Årsager til proces-ustabilitet i biogasanlæg og strategier for forebyggelse og genopretning af processen

EFP-2005.J.nr.:33031-0029

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Publication date:
2009

Document Version
Også kaldet Forlagets PDF

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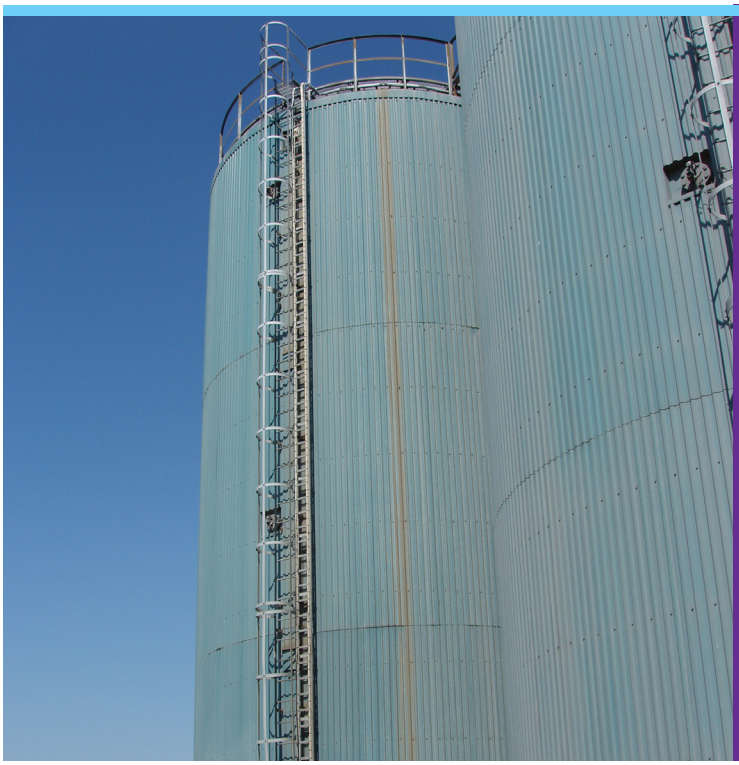

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ISBN.nr.: 978-87-91855-75-7

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Indledning:

Biogas har været anvist som et at de områder hvor der er positiv samfundsøkonomisk regnskab. Udbredelse af biogas kræver dog fortsat optimering og forbedring af anlæggets økonomi. Uforudsete procesudfald forekommer med mellemrum på biogasanlæg, hvor processen pludselig hæmmes og biogasproduktionen ophører helt eller delvist, ofte uden at årsag kan identificeres entydigt. Disse procesuheld varer ofte relativt lang tid med alvorlige økonomiske konsekvenser for anlæggene. Der mangler stadig grundlæggende viden med henblik på at identificere årsager og mekanismer, dels for at kunne forebygge og, hvis uheldet indtræffer, hurtigst muligt at kunne genoprette processen.
Det overordnede mål med projektet har været at udvikle værktøjer til at forstå og undgå procesudfald og opnå en mere stabil drift i danske biogasanlæg. Man kan dog stadig, på trods af agtpågivenhed, forvente at procesudfald ind i mellem vil opstå på de danske biogasanlæg, og projektet her derfor også fokuseret på udvikling af forskellige strategier til genopretning af processen når uheldet har været ude.

I denne rapport er resultater fra projektet EFP 05 med titel: ”Årsager til proces-ustabilitet i biogasanlæg og strategier for forebyggelse og genopretning af processen” rapporteret.

Sammenfatning:

Arbejdet i projektet har været koncentreret omkring 3 emner:

1: Kortlægning af årsager til ubalance i Danske biogasanlæg
Det indledende arbejde var koncentreret om indsamling af data og en række interviews af forskellige biogasfællesanlæg. Fra dette kortlægningsarbejde kom det frem at de hyppigste årsager til ubalancer var:

- høj koncentration af ammoniak
- høj koncentration af langkædede fedtsyrer
- skumning i forlager- og rådnetanke
- temperaturforstyrrelser

En korrelation mellem øget rest-biogasproduktion (suboptimale proces betingelser) og høj fraktion af industriaffald i råvare blev også observeret. Proces-ubalancer og suboptimal drift opstår hovedsageligt på grund af:

- utilstrækkelig viden om biomassens sammensætning,
- utilstrækkelig viden om biomassens nedbrydning karakteristika,
- utilstrækkelig proces overvågning, især med hensyn til flygtige fedtsyrer, og
- utilstrækkelig forlagerkapacitet hvilket forårsager uhensigtsmæssig blanding og hindrer nøjagtig dosering af de forskellige biomasser.

2: Strategier for etablering af biogas processen efter ammoniumhæmning

Formålet med denne undersøgelse var at afprøve forskellige strategier for at finde den bedste strategi mht. den hurtigste proces-genoprettelse efter ammoniumhæmning. Der blev både udført batch og kontinuerlige reaktoreksperimenter. Biogasprocessen blev hæmmet med tilsætning af ammonium direkte i reaktor og efter 3-5 dage blev følgende strategier forsøgt:

a) Fortynding med vand (50% fortynding)
b) Fortynding med podemateriale (50% fortynding)
c) Fortynding med frisk gylle eller
d) Ingen fortynding (vente på at processen selv reetablerer sig).

Strategierne a) til c) med forskellige former for fortynding medførte den hurtigste procesgenoprettelse i forhold til d) uden fortynding. Den største methanproduktion under proces-genoprettelsen blev opnået ved fortynding med frisk gylle. Processen var dog ikke stabil og en stor koncentration/ophobning af propionat blev observeret under forløbet. Dette kan tyde på en usikkerhed ved at anvende denne strategi, idet den potentielt kan medføre yderligere procesustabilitet hvis genopretningsforsøg ikke lykkes umiddelbart. Derfor anses den bedste og sikreste strategi for oprettelse af biogasprocessen at være fortynding af den hæmmede proces med podemateriale (udrådnet gylle) for en hurtig og stabil reetablering af processen. Denne metode kan evt. kombineres med vand og/eller

frisk gylle fortynding, afhængigt af omstændighederne og tilgængelighed af egnet podemateriale.

Udover laboratorieforsøgene fulgtes processen i et fuld-skala biogasanlæg som var ammoniumhæmmet pga tilsætning af minkgylle. Som reetableringsstrategi blev anlægget fodret med frisk gylle (uden minkgylle) hvilket gradvist reducerede ammoniumkoncentrationen i reaktoren. En fuldstændig reetablering af processen blev opnået efter 31 dage, hvilket er signifikant længere tid end i laboratorie eksperimenterne. Fra både laboratorie og fuldskala observationer kan man konkludere at man med fordel kan genoprette processen med en fortyndingsstrategi. Strategien med at vente (til at processen selv stabiliseres) var det dårligste valg.

3: Strategier for genetablering af biogasprocessen efter lipidhæmning

Formålet med denne undersøgelse var at forsøge forskellige strategier for at finde den bedste måde til at genoprette processen efter hæmning ved tilsætning af fedtstoffer. Biogasprocessen blev hæmmet ved tilsætning af 5 g/l oleat direkte i reaktoren. Reetablerings strategier der blev anvendt kan deles i følgende typer:

- Indfødningsstrategier:
    - ingen indfødning eller,
    - kontinuerlig indfødning med frisk gylle (HRT 20 dage).
- Fortyndingsstrategier - Erstatning af 40% af reaktorindholdet med:
    - frisk gylle
    - podemateriale (udrådnet materiale fra reaktorer før hæmningen)
    - vand
- Absorptionsstrategier - Tilsætning af:
    - fibre (filtreret udrådnet gylle)
    - bentonit, i samme mængde som den tilsatte oleat dvs. 5 g-VS/l.

Eksperimenterne blev udført i 2 faser, hvor indfødningen af reaktorerne med gylle blev stoppet i fase 1 efter introduktion af hæmningen, hvor imod fase 2 koncentrerede sig om

at finde genoprettelsesstrategier hvor indfødning af reaktorerne med gylle ikke blev stoppet efter introduktion af hæmningen, men derimod fortsat blev fodret med gylle.

Resultaterne kan opsummeres som følgende:
Samudrådning af gylle med fedtholdigt affald kan forbedre biogasproduktionen og dermed økonomien i gyllebaserede biogasanlæg. Fedt er dog potentielt hæmmende for biogasprocessen og biogasanlæg kommer til tider ud for ubalance pga af tilsætning af fedtholdigt affald. Langkædede fedtsyre (LCFA) koncentrationer højere end 1.0 g L^{-1} hæmmede gylleudrådning i batch og semi-kontinuerte forsøg, som resulterede i midlertidigt ophør/reduktion af biogasproduktionen. LCFA hæmningerne var reversible.
Af de undersøgte genopretningsstrategier, viste det sig at den mest anvendte strategi, som er at stoppe indfødning og **afvente** processens selv-stabilisering, var den dårligste strategi. Proces genopretning var langsomst og processen var mest ustabil med meget høje VFA niveauer. Reetableringsstrategier med f**ortydning** af reaktorerne med **aktivt podemateriale** fra en "sund" reaktor, for at forøge biomasse/LCFA forhold, eller tilsætning af **lipidabsorberende materiale** for at adsorbere LCFA og dermed reducere den aktive LCFA koncentration, var de bedste genoprettelses strategier. Effekten af fiber tilsætning var sammenlignelig med bentonittilsætning.
Gentagen udsættelse af processen for oleat-belastning **medførte større robusthed** i processen mod hæmning. Dette er konsistent med tidligere undersøgelser som viste at det var ophobning af fri LCFA som var den hæmmende komponent, når mikroflora ikke tidligere var tilvænnet lipid og dermed havde opbygget fornøden kapacitet til at nedbryde fri LCFA i takt med frigivelse fra indledende nedbrydning af lipid.

I løbet af projektet er udarbejdet et antal artikler.

Disse er vedlagt denne rapport, hvor en mere detaljeret beskrivelse af forsøg og resultater kan findes.

Følgende artikler er vedlagt:

1) Nielsen H.B. and I. Angelidaki (2008). Codigestion of manure and industrial organic waste at centralized biogas plants: process imbalances and limitations. Water Science Technology; 58.7:1521-1528.
2) Nielsen H.B. and I. Angelidaki (2008). Strategies for an optimized recovery of the biogas process following ammonia inhibition. Bioresource Technology; 99(17):7995-8001.
3) Palatsi J.; Laureni M.; Andres M.V.; Flotats X., Nielsen H.B., Angelidaki I. (2009). Strategies for recovering inhibition caused by long-chain fatty acids on anaerobic thermophilic biogas reactors. Bioresource Technologt; 100:4588–4596.
4) Palatsi, J., Illa, J., Prenafeta-Boldu, F.X., Laureni, M., Fernandez, B., Angelidaki, I and Flotats, X. (2009). Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: Batch tests, microbial community structure and mathematical modelling. Accepteret for publicering i Bioresource Technology.
5) Nielsen H.B. and Angelidaki. Genetablering af biogasprocessen. FiB (Forskning i Bioenergi), 22: Dec. 2008

Codigestion of manure and industrial organic waste at centralized biogas plants: process imbalances and limitations

H. B. Nielsen and I. Angelidaki

ABSTRACT

The present study focuses on process imbalances in Danish centralized biogas plants treating manure in combination with industrial waste. Collection of process data from various full-scale plants along with a number of interviews showed that imbalances occur frequently. High concentrations of ammonia or long chain fatty acids is in most cases expected to be the cause of microbial inhibitions/imbalances while foaming in the prestorage tanks and digesters is the most important practical process problem at the plants. A correlation between increased residual biogas production (suboptimal process conditions) and high fractions of industrial waste in the feedstock was also observed. The process imbalances and suboptimal conditions are mainly allowed to occur due to 1) inadequate knowledge about the waste composition, 2) inadequate knowledge about the waste degradation characteristics, 3) inadequate process surveillance, especially with regard to volatile fatty acids, and 4) insufficient pre-storage capacity causing inexpedient mixing and hindering exact dosing of the different waste products.

Key words | centralized biogas plants, codigestion, industrial waste, process imbalances

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INTRODUCTION

Today, 20 centralized biogas plants (Figure 1) and more than 60 farm-scale plants are in operation in Denmark. The main purpose of the centralized plants is to treat livestock manure and reuse the material as fertilizer (Ahring *et al.* 1992; Hjort-Gregersen 1999; Seadi 2000). The methane yield from manure is relatively small and in order to increase the biogas production, the plants co-digest manure together with other organic waste from food industries and municipalities (Angelidaki & Ellegaard 2003). The co-substrates–rich in lipids, proteins and carbohydrates–are essential for the plant's economy, but might lead to disturbances if not handled properly. Several of the Danish centralized biogas plants have been exposed to process imbalances that could be directly related to the composition of the substrate. However, the significance of the problem is unknown and only a few studies has been carried out (Planenergi 2001; Hartmann *et al.* 2004; Angelidaki *et al.* 2005). In the present study we, therefore, focus on this topic. We present data obtained from several of the Danish centralized biogas plants and give examples of imbalances caused by the treatment of organic industrial waste. We propose reasons for the cause of the imbalances on a practical and microbial level and verify our theories with data from experiments in our laboratory.

doi: 10.2166/wst.2008.507

MATERIALS AND METHODS

Biogas output and screening of process imbalances at the biogas plants

Process data, i.e. biogas production, from the plants was obtained directly from the plants (measured daily) or via the Danish magazine "Dansk Bioenergi" (monthly average).

