolysis of neutral fats under acidogenic conditions as compared to methanogenic conditions is governed by the difference on the available lipid surface for hydrolysis (Sanders, 2001). In this regard, this author suggests that methane production had a positive effect, because it reduces the coagulation of the lipid spheres which increases the lipid-water interface.

Because lipid hydrolysis proceeds relatively fast under methanogenic conditions (Hanaki *et al.*, 1981; Broughton *et al*, 1998; Salminen *et al.*, 2000), it is reasonable to expect that LCFA, which contain approximately 95% of the original lipid COD, will prevail

in lipid-containing wastewaters. Palmitic acid (hexadecanoic) and oleic acid (cis-9-octadecenoic) are, respectively, the most abundant saturated and unsaturated LCFA present is waste/wastewater (Table 1.1).

At neutral pH, e.g. in a bioreactor, LCFA become ionised, being appropriate to refer to them in that form, for instance oleate instead of oleic acid. Both terms are used throughout the present thesis.

Table 1.1 LCFA composition in various common raw materials and wastewater (% of total LCFA).

Common name (structure[1])	Palm oil [a]	Olive oil [a]	Soy bean oil [a]	Cotton seed oil [a]	Cocoa butter [a]	Whole milk [b]	Chicken fat [a]	Beef tallow [a]	Raw sewage [c]	Domestic wastewater [d]
Lauric (12:0)						7.0		1.0		
Myristic (14:0)	1.4		1.0	1.4		6.0	1.4	2.6		2.2
Palmitic (16:0)	42.9	14.3	11.0	25.7	26.7	21.0	21.0	28.1	27.6	16.4
Palmitoleic (16:1)	0.7	1.4		1.0	0.5	2.0	6.7	3.8		0.9
Stearic (18:0)	4.8	2.4	4.8	2.9	32.9	6.0	4.3	20.0	16.7	8.1
Oleic (18:1)	39.0	71.4	21.9	15.2	33.8	39.0	42.4	37.6	48.3	30.5
Linoleic (18:2)	10.0	5.5	49.0	51.9	4.3	13.0	20.0	2.9	5.1	29.2

[1]Number of carbon atoms:number of double bonds.

[a]Taylor, 1965; [b]Hanaki et al., 1981; [c]Viswanathan et al., 1962; [d]Quéméneur and Marty, 1994.

1.2.1.2 ACIDOGENESIS/FERMENTATION

Acidogenesis is the first energy yielding step, where soluble substrates such as amino acids and sugars, after being transported through the membrane into the cell, are degraded to organic acids and alcohols. In general, the acidogenic (fermentative) population represents about 90% of the total microbial population present in anaerobic digesters (Zeikus, 1980). They have short doubling time (Mosey, 1983), and therefore acidogenesis is not a limiting step in the global process of anaerobic digestion (Gujer and Zehnder, 1983). In environments with efficient H_2 sinks, such as anaerobic digesters, many of the acidogenic

species direct their metabolism to acetogenesis. This facultative change in metabolism has been demonstrated in defined methanogenic cocultures degrading lactate, pyruvate, cellobiose, glucose, fructose and cellulose (Mah, 1982).

Hydrogenation of unsaturated fatty acids

Acidogenic bacteria cannot degrade LCFA, although some species are capable of hydrogenation of unsaturated fatty acids (Dawson and Kemp, 1970). Hydrogenation of unsaturated fatty acid bonds is known to occur in the rumen. However, the importance of hydrogenation in anaerobic digesters has not been clarified. According to Novak and Carlson (1970), unsaturated LCFA needs to be saturated (hydrogenated) before entering the cycle reaction of β-oxidation. Weng and Jeris (1976) also proposed that, during the degradation of oleic acid (C18:1), the first step is the hydrogenation of the double bond and only afterwards stearate undergoes β-oxidation to finally form acetate. An alternative mechanism was proposed by these authors where oleic acid is firstly converted to pelargonic acid which afterwards undergoes β-oxidation to form acetate and propionate. However these authors concluded that this mechanism had a secondary role in the degradation of oleic acid.

The hypothesis that β-oxidation of unsaturated LCFAs can occur was also suggested by Roy *et al.* (1986), who reported the capacity of direct oxidation of unsaturated LCFA by isolated *Syntrophomonas* sp.. On the other hand, the detection of palmitoleic acid (C16:1) as a product of linoleic (C18:2) and oleic acid degradation also suggests the possibility of this mechanism to occur (Canovas-Diaz *et al.*, 1991; Lalman and Bagley, 2000). According to Lalman and Bagley (2001) this hypothesis is also supported by the free energies involved in LCFA degradation, since the production of shorter chain LCFAs from oleic and linoleic acid is energetically more favourable than from stearic acid.

1.2.1.3 SYNTROPHIC ACETOGENESIS

The fermentation products (short chain fatty acids and alcohols) are further oxidised to acetate by obligate hydrogen producing acetogens (OHPA), while reducing hydrogen ions to hydrogen (or bicarbonate to formate). Under thermodynamic standard conditions

these reactions are unfavourable, since they exhibit positive Gibbs free energy values (ΔGº') (Table 1.2). It was concluded that these reactions proceed only if the reaction products (mainly H_2) will be sufficiently reduced in concentration to yield a negative net value for the free energy change. This is achieved by syntrophic association with hydrogen (formate)-utilizing bacteria, usually hydrogenophilic methanogens that serve as hydrogen (formate) scavengers (Dolfing, 1988; Boone *et al*., 1989).

Table 1.2 Thermodynamics of some of the reactions involved in syntrophic conversions during methanogenic decomposition (Drake, 1994).

	ΔGº' (kJ/mol) [(a)]
Ethanol + $H_2O \rightarrow$ Acetate$^-$ + H^+ + $2H_2$	+9.6
Butyrate$^-$ + $2H_2O \rightarrow$ 2Acetate$^-$ + H^+ + $2H_2$	+48.1
Propionate$^-$ + $3H_2O \rightarrow$ Acetate$^-$ + HCO_3^- + H^+ + $3H_2$	+76.1
HCO_3^- + $4H_2$ + $H^+ \rightarrow$ CH_4 + $3H_2O$	-135.6

[(a)] 298ºK, pH 7, 1M for solutes and 1 atm for gases.

According to the data presented in this Table, acetogenesis from propionate is the less favourable conversion. The digestion pathway of propionate is thought to mainly occur via the randomising methyl-malonyl-CoA, by reversing the digestion pathway to pyruvate and hence to acetyl-CoA and acetate (Stams and Plugge, 1994). However, other pathways of propionate degradation via butyrate can also occur in methanogenic environments (Tholozan *et al.*, 1988; Lens *et al*., 1996; de Bok *et al.*, 2001). *Syntrophobacter* species, such as *S. wolinii* (Boone and Bryant, 1980), *S. pfennigii* (Wallrabenstein *et al*, 1995) and *S. fumaroxidans* (Harmsen *et al.*, 1998) are the most commonly reported syntrophic propionate-oxidizing bacteria.

Acetate can also be synthesised by carbon dioxide reducing acetogenic bacteria, also called homoacetogenic bacteria. However, under low hydrogen concentration environments, such as bioreactors, hydrogenophilic archaeal species, which have a higher affinity for hydrogen, outcompet homoacetogenic bacteria (Katinka, 1994). Also thermodynamically,

H_2/CO_2-methanogenesis ($\Delta G^{o'}$=-135.6 kJ/mol) is a more energy-yielding process than H_2/CO_2-acetogenesis ($\Delta G^{o'}$=-95 kJ/mol) and, therefore the activities of methanogenic bacteria can be so high that homoacetogens are essentially not detected (Dolfing, 1988). This was evident in the work of Mackie and Bryant (1981), how found under mesophilic conditions, that methanogens successfully outcompeted acetogens for H_2, as acetate production from H_2/CO_2 represented less then 2% of the total acetate production during anaerobic degradation of manure.

β-oxidation of C_{4+} fatty acids

During this process, pairs of carbon atoms are removed from the carboxyl group end in a cycle until the chain is fully degraded to acetate (Figure 1.2).

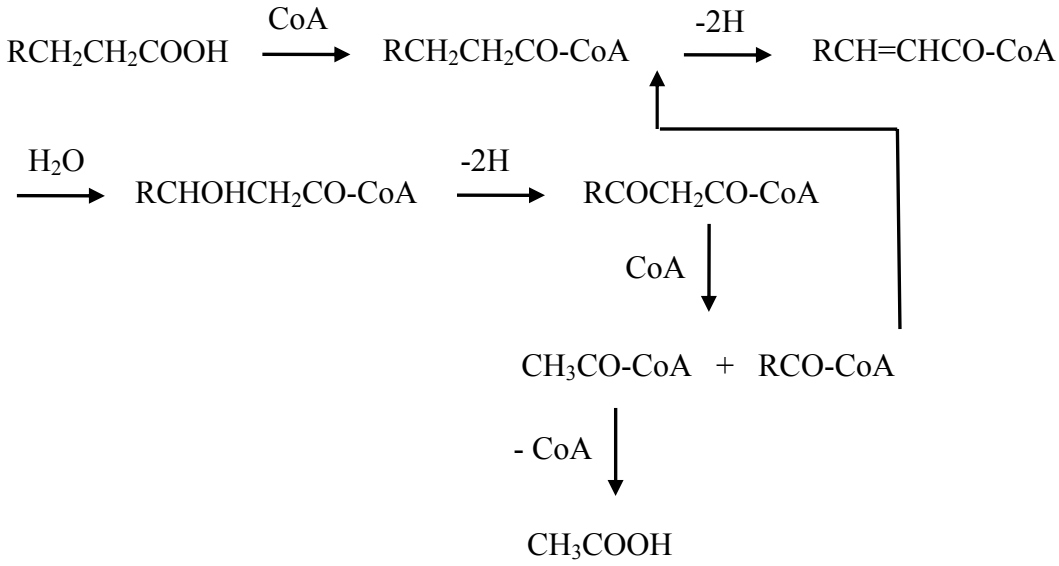

Figure 1.2 Mechanism of the β-oxidation cycle in anaerobic organisms (Ratledge, 1994).

The initial step is the activation with coenzyme A, followed by the removal of two hydrogen atoms (dehydrogenation) and formation of a double bond between the second and the third carbon. Afterwards in this double bond a hydroxyl group is fixed. Then, a second dehydrogenation takes place: two hydrogen atoms are removed from the third carbon atom and a ketone group is formed. Then, a coenzyme A splits the chain into acetic acid and a two carbons shorter fatty acid, which follows through repeated cycles until is fully degraded to acetic acid. Odd chained organic acids, such as valerate, will produce propionate in the final cycle. Fatty acids up to C_{11} may be able to diffuse through the cell membrane, but those above that, require adsorption on to a cell wall transport protein (Ratledge, 1994).

A variation of the β-oxidation mechanism has been proposed for the oxidation of double bonded fatty acids (Ratledge, 1994). Since most monounsaturated fatty acids have the double bound in the 9 carbon atom (from the carboxyl group), after 3 cycles, the double bound will be between the third and forth carbon atoms (*cis* configuration). For the cycle to continue, the double bound needs to be between the third and second carbon atoms (*trans* configuration). At this point, a *cis*-3-*trans*-2-enoyl-CoA isomerase switches the bond and β-oxidation proceeds with hydratase. However, the occurrence of this mechanism in anaerobic digestion is not yet clear.

The process of β-oxidation seems to be the rate limiting step for LCFA degradation (Novak and Carlson, 1970; Hanaki *et al.*, 1981; Rinzema 1988; Angelidaki and Ahring, 1995). *Syntrophomonas sapovorans* (Roy *et al.*, 1986), *Syntrophomonas wolfei* subsp. *saponavida* (Lorowitz *et al.*, 1989), *Thermosyntropha lipolytica* (Svetlitshnyl *et al.*, 1996) are reported syntrophic acetogenic bacteria able to degrade LCFA.

1.2.1.4 METHANOGENESIS

This is the final step and represents, in many cases, the rate-limiting conversion in the whole AD process (Lawrence, 1971). Methanogenic archaea are the highly specialised trophic group involved in this last step, which metabolise the end products of the previous reactions (mainly acetate, H_2, formate and CO_2) to form methane. Approximately 70% of the methane is produced by cleavage of acetate (Jeris and McCarty, 1965), through a reaction commonly called as aceticlastic (or acetoclastic) methanogenesis (equation 1.1).

Just a limited number of species have been isolated in anaerobic conditions that utilise acetate, belonging to *Methanosaeta* genus (3 species, homotrophic) and *Methanosarcina* genus (5 species, can also utilise H_2 and CO_2, methylated C-1 compounds and methanol). Morphologically these two genera are very distinct: *Methanosaeta* includes large sheathed rods, often forming long filaments and large aggregates whereas *Methanosarcina* contains species of cocci, which may occur singly, in packets, or in large pseudoparenchyma (Whitman *et al*., 1992).

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad \textbf{(1.1)}$$

The uptake of hydrogen or formate to produce methane is commonly referred to as hydrogenotrophic methanogenesis. As referred before, the hydrogenophilic bacteria control the redox potential of the media, maintaining hydrogen concentration at low levels, and thus conditioning syntrophic acetogenesis. This trophic group includes large numbers of species within 5 orders in the archaea (Boone *et al*.,1993).

1.2.2 MONITORING ANAEROBIC DIGESTION

1.2.2.1 PERFORMANCE INDICATORS

Parameters such as Chemical Oxygen Demand (COD) removal, Volatile Suspended Solids (VSS) and CH_4 yields are usually used in assessing the operation of a digester. Also the pH is an important parameter to be measured directly and in relation to the Volatile Fatty Acid (VFA) concentrations and alkalinity. The concentration of H_2 determines the rate of degradation of some intermediate compounds by syntrophic cocultures (Sakharova and Rabotnova, 1976) and is a potentially useful monitoring parameter in process control. In the case of an imbalance between acidogenesis and methanogenesis a neutral pH may be maintained at the expense of buffering capacity (Grady and Lim, 1980) which would lead ultimately to acidification. Alkalinity is therefore useful in conjunction with pH measurements.

1.2.2.2 BIOLOGICAL INDICATORS

Measurements of the biomass content in terms of VSS, although useful to asses (in a simple and easy way) the global amount of biomass present in the bioreactor, does not distinguish between biomass and other particulate organic matter, neither provides detailed information on the content of specific trophic groups. Classical culture-dependent sludge characterisation methods, such as the Most Probable Number (MPN) technique, can give overall information about changes occurring in the consortia, but is slow (due to the slow growth rate of anaerobic bacteria) and often unreliable, since easily cultivable microorganisms are detected but not those that only grow on specific media, require unknown growth conditions, or have obligate syntrophic interactions with other microorganisms (Dolfing and Bloemen, 1985). Direct microscopic analyses do not distinguish between viable and non-viable cells and is usually based on cell morphology only, which for most bacteria is not very distinctive. For methanogenic bacteria, assay of coenzyme F_{420} by microscopic fluorescence detection can give an estimate of the methanogenic biomass in digesters, since this coenzyme is found in all methanogenic bacteria but less commonly and in lower concentration in non-methanogenic bacteria (Vogels *et al*., 1988). However, some methanogens, such as *Methanosaeta*, exhibit low autofluorescence (Dolfing *et al.*, 1985) which limits the practical interest of using this method to give quantitative information (Colleran *et al*., 1992).

Microbial activity tests give an indication of the ability of the test system to produce methane from a particular substrate, without need of direct isolation and cultivation of specific anaerobic bacteria. In most anaerobic digestion applications, activity of a particular microbial population is generally defined with respect to substrate consumption or product formation, monitored in batch conditions, as a function of time. There are several different methodologies available to assess activity of anaerobic microorganisms, recently reviewed by Rozzi and Remigi (2002). Those techniques are grouped in three main classes: (a) substrate consumption methods, which includes techniques based in assessing the extent of substrate consumption, in terms of depletion of dissolved organic carbon (DOC), total organic carbon (TOC) or chemical oxygen demand (COD); (b) gas chromatographic methods, in which the mass and composition of biogas, as methane and carbon dioxide, is assessed; and (c) volumetric/manometric methods which assess the amount of produced

biogas. In the volumetric methods, the vessel for the activity test is kept at constant pressure and the biogas is released when it is produced, whereas in the manometric methods the vessel is sealed and biogas production induces a pressure increase. In most of the reported techniques, activity assessment is based on biogas/methane production measurements, which assign easier and/or less time consuming methods. The first methods developed for measuring biogas production were based on syringe piston displacement (Owen *et al.*, 1979) or liquid displacement in a reversed cylinder (bell) (Valke and Verstraete, 1983) due to the pressure increase from the biogas produced inside the reactor. These methods only measure total biogas produced, unless a CO_2 adsorption device is inserted into the system. Shelton and Tiedje (1984) used, for the first time, pressure transducers to measure biogas production, a method that was later adapted by Reynolds (1986) and Concannon *et al.* (1988) to apply to methanogenic activity tests using liquid substrates (acetate, propionate, butyrate, ethanol, methanol and formate). The pressure transducers used to electronically measure the pressure developed during the assay, were specially designed according to the conditions used in the tests (volume, biomass and substrate content), in order to guarantee maximum accuracy in the pressure readings. In this method, the measurement of methane content in the end of the assay allows the establishment of a correlation between the pressure developed and the methane produced during the assay. Later on, the method was extended to the measurement of methanogenic activity using gaseous substrates, thus allowing the evaluation of the hydrogenophilic activity (Coates *et al.*, 1996).

The application of bacterial and archaeal small subunit ribosomal RNA (SSU rRNA or 16S rRNA) sequence information to study microbial communities, in a culture-independent way, has shown an explosive increase in the past decade, and its application in ecological studies is still increasing. Along with that, new molecular-based techniques have been developed, which are expected to outcompete/complement conventional culture techniques for sludge characterisation. With the Polymerase Chain Reaction (PCR) amplification method, certain target rRNA genes can be amplified to make them detectable (Giovannoni, 1991). Cloning and sequencing of 16S rRNA genes provides information about the corresponding nucleotides sequence, and therefore, about the genetic diversity and phylogenetic relationships between microorganisms present in a particular ecosystem (Amann *et al.*, 1995; Godon *et al.*, 1997). Fingerprinting methods such as Denaturing or

Temperature Gradient Gel Electrophoresis (DGGE or TGGE, respectively) are fast and reliable methods for studying the dynamics of ecosystems, based on a sequence-specific separation of 16S rRNA and rDNA amplicons. This analysis allows to get an overall impression of the heterogeneity of the microbial community as well as to monitor individual differences and temporal changes in the predominant community (Zoetendal *et al*, 2001). Both DGGE and random cloning of 16S rRNA genes are PCR–based approaches and therefore the outcome results cannot be converted to bacterial numbers. Fluorescent *in situ* hybridisation (FISH) can be used to specifically detect and quantify bacteria using 16S rRNA target probes (Raskin *et al.*, 1994). Moreover, since it is an *in situ* technique, it makes it possible to study the spatial organization of the microorganisms and to locate certain phylogenetic groups in sludge aggregates (Harmsen *et al.,* 1996; Sekiguchi *et al.*, 1999). This analysis can be hampered by cross-reactions during hybridisation, inaccessibility of the target and low number of target molecules (Oude Elferink *et al.*, 1998) and, on the other hand, concerning specific probe design, this technique is dependent on the available sequences in the DNA databases. All methods have their limitations and complement each other with valuable information. Hence, a combined approach should be used to provide an accurate analysis of microbial composition and diversity in a complex community. Furthermore, when combined with other techniques, chemical, biochemical and/or physiological assays, they can provide considerable information and improve our understanding about the role and dynamics of microorganisms (Verstraete *et al.*, 1996; Oude Elferink *et al.*, 1998) in the complex anaerobic digestion process.

1.3 PROBLEMS IN ANAEROBIC TREATMENT OF LIPID/LCFA BASED WASTEWATERS

In the last few years, anaerobic wastewater treatment technology was markedly improved due to the development of the Upflow Anaerobic Sludge Blanket (UASB) concept and its world-wide application, along with the more recent designs of the Expanded Granular Sludge Bed (EGSB) and the internal circulation (IC) reactors. In such systems, biomass immobilization is achieved by self granulation, a crucial requirement that, when unsuccessful, irreversibly affects the overall performance. The application of UASB or EGSB reactors to lipid/LCFA containing wastewaters has been the subject of recent research. Although there is evidence that granular sludge is more resistant to LCFA toxicity than suspended or flocculent sludge (Hwu, 1997), it is also evident that granulation and/or granule stability is problematic for lipid containing wastewaters (Hawkes *et al*., 1995; Sam-Soon *et al.*, 1991; Hwu, 1997). Samson *et al.* (1985) referred the treatment failure of an industrial scale UASB reactor treating milk fat, due to sludge flotation. Hawkes *et al.* (1995) found that, for an ice-cream wastewater, granulation was unsuccessful, and concluded that the fixed bed system was the most adequate for that kind of wastewater. Rinzema (1988) observed that the application of conventional UASB reactors to LCFA containing wastewaters resulted in local overloading of LCFA and severe washout caused by flotation. This author found that for LCFA containing wastewaters, an EGSB reactor could be applied, but for lipid containing wastewaters the system would only be feasible with some design modifications by introducing a sieve-drum to prevent the washout of granular sludge. Sam-soon *et al.* (1991) used a UASB reactor to treat oleate as a unique carbon source and reported that the original inoculated pellets suffered from disintegration and encapsulation by a gelatinous and whitish mass. From a thermodynamic viewpoint, disintegration of granules is predictable because at neutral pH, LCFA act as surfactants, lowering the surface tension. Consequently the aggregation of hydrophobic bacteria, like most of acetogens (LCFA-degraders), is unfavourable (Daffonchio *et al*., 1995; Hwu, 1997), thus increasing the washout probability of these bacteria. Hwu (1997) concluded that typical operating parameter of EGSB reactors (Vup>4 m/h, HRT<10h) inhibited the

treatment performance of LCFA containing wastewaters. The recirculation of the washed out biomass, which had high specific oleate conversion rate, was beneficial to enhance the overall performance. Furthermore, it is known that recycling induces a protective effect when dealing with toxic wastewaters (Young, 1991), improving the stability by decreasing the toxic concentration.

From the existing literature it is suggested that LCFA exert a bactericidal effect on methanogenic bacteria and no adaptation was observed. The recovery after a lag phase usually observed in batch assays is attributed to the growth of few survivors (Rinzema *et al*., 1994). These authors found that acetoclastic bacteria do not adapt to LCFA neither upon repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic concentrations. The acetate-utilizers (aceticlastic) seem to be more affected by LCFA than the H_2-utilizers (hydrogenophilic) bacteria (Hanaki *et al.*, 1981). Koster and Cramer (1987) reported that aceticlastic methanogens were inhibited by oleic, myristic, lauric, capric and caprylic acids at 33ºC, in the range of concentrations between 300 and 900 mg/l. In later studies, Angelidaki and Ahring (1992) reported that, under thermophilic conditions, concentrations between 100 and 200 mg/l of oleic acid and higher than 300 mg/l of stearic acid inhibited the degradation of acetate, propionate and butyrate. The lag phase on methane production increased with the increase of the added LCFA concentrations until no growth was found at concentrations of 500 mg/l for oleate and 1000 mg/l for stearate. Lalman and Bagley (2001) found that oleic acid at a concentration as low as 30 mg/l inhibited aceticlastic methanogens at 21ºC, whereas hydrogenophilic methanogens were only slightly inhibited by concentrations up to 100 mg/l of oleic and stearic acid. A higher susceptibility of aceticlastic than hydrogenophilic bacteria to oleic acid, at 37ºC, was also reported by Alves *et al* (2001). These authors found that 50 mg/l of oleic acid caused a 50% relative aceticlastic activity loss, whereas for the same reduction of the hydrogenophilic activity, a concentration of oleic acid 4 fold higher was required.

The mechanism of LCFA toxicity is related with the adsorption of the surface active acids onto the cell wall, which affects its transport and/or protective functions (Demeyer and Henderickx, 1967; Galbraith *et al.*, 1973; Rinzema, 1988). This could suggest that toxicity of LCFA was related with concentration: biomass ratio. However, results of Koster and Cramer (1987) and Rinzema *et al*. (1994) showed that the toxic effect is merely

concentration dependent. Among other conclusions, Hanaki *et al.* (1981) found, in batch assays, that: (i) glucose fermentation was not affected by the presence of LCFA; (ii) the addition of acetate and butyrate intensified the toxic effect of LCFA and (iii) oleate was less inhibitory than a LCFA mixture. Angelidaki and Ahring (1992) suggested that the response to the addition of neutral lipids may depend upon the degree of adaptation to lipids, whereas the addition of free LCFA above a certain concentration may directly result in process failure. Alves *et al*. (2001) recently concluded that, to treat oleate-based effluents, it is advantageous to pre-expose the biomass to lipids. The observed increasing aceticlastic tolerance to oleic acid toxicity was interpreted as an acclimation process. Therefore, the use of an acclimated inoculum when treating LCFA-based wastewaters should enhance reactor performance. In this regard, Pereira *et al*. (2001) found that, if a gradual start up with a co-substrate was performed, sludge previously acclimated with oleic acid was more resistant to oleic acid toxicity than a non acclimated one and allowed higher methane yields when incubated in continuous reactors.

More than inhibition, flotation of granular biomass conducting to washout, was found to be the most important operational problem of UASB reactors (Rinzema, 1988). Hwu (1997) studied the adsorption of LCFA in relation with granular sludge flotation in a UASB reactor and concluded that granular sludge flotation occurred at concentrations far below the toxicity limit. A critical flotation occurred at sludge loads of about 200 mg COD/(gVSS.day), when operating a continuous sludge bed reactor at HRT between 16 and 27 hours. This might suggest that complete washout of granular sludge would occur prior to inhibition. Furthermore, it is known that addition of calcium salts prevents, to some extent, inhibition problems, but has no effect upon flotation problems (Hanaki *et al*, 1981; Rinzema, 1988).

Since sorption of LCFA onto the surface of granules leads to sludge flotation and inhibition, the prevention of excessive LCFA adsorption onto granule surfaces is suggested by Hwu (1997), as a potential option to guarantee efficiency and reliability of high-rate treatment systems. According to this author, this requires enhancement of LCFA biodegradation, which can be achieved by applying a longer substrate-biomass contact time, i. e., a prolonged HRT. However, in later studies, Alves *et al.* (2001) observed that after continuous feeding with oleic acid at concentrations up to 12 g COD/l, anaerobic sludge

became encapsulated by a whitish matter, was able to efficiently mineralise the accumulated substrate (2103 mg COD-CH_4/gVSS) at a maximum methane production rate of 283 mg COD-CH_4/(gVSS.day), when incubated in batch vials at 37ºC without any added carbon source. Therefore, the ability of the encapsulated sludge to efficiently mineralise high amounts of adsorbed/accumulated LCFA may represent a potential challenge for the optimisation of methane production when treating effluents with high lipid/LCFA content, worthy of further investigation.

1.4 THESIS OUTLINE

This work focuses on the anaerobic biodegradation of LCFA associated to anaerobic sludge by mechanisms of adsorption, precipitation or entrapment.

In **Chapter 2,** the behaviour of granular and suspended sludge is compared, during a continuous run in EGSB reactors, with oleic acid as substrate. The amount of accumulated LCFA, its composition, and the corresponding biomethanisation rate in batch assays are presented. **Chapter 3** describes the potential of image analysis techniques to monitor the morphological changes in granular and suspended sludge collected during the continuous experiment with the EGSB reactors described in **Chapter 2**. Relationships between the average equivalent diameter of aggregates and the amount of biomass-associated LCFA, as well as between sludge dispersion level and the amount or the mineralisation rate of biomass-associated LCFA, are demonstrated. In **Chapter 4**, the application of a molecular approach to evaluate the microbial diversity of bacteria and archaea in the same sludge samples is presented. This molecular approach complements physiological (**Chapter 2**) and morphological (**Chapter 3**) characterisation of the seed and EGSB reactor sludges.

Chapter 5 compares sludge biochemical characteristics, namely, methanogenic activity, tolerance to oleic or palmitic acid toxicity and the capacity for oleic or palmitic acid biodegradation, before and after degrading the accumulated (biomass-associated) LCFA. The relative importance of transitory toxic effects and transport limitation effects caused by the accumulated LCFA is discussed.

In **Chapter 6,** the kinetics of biomethanisation of biomass-associated LCFA is presented. The effect of adding individual VFA (acetate, propionate and butyrate) to a LCFA loaded sludge was studied, and the interaction between biomass-associated LCFA degradation and VFA depletion is discussed for each particular case.

Finally, **Chapter 7** presents the concluding remarks and the perspectives for further research on this topic.

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2 METHANISATION OF LCFA ACCUMULATED ONTO ANAEROBIC SLUDGE LOADED WITH OLEIC ACID

Abstract
The maximum potential methane production of sludge samples taken during the operation of two EGSB reactors fed with increasing oleic acid concentrations between 2 and 8 g COD/l (HRT=1 day), was studied in batch assays. RI was inoculated with granular sludge and RII with suspended sludge. After removing the residual (soluble) substrate, the loaded sludge was incubated in batch vials without any added carbon source. A maximum methane production rate of 434±60 mg COD-CH_4/(gVSS.day) was obtained for the suspended sludge taken on day 70, when oleate, at a concentration of 2 g COD/l, was fed with skim milk as co-substrate (50% COD). The maximum plateau achieved in the methane production curve, considered as an indirect measurement of the amount of accumulated LCFA, was 3272±877 mg COD-CH_4/gVSS, obtained for the suspended sludge taken on day 162, when oleate was fed as the sole carbon source at 6 g COD/l. The methanisation rate of the accumulated (biomass-associated) LCFA was enhanced under stirring conditions and was inhibited by adding oleic acid. Extraction and gas chromatography analysis confirmed that the main accumulated LCFA was palmitate, and not oleate. Palmitate accumulated onto the sludge and further β-oxidation was inhibited when in presence of oleic acid. If oleic acid was removed from the medium β-oxidation proceeded with methane production. The maximum accumulated palmitate and the rate of methane production were highly dependent on biomass structure, suspended sludge being more efficient than granular sludge for that purpose. The results presented in this Chapter therefore suggest that, in order to enhance methane production, it would be advantageous to sequence cycles of adsorption/accumulation and degradation, when treating effluents with high lipid/LCFA content.

2.1 INTRODUCTION

Lipids are one of the major components of organic matter in wastewater. Along with slaughterhouses and edible oil and fat refineries, dairy products industries are important contributors for the total lipid emission (Rinzema, 1988). Lipids are easily hydrolysed to LCFA, which are further converted to acetate and hydrogen through β-oxidation mechanism by the proton reducing acetogenic bacteria (Weng and Jeris, 1976). LCFA are especially problematic compounds for anaerobic wastewater treatment. Tentative application of granule-based digesters to lipid-containing wastewaters revealed that, although granular sludge was more resistant to LCFA toxicity than suspended or flocculent sludge (Hwu, 1997), physical stability of granules is critical (Sam-Soon *et al*., 1991; Hawkes *et al*., 1995). This problem arises because these compounds adsorb onto the biomass and, besides the acute toxic effect, induce granular sludge flotation, which occurs at concentrations far below the toxicity limit (Hwu, 1997).

Although for treating LCFA-based wastewaters, the typical EGSB operating conditions (Vup>4 m/h and HRT<10 h) were found to inhibit the treatment performance due to washout in particulate form, when operating at a higher HRT, i. e. 24 hours, with a co-substrate, high COD removal efficiencies (82-89%) can be obtained without a significant washout of granules or fatty matter (Hwu, 1997). Moreover, the use of biomass recycling was also found to promote digester performance, since the washed-out biomass exhibited a higher oleate degradation capacity than the biomass remaining inside the reactor.

Results from batch assays suggested that LCFA exert an irreversible and non-adaptable toxic effect on anaerobic digestion (Rinzema *et al.*, 1994; Angelidaki and Ahring, 1992). However, results from continuous experiments with a gradual replacement of a co-substrate by oleic acid, revealed that aceticlastic bacteria increased the tolerance to oleic acid toxicity (Alves *et al*., 2001). Moreover, a digester inoculated with an acclimated sludge exhibited higher methane yields than a digester inoculated with a non-acclimated sludge (Pereira *et al*., 2001).

In a previous work, when continuously fed with oleate at concentrations up to 12 g COD/l, the anaerobic sludge became encapsulated by a whitish matter, which was *a priori*

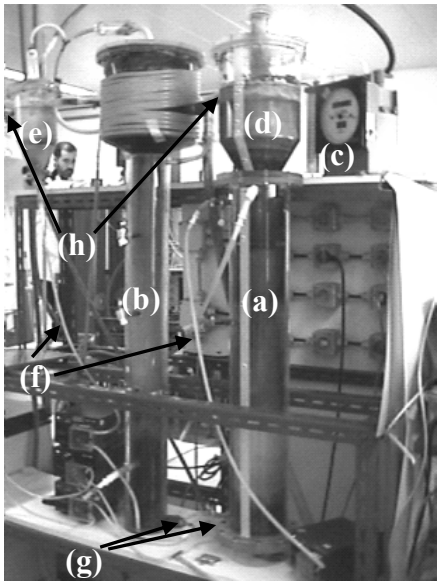
supposed to be oleic acid. When this encapsulated sludge was incubated in batch vials, after two consecutive washings for removing the residual (soluble/colloidal) substrate, methane was produced at a rate of 283 mg COD-CH_4/(gVSS.day) (99 ml $CH_{4(STP)}$/(gVSS.day)), achieving a plateau of 2103 mg COD-CH_4/gVSS (736 ml$CH_{4(STP)}$/gVSS), without any added carbon source. This methane production rate seemed to be delayed when oleic acid was added to the vials (Alves *et al*., 2001).

The aim of this work was to characterise the potential maximum methane production from the accumulated substrate exhibited by the sludge of two EGSB reactors fed with increasing loads of oleic acid. The behaviour of granular and suspended sludge was compared. Sodium oleate was used as a LCFA model since it is, in general, the most abundant of all LCFA present in wastewater (Komatsu *et al*., 1991), has a good solubility and it is one of the more toxic (Galbraith *et al*., 1971).

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL SET-UP AND OPERATION MODE

Two 10 l EGSB reactors (RI and RII) were operated in parallel with increasing oleate concentrations between 2 and 8 g COD/l, at a HRT of 1 day. Temperature was kept constant at 37±1 ºC. RI was inoculated with granular sludge and had an internal settler. RII was inoculated with suspended sludge and was equipped with an external settler (Figure 2.1). In both reactors a recycling rate of 14 l/day was applied.



Legend:
- (a) RI
- (b) RII
- (c) Gas meter
- (d) Internal settler
- (e) External settler
- (f) Recycle
- (g) Feeding
- (h) Treated effluent

Figure 2.1 Experimental set-up.

SEED SLUDGE

The granular sludge was obtained from an UASB treating a brewery effluent located in UNICER, Oporto, Portugal. Suspended sludge was collected from a laboratory digester treating an oleic acid-based synthetic effluent. The appearance of this sludge was gelatinous, seeming to be "encapsulated" by a whitish matter. Therefore, before inoculation it was allowed to degrade the biomass-associated substrate in batch mode at 120 rpm and 37ºC, until no more biogas production was detected (this lasted 15 days). 1.6 l of granular

sludge (20.2 g VSS/l) and 2 l of suspended sludge (18.0 g VSS/l) were added to RI and RII, respectively.

SUBSTRATE

In the first 70 days, the substrate consisted of skim milk (50% COD) and sodium oleate (50% COD) diluted with tap water and supplemented with macro and micronutrients:

Macronutrients – $MgSO_4.7H_2O$: 30 g/l; KH_2PO_4: 28.3 g/l; KCl: 45 g/l. 0.6 ml of this solution was added per gram of COD fed.

Micronutrients – $FeCl_2.6H_2O$: 2g/l; H_3BO_3: 0.05 g/l; $ZnCl_2$: 0.05 g/l; $CuCl_2.2H_2O$: 0.038 g/l; $MnCl_2.4H_2O$: 0.5 g/l; $(NH_4)_6Mo_7O_{24}.4H_2O$: 0.05 g/l; $AlCl_3.6H_2O$: 0.09 g/l; $CoCl_2.6H_2O$: 2 g/l; $NiCl_2.6H_2O$: 0.092 g/l; $Na_2SeO_3.5H_2O$: 0.164 g/l; EDTA: 1 g/l; Resazurin: 0.2 g/l; HCl 37%: 1 ml/l. The composition of this solution was based in the work of Zehnder *et al*. (1980) and was supplemented to the influent feed by addition of 1 ml per litre.

To give suitable alkalinity 5 g $NaHCO_3$/l were added to the feed.

From the day 70 on, the carbon source was exclusively composed by sodium oleate, and a nitrogen supplement was added to the macronutrients solution, in order to maintain a ratio COD/N/P of 200:5:1.1.

2.2.2 ROUTINE ANALYSIS

Routine reactors performance was monitored by determining influent and effluent total and soluble (centrifuged 10 min at 15 000 rpm) Chemical Oxygen Demand (COD), influent flow rate, methane production and Volatile Suspended Solids (VSS).