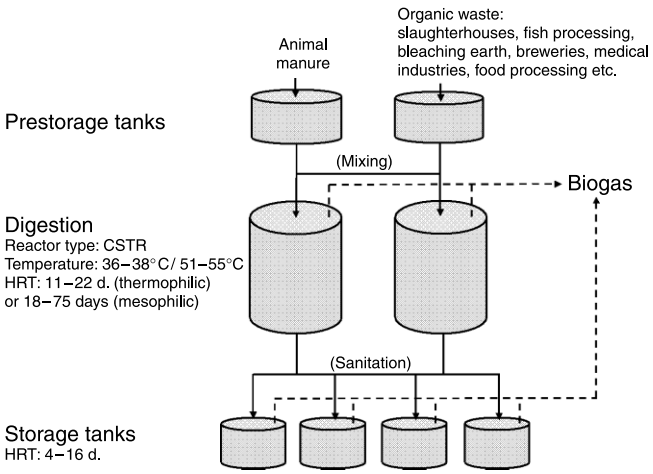

Figure 1 | The typical process flow at Danish centralized biogas plant. The plants range in digester size from 750 m^3 to 8500 m^3. Approximately 75% of the bio mass treated is animal manure while the remaining 25% mainly consists of waste from the food processing industry. It should be noted that variations from this illustration exists. For instance, is the number of pre-storage tanks and digesters between 1 and 3.

A number of interviews with plant managers were also carried out. The obtained data and information was analyzed and used for estimating the frequency of the process failures and for setting up additional experiments (case studies) investigating relevant topics related to specific inhibition incidents at the plants.

Case studies

Effect of codigestion of manure together with blood

The effect of temperature on the process stability during codigestion of blood and cattle manure was investigated in a lab-scale experiment consisting of two 4.5 litre continuously stirred tank reactors (CSTR). One reactor was operated at mesophilic conditions (37°C) with a hydraulic retention time (HRT) of 20 days and 4 litres working volume. The reactor was inoculated with digested material from a mesophilic full-scale biogas plant. The second reactor was operated at thermophilic conditions (53°C) with a HRT of 15 days and a working volume of 3 litres. This reactor was inoculated with digested material from a thermophilic full-scale biogas plant. Both reactors were fed once a day with cattle manure (7.0% TS, 5.5% VS, pH 7.21) that was diluted with distilled water in a ratio of 10:7. During start up (approximately 4 weeks) the feed volume was slowly increased to 100 ml/d. From day 0–17 of the experimental period the loading was 100 ml/d (period 1) and from day 16–39 full loading–200 ml/d–was applied (period 2–4). From day 40 the reactors was fed 160 ml manure/d supplemented with 40 ml blood/d (19.1% TS, 18.0% VS, 16.0 g-N/l) (period 3). This procedure was continued until the end of the experiment in the mesophilic reactor while blood was omitted from the feedstock of the thermophilic reactor from day 60 due to a low methane production and high volatile fatty acid levels (VFA). From day 60 to the end of the experiment the thermophilic reactor was, therefore, only fed with 200 ml manure/d (period 4).

Toxicity test of tall oil

The toxicity effect of tall oil on the anaerobic digestion of cattle manure was tested in batch experiments. Tall oil is a viscous yellow-black odorous liquid obtained as a byproduct of the Kraft process of wood pulp manufacture. Tall oil contains rosins, unsaponifable sterols (5–10%), resin acids (mainly abietic acid), long chain fatty acids (mainly palmitic acid, oleic acid and linoleic acid, fatty alcohols, sterols, and other alkyl hydrocarbon derivates. To 1 litre serum bottles was added 150 ml cattle manure (7.0% TS, 5.5%) and 250 ml inoculum (3.6% TS, 2.8% VS) from a pilot-scale reactor treating cattle manure. The bottles were flushed with N_2, closed with butyl rubber stoppers and aluminium crimps, and incubated at 55°C. Eight days after when a steady methane production was obtained, the bottles were opened and different concentrations of tall oil were added: 0.1 g/l, 1.2 g/l, 3 g/l, 6 g/l, 10 g/L. Finally the bottles were flushed and closed as explained before, vigorously agitated and incubated at 55°C. Control bottles had no added tall oil and blanks consisted of 150 ml water and 250 ml inoculum without added tall oil. The experiment was performed in triplicates. The methane production was measured frequently during the entire experiment.

Estimation of the methane potential left in the residuals

Estimation of the residual methane production, left over in the effluent-biomass, was determined in digested biomass

from the main digestion step as well as from down stream digestion/storage steps from a number of centralized biogas plants. Samples of 300 ml was transferred to 1 litre serum bottles flushed with 80%/20% N_2/CO_2 and incubated at the same temperature as the main reactor were operating under. The methane production was measured frequently over a period of approximately two months. The ammonia concentration of the biomass was determined before incubation.

Analytical methods

pH and ammonia-N/total-N content were determined using standard methods (*Standard Methods for the Examination of Water and Wastewater 1995*). CH_4 production in batch experiments was measured by GC using flame ionization detection. CH_4 and CO_2 production from lab-scale reactors were determined by GC using thermal conductivity detection. For VFA determination, 1 ml samples was acidified with 70 μl 17% phosphoric acid, centrifuged at 10,500 rpm for 20 min, and analyzed on GC equipped with flame ionization detector.

RESULTS AND DISCUSSION

Examples of biogas output from centralized biogas plants and unknown process imbalances

As mentioned, the frequency of process imbalances is unknown but the overall impression from the data collection and interviews was that imbalances in average occurs approximately once per year at the plants. Typical examples of the biogas output from three different plants are illustrated in Figure 2a–c. During a period of 3 years one plant (Figure 2a) had 4 production failures all lasting 3–6 weeks while another plant (Figure 2b) had one severe process imbalance lasting for several months. The third plant that is illustrated (Figure 2c) was exposed to two severe imbalances during a period of 10 years. The cause of imbalance in all examples was unknown but according to the interviews inhibition by long chain fatty acids (LCFA) was suspected in Figure 2b, while ammonia inhibition was suspected in Figure 2c.

Examples of well defined process imbalances and case studies

Example 1: ammonia inhibition caused by degradation of blood

Figure 3a shows the reactor performance of a full-scale plant during digestion of blood. The plant has a reactor capacity of 7,600 m^3 and consists of three equal sized reactors that are operated at 53°C with a HRT of approximately 17–18 days. The plant treats approximately 362 tons manure/d together with approximately 75 tons/d alternative waste (organic industrial waste). From the beginning of September 2005 the organic industrial waste consisted of blood from pigs. An increase in ammonia concentration and VFA was seen immediately and from the middle of October a decrease in biogas production of approximately 32% was observed. The blood was omitted from the feedstock from the 10th of November and approximately 2 weeks after the biogas production was back at the original level. The whole inhibition period of the methane production lasted for approximately 6 weeks. Not surprisingly the data from the plant shows that the process imbalance could have been avoided if the warning by the increasing VFA concentrations had been applied in the operation procedures. Besides this, the sudden sharp increase in ammonia concentration also gave an indication of a rather unrestricted reactor operation and an unbalanced process. The data also raises the question if the operation temperature of the plant was suitable for treatment of the blood and if blood should have been added to the reactors at all. It is well known that the inhibitory effect of ammonia increases with temperature (Anthonisen *et al.* 1976). In this context, the process at the full-scale plant was simulated in a lab-scale reactor experiment at mesophilic and thermophilic temperatures (Figure 3b–d). The loading with blood was approximately the same as in the full-scale plant (18–20% w/w). As in the full-scale plant an immediate significant increase in VFA concentration in the thermophilic reactor was observed when blood was added, while a more moderate increase was seen in the mesophilic reactor. A clear increase in methane production was also observed (highest in the mesophilic reactor) due to an increase of the organic loading with easily degradable blood. This pattern was also seen in the full-scale plant. The methane

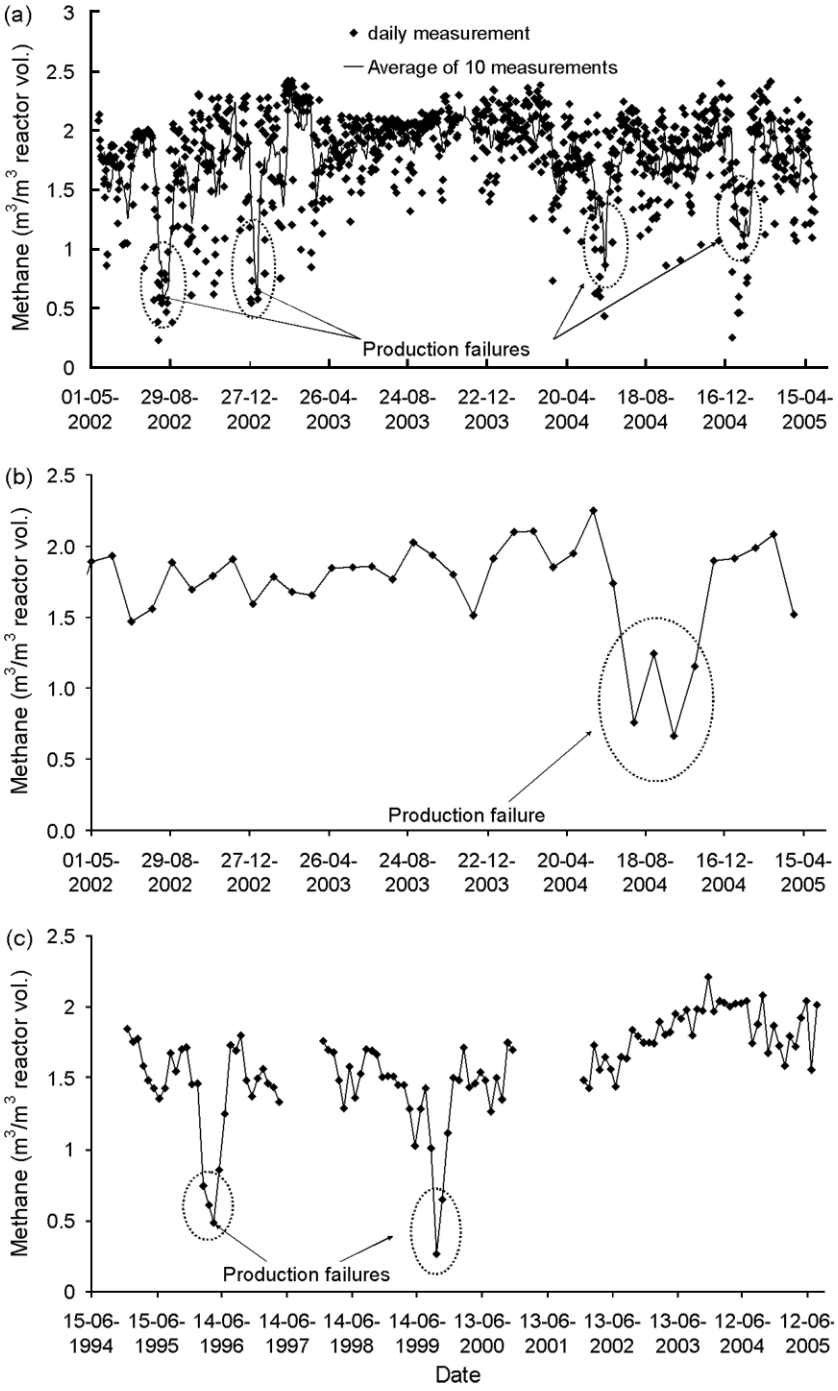

Figure 2 | Typical methane production profiles at three centralized biogas plants in Denmark. Several process inhibitions can be distinguished.

production in the thermophilic reactor started to decrease after only 6 days of feeding with blood and the production never fully recovered during the experimental period, despite the fact that the reactor was not added blood from day 60. An inhibition/decrease of the methanogenesis in the mesophilic reactor was also seen from approximately day 55. Interestingly, the free ammonia concentration (NH_3) in that reactor were not high and well below the inhibitory

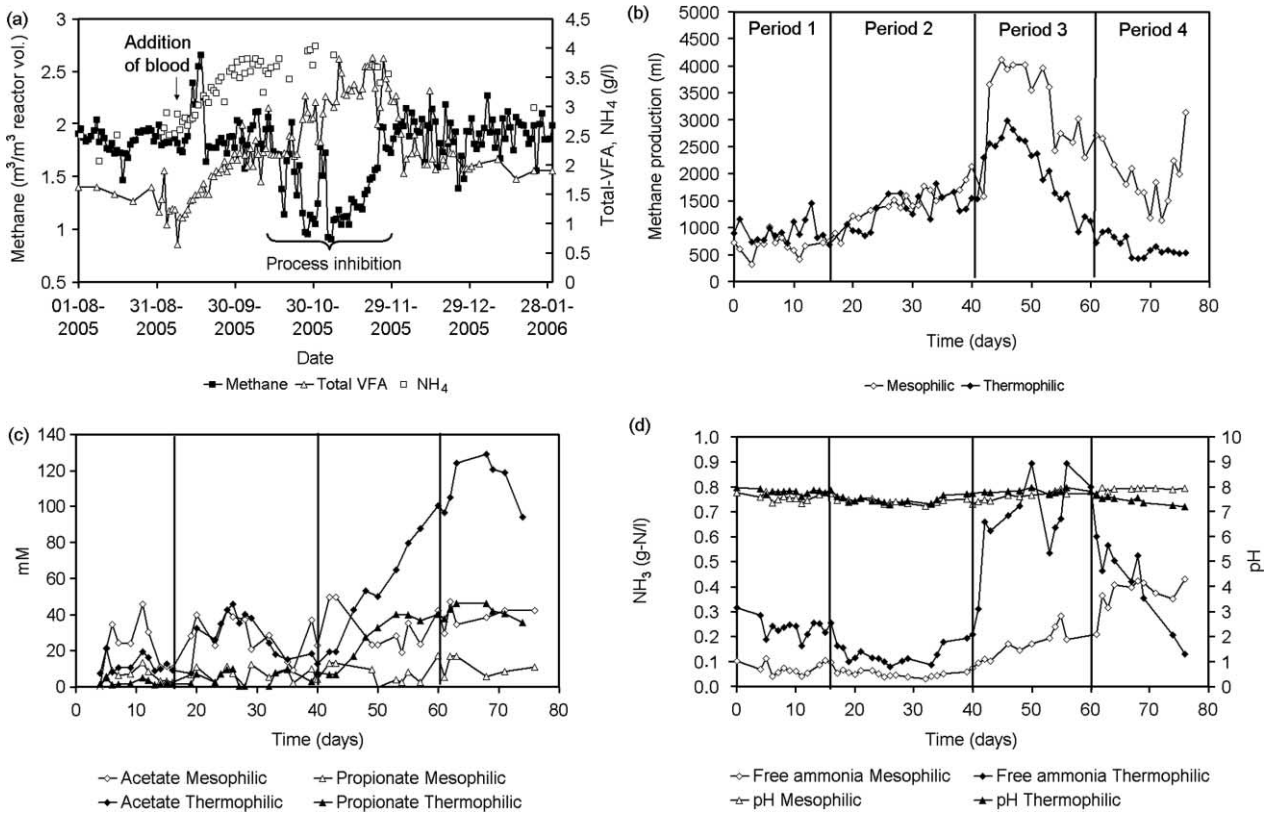

Figure 3 | Anaerobic co-digestion of pigs blood and cattle manure at (a) full-scale conditions and (b–c) lab-scale conditions. (b–d) Period 1: half loading (100 ml manure/d); Period 2: full loading (200 ml manure/d); Period 3: 40 ml blood/d and 160 ml manure/d; Period 4: 40 ml blood/and 60 ml manure/d in the mesophilic reactor, 200 ml manure/d in the thermophilic reactor.

level of 0.7–1.0 g-N/l that previously has been suggested (Angelidaki & Ahring 1993; Hansen *et al.* 1998). This pattern illustrates that not only free ammonia but also other components in the blood affected the process stability of the reactors.

The results of this case study show that operation temperature has a high impact on process stability during codigestion of manure with pig's blood. The results also show that it was not possible to obtain a stable codigestion of manure with blood neither at thermophilic nor mesophilic temperatures when applying the same loading conditions as at the full-scale plant. Therefore, we conclude that blood should only be added in small amounts and under careful process monitoring in order to avoid process inhibition at the plant. However, the present case study also illustrates one of the practical problems that many of the biogas plants are facing. Due to contract obligations the plants are sometimes forced to take in large amounts of industrial waste at inappropriate moments. Because of a limited prestorage capacity (Figure 1) the waste is subsequently fed to the reactors at a loading rate that is unsuitable for obtaining a stable process.

Example 2: acute inhibition by tall oil

During spring 2006 two mesophilic centralized biogas plants were subject to severe process inhibitions. In one of the plants, the reactors needed to be emptied and re-inoculated with digested biomass in order to re-establish the production. Prior to the inhibition the plant had been added tall oil twice within a few days in an amount of 6 g/l. Apparently, the tall oil had an acute toxic effect to the process. The methane potential of tall oil was estimated by the supplier to be "high", but no practical evaluation of the degradability/toxicity of the product was performed before it was added to the plant. The inhibitory threshold level of tall oil was evaluated in our laboratory (batch tests) and found to be as low as between 0.1 to 1.2 g/l (Figure 4).

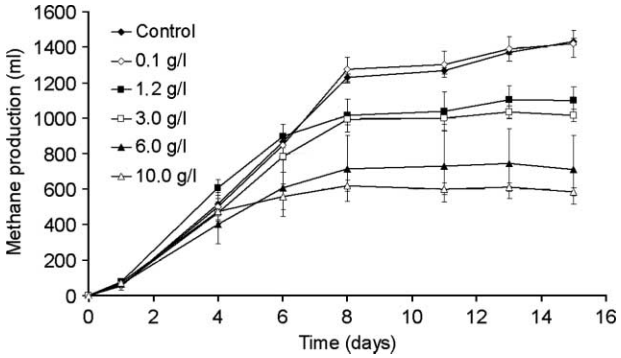

Figure 4 | Toxicity effect of various concentrations of tall oil on the anaerobic digestion of cattle manure in batch vials. The figure shows the development of methane production following addition of tall oil. Values are given as means of triplicates with standard deviations.

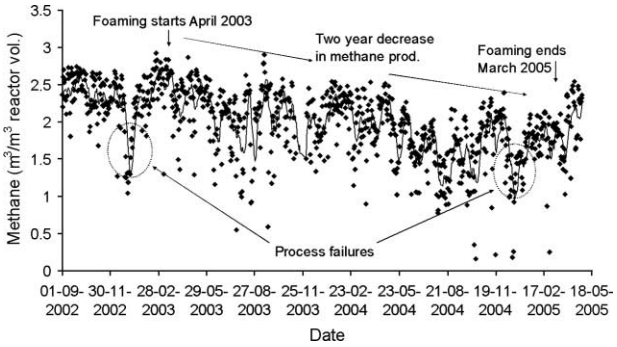

Figure 5 | Methane production profile of a centralized biogas plants during a period of frequent foaming in the pre-storage tanks and the reactors.

Although inhibitions caused by LCFA sometimes can be easily distinguish in batch experiments than in reactor systems (Nielsen & Ahring 2006), the results shows that the knowledge about the waste composition and its degradation characteristic was inadequate. This example of a process imbalance is also a result of a practical problem that the centralized biogas plants often are facing. The amount of industrial organic waste is inadequate and strong competition for this limited resource exists. In order to withhold an acceptable biogas production some plants are, therefore, willing to take risks and treat unknown waste products.

Example 3: foaming in pre-storage tanks and reactors

Foaming in the pre-storage is a problem repeatedly observed at the Danish biogas plants. A sudden lowering of pH due to inexpedient mixing of different waste types leading to a CO_2-stripping is normally considered as the main reason for foaming incidents. The practical reason for most of the foaming problems is a limited number (1–3) of pre-storage tanks forcing the plants to mix the different waste products before feeding to the reactors (Figure 1). Therefore, construction of more prestorage tanks would have helped in limiting this problem.

Sometimes foaming is not only observed in the pre-storage tanks but also occurs inside the reactors. This is illustrated in Figure 5. In this plant the foaming also affected the biogas production. Foaming started in the beginning of April 2003 and happened frequently during a period of almost 2 years. As a consequence of the foaming a slow but long term decrease in methane production was observed. Thus from June 2004 to March 2005 the production was 32% lower than before the foaming problems started. According to an interview with the plant the foaming could not be related to a specific substrate and the problem ended just as suddenly as it had started.

From the results of the case studies and data collection, it is our impression that more knowledge about the waste products is needed at the biogas plants. This is especially important with regard to different degradation characteristics such as the toxicity levels (exemplified in case study 2) and the formation of inhibitory by-products such as ammonia (exemplified in case study 1). This can be obtained by some of the simple experiments that were used in the present study. From a "practical point of view" other improvements could be obtained by constructing more pre-storage tanks. This action would help on foaming problems and at the same time ensure a more precise dosing of the individual waste product, for instance blood, which would help increasing the process stability. Finally, a more precise identification and removal of the complex/inhibiting waste types, such as tall oil, would be possible if more pre-storage tanks were build, since the product could be analyzed/tested before feeding it to the reactors. All in all, it is our believe that construction of more prestorage tanks would be very helpful and seems as a simple way for lowering the number of process imbalances at the centralized biogas plants in Denmark.

Methane potential left in the residuals

Besides actual process failures, approximately 25% of the biogas plants had an unexploited methane potential of 20 to 30% in the residual (Table 1). Such suboptimal

Table 1 | Methane loss (%) at the Centralized biogas plants related to various operation conditions. The loss was estimated as the amount of methane produced from the residuals compared to the methane production of the plant

Plant	Methane loss (%)	Temperature (m/t)*	Manure content (%)	HRT reactor (days)	HRT post-storage (days)	Ammonia-N (g/l)
<10% methane loss						
Filskov	2.9	t	68	9	50	3.3
Studsgaard	3.6	t	91	20	15	3.6
Vegger	4.4	t	81	19	34	3.1
Vaarst-Fjellerad	6.1	t	73	12	53	1.6
Blåhøj	8.3	t	83	15	16	3.1
Revninge	9.8	m	82	67	67	4.3
Average	5.9		79.7	23.7	39.2	3.2
10–20% methane loss						
Snertinge	10.3	t	59	20	6	3.0
Fangel	10.5	m	82	21	15	3.5
Lemvig	11.0	t	73	15	3	2.3
Hashøj	11.8	m	67	20	5	5.6
Nysted	14.0	m	87	32	15	4.4
Thorsø	15.0	t	94	16	6	3.8
Sinding-Ørre	17.4	t	72	18		1.7
Average	12.9		76.3	20.3	8.3	3.5
>20% methane loss						
Vester Hjermitslev	20.1	m	67	23	41	6.4
Lintrup	21.2	t	76	19	3	3.1
Blåbjerg	27	t	63	15	4	3.8
Ribe	30.7	m	71	11		2.8
Average	24.8		69.3	16.0	16.0	4.0

*m = mesophilic, t = thermophilic.

process conditions are often long term and more difficult to recognize than actual process failures. Identification requires either correlation of the plant's methane production with the expected methane production based on the influent feedstock or estimation of the process stability as indicated by VFA levels or by estimation of the residual methane potential of effluent biomass.

In Table 1 the estimated methane loss has been related to various operation parameters that potentially could affect the significance of the methane loss. Although the loss is a product of the listed parameters and that large variations in the parameters occur between the plants some tendencies can be seen. Low manure fractions in the feedstock (high fractions of industrial waste) were connected to large methane potential losses possibly due to a higher organic loading of the reactors. Additionally, short HRT's in the reactors could also be connected to high residual methane potentials although some plants with low HRT in the reactors had a lower methane loss (Filskov and Vaarrst-Fjellerad) than that some plants with a longer HRT (Blåbjerg and Lintrup). The reason for this inconsistency is that, some of the plants with a short HRT in the reactors has a long HRT in the post-storage tanks and a therefore a rather large fraction of the methane production is obtained via this second digestion step. No connection was observed between the operation temperature and the methane loss because the retention times in general has been correctly incorporated in the reactor configuration, i.e. mesophilic plants on average has a longer retention time (29 days) than thermophilic (16 days) plants.

Suboptimal process conditions caused by high ammonia concentrations in the reactors (>4 g-N/l) have previously

been reported as a reason for high methane potentials in the residuals (Angelidaki *et al.* 2005), which often is used as a guideline at the Danish centralized biogas plants. Such pattern—although weak—was also seen in the present study. Plants with a residual methane potential below 10% had on average an ammonia concentration of 3.2 g-N/l while in plants with a residual methane potential of more than 20% the average concentration was 4.0 g-N/l. In this context, estimation of ammonia might in some cases be useful—as seen in case study 1 with digestion of blood—and regular measurement of the ammonia concentration is performed at a few plants. However one should not forget that ammonia concentration does not reflect the state of the process, but are a cause of an unrestricted reactor operation. Furthermore, the high impact of ammonia adaptation on the inhibitory level (Angelidaki & Ahring 1993; Hansen *et al.* 1998) makes ammonia concentration somewhat difficult to use as an indicator of suboptimal reactor performance.