COD and VSS were determined according to Standard Methods (APHA *et al*., 1989). COD removal efficiency was calculated according to the equation 2.1:

$$\text{COD removal}(\%) = \frac{\text{COD}_{\text{influent}} - \text{COD}_{\text{effluent}}}{\text{COD}_{\text{influent}}} \times 100 \quad \textbf{(2.1)}$$

Biogas flow rate was measured by a "Shinagawa Seiki DC-1C Dry test gas meter" (SHINAGAWA CORPORATION TOKYO, Japan). Methane content of the biogas was determined by gas chromatography using a Chrompack Haysep Q (80-100 mesh) column, with N_2 carrier gas at 30 ml/min and a flame-ionisation detector. Temperatures of the injection port, column, and flame-ionisation detector were 120, 40 and 130 ºC, respectively. Biogas samples were analysed in triplicate and the results average expressed as percentage methane by calibration against standards of 50% (v/v) methane/air.

2.2.3 SLUDGE CHARACTERISATION

2.2.3.1 METHANOGENIC ACTIVITY MEASUREMENTS

These tests were performed according to the method developed at the Microbiology Department of the National University of Ireland (Galway), under the supervision of Professor Emer Colleran (Colleran *et al.*, 1992; Coates *et al.*, 1996).

The test involves the monitoring of the pressure increase developed in sealed vials fed with non gaseous substrates (acetate, propionate, butyrate and ethanol) or pressure decrease in vials previously pressurized with gaseous substrates (H_2/CO_2). A hand held pressure transducer (Centrepoints Electronics, Galway, Ireland) capable of measuring a pressure variation of two bar (≈ 2 atm) relative to the atmospheric pressure (0 to ± 2 atm) over a range of -200 to +200 mV, with a minimum detectable variation of 0.005 bar, corresponding to 0.05 ml biogas in 10 ml headspace was used (Figure 2.2). A volume of 30 µl (approx) of biogas is released by each pressure measurement, which represents an insignificant fraction of the total biogas produced in the test. Blank controls were used for liquid substrates (no added substrate) and for gaseous substrates (pressurized with N_2/CO_2).

The basal medium used in the tests was made up with demineralised water and was composed of cysteine-HCL (0.5 g/l), resazurin (1ml/l) and sodium bicarbonate (3 g/l). The

pH was adjusted to 7.0-7.2 with NaOH 8N and the medium was prepared under strict anaerobic conditions.

Activity tests were carried out in 25 ml vials when using liquid substrates and 70 ml vials when using gaseous substrates. In both cases, the working volume was 12.5 ml and the VSS concentration was in the range of 2 to 5 g/l. After overnight acclimation (37 ºC, 150 rpm), the excess pressure was vented, the substrates were added to the vials and the time course of pressure change was monitored. For the liquid substrates a volume of 0.125 ml was dispensed from stock solutions 100 fold concentrated, in order to obtain in the test vial concentrations of 30 mM for acetate, propionate and ethanol and 15 mM for butyrate. For the gaseous substrates, H_2/CO_2 80/20 (vol/vol) and N_2/CO_2 80/20 (vol/vol) were pressurized at 1 bar in the test and blank vials, respectively.

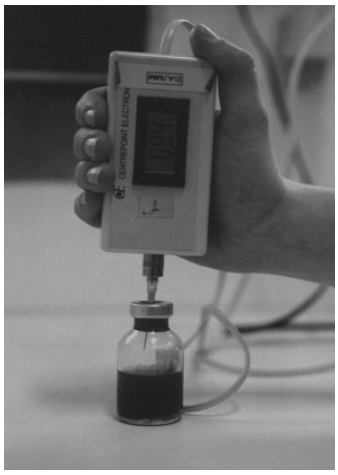


Figure 2.2 Hand held pressure transducer.

In the test and blank vials of liquid substrates, the methane content of the biogas produced was determined by gas chromatography, as described in sub-chapter 2.2.2 (p.56)). Then, for all the vials, the headspace volume was individually determined by recording the mV change per ml of air (mV/ml) when a known volume of air was injected in the closed vial. This headspace correction factor (mV/ml) is used to convert recorded millivolt readings to ml of biogas produced. Finally, the VSS content of each vial was determined. All the batch experiments were performed in triplicate assays.

Quantification of methane produced during the tests:

- ***Liquid substrates***

The percentage methane (Mp) of the biogas produced in each vial during the test was determined considering the following molar balance applied to the methane (equation 2.2):

$$\frac{P_{(i)}Vh}{RT}\times \%CH_{4(i)}+\frac{\Delta PVh}{RT}\times Mp=\frac{\left(P_{(i)}+\Delta P\right)Vh}{RT}\times \%CH_{4(f)} \qquad \textbf{(2.2)}$$

Where:

R – Perfect gas law constant

T – Absolute Temperature

P(i) – Initial absolute pressure (≈ 1 atm)

ΔP – Pressure increase during the test

Vh – Volume of head space (constant)

$\%CH_{4(i)}$, $\%CH_{4(f)}$ – Initial and final methane content in the headspace

Mp – Methane content in the biogas produced during the test

Considering that temperature and headspace volume were constant through the test, and that the initial methane content is negligible, equation 2.2 can be rearranged as:

$$Mp=\frac{P_{(i)}+\Delta P}{\Delta P}\times \%CH_{4(f)} \qquad \textbf{(2.3)}$$

ΔP corresponds to the pressure increase due to the biogas produced which is equivalent to the final mv reading. Dividing by the headspace correcting factor (mV/ml), the volume of biogas produced (Vc) is obtained (equation 2.4). $P_{(i)}$ is the initial absolute

pressure obtained when the vial are vented (≈ 1atm). The corresponding 0 mV reading of relative pressure, in fact represent ideally 100 mV of absolute pressure. Therefore dividing this value by the correcting headspace factor mV/ml the headspace volume (Vh) is obtained (equation 2.5).

$$Vc = \frac{\text{Final mV reading (mV)}}{\text{Headspace correction factor (mV/ml)}} \quad \textbf{(2.4)}$$

$$Vh = \frac{\text{mV reading at 1atm relative pressure (ideally=100)}}{\text{Headspace correction factor (mV/ml)}} \quad \textbf{(2.5)}$$

Taking into account these definitions, equation 2.3 can be written as:

$$Mp = \frac{Vh+Vc}{Vc} \times \%CH_{4(f)} \quad \textbf{(2.6)}$$

The specific methanogenic activity is then determined according to equation 2.7 and expressed as $mlCH_{4(STP)}/(gVSS.day)$:

$$\text{Specific methanogenic activity} = \frac{GP \times Mp \times CF}{VSS} \quad \textbf{(2.7)}$$

where:

GP = volume of biogas produced per day, determined by equation 2.8.

CF = calibration factor to convert to Standard Temperature and Pressure conditions (STP), determined by equation 2.9 (Appendix A).

VSS = volatile suspended solids in each vial

$$GP = \frac{\text{initial slope of the plot mV readings } vs \text{ time } (\text{mV/h})}{\text{Headspace correction factor } (\text{mV/ml})} \times 24 \quad \textbf{(2.8)}$$

$$CF = \frac{100}{\text{mV reading at 1atm}} \times \frac{273}{(273+37)} \quad \textbf{(2.9)}$$

- ***Gaseous substrates***

The conversion of H_2/CO_2 to CH_4 is accompanied by a decrease in gas volume and a reduction of headspace pressure in the test vials, which corresponds to the transformation of 4 moles of H_2 and 1 mole of CO_2 into 1 mole of methane, according to equation 2.10:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \textbf{(2.10)}$$

The pressure decrease was directly converted to ml of methane produced by using equation 2.11:

$$\text{Volume } CH_4 (\text{ml}) = \frac{P_{(n-1)} - P_{(n)}}{\text{mV/ml} \times 4} \quad \textbf{(2.11)}$$

where:

$P_{(n-1)}$ = mV reading at time n-1

$P_{(n)}$ = mV reading at time n

mV/ml = headspace correction factor

The cumulative methane production is calculated by addition of the CH_4 volumes produced between each reading. The initial slope of the methane production curve (ml CH_4/h) was determined and the specific methanogenic activity was calculated according to equation 2.12 and expressed as ml $CH_{4(STP)}$/(gVSS.day):

$$\text{Specific methanogenic activity} = \frac{\text{ml}CH_4/\text{h} \times 24 \times \text{CF}}{\text{VSS}} \quad \textbf{(2.12)}$$

Specific methanogenic activity values were converted to mg COD-CH_4/(gVSS.day) (350 ml CH_4 are theoretically produced for each g of COD) and background methane production due to the residual substrate (blank controls) was discounted.

It should be noticed that, concerning the specific methanogenic activity in the presence of indirect methanogenic substrates (propionate, butyrate or ethanol), the methane production rate will only be a valid measurement of the corresponding indirect substrate consumption rate when the aceticlastic and hydrogenophilic activities are not rate limiting (Dolfing and Bloemen, 1985).

2.2.3.2 TOXICITY MEASUREMENTS

The effects of a potential inhibitor on the activity of an individual trophic group present in the microbial consortium is determined by monitoring the methane production rates in sealed vials to which, besides the specific substrate of the trophic group under study, the toxic compound is added. The tests were performed according to the procedure described by Colleran and Pistilli (1994) using the same pressure transducer technique previously described for the methanogenic activity measurements.

Oleate was the potential toxicant at concentrations in the ranged of 100 to 900 mg/l. Acetate (30 mM) was added, in order to evaluate the influence of oleate concentration on the aceticlastic activity. The choice of this trophic group for the toxicity studies was based on its generalised higher susceptibility to adverse conditions (Yang and Speece, 1986) and specifically to the presence of oleic acid (Hanaki *et al*., 1981; Alves *et al.*, 2001), as well as their important metabolic role on the anaerobic digestion process (Gujer and Zehnder, 1983). The oleate concentration that caused a 50% relative methanogenic aceticlastic activity loss was defined as the toxicity index (IC_{50}).

In some tests, lag phases preceded the initial methane production and their length was determined graphically according to Figure 2.3. In those cases, initial methane production rate was null or of the same magnitude as the methane production rate in the blank controls, resulting in null or insignificant initial activity values.

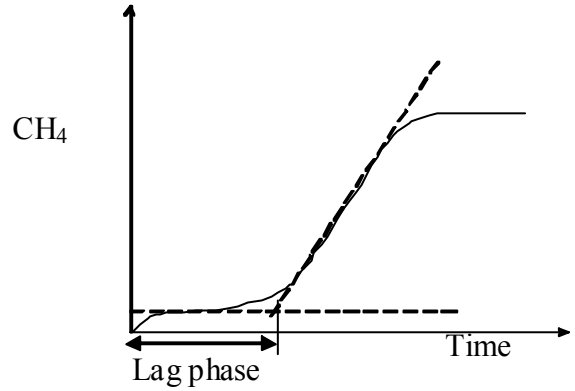


Figure 2.3 Example of lag phase determination.

2.2.3.3 LCFA BIODEGRADATION MEASUREMENTS

These tests were performed in order to evaluate the capacity of the microbial consortium to mineralise oleate to methane, using the same methodology previously described for the methanogenic activity measurements. Oleate was the sole substrate added to the vials at concentrations ranging from 100 to 900 mg/l. The specific methane production was also determined according to equation 2.7 (p.60).

2.2.3.4 MICROSCOPIC OBSERVATIONS

Phase contrast microscopy was performed with a Zeiss (Oberkochen, Germany) Axioscop microscope. The same microscope was used to visualise the auto fluorescence of methanogenic bacteria (420 nm).

Scanning electron microphotographs were obtained in a Leica S360 microscope (Leica Cambridge, The Netherlands). Samples were fixed with 3% (wt/vol) glutaraldehyde solution in cacodylate buffer, pH 7.2, and dehydrated with ascending concentrations of

ethanol solutions (10, 25, 50, 75, 90 and 100%). Prior to microscopic examination, samples were sputter- coated with gold. Chemical composition of the sludge aggregates was determined by EDS (Energy Dispersion Spectroscopy).

2.2.3.5 METHANISATION OF LCFA ACCUMULATED ONTO THE SLUDGE

After two consecutive washings and centrifugations (4000 rpm, 10 min) with anaerobic basal medium, the sludge samples were incubated in batch vials of 25 ml at 37 °C, 150 rpm under strict anaerobic conditions, without any added carbon source, calcium or nutrients. One of the experiments was also performed under static conditions for comparative purposes. The methane production was followed by measuring the pressure developed in each vial, using the above described pressure transducer technique. The initial methane production rate and the maximum plateau were determined for each vial according to Figure 2.4.

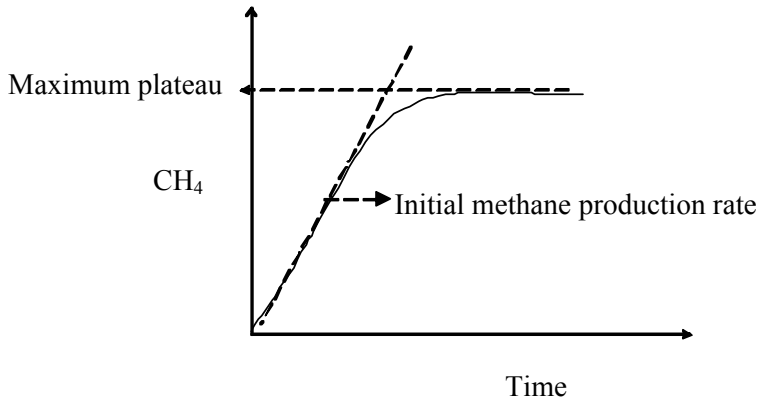


Figure 2.4 Example of a curve obtained in the batch assays.

The cumulative methane production at the end of the batch assays (maximum plateau) was considered an indirect measurement of the amount of accumulated (biomass-associated) LCFA. Specific methane production rate was obtained by dividing the initial slope of each curve by the VSS content of each vial at the end of the experiment and were expressed as mg COD-CH_4 /(gVSS.day). The amount of accumulated LCFA per VSS unit was

determined by dividing the maximum plateau by the VSS content of each vial at the end of the experiment and was expressed as mg COD-CH_4/gVSS. All the batch experiments were performed in triplicate assays.

2.2.4 EXTRACTION AND GC ANALYSIS OF LCFA ACCUMULATED ONTO THE SLUDGE

After two consecutive washings and centrifugations (4000 rpm, 10 min) with anaerobic basal medium, an aliquot of each sludge sample was dried at 105ºC, weighed and placed into separating funnels. A solution of internal standard (pentadecanoic-C15) was added to the sample, and, after acidification to pH 2, a multiple extraction with 5x1 ml of petroleum ether was applied. The ether phase was transferred to glass vials, immediately capped, and stored at –20ºC. LCFA concentration was determined by a gas chromatograph (CP-9001 Chrompack) equipped with a flame ionisation detector (FID) and a split/splitless injector. LCFA's were separated on a FFAP-CB 25m x 0,32mm x 0,3µm column (Chrompack), using nitrogen (N_2) as carrier gas at 35KPa, 31:1 split rate. Oven temperature was 40ºC for 2 min, with a 5ºC/min ramp to 250ºC, and a final hold at 250ºC for 15 min. A more detailed description of this method is presented in Appendix B.

2.2.4.1 EVALUATION OF LCFA ACCUMULATED ONTO ACTIVE AND INACTIVATED ANAEROBIC SLUDGE

In a separate experiment, the two EGSB reactors previously described, RI and RII, were also operated during 51 and 38 days, respectively with a constant oleate concentration of 6 g COD/l and HRT of 1 day. Routine reactor performance was monitored by determining influent and effluent COD, pH and % of CH_4 in the biogas (as described before, p. 56).

RI was inoculated with 1 l of suspended sludge (43.7 gVSS/l) obtained from the previous experiment using the same reactors (loaded with increasing oleate concentrations between 2 and 8 g COD/l). This sludge, collected at the end of the referred operation, was kept under anaerobic conditions at 4ºC until the beginning of the actual experiment, during

a total period of 5 months. The appearance of the sludge was gelatinous, seeming to be "encapsulated" by a whitish matter and prior to inoculation it was let to degrade the biomass-associated substrate in batch mode at 150 rpm and 37ºC, until no more biogas was produced. RII was inoculated with 1 l of the same sludge (43.7 gVSS/l) inactivated by autoclaving, as described by Hwu (1997).

Biomass samples were collected from the reactors on days 28, 30 and 37 and the LCFA content (biomass-associated) determined as described above (sub-chapter 2.2.4).

2.3 RESULTS AND DISCUSSION

2.3.1 INOCULA CHARACTERISATION AND REACTOR PERFORMANCE

Both inocula were characterised in terms of specific methanogenic activity, oleic acid toxicity towards aceticlastic bacteria and oleic acid biodegradation capacity. Table 2.1 summarises the corresponding results.

Table 2.1 Specific methanogenic activity, oleic acid toxicity against aceticlastic bacteria and oleic acid biodegradation capacity for the granular and suspended seed sludge (Mean±SD)

		granular	suspended
Initial methane production rate in the presence of:		(mg COD-CH_4/(gVSS.day))	
	Acetate	934±32	306±17
	Propionate	457±29	137±40
	Butyrate	(n.d.)	149±9
	Ethanol	1469±269	303±6
	H_2/CO_2	1706±46	1391±89
Oleic acid toxicity limit (IC_{50}) (mg/l)		345±26	133±16
Biodegradation for oleate concentrations:		(mg COD-CH_4/(gVSS.day))	
	100 mg/l	(n.d.)	17±3
	300 mg/l	40±3	20±6
	500 mg/l	29±3	26±3
	700 mg/l	23±3	23±6
	900 mg/l	29±6	20±6

SD – Standard deviation of triplicates
n.d.- non-detectable.

The granular sludge exhibited activities significantly higher than the suspended sludge for aceticlastic, hydrogenophilic and syntrophic propionate and ethanol degrading bacteria. Only methanogenic activity with butyrate as substrate was non- detectable in this sludge,

whereas a value of 149 mg COD-CH_4/(gVSS.day) was obtained for the suspended sludge. The toxicity limit (IC_{50}) of oleic acid towards aceticlastic methanogens was higher for the granular than for the suspended sludge, indicating the higher resistance of the granular inoculum to the toxicant studied. This result is consistent with the higher resistance to LCFA toxicity of granular sludge when compared with suspended or flocculent sludge previously reported by Hwu (1997). Concerning oleic acid biodegradation capacity, and for the range of concentrations studied, the granular sludge exhibited slightly, but not significantly higher biodegradation rates than the suspended sludge.

Table 2.2 summarises the average pseudo steady-state operation parameters for RI (granular sludge) and RII (suspended sludge).

Table 2.2 Operating conditions and performance of RI and RII (Mean±SD).

Time (days) **[PERIOD]**	**HRT (±0.01)** (days)	**Influent COD** (g/l)	**Influent oleate COD** (g/l)	**COD Removal efficiency** (%)		**Effluent VSS** (g/l)		**Methane production** (l CH_4/(l.day))	
				RI	RII	RI	RII	RI	RII
0-70 **[I]**	1.01	3.8 (±0.3)	1.9 (±0.2)	96.5 (±0.6)	85.8 (±3.2)	0.38 (±0.07)	0.65 (±0.04)	1.06 (±0.1)	0.79 (±0.1)
70-119 **[II]**	1.01	3.8 (±0.3)	3.8 (±0.3)	83.4 (±4.8)	74.4 (±5.5)	0.85 (±0.22)	0.72 (±0.15)	0.23 (±0.05)	0.26 (±0.05)
119-162 **[III]**	1.01	6.2 (±0.7)	6.2 (±0.7)	74.2 (±3.8)	74.6 (±2.9)	1.96 (±0.43)	1.57 (±0.17)	0.16 (±0.02)	0.20 (±0.08)
162-219 **[IV]**	1.01	8.2 (±0.5)	8.2 (±0.5)	68.8 (±3.4)	69.4 (±5.5)	2.71 (±0.57)	2.50 (±0.58)	0.15 (±0.02)	0.22 (±0.06)

During the first 70 days skim milk was introduced as a co-substrate, representing 50% of the total COD fed. For the two first operating conditions, RI exhibited higher removal efficiencies and higher methane production than RII, but in the two last operation periods, higher methane productions and lower VSS levels were obtained in RII. From the day 70 on, oleate was the sole carbon source fed to both digesters, and the methane production decreased to 20-30% of the initial value. Along the trial period the methane yield decreased

from 289 and 242 l CH_4/kg $COD_{removed}$ to 27 and 39 l CH_4/kg $COD_{removed}$ in RI and RII, respectively. This large discrepancy between COD removal and methane production rate is expected to be found when treating effluents with high lipid/LCFA content, as a result of a significant physicochemical COD removal from the aqueous phase to the solid phase (Sayed *et al*, 1987; Hwu, 1997; Pereira *et al*, 2001). In fact, in the last operation period (organic loading rate at 8 kg oleate-COD/(m^3.day)) only 7.7% to 11% of the removed oleate-COD was used for methane production revealing a high accumulation of substrate. Phenomena such as precipitation with calcium ions and adsorption onto the biomass are known to be responsible for LCFA's removal (Hanaki *et al*., 1981; Roy *et al.*, 1985; Rinzema *et al*., 1988; Hwu, 1997) and thus justify this COD accumulation. Assuming that magnesium ions can exhibit a similar effect as calcium ions, the molar ratio of oleate to divalent ions (Ca^{2+} + Mg^{2+}) present during the trial period was determined (Table 2.3). These values were calculated considering the contribution of the calcium content present in the skim milk and tap water and the magnesium supplied in the macronutrients. Because each divalent ion can theoretically precipitate two oleate molecules, a total precipitation of the existing oleate would be possible for a molar ratio less than 2, a condition that prevailed only during the first operation period (first 70 days). Based on this stoichiometric ratio, the maximum oleate-COD that could be precipitated during operation was determined and compared to the total COD accumulated in the reactors (Table 2.3).

Table 2.3 Molar ratio of oleate to calcium and magnesium ions during the trial period.

Time (days)	**Molar ratio Oleate/(Ca^{2+}+Mg^{2+})**	**[(a)]Oleate-COD removed by precipitation** (g/(l.day))	**Total COD accumulated** (g/(l.day))	
			RI	***RII***
0-70	1.33	1.9	0.64	1.00
70-119	4.03	2.26	2.51	2.08
119-162	5.70	2.60	4.14	4.05
162-219	6.79	2.89	5.21	5.06

[(a)] Theoretical values.

Excluding the two first periods, where precipitation phenomena could be responsible for the total COD accumulation, from day 119 on, even if all the calcium and magnesium ions had stoichiometrically precipitated the oleate, the maximum fraction removed by precipitation would correspond to 55-65 % of the total COD accumulated. This suggested the adsorption of a considerable fraction of oleate or any intermediate of its degradation. Therefore, since, precipitation/adsorption phenomena could have an important contribution on LCFA accumulation, the term "biomass-associated" LCFA was considered to be more appropriate to assign to the non-methanised substrate accumulated onto the sludge, either due to precipitation or adsorption. In Figure 2.5 (a) and (c) the typical sludge aspect is shown, where clear whitish zones, which represent the biomass-associated LCFA, can be observed entrapping the biomass. These white zones seem to act as a light emission barrier decreasing the visible auto fluorescence of the methanogenic population (Figure 2.5 (b) and (d)). Also scanning electron inspection of the aggregates, suggest the presence of a non biological matrix entrapping the microorganisms (Figure 2.6 (b)), in which traces of calcium were detected (Figure 2.6 (c)).

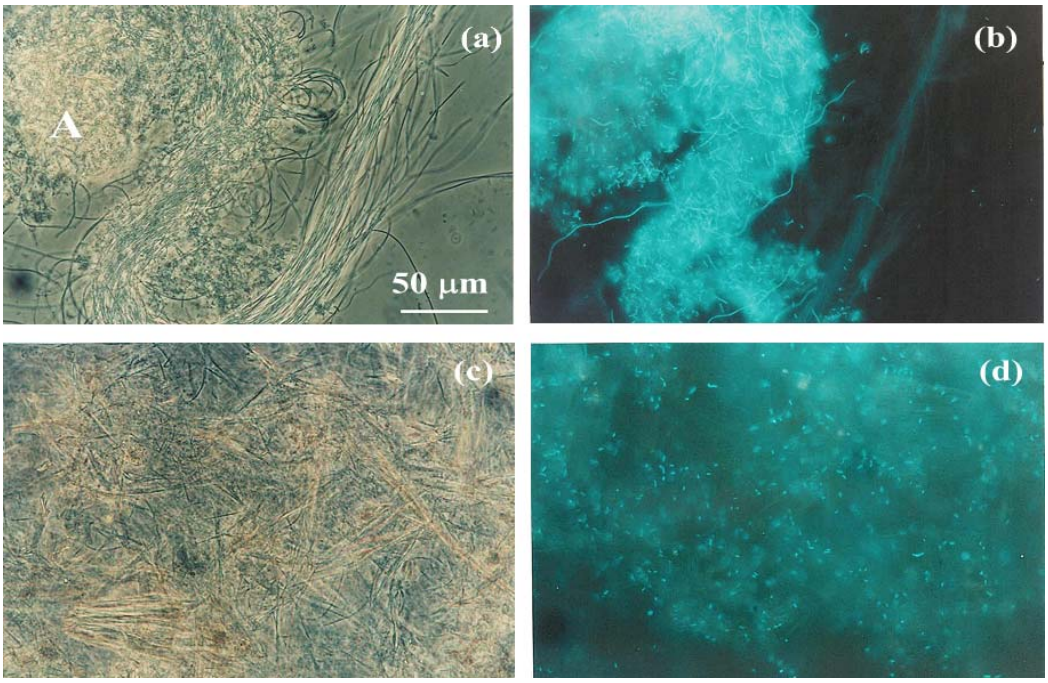


Figure 2.5 Typical microscopic aspect of the loaded sludge: (a) and (c) phase contrast (A-whitish matter); (b) and (d) under fluorescence at 420 nm.

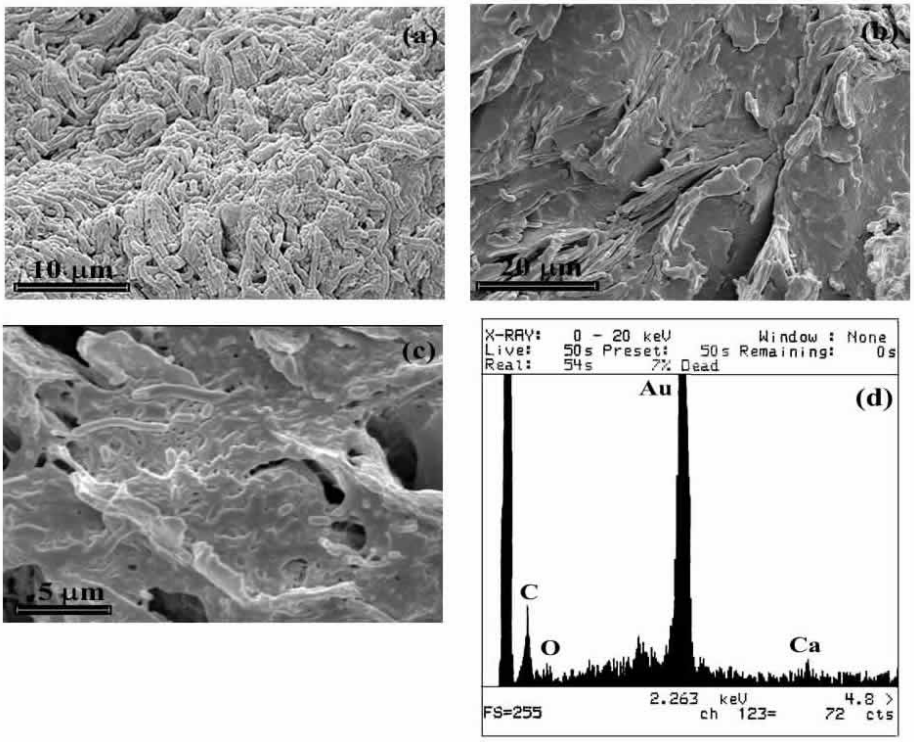


Figure 2.6 Scanning electron microphotographs of the sludge: (a) before (suspended sludge inoculum), (b) and (c) after being loaded with oleate. (d) Elemental composition spectrum of the matrix visualized in (b) obtained by EDS analysis.

2.3.2 METHANISATION OF LCFA ACCUMULATED ONTO THE SLUDGE

During the operation, the sludge in both reactors was segregated in two layers: a bottom settled layer and a top floating one. For each applied oleate loading rate, samples from the bottom and top layers were collected from both reactors and characterised in terms of potential methane production due to the methanisation of the accumulated (biomass-associated) LCFA, in batch assays.

Figure 2.7 represents the results from the batch experiments for the sludge taken from RI and RII in the bottom and top layers at the end of operating periods I, II, III, and IV. Table 2.4 summarises the obtained results.

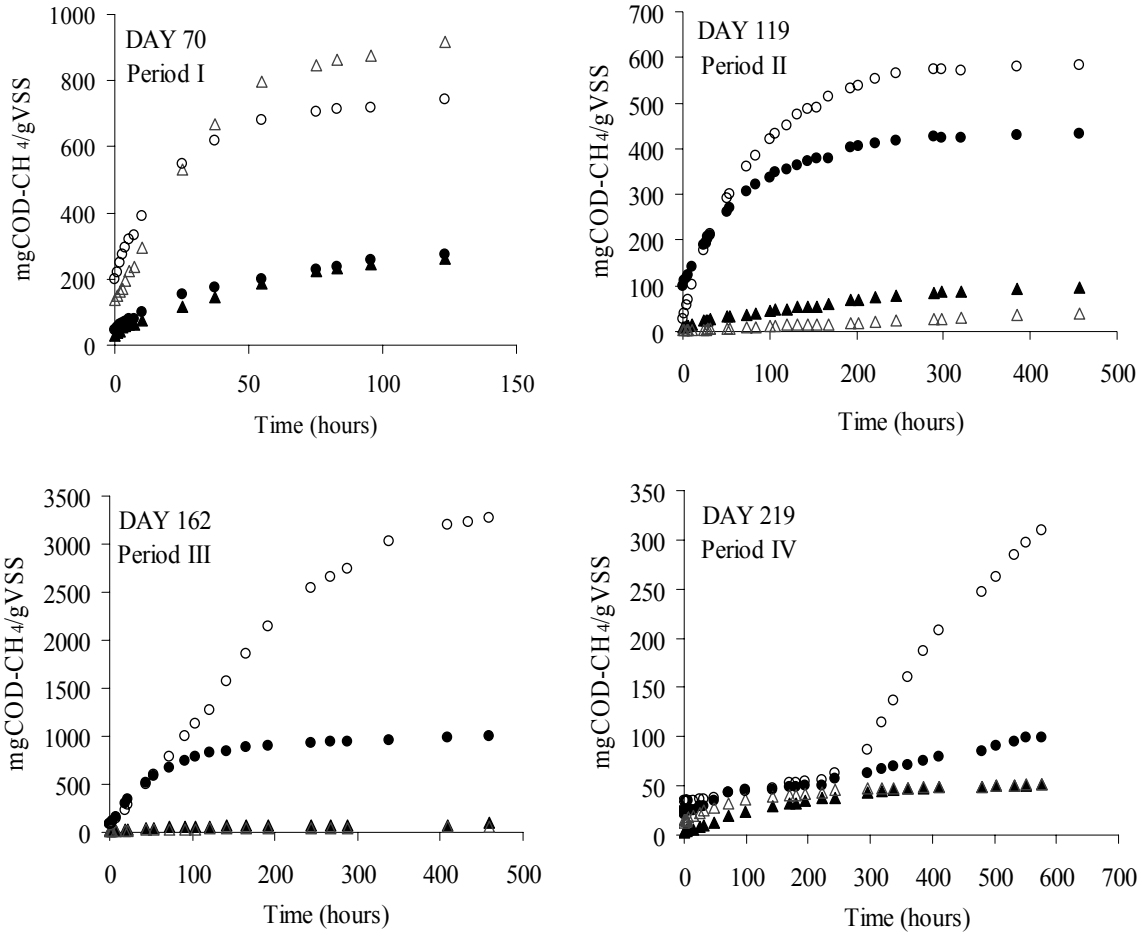


Figure 2.7 Specific methane production in the batch assays. (●) RI top, (o) RII top, (▲) RI bottom, (△) RII bottom.

On day 162, when oleate was fed at 6 kg COD/(m^3.day), the highest plateau in the methane production curve was obtained for the RII-top sludge (3271±877 mg COD-CH_4/gVSS) (1145±307 ml $CH_{4(STP)}$/gVSS)) which was more than three fold the obtained in the RI-top sludge. For the bottom sludges, no significant differences were found (Table 2.4). For the oleate organic load of 8 kg COD/(m^3.day) (Period IV), a clear delay on the biomass-associated LCFA mineralisation was observed, as a lag-phase of 300 hours preceded the initial methane production.

Table 2.4 Maximum plateau (mg COD-CH_4/gVSS) and methane production rate (mg COD-CH_4/(gVSS.day)) obtained in the batch experiments (Mean±SD).

		RI top	**RII top**	**RI bottom**	**RII bottom**
DAY 70	Maximum "plateau"	276±37	743±80	263±17	917±120
Period I	Methane production rate	114±12	434±60	100±17	380±49
DAY 119	Maximum "plateau"	434±17	583±117	97±34	40±6
Period II	Methane production rate	86±9	143±20	11±3	3±3
DAY 162	Maximum "plateau"	997±109	3271±877	97±6	54±9
Period III	Methane production rate	200±6	243±9	14±3	9±3
DAY 219	Maximum "plateau"	100±11	317±69 (*)	51±3	51±9
Period IV	Methane production rate	4±1	7±1	3±1	2±1

(*) This value is underestimated

When comparing the methane production rates in continuous operation and in batch mode for the oleate loading rate of 6 kg COD/(m^3.day), in RII, it is concluded that more methane would be produced per day if the feed was suppressed, provided that the conditions prevailing in the batch experiments were assured. In fact, considering the amount of VSS present in the RII top layer (30 g), 2.6 l CH_4 would be produced per day, whereas in continuous mode only 2 l CH_4 were produced in the whole reactor (Table 2.2). This suggests that accumulated LCFA can be degraded more efficiently, when no oleate is present in the medium (as was the case of the batch tests). This behaviour was confirmed in batch assay where washed-encapsulated sludge was incubated and oleate was added at concentrations ranging from 100 to 900 mg/l, (Figure 2.8). A significant decrease from 117 mg COD-CH_4/(gVSS.day) to 23 mg COD-CH_4/(gVSS.day), was observed when 100 mg/l oleic acid were added.

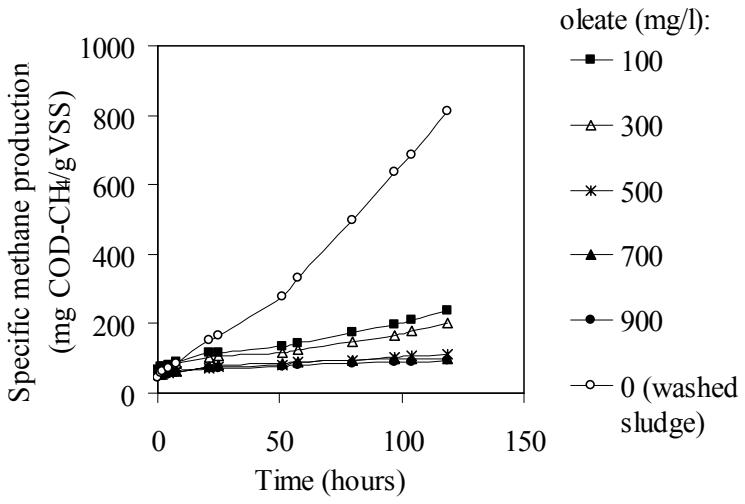


Figure 2.8 Effect of adding oleate in the specific methane production in the batch assays.

The strong effect of oleic acid on the methanisation rate of the biomass-associated LCFA, may suggest that this accumulated substrate is no more oleic acid, but probably an intermediate formed during its degradation, such as stearate or palmitate, which are, according to Weng and Jeris (1976), the first saturated LCFA involved in the anaerobic β-oxidation of oleic acid. Although accumulation of stearic acid is not expected to occur to a significant extent, evidence of palmitic acid accumulation is reported in the literature. Salminen *et al*. (2000) reported that during the batch degradation of solid poultry slaughterhouse wastes, stearate was consumed faster than it was produced and also that palmitic acid was the most abundant LCFA detected in the medium. Beccari *et al.* (1998) applied a two-reactor system with partial phase separation for treating olive oil effluents and reported that in the conversion between oleic acid and palmitic acid, the saturation from oleic to stearic was the limiting step, whereas the first step of β-oxidation (stearic to palmitic) proceeded quickly. Also, Lalman and Bagley (2000, 2001) reported that palmitic acid was the primary detected product from oleic and linoleic acids, and that stearic acid was not detected. In opposition to Beccari *et al* (1998), they reported that stearic acid degradation is slower than oleic acid degradation. However, this conclusion was only based on the monitoring of oleic/stearic acids depletion in the medium, in batch assays. Although their conclusion is reinforced by the free energy values ($\Delta G^{0'}$) involved in the first step of

both conversions (-78.6 kJ/mol for the reaction between oleic and palmitic acids and +50 kJ/mol for the reaction between stearic and palmitic acids), LCFA adsorptive removal was not taken into account in their studies.

Experiments made under static and stirring conditions led to the conclusion that the methane production rate is significantly enhanced by stirring as can be observed in Figure 2.9. A lag phase of about 300 hours was observed preceding the initial methane production under static conditions. As the substrate is already in intimate contact with the biomass, substrate diffusion limitations are not expected to limit the degradation rate. However, product diffusion limitation, e.g. biogas release, was observed to be difficult under static condition, which may justify the different methane production patterns.

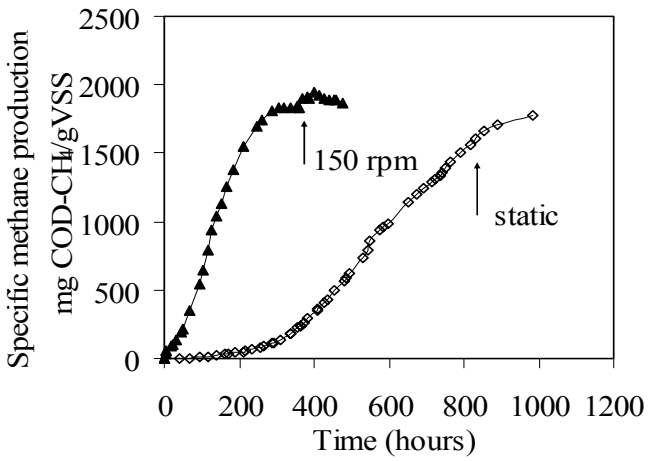


Figure 2.9 Effect of stirring in the specific methane production in the batch assays.

2.3.3 METHANOGENIC ACTIVITY OF THE SLUDGE LOADED WITH OLEATE

At the end of operation, the biomass from both reactors was also characterised in terms of methanogenic activity with acetate, propionate, butyrate, ethanol and H_2/CO_2. Both sludge exhibited no detectable activity on acetate, propionate and butyrate and an insignificant activity on ethanol (<10 mg COD-CH_4/gVSS.day). The measurement of the methanogenic activity in the presence of propionate, butyrate and ethanol (indirect methanogenic substrates) can be underestimated, since, in this case, aceticlastic activity can

be limiting. The granular sludge (from RI) exhibited higher methanogenic activity for hydrogenotrophic methanogens (80±3 mg COD-CH_4/(gVSS.day)), than the suspended one (36±6 mg COD-CH_4/(gVSS.day)). Furthermore, in this sludge methane production from H_2/CO_2 proceeded without delay, whereas in the suspended sludge (from RII) a lag-phase of 520 hours was found. In the measurement of the specific methanogenic activity, the accumulation of LCFA onto the biomass may, besides the potential toxic effect, hinder the transport of substrate and products, inducing a delay on the initial methane production as well as a reduction on the methane production rate. In general, suspended sludge accumulated higher amounts of biomass-associated LCFA (Table 2.4), which can explain the lower methane production rates and higher delays on initial methane production found for this sludge in the activity tests.

2.3.4 EXTRACTION AND GC ANALYSIS OF THE LCFA ACCUMULATED ONTO THE SLUDGE

The extraction and GC analysis of the accumulated matter confirmed the previous suspicion that it was not mainly composed of oleic acid. In fact, only traces of this LCFA were detected and palmitic acid (C16:0) was the main compound present in both sludges (Figure 2.10). To support this result, a continuous experiment was made using the same EGSB reactors fed with oleate as the sole carbon source at 6 kg COD/(m^3.day). The suspended sludge collected at the end of the previous continuous experiment was used as inoculum, after allowing the depletion of the biomass-associated LCFA accumulated during that previous experiment. This sludge had shown a certain degree of inhibition due to the overload with oleate at concentrations of 8 g COD/l (Figure 2.7, p. 72). One of the reactors was inoculated with active sludge (RI) and the other with inactivated sludge (RII).

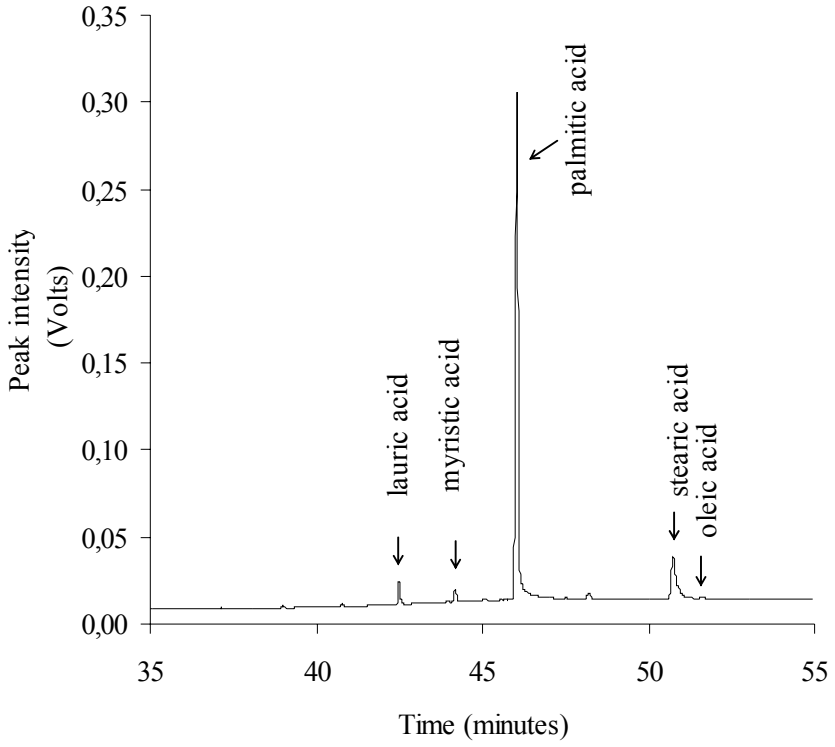
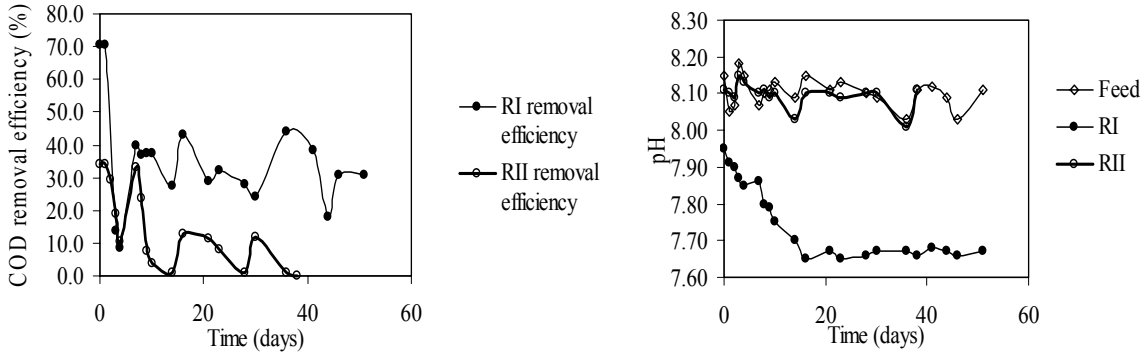


Figure 2.10 Example of a chromatogram obtained by GC analysis after extraction of the accumulated matter.

Figure 2.11 represents the time course of the COD removal efficiency of RI and RII, as well as the pH in the feed and in RI and RII. Although removal efficiency and % of methane were very low during the trial period, a clear difference between RI and RII was observed both in terms of removal efficiency (average value of 35% in RI and 14% in RII) as well as in terms of methane content (20% and 0% in RI and RII, respectively). Also the different pH values revealed that at least some biological activity was detected in RI, but not in RII. These results clearly demonstrate that the sludge previously inhibited by accumulation of LCFA was not able to retrieve the performance previously exhibited in similar operating conditions (Table 2.2, p.68). However, it should be noted that RI and RII were operated at an extremely high load of oleate as the sole carbon source (6 kg COD/(m^3.day)) without any start-up strategy. In previous experiments, there was evidence that if a gradual start up with a co-substrate was performed, a similar sludge, previously

overloaded with oleic acid, was more resistant to oleic acid toxicity and allowed higher methane yields when incubated in continuous reactors (Pereira *et al*., 2001).



(a) (b)

Figure 2.11 Time course of (a) COD removal efficiency and (b) pH, of feed and of RI and RII.

On day 28, sludge samples from RI and RII were taken for extraction and GC analysis. The LCFA content of the feed was also analysed in the same way and confirmed within an error of 10% the obtained value from the COD analysis. Table 2.5 summarises the corresponding results. In the active sludge (RI) the main LCFA detected was palmitic acid, at a concentration 2.8 times higher than the exhibited by oleic acid. In the inactivated sludge, however, oleic acid was the main LCFA accumulated, at a concentration 8 times higher than palmitc acid. The analysis to active sludge, repeated on days 30 and 37, revealed the same patter with palmitic acid to be 3.6 and 52 times the concentration detected for oleic acid, respectively. This increasing accumulation of palmitic acid along the operation can be due to an inhibition of palmitate degradation by the continuously fed oleic acid. From the present results it is evident that the transformation of oleic acid to palmitic acid is a fast and non-limiting step in oleic acid degradation. The accumulation of palmitic acid onto the sludge suggests that its further degradation is a difficult step at least under continuous operation. Hanaki *et al*. (1981) also studied the accumulation of lipids and LCFA onto anaerobic sludge and concluded that a fast adsorption of LCFA occurred. LCFA

accumulation was attributed to their inhibitory effects to its own β-oxidation. However, these experiments were in batch assays during whole milk degradation and no relevant information or discussion were addressed to the identification of individual LCFA.

Table 2.5 Analysis of LCFA content on the feed and on the sludge samples collected from RI and RII, on day 28.

	mg/mg dry weight		
LCFA	**Feed**	**RI**	**RII**
Lauric acid (C12:0)	n.d	n.d.	n.d.
Myristic acid (C14:0)	0.012	0.013	0.011
Palmitic acid (C16:0)	0.004	0.254	0.035
Stearic acid (C18:0)	0.007	0.040	0.016
Oleic acid (C18:1)	0.341	0.090	0.277

n.d.- non-detectable.

2.4 CONCLUSIONS

During the continuous load of two EGSB reactors (RI inoculated with granular sludge and RII inoculated with suspended sludge) with oleic acid as the sole carbon source up to 8 kg COD/(m^3.day), methane yields as low as 25 l CH_4/kg $COD_{removed}$ were obtained. COD removal revealed to be mainly due to LCFA accumulation onto the sludge. When comparing methane production rates in batch mode (exclusively due to methanisation of the accumulated LCFA) and in continuous operation (e. g., oleate loading rate of 6 kg COD/(m^3.day)), it was concluded that more methane would be produced per day if the feed was suppressed. Palmitic acid was the main LCFA accumulated onto the sludge and further β-oxidation was inhibited when in presence of oleic acid. When oleic acid was removed from the medium, β-oxidation proceeded with subsequent methane production. The accumulation of palmitate under continuous load of oleic acid and the rate of its methanisation in batch assays, were highly dependent on biomass structure, suspended sludge being more interesting for that purpose than granular one.

From these results it can be concluded that for treating effluents with high lipid/LCFA content, it should be advantageous to sequence cycles of adsorption/accumulation and degradation in order to enhance methane production.

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3 MORPHOLOGICAL CHANGES IN ANAEROBIC SLUDGE LOADED WITH OLEIC ACID

Abstract

In this chapter, quantitative image analysis techniques are applied to monitor the morphological changes in the suspended and granular sludge, from the two EGSB reactors fed with oleic acid, described in Chapter 2. Deterioration of granular sludge was quantified during the trial period by the percentage of aggregates smaller than 1 mm (Feret Diameter) either in terms of projected area or in terms of number of aggregates. A good correlation was obtained between these values and the % of aggregates smaller than 1 mm physically sorted and quantified by the VSS content. A morphological parameter relating the amount of free filaments and the projected area of aggregates (Lfa) was defined to quantify the dispersion level of the granular sludge, which increased until day 141 and remained almost invariable afterwards. As expected, the total free filament length was significantly higher in the suspended than in the granular sludge, attaining a maximum value as high as 250 m/mg VSS. The equivalent diameter of the bottom aggregates larger than 1 mm (in terms of Feret diameter) increased with the increase on the amount of accumulated LCFA. After a threshold value of about 200 mg COD-CH_4/gVSS, the aggregates migrated to the top floating layer. In the granular sludge the amount of accumulated LCFA and their mineralisation rate to methane exponentially increased with the total free filament length, but exponentially decreased with the increase on the percentage of aggregates smaller than 1 mm. This evidenced the importance of filamentous bacteria on the process of LCFA accumulation/degradation and the relative irrelevance of the surface area for the phenomenon.

3.1 INTRODUCTION

Usually the processes of granulation and granule-disintegration are coupled with a macroscopic transformation of size and morphology. These changes can be quantified by image analysis, although most of the published works are mainly focused on size determinations (Dudley *et al.*, 1993; Jeison and Chamy, 1998). Bellouti *et al.* (1997), by using image analysis to measure the fractal dimension, differentiated anaerobic flocs and granules and Howgrave-Graham and Wallis (1993), quantified the bacterial morphotypes within anaerobic granules from transmission electron micrographs also using image analysis. Systematic microscopic examinations have not been used so far to follow the granulation/disintegration process, because they are tedious and difficult to implement in a quantitative way. Classical methods to characterize microbial aggregates and evaluate the content in filamentous bacteria were based on manual counting, under a microscope with an eyepiece equipped with a micrometer and have been mostly developed for activated sludge processes to alert for bulking problems. Sezgin *et al*. (1978) developed a procedure to determine the total length of filamentous bacteria and Jenkins *et al*. (1993) proposed another technique to quantify *Nocardia* sp. Manual counting techniques are, however, rather laborious, imprecise and time-consuming methods which make them not feasible for routine monitoring. Hence, automated image analysis seems an appropriate method to characterise quantitatively flocs and filamentous bacteria. Reliable information of this type should enable to improve the daily operation of wastewater treatment plants. Such a method has been proposed by da Motta *et al*. (2001) and Amaral *et al* (2002) and subsequently used to monitor bulking events in pilot plants (da Motta *et al*., 2001).

One application of image analysis to anaerobic wastewater treatment was done previously by Alves *et al.* (2000), to follow the number and the total length of filaments present in anaerobic sludge when exposed to oleic acid shock loads. Bacterial filamentous forms are known to play a key role in the process of granulation (Wiegant, 1988) and, therefore, are possibly released to the bulk medium in the process of granules disruption. The quantification of filaments and filaments to aggregates ratio can thus give important insights on process stability and are potentially good alert indicators to be included in expert systems for supervision and control of high rate anaerobic wastewater treatment.

When fed with some problematic substrates, the stability of granule-based anaerobic reactors, such as the UASB reactor or the EGSB reactor, is largely affected. Lipidic compounds are one of these problematic substrates. They are easily hydrolysed to LCFA, that adsorb onto the biomass, inducing granule disruption, flotation and washout (Hwu, 1997). This occurs at concentrations far below the toxicity limit, which might suggest that complete washout of granular sludge would occur prior to inhibition. Furthermore, the addition of calcium salts prevents inhibition to some extent, but does not prevent flotation (Hanaki *et al.*, 1981).

In Chapter 2, the maximum potential methane production due to the degradation of the accumulated (biomass-associated) LCFA exhibited by the sludge of two EGSB reactors fed with increasing loads of oleic acid was studied in batch assays. The maximum accumulated LCFA (mainly palmitate) under continuous oleate load, and the rate of its further methanisation in batch assays, were found to be highly dependent on biomass structure, being suspended sludge more efficient than granular one.

In the present work, image analysis techniques were applied to monitor the morphological changes in granular and suspended sludge, collected during the continuous oleate load of the EGSB reactors described in Chapter 2. Morphological parameters relating the total free filament length and the total projected area of aggregates were determined and the average equivalent diameter of aggregates related to the amount of biomass-associated LCFA. Tentative relationships between sludge dispersion level and the amount or the mineralisation rate of biomass associated LCFA, were searched.

3.2 MATERIALS AND METHODS

3.2.1 SLUDGE SOURCES

Biomass was sampled from the two EGSB reactors previously described in Chapter 2 (p.55). One of the reactors was inoculated with granular sludge (RI) and the other reactor was inoculated with suspended sludge (RII). Biomass recycling was applied in both reactors. The influent oleate concentration increased from 2 to 8 g COD/l and the HRT was set at 1 day. Four operation periods were identified: Period I, II, III and IV (Chapter 2, Table 2.2 p.68). In both reactors, the biomass was segregated in two layers, a bottom layer where a little amount of biomass-associated LCFA was present, and the top layer, which exhibited large amounts of biomass-associated LCFA (Chapter 2, Table 2.4 p.73).

Sludge samples for image analysis were taken on days 70, 92, 119, 141, 162, 191, and 219, from both layers. For all the samples, the Volatile Suspended Solids (VSS) content was determined as previously described in the sub-chapter 2.2.2 (p. 56). Sludge from RI was also characterised by the % of fine solids, determined by the ratio between the VSS that could be removed using a syringe equipped with a 20 Gx1″ needle, and the total VSS. The breadth of the largest particle that could enter the needle was set at 1 mm and was determined by image analysis using the minimal Feret diameter (*minFD*) (Russ, 1995). The minimal Feret Diameter is the minimum distance between parallel tangents touching opposite sides of an object (Figure 3.1)

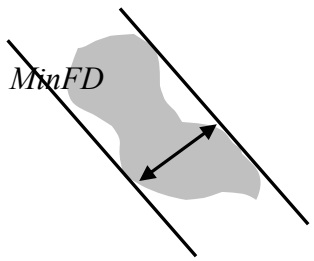


Figure 3.1 Definition of the Minimum Feret Diameter (minFD).

All samples were then classified in two categories: small aggregates which were all particles with a *minFD* smaller than 1 mm and large aggregates which had a *minFD* bigger than 1 mm. The granular inoculum was characterised in terms of size and morphological characteristics. The average equivalent diameter of the large aggregates (MinFD>1mm) was 1.59±0.07 mm and the average equivalent diameter of the small aggregates (MinFD<1mm) was 0.184±0.008 mm. The percentage of the projected area of the large aggregates accounted for 46.5%. The ratio between the total filament length and the total area of aggregates was 37.7 mm^{-1}.

3.2.2 IMAGE ANALYSES

All the methods developed for image acquisition and processing, including the Filament and Floc programmes were done by Luís Amaral and are fully described in his Ph.D. thesis (Amaral, 2003).

3.2.2.1 IMAGE ACQUISITION

Image acquisition for filaments was accomplished through phase contrast microscopy on a Diaphot 300 Nikon microscope (Nikon Corporation, Tokyo) with a 100 times magnification. For aggregates with an equivalent diameter larger than 50 μm, images were acquired through visualisation on an Olympus SZ 40 stereo microscope (Olympus, Tokyo) with a 15 times magnification. All the images were digitised with the help of a CCD AVC D5CE Sony grey scale video camera (Sony, Tokyo) and a DT 3155 Data Translation frame grabber (Data Translation, Marlboro), with a 768 x 576 pixel size in 8 bits (256 grey levels) by the Image Pro Plus 3.0 (Media Cybernetics, Silver Spring) software package.

In the procedure of image acquisition for filaments, a volume of 20 μl was put on the slide and covered with a 20x20 mm cover slip for visualization and image acquisition in phase contrast and direct light, respectively. A total of 18 images per slide were acquired, according to Figure 3.2. This methodology intends to minimize non-uniform flocs deposition in the slide. For the larger aggregates, a volume of 2.8 ml was transferred to a Petri dish for visualization and image acquisition. The Petri dish was thoroughly screened, according to Figure 3.3, from left to right and top to bottom with optimal focusing in the

aggregates borders. About 50 images per sample were acquired in both acquisition procedures. The pixels were further calibrated to metric unit dimensions for each magnification.

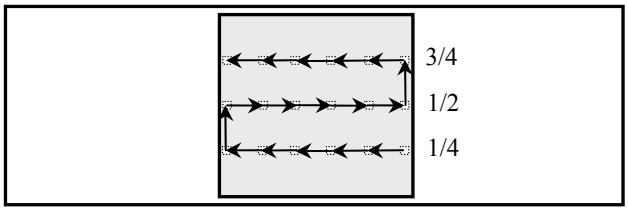


Figure 3.2 Image acquisition methodology within each slide.

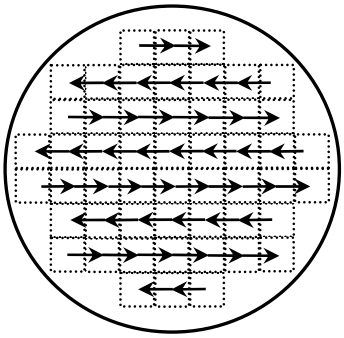


Figure 3.3 Image acquisition methodology within each Petri dish.

Image analysis and processing was accomplished by using two dedicated programmes developed in Matlab (The Mathworks, Inc., Natick) by Luís Amaral (Amaral, 2003). In the next two sub-chapters a brief description of these programmes is presented.

3.2.2.2 FILAMENTS PROGRAMME

The total free filament length was determined by this programme. The original image was first enhanced by means of a Mexican-hat filter (Russ, 1995), followed by a background homogenisation, Wiener filtering and histogram equalization. Subsequently, a defined threshold binarises the image. In order to identify the filaments skeletonisation and

a 10 pixels length end-point removal is performed. Free filaments are not only the dispersed bulk filaments, but also those that are attached to an aggregate and still have one free extremity (Figure 3.4).

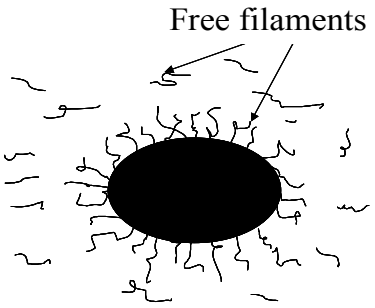
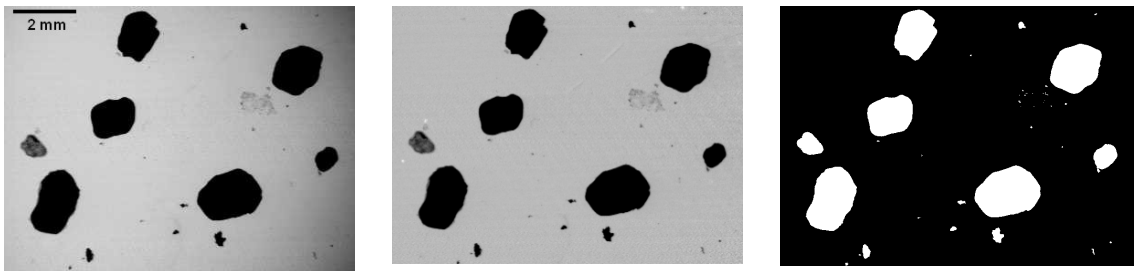


Figure 3.4 Schematic representation of an aggregate and free filaments.

3.2.2.3 FLOC PROGRAMME

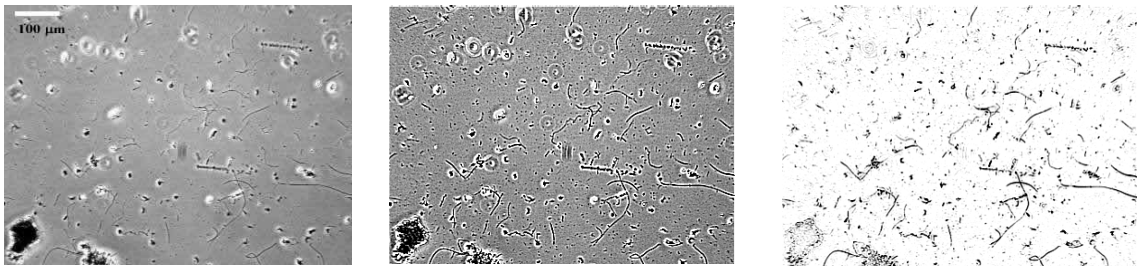
This programme was applied only to the granular sludge. The background from the original image is subtracted and then a previously defined threshold binarises the image. In the binarised image only the objects larger than 5x5 pixels are treated, discarding the debris present in the image. The particle size was evaluated by its equivalent diameter (D_{eq}), calculated from its projected area (A) by $D_{eq} = 2\sqrt{A/\pi}$, and by its minimum Feret diameter (*minFD*). *minFD* is the minimum distance between parallel tangents touching opposite sides of an object.

Figure 3.6 and Figure 3.5 present examples of images from the filament and floc programmes, respectively.

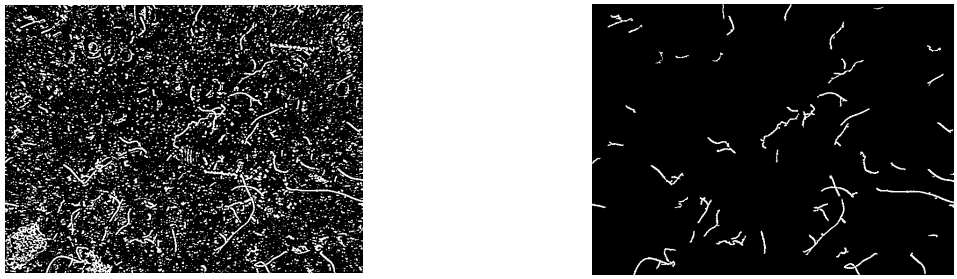


a) Original image b) Background subtracted image c) Binary image

Figure 3.5 Images from the Floc program.



a) Original image b) Mexican-hat image c) Background homogenisation image



d) First binary image e) Final binary image

Figure 3.6 Images from the Filament program.

3.2.3 BATCH EXPERIMENTS

Some of the samples characterised by image analysis, had also been characterised for the amount of biomass-associated LCFA and for the corresponding methanisation rate, in batch tests as previously described in the sub-chapter 2.2.3.5 (p.64).

3.3 RESULTS AND DISCUSSION

Figure 3.7 represents the results obtained from the filament programme. The total free filament length per mg VSS is presented along the operation time for both reactors and for the bottom and top layers. In RII a value of total free filament length as high as 250 m/mg VSS was obtained, which seems to be an extremely high value. However, this is a reasonable value, according to the following considerations: if the average diameter of filaments is 0,5 μm, with a length of 250 m, the total cell volume will be 5E-5 cm^3 and the corresponding cell mass can be estimated as 5.2E-5 g i.e. 0.05mg filaments/mg cells. If the average diameter of free filaments is 1 μm this will represent about 0.20 mg filaments/mg cells. As expected, the total free filament length was significantly higher in the suspended (from RII) than in the granular sludge (from RI). In the top section of both reactors the free filament concentration was higher than in the bottom section, evidencing a stratification of sludge dispersion, higher in the top than in the bottom layer of both reactors.

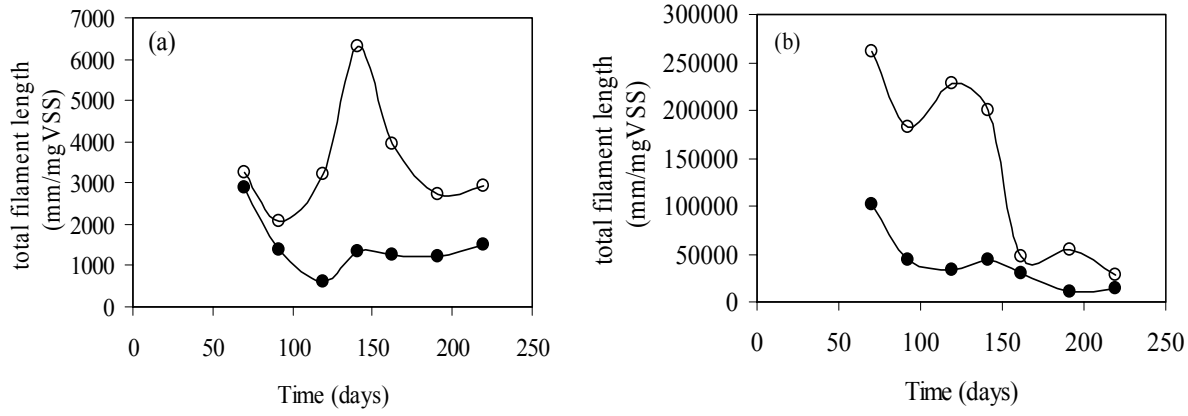


Figure 3.7 Total free filament length in RI (a) and RII (b) sludges. (•) bottom, (o) top.

This parameter provides quantitative information about the aggregation state of biomass. Granules are known to form agglomerates with a relatively smooth surface and with a higher strength of aggregation than flocs or suspended sludge (Hulshoff Pol, 1989).

When granular sludge deteriorates, a release of filamentous microbial forms can occur, simultaneously with the decrease in size of the aggregates. In both situations, washout should be an immediate consequence, due to the low hydraulic retention times usually applied to UASB and EGSB reactors. On day 150, a significant increase of the number of free filaments was found in RI, which is possibly related to the disintegration of granular sludge, as a result of oleate loading rate increase to 6 g COD/l.day on day 119. The subsequent decrease can be due to the washout of the dispersed fraction of biomass, as a significant increase on RI effluent VSS content was observed from day 150 on (Figure 3.8). Values as high as 3.5, 4.8 and 4.3 g VSS/l were attained on days 158, 190 and 203, respectively. Also in RII (suspended sludge), the decrease of the number of free filaments (Figure 3.7 (b)) was followed by a progressive increase on effluent VSS content, supporting the occurrence of a considerable washout of filamentous forms along the trial period. It should be mentioned that the values of VSS measured in the effluent accounted not only for the cells that were washed out, but also for the substrate that was associated to these cells, which was quantified in Chapter 2.

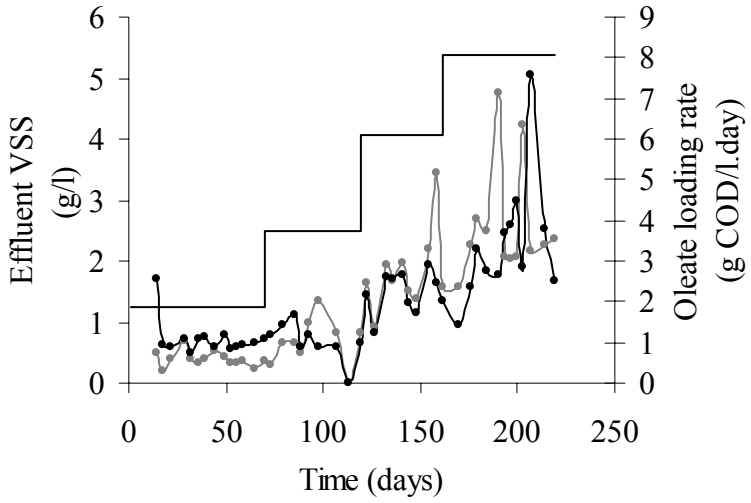


Figure 3.8 Time course of RI (•) and RII (•) effluent VSS content, during the trial period.

It is known that bacterial filamentous forms represent a large diversity of acidogenic and methanogenic bacteria. For instance, *Methanosaeta* is a filamentous methanogen and the unique known filamentous aceticlastic bacteria. Aceticlastic bacteria are responsible for about 70% of the total methane production (Jeris and McCarty, 1965) being, thus, an important group in the anaerobic degradation process. It is, therefore, acceptable that the selective washout of filamentous bacteria will hinder reactor performance and can be related to the absence of methanogenic activity in the presence of acetate, propionate, butyrate and ethanol exhibited by both sludge at the end of operation (Chapter 2, p. 75).

Besides filament release to the bulk medium, a macroscopic transformation of size and morphology of granules was expected along the operating period. Results concerning aggregate measurements were only performed for the granular sludge present in Reactor RI. Figure 3.9 (a) shows the time course of the percentage of small aggregates in terms of projected area, number of particles and also by applying the physical separation previously described in sub-chapter 3.2.1, followed by assessment of the VSS of each fraction. These results represent average values between bottom and top layers. This figure clearly shows that granule disintegration occurred along the trial period, as the fraction of small aggregates increased steadily, whatever the method used for its determination. With the single exception of one experimental point it is relevant that an acceptable correlation was found between the percentage of small aggregates determined by the physical separation and by image analysis. (Figure 3.9 (b)).

Although the above presented figures clearly evidenced that filaments were released to the medium and also that aggregates became smaller during the reactor operation, the relative importance of each process for granule deterioration is not clear. The ratio between the total free filament length and the total area of aggregates (Lfa) is a sensitive morphological parameter that gives directly the proportion between filamentous dispersed and aggregated fractions. This parameter was defined before to recognize anaerobic granulation time (Araya-Kroff *et al*., 2002). Figure 3.10 presents values of Lfa determined for RI in the bottom and top layers.

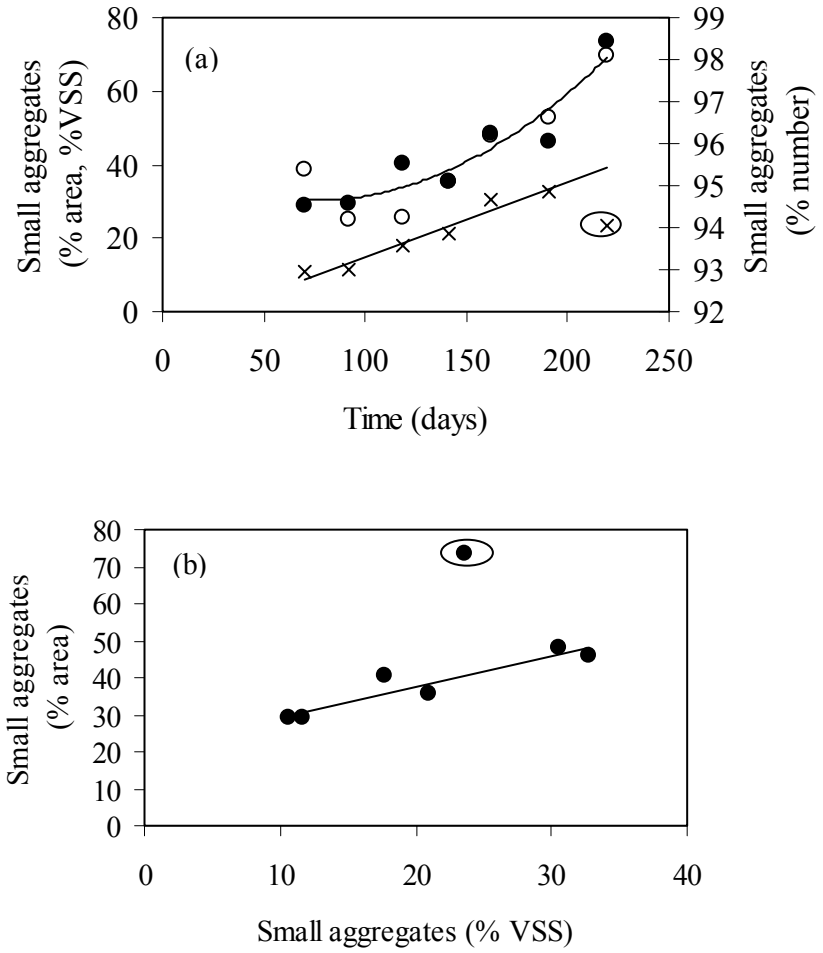


Figure 3.9 (a) Time course of the ratio of small aggregates determined by weight (×- % of VSS) and determined by image analysis (o - % of number, • - % of projected area). (b) Correlation between a method of image analysis and the physical separation method based on the weight of VSS. Marked point was not taken into account for the correlation.

Figure 3.10 clearly illustrates the occurrence of a morphological stratification within the reactor, with a significantly higher filaments-to-aggregates ratio in the top than in the bottom layer, from day 100 on. After day 140, the morphological structure in terms of filaments-to-aggregates ratio remained approximately invariant in both sections. It is interesting to note that an inverse behaviour, with an initial increase followed by a decrease, was found for a granulation process by Araya-Kroff *et al*. (2002). The evolution of filaments and small aggregates during the operating time, in the present study, did not

follow similar trends. Filaments showed a maximum value around the day 140 and a further decrease was observed. However, the relative amount of small aggregates increased steadily all along the operating period. This is not surprising, since these small aggregates should be fragments of granules that have still granular properties of density which, notwithstanding the smaller size, may allow their permanence inside the reactor.