CONCLUSIONS

From our interviews with various plant managers together with our data-collection and lab-results we conclude that the most frequent process imbalances that occurs at the Danish centralized biogas plants are related to the composition and handling of the substrates. High concentrations of ammonia and long chain fatty acids is often the cause of inhibition but foaming might also affect the biogas output of the process. The high concentrations of inhibitory compounds are allowed to occur as a result of:

(a) Inadequate knowledge about the substrate composition.
(b) Inadequate knowledge about the degradation characteristics of the waste, especially with regard to toxicity level, formation of by-products and biogas potential.
(c) Inadequate process surveillance, especially with regard to volatile fatty acids.
(d) Insufficient pre-storage capacity and inexpedient mixing of the different waste products in pre-storage tanks inducing foaming, and hindering exact dosing of specific waste types to the reactors.

ACKNOWLEDGEMENTS

Henard de Meer, Elena Pueyo Abad, Victoria Andrés, and Hector Garcia are acknowledged for technical assistance. We thank the centralized biogas plants that participated in the project. This work was supported by the Danish Energy Council EFP-05 Journalnr.:33031-0029.

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Bioresource Technology 99 (2008) 7995–8001

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Strategies for optimizing recovery of the biogas process following ammonia inhibition

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ARTICLE INFO

Article history:
Received 4 December 2007
Received in revised form 18 March 2008
Accepted 22 March 2008
Available online 6 May 2008

Keywords:
Biogas
Ammonia
Recovery strategies
Anaerobic digestion
Inhibition

ABSTRACT

Strategies for recovery of ammonia-inhibited thermophilic biogas process, were evaluated in batch and lab-scale reactors. Active methane producing biomass (digested cattle manure) was inhibited with NH_4Cl and subsequently, 3–5 days later, diluted with 50% of water, or with 50% digested manure, or with 50% fresh manure or kept undiluted. Dilution with fresh cattle manure resulted in the highest methane production rate during the recovery period while dilution with digested cattle manure gave a more balanced recovery according to the fluctuations in volatile fatty acids. Furthermore, the process recovery of a 7600 m^3 biogas plant suffering from ammonia inhibition was observed. The ammonia concentration was only gradually lowered via the daily feeding with cattle manure, as is the normal procedure at Danish full-scale biogas plants. Recovery took 31 days with a 40% methane loss and illustrates the need for development of efficient process recovery strategies.

1. Introduction

Anaerobic digestion is a technology widely used for treatment of organic waste. During the process the waste is degraded with a simultaneous energy production in the form of biogas (CH_4, CO_2). In Denmark alone, 20 centralized biogas plants - with a reactor volume of 550–8500 m^3 – are in operation along with more than 80 farm-scale plants. The main purpose of the plants is to digest livestock manure together with organic industrial waste from slaughterhouses, food processing industries etc. (Ahring et al., 1992). A drawback of co-digesting manure and industrial waste is the presence of high ammonia (NH_4^+/NH_3) concentrations in the reactors, emerging from a high natural ammonia concentration in manure and from the production of ammonia during the degradation of proteins, often present in high concentrations in industrial waste (Nielsen and Ahring, 2007; Ramsay and Pullammanappallil, 2001). Ammonia is essential for bacterial growth but also inhibits the anaerobic digestion process if present in high concentration. Free (un-ionised) ammonia (NH_3) has been pointed out as the cause of inhibition in high ammonia loaded processes (Sprott et al., 1984). The free ammonia concentration is a function of total ammonia concentration ($NH_4^+ + NH_3$) of temperature, pH (Anthonisen et al., 1976) and pressure (CO_2) (Vavilin et al., 1995). Thus, an increase in temperature or pH will lead to an increase in the fraction of free ammonia while increasing total gas pressure leads to decreasing inhibition from free ammonia due to a lowering of pH. Studies have suggested that adapted anaerobic digestion of livestock manures is inhibited at a NH_3-concentration of 0.7–1.1 g-N L^{-1}(Angelidaki and Ahring, 1993a; Hansen et al., 1998) while the concentration needed for inhibition of an unadapted process can be as low as 0.08–0.10 g L^{-1} (Braun et al., 1981; de Baere et al., 1984). Inhibition might also be related to total ammonia concentration (Kayhanian, 1999; Sprott and Patel, 1986; Wiegant and Zeeman, 1986). In this context, inhibition has been reported to start at a total ammonia-N level of 1.5–2.0 g L^{-1} (Hashimoto, 1986; Van Velsen, 1979). However, an ammonia-N tolerance of up to 3–4 g L^{-1} for an adapted process has also been reported (Angelidaki and Ahring, 1993a).

Ammonia inhibition might affect the digestion process to different levels ranging from mild suboptimal reactor performances (“inhibited steady state”) where mainly the methanogens are inhibited and VFA are accumulated to severe inhibition affecting all stages of the digestion process (Angelidaki and Ahring, 1993a; Hansen et al., 1998; Nielsen et al., 2007). In worst case the inhibition might last for several months resulting in serious economical losses to the biogas plants. Numerous studies have focused on the prevention of various process imbalances, particularly via development of different process control strategies and via automation and enhancement of process monitoring (Ahring et al., 1995; Boe et al., 2007; Cord-Ruwisch et al., 1997; Hansson et al., 2002, 2003; Hill and Holmberg, 1988; Hill et al., 1987; Marchaim and

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doi:10.1016/j.biortech.2008.03.049

Krause, 1993; Nielsen et al., 2007; Pind et al., 2002; Sterling et al., 2001; Steyer et al., 1999; Switzenbaum et al., 1990). Other studies have tried to come up with practical solutions for overcoming inhibition. Addition of materials – such as bentonite, glauconite and phosphorite – with ion exchange capacity, have been able, to some extent counteract inhibition and aid process recovery (Angelidaki and Ahring, 1993b; Hansen et al., 1999; Krylova et al., 1997). Lowering the temperature from thermophilic (55 °C) to more moderate conditions (40–50 °C) resulted in increase of the methane yield of an inhibited reactor. This process improvement was ascribed to the lowering of the free ammonia concentration due to temperature decrease (Angelidaki and Ahring, 1994). However, the total ammonia concentration will not be changed during such procedures and many Danish biogas plants prefer to operate at thermophilic temperatures, due to the generally higher degradation rates and better sanitation effect (Angelidaki and Ellegaard, 2003). Increasing the C/N ratio of the feedstock has been used to prevent ammonia inhibition and shift slightly elevated ammonia concentrations into the range necessary for optimum biogas production. However, during more serious inhibition levels this procedure will contribute further to inhibition and makes recovery of the process difficult (Kayhanian, 1996, 1999). Finally, dilution of the reactor content with fresh water can be an effective method for lowering the ammonia concentration. A side effect of this procedure can be a serious decrease in biogas production and biotransformation capacity depending on the reactor system (high-solids reactor) (Kayhanian, 1999).

Despite the significant amount of literature on the subject, ammonia inhibition is still an everyday threat at biogas plants performing codigestion and process imbalances caused by ammonia is frequently reported. Careful substrate management and early detection of inhibitions is, of cause, essential in order to minimize the economic losses. However, since these preventive measures often fail; it is important to establish solid knowledge for recovering the process as quickly as possible. Therefore, the purpose of the present study was to test different strategies for obtaining a fast recovery of the biogas process in manure based biogas plants suffering from ammonia inhibition.

2. Methods

2.1. Recovery strategies

The general outline of the experiments in the study was to impose ammonia inhibitions during anaerobic digestion of cattle manure and subsequently test different strategies in order to facilitate the recovery of the process. The tests were carried out in batch and continuously fed lab-scale reactor experiments and one of the strategies was also applied on a full-scale biogas plant suffering from ammonia inhibition. Since ammonia is not degraded during anaerobic digestion we decided to base the recovery strategies on simple dilution methods. The strategies were as follows:

- *Recovery strategy 1 (RS1)*. In this strategy no changes were made in the original operation parameters following a pulse load ammonia inhibition. i.e., in the batch tests no dilution was applied (self-recovery), and in the continuously fed reactor experiments the daily feeding with fresh manure was continued and thus the ammonia concentration was only gradually lowered through effluent wash out.
- *Recovery strategy 2 (RS2)*. In this strategy the inhibited biomass was diluted with distillated water in order to obtain a well defined lowering of the ammonia concentration (Kayhanian, 1996).
- *Recovery strategy 3 (RS3)*. The biomass was diluted with effluent (digested biomass) that had been saved from a reactor treating cattle manure. The design of the strategy was to make a moderate lowering of the ammonia concentration (effluent contains ammonia) with simultaneous addition of a non-inhibited active biomass.
- *Recovery strategy 4 (RS4)*. The biomass was diluted with fresh manure. The intension of this strategy was to make a moderate lowering of the ammonia concentration (as in RS3) with simultaneous addition of easily degradable material to stimulate a high methane production concurrent with recovery.

2.2. Batch experiments

Blended cattle manure and the effluent from an anaerobic thermophilic (55 °C) lab-scale digester treating cattle manure was mixed in the ratio 3:5. The total solids concentration (TS) and volatile solids (VS) of the mixture was 23.6 g^{-1} and 16.2 g L^{-1}, respectively. The total-N concentration was 3.0 g L^{-1} and the ammonia-N concentration was 2.4 g L^{-1}. The mixture was distributed in amounts of 40 ml in 116-ml vials and the vials were incubated at 55 °C. Following nine days of steady methane production, NH_4Cl was added to the vials to obtain a final concentration of 7.0 g total-N $L-^{1}$ and 6.4 g ammonia-N L^{-1}. The vials were then incubated for another 3 days at 55 °C and the different recovery strategies were subsequently carried out: RS(1) continued incubation at 55 °C and no further changes. RS(2) the vial contents were diluted with 40 ml of distillated water, resulting in a total nitrogen concentration of 3.5 g L^{-1}. RS(3) the vial contents were diluted with 40 ml of effluent, resulting in a total nitrogen concentration of 5.2 g L^{-1}. RS(4) the vial contents were diluted with 40 ml fresh cattle manure, resulting in a total nitrogen concentration of 4.6 g L^{-1}.

Following the recovery attempts, the vials were incubated at 55 °C for a period of 29 days. Vials that were only added effluent:manure mixture (no NH_4Cl) were incubated at 55 °C during the whole experimental period and served as control vials. All experimental series were conducted in triplicates. The methane production was measured 3–4 times a week in all vials of each series and the volatile fatty acids concentration (VFA) was measured 1–2 times a week in one vial of each series. Before each incubation the vials were flushed with N_2/CO_2 (80%/20%), to obtain anaerobic conditions, and subsequently closed with butyl rubber stoppers and aluminum crimps.

2.3. Reactor experiments

Four 4.5 L continuously stirred tank reactors (CSTR) with a working volume of 3.0 L (Angelidaki and Ahring, 1993a) were inoculated with effluent from a stable pilot-scale CSTR operating on cattle manure at 55 °C. The reactors were named R1, R2, R3, R4 according to the different recovery strategies that later were to be tested. The reactors were stirred by a propeller every third minute for one minute at 100 rpm and operated at 52–54 °C. Two different batches of cattle manure were used as feedstock. Both batches were kept at 4 °C and blended before use. The blended manure was mixed with tap water in the ratio 1:2 in order to enable automatic feeding. During start up and from day 0–26 of the experimental period the TS/VS content of the diluted manure were 16.5/12.0 g L^{-1} and the ammonia-N concentration 0.86 g L^{-1}. From day 26–53 of the experimental period the TS/VS content of the diluted manure were 27.1/21.2 g L^{-1} and the ammonia-N concentration 1.7 g L^{-1}.

Start-up took two weeks. During this period the reactors were gradually fed with 0–150 ml per day cattle manure. The reactors were subsequently operated at full loading (200 ml d^{-1}) for another two weeks before initiation of the experiment (day 0). The hydraulic retention time (HRT) of the reactors during full loading was 15 days. The reactors were feed four times every 6 h.

Ammonia inhibition was induced at day 16 of the experimental period. The reactors were added NH_4Cl in an amount resulting in a free ammonia concentration (NH_3) of 1.2 g-N L^{-1}. This value was chosen in order to exceed the level of 1.1 g-N L^{-1} that was found to cause inhibition by Hansen et al. (1998). Specifically the reactors were added the following amounts of NH_4Cl: RS(1) 30.0 g L^{-1}. RS(2) 36.2 g L^{-1}. RS(3) 38.7 g L^{-1}. RS(4) 39.3 g L^{-1}. The amounts were slightly different in each case due to differences in initial pH level. Following inhibition the daily feeding with 200 ml cattle manure was continued.