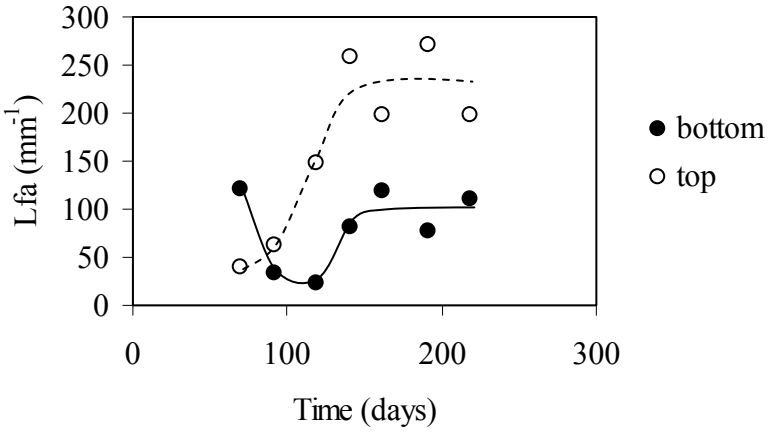


Figure 3.10 Time course of the ratio between the total free filament length and the total projected area of aggregates (Lfa)

In Figure 3.11, an aspect of granule deterioration into smaller fragments is shown. The decrease of the visible auto-fluorescence of the methanogenic population as a result of a light emission barrier caused by the accumulation of LCFA onto the sludge, as previously observed in Chapter 2 (Figure 2.5, p.70), can also be seen.

For filaments, especially for those that are not linked to any fragment of granule, it is comprehensible that they could be selectively washed out from the reactor. The relationship between the parameter Lfa and the % of small aggregates (% projected area) evidences this behaviour (Figure 3.12). When granules started to fragment (for a relatively low percentage of small aggregates), there was a linear trend between the fragmentation and the release of filaments to the medium, especially in the top layer. After a certain degree of fragmentation (% of small aggregates higher than about 38%), the ratio of filaments/aggregates became approximately constant and independent of the % of small aggregates. This evidence makes

clear the relative importance of fragmentation and filaments abundance during the process of granules deterioration, in the conditions applied in this reactor, and accounting for the possible selective washout of filaments.

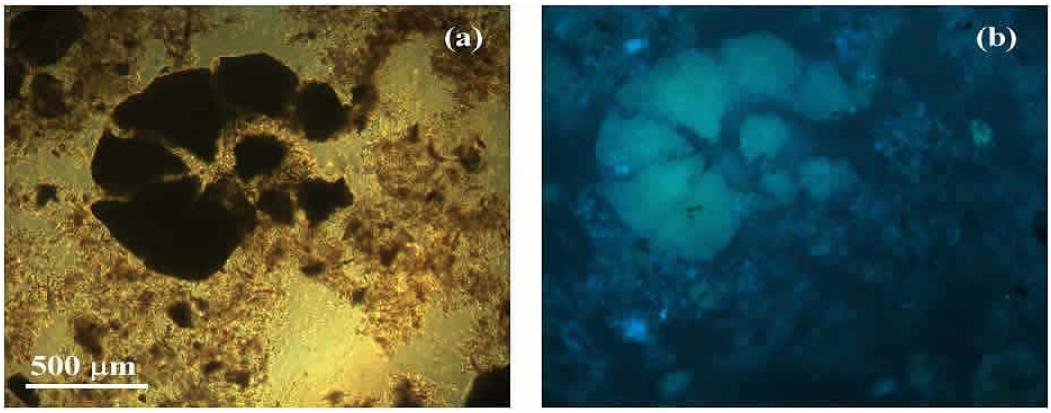


Figure 3.11 Typical microscopic aspect of the granular sludge present in the top layer (day 219) showing: (a) granule fragmentation and (b) auto-fluorescence of methanogenic bacteria at 420 nm.

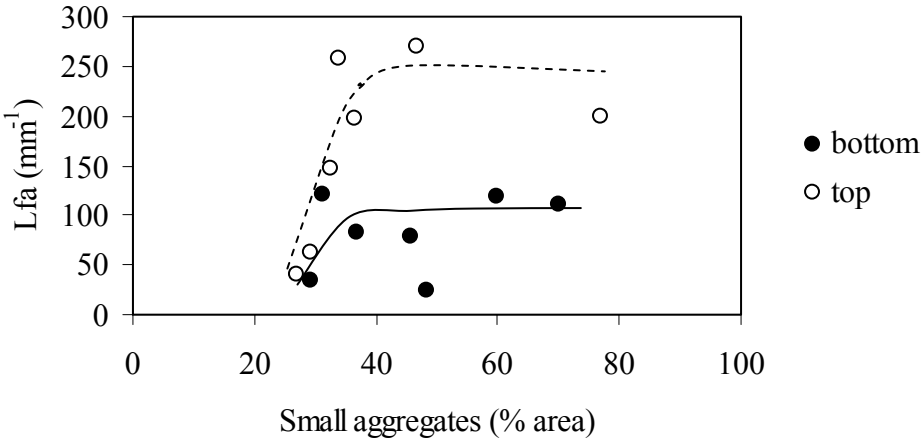


Figure 3.12 Relationship between the total free filaments length per total area (Lfa) and the percentage of small aggregates in terms of projected area.

As the oleate loading rate increased, it was expected that more LCFA could accumulate onto the sludge. Figure 3.13 represents the effect of the amount of biomass-associated LCFA on the average equivalent diameter of the larger aggregates (>1 mm).

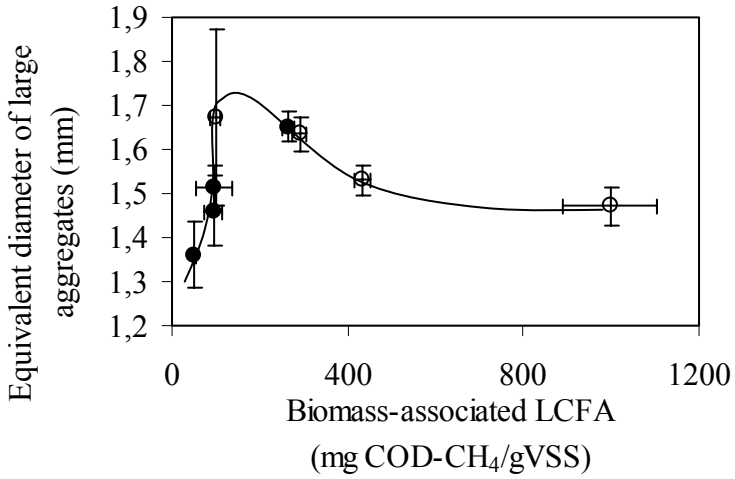


Figure 3.13 Effect of the accumulated (biomass-associated) LCFA on the average equivalent diameter of non-fine aggregates present in reactor RI (inoculated with granular sludge). (•) bottom, (o) top.

In the bottom sludge, the maximum amount of accumulated LCFA was about one fourth of the maximum determined in the top sludge. It is accepted that adsorption of LCFA induces biomass flotation (Hwu, 1997) and consequently higher amounts of adsorbed substrate were expected in the top sludge. From Figure 3.13 it seems that when the biomass-associated LCFA increased up to 100 to 200 mg COD-CH_4/gVSS the larger aggregates present in the bottom also increased in size. When the amount of accumulated LCFA was higher than this value, the aggregates migrated to the top layer, and decreased somewhat in size. It is understandable that aggregates that accumulated more LCFA migrated to the top section, due to a decrease in the overall density. From this Figure, the definition of the threshold value of biomass-associated LCFA that originates this migration is of practical interest, since the top sludge in this type of reactor is primarily condemned to be washed out. Hwu (1997) also studied the problems of washout in EGSB reactors fed with an oleic-acid based effluent and concluded that the typical operating parameters of EGSB reactors

(Vup>4 m/h, HRT<10h) inhibited the treatment performance of LCFA containing wastewaters, being, in that case, washout problems more acute than toxicity problems of these compounds. He found that for an EGSB reactor operated at an HRT between 16 and 27.6 hours, sludge flotation started occurring at a specific organic load of 86 mg COD/(gVSS.day) (corresponding to a value of 98.9 mg COD/gVSS) and complete flotation occurred at a specific organic load of 203 mg COD/(gVSS.day) which corresponds to about 140 mg COD/gVSS. It is relevant to note that the threshold value that induced flotation, obtained in the present work (Figure 3.13), is in the same range of those reported by Hwu (1997).

Other important aspects that should be studied are the influence of filaments on the accumulation of biomass-associated LCFA or vice-versa and the effect of the accumulated LCFA on the abundance of free filaments. Being difficult to decide which should the independent variable be in this case, it seems more interesting to observe the influence of the total filament length on the accumulation of the biomass associated LCFA, as well as in the corresponding degradation rate to methane (Figure 3.14). The obtained exponential relationship between the accumulation of biomass-associated LCFA and the total free filament length clearly evidenced the importance of filaments on the accumulation of LCFA. On the other hand, the degradation rate of these LCFA was highly enhanced when filaments increase in the sludge which may also suggest that filamentous bacterial forms are involved in LCFA degradation.

It should be remembered that these results concern only sludge from reactor RI, which was inoculated with a granular inoculum. In the suspended sludge that was inoculated in reactor RII, only filaments were measured and hence no discussion was addressed to this sludge about aggregates and fragmentation, etc. Due to the huge capacity of that suspended sludge to accumulate LCFA, the influence of total free filament length on the LCFA accumulation was also studied in that sludge. However, no trend was observed between these two variables. The reason is probably because by image analysis it was possible to measure free filaments, but not filaments that are completely enclosed inside a floc. Suspended sludge was composed of small aggregates which are called flocs to distinguish them from pellet or granules, according to the classification of Dolfing (1987). These flocs are fluffy structures where filaments form an interlinking grid which is expected

to be highly efficient for entrapment of biomass-associated LCFA, especially because filamentous bacteria have a high surface area, by comparison with cocci, or rod shaped cells. This is evident from Figure 3.15 (a), where white zones can be observed in between the aggregate, which are likely account for entrapped LCFA. In Figure 3.15 (b) a reduction of F_{420} autofluorescence is observed in these zones as was previously shown in Figure 3.11. Therefore, the better performance of suspended sludge to accumulate LCFA could be due not only to the higher surface area resulting from the smaller size of flocs, when compared to the granules, but especially from the internal filamentous microstructure, which was impossible to quantify by image analysis.

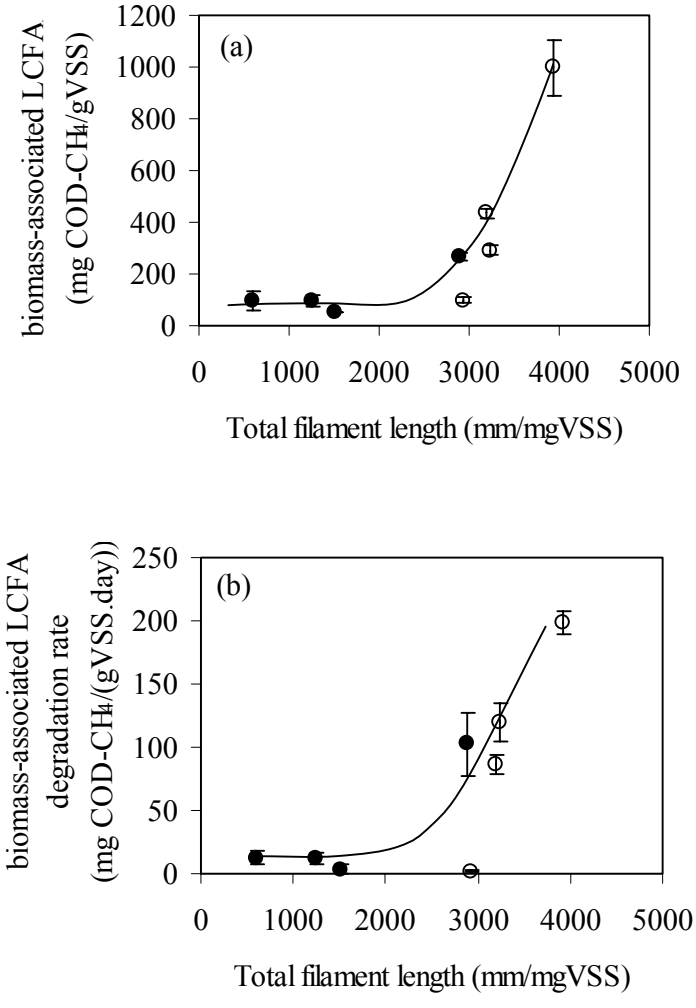


Figure 3.14 Effect of the total filament length on (a) the amount of biomass-associated LCFA and (b) the biomass-associated LCFA degradation rate. (•) bottom, (o) top.

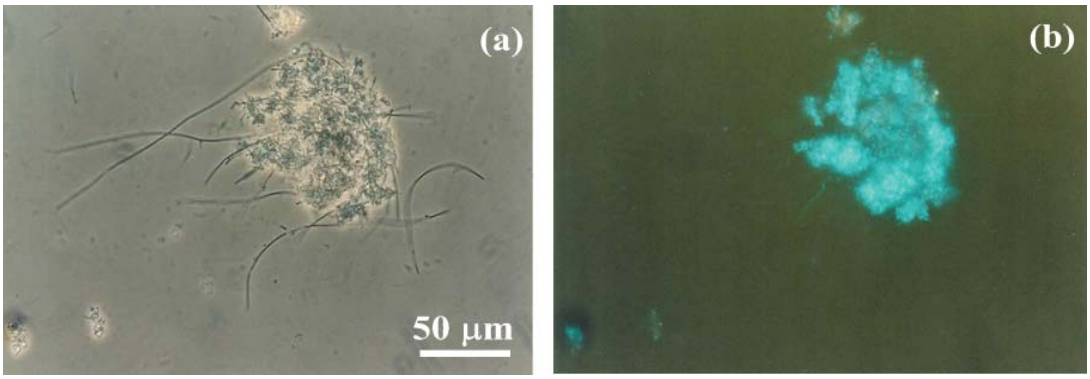


Figure 3.15 Example of a floc: (a) phase contrast and (b) auto-fluorescence of methanogenic bacteria at 420 nm.

The relative importance of filaments and surface area for the phenomenon of LCFA accumulation and degradation can be more deeply understood by comparing Figure 3.14 and Figure 3.16. In Figure 3.16, the amount and degradation rate of the biomass-associated LCFA are related to the relative amount of aggregates smaller than 1 mm, expressed in percentage of their projected area. It was not expected to obtain a decreasing trend between these variables, since an increase in the relative amount of small aggregates should correspond to an increase in the surface area available for adsorption. However, these clear trends suggest the accumulation of LCFA by mechanisms different from adsorption, evidencing the relative unimportance of the available surface area of (macro) aggregates on the accumulation and degradation of biomass-associated LCFA, when compared to the role of the (micro) filamentous structures.

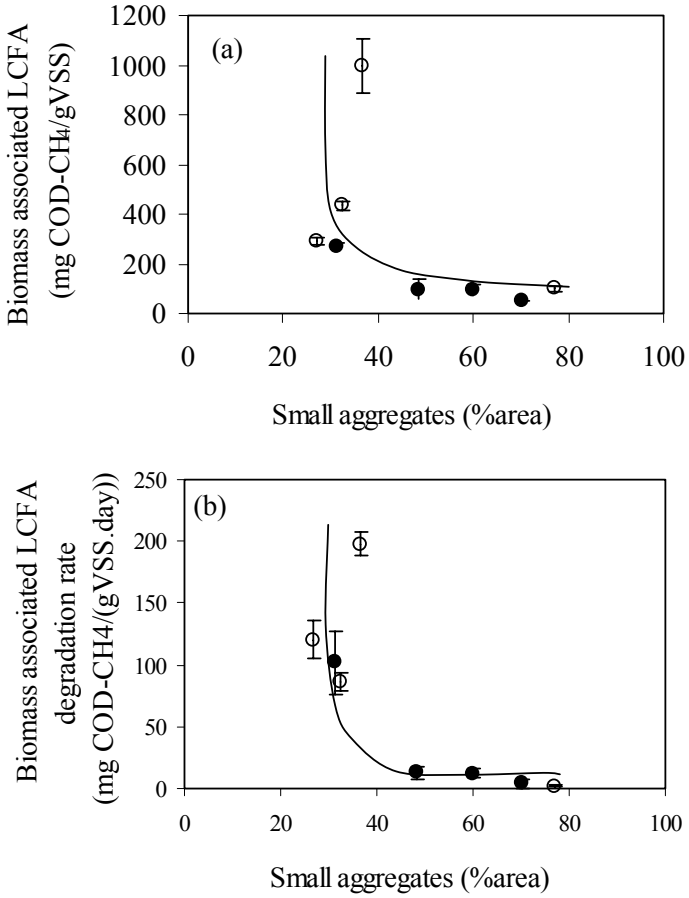


Figure 3.16 Effect of the percentage of small aggregates on (a) the amount of biomass-associated LCFA and on (b) the biomass-associated LCFA degradation rate. (•) bottom, (o) top.

3.4 CONCLUSIONS

The sludge from the two ESGB reactors described previously in Chapter 2 (RI-granular inoculum and RII-suspended inoculum) was morphologically characterised using quantitative image analysis techniques that proved to be a powerful tool to monitor and quantify morphological changes in the anaerobic sludge. Deterioration of granular sludge was quantified along the trial period by the percentage of aggregates smaller than 1 mm either in terms of projected area or in terms of number of aggregates. A good correlation was obtained between these values and the % of aggregates smaller than 1 mm physically sorted and quantified by the VSS content. A morphological parameter relating the amount of free filaments and the projected area of aggregates (Lfa) was defined to quantify the dispersion level of the granular sludge, which increased until day 141 and remained almost invariable afterwards. The total free filament length was significantly higher in the suspended than in the granular sludge attaining a maximum value as high as 250 m/mg VSS. The equivalent diameter of the bottom aggregates larger than 1 mm (in terms of Feret diameter) increased with the increase of the amount of accumulated LCFA. After a threshold value of about 200 mg COD-CH_4/gVSS, the aggregates migrated to the top floating layer, evidencing the practical interest of this threshold value as far as the continuous operation of sludge bed reactors with LCFA based effluents is concerned. In the granular sludge, the amount of accumulated LCFA and their mineralisation rate to methane exponentially increased with the total free filament length, but exponentially decreased with the increase of the percentage of aggregates smaller than 1 mm. This evidenced the importance of filamentous bacteria on the process of LCFA accumulation/degradation and the relative irrelevance of the surface area for the phenomenon.

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4 MOLECULAR MONITORING OF MICROBIAL DIVERSITY IN ANAEROBIC SLUDGE LOADED WITH OLEIC ACID

Abstract

In this chapter, a molecular approach is used to evaluate the microbial diversity of bacteria and archaea in two EGSB reactors fed with increasing oleic acid loading rates up to 8 kg COD/(m^3.day) as the sole carbon source (Chapter 2). One of the reactors was inoculated with granular sludge (RI) and the other with suspended sludge (RII). During operation, the sludge in both reactors was segregated in two layers: a bottom settled one and a top floating one. For each applied oleate load, sludge samples from both layers where analysed. The composition of the bacterial community, based on 16S rDNA sequence diversity, was affected most during the oleate loading process in the two reactors. The archaeal consortium remained rather stable during operation in RI (granular sludge), whereas in RII (suspended sludge) the relative abundance of *Methanosaeta*-like organisms became gradually weaker, starting in the bottom layer. In the range of oleate loads evaluated, 6 kg of COD/(m^3.day) was found as the maximum value that should be applied to the system. A further increase to 8 kg of oleate-COD/(m^3.day) induced a maximal shift on the microbial structure of the sludge. This shift was concomitant with a sharp decrease of the sludge capacity to mineralise the biomass-associated LCFA to methane. At this time point, methanogenic aceticlastic activity was not detected and only very low methanogenic activity on H_2/CO_2 was exhibited by the sludge.

4.1 INTRODUCTION

LCFA result from lipids hydrolysis and are especially problematic for anaerobic wastewater treatment. Besides their direct toxicity to both methanogens and acetogens, the two main trophic groups involved in LCFA degradation, they can adsorb onto biomass particles causing biomass flotation and washout (Koster and Cramer, 1987; Rinzema *et al.*, 1994; Hwu, 1997). The study of these phenomena, as well as reactor operation and alternative configurations in the application of the anaerobic digestion technology to effluents with high lipid/LCFA content, have been the subject of considerable research (Rinzema, 1988; Sam-Soon *et al.*, 1991; Hwu, 1997; Alves *et al.*, 2001a; Alves *et al.*, 2001b; Pereira *et al.*, 2001; Pereira *et al.*, 2002). However, there is still a lack of knowledge regarding the microbiological aspects of the complex consortia involved in degradation.

Recent developments in molecular ecology have provided new molecular techniques that make it feasible to investigate complex microbial communities, overcoming the problems associated with the traditional cultivation-dependent methods (Amann *et al.*, 1995). Especially in anaerobic bioreactors, where stability and performance is strongly dependent on complex microbial interactions, this development can provide an opportunity to establish the connection between the microbial structure and the functional characteristics of the system. The use of 16S rDNA-based methods employing Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer and Smalla, 1998), molecular cloning and sequencing (Godon *et al.*, 1997; Wu *et al.*, 2001) and fluorescent *in situ* hybridisation (Raskin *et al.*, 1994 a; Harmsen *et al.,* 1996; Sekigushi *et al.*, 1999) can provide an accurate estimate of the microbial composition and diversity in a complex community. Furthermore, when combined with other techniques, chemical, biochemical and/or physiological assays, they can provide considerable information and improve our understanding about the role and dynamics of microorganisms (Raskin *et al.*, 1994 b; Verstraete *et al.*, 1996; Oude Elferink *et al.*, 1998).

In previous work, it was found that after feeding a reactor with oleate as the sole carbon source, the biomass became encapsulated by a whitish matter. When this biomass was washed to remove the residual (not biomass-associated) substrate, and incubated in batch vials, it was able to produce methane by degradation of the accumulated substrate

(Alves *et al*., 2001b; Pereira *et al.*, 2001). The maximum potential methane production due to the degradation of the accumulated (biomass-associated) LCFA exhibited by the sludge of two EGSB reactors fed with increasing loads of oleic acid was studied in batch assays (Chapter 2). The behaviour of granular and suspended sludge was compared since, although being more resistant to LCFA toxicity than the suspended or flocculent sludge, granular stability is critical for lipid/LCFAs containing wastewaters (Sam-Soon *et al.*, 1991; Hawkes *et al.*, 1995; Hwu, 1998; Chapter 3).

The aim of this work was to evaluate the microbial diversity of bacteria and archaea in the granular and suspended sludge collected during the continuous oleate load of those EGSB reactors, using a molecular approach.

4.2 MATERIALS AND METHODS

4.2.1 SLUDGE SOURCES

Sludge samples were obtained from the two 10 l EGSB reactors operated at mesophilic conditions (37ºC), previously described in Chapter 2 (p.55). One reactor (RI) was inoculated with granular sludge whereas another reactor (RII) was inoculated with suspended sludge. During the operation a significant amount of sludge accumulated as a floating top layer in both digesters. At the end of each period, samples from the bottom and top layers were collected from each reactor, washed and centrifuged (4000 rpm, 10 min) twice with the same anaerobic basal medium used in the methanogenic activity measurements (Chapter 2, p.57).

4.2.2 MICROBIAL COMMUNITY ANALYSIS

4.2.2.1 DNA EXTRACTION AND AMPLIFICATION

Total DNA was extracted from approximately 1ml of homogenised sludge sample as previously described by Harmsen *et al*. (1995). The 16S rRNA-genes were amplified by Polymerase Chain Reaction (PCR) using a *Taq* DNA polymerase kit (Life Technologies, Gaithersburg, Md.). Complete bacterial 16S rDNA was selectively amplified for cloning and sequencing using 7-f (5'-AGAGTTTGAT(C/T)(A/C)TGGCTCAG-3') and 1510-r (5'-ACGG(C/T)TACCTTGTTACGACTT-3') primers (Lane, 1991) with the following thermocycling program: 94ºC for 5 min; 25 cycles of 94ºC for 30 s, 52ºC for 20 s, and 68ºC for 40 s; and 68ºC for 7 min. The reactions were subsequently cooled to 4ºC. For DGGE a specific region of eubacteria 16S rDNA (V6-V8 region) was amplified using 968-GC-f (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC CTTAC-3') and 1401-r (5'-CGGTGTGTACAAGACCC-3') primers (Nübel et *al.*, 1996) with the same thermocycle program, but increased number of cycles to 35 and an annealing temperature of 56ºC. For archaea, primers A109-f (5'-AC(G/T)GCTCAGTAACAGTAACACGT-3') (Grosskopf *et al*., 1998) and 1510-r were used for complete 16S rDNA amplification and A109(T)-f (original Grosskopf *et al*. (1998)

, 3rd bp changed into T only, G.H.J. Heilig personal communication) and 515-GC-r (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGCTGCTGGCAC-3')
(Lane, 1991) for V2-V4 region amplification for DGGE use. Both reactions were performed with the following thermocycle program: 94ºC for 5 min; 24 (34 for DGGE use) cycles of 52ºC for 40 s, 68ºC for 1 min and 94ºC for 30 s; 52ºC for 40 s and 68ºC for 7 min. All primers were purchased from MWG-Biotech (Ebersberg, Germany). The size and amount of PCR products were estimated by 1% agarose gel (wt/vol) electrophoresis and ethidium bromide staining.

4.2.2.2 DGGE ANALYSIS

DGGE analysis of the amplicons was done as described by Zoetendal *et al.* (2001). Denaturant gradients from 35 to 50% for bacterial amplicon separation and from 30 to 45% for the archaeal ones, were used. DGGE gels were scanned at 400 dpi and the DGGE profiles compared using the Molecular Analyst 1.12 software package (BioRad, Hercules, CA, USA). Similarity indices of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient (Häne *et al*., 1993). Community shifts were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

4.2.2.3 CLONING AND SEQUENCING

Cloning and sequencing with the Sp6 primer (5'-GATTTAGGTGACACTATAG-3') (MWG-Biotech, Ebersberg, Germany), was done as described by Heilig *et al*. (2002). Similarity search of the partial 16S rDNA sequences derived from the sludge clones was performed using the NCBI sequence search service available in the internet (http.//www.ncbi.nlm.nih.gov/blast/).

4.2.2.4 CELL FIXATION AND FLUORESCENT IN SITU HYBRIDISATION.

After being washed and resuspended in phosphate buffered saline (PSB), sludge samples were fixed overnight according to Amann (1995). Fixed samples were sonicated for 5 min at 150 Watt, spotted to wells on gelatine-coated slides, dried for 20 min at 45ºC and dehydrated (Amann, 1995). Thereupon, *in situ* hybridisation was performed with the MX825-CY3 probe (5'-TCGCACCGTGGCCGACACCTAGC-3', target group: *Methanosaeta*; Raskin *et al.*, 1994 a) as detailed by Manz *et al*. (1992). For detection of all DNA, 4,6-diamidino-2-phenylindole (DAPI) was added to the wash buffer at a final concentration of 100 ng/ml. After rinsing the slides in water, they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA). Digital images of the slides, viewed with a Leica (Wetzlar, Germany) DMR HC epifluorescence microscope, were taken with a Leica DC 250 digital camera. These images were analysed with Leica QFluoro image analysis software at a Leica Q550 FW computer.

4.3 RESULTS AND DISCUSION

4.3.1 POPULATION DYNAMICS

DNA extractions from the granular samples (I) and suspended sludge samples (II), collected from the bottom (b) and the floating top layer (t) of both reactors (RI and RII, respectively) at the end of each operation period (1, 2, 3 and 4), were used as template for amplification of the V6-V8 bacterial regions and the V2-V4 archaeal regions. These amplicons were separated by DGGE and the obtained band patterns of each lane, which corresponded to each sample, were compared. Figure 4.1 and Figure 4.2 present the obtained results.

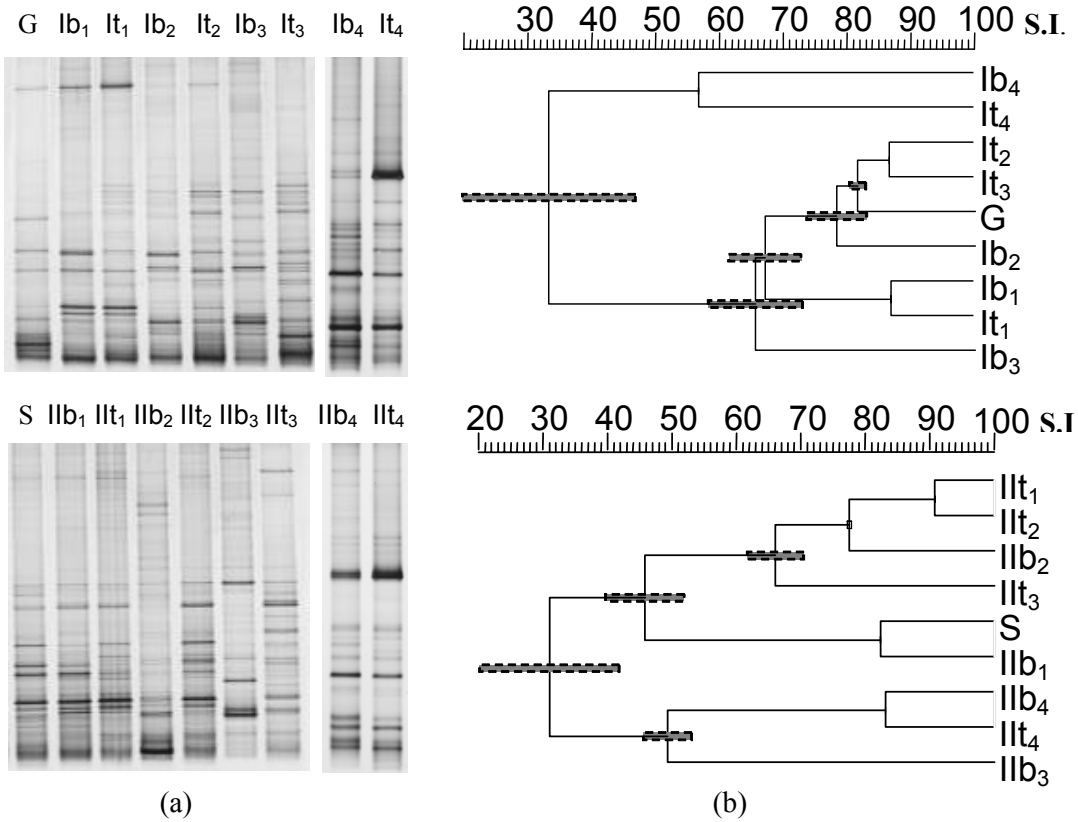
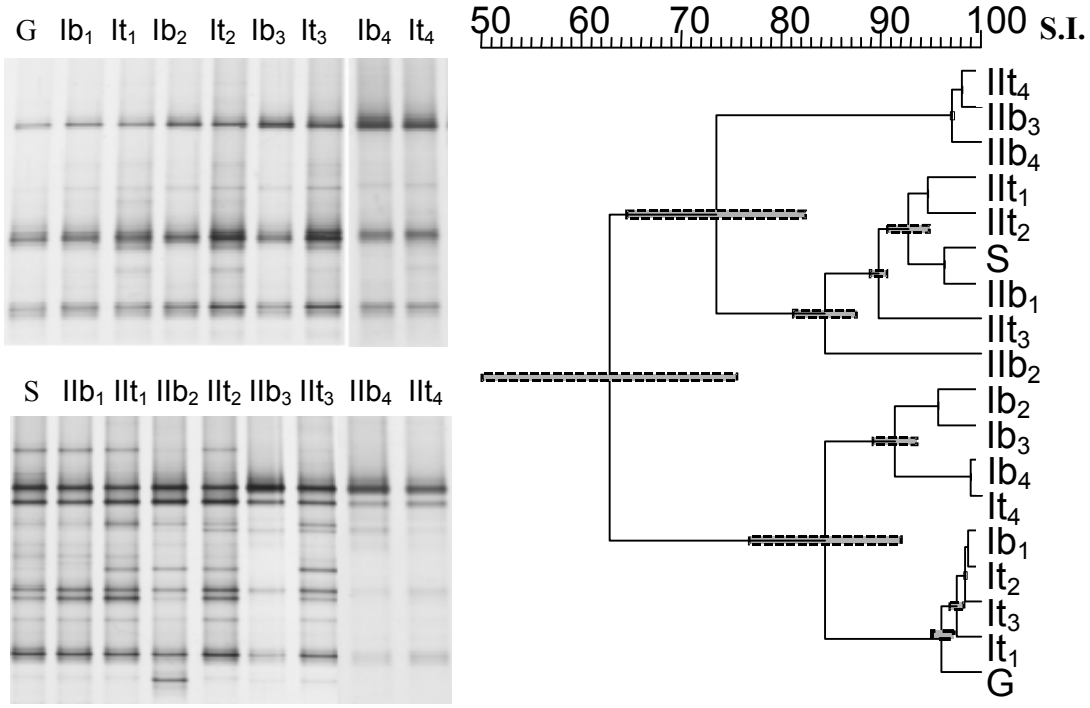


Figure 4.1 DGGE profiles of bacterial amplicons from sludge samples (a) and corresponding similarity index (S.I.) dendogram (UPGMA clustering) (b). I-granular sludge, II-suspended sludge, b-bottom layer, t-top layer, 1,2,3 and 4-operation periods, G-granular inoculum, S-suspended inoculum.

For the bacterial domain (Figure 4.1), comparison of DGGE band-patterns revealed a clear shift in the community structure with a decrease in the similarity indices between the bottom and top of RI-granular sludge from 86.8, in period 1, to 56.7 in period 4. At the end of the operation, the similarity index between bottom sludge and the inoculum was 42.8, and between top sludge and the inoculum was 17.3, suggesting that a higher shift in the community structure occurred in the top than in the bottom sludge. This shift was maximal when the oleate loading rate was increased from 6 to 8 kg COD/(m^3 day) (the similarity index between the top sludge at the end of period 3 and 4 was only 14.9). In RII-suspended sludge, the similarity index between bottom and top sludge attained a minimum value of 29.4 at the end of period 3 and increased to 83.1 at the end of the operation, indicating that at the end of period 3 the microbial structure from the top sludge was significantly different from the bottom sludge. This was not the case at the end of the operation, when both layers exhibited low similarity indices to the inoculum (28.7 between bottom and inoculum and 15.2 between top and inoculum). Furthermore, the suspended top sludge characterised at the end of period 3 revealed the highest methane production capacity from the accumulated (biomass-associated) LCFA (3271±877 mg COD-CH_4/gVSS) (Chapter 2, Table 2.4, p. 73), suggesting that the microbial structure of this sludge includes groups of microorganisms particularly important for the degradation of LCFA to methane. The lowest similarity index among the community patterns of the top layer sludge was found between period 3 and 4, i.e. 27.3. This maximal shift induced in the predominant bacterial composition may include the loss of microorganisms important for the degradation of LCFA to methane, and thus explain the sharp decrease of the capacity to methanise the biomass-associated LCFA, exhibited by this sludge at the end of period 4 (317±69 mg COD-CH_4/gVSS) (Chapter 2, Table 2.4, p. 73). In fact, in both reactors a significant increase of the effluent VSS content along the oleate load increase, and especially during the last operation period (Chapter 3, Figure 3.8, p.96), indicated a considerable washout of the dispersed fraction of biomass. The selective washout of acetogenic bacteria during the operation may also be related to the absence of methanogenic activity in the presence of acetate, propionate, butyrate and ethanol exhibited by both sludge at the end of operation (Chapter 2, p. 75).

In the archaeal consortium (Figure 4.2) no significant shift in RI-granular sludge community patterns was detected indicating that the dominant microbial composition

remained rather stable over operation, whereas in RII-suspended sludge the diversity decreased, starting in the bottom layer.



(a) (b)

Figure 4.2 DGGE profiles of archaeal amplicons from sludge samples (a) and corresponding similarity index (S.I.) dendogram (UPGMA clustering) (b). I-granular sludge, II-suspended sludge, b-bottom layer, t-top layer, 1,2,3 and 4-operation periods, G-granular inoculum, S-suspended inoculum.

The differences observed between the two sludges can be understood based on their different morphological and physiological structure. In fact, contrary to the weak and random microbial aggregation of suspended and flocculent sludge, granules form dense aggregates with a layered microbial organization, in which the internal core consists mostly of aceticlastic methanogens, surrounded by a second layer of acetogenic and hydrogenotrophic bacteria, with a peripheral layer comprising acidogenic, sulfate-reducing and hydrogenotrophic bacteria (MacLeod *et al.*, 1990; Guiot *et al.*, 1992; Fang *et al.*, 1994; Quarmby and Forster, 1995). This layered structure was observed in granules fed with

various substrates including brewery wastes, as was the case of the granular inoculum used in RI. Thus, being located in the inner core of the granules, methanogenic organisms (archaeal domain) can be more protected from the toxic effect and from the hydraulic shear stress, which can explain the insignificant shift in archaeal population observed in this sludge as opposed to the case of suspended sludge. The lower protection offered to the archaeal consortium in the case of the suspended sludge did not prevent the toxic effect, mainly on the community present at the feed inlet, i.e. the bottom layer.

Besides microbial organization, good settling properties are also pointed as an advantage of biomass aggregation (MacLeod *et al.*, 1990). However, for lipid/LCFA containing wastewaters, granule stability is very problematic and disintegration is often observed (Sam-Soon *et al.*, 1991; Hawkes *et al.*, 1995). In the thermodynamic respect, the disintegration of granules is predictable, when in contact with this compound, because at neutral pH, LCFA act as surfactants, lowering the surface tension, compromising the aggregation of hydrophobic bacteria, like most acetogens (LCFA-degraders) (Daffonchio *et al.*, 1995). Thus, the decrease in the similarity indices between the bacterial consortium (located in the outer layer) from bottom and top layers on RI-granular sludge with the increase in the toxicant fed to the reactors can be a result of disintegration. The granules suffering from disintegration by contact with oleate would become lighter and accumulated, together with smaller fragments from disintegration, in a floating top layer. The results obtained in Chapter 3, by applying image analysis to characterise the morphology of the granules during the operation of this reactor (RI), confirmed the occurrence of granular disintegration along the trial period.

4.3.2 DGGE BANDS IDENTIFICATION

To identify the prominent bands in the DGGE patterns of sludge samples from the last operation period, bacterial and archaeal 16S rRNA-genes from samples Ib_4, It_4, IIb_4 and IIt_4 were amplified, cloned and sequenced. The DGGE mobility of the amplicons obtained from the clones were compared to the DGGE profiles of the sludge samples in order to determine to which fragments they corresponded. Due to the considerable shift induced on the bacterial structure during the last operation period and, to retrieve more information, the

same procedure was applied to sample IIt_3. This sample, taken from the top of RII-suspended sludge at the end of period 3, when oleate was fed at 6 g COD/l, was chosen since it exhibited the highest methane production capacity from the adsorbed substrate (Chapter 2, Table 2.4, p. 73), and thus, would include groups of microorganisms particularly important for the degradation of LCFAs to methane. Table 4.1 summarises the sequencing results and Figure 4.3 illustrates their corresponding position in each DGGE profile.

Table 4.1 Sequencing results of the sludge clones.

Clone	GenBankAccession number	Sequence length (bp)	Closest relatives (% sequence similarity)
B1	AF455055	811	*Uncultured eubacterium* WCHB1-71 (94%), *Syntrophomonas sapovorans* (94%)
B2	AF455056	813	*Uncultured bacterium* clone C (97%), *Buchnera aphidicola* (91%)
B3	AF455057	800	*Uncultured bacterium* mle l-42 (97%), *Aminomonas paucivorans* (88%)
B4	AF455058	903	*Desulfovibrio mexicoense* (98%)
B5	AF455059	736	*Pseudomonas stutzeri* (96%)
B6	AF455060	904	*Gram-positive bacterium* MOL361 (87%), *Erysipelothrix rhusiopathiae* (88%)
B7	AF455061	657	*Unidentified eubacterium* cloneVadinBA43 (93%), *Spirochaeta africana* (88%)
B8	AF455062	906	*Syntrophomonas sapovorans* (97%)
B9	AF455063	902	*Uncultured bacterium* SJA-88 (90%), *Clostridium cellobioparum* (87%)
B10	AF455064	804	*Syntrophomonas sp.* MGB-C1 (97%)
B11	AF455065	812	*Trichlorobacter thiogenes* (97%)
A1	AF455066	441	*Methanobacterium formicicum* (97%)
A2	AF455067	815	*Methanosaeta concilli* (97%)
A3	AF455068	775	*Methanobacterium sp.* DSM 11106 (95%)
A4	AF455069	884	*Methanosaeta concilli* (98%)
A5	AF455070	827	*Uncultured archaeon* TA05 (98%), *Methanosaeta concilli* (98%)
A6	AF455071	852	*Methanobacterium formicicum* strain FCam (98%)
A7	AF455072	774	*Methanobacterium sp.* DSM 11106 (96%)

Sequencing and blast searching of the bacterial clones resulted mainly in matches with unknown and uncultured microorganisms assigned to the Gram-positive group (clones B1, B3, B6, B9), Proteobacteria (clone B2) and Spirochaetales (clone B7). Close relatives to *Desulfovibrio mexicoense* (clone B4) and *Trichlorobacter thiogenes* (clone B11) belonging to the delta subdivision of Protebacteria were also found, although corresponding to very diffuse fragments on the DGGE profiles (Figure 4.3 (a)). Clones B8 and B10 were closely related to the *Syntrophomonas* genus. The presence of saturated fatty acid-β-oxidizing syntrophic bacteria is desirable to maximize the consumption of butyrate and higher fatty acids resulting from LCFA conversion into acetate and hydrogen through β-oxidation mechanism by the proton reducing acetogenic bacteria (Weng and Jeris, 1976). *Syntrophomonas*-like organisms were found in sample IIt_3 but corresponded to very diffuse fragments in the DGGE profiles from the other samples (end of operation) (Figure 4.3 (a)), suggesting that the presence of these microorganisms became weaker by increasing the oleate concentration from 6 to 8 g COD/l. *Pseudomonas*-like organisms (clone B5) corresponded to strong DGGE-bands in the suspended sludge at the end of digesters operation, whereas in the granular sludge they were found only in the top layer.

For the archaeal domain, the clone sequences were affiliated with the two main groups, the aceticlastic *Methanosaeta* and the hydrogenotrophic *Methanobacterium.* In the last operation period, *Methanosaeta*-like organisms (clones A2, A4 and A5), known to be sensitive to LCFA (Hanaki *et al.*, 1981; Alves *et al.*, 2001(b)) were clearly present in the granular biomass, but corresponded to very faint bands in the suspended sludge profiles (Figure 4.3 (b)). Along the operation, these DGGE bands exhibited stable relative intensities in the RI-granular sludge profiles, whereas in RII-suspended sludge they gradually faded, indicating that the relative abundance of this group became weaker in this sludge during the operation but remained quite constant in the granular one (Figure 4.2 (a)). This fact can be related to the layered granular organization referred to before, where M*ethanosaeta-like* organisms, being located in the internal granule core, take advantage of a more protected environment against the toxic effect and hydraulic shear stress. This protective concept is also sustained by the higher toxicity limit (IC_{50}) of oleic acid towards aceticlastic bacteria exhibited by the granular inoculum when compared with the suspended sludge (Chapter 2, Table 2.1, p. 67).

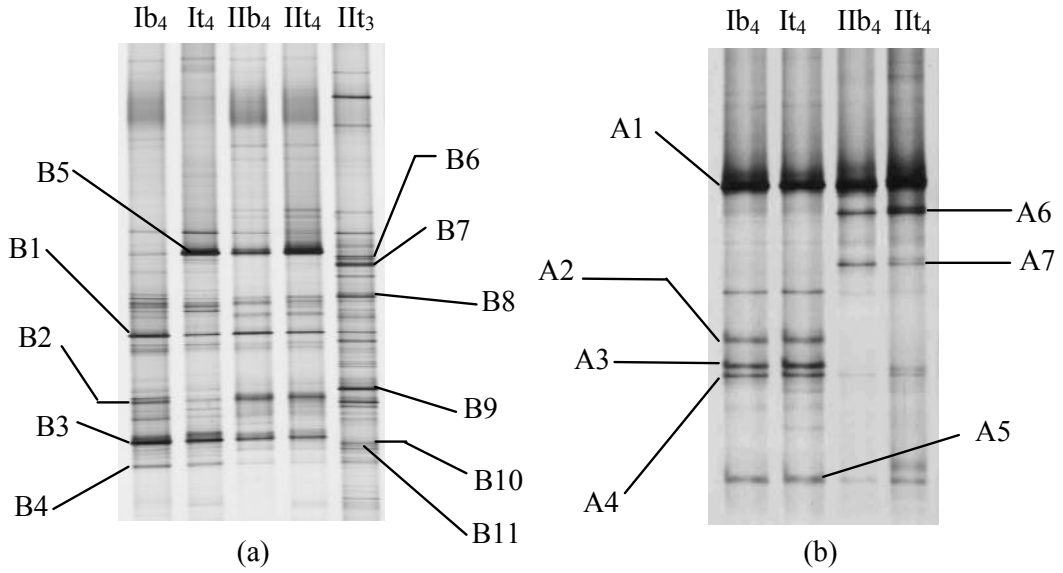


Figure 4.3 Correspondent position of each bacterial (a) and archaeal (b) sludge clone in the total DGGE profiles of the analysed samples. I-granular sludge, II-suspended sludge, b-bottom layer, t-top layer, 3,4-operation periods.

In a previous work, Zheng and Raskin (2000) used a genus specific probe to evaluate the levels of *Methanosaeta sp.* in a number of bioreactor samples and found that they were more abundant in granular sludge than in flocculent sludge. In this work, the *Methanosaeta* genus specific probe MX825 was also used to evaluate the levels of this group of microorganisms in both reactors at the end of operation (samples Ib_4 and IIb_4). The results obtained by fluorescent *in situ* hybridisation were consistent with previous data found by DGGE/sequencing. In the suspended sludge only a weak fluorescent signal was observed whereas in the granular sludge small chains of *Methanosaeta*-like organisms were still detected (Figure 4.4). However, in both sludges no aceticlastic activity was detected at the end of operation (Chapter 2, p. 75). Besides the acute toxicity of oleate to this group of microorganisms (Alves *et al.*, 2001b), substrate unavailability either due to diffusion limitations through the LCFA accumulation on the aggregates or due to the selective

washout of acetogenic bacteria (responsible for the acetate production) can be responsible for aceticlastic activity loss during the oleate load operation.

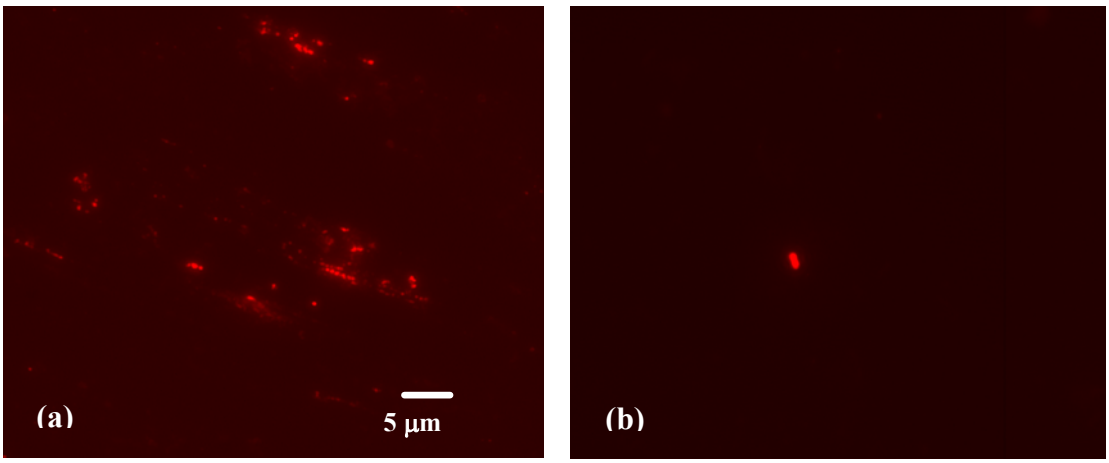


Figure 4.4 Epifluorescence photographs showing *in situ* hybridisation with probe MX825-CY3 of (a) granular and (b) suspended sludge present in the bottom layer of reactors, at the end of operation.

4.4 CONCLUSIONS

In this work, it is shown that the combination of molecular-based sludge characterisation with physiological (Chapter 2) and morphological (Chapter 3) characterisation can be a powerful approach to study and optimise the anaerobic digestion process for treating lipids/LCFA based effluents. Furthermore, it allowed a better understanding of the mechanisms of sludge disintegration, flotation and washout, in relation with the contact/accumulation of LCFA. Although in general, the methanogenic sludge population can be characterised relatively well, for the group of microorganisms involved in β-oxidation, the presence of many unidentified species in the sludge underlines the need for future research on classical isolation and characterisation studies. This need is of utmost importance as it was observed that the bacterial domain was more affected by the contact with increasing oleic acid concentrations. Furthermore, the increase in the organic loading rate to 8 kg oleate-COD/(m^3.day) induced a maximal shift on the microbial community structure of the sludges. Also at this time point, methanogenic aceticlastic activity was not detected and only a very low methanogenic activity on H_2/CO_2 was still exhibited by both sludge. These results, together with the higher methane production capacity from the biomass-associated LCFA exhibited by the sludges in the previous organic loading rate evaluated, i.e. 6 kg oleate-COD/(m^3.day) (Chapter 2), suggests that this should be the maximum organic loading rate applied to these reactors when treating oleic acid based effluents.

4.5 REFERENCES

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5 RETRIEVAL OF SLUDGE BIOCHEMICAL CHARACTERISTICS AFTER METHANISATION OF THE ACCUMULATED LCFA

Abstract

In this Chapter, the effect of the accumulated LCFA on the methanogenic activity, oleic or palmitic acid toxicity and biodegradation capacity is studied. The specific methanogenic activity in the presence of individual substrates (acetate, propionate, butyrate, ethanol and H_2/CO_2) measured before and after the depletion of the accumulated LCFA (mainly palmitic) strongly contradicted the accepted theory of bactericidal effect of LCFA towards anaerobic consortia as well as their permanent toxic effect. Although the encapsulated sludge had only detectable methanogenic activity in H_2/CO_2 and ethanol (24±6 and 437±12 mg COD-CH_4/(gVSS.day), respectively), after allowing the mineralisation of the accumulated LCFA, all the measured activities increased significantly, attaining values of 533±95, 16±4, 222±71, 67±1 and 2709±38 mg COD-CH_4/(gVSS.day), for acetate, propionate, butyrate, ethanol and H_2/CO_2, respectively. Aceticlastic tolerance to LCFA toxicity and capacity of LCFA biodegradation were also improved after allowing mineralisation of the biomass-associated LCFA. The results obtained put in evidence the possible strong effect of diffusion limitations imposed by the LCFA layer that could hamper the access of the added substrates to the cells as well as the subsequent biogas release. This physical effect could easily mislead the data obtained in previous works that suggest severe conclusions about LCFA inhibitory effects. On the other hand, these results support the practical interest of using sequential adsorption (accumulation) and degradation (methanisation of the accumulated LCFA) steps for the treatment of lipid/LCFA based effluents, as suggested in Chapter 2, because besides the sludge ability to mineralise large amounts of biomass-associated LCFA, its activity is enhanced after the degradation step.

5.1 INTRODUCTION

Adsorption of LCFA onto anaerobic sludge has been often reported in the literature (Hanaki *et al.*, 1981; Rinzema *et al.*, 1994; Hwu, 1997). Besides problems of sludge flotation that hinder reactor performance, this phenomenon was considered to be the main factor of toxicity due to physical interactions between the LCFA and the cell wall, affecting its transport and protective functions (Demeyer and Henderickx, 1967; Galbraith and Miller, 1973). According to Galbraith *et al.* (1971), this is particularly acute for gram positive bacteria since gram negative microorganisms are, in part, protected by the lipopolysaccharide layer in their cell wall. As acetogens involved in β-oxidation are gram positive and methanogens resemble gram positive bacteria it is, in fact, expected that LCFA may be potential inhibitors of the metabolic pathways involved in its anaerobic degradation (Koster and Cramer, 1987). According to Rinzema *et al.* (1994), LCFA exert a bactericidal effect on methanogenic bacteria and no adaptation occurs. The recovery after a lag phase usually observed in batch assays is attributed to the growth of few survivors. These authors reported also that aceticlastic bacteria do not adapt to LCFA neither upon repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic concentrations. Angelidaki and Ahring (1992) reported oleate and stearate inhibition of all steps of the anaerobic digestion of cattle manure and concluded that the toxic effect of both LCFA was permanent and irreversible. Furthermore, these authors suggested that the response to the addition of neutral lipids may depend upon the degree of adaptation to lipids, whereas the addition of free LCFA above a certain concentration may directly result in process failure.

Although in these previous works the obtained results clearly suggest the above-mentioned conclusions about inhibition and toxicity of LCFA, the results obtained in Chapter 2 evidenced that mineralisation of the accumulated (biomass-associated) LCFA can be efficient in batch assays, provided no external substrate is present in the medium. Sludge taken from an EGSB reactor loaded with oleate up to 6 Kg COD/ (m^3.day), as the sole carbon source, was able to produce 3270±880 mg COD-CH_4/gVSS exclusively from the accumulated (biomass-associated) LCFA at a specific rate of 240±8 mg COD-CH_4/(gVSS.day), evidencing metabolic activity of the consortium in these conditions. Palmitic acid, described as the second saturated LCFA resulting from oleic acid β-oxidation

(Weng and Jeris, 1976), was found to be the main LCFA accumulated onto the sludge and its further degradation to methane was inhibited when in the presence of oleic acid. The obtained results suggested the potentiality of using a sequential Fed-batch operation when treating effluents with high lipid/LCFA content. However, the effects of the transitory accumulation of LCFA on the biochemical properties of anaerobic consortia, namely, on the methanogenic activity, the tolerance to oleic acid toxicity and on its capacity of LCFA biodegradation, are not yet clear.

The main purpose of the experiments described in this chapter was to investigate the possible retrieval of sludge biochemical characteristics, namely, the methanogenic activity, the tolerance to oleic acid toxicity and the capacity of oleic acid biodegradation, after allowing the methanisation of the biomass-associated LCFA. For that, a continuous reactor was fed with oleic acid, in order to obtain anaerobic sludge loaded with LCFA, as previously performed in Chapter 2. Since palmitic acid was found to be the most important intermediate that accumulated onto the sludge in those conditions, a parallel reactor was run feeding palmitic acid as the sole carbon source. Besides the interest of comparing reactor performance in terms of methane yield, it was also a purpose of this study to assess the possible accumulation of shorter LCFA and to compare the behaviour of each sludge when in the presence of the corresponding fed LCFA.

5.2 MATERIALS AND METHODS

5.2.1 EXPERIMENTAL SET-UP AND OPERATION MODE

Suspended sludge, previously acclimated to oleic acid, was continuously loaded in two 1 l EGSB reactors (RO and RP) at a constant influent concentration of 4 g COD/l (HRT≅1 day), under mesophilic conditions (37±1 ºC). RO was feed with oleic acid and RP with palmitic acid. Both reactors were equipped with an external settler (vol= 200 ml) from which biomass was recycled at a rate of 4 l/day.

SEED SLUDGE

The seed sludge was obtained from a local municipal sludge digester (Braga ETAR, Portugal) and was allowed to acclimate to oleate in the presence of skim milk as co-substrate (50% COD). The acclimation procedure was carried out in a 4 l Fed-batch reactor (at 37±1 ºC), containing 18.26 g/l VSS of seed sludge, during 103 days. The substrate was supplied to the reactor every time no more biogas production was detected and was made-up in liquid collected from the reactor (to maintain a constant reactor volume). Substrate (oleate+skim milk) concentrations of 1 g COD/l and 2 g COD/l were applied to the reactor during the first 50 days and the following acclimation time, respectively. A supplement of macro (0.6 ml/g COD) and micronutrients (1 ml/l) (composition described in sub-chapter 2.2.1, p.55) was added, as well as 5 g $NaHCO_3$/l (to give suitable alkalinity). During the acclimation process, the reactor average pH value, measured before each new feed supply, was of 7.6±0.2.

After washed and centrifuged (4000 rpm, 10 min) twice with anaerobic basal medium (composition described in sub-chapter 2.2.3.1, p.57) to remove the residual substrate, 500 ml of acclimated suspended sludge (7.75 g VSS/l) were added to RO and to RP.

SUBSTRATE

During the first 28 days (start-up period) the substrate consisted of skim milk (50% COD) and the corresponding individual LCFA (50% COD) diluted with tap water and supplemented with macro and micronutrients (composition described in sub-chapter 2.2.1,

p.55). 0.6 ml of macro solution was added per gram of COD fed and 1 ml of micro solution per litre of influent feed. To give suitable alkalinity, 5 g $NaHCO_3$/l was added to the feed. From day 28 on, the carbon source was exclusively composed by sodium oleate in RO and palmitic acid in RP. During this time, a nitrogen supplement was added to the macronutrients solution in order to maintain a COD/N/P ratio of 200:5:1.1.

Due to the low solubility of palmitic acid in aqueous media (7.2 mg/l in water at 20ºC, Lide, D.R. (1991-92)) and the unavailability of its salt form, this feed stock was homogenised in a Euroturrax T_{20} (IKA Labortechnik, Germany) to form a fine emulsion. The feed stock was continuously stirred to promote a homogeneous emulsion and the monitoring of the influent COD revealed that the desired value was assured. The influent pH was kept near 8.

5.2.2 ROUTINE ANALYSIS

Routine reactor performance was monitored by determining influent and effluent total and soluble (centrifuged 10 min at 15 000 rpm) COD, influent flow rate, methane production, effluent VFA (membrane-filtered, 0.45 μm) and VSS.

COD and VSS were determined according to standard methods (APHA *et al*., 1989). COD removal efficiency was calculated according to equation 2.1 (p.57).

Biogas production was measured by using an impulse gas meter similar to that previously described by Veiga *et al*. (1990). Methane content of the biogas was determined by gas chromatography as previously described in sub-chapter 2.2.2 (p.56).

VFA (acetic, propionic and butyric) were determined by high-performance liquid chromatography using a Chrompack column (300×6.5 mm) and a mobile phase of 5 mM sulphuric acid (H_2SO_4) at 0.7 ml/min. The column was set at 40°C and the detection was spectrophotometric at 220 nm.

5.2.3 SLUDGE CHARACTERISATION

5.2.3.1 METHANOGENIC ACTIVITY MEASUREMENTS

Methanogenic activity against acetate, propionate, butyrate, ethanol and H_2/CO_2 was determined using the procedure previously described in the sub-chapter 2.2.3.1, p.57.

5.2.3.2 TOXICITY OF OLEIC OR PALMITIC ACID TOWARDS ACETICLASTIC METHANOGENS

These tests were performed as previously described in sub-chapter 2.2.3.2, p.62. Due to the lower toxicity of palmitic acid towards the trophic group under study, concentrations up to 2500 mg/l were tested.

5.2.3.3 LCFA BIODEGRADATION MEASUREMENTS

The capacity of the microbial consortium to biodegrade oleic or palmitic acid was determined as previously described in sub-chapter 2.2.3.3, p.63.

5.2.3.4 MICROSCOPIC OBSERVATIONS

Optic and scanning electron microscopy (including Energy Dispersion Spectroscopy) was performed as previously described in sub-chapter 2.2.3.4, p.63.

5.2.4 EXTRACTION AND GC ANALYSIS OF LCFA ACCUMULATED ONTO THE SLUDGE

The LCFA content accumulated onto the sludge was determined as previously described in sub-chapter 2.2.4, p.65.

5.2.5 EFFECT OF ALLOWING THE DEPLETION OF THE BIOMASS-ASSOCIATED LCFA ON SLUDGE BIOCHEMICAL CHARACTERISTICS

Specific methanogenic activity of the sludge before and after degrading the biomass-associated LCFA accumulated during the continuous load was compared using parallel assays. After two consecutive washings and centrifugations (4000 rpm, 10 min) with anaerobic basal medium (composition described in the sub-chapter 2.2.3.1 (p.57)), the sludge was incubated in 20 vials of 25 ml and 8 vials of 70 ml at 37 °C, 150 rpm under strict anaerobic conditions. Two sets of 14 vials were separated for parallel assays. In the first set, the methanogenic activities against acetate, propionate, butyrate, ethanol and H_2/CO_2 were determined in duplicate as mentioned above. In the other set of 14 vials, no substrate was added but the degradation of the biomass-associated LCFA was followed until stabilisation. After this stabilisation, the vials were depressurised, vented with N_2/CO_2 (80:20 vol/vol) and the methanogenic activity against acetate, propionate, butyrate, ethanol and H_2/CO_2 was measured in duplicate, as described for the first set of vials.

The same approach of setting parallel assays was used to characterise the sludge, before and after degrading the biomass-associated LCFA, in terms of oleic or palmitic acid toxicity towards the aceticlastic bacteria and the corresponding added LCFA biodegradation capacity.

It should be noticed that in these assays, the values of VSS, which account for all the organic matter, represent both biomass and biomass-associated LFA. Therefore, in the vials where complete degradation of the biomass-associated LCFA was not achieved, VSS values obtained at the end of the experiment were corrected with the average value obtained in the "blank" vials (controls for liquid substrates), where no substrate was added and the methanisation of biomass-associated LCFA was followed until no more biogas was produced.

5.3 RESULTS AND DISCUSION

5.3.1 INOCULA CHARACTERISATION

Prior to inoculation, the acclimated seed sludge (inocula) was characterised in terms of specific methanogenic activity, oleic and palmitic acid toxicity towards the aceticlastic trophic group, as well as in terms of biodegradation capacity of these two LCFA by the microbial consortium. Table 5.1 summarises the obtained results for the methanogenic activity.

Table 5.1 Specific methanogenic activity exhibited by the inocula (Mean±SD).

Initial methane production rate in the presence of:	(mg COD-CH_4/(gVSS.day))
Acetate	146±15
Propionate	(n.d.)
Butyrate	80±18
Ethanol	72±4
H_2/CO_2	581±33

n.d.- non-detectable.

As shown in Table 5.1, the sludge exhibited no detectable activity against propionate and relatively low specific methanogenic activity values against the other substrates.

Figure 5.1 (a) and (b) present the long-term course of specific methane production during the assessment of oleic and palmitic acid toxicity towards the aceticlastic bacteria. LCFA toxicity to anaerobic sludge is expressed as IC_{50} values, defined as the LCFA concentration that causes a 50% relative methanogenic aceticlastic activity loss. In terms of toxicity limit to the LCFA under study, a higher IC_{50} value (1100±50 mg/l) was found for palmitic than for oleic acid (70±10 mg/l) revealing a higher tolerance of the aceticlastic methanogens to this LCFA than to oleic acid. This result is consistent with the lower inhibitory effect of saturated than unsaturated LCFA to anaerobic microorganisms suggested in the literature (Demeyer and Henderickx, 1966; Galbraith *et al*, 1971; Roy *et al*., 1985; Komatsu *et al*., 1991; Angelidaki and Ahring, 1992; Beccari *et al.*, 1998). In this

study, a concentration of palmitate sixteen-fold higher than oleate was required to cause a 50% relative methanogenic aceticlastic activity loss.

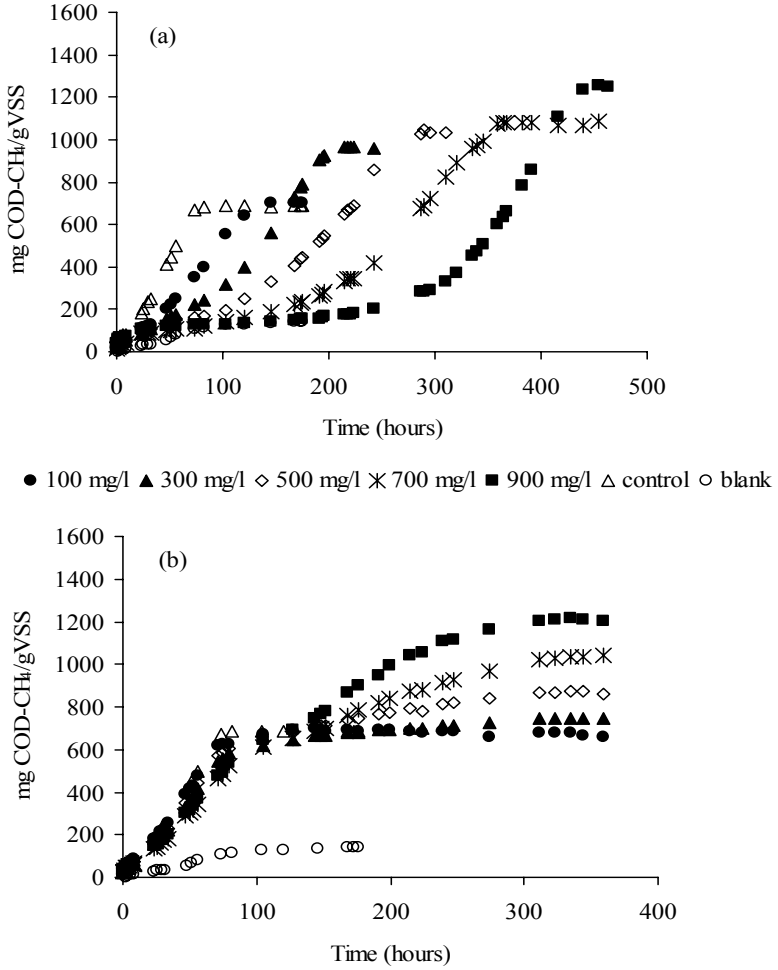

Figure 5.1 Long-term course of specific methane production during the toxicity batch experiment with (a) oleate and (b) palmitate. Control - only acetate added.

The different effect of the increasing oleic or palmitic acid concentrations on acetate methanisation is clearly evidenced in the specific methane production curves obtained during the toxicity batch experiments. The sludge showed a lag phase of increasing length prior to methane production for oleate concentrations exceeding 300 mg/l (Figure 5.1 (a)). After the lag phase, acetate and oleate undergo a virtual simultaneous degradation to methane. When increasing concentrations of palmitate were added (Figure 5.1 (b)), the

methanisation of acetate and palmitate followed a diauxic behaviour and no lag phases were observed in the range of concentrations under study. Furthermore, a constant methane production corresponded to the first plateau, which can be correlated with the constant initial added acetate concentration. The same methane production was also achieved in the control curves (only acetate). For higher palmitate concentration, i.e. 2000 and 2500 mg/l, acetate and palmitate were virtually degraded simultaneously after lag phases of 0 and 600 hours (data not shown).

Figure 5.2 represents the methane production curves obtained during the biodegradation batch experiments of oleic and palmitic acids and Table 5.2 summarises the obtained results.

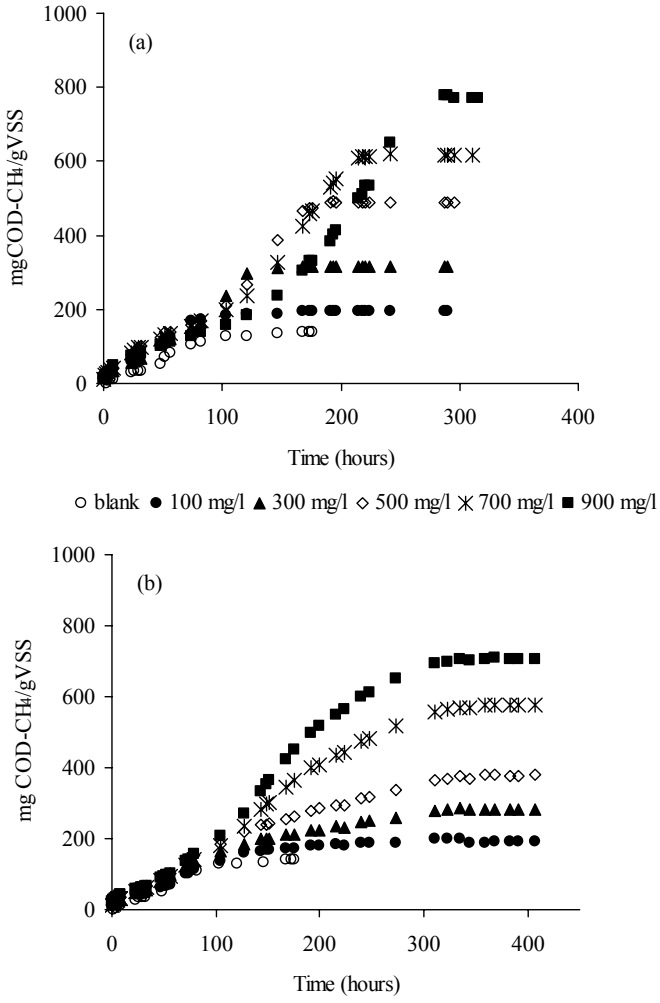

Figure 5.2 Specific methane production during the (a) oleic and (b) palmitic acid biodegradation batch experiment.

The sludge exhibited slightly higher biodegradation rates for oleic than for palmitic acid, but during oleic acid biodegradation, significantly higher lag phases preceding the maximum methane production were detected.

The comparison between Figure 5.2 (b) and Figure 5.1 (b) also support the diauxic behaviour found in the methanisation of acetate + palmitate observed in the toxicity batch experiments, as palmitate biodegradation curves showed the same behaviour as the second branch (after 100 hours) of the toxicity curves.

Table 5.2 Results from the oleic and palmitic acid biodegradation batch experiments (Mean±SD).

Maximum specific methane production rate (mg COD-CH_4/(gVSS·day))		Lag phase (hours)
Oleate concentrations:		
100 mg/l	17±7	0
300 mg/l	25±1	0
500 mg/l	56±5	70±5
700 mg/l	62±2	90±2
900 mg/l	62±8	110±10
Palmitate concentrations:		
100 mg/l	3±2	0
300 mg/l	15±8	0
500 mg/l	12±7	0
700 mg/l	23±4	50±2
900 mg/l	40±4	60±3

5.3.2 CONTINUOUS LOAD WITH OLEATE/PALMITATE

After a start-up of 28 day with skim milk as co-substrate (50% COD) and progressive decrease of the HRT, the acclimated seed sludge was continuously loaded with oleate (in RO) or palmitate (in RP) at a constant concentration of 4 g COD/l. Table 5.3 summarises the average operation conditions and performance for RO and RP. Acetate was the only VFA detected in the effluent of both reactors representing 39 and 29% of the total soluble effluent COD, in RO and RP, respectively.

RP exhibited a better performance in terms of removal efficiency and VFA and VSS effluent levels. However, in RO a methane production 25% higher than that achieved in RP was obtained. Nevertheless, in both reactors a considerable low methanisation of the fed LCFA was achieved, with methane yields as low as 33 and 29 l CH_4/kg $COD_{removed}$, in RO and RP respectively, revealing an important accumulation of non-methanised substrate, more pronounced in RP.

Table 5.3 Operating conditions and performance of RO and RP (Mean±SD).

Time (days)	**HRT (±0.01)** (days)	**Influent COD** (g/l)	**COD Removal efficiency** (%)	**Effluent VFA-COD** (g/l)	**Effluent VSS** (g/l)	**CH_4** (%)	**Biogas** (l/(l.day))
RO							
0-28	2.7-1.18	3.9 (±0.3)	65.4-93.1	(*)	(*)	48.4-69.4	(*)-0.28
28-75	1.18	3.9 (±0.3)	80.3 [a] (±5.4)	0.32 [a] (±0.09)	0.88 [a] (±0.24)	54.9 [a] (±8.3)	0.16 [a] (±0.05)
RP							
0-28	2.7-1.14	3.7 (±0.3)	61.6-90.1	(*)	(*)	47.2-62.4	(*)-0.25
28-75	1.14	3.7 (±0.3)	92.8 [a] (±2.5)	0.08 [a] (±0.03)	0.76 [a] (±0.46)	51.1 [a] (±6.4)	0.13 [a] (±0.07)

(*) not determined.
[a] pseudo steady-state values.

With the exception of a short feeding period with skim milk as co-substrate, RO was operated at similar conditions to those applied to RII during period II, in the previous study reported in Chapter 2 (Table 2.2, p.68). Although in terms of COD removal efficiency and effluent VSS, no major differences were found between RO and RII, concerning methane production a large divergence was observed. Indeed, in RO a methane production rate as low as 0.09 l/(l.day) was obtained which corresponded to only 38% of that previously achieved in RII. The inoculum used in RII exhibited better characteristics, namely a significantly higher specific methanogenic activity against acetate and a higher tolerance to oleic acid toxicity of the aceticlastic trophic group (Table 2.1, p.67). As this trophic group is responsible for about 70% of the total methane production (Jeris and MacCarty, 1965), this

may justify the higher methanisation achieved in this reactor. The lower methanisation attained in RO indicates that more substrate was retained in this reactor by mechanisms of adsorption, precipitation or entrapment, than in RII. It is therefore expected a considerably higher amount of biomass-associated LCFA in this sludge than that previously accumulated in the RII-top sludge on day 119 (583±117 mg COD-CH_4/gVSS, Table 2.4, p.73).