The recovery strategies were carried out at day 21 of the experimental period: RS(1) no changes in operation. RS(2) 50% of the reactor volume was removed and substituted with water. The dilution ratio of 50% was chosen in order to obtain a theoretical free ammonia concentration below the inhibitory level of 0.7 g-N L^{-1} suggested by Angelidaki and Ahring, 1993a. RS3) 50% of the reactor volume was removed and substituted with effluent that had been collected during the period of stable operation. The total-N and ammonia-N concentration of the effluent was 1.77 and 1.13 g L^{-1}, respectively. RS4) 50% of the reactor volume was removed and substituted with undiluted fresh cattle manure with a TS/VS content of 49.6 g L^{-1} and 36.0 g L^{-1}, respectively. The total-N and ammonia-N concentration of the manure was 4.22 and 2.57 g L^{-1}, respectively.

In all four strategies the daily feeding with 200 ml diluted cattle manure was continued until the termination of the experiment at day 53. During the experiment the reactor performance was analyzed with regard to methane production, VFA concentration, pH and ammonia concentration.

2.4. Full-scale observations

During a period of one and a half year the process at a full-scale biogas plant was followed in order to observe possible process imbalances caused by ammonia inhibition. The plant has a reactor capacity of 7600 m^3 and consists of three equal sized reactors that are operated at 53 °C with a HRT of approximately 17–18 days. The plant treats approximately 362 tons manure d^{-1} together with approximately 75 tons d^{-1} alternative waste (organic industrial waste). Samples from reactor one were send to our laboratory 3–5 times a week. The samples were frozen and analyzed once a month with regard to VFA concentration. Monitoring of the biogas production was done at the plant as well as a weekly measurement of ammonia concentration and pH of the reactor. From the 10th of October 2006 a significant increase in VFA concentrations was observed and from the 16th of October a decrease in biogas production was also seen. Before the imbalance (middle of September) the substrate had been supplemented with waste from a mink farm. This waste normally has an high ammonia content and was possibly the cause of the imbalance. However, no analysis of the waste was made by the plant, but a strong indication of an ammonia inhibition was given by a clear increase in ammonia concentration simultaneous with the increase in VFA. A strategy similar to RS1 was used to mitigate the ammonia inhibition and facilitate the recovery of the process. Thus feeding with industrial waste, including mink farm waste was immediately stopped from the 18th of October and replaced by manure. RS1 was chosen as the preferred strategy since the plant found this procedure to be safer with regard to overloading and easier to perform than the other strategies.

2.5. Analytical methods

TS, VS, pH and ammonia content were determined using standard methods (Greenberg et al., 1998). CH_4 production from the batch experiments was measured by gas chromatography using flame ionization detection. CH_4 and CO_2 production from the reactors were determined by gas chromatography using thermal conductivity detection. For manual VFA determination 1 ml of sample was acidified with 70 μl 17% phosphoric acid, centrifuged at 10,500 rpm for 20 min, and analyzed on a GC equipped with flame ionization detector.

3. Results and discussion

3.1. Batch experiments

Addition of NH_4Cl resulted in a 53% decrease in methane production from day 0 to day 3 (Fig. 1). Thus, the control vials produced 35 ml CH_4 while an average production of 16.5 ml CH_4 were obtained from the test vials. Shortly after the initiation of the recovery strategies a further aggravation of the process was observed for RS2 (water dilution) and RS3 (effluent dilution) vials when compared to RS1 (no dilution) vials. However, at the end of the experiments the accumulated methane production in RS2 vials were comparable to the control vials while the methane production of RS1 and RS3 vials only corresponded to 64% and 83% of the control vials, respectively. In RS4 vials (fresh manure dilution) a complete recovery of the accumulated methane production was observed only three days after the initiation of the recovery strategy. Furthermore, the strategy led to a significant increase in methane production and at day 29 RS4 vials had produced 476 ml CH_4 corresponding to 420% of the control vials. However, in batch experiments the addition of extra substrate without loss of bacterial culture does not match the situation in a continuously operated process.

From the results of the batch experiments, RS4 seems as the best strategy due to the fast recovery and increase of the methane production. A full recovery of the process was also achieved with RS2 but it took a considerably longer time than with RS4, although

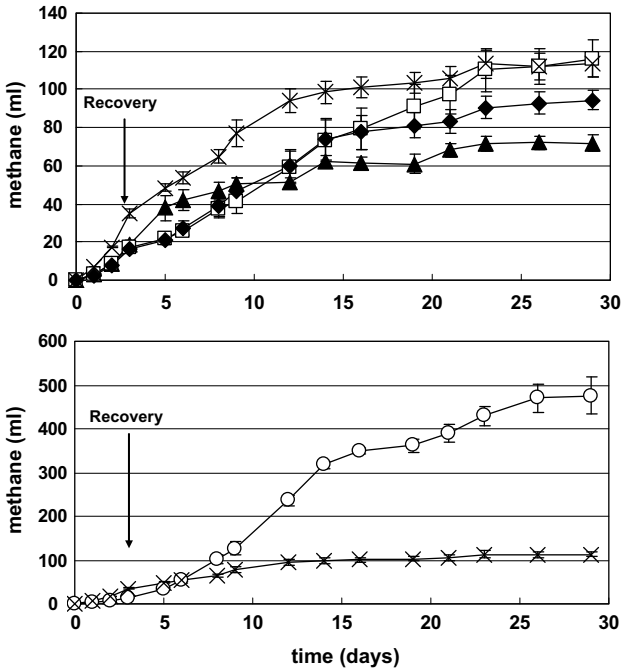

Fig. 1. Batch experiments. A 3:5 mixture of cattle manure and digested manure were incubated in batch vials at 55 °C. NH_4Cl was added when a steady methane production was observed in order to induce an ammonia inhibition. Three days later different recovery strategies were carried out in order to mitigate the ammonia inhibition and facilitate the recovery of the process. The figure shows the methane production with standard deviations following addition of NH_4Cl. ▲: RS1, □: RS2, ♦: RS3, ○: RS4, ×: Control vials.

the ammonia concentration was lower in RS2 vials than in RS4 vials. The recovery efficiency of the methane production was, therefore, not only related to the ammonia concentration but also to the substrate composition. Complete recovery of the methane production was not possible by application of RS1 and RS3. In RS1 vials the process was still inhibited at the end of the experiment because the ammonia concentration was not lowered. Insufficient lowering of the ammonia concentration was possibly also the reason why the process never totally recovered in RS3 vials.

3.2. Reactor experiments

The output of the lab-scale reactor experiment is illustrated in Figs. 2 and 3. Before addition of NH_4Cl a stable process (i.e. stable methane production and stable VFA levels) was observed for all reactors. The average methane production was 234 ml g VS^{-1} for R1, 226 ml g $VS^{-1}$for R2, 263 ml g $VS^{-1}$for R3 and 246 ml g $VS^{-1}$for R4. The acetate and propionate concentration was below 1 mM for all reactors. Addition of NH_4Cl (day 16) resulted in an immediate inhibition of methanogenesis in all four reactors, and a slight drop in pH from approximately 7.6 at day 16 to 7.3 at day 19. No remarkable increase in the VFA concentrations was observed, which illustrates that the ammonia inhibition was an overall inhibition of the process and not only an inhibition of methanogenesis.

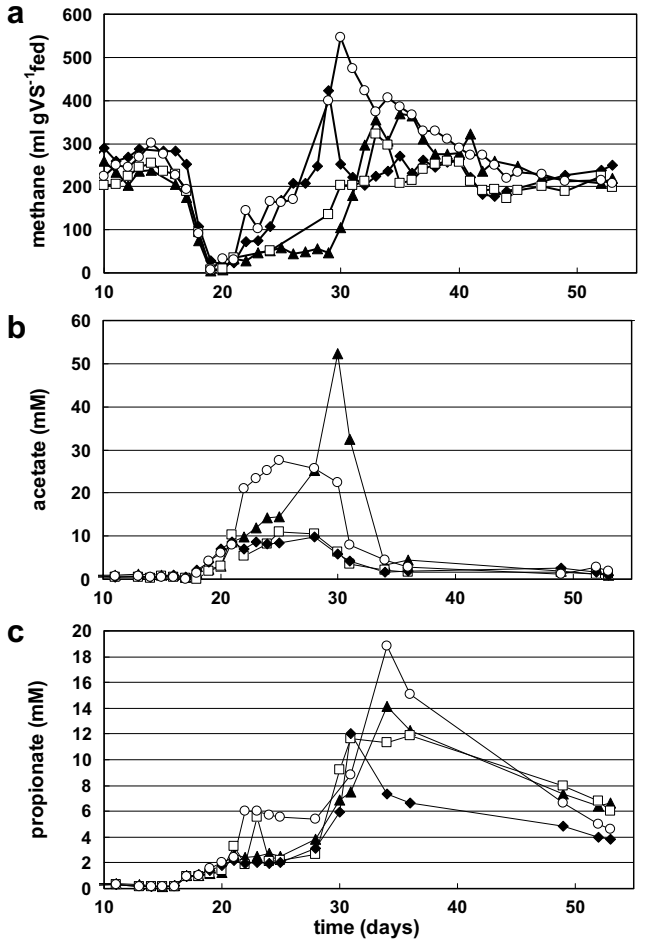

Fig. 2. Lab-scale reactor experiments. The anaerobic digestion process in four digesters was inhibited by addition of NH_4Cl at day 17. At day 21 different recovery strategies were carried out in order to mitigate the ammonia inhibition and facilitate the recovery of the process. The figure shows the methane production and VFA concentrations. (a) methane production; (b) acetate concentration; (c) propionate concentration. ▲: R1, □: R2, ♦: R3, ○: R4.

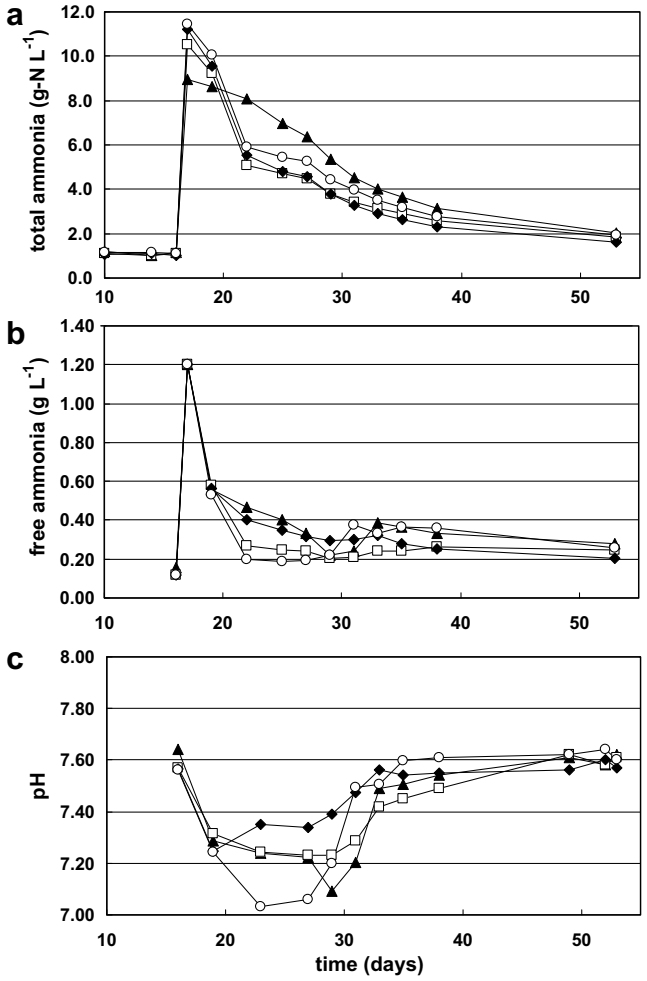

Fig. 3. Lab-scale reactor experiments. Development of free ammonia (NH_3), total ammonia (NH_4^+/NH_3) and pH following addition of NH_4Cl and initiation of different recovery strategies. (a) Total ammonia-N; (b) free ammonia-N; (c) pH. ▲: R1, □: R2, ♦: R3, ○: R4.