At the end of the continuous load (day 75), the sludge loaded with oleate (from RO), exhibited the typical whitish greasy aspect (Figure 5.3 (a)) previously observed in Chapter 2 (p.70) and referred to in the literature as related to LCFA adsorption onto the sludge (Rinzema, 1988; Sam-Soon *et al*., 1991; Hwu, 1997; Pereira *et al.*, 2001). However, the sludge collected from RP, loaded with palmitate, kept its original darker aspect in which whitish spots were eye perceptible (Figure 5.3 (b)), suggesting the occurrence of precipitation of this LCFA or any intermediate of its degradation mainly in individualized aggregates rather than entrapping the sludge. Microscopic examination of this sludge confirmed the presence of several crystal-like forms of variable size amid the sludge (Figure 5.3 (c), (d) and (e)), in which traces of calcium were detected (Figure 5.3 (f)). Precipitation with divalent cations, such as Ca^{2+} and Mg^{2+}, may thus justify LCFA accumulation in this sludge, in the form of precipitates. However, it is unlikely that this phenomenon accounted for all COD accumulated in RP, since even if all the existing calcium and magnesium ions had stoichiometrically precipitated the fed palmitate, the maximum fraction removed by precipitation would correspond to 62 % of the total COD accumulated in this reactor (Table 5.4).

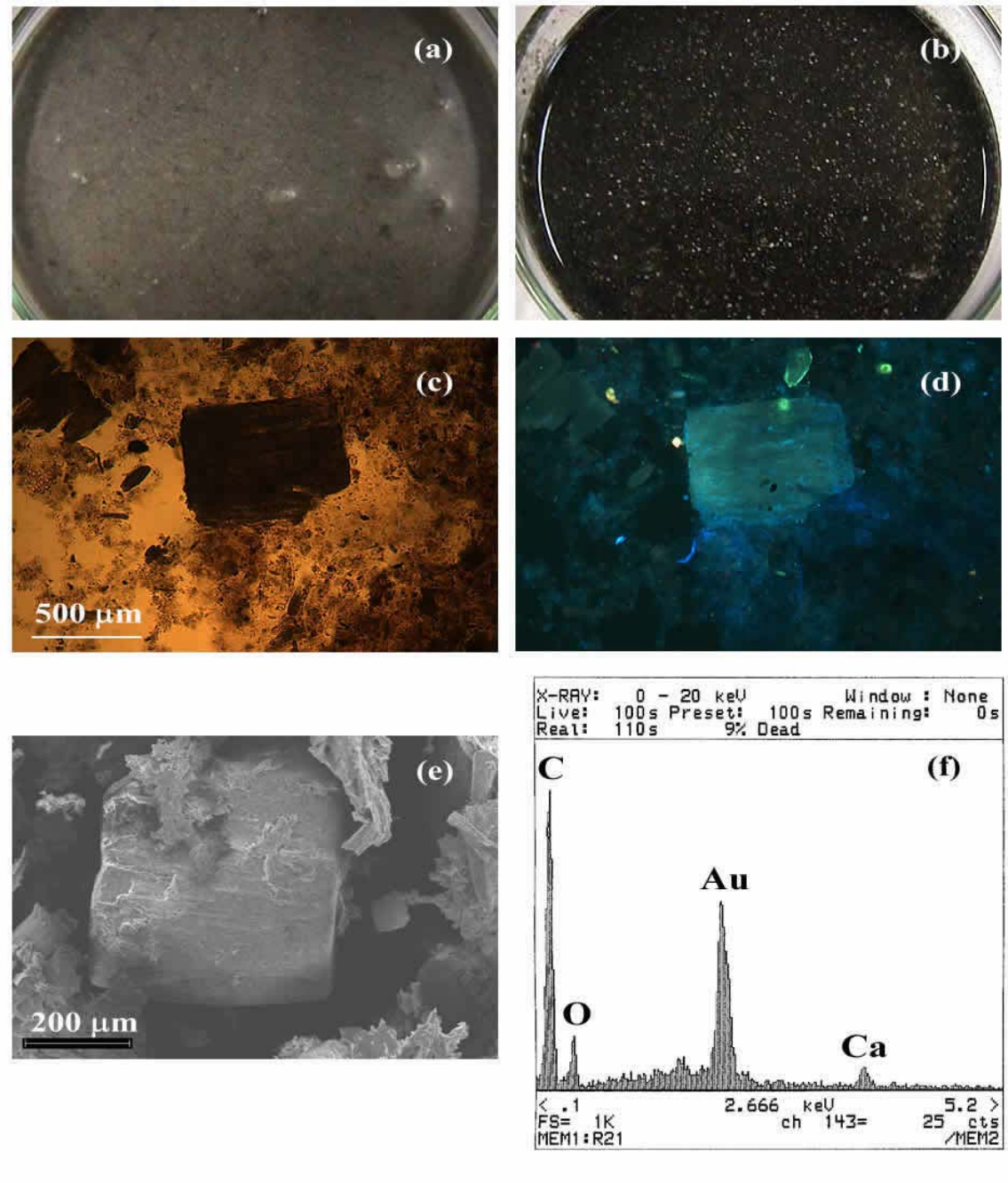

Figure 5.3 Sludge aspect after the continuous load with (a) oleate and (b) palmitate. Optic microphotographs of the sludge loaded with palmitate, (c) showing the crystal-like forms and (d) under fluorescence at 420 nm. (e) Scanning electron microphotograph of the crystal-like matter and (f) corresponding elemental composition spectrum, obtained by EDS analysis.

Table 5.4 Molar ratio of each LCFA to calcium and magnesium ions during the trial period (days 28 to 75).

	Molar ratio LCFA/(Ca^{2+}+Mg^{2+})	**[(a)]LCFA-COD removed by precipitation** (g/(l.day))	**Total COD accumulated** (g/(l.day))
RO - oleate	4.10	1.93	2.40
RP - palmitate	4.47	1.76	2.82

[(a)] Theoretical values.

The results obtained after extraction and GC analysis of the accumulated matter present in both sludges (from RO and RP) are presented on Table 5.5. Figure 5.4 shows the typical GC chromatograms obtained. As expected, based on the result previously reported in Chapter 2, palmitic acid was the main LCFA accumulated onto the sludge fed with oleic acid (from RO). In the sludge fed with palmitic acid (from RP) the main LCFA accumulated was also palmitic acid. No other intermediates longer than capric (C10:0) could be detected, or if any, only in trifling concentrations (Figure 5.4 (b)).

Table 5.5 LCFA content of the accumulated matter present in the sludge from RO and RP, at the end of the continuous run (Mean±SD).

	mg/mg dry weight	
LCFA	**RO**	**RP**
Lauric acid (C12:0)	n.d.	n.d.
Myristic acid (C14:0)	0.015±0.00	n.d.
Palmitic acid (C16:0)	0.265±0.01	0.286±0.21 [(a)]
Stearic acid (C18:0)	0.019±0.00	n.d.
Oleic acid (C18:1)	0.019±0.00	n.d.

n.d.- non-detectable.
[(a)] The high SD value is due to the highly heterogeneous distribution of the LCFA among the sludge.

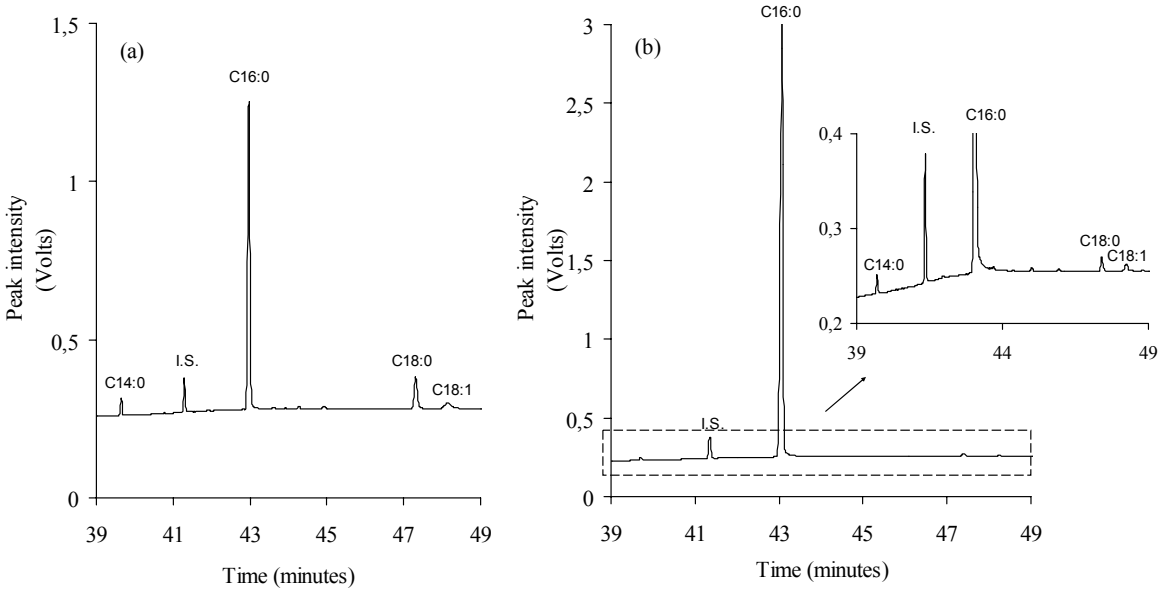

Figure 5.4 GC chromatograms showing LCFA peaks detected in: (a) sludge from RO and (b) sludge from RP. I.S. - internal standard (pentadecanoic acid, C15).

The high accumulation of palmitic acid in RP, was apparently not by adsorption or entrapment and was not completely justified by precipitation with divalent ions. Due to commercial unavailability, the palmitic acid fed to RP was in the acid form. The pH was verified to fit neutrality requirements, but a strong insolubility of this LCFA was evident when preparing the feeding. To overcome problems of solid sedimentation in the feed tank, a continuous stirring was promoted, in order to guarantee the desired influent COD. Hence, besides a possible biochemical inhibition of palmitate degradation, bioavailability limitations as a cause for substrate accumulation should not be excluded in this study. In the literature, the addition of surfactants, such as sodium dodecylbenzenesulfonate (Petruy and Lettinga, 1997), or diethyl ether (Lalmam and Bagley, 2000), are pointed out as efficient approaches to solve LCFA solubility problems in biodegradability and toxicity studies. However, the high toxicity to methanogenic bacteria of the emulsifiers/solvents is also reported by the same authors. Even at a sub-toxic concentration of 9.5 mg/l, sodium dodecylbenzenesulfonate used by Petruy and Lettinga (1997) was able to cause a 38% inhibition of methanogenic activity. In the work of Lalmam and Bagley (2000 and 2001), in the presence of a diethyl ether concentration of 14.2 g/l, the maximum acetic acid

degradation rate was severely retarded (from 36 μg/(gVSS.day) to 5.5 μg/(gVSS.day)), and complete consumption was accomplished within approximately 14 days, whereas in the absence of diethyl ether acetate was consumed after 2 days. However, although reported as non-biodegradable by the anaerobic sludge, it is likely that the inhibitory effect of these compounds on methanogenic activity can introduce a synergetic effect on LCFA biodegradation and inhibition studies, leading to a mismatch in the data interpretation. On the other hand, from a practical viewpoint, the addition of extra "pollutants" (non-biodegradable by the anaerobic sludge) to promote the solubility of LCFA, seems to be an unreasonable approach for the treatment purpose. Therefore, in this work it was considered preferable to avoid the use of such compounds.

The biomass with palmitate accumulated mainly as individualised "aggregates" and not entrapping the sludge may represent a source for further investigation. Besides the potential toxic effect, it is reasonable to expect that the accumulation of LCFA onto the biomass can create a physical barrier and hinder the transfer of substrates and products (e.g. biogas release) through the LCFA layer, inducing a delay on initial methane production as well as a reduction of the methane production rate. Comparing both of these sludges, where palmitate accumulated in different ways of association with the biomass, may give some insights on the relative importance of diffusion limitations caused by the biomass-associated LCFA on the activity measurements.

5.3.3 METHANOGENIC ACTIVITY BEFORE AND AFTER DEGRADING THE BIOMASS-ASSOCIATED LCFA

After the continuous oleate load (day 75), the sludge collected from RO was characterised in terms of specific methanogenic activity for acetate, propionate, butyrate, ethanol and H_2/CO_2, before and after the degradation of the biomass-associated LCFA. This sludge contained a high LCFA content (mainly palmitic acid) entrapping the biomass and will, therefore, be entitled as "encapsulated sludge" in the course of this study. Figure 5.5 presents the specific methane production curves obtained during the batch activity experiments and Table 5.6 summarises the corresponding specific methane production rates.

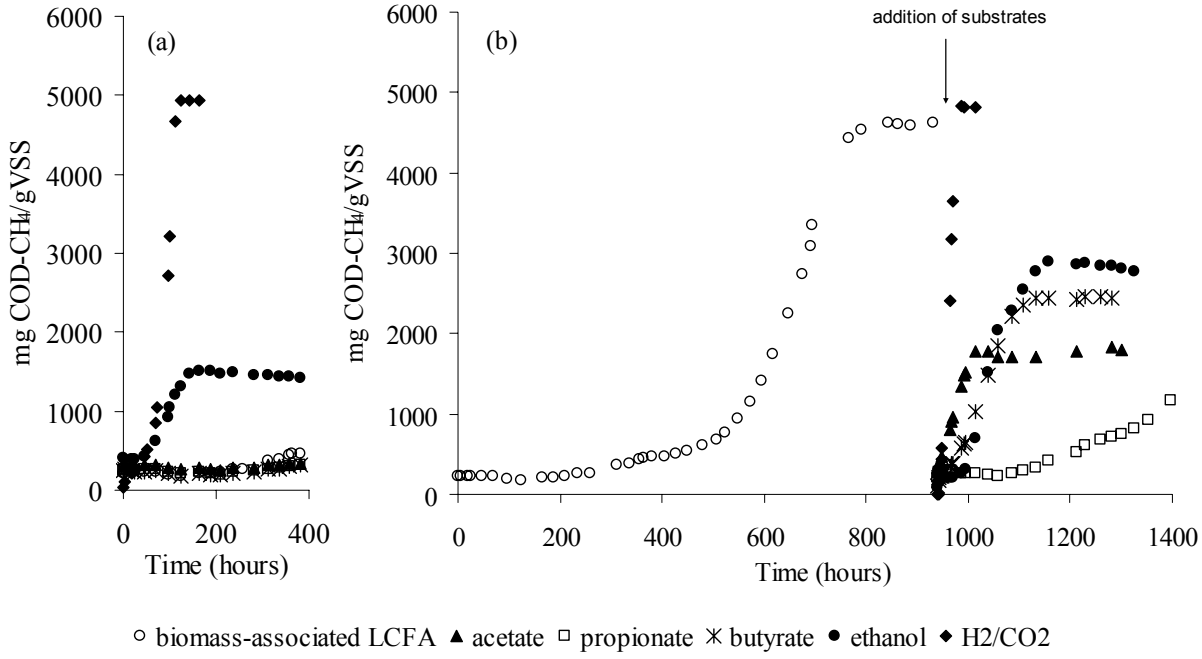

Figure 5.5 Specific methane production during the activity batch experiments exhibited by the encapsulated sludge (from RO), before (a) and after (b) degrading the biomass-associated LCFA.

In the experiment before degrading the accumulated LCFA, the encapsulated sludge exhibited no initial methanogenic activity with acetate, propionate or butyrate as substrates. The initial methane production rate was lower than the one measured in the blank controls (no added substrate), that corresponded to the degradation of the biomass-associated LCFA. Only the initial activity against H_2/CO_2 and ethanol presented measurable values of 434±12 and 24±6 mg COD-CH_4/(gVSS.day), respectively. After allowing the depletion of the biomass-associated LCFA, the initial methane production rate exhibited by the same sludge, from all the added substrates, was significantly increased (Figure 5.5 (b)), Table 5.6). These results clearly show that, after degrading the biomass-associated LCFA, the methanogenic activity was enhanced, contradicting the well reported and accepted theories of the bactericidal and permanent toxic effect of LCFA towards anaerobic bacteria reported by Rinzema *et al*. (1994) and by Angelidaki and Ahring (1992), respectively. On the other hand, these results support the practical interest of using sequential accumulation and degradation (methanisation of the accumulated LCFA) steps for the treatment of

lipid/LCFA based effluents, as suggested in Chapter 2, because besides the sludge ability to mineralise large amounts of biomass-associated LCFA, its activity is enhanced after the degradation step.

Table 5.6 Specific methanogenic activity in the presence of acetate, propionate, butyrate, ethanol and H_2/CO_2, exhibited by the encapsulated sludge (from RO), before and after degrading the biomass-associated LCFA (Mean±SD).

Initial methane production rate in the presence of: (mg COD-CH_4/(gVSS.day))	Before degrading accumulated LCFA	After degrading accumulated LCFA
Acetate	0	533±95
Propionate	0	16±4
Butyrate	0	224±71
Ethanol	24±6	67±1
H_2/CO_2	437±12	2709±38
No added Substrate	35±4	(n.d.)

n.d. - non-detectable.

Although it is proven that the toxic effect of the biomass-associated LCFA is not permanent, it is not completely clear from these results if there is or not a transitory metabolic inhibition when the LCFA are in intimate association with the sludge. In fact, the null activities measured for the encapsulated sludge can represent simply a delay on methane production measurement due to transport limitation effects. Transport of substrates form the bulk liquid to the cells can be hindered by the accumulated layer of LCFA, as well as the subsequent biogas release. This hypothesis is reinforced by the fact that H_2, the smallest and the most hydrophobic of the tested substrates, was rapidly transformed into methane, which may suggest that its diffusion through the LCFA layer was faster than for the other substrates. This is in accordance with the reported absence of lag phases on the assessment of hydrogenophilic activity in the presence of LCFA (Hanaki *et al*., 1981). In the case of ethanol, methane production was also observed, likely because this substrate dissolved the accumulated LCFA, overcoming, to some extent, substrate and product diffusion limitations. The differentiation between a transitory toxic effect and a transport

limitation effect needs to be investigated. Probably both phenomena are involved, since, for instance, the methanogenic activity with propionate as substrate was still considerably low after the depletion of the biomass-associated LCFA, suggesting that this particular trophic group can be affected. However, to be sure about that, the long term history of the sludge should be considered. In this case, before the continuous oleate load, the seed sludge (inoculum) exhibited already a null methanogenic activity with propionate as substrate (Table 5.1, p.137). When extending this comparison to the other tested substrates, one can not disregard the significantly higher activity with acetate, butyrate and H_2/CO_2 exhibited by the depleted sludge, when compared to that previously exhibited by the seed sludge (Table 5.6 and Table 5.1). Enhancement of sludge activity against these particular substrates seems to find a feasible explanation based on the mechanism of LCFA β-oxidation. In fact, during oleate/palmitate β-oxidation both butyrate and acetate are formed (Weng and Jeris, 1976), which may result in the enrichment of both aceticlastic and acetogenic butyrate-degrading bacteria. The same can occur with hydrogenotrophic bacteria, an important group that acts syntrophically with hydrogen producing acetogenic bacteria during LCFA degradation. Since propionate and ethanol are improbable intermediates of oleate/palmitate β-oxidation, an enhancement of sludge activity in these substrates may, therefore, not be promoted.

One important aspect that should be pointed out when considering the specific activity values presented in this and subsequent biochemical studies is that, due to the presence of a high amount of biomass-associated LCFA in the sludge under study, the VSS content assigning only biomass present in the batch experiments was significantly low, i. e. 1-1.5 gVSS/l. As described in the Methods (sub-chapter 2.2.3.1, p.57), a concentration of 2-5 gVSS/l is recommended for those tests. In this study, as the VSS accounting for the biomass were lower, an excess of substrate was present, which may result in underestimated activity values, due to enzyme saturation and/or inhibition by substrate.

The sludge collected from RP was also characterised in terms of specific methanogenic activity with acetate, propionate, butyrate, ethanol and H_2/CO_2. This sludge contained a high LCFA content (palmitic acid) in the form of differentiated particles amid the biomass, instead of entrapping the sludge, being thus entitled as “non-encapsulated loaded sludge” in the course of this study. Figure 5.6 presents the long-term methane

production curves obtained when specific methanogenic activity was measured in the encapsulated loaded sludge and in the non-encapsulated one.

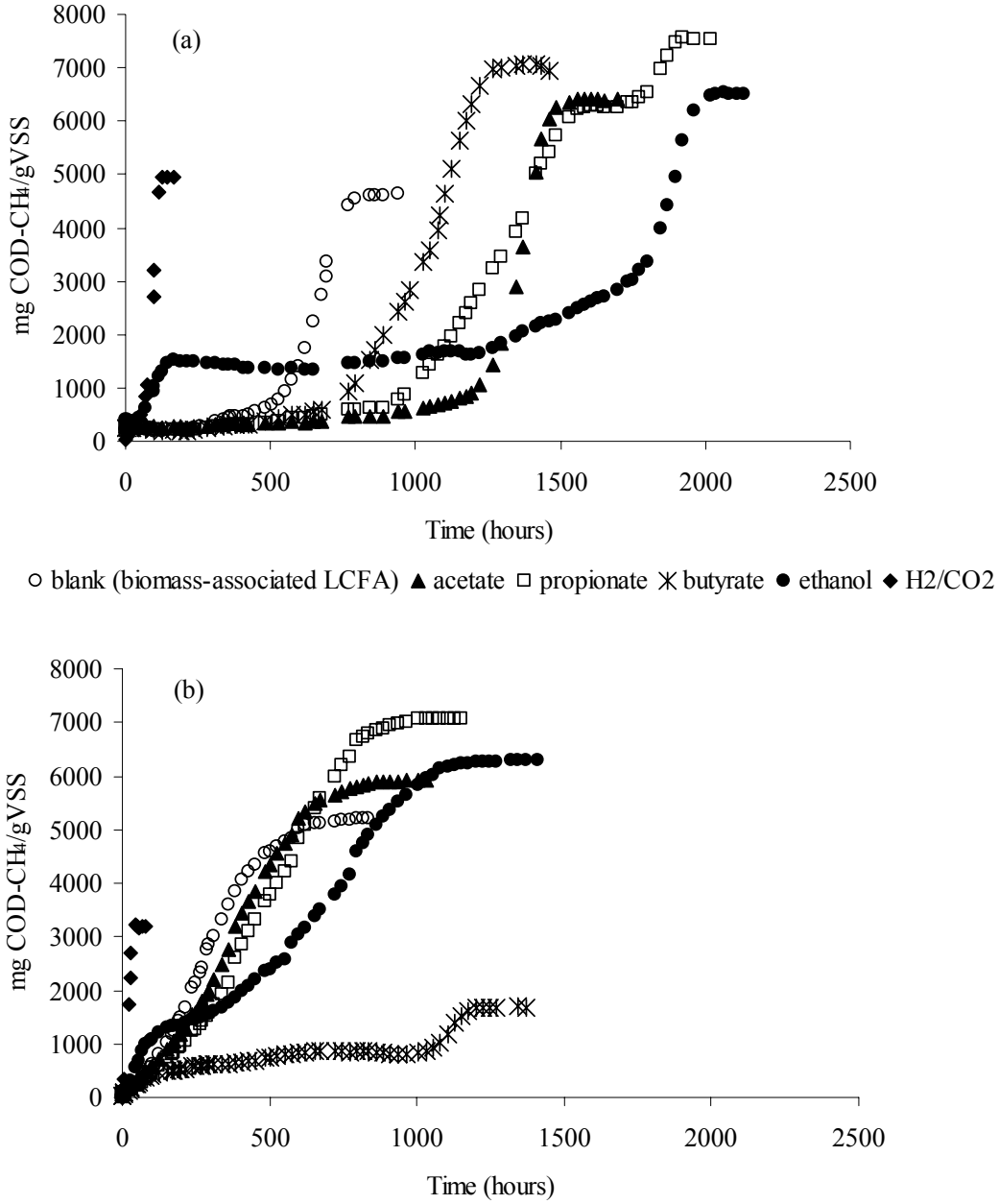

Figure 5.6 Prolonged monitoring of specific methane production during the methanogenic activity batch experiments: (a) encapsulated loaded sludge (from RO), (b) non-encapsulated loaded sludge (from RP).

During the methanogenic activity measurements, the non-encapsulated loaded sludge exhibited a considerable initial methanogenic activity on all the substrates tested with the single exception of butyrate (Figure 5.6(b)). Also the blank control test, where only the accumulated LCFA was present, exhibited a significant initial methane production. However, for the encapsulated loaded sludge, only methane production from ethanol and H_2/CO_2 was detected during the first 500 hours of the test (Figure 5.6(a)). Moreover, in this sludge lag phases up to 1200 hours were detected preceding initial methane production from the other tested substrates. As referred before, in this sludge the accumulated LCFA was in intimate association with the biomass, and thus it is reasonable to expect that LCFA accumulation can hinder the transfer of substrates and products between the bulk liquid and the cells, inducing a delay on initial methane production. In the non-encapsulated loaded sludge, LCFA was accumulated as individualised precipitates rather than adsorbed onto the sludge, and thus diffusion limitation effects are not expected to occur. The different behaviour exhibited by both sludges, in terms of specific methane production curves, therefore suggests that diffusion limitations caused by the intimate association of the accumulated LCFA with the biomass have indeed an important role in the measured methanogenic activities. This physical effect could easily mislead the data obtained in previous works that suggest severe conclusions about LCFA inhibitory effects. Besides transport of substrates from the bulk media to the cells, subsequent biogas release can be hindered by the physical "barrier" created by the accumulated LCFA, and thus significantly hamper the measured initial activity. This effect may justify the lag phase of about 500 hours preceding the initial methane production observed during the mineralisation of the biomass-associated LCFA (blank control) by the encapsulated loaded sludge, since in this case the substrate is already in intimate contact with the cells. In the non-encapsulated loaded sludge this effect was not observed.

The maximum plateau achieved in the cumulative methane production curve during the mineralisation of the biomass-associated LCFA was considered an indirect measurement of the amount of accumulated (biomass-associated) LCFA (as described in sub-chapter 2.2.3.5, p.64). From those curves, a specific LCFA content of 4570±257 and 5200±9 mg COD/gVSS were found to be present in the encapsulated and non-encapsulated sludge, respectively. The higher specific LCFA content present in the non-encapsulated sludge is in

accordance with the higher COD accumulated in RP, during reactor continuous LCFA load (Table 5.4). Furthermore, it supports the relative importance of the degree of intimate state of association of the accumulated LCFA with the sludge, than the specific LCFA content present in that sludge, in terms of methanogenic activity measurements.

Notwithstanding the importance of a virtual inhibition due to physical transport limitations, the effect of a true metabolic inhibition should not be disregarded. In fact, in the non-encapsulated sludge the presence of butyrate severely inhibited methane production, and the presence of acetate and propionate induced a delay on the degradation of the biomass-associated LCFA, although not as pronounced as for the encapsulated sludge. This behaviour suggests a possible inhibition by these acids in the β-oxidation process. More discussion on this matter, in connection with the methanisation kinetic of the biomass-associated LCFA, will be further presented in Chapter 6.

As described in the Methods (sub-chapter 2.2.3.1, p.57), methanogenic activity is measured by the initial slope of each curve, discounting the background methane production obtained in the blank controls that corresponds in this case to the initial degradation rate of the biomass-associated LCFA. For the non-encapsulated loaded sludge, the initial methane production rate from acetate, propionate and butyrate was lower than the one measured in the blank controls (Figure 5.6(b)), resulting in null methanogenic activities against those substrates. In the same way as for the encapsulated loaded sludge, the non-encapsulated one exhibited only a measurable activity with ethanol and H_2/CO_2 (155±8 and 2351±33 mg COD-CH_4/(gVSS.day), respectively). After allowing the depletion of the biomass-associated LCFA, the initial methane production rate exhibited by this sludge, from all the added substrates except ethanol, was also increased (Figure 5.7 (b), Table 5.7). A major enhancement was found in the activity with acetate, butyrate and H_2/CO_2, important intermediates of palmitate degradation.

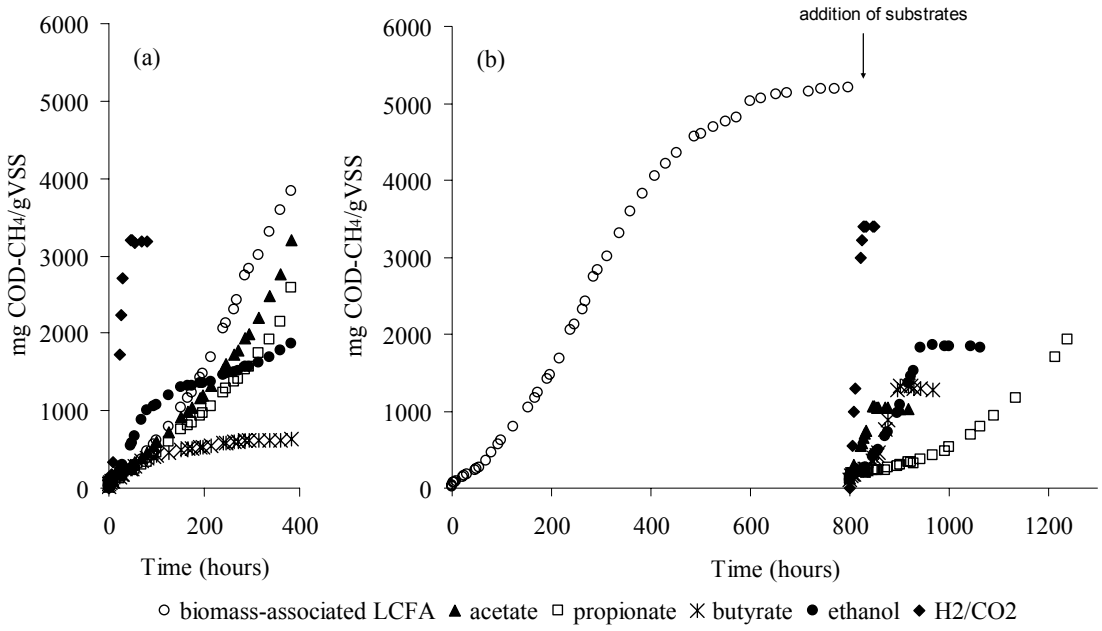

Figure 5.7 Specific methane production during the activity batch experiments exhibited by the non-encapsulated sludge (from RP), before (a) and after (b) degrading the biomass-associated LCFA.

It is remarkable that, after depletion of the biomass-associated LCFA, both the encapsulated and the non-encapsulated loaded sludges exhibited activities of similar order of magnitude (Table 5.6 and Table 5.7). One may therefore hypothesize that both sludges had also similar activities before degrading the accumulated LCFA - mainly palmitic acid in both cases - and that the different pattern of the specific methane production curves, before degrading the biomass-associated LCFA (Figure 5.6), resulted mostly from the different physical state of LCFA interaction with the biomass. Since the encapsulated sludge resulted from the continuous load with oleic acid, apparently more toxic than palmitic acid (Figure 5.1, p.138), the possibility of being submitted to higher toxic load which may have induced a lower activity, could therefore be speculated. However, the transformation of oleic acid to palmitic acid was found to be a fast and non-limiting step in oleic acid degradation (Chapter 2), and thus, palmitic acid was, in fact, the main LCFA that contacted for a long term with both sludges. Hence, the hypothesis that they may have a similar state of intrinsic activity seems to be valid.

Table 5.7 Specific methanogenic activity in the presence of acetate, propionate, butyrate, ethanol and H_2/CO_2, exhibited by the non-encapsulated sludge (from RP), before and after degrading the biomass-associated LCFA (Mean±SD).

Initial methane production rate in the presence of: (mg COD-CH_4/(gVSS.day))	Before degrading accumulated LCFA	After degrading accumulated LCFA
Acetate	0	454±5
Propionate	0	41±3
Butyrate	0	110±9
Ethanol	155±8	138±11
H_2/CO_2	2351±33	3224±182
No added Substrate	129±3	(n.d.)

n.d. - non-detectable.

Besides the elimination of LCFA reversible inhibitory/transport limitation effects by allowing the mineralisation of the biomass-associated LCFA, the possible enrichment of specific populations in the consortium (involved in LCFA degradation) during this mineralisation process, may have been also promoted. Scanning electron microscopic inspection of both sludges revealed a significant increase in coccoid-like forms after degrading the accumulated LCFA (Figure 5.8), suggesting that a possible shift in the populations present in the sludge may have occurred. However, microbial diversity before and after degrading the accumulated LCFA should be further investigated, using for instance 16S rDNA-based methods (Chapter 4). In the encapsulated sludge the presence of a non biologic matrix entrapping the microorganisms (Figure 5.8 (a)), as previously observed in Chapter 2 (p.71) could be also detected.

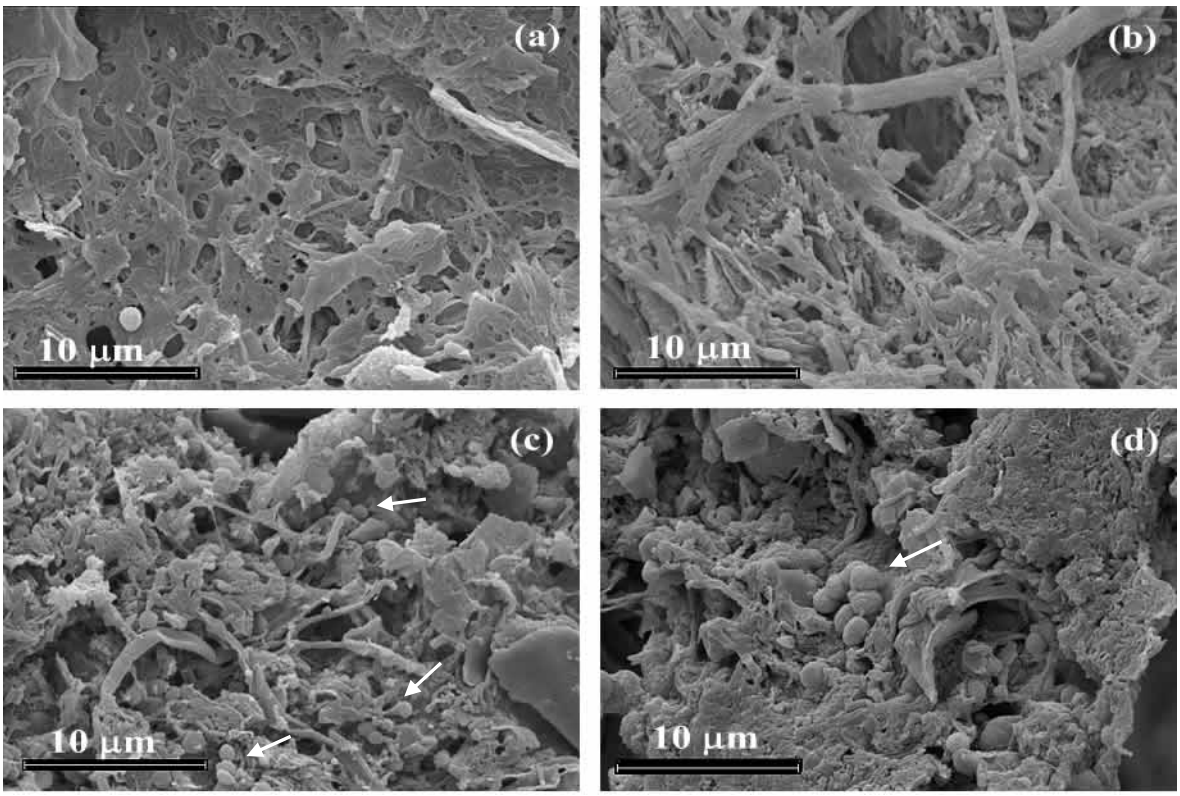

Figure 5.8 Scanning electron microphotographs of: encapsulated sludge (from RO) (a) before and (c) after allowing the methanisation of the biomass-associated LCFA, non-encapsulated sludge (from RP) (b) before and (d) after allowing the methanisation of the biomass-associated LCFA.

5.3.4 ENHANCEMENT OF ACETICLASTIC TOLERANCE TO LCFA TOXICITY AFTER DEGRADING THE BIOMASS-ASSOCIATED LCFA

The sludge from RO (fed with oleate) was also characterised in terms of oleic acid toxicity towards aceticlastic bacteria, before and after degrading the biomass-associated LCFA (mainly palmitic acid). Figure 5.9 presents the long-term specific methane production curves obtained in both assays.

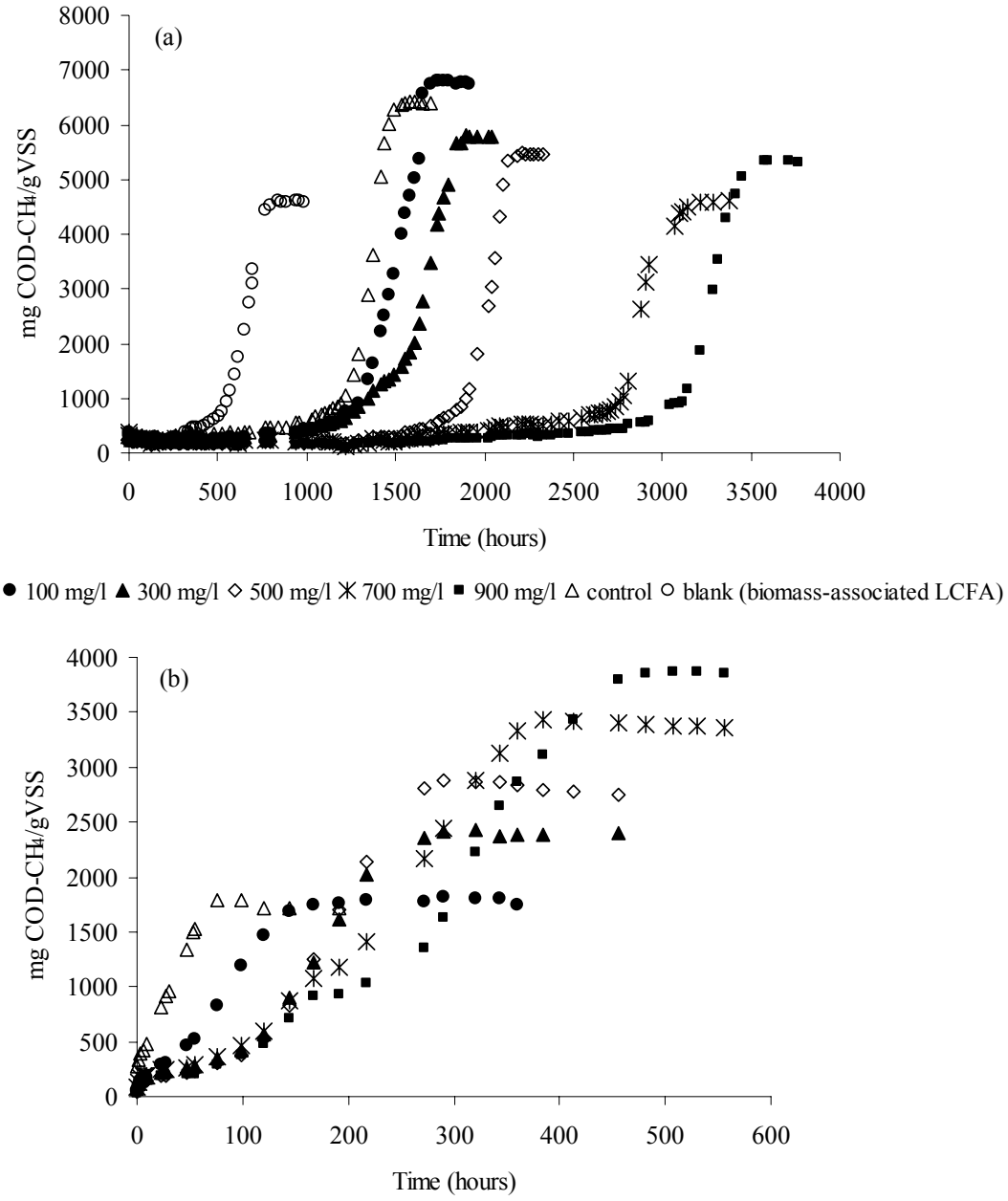

Figure 5.9 Long-term course of specific methane production during oleic acid toxicity batch experiments exhibited by the sludge from RO (encapsulated), before (a) and after (b) degrading the biomass-associated LCFA.

Oleate toxicity to anaerobic sludge is expressed as IC_{50} values, defined as oleate concentration that causes a 50% relative methanogenic aceticlastic activity loss. Before degrading the biomass-associated LCFA, this sludge exhibited no initial methanogenic

activity with acetate (control) because the initial methane production rate was lower than the one measured in the blank controls (no added substrate), that corresponded to the degradation of the biomass-associated LCFA. Therefore, in this case the toxicity limit (IC_{50}) could not be determined. Furthermore, the addition of increasing oleic acid concentrations (100 to 900 mg/l) induced a considerable increase, from 50 to 800 hours, in the already long lag phase that preceded methane production in the presence of acetate (control). The low plateau obtained in the methane production curves revealed the low substrate mineralisation achieved in these conditions. After mineralisation of the biomass-associated LCFA (Figure 5.9 (b)), methane production due to the degradation of acetate (control) proceeded immediately and the maximum delay on methane production obtained was of about 80 hours, when 900 mg/l of oleic acid were added. This means that, after depletion of the accumulated LCFA, a 100 fold lower delay on the aceticlastic activity (methane production) is induced by the presence of 900 mg/l of oleic acid (higher concentration tested), thus evidencing the advantage of allowing the sludge to mineralise the biomass-associated LCFA. Besides a potential LCFA concentration dependent inhibition of the activity of the sludge via a biochemical mechanism, transport limitation effects caused by the physical barrier created by the LCFA layer can, as referred before, hinder the transport of substrates from the bulk media to the cells as well as subsequent biogas release, and thus significantly hamper the measured initial methane production. After depletion of the biomass-associated LCFA, these reversible inhibitory/transport limitation effects can be eliminated. However, sludge microstructure where filaments form an interlinking grid, is now "free" to further accumulate the added LCFA, which may induce once more, delays on the measured initial methane production (lag phases).

After depletion of the biomass-associated LCFA, the sludge exhibited a toxicity limit (IC_{50}) to oleic acid of 80±10 mg/l (Figure 5.9 (b)). A similar IC_{50} value (70±10 mg/l) was previously exhibited by the sludge before being continuously loaded with oleic acid (inoculum, Figure 5.1, p.138), suggesting that no significant increase on the resistance to oleic acid toxicity occurred by the LCFA contact/degradation. However, some care should be taken when comparing these values since, as referred before, the correspondent determinations were not performed in similar condition of VSS concentration.

The sludge from RP (fed with palmitic acid) was characterised in terms of palmitic acid toxicity towards aceticlastic methanogens, before and after degrading the biomass-

associated LCFA (palmitic acid). Figure 5.10 presents the long-term specific methane production curves obtained in both assays. This sludge exhibited also no initial methanogenic activity with acetate (control) before degrading the biomass-associated LCFA, because the initial methane production rate was lower than the one measured in the blank controls (no added substrate), that corresponded to the degradation of the biomass-associated LCFA. Consequently, the palmitic acid toxicity value (IC_{50}) towards aceticlastic bacteria could not be determined. In this sludge, inhibition of aceticlastic bacteria by the presence mainly of palmitic acid caused a decrease in the methane production rate. After mineralisation of the biomass-associated LCFA, no aceticlastic inhibition was observed in the range of palmitic acid concentrations under study (Figure 5.10 (b)). The specific methane production curve patterns clearly evidence that the presence of palmitic acid had no interference on acetate degradation rate, which was preferentially consumed by the consortium. A similar behaviour had been show by the sludge before being continuously loaded with palmitic acid (inoculum, Figure 5.1 (b)). These results, therefore, evidenced that, as found for the sludge that was continuously loaded with oleic acid (from RO), for the sludge that was continuously loaded with palmitic acid (from RP), it is advantageous to allow the mineralisation of the accumulated LCFA, since an enhancement on its tolerance to palmitic acid toxicity is promoted.

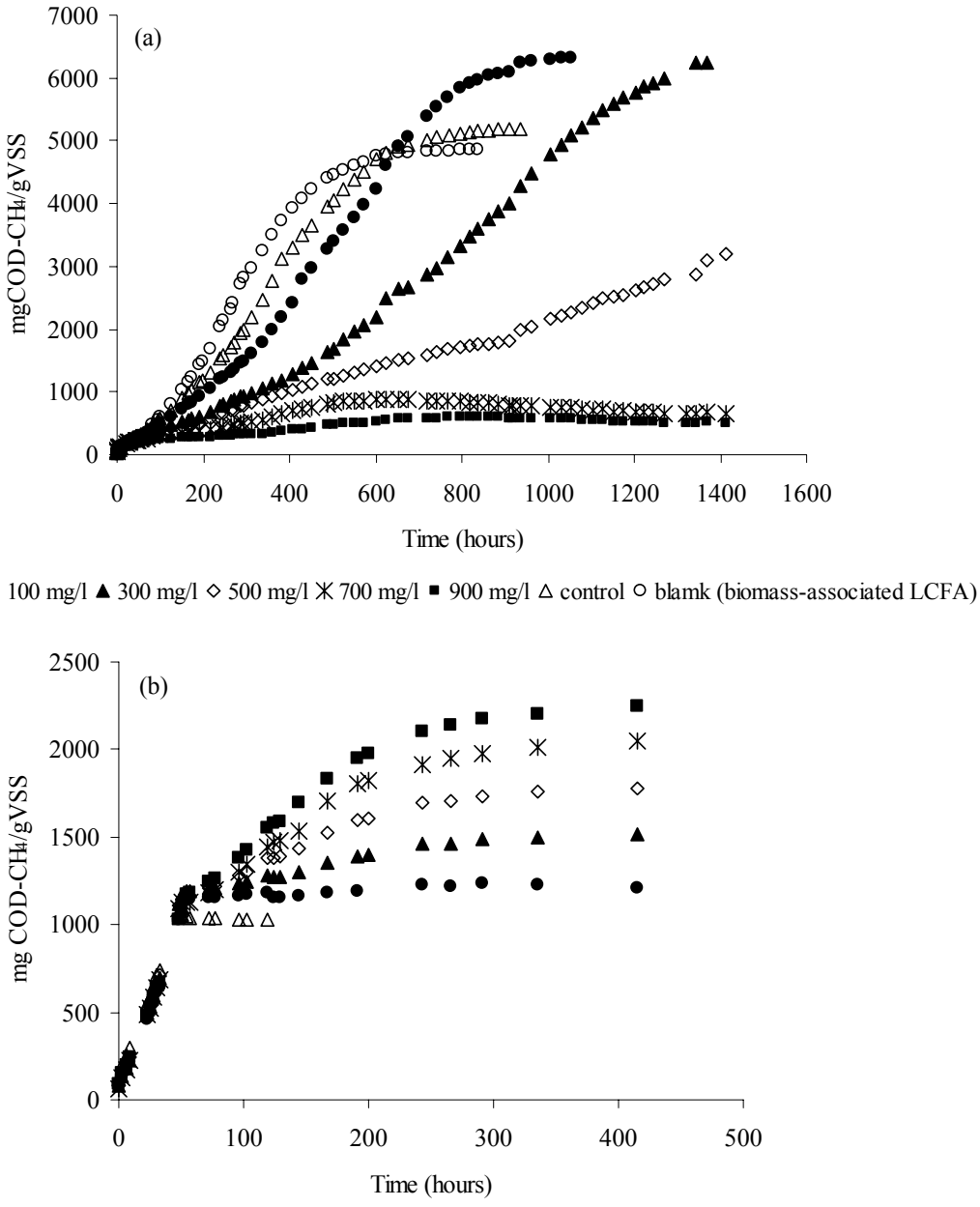

Figure 5.10 Long-term course of specific methane production during palmitic acid toxicity batch experiments exhibited by the sludge from RP (non-encapsulated), before (a) and after (b) degrading the biomass-associated LCFA.

5.3.5 ENHANCEMENT OF LCFA BIODEGRADATION CAPACITY AFTER DEGRADING THE BIOMASS-ASSOCIATED LCFA

Both sludges were also characterised in terms of biodegradation capacity of the correspondent fed LCFA, before and after mineralisation of the biomass-associated LCFA.

Figure 5.11 presents the course of specific methane production during oleic acid biodegradation batch experiments exhibited by the sludge from RO (fed with oleic acid), before (a) and after (b) degrading the biomass-associated LCFA. In the first assay, the sludge was encapsulated by a high amount of LCFA, mainly palmitic acid, and thus these curves represent the effect of adding oleic acid on the methanisation of the biomass-associated LCFA (Figure 5.11 (a)). As can be seen, the addition of oleic acid concentrations between 100 to 900 mg/l induced a considerable delay on methane production up to about 1000 hours. This strong effect of oleic acid on the methanisation rate of the accumulated palmitic acid was already observed in Chapter 2 (Figure 2.8, p.74). After mineralisation of the biomass-associated LCFA (Figure 5.11 (b)), methane production due to the degradation of the added oleic acid proceeded relatively faster, although lag phases up to about 200 hours were observed preceding initial methane production which can be consequence of a new accumulation of added LCFA (Table 5.8).

The advantage of allowing the mineralisation of the biomass-associated LCFA is clearly evidenced when comparing the time required for LCFA mineralisation in both situations. For instance, if oleic acid at 900 mg/l (highest concentration tested) is added to the encapsulated sludge, initial methane production starts only after 1500 hours and seems to be concluded in a total of 2700 hours. By contrast, if the biomass-associated LCFA is allowed to be first mineralised (about 900 hours) and only after the added oleic acid (500 hours) a total of 1400 hours are required. On the other hand, besides eliminating LCFA reversible inhibitory/transport limitation effects by allowing their methanisation, the possible enrichment of specific populations in the consortium (involved in β-oxidation process) may, as suggested before, be also promoted during LCFA degradation.

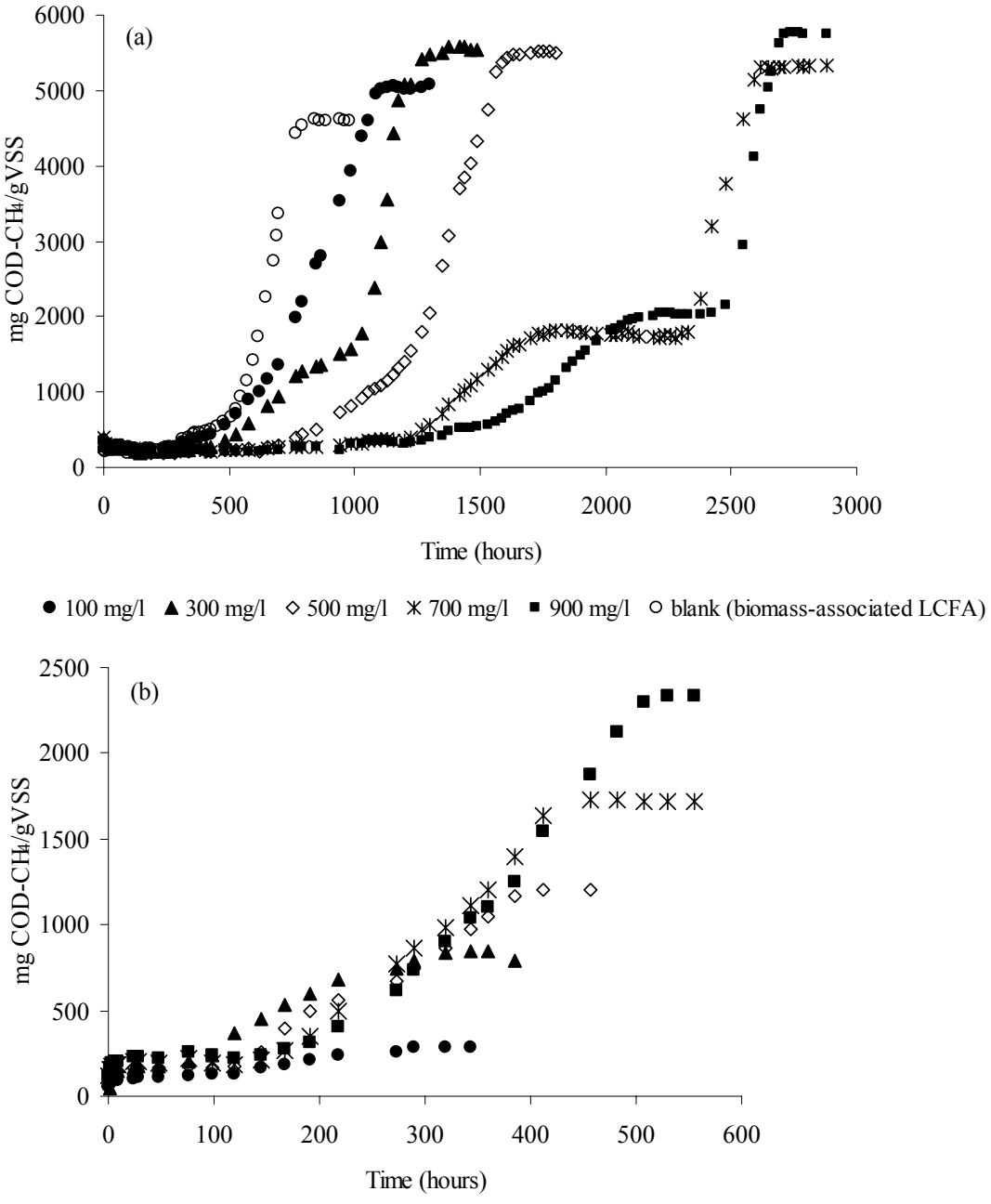

Figure 5.11 Specific methane production during oleic acid biodegradation batch experiments exhibited by the sludge from RO, before (a) and after (b) degrading the biomass-associated LCFA.

Table 5.8 Results from oleic acid biodegradation batch experiments, performed in the sludge from RO, after depletion of the biomass-associated LCFA (Mean±SD).

	Maximum specific methane production rate (mg COD-CH_4/(gVSS·day))	Lag phase (hours)
Oleic acid concentrations:		
100 mg/l	16±7	50±5
300 mg/l	71±3	50±10
500 mg/l	81±4	100±5
700 mg/l	114±4	150±5
900 mg/l	158±5	180±20

Figure 5.12 presents the course of specific methane production during palmitic acid biodegradation batch experiments exhibited by the sludge from RP (fed with palmitic acid), before (a) and after (b) degrading the accumulated LCFA. This sludge also contained a high amount of biomass-associated LCFA, namely palmitic acid and thus, the assay performed before allowing the mineralisation of the biomass-associated LCFA (Figure 5.12(a)) presents the effect of adding palmitic acid on the methanisation of the biomass-associated palmitic acid. A similar effect to that previously observed when the added substrate was oleic acid (Figure 5.11(a) and Chapter 2) was found, although less pronounced. The addition of 100 mg/l of palmitic acid did not significantly decrease the methanisation of the biomass-associated LCFA. A maximum decrease on the methanisation rate, from 240 mg COD-CH_4/(gVSS.day) to 32 mg COD-CH_4/(gVSS.day), was achieved when 700 mg/l of palmitic acid were added. After allowing the mineralisation of the biomass-associated LCFA (Figure 5.11(b)), an increase on the methanisation rate was obtained for added palmitic acid concentrations higher than 500 mg/l. Lag phases preceding initial methane production were not observed in this assay (Table 5.9).

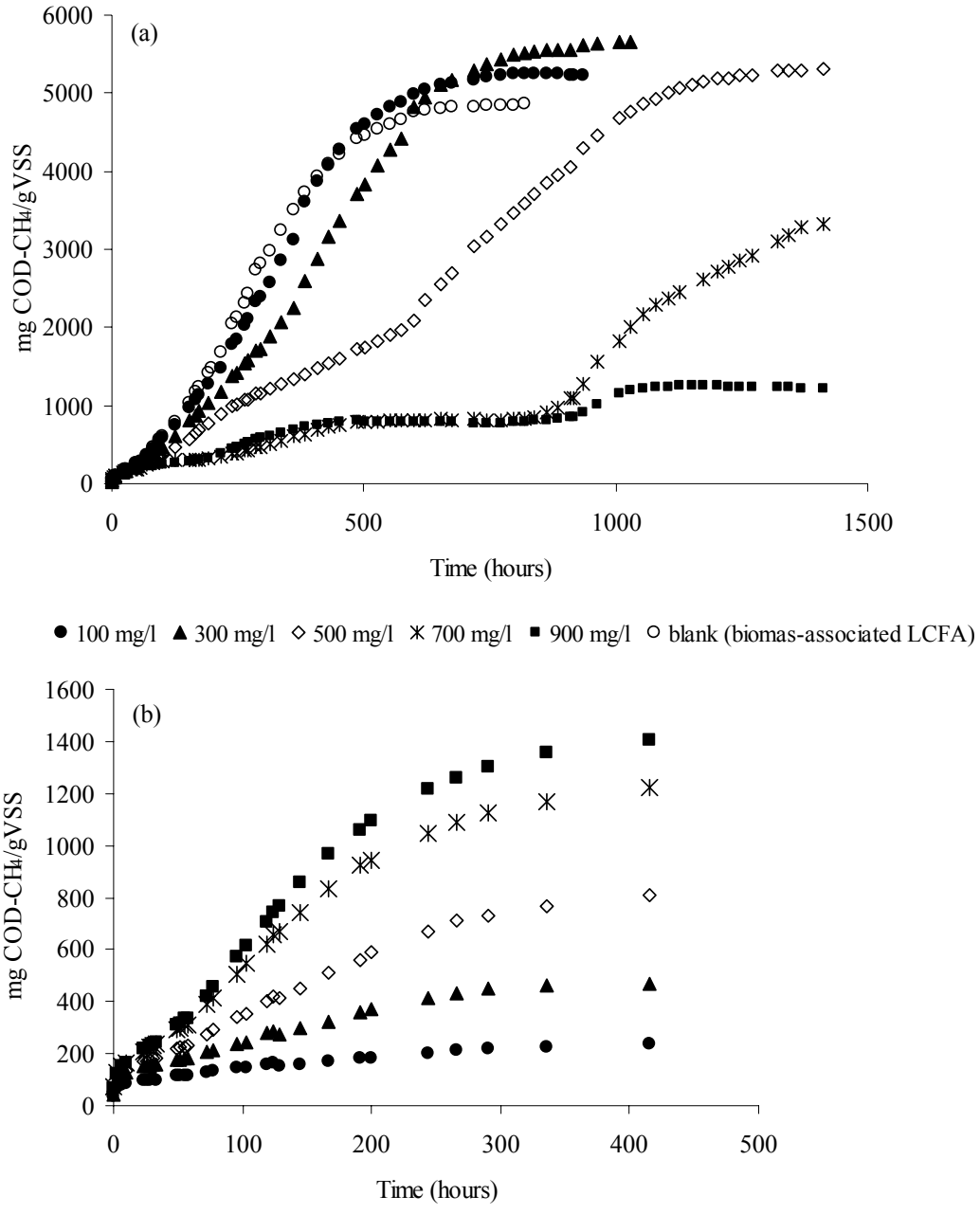

Figure 5.12 Specific methane production during palmitic acid biodegradation batch experiments exhibited by the sludge from RP, before (a) and after (b) degrading the biomass-associated LCFA.

Table 5.9 Results from palmitic acid biodegradation batch experiments, performed in the sludge from RP, after depletion of the biomass-associated LCFA (Mean±SD).

	Maximum specific methane production rate (mg COD-CH_4/(gVSS·day))	Lag phase (hours)
Palmitic acid concentrations:		
100 mg/l	19±3	0
300 mg/l	36±5	0
500 mg/l	59±22	0
700 mg/l	105±12	0
900 mg/l	125±39	0

5.4 CONCLUSIONS

Specific methanogenic activity in the presence of individual substrates (acetate, propionate, butyrate, ethanol and H_2/CO_2) measured before and after the depletion of the accumulated LCFA (mainly palmitic) strongly contradicted the accepted theory of the bactericidal effect of LCFA towards anaerobic consortia as well as their permanent toxic effect. The anaerobic consortia remained active after being loaded with amounts of LCFA up to 5200 mg COD/gVSS. In the loaded sludge, only a methanogenic activity in H_2/CO_2 and ethanol was detected. However, after allowing mineralisation of the accumulated LCFA, all the measured activities were significantly enhanced. Tolerance of aceticlastic bacteria to LCFA toxicity and capacity of LCFA biodegradation exhibited by the consortium were also improved after allowing mineralisation of the biomass-associated LCFA. The results obtained put in evidence the possible strong effect of diffusion limitations imposed by the LCFA layer that could hamper the access of the added substrates to the cells as well as the subsequent biogas release. This physical effect could easily mislead the data obtained in previous works that suggest severe conclusions about LCFA inhibitory effects. On the other hand, these findings support the practical interest of using sequential adsorption and degradation steps for the treatment of lipid/LCFA based effluents, as suggested in Chapter 2, because besides the sludge ability to mineralise high amounts of biomass-associated LCFA, its activity is enhanced after the degradation step.

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6 METHANISATION OF BIOMASS-ASSOCIATED LCFA: KINETICS AND EFFECT OF ADDED VFA

Abstract

So far, regarding the research in this thesis, LCFA accumulated onto anaerobic sludge can be efficiently biodegraded to methane provided no external substrate is present in the medium. After allowing the mineralisation of the biomass-associated LCFA sludge, activity was significantly improved. From a practical viewpoint, and considering the interest of using sequential adsorption (accumulation) and degradation (methanisation of the accumulated LCFA) steps for the treatment of lipid/LCFA based effluents, previously suggested in Chapter 2, the conditions that allow optimal mineralisation of the accumulated LCFA need to be assessed. In this chapter, the new methodology to quantify, in batch assays, the amount of LCFA accumulated onto the sludge, presented on Chapter 2, was explored, which allowed the establishment of the mineralisation kinetics of biomass-associated LCFA (mainly palmitic), according to an inhibition model based on Haldane's enzymatic inhibition kinetics. A total of 38 samples of encapsulated-loaded sludge, taken from different types of reactors fed with oleic acid, were used. An optimal value of 1080 mg COD-CH_4/gVSS was determined for the amount of accumulated LCFA that could be mineralised at the maximal rate of 250 mg COD-CH_4/(gVSS.day). Near this optimal specific concentration of LCFA, the effect of adding VFA to the medium was studied, in order to evaluate their interactions with the methanisation of the biomass-associated LCFA. Different patterns were obtained for each individual substrate. Although acetate and butyrate were preferentially consumed by the consortium, in the case of propionate no evidence of a sequential consumption pattern was observed. An important information that can be withdrawn from the obtained results is that, in these conditions, if the sludge is allowed to mineralise the accumulated LCFA in the presence of acetate, propionate or butyrate, no significant delay in the overall methane production is induced.

6.1 INTRODUCTION

Although lipids are potentially attractive for biogas production due to their high theoretical methane yield, in practice a physico-chemical pre-treatment is usually applied. This arises because neutral fats are easily hydrolysed to LCFA (Hanaki *et al.*, 1981), which are reported to exert an acute toxic effect towards syntrophic acetogenic and methanogenic populations, involved in the β-oxidation and methanogenic steps (Koster and Cramer, 1987; Angelidaki and Ahring, 1992; Rinzema *et al.*, 1994; Hwu, 1997). More than inhibition drawbacks, Rinzema (1988) and, more recently, Hwu (1997) demonstrated that flotation and washout were associated to the adsorption of LCFA onto anaerobic sludge. Hwu (1997) added some insights to this phenomenon by assessing the critical LCFA specific organic load that induced flotation and complete washout during the operation of EGSB reactors, concluding that it corresponded to a concentration far below the toxicity limit. Following these conclusions, he attributed to LCFA sorption a negative effect that should be prevented to guarantee efficiency and reliability of high-rate continuous treatment systems.

However, the research described in Chapter 2 showed that, LCFA accumulated onto the sludge by mechanisms of adsorption, precipitation or entrapment, can be efficiently mineralised, in batch assays, provided no external substrate is present in the medium. Furthermore, in Chapter 5, it was observed that the anaerobic consortia remained active after being loaded with amounts of LCFA up to 5200 mg COD/gVSS. Only methanogenic activity from H_2/CO_2 and ethanol was detected in the sludge containing biomass-associated LCFA. After allowing the mineralisation of this accumulated LCFA, all the measured activities were significantly enhanced, strongly contradicting the accepted theory of the bactericidal effect of LCFA towards anaerobic consortia as well as their permanent toxic effect.

Previous works dealing with tentative application of sludge bed-based continuous reactors to effluents containing lipids/LCFA, searched for reasonable solutions to overcome flotation problems that usually impair process efficiency (Rinzema, 1988, Hwu, 1997). Partial phase separation was also applied to this type of effluent (Hamdi *et al.,* 1992; Beccari *et al.,* 1998).

The results obtained throughout the present thesis support the interest of using a different reactor design, not continuous, but sequential, where accumulation of LCFA is followed by its mineralisation. In this way, it seems to be possible to enhance biomethanisation of LCFA, which are mainly associated to the biomass. In this regard, the conditions that allow optimal mineralisation of the accumulated LCFA need to be assessed, since long lag phases preceding initial methane production may be observed and should be avoided in practice. For instance, as observed in Chapter 5, in sludge with a specific LCFA-load as high as 4500 mg COD/gVSS, a lag phase of about 500 hours was detected before the initial methane production. On the other hand, the presence of other substrates may interfere with the degradation of the LCFA accumulated onto the sludge and thus hamper the obtained methane production. Also in Chapter 5, this behaviour was observed when oleic acid, palmitic acid or VFA (acetate, propionate and butyrate) were added to the LCFA-loaded sludge.

The aim of this work was to establish the methanisation kinetics of the biomass-associated LCFA and to evaluate the effect of adding individual VFA to the medium, in order to evaluate their possible interaction with the methanisation of the biomass-associated LCFA.

6.2 MATERIALS AND METHODS

6.2.1 SLUDGE SOURCES

Anaerobic sludge samples were obtained from lab-scale reactors operated at mesophilic conditions (37ºC) with increasing influent sodium oleate concentrations. Table 6.1 presents the operating conditions prevailing at the time of sample collection. The fixed bed reactor configuration was designed to allow the sampling of entrapped biomass during reactor operation (Alves *et al.*, 1998; Alves *et al.*, 2001; Cavaleiro *et al.*, 2001). Prior to characterisation, all the sludge samples collected were washed and centrifuged (4000 rpm, 10 min) twice, with the same anaerobic basal medium used in the methanogenic activity measurements (sub-chapter 2.2.3.1, p.57).

Table 6.1 Sources of the anaerobic suspended sludge used in this work.

Reactor type	**Operation mode**	**Influent COD** (g/l)	**HRT** (day)	**Type of substrate**	**Oleate loading rate** (kg COD/ (m^3.day))	**Reference**
Fixed bed	Steady state	12	1.4	SM (50%COD)+ SO (50%COD)	4.3	Alves *et al.*, 2001
Fixed bed	Steady state	12	1.4	SO (100%COD)	8.6	Alves *et al.*, 2001
Fixed bed	Organic shock [a]	20	0.67	SM (50%COD)+ SO (50%COD)	15	Cavaleiro *et al.*, 2001
Fixed bed	Hydraulic shock [b]	4	0.13	SM (50%COD)+ SO (50%COD)	15	Cavaleiro *et al.*, 2001
EGSB	Steady state	4	1.0	SM (50%COD)+ SO (50%COD)	2	Chapter 2
EGSB	Steady state	4	1.0	SO (100%COD)	4	Chapter 2
EGSB	Steady state	6	1.0	SO (100%COD)	6	Chapter 2
EGSB	Steady state	8	1.0	SO (100%COD)	8	Chapter 2
EGSB	Steady state	4	1.1	SO (100%COD)	4	Chapter 5
EGSB	Steady state	4	1.0	SO (100%COD)	4	This work

SM-skim milk, SO-sodium oleate.

[a] 7 samples were taken during four days.

[b] 10 samples were taken during four days.

The sludge referred in the last row of the previous table, was loaded with increasing sodium oleate concentrations between 2 and 4 g COD/l, as previously described in Chapter 2 (sub-chapter 2.2.1, p. 55), in order to obtain anaerobic sludge with a specific LCFA load of about 1000 mg COD/gVSS. Prior to inoculation, the sludge exhibited a specific methanogenic activity with acetate, propionate, butyrate and H_2/CO_2 of 129±5, 7±4, 89±3 and 273±18 mg COD-CH_4/(gVSS.day), respectively.

6.2.2 SLUDGE CHARACTERISATION

6.2.2.1 METHANISATION OF LCFA ACCUMULATED ONTO THE SLUDGE

The amount of biomass-associated LCFA and the correspondent methanisation rate in batch assays were determined as previously described in the sub-chapter 2.2.3.5 (p. 64).

6.2.2.2 METHANOGENIC ACTIVITY MEASUREMENTS

For most of the collected samples, methanogenic activity tests against acetate, propionate, butyrate and H_2/CO_2 were performed using the procedure previously described in sub-chapter 2.2.3.1 (p. 57).

6.2.2.3 EFFECT OF ADDED VFA ON BIOMASS-ASSOCIATED LCFA METHANISATION RATES

Specific methanogenic activity of the sludge (against acetate, propionate, butyrate and H_2/CO_2) before and after depletion of the biomass-associated LCFA was determined as previously described in the sub-chapter 5.2.5 (p.136). Triplicate assays were performed.

In an extra set of vials, with a 500 ml working volume, specific methanogenic activity (against acetate, propionate and butyrate) before degrading the biomass-associated LCFA was measured by VFA depletion in the medium, in the same conditions used in the first set (gas measurement). Possible VFA released to the medium during the methanisation of the biomass-associated LCFA was also assessed. VFA concentration was determined by high-

performance liquid chromatography as previously described in the sub-chapter 5.2.2 (p.134). Duplicate assays were performed.

6.3 RESULTS AND DISCUSION

6.3.1 KINETICS OF BIOMASS-ASSOCIATED LCFA DEGRADATION

When the sludge samples were incubated in batch vials without any added carbon source, calcium or nutrients, methane was produced due to the mineralisation of the LCFA (mainly palmitic acid) accumulated onto the sludge during the continuous oleate load. The specific rate of methane production and the amount of biomass-associated LCFA per VSS unit were calculated for each sample as described in sub-chapter 6.2.2.1, and are plotted in Figure 6.1. This graph represents the influence of the available substrate on the kinetics of its own degradation, measured by the corresponding methane production. It is not a traditional plot of a kinetic behaviour as the substrate is not in the medium, but instead it is accumulated onto the sludge. In spite of that, experimental data were fitted by equation 6.1, which represents an adaptation of an inhibition model used before by Andrews (1971) to describe the inhibition of volatile fatty acids in the anaerobic digestion process. This model is based on the substrate inhibition enzymatic kinetics developed by Haldane (1930).

$$q_p = \frac{\hat{q}_p \times S_{ba}}{K_S + S_{ba} + \frac{S_{ba}^2}{K_i}} \qquad \textbf{(6.1)}$$

where:

q_p – specific methane production rate [$M_{substrate}/(M_{biomass}.t)$]

S_{ba} – biomass-associated substrate per VSS unit [$M_{substrate}/M_{biomass}$]

$\hat{q}_p, K_S, K_i$ are model parameters

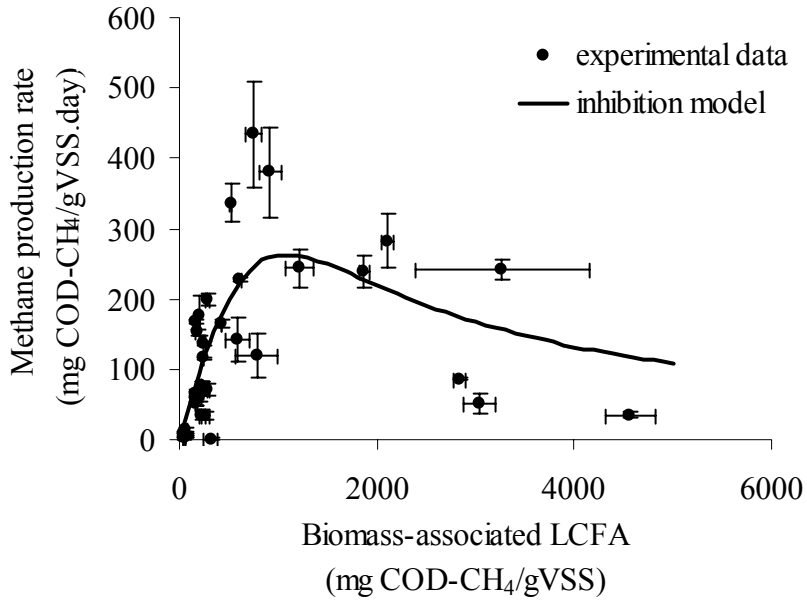

Figure 6.1 Kinetics of methane production in batch assays due to the degradation of the biomass-associated LCFA. Bars represent standard deviation.

Table 6.2 summarises the results obtained by the fitting as well as the corresponding correlation coefficient (determined by the minimum least square method). The relatively low correlation coefficient obtained (0.56) suggests that the mechanism of inhibition is possibly more complex than a simple substrate inhibition phenomenon, in part because β-oxidation process involves a sequence of substrates and products that can compete with each other. In fact, an inhibitory effect of oleic acid (C18:1) in the methanisation of accumulated palmitic acid (C14:0) was previously observed in Chapter 2. Rinzema *et al.* (1994) suggested the existence of a higher affinity of β-oxidation enzymes for fatty acids with a longer carbon chain, which may, in part, justify this finding. Also, the presence of acetate, propionate and butyrate was found to induce a delay on the mineralisation of the biomass-associated LCFA, suggesting a possible inhibition of these acids in the β-oxidation process (Chapter 5). On the other hand, the method used to measure the amount of LCFA accumulated onto the sludge has some limitations because the measured product (methane) depends on the metabolic activity of the sludge. When an excess of biomass-associated LCFA is present, a possible inhibition of its own degradation can lead to an underestimation

of the existing LCFA. In this way, experimental data corresponding to sludge where high and possibly inhibitory amounts of biomass-associated LCFA are present will exhibit a lower plateau in the methane production curve and then will be placed in a region of lower amounts of accumulated LCFA. Moreover, the results obtained in Chapter 5 suggest the possible strong effect of diffusion limitations imposed by the LCFA layer, which are not taken into account in this model. In this case, as the substrate is already in intimate contact with the biomass, substrate diffusion limitations are not expected to limit the degradation rate. However, product diffusion limitation, e.g. biogas release, can be hampered by the LCFA layer and thus, may induce a delay on the measured methane production.