The initiation of the different recovery strategies at day 21 gave a performance pattern resembling the one in the batch experiment, but with some differences:

R1 (no changes in operation parameters). The inhibition in R1 continued until day 29 because of the rather slow dilution of the reactor content that was obtained via the continued daily feeding with cattle manure. From day 29 the methane production started to recover and at day 31–32 the production was at a level similar to the one before inhibition. A relatively high methane production was observed in the days following termination of the inhibition (32–42), possibly because of a surplus of fresh manure that had not been degraded during the inhibition period. At the end of the experiment the production had stabilized at the original level. A significant increase in acetate was observed from 15 mM to 52 mM (day 25–30) just before the methane production started to recover, which gave evidence that the fermentation processes recovered before methanogenesis. The delayed increase in propionate concentration and slow return back to the normal level (day 28–53) indicated that the syntrophic bacteria degrading propionate were the slowest growing and the last microorganisms to recover. In this context, it can be concluded that propionate gave the best indication of when the entire process had stabilized, which is in accordance to other studies (Nielsen and Ahring, 2006; Nielsen et al., 2007). The free ammonia concentration was 1.2 g-N L^{-1}

when the inhibition was initiated but three days after the inhibition the concentration had decreased to a level of approximately 0.55 g-N L^{-1} (in all four reactors) due to a lowering in pH. The inhibition in R1 continued from day 19 to day 29 although the NH_3-concentration was well below the suggested levels of 0.7 g-N L^{-1} to 1.1 g-N^{-1} (Angelidaki and Ahring, 1993a; Hansen et al., 1998). This pattern illustrates that not only free ammonia but also additional factors might have been causing inhibition of the process. This could have been the increase in NH_4^+ concentration (Sprott and Patel, 1986), lowering of pH or an increase in salinity (Cl^-) (Gebauer, 2004; Jackson-Moss et al., 1989; Panswad and Anan, 1999; Vijayaraghavan and Ramanujam, 1999).

R2 (water dilution). Diluting with water gave an efficient lowering of the ammonia concentration, although not a 50% decrease as expected, probably due to a shift in ionic balance. In addition to this, the dilution rate of ammonia from day 22–53 was lower in R2 than in the other reactors, despite the similar feeding rate and feedstock. Nevertheless, the recovery of the methane production started earlier in R2 than in R1 because of the more efficient lowering of the ammonia concentration. Full recovery of the methane production in R2 was, however, obtained only one day before R1, corresponding to nine days after initiation of the recovery strategy. Furthermore, no peak production was observed in the days following termination of the inhibition (32–42) as seen in R1. As a consequence of this the total methane production in R2 during the recovery periods (day 21–42) was 8% lower than in R1. This illustrates a loss of methane potential when the reactor content was diluted with water, a problem also reported by Kayhanian (1999). In comparison, this problem was not observed in the batch experiment because the substrate was retained in the vials when performing the recovery strategy. The increase in propionate concentration in R2 during the recovery was comparable to the propionate increase in R1, but the increase in acetate concentration was more moderate in R2 because of the lower substrate concentration and lower fermentation.

R3 (effluent dilution). An efficient reestablishment of the methane production in R3 was observed following initiation of RS3. The production started to increase immediately after effluent was added and had returned to the original level after only five days (day 26–28). The total methane production in R3 during the recovery periods (day 21–42) were 13% and 23% higher than the productions in R1 and R2, respectively. A small peak in methane production was observed at day 29 possibly because of a small surplus of fresh manure that had not been degraded during the inhibition (like in R1). However, the biogas production from day 26 to day 53 was comparable to the production before the inhibition because the dilution with effluent did not change the composition of the reactor content with the exception of the ammonia concentration. The increase in acetate concentration following inhibition was in the same range as during the water dilution (R2) and much more moderate than in R1. A peak in propionate was also observed during the recovery in R3 but in contrast to the other reactors the increase started earlier and the return was faster. The observed methane and VFA patterns illustrate a fast recovery of not only the methanogens but also the syntrophic consortia in R3, when compared to the other reactors. The reason was possibly due to the concept of RS3, i.e. addition of a non-inhibited active biomass in combination with a lowering of the ammonia concentration.

R4 (fresh manure dilution). Reestablishment of the methane production in R4 following initiation of RS4 was in the same range as in R3 and took only 5–6 days. However, a significant peak in the production from day 29 to 39 gave an additional methane production during the recovery periods (day 21–42) of 60%, 74% and 42% in comparison to R1, R2 and R3, respectively. The increased production was a consequence of the easily degradable extra organic matter in the fresh manure used for dilution. This is a rather positive result with regard to minimization of the economic losses following the ammonia inhibition. However, reactor R4 was clearly more unstable than R2 and R3 as indicated by the higher VFA and lower pH. The drop in pH from 7.3 at day 19 to 7.0–7.1 from day 23–27 was a consequence of the increase in acetate concentration and a rather low buffer capacity of the reactor content due to the dilution of the feedstock. Most methanogens have a pH optimum between 6.5 and 8.0 and the drop was as such not critical. However, a further decrease in pH as a consequence of an increased loading and a subsequent increase in VFA could have been fatal. It is our believe that the process of reactor R4 was more unstable than in R2 and R3 during the recovery period because of the high organic loading, obtained from the dilution of the reactor content with fresh manure. When compared to R1 the process in R4 did on the other hand not seem more negatively affected.

3.3. Full-scale observations

From the 1st of August until the 15th of October the biogas production at the full-scale plant was 20,000–25,000 m^3 d^{-1} with a methane content of approximately 65% (Fig. 4a). From the middle of September a gradual increase was observed in ammonia concentration, probably due to the feeding with organic waste from a mink farm. The VFA concentration started to increase from the 10th of October and from the 16th of October a sudden decrease in biogas was observed (Fig. 4b,c). The recovery attempt (similar to RS1) was initiated at the 18th of October. This resulted in an immediate reduction of the ammonia concentration but no clear impact on the biogas production was observed until the 21st of November. As a consequence of this, the recovery time of the overall process at the full-scale plant was considerably longer than in R1 of the lab-scale reactor experiments. This may be due to dissimilarities between the full-scale plant and the lab-scale reactors with regard to operating conditions, feedstock composition and reactor characteristics. Nevertheless, strategies based on the same principles as RS1 are normally used at Danish biogas plants and the data obtained from the full-scale plant stresses out the need for development of efficient recovery strategies following ammonia inhibition.

3.4. Evaluation of the recovery strategies

In the present study the recovery strategies were based on two different parameters: the ammonia concentration following initiation of the recovery and the substrate composition. However, when evaluating the various strategies it is difficult to relate the efficiency of the strategy directly to either the ammonia concentration or the substrate composition. This was particularly seen in the batch experiments where the recovery was more efficient with manure dilution than with water dilution although the ammonia concentration was higher. Furthermore, various counter effects of the different processes occurring during anaerobic digestion might obscure the evaluation. For instance, addition of easily degradable substrate (manure) might result in overloading of the reactor and result in increase of the VFA level and thereby decrease of the pH. This was particularly illustrated in the R4 reactor experiment. A lowering of pH will reduce the negative effect of free ammonia via a decrease in free ammonia concentration but might in extreme cases also deteriorate the process by and inhibiting methanogenesis.

It is also important to mention that the scope of the study was to compare the effect of diluting the biomass of ammonia-inhibited reactors with different substances. Due to the workload of a CSTR experiments only one dilution rate was selected (50%) in which the free ammonia concentration in theory was lowered from a

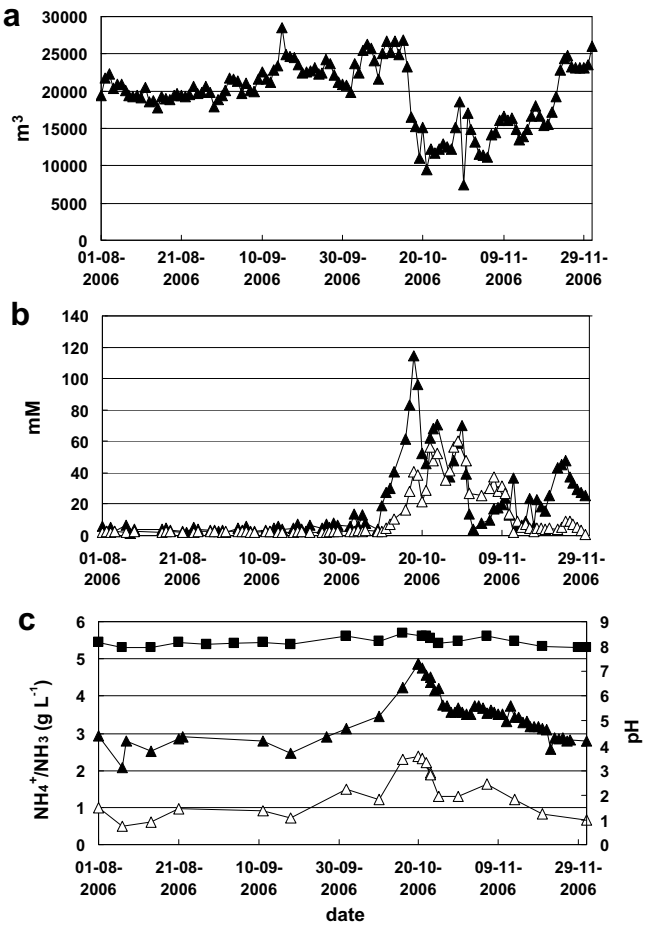

Fig. 4. Full-scale observations showing biogas production, VFA concentrations and pH from august 2006 to November 2006. During the observation the plant was exposed to a process imbalance presumably caused by ammonia. (a) Biogas production; (b) acetate concentration: ▲ and propionate concentration: △; (c) pH: ■, total ammonia (NH_4^+/NH_3): ▲, free ammonia (NH_3): △.

level of 1.2 g-N L^{-1} which is higher than the inhibitory level (1.1 g-N L^{-1}) suggested by Hansen et al. (1998) to 0.6 g-N L^{-1} which is lower the inhibitory level suggested by Angelidaki and Ahring (1993a). The outcome of the strategies could have been different if other dilution rates had been used or if the ammonia concentrations in the reactors had differed.

In the batch experiments a full methane production recovery was observed via application of RS2 and RS4. In comparison, a full recovery was observed for all strategies in the reactors. The methane production of R1 and R2 showed almost the same recovery time of app. 9–10 day. However, the lower methane production in R2 does not favor RS2 and the very slow full-scale recovery excludes RS1 as a recommendable strategy. R3 and R4 had a similar methane recovery time of 5–6 days but R4 had a significant higher methane production in the days following recovery. This fact favors RS4 seen from a "plant-managers point of view" where a high biogas production is preferable due to the economic aspects. However, the pronounced fluctuations in other process parameters (pH and VFA) give evidence of an unstable process following the recovery of methane production. Application of RS4 requires full focus on all process parameters, which are unusual at large scale biogas plants. A strategy similar to RS4 with several consecutive dilution steps in stead of one, could perhaps stabilize the recovery process. The more moderate fluctuations in process parameters in R3, especially propionate, show a more stable recovery process and favor RS3 in comparison to the other strategies. However, various practical obstacles might to some extent be associated with this strategy. Typically, the effluent storage capacities immediately available at plant premises (Danish biogas plants) makes up only approximately 1/4–1/3 of the reactor volume, which restricts the dilution potential. As a solution to this problem, older digested manure is often available from seasonal storage tanks, but at some distance requiring costly road transport. Contamination of the effluent with the inhibiting component – in this case ammonia – might be another obstacle. Early detection of the process imbalance is, therefore, crucial in order to moderate the ammonia increase in the effluent. The ammonia concentration level in the reactors during normal operation is also important for the applicability of the strategy. In the present study the ammonia concentration of the effluent was only 1.1 g-N L^{-1} due to the dilution of the manure, but the average ammonia concentration in Danish centralized biogas plants is usually in the range 2.5–3.5 g-N L^{-1} (Angelidaki et al., 2005; Nielsen and Ahring, 2007). It is therefore expected that the lowering of the ammonia concentration via dilution with effluent will be less pronounced at full-scale plants than in the reactor experiments of the present study. The ammonia concentration in fresh manure is normally slightly lower than the effluent, but still in the range of 2–4 g-N/L. It is however, easier to find "fresh" manure with relatively low ammonia concentration (eg. cattle manure instead of pig manure) and use it for dilution of ammonia-inhibited processes. Therefore, for practical applications, the best solution for the plants seems to be RS3 in combination with either RS2 or RS4. The ratio between the different strategies depends on the ammonia concentration in the reactor/effluent/fresh manure, the effluent storage capacity of the plant, the delivery capacity of fresh manure and the organic loading of the reactors. It should also be emphasized that efficient monitoring of process parameters such as VFA is not only important for early indication of process inhibitions but also during recovery of an inhibited process.

4. Conclusions

The results of the present study show that it is possible to improve the recovery speed and stability of an ammonia-inhibited anaerobic digester treating cattle manure by dilution of the biomass with water, reactor effluent and manure. From an economical point of view, dilution with manure was the most efficient strategy because of a high methane production during the recovery period. However the most stable recovery process was observed when the biomass was diluted with reactor effluent.

Acknowledgements

We thank Sonia Guldener for performing some of the experiments during her master thesis and Hector Garcia for technical assistance. All experiments where carried out at the Institute of Environment & Resources, DTU. This work was supported by the Danish Energy Council EFP-05 Journal No.: 33031-0029.

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Bioresource Technology 100 (2009) 4588–4596

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

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

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 21 April 2009
Accepted 22 April 2009
Available online 26 May 2009

Keywords:
Anaerobic
Lipids-manure co-digestion
LCFA inhibition
Recovery strategies

ABSTRACT

Long chain fatty acids (LCFA) concentrations over 1.0 g L^{-1} were inhibiting manure thermophilic digestion, in batch and semi-continuous experiments, resulting in a temporary cease of the biogas production. The aim of the work was to test and evaluate several recovery actions, such as reactor feeding patterns, dilution and addition of adsorbents, in order to determine the most appropriate strategy for fast recovery of the reactor activity in manure based plants inhibited by LCFA. Dilution with active inoculum for increasing the biomass/LCFA ratio, or addition of adsorbents for adsorbing the LCFA and reducing the bioavailable LCFA concentration, were found to be the best recovery strategies, improving the recovery time from 10 to 2 days, in semi-continuously fed systems. Moreover, acclimatization was introduced by repeated inhibition and process recovery. The subsequent exposure of the anaerobic biomass to an inhibitory concentration of LCFA improved the recovery ability of the system, indicated as increasing degradation rates from 0.04 to 0.16 g COD_CH_4/g VS day. The incubation time between subsequent pulses, or discontinuous LCFA pulses, seems to be a decisive process parameter to tackle LCFA inhibition in manure anaerobic co-digestion.

1. Introduction

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

In anaerobic treatment systems, lipids are rapidly hydrolysed by extracellular lipases to long chain fatty acids (LCFA) and glycerol. LCFA are further degraded to acetate and hydrogen through β-oxidation process (Weng and Jeris, 1976). Exploitation of the biogas potential of lipids is difficult, because lipid containing wastes often have low content of nutrients, low alkalinity (Angelidaki and Ahring, 1997a,b) and, mainly, due to their toxicity towards the anaerobic digestion process (Hanaki et al., 1981; Hwu et al., 1996; Rinzema et al., 1994). Moreover, problems with anaerobic treatment of lipids are caused by the adsorption of light lipid layer around biomass particles causing biomass flotation and wash-out (Hwu et al., 1997).

Adsorption of LCFA onto the microbial surface has been suggested as the mechanism of inhibition, affecting transportation of nutrients to the cell (Alves et al., 2001a,b; Hwu et al., 1998). The LCFA inhibition is dependent on the type of microorganism, the specific surface area of the sludge, the carbon chain length and of the saturation (C=C) of LCFA (Hwu et al., 1996; Salminen and Rintala, 2002a). It has been reported that LCFA are inhibiting anaerobic microorganisms at very low concentrations, with IC_{50} values for oleate over 50 and 75 mg L^{-1} (Alves et al., 2001b; Hwu et al., 1996), palmitate over 1100 mg L^{-1} (Pereira et al., 2005) or stearate over 1500 mg L^{-1} (Shin et al., 2003), at mesophilic temperature range. Although thermophiles are more susceptible to LCFA toxicity compared to mesophiles, they recover faster after LCFA-inhibition due to their faster growth rates (Hwu and Lettinga, 1997). Methanogens were reported to be more susceptible to LCFA inhibition compared to acidogens (Lalman and Bagley, 2002; Mykhaylovin et al., 2005; Pereira et al., 2003). Fortunately,

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doi:10.1016/j.biortech.2009.04.046

inhibition caused by LCFA is a reversible process; neither syntrophic acetogenic nor methanogenic activities were irreversibly damaged, since the rate of methane formation increased dramatically within a short time after the LCFA-biomass associated degradation had recommenced (Pereira et al., 2003, 2005).

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

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

2. Methods

2.1. Analytical methods

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

For determination of LCFA in biological samples, some direct procedures based on direct methanolic–HCl solution were tested with good results (Neves et al., 2009; Sönnichsen and Müller, 1999). In the present study, a new method using clorotrimethylsilane (CTMS) as fatty acids methyl esters (FAME) catalyst, without prior extraction over lyophilized samples, was developed, based on Eras et al. (2004) methodology. This methodology can be used to determinate total fats and LCFA in solid, liquid or paste samples. Moreover, the method allows small amount of sample to be used, reducing the reaction temperature and processing time, characteristics often needed on biological samples. Anaerobic reactor samples, from 0.5 to 1 mL, were transferred together with Extraction Standard (ES), heptadecanoic acid (C17:0, 51610 Fluka puriss >99.0%), to screwed pirex glass tubes (10 mL) and lyophilized overnight at −40 °C. For soluble LCFA ($LCFA_S$) determination, samples were previously centrifuged (2 × 3500 rpm) and only soluble fraction was placed on the pirex tubes. Afterwards a magnetic stir bar was introduced together with 0.5 mL of CTMS (CTMS GC Panreac 352776.0207) and 1 mL of N_2 saturated methanol, under a hood fume, tighten the vials with Teflon screw cup and shacked at vortex for 1 min. The tubes were introduced into aluminium block and maintained in stirring and heating (90 °C) for 1.5 h reaction time. When the vials were at room temperature, were opened and 1–5 mL of hexane was added (dilution in order to obtain the desired concentration of 0.5–600 mg L^{-1}). Commercial powder $NaHCO_3$ was added till no reaction (effervescence) was detected, and finally 2 mL of saturated solution of $NaHCO_3$ was added. The vials were shaken in vortex again and centrifuged (10 min 3500 rpm) till phase separation. 900 μL of the organic phase were directly transferred to GC vial, together with 100 μL of methyl pentadecanoate (C15:0 FAME, Fluka 76560 puriss. p.a. standard for GC) as internal standard (IS).

FAME were identified and quantified by GC 3800 gas chromatograph (Varian, USA), fitted with CP7489:CP-Sil 88 FAME capillary column (50m × 0.25mm × 0.2 μm, Varian, USA), flame ionization detector (FID) and equipped with auto sampler (CP 8400. Varian, USA). The FID was supplied with H_2 and synthetic air, while He was used as carrier and make-up gas with a flow rate of 2 mL min^{-1}. Samples of 1 μL were injected in split mode. The oven initial temperature was 60 °C during 1 min, then increased to 100 °C at 25 °C min^{-1}, to 160 °C at 10 °C min^{-1}, to 240 °C at 4 °C min^{-1}, with a final isotherm step of 5 min. Injector and detector temperature were set constant at 270 °C and 300 °C, respectively. 36 different FAME from C6:0 to C24:1 were calibrated using FAME GC mixture (Supelco 18919-1AMP FAME Mix C4–C24) and IS, from 0.5 to 600 mg/L, The recovery of LCFA, was determined by the ES (C17:0) recovered in blanks and real digested manure samples, and it was always over 87.5% in all determinations.

2.2. Substrates and inoculum

Cow manure was used as basis substrate. The manure was diluted with distilled water in order to decrease the ammonia level and ensure that LCFA was the only inhibitor in the experiments. The diluted manure used had an average concentration of 2.5% TS and 2.0% VS (Table 1).

Digested thermophilic effluent from a biogas pilot-scale plant (PP), digesting cow manure located at DTU (Kongens Lyngby, Denmark), with an average concentration of 3.0% TS and 2.2% VS, was used as initial inoculum for experiments. In the subsequent experiments, inoculum was provided from the effluent of the reactors

Table 1
Analysis of substrate, inoculum and adsorbents used in the experiments.

	Diluted manure			Inoculum			Fibers		Bentonite
	BTA	E1	E2	BTA	E1	E2	E1	E2	E1&E2
TS (%w/w)	2.40 ± 0.05	2.45 ± 0.42	2.34 ± 0.73	3.02 ± 0.01	2.05 ± 0.29	2.04 ± 0.15	59.60 ± 7.96	21.01 ± 0.72	94.04
VS (%w/w)	2.0 ± 0.05	1.98 ± 0.38	1.93 ± 0.65	2.25 ± 0.01	1.47 ± 0.20	1.44 ± 0.18	34.80 ± 4.87	18.80 ± 0.70	5.09
TKN (g/kg)	–	1.31	1.41 ± 0.25	–	–	1.32 ± 0.06	–	5.17 ± 0.20	0.28 ± 0.21
NH_4^+–N (g/kg)	–	1.05	0.92 ± 0.03	–	–	0.91 ± 0.05	–	1.76 ± 0.34	–
pH	–	7.52	7.49 ± 0.24	–	–	7.68 ± 0.19	–	8.21 ± 0.01	~8 (10% H_2O)

used in the present experiments. Table 1 summarizes the characteristics of substrates, adsorbents and inoculum used in batch and semi-continuously fed reactors.

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

Commercial powder bentonite ($Al_2O_3 \cdot 4SiO_2 \cdot H_2O$ Prod 18609 Sigma–Aldrich, St. Louis, USA) and fibers, obtained from filtered digested manure, were used as absorbents for the experiments testing adsorption strategies. Initially, fibers were obtained from a Danish manure centralized biogas facility, while in the subsequent experiments were manually obtained by filtration of digested manure from a pilot-scale plant (Kongens Lyngby, Denmark). This caused some changes in composition (Table 1), however; the same VS amount of fibers were added to the reactors in all experiments.

2.3. LCFA toxicity assay (BTA)

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

120 ml vials were used in the BTA with a working volume of 40 ml. The assay included: blanks (30 ml of inoculum and 10 ml of distilled water), controls (30 mL of inoculum and 10 mL of diluted manure) and test vials with 30 ml inoculum and 10 ml of different dilutions of LCFA. The vials were inoculated under anaerobic conditions, while gassing with N_2 gas. Subsequently, the vials were closed with rubber stopper and aluminium crimps and were incubated at 55 °C without agitation. The methane production in the head space of the vials was monitored by gas chromatography until biogas production ceased. Each LCFA concentration was conducted in triplicate.

LCFA was added in the vials as a pulse, when the methane production from manure was increasing exponentially (at day 5). LCFA was added to a total concentration of 1.0, 2.5, 4.0 and 6.0 g L^{-1} corresponding to 2.8, 7.0, 11.2 and 16.8 g COD L^{-1}. Subsequently, vials were vigorously agitated until the LCFA was dissolved/emulsified. No LCFA was added in blanks and controls.

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

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

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

- *Feeding strategies*: (a) No-feeding ($R_{no\text{-}feed}$) and (b) continuous feeding (R_{feed}) with fresh manure, and HRT of 20 days corresponding to an OLR of 1.0 g VS L^{-1} day^{-1}.
- *Dilution strategies*: Replacement of 40% of the reactor content by: (a) fresh manure (R_{manure}); (b) digested manure or effluent from reactors before inhibition ($R_{inocula}$) and (c) water (R_{water}).
- *Adsorption strategies*: (a) Addition of fibers (R_{fiber}), obtained from filtered digested manure and (b) addition of bentonite powder ($R_{bentonite}$), both in the quantity of 5 g VS L^{-1}.

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

Recovery experiment 2 (E2) was aiming to test recovery strategies in the same reactors, pre-exposed to LCFA from E1. The reactors in E2 were daily fed with manure, and were subsequently exposed to inhibition by pulse addition of LCFA. The main difference between E1 and E2 was that in experiment E1 daily feeding with manure was ceased after LCFA was applied (except for R_{feed}), while in experiment E2 the daily feeding of the reactors with manure continued also after the initiation of the recovery strategy (except for $R_{no\text{-}feed}$). E2 was repeated twice (RUN3 and RUN4), or two subsequent LCFA pulses were applied. Analysis of LCFA-FAME time course was only monitored in E2 by GC-FID.

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

To compare process performance in consecutive inhibited-recovered reactors, recovery time (days), the maximum methane production rate (g COD_CH_4 g^{-1} VS day^{-1}) and acetate maximum consumption rate (g COD_Ac g^{-1} VS day^{-1}) were calculated, per unit of initial measured VS (biomass). The recovery time was calculated as the time between the initiation of the recovery action and the time when the methane production rate exceeded the mean value of control reactor ($R_{control}$). The maximum methane production rate was calculated as the maximum slope of the methane yield curve, while the acetate consumption rate was calculated

Table 2
Summary of the experimental set-up.

Exp	Reactor configuration	Temp. (°C)	Agitation	LCFA pulse (g L^{-1})	RUN	Recovery strategies	Manure after recovery action
BTA	Batch	55	No	1.0, 2.5, 4.0 and 7.0	–	–	–
E1	Semi-continuous	55	Shaker	4	RUN1 & RUN2	No-feed, feed, inocula, manure, water, bentonite, fiber	No (except R_{feed})
E2	Semi-continuous	55	Shaker	4	RUN3 & RUN4	No-feed, feed, inocula, manure, water, bentonite, fiber	Yes (except $R_{no\text{-}feed}$)

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

3. Results and discussion

3.1. LCFA toxicity assay

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

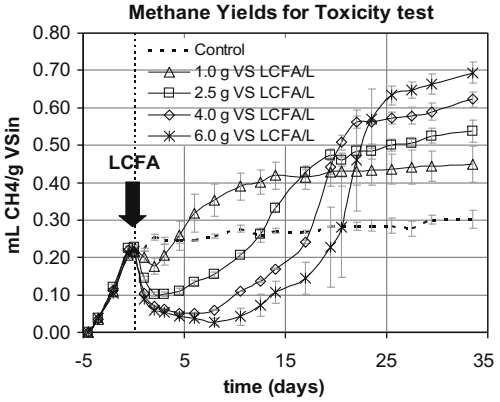

Fig. 1. Accumulated specific net methane production (mL CH_4/g VS_{in}) at different LCFA concentrations tested in batch experiment. Methane production of control vials was subtracted from methane production of test vials with LCFA addition. Arrows indicate the LCFA time application.