From a practical viewpoint, however, the advantage of this method is to quantify the potential mineralisation capacity of the accumulated LCFA exhibited by an encapsulated sludge.

Table 6.2 Kinetic constants for the applied inhibition model [a].

$\hat{q}$ (mg COD-CH_4/(gVSS.day))	384361
K_S (mg COD-CH_4/gVSS)	790495
K_i (mg COD-CH_4/gVSS)	1.48
Correlation coefficient	0.56

[a] based on Haldane (1930) enzymatic kinetics

According to the inhibition model a critical specific concentration of 1080 mg COD-CH_4/gVSS (equation 6.2) represents the amount of accumulated LCFA after which inhibition of its own mineralisation occurs.

$$S_{ba}^{crit} = \sqrt{K_S \times K_i} \qquad \textbf{(6.2)}$$

6.3.2 EFFECT OF THE BIOMASS-ASSOCIATED LCFA ON THE SPECIFIC METHANOGENIC ACTIVITY

The quantification of the amount of accumulated (biomass-associated) LCFA in the sludge samples that were also characterised in terms of specific methanogenic activity on different individual substrates (acetate, propionate, butyrate and H_2/CO_2) allowed the establishment of a direct relationship between the accumulated LCFA and the methanogenic activities (Figure 6.2). A clear decreasing trend between methanogenic activities and the amount of biomass-associated LCFA was observed. This effect was more sharp for the acetogenic activity against propionate and butyrate (Figure 6.2 (b) and (d)) than for aceticlastic or hydrogenophilic activities (Figure 6.2 (a) and (c)).

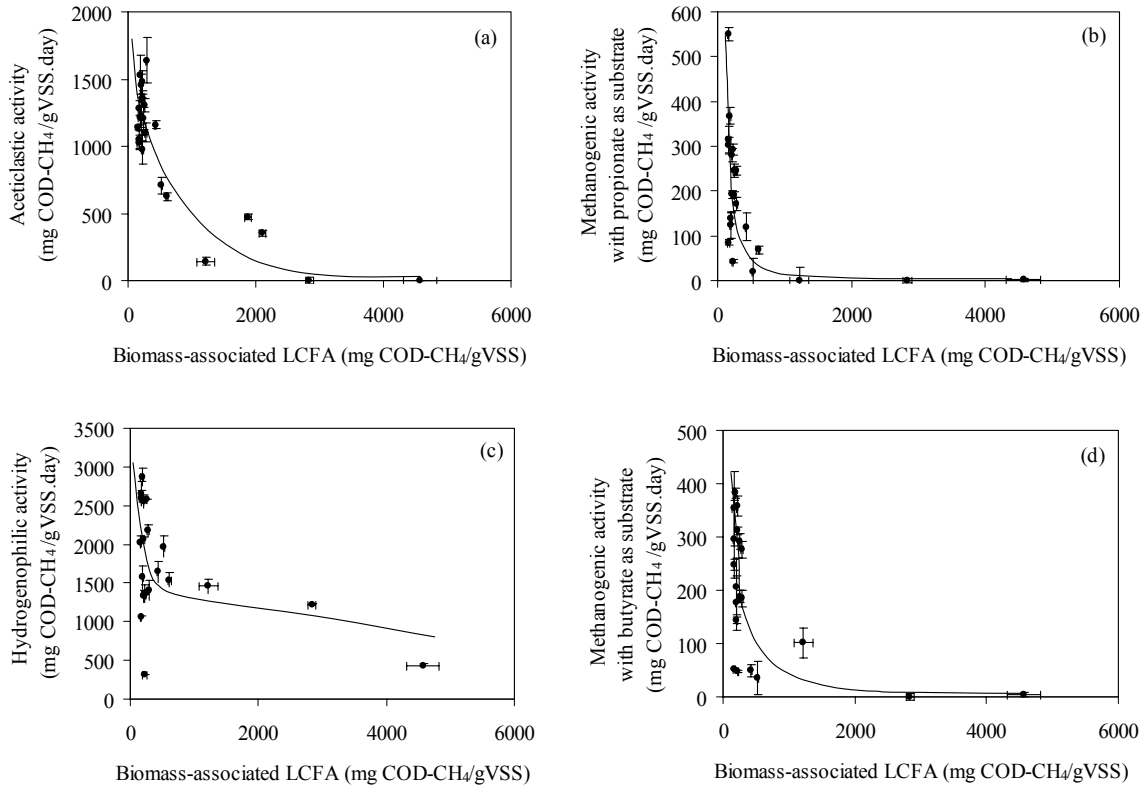

Figure 6.2 Effect of the biomass-associated LCFA on the specific methanogenic activity with acetate (a), propionate (b), H_2/CO_2 (c) and butyrate (d) as substrates. Bars represent standard deviation.

Toxicity measurements are usually performed by adding increasing concentrations of a toxic compound to different vials, simultaneously with a constant concentration of the specific substrate that is consumed by the trophic group under study. Ideally, a constant toxic concentration should be present in the bulk medium while the specific substrate is consumed. When this method is applied for an adsorbable and biodegradable substrate such as LCFA, the toxic concentration in the medium may change before and/or during the consumption of the substrate, due to adsorption onto the sludge and/or to biodegradation. The direct relationship between the amount of biomass-associated LCFA and the methanogenic activities against the different substrates (Figure 6.2) may be a more representative way to express the toxicity of these compounds, since the adsorption of LCFA is an important and well reported phenomenon. As illustrated in Figure 6.2, it is possible to estimate the values of accumulated LCFA per sludge (VSS) unit that reduces the activities to 50% of the maximum detected, these values being about 500 and 200 mg COD/gVSS for aceticlastic activity and acetogenic activity (on propionate and butyrate), respectively. For the hydrogenophilic activity, the value is not well defined, since the 50% reduction is maintained in the range between 200 and 1000 mg COD/gVSS.

However, as shown in Chapter 5, even when methanogenic activity measurements indicated a severe inhibition, the anaerobic consortia remained active, and after depletion of the biomass-associated LCFA all measured activities were significantly enhanced. The results thereby obtained put in evidence the possible strong effect of diffusion limitations imposed by the LCFA layer that could hinder the access of the added substrates to the cells as well as the subsequent biogas release, and thus hamper the measured methane production. On the other hand, as described in the Methods (sub-chapter 2.2.3.1, p.57), methanogenic activity is measured by following the methane production along the time when each individual substrate is added to the LCFA-loaded sludge. Both substrates can be degraded by the anaerobic consortium and thus the measured methane production may be a result of the interaction between both conversions. Furthermore, in this procedure, the background methane production of the blank test, which corresponds in this case to the initial degradation rate of the biomass-associated LCFA, is discounted which can mislead the obtained activity values.

In this regard, it seems that until a threshold specific LCFA load is attained, the decrease of the activity is merely a decrease on the initial methane production rate, obtained by the difference between test and "blank" vials. After that threshold load, the decrease of the activities is a result of a clear lag phase preceding methane production. In this case, the initial methane production when the individual VFA are added is delayed, compared to the situation where no substrate is added, resulting in null measured activities (see for instance Figure 5.6 (a), p.150). A similar effect was already reported by Hanaki *et al.* (1981) who found that the addition of acetate or n-butyrate intensified the inhibitory effect of a fatty acid mixture. From Figure 6.2 it can be seen that, although the trend of the methanisation rate from propionate and butyrate is to attain null values for amounts of biomass-associated LCFA higher than about 1000 mg COD-CH_4/gVSS and 2000 mg COD-CH_4/gVSS, respectively, acetate methanisation rates can still be measured for 2000 mg COD-CH_4/gVSS, and have a tendency to achieve null values for 3000 mg COD-CH_4/gVSS. The hydrogenophilic activity, however, exhibited a significant value of 1200 mg COD-CH_4/(gVSS.day) in the range between 2000 and 3000 mg COD-CH_4/gVSS of biomass-associated LCFA. The more acute "virtual" inhibition of acetogens when compared to aceticlastic and hydrogenophilic methanogens may suggest that a stronger interaction of propionate and butyrate with the methanisation of the biomass-associated LCFA may occur.

In order to clarify this point, the interaction between added individual VFA (acetate, propionate and butyrate) and the methanisation of the biomass-associated LCFA was studied. As discussed above, different behaviours may be observed, depending on the amount of LCFA accumulated onto the sludge. However, from a practical viewpoint, it is important to better understand the role of added VFA in the LCFA-loaded sludge conditions that allow optimum methanisation of the accumulated LCFA, as determined according to the inhibition model based on Haldane's enzymatic inhibition kinetics (Figure 6.1). Therefore, a sludge containing a specific LCFA content near this optimum value was selected for this study.

6.3.3 EFFECT OF ADDED VFA ON THE BIOMASS-ASSOCIATED LCFA METHANISATION

A sludge containing 1200 mg COD-CH_4/gVSS of associated LCFA was characterised in terms of specific methanogenic activity with acetate, propionate, butyrate and H_2/CO_2, before and after allowing the mineralisation of accumulated LCFA, which was mainly palmitic acid. In the assay made before allowing the mineralisation of the biomass-associated LCFA, specific methanogenic activity with acetate, propionate and butyrate was simultaneously assessed by VFA depletion in the medium (Figure 6.3). VFA were also monitored during the mineralisation of the biomass-associated LCFA ("blank" assay), which revealed that there was no detectable release of volatile fatty acids to the medium during the degradation process. Hence, in the interpretation of the subsequent result, it was assumed that this condition also prevailed. During propionate degradation, acetate and butyrate were detected in concentrations not exceeding 140 mg/l, which corresponded to a specific concentration of 55 mg COD/gVSS, and 74 mg/l (49 mg COD/gVSS), respectively. Acetate was also detected during butyrate degradation, in concentrations lower than 170 mg/l (66 mg COD/gVSS). In order to allow a direct comparison with the correspondent methane production, VFA depletion was expressed in terms of specific concentration and includes the total VFA present, not only that initially added but also possible intermediates. Table 6.3 summarises the results obtained for the specific methanogenic activity, before (measured by VFA depletion and CH_4 production) and after mineralisation of the biomass-associated LCFA (measured by CH_4 production).

From the obtained results it can be seen that, when acetate was added to the LCFA-loaded sludge (Figure 6.3) (a)) initial methane production was mainly due to the consumption of the added acetate, since acetate depletion rate was similar to the corresponding methane production (Table 6.3). The slight difference between the two rates may indicate that some mineralisation of the accumulated LCFA occurred simultaneously, which proceeded after extinction of acetate in the medium. When acetate was not present, the mineralisation of the LCFA proceeded at a lower rate, which seams reasonable since β-oxidation of LCFA is a slower process (breakage of acetate from the fatty acid chain before aceticlastic metanogenesis). A similar pattern was observed when the added VFA was butyrate (Figure 6.3 (c)).

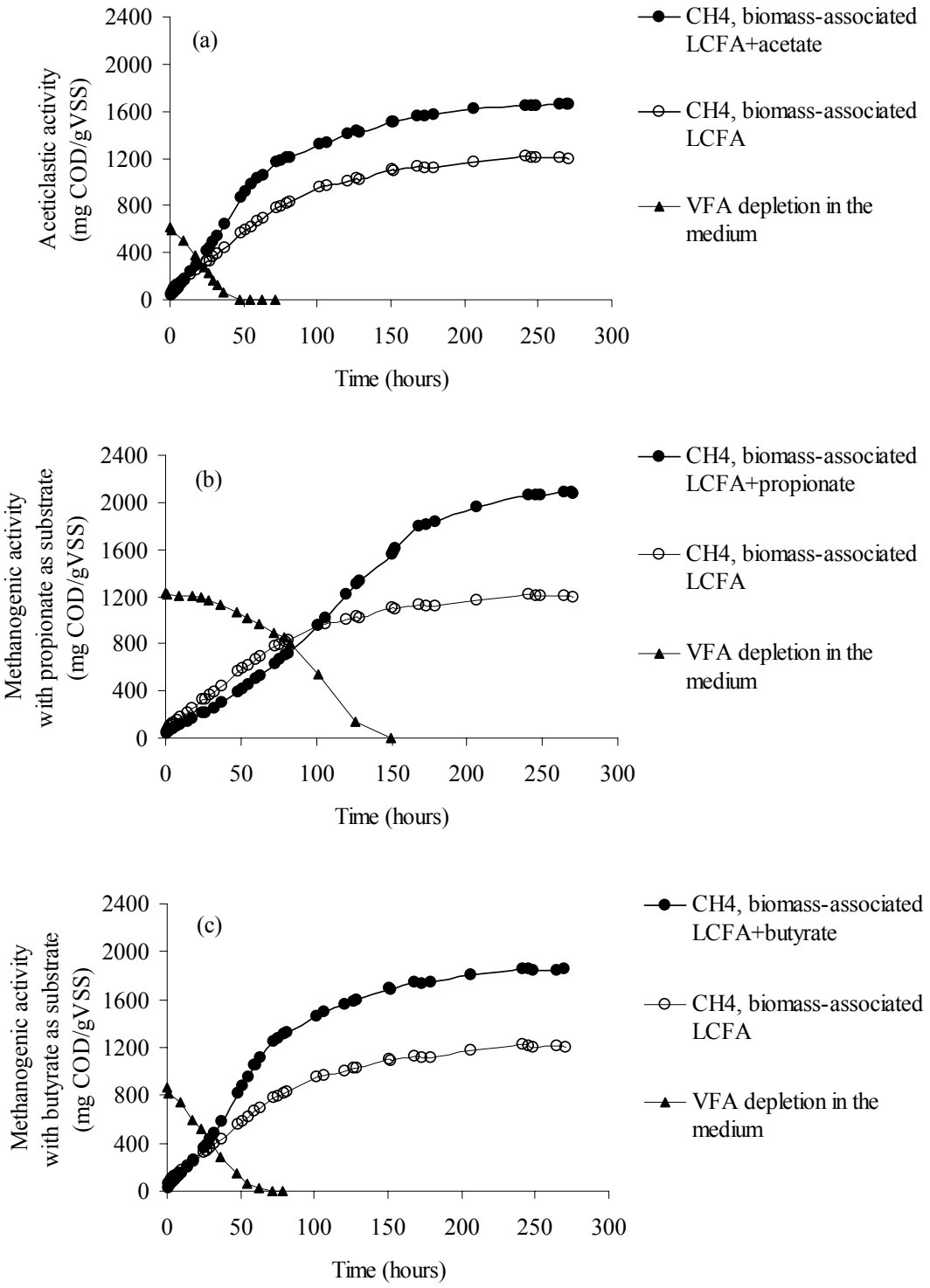

Figure 6.3 Specific methanogenic activity in the presence of acetate (a), propionate (b) and butyrate (c), before degrading the biomass-associated LCFA.

Table 6.3 Specific methanogenic activity before (measured by VFA depletion in the medium and CH_4 production) and after degrading the biomass-associated LCFA (measured by CH_4 production) (Mean±SD).

Specific methanogenic activity (mg COD/(gVSS.day))			
In the presence of:	Before degrading accumulated LCFA		After degrading accumulated LCFA
	VFA depletion	CH_4 production[(b)]	CH_4 production
Acetate	362±3	387±8	326±13
Propionate	47±5	172±11	43±2
Butyrate	359±13	345±6	146±6
H_2/CO_2	-	1424±94	1669±81
No added substrate[(a)]	(n.d.)	244±27	(n.d.)

n.d.- non-detectable.

[(a)] corresponds to the methanisation of the biomass-associated LCFA.

[(b)] without discounting the production of the blank controls (no added substrate).

Concerning propionate a different behaviour was found (Figure 6.3 (b)). The initial methane production was lower than in the situation where the sludge was allowed to mineralise the accumulated LCFA (no added substrate, Table 6.3). In the former situation, methane production seems to be a result of the interaction between both substrates (propionate+LCFA) degradation, since there was no evidence of a sequential consumption pattern.

In this experiment, if the background methane production of the "blank" was discounted, significantly lower values of specific activity with acetate, propionate and butyrate were obtained, i. e. 143, 0, and 101 mg COD/(gVSS.day), respectively. These results could therefore mislead conclusions about the severe LCFA inhibitory effect presented in Figure 6.2. This also points out the importance of measuring the methanogenic activity by following methane production, only when blank control assays do not exhibit a significant background methane production as is the case of adsorbable biodegradable substrates.

It should be noticed that the present results correspond to a situation of sludge specific LCFA-load that allows optimum methanisation of the accumulated LCFA, and in which the

decrease of the activity with the specific substrates seems to be merely a decrease on the initial methane production. For higher amounts of accumulated LCFA, the decrease of the activities is a result of lag phases preceding methane production which are likely caused by reversible inhibitory effects or by transport limitation effects. This was previously observed in Chapter 5.

After mineralisation of the biomass-associated LCFA, the specific methane production rate from the added acetate did not change significantly when compared to acetate consumption in the presence of the accumulated LCFA (Table 6.3). The same behaviour was found for propionate. In the case of butyrate, a lower mineralisation of this substrate was found after depletion of the accumulated LCFA. Concerning hydrogenophilic activity, an increase was also found after mineralisation of the biomass-associated LCFA. In the former situation, i. e. in the presence of the accumulated LCFA, the added H_2/CO_2 was converted to methane within 30 hours. During this time period, methane production in the blank controls (N_2/CO_2) was not significant, which may suggest that this substrate is also preferentially consumed.

It is also important to notice that, for all the tested substrates, the sludge either when loaded with LCFA or after mineralisation of this accumulated LCFA, exhibited higher activities than before being submitted to the contact with LCFA, i. e. the seed sludge (sub-chapter 6.2.1). This effect was more pronounced for hydrogenophilic and aceticlastic activity.

6.4 CONCLUSIONS

The methanisation kinetics of the biomass-associated LCFA (mainly palmitic acid) was established according to an inhibition model based on Haldane's enzymatic inhibition kinetics, using a total of 38 samples of encapsulated-loaded sludge taken from different types of reactors fed with oleic acid. Based on this model, 1080 mg COD-CH_4/gVSS was found to be the critical amount of accumulated LCFA, after which inhibition of its own degradation to methane occurs. For this optimal value of LCFA-loaded sludge, a maximal mineralisation rate of 250 mg COD-CH_4/(gVSS.day) could be achieved. Near this optimal specific concentration of LCFA, different patterns of interaction of the added VFA with the degradation of the biomass-associated LCFA were found. Although acetate and butyrate were preferentially consumed by the consortium, in the case of propionate no evidence of a sequential consumption pattern could be withdrawn. However, in terms of overall mineralisation, the presence of these VFAs did not significantly delay the obtained methane production.

From a practical viewpoint, and considering the interest of using sequential adsorption (accumulation) and degradation (methanisation of the accumulated LCFA) steps for the treatment of lipid/LCFA based effluents, previously suggested in Chapter 2, these results suggest that, in order to optimise methane production due to the mineralisation of LCFA accumulated onto the sludge, a maximum specific LCFA-load of about 1000 mg COD/gVSS should be promoted before the degradation step. In these conditions, if during the degradation step acetate, propionate or butyrate is present in the medium, and at least in concentrations not higher than 30 mM for acetate and propionate or 15 mM for butyrate, no significant delay in the obtained methane production is induced.

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7 CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH

From the work presented in this thesis some major conclusions can be concluded:

1. In reactors continuously fed with oleic acid, palmitic acid accumulated onto anaerobic suspended and granular sludge. This LCFA was efficiently biomethanised in batch assays, but the addition of oleic acid inhibited this conversion. The application of stirring conditions (150 rpm) enhanced the biomethanisation rate, when compared to static conditions. The maximum accumulated palmitate and the rate of methane production were highly dependent on biomass structure, suspended sludge being more efficient than granular sludge for that purpose.

2. The application of image analysis techniques gave important insights on the relative importance of fragmentation and filament release during granular sludge deterioration. Free filaments (those that have at least one free extremity) have an important role in the accumulation of biomass-associated LCFA as well as in their degradation rate. In granular and suspended sludge, the composition of the bacterial community, based on 16S rDNA sequence diversity, was more affected during the oleate loading process than the archaeal consortium. The archaeal consortium remained rather stable in the granular sludge, whereas in the suspended sludge, the relative abundance of *Methanosaeta*-like organisms became gradually weaker. The combination of molecular-based sludge characterisation with physiological and morphological characterisation allowed a better understanding of the mechanisms of sludge disintegration, flotation and washout, in relation with the contact/accumulation of LCFA.

3. The anaerobic consortia remained active after being loaded with high amounts of LCFA. In a loaded sludge, only methanogenic activity against H_2/CO_2 and ethanol were detected. However, after allowing the mineralisation of the accumulated LCFA, all the measured activities were significantly enhanced. The results obtained put in evidence the possible strong effect of diffusion limitations imposed by the LCFA layer that could hamper the access of the added substrates to the cells as well as the subsequent biogas release.

4. Based on the kinetics of methanisation of the biomass-associated LCFA, an optimal specific LCFA-load of about 1000 mg COD/gVSS should be promoted in order to achieve a maximal mineralisation rate of this substrate. Near this optimal value, the addition of acetate, propionate or butyrate did not significantly decrease the methane production rate obtained for the biomass-associated LCFA.

5. From a practical viewpoint, the results presented in this work suggest that, in order to enhance methane production, it should be advantageous to sequence cycles of adsorption/accumulation and degradation, when treating effluents with high lipid/LCFA content.

This work therefore constitutes a basis for the technological development of an efficient sequential system that allows the biomethanisation of such effluents. However, further research is needed to better understand the mechanisms involved in LCFA biodegradation, the interactions with different co-substrates and the relative importance of transport limitation and metabolic inhibition phenomena. Furthermore, modelling of the process and development of expert systems will be of utmost importance for the application to particular real cases.

APPENDIX A

Calibration of the pressure transducer

Before starting an activity, toxicity or biodegradation test, the calibration factor of the hand-held pressure transducer was determined, according to the following procedure:

1. A 70 ml vial was totally filled with water, sealed with a gas-tight butyl rubber bung and caped with a one piece aluminium seal. Possible air bubbles present in the vial were removed by injecting water into the vial using a syringe and allowing air bubbles to escape through a needle injected simultaneously with the syringe.

2. Exactly 10 ml of water was then removed from the vial (using a syringe). This procedure is made easier by injecting a second needle through the bung.

3. Afterwards, the vial was vented and the mV reading recorded. This 0 ml reading measures the background pressure in the vial at atmospheric pressure.

4. 1ml air was injected into the vial and the correspondent mV reading recorded.

5. Steps 3 and 4 were repeated using volumes between 1 and 20 ml. Triplicate readings were done for each volume of air being injected. The mV values obtained at 0 ml reading (after ventilation of the vial) were subtracted to each correspondent mV reading at the particular volume being measured.

Figure A.1 presents an example of a pressure transducer calibration curve where the linear correlation, in the range of pressures until 2 bar, can be seen.

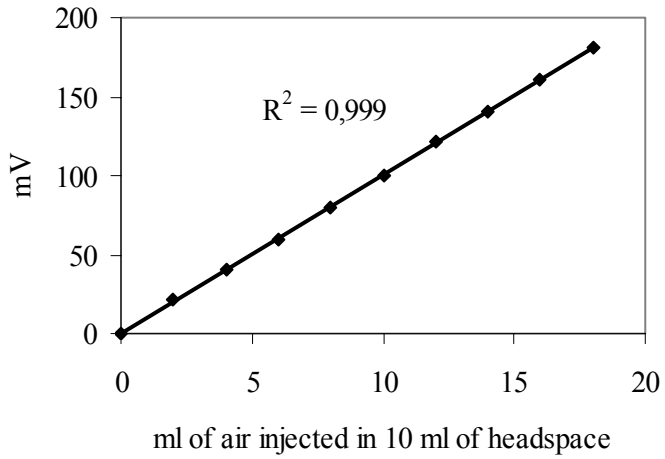

Figure A.1 Example of a pressure transducer calibration curve.

Ideally, when 10 ml of air are injected in 10 ml of headspace, a value of 100 mV should be read. However, a divergence on the reading may occur, for instance 103.4 instead of 100 mV. This deviation is taken into account and corrected in the calibration factor, as well as the conversion to Standard Temperature and Pressure conditions (STP) (equation A.1).

$$FC = \frac{100}{103.4} \times \frac{273}{(273+37)} = 0.852 \qquad \text{(A.1)}$$

APPENDIX B

Extraction Method to Analyse LCFA Accumulated onto Anaerobic Sludge

Introduction

The adsorption of oleic acid and other long chain fatty acids (LCFA) on the surface of anaerobic sludge is described in the literature (Hanaki *et al*, 1981, Alves *et al*., 2001). Ning *et al.* (1996) postulated that anaerobic bio-sorption is mainly a physico-chemical process, but experiments performed by Hwu (1997) have shown that oleate became adsorbed prior to be biodegraded and that after starting the methane production, a certain amount of oleate was desorbed due to biogas release. Alves *et al*., 2001 found that, when feeding an anaerobic sludge with oleic acid as a sole carbon source, it became encapsulated by a whitish matter, which was efficiently degraded to methane in batch vials without any added substrate. Important information about these adsorption/degradation phenomena and their mechanisms could be retrieved by the identification of the adsorbed matter composition. An oleic acid extraction method applied to the liquid medium using a methylation reagent was reported by Hwu (1997). Through methylation acids are transformed in methyl esters, which are more easily detected on Gas Chromatography allowing a fast analysis (Hwu. 1997). However this method was only applied to the liquid medium and not to the adsorbed matter.

The aim of this work was to develop an extraction method to analyse the LCFA adsorbed onto anaerobic sludge.

Materials and Methods

Samples source

Extraction methods were assayed in anaerobic biomass collected at the end of the operation of a EGSB reactor (RII) fed with oleic acid at concentrations up to 8 g of chemical oxygen demand (COD)/l as sole carbon source (Chapter 2, p.51). The choice of this particular sludge was specially made since this sludge exhibited a considerable amount of adsorbed fatty matter.

Extraction Methods

Four different methods were assayed in order to choose the best one or an adaptation to apply on this particularly case.

Method 1 - Method reported by Hwu (1997) to extract oleic acid from the aqueous phase.

Method 2 - Samples from the reactor were collected and centrifuged (4000 rpm, 5 min). A known volume of supernatant was taken, and placed into separating funnels. A solution of internal standards (heptanoic-C7 and pentadecanoic acid-C15) was added to the sample, and, after acidification to pH 2, a multiple extraction with 5x1 ml of petroleum ether was applied. The ether phase was transferred to glass vials, immediately capped, and stored at –4ºC. Two different fatty acids were used as internal standards in order to cover a high range of chain sizes. Heptanoic acid (C7) was used as a standard in the analysis from C2 to C8 and pentadecanoic acid (C15) was used to determine C10 to C18 LCFA concentrations. When applied to the biomass the procedure was the same, but a known amount of biomass was used instead of the supernatant. The biomass was washed twice with anaerobic buffer during centrifugation.

Method 3 - The extraction procedure in this case was similar to method 2, but with some modifications in order to apply it to the biomass. Samples from the reactor were collected, washed with gluteraldehyde 0.3 % and centrifuged (4000 rpm, 5 min). A known amount of biomass was suspended in a known volume of gluteraldehyde buffer, stirred for 20 minutes and sonicated for 10 minutes in order to detach the adsorbed fatty matter from the biomass. The samples were centrifuged and washed in the same conditions as described in method 2.

Method 4 - Samples from the reactor were collected, washed and centrifuged (4000 rpm, 10 min) twice with anaerobic basal medium. A known volume of each sample was dried at 105ºC. Dried samples were collected, and placed into separating funnels. Due to the drying step at 105ºC, detection of volatile fatty acids is not expected and thus solution of internal standard containing only C15 was added to the sample. After acidification to pH 2,

a multiple extraction with 5x1 ml of petroleum ether was applied. The ether phase was then transferred to glass vials, immediately capped, and stored at –4ºC.

Gas chromatography (GC) analysis

Volatile and LCFA concentration were determined by a gas chromatograph (CP-9001 Chrompack) equipped with a flame ionisation detector (FID) and a split/splitless injector. LCFAs were separated on an FFAP-CB 25m x 0,32mm x 0,3µm column (Chrompack), using nitrogen (N_2) as carrier gas at 35KPa, 31:1 split rate. The oven temperature was 40ºC for 0.2 min, with a 5ºC/min gradient to 250ºC, and a final hold in time of 15 min of 250ºC. Figure B.1 illustrates the retention times of a standard LCFA solution from C2 to C18 separated under these conditions.

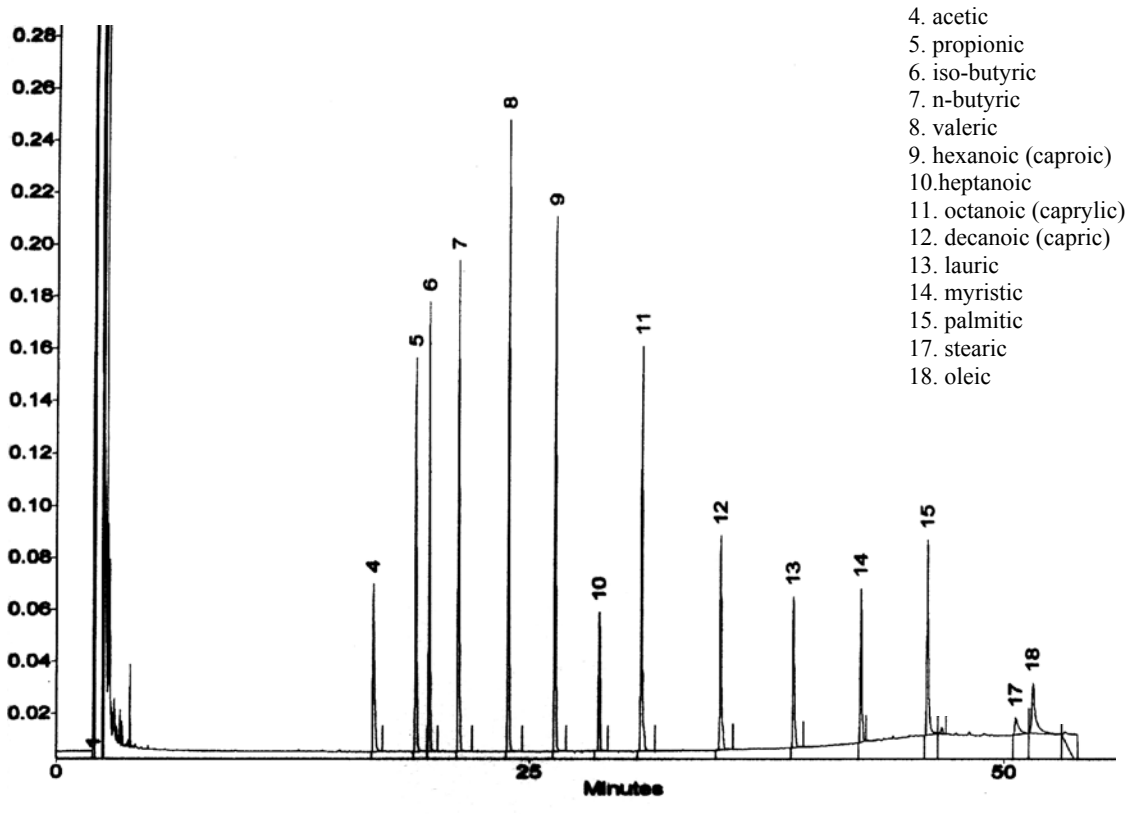

Figure B.1 Chromatogram obtained from de injection of 1µl of a sample with 100 mg/l of LCFA from C2 to C18.

Results and Discussion

Method 1 was assayed on aqueous samples with a known amount of LCFAs from C2 to C18, which were methylated under the treatment. A very complex mixture was obtained and the identification under GC analysis became difficult. In other hand, it was no way to ensure the methylation of all LCFAs presents on the samples, which gave rise to the detection of both methyl esters and acids by chromatography. When method 2 was applied to the same sample better results were obtained. Compounds were well separated with high peak signals, which allowed a good identification. However, this method did not result when applied to the biomass. In this case after the extraction with petroleum ether, a clear whitish layer (Figure B.2 (a)) could be observed around the darker biomass layer, separating the organic and the aqueous phases formed.

Method 3 did not result in any further improvement. A reproducible detachment from the biomass of adsorbed matter was not guaranteed. It was then decided to develop a new method (method 4) based on samples drying. The dried residue exhibited an efficient dissolution on the organic solvent, ensuring a more efficient LCFA extraction. Two phases separation was clearly observed, i.e., an organic phase with a transparent yellowish colour and a solid phase containing the biomass (Figure B.2 (b)).

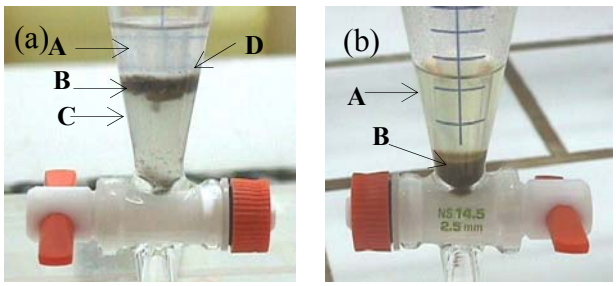

Figure B.2 Aspect of phase separation when extracting the fatty matter from a biomass sample using (a) method 2 and (b) method 4. A- organic phase; B- biomass; C- aqueous phase; D- whitish layer.

Table B.1 summarises the results obtained after extraction and GC analysis of the LCFA content of the sludge dried residue and Figure B.3 shows an example of one chromatogram obtained.

Table B.1 LCFA content of the accumulated matter present in the sludge (Mean±SD of triplicates).

LCFA	mg/mg dry weight
Lauric acid (C12:0)	n.d.(*)
Myristic acid (C14:0)	0.015±0.003
Palmitic acid (C16:0)	0.279±0.028
Stearic acid (C18:0)	0.007±0.002
Oleic acid (C18:1)	0.009±0.006

(*)value <0.005, considered non-detectable.

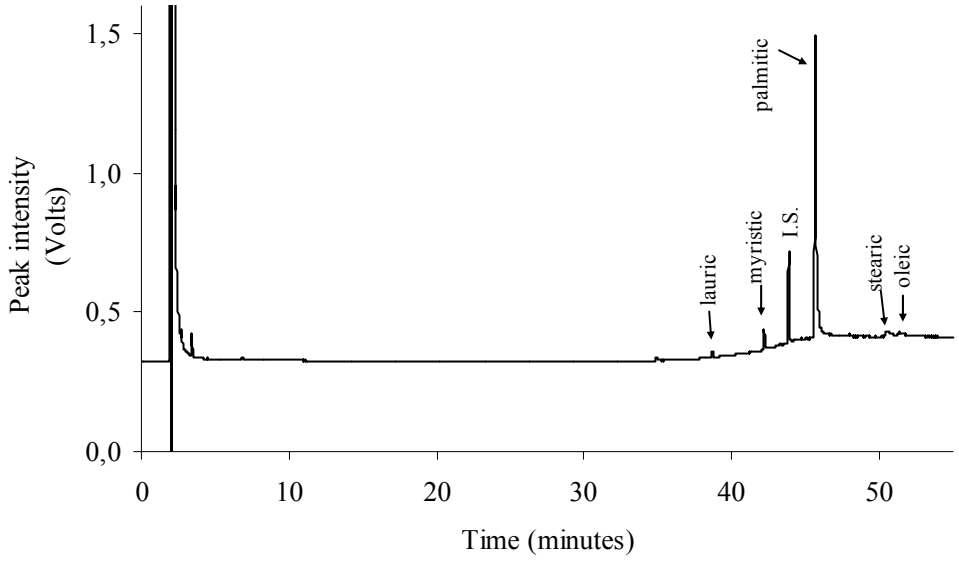

Figure B.3 Chromatogram obtained from de injection of 1µl of the organic phase resulting from the extraction of a dried sample taken from the EGSB reactor (I.S. – internal standard (C15)).

Conclusions

A method to extract fatty matter adsorbed onto anaerobic sludge was developed after comparing several alternatives. The best results were obtained by drying the biomass sample for 24 h at 105ºC, following by acidification at pH 2 and applying a multi-stage petroleum ether extraction. Gas chromatography analysis after this extraction method allowed consistent detection and quantification of the different long chain fatty acids. Due to the drying step at 105ºC, this method can no be applied for detecting volatile fatty acids.

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PUBLICATIONS LIST

The results presented in this thesis are partially published/submitted in:

INTERNATIONAL JOURNALS

Amaral, A.L., Pereira, M.A., da Motta, M., Pons M.-N., Vivier, H., Mota, M., Ferreira, E.C., Alves, M.M. (2003) Image analysis as a tool to detect and quantify morphological changes in anaerobic sludge: II. Application to a granule deterioration process triggered by the contact with oleic acid. *Biotechnol Bioeng*. (submitted).

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