After the initial inhibition, the process self-recovered for all tested concentrations (Fig. 1). This is in accordance with previous results, where the same pattern was observed, a temporary inhibition that was monitored as a lag-phase. This phenomenon was reported to be adscribed to surface adsorption and transport sites (Cavaleiro et al., 2008; Pereira et al., 2005).

3.2. E1: LCFA inhibition of un-adapted semi-continuous reactors and subsequent application of recovery strategies (no feeding after recovery action was applied)

As a part of the recovery strategy, the daily feeding with manure was ceased in all the reactors, after application of the LCFA pulse, except for the R_{feed} strategy and the $R_{control}$, which were fed daily with diluted fresh manure with an HRT of 20 days. It was clear that the strategy of self-recover process ($R_{no\text{-}feed}$) was the strategy that resulted in the slowest recovery time, which was over 40 days, compared to 9 or 7 days in R_{feed} for RUN1 and RUN2, respectively (Fig. 2, and Table 3). Additionally, VFA accumulation in $R_{no\text{-}feed}$ was significantly higher, 92.8 mM compared to 47.2 mM or

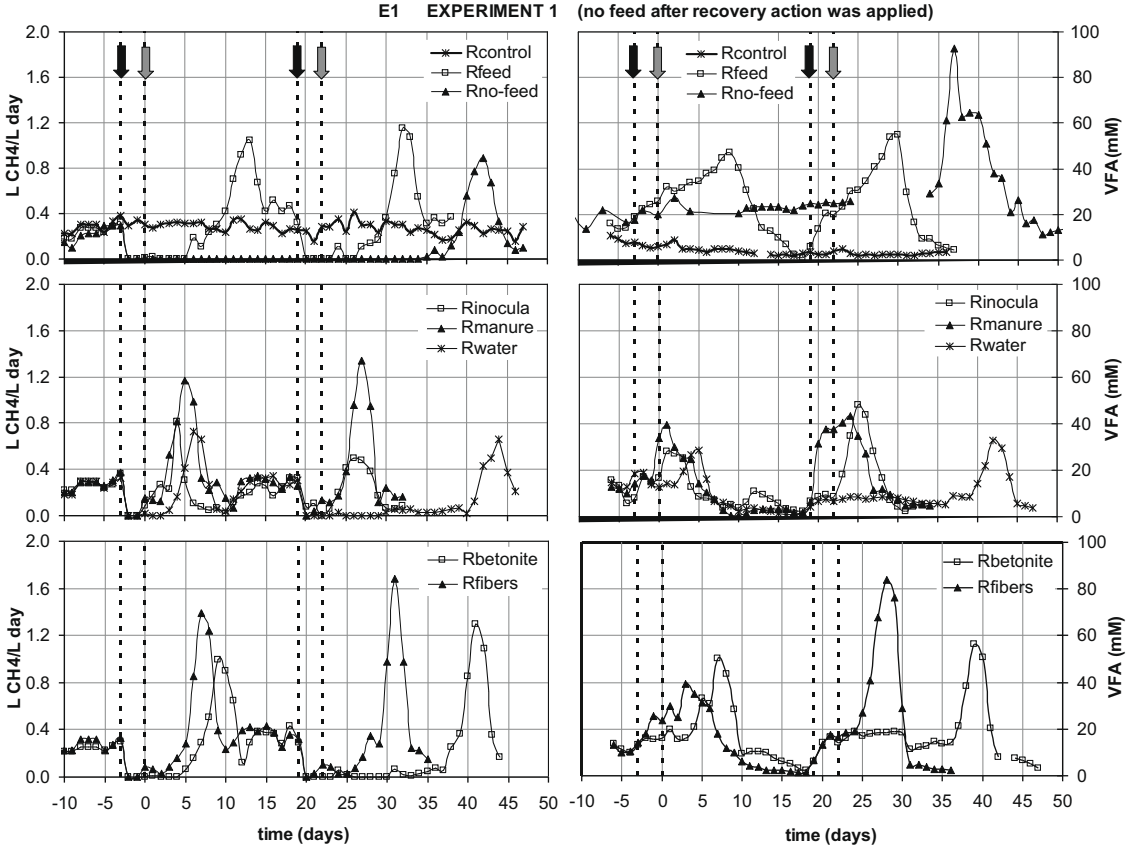

Fig. 2. Methane production (L CH_4/L day) and VFA concentration (mM) during E1 (no feed after recovery action was applied). Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.

Table 3
Process parameters obtained during E1 (RUN1 and RUN2).

RUN	Max Prod. Rate (L CH_4/L day)		Max VFA (mM)		Recovery time (days)	
	1	2	1	2	1	2
$R_{control}$	0.38	0.41	09.0	05.1		
$R_{no\text{-}feed}$	0.89		92.8		40	
R_{feed}	1.04	1.15	47.2	54.8	9	7
$R_{inocula}$	0.81	0.50	28.3	47.9	3	3
R_{manure}	1.17	1.34	39.7	43.3	4	3
R_{water}	0.72	0.66	28.6	32.7	5	20
$R_{bentonite}$	0.99	1.29	50.1	56.3	7	17
R_{fiber}	1.39	1.68	39.2	83.7	5	6

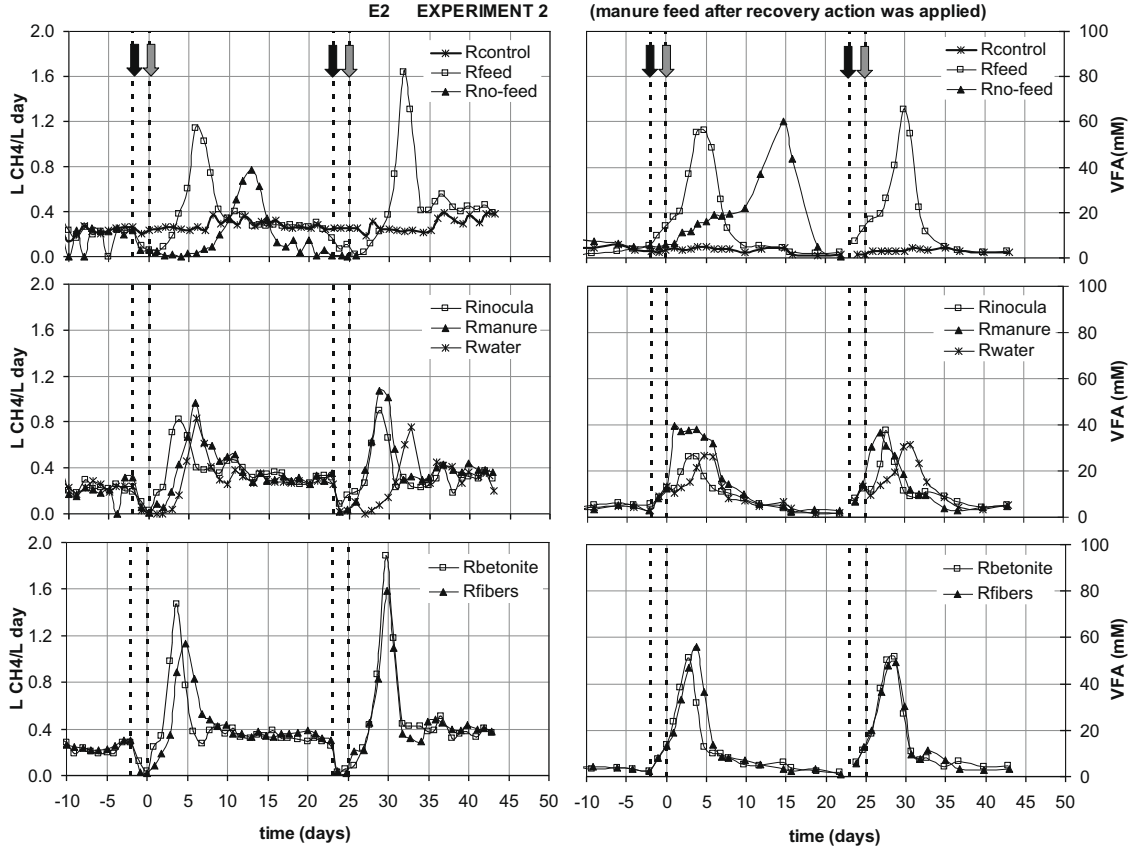

Fig. 3. Methane production (L CH_4/L day) and VFA concentration (mM) during E2 (semi-continuous feeding with manure after recovery action was applied). Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.

54.8 mM in R_{feed} for RUN1 and RUN2, respectively. The daily feeding of the reactor with manure (R_{feed}), resulted in reduction of the inhibitory LCFA concentration, due to dilution by feeding. By calculating the expected methane production from the substrates introduced in $R_{no\text{-}feed}$ (methane production measured/theoretical production expected), it was found that over 90% of the expected methane production was achieved. Oppositely, low methane recovery was obtained in R_{feed}, indicating that part of the LCFA was washed undegraded out of the reactor, allowing the system to recover faster.

The fastest recovery time was obtained, as expected, when the inhibited reactor was diluted with inoculum ($R_{inocula}$). 3 days after the application of the recovery action, the process recovered and the lowest VFA accumulation was registered, 28.3 mM (Fig. 2 and Table 3). Dilution strategies, with the replacement of 40% of reactor content, resulted in dilution of the initial LCFA concentration, estimated on 2.4 g L^{-1} (60% compared to the initial concentration). The reactor diluted with manure (R_{manure}) also showed a fast recovery time (4 days), but the maximum methane production rate and the maximum VFA accumulated levels in R_{manure} were also higher, due to the extra organic material contained in the fresh manure compared to $R_{inocula}$ (Fig. 2 and Table 3). However, in the second run (RUN2) those differences disappeared, with a very similar behaviour of $R_{inocula}$ and R_{manure}. The dilution introduced in R_{water} had a positive effect on the first run (RUN1) over inhibition, but the recovery time increased on the second run (RUN2), from 5 to 20 days (Table 3), by the consecutive wash out of biomass and residual organic matter (2 consecutive dilutions by water introduced in only 21 days without feeding the system). The longer recovery time in the R_{water} was attributed to the decrease also in the biomass content of the reactor which was not the case when dilution was made by inoculum ($R_{inocula}$) and fresh manure (R_{manure}). The content of biomass relative to LCFA concentration has been described as critical for the hydrolysis and acidification

of lipids (Miron et al., 2000; Salminen and Rintala, 2002b). The lipid-to-inoculum ratio has been previously shown to affect specific methanogenic activity during slaughterhouse waste digestion and LCFA inhibition (Salminen et al., 2000). Similarly, we can conclude that the inhibitory effect of LCFA was not only depended on the LCFA concentration, as it was shown in batch toxicity assays (Fig. 1), but also on the LCFA/biomass ratio, as it was shown by recovery time (Table 3) during discontinuous reactors operation when dilution with inoculum was applied.

The addition of adsorbents such as bentonite ($R_{bentonite}$) or fibers (R_{fibers}) had a positive effect on the recovery of the LCFA pulse, compared to the R_{feed} (reduction of the recovery time from 9 days in R_{feed} to 7 or 5 days in $R_{bentonte}$ or R_{fiber} in RUN1, respectively), with similar or lower VFA levels in reactors where absorbent were added (Fig. 2 and Table 3). Another advantage of using adsorbents as process recovery agents, compared to dilution strategies was the possibility of utilization of the total biogas potential contained in the LCFA, as LCFA was retained in the reactor, contrary to the dilution strategies, where a significant part of the initial LCFA concentration (40%) was removed undegraded from the system. An exception of adsorption recovery actions behaviour was reported in E1, in the second run (RUN2), with an increase in recovery time (6–17 days). This was due to the lower amount of bentonite and fibers (2.22 g VS L^{-1}) that were used in RUN2 compared to the RUN1, as it was assumed that fibers and bentonite were still inside the reactors in significant amounts (reactors were not fed during E1, and only small amounts were retrieved for sampling analyses). This behaviour would be discussed later, together with E2 results.

3.3. E2: LCFA inhibition of pre-exposed biomass in semi-continuously fed reactors and subsequent application of recovery strategies (daily feeding with manure after recovery action was applied)

This experiment was started approx. 2 months after experiment E1 was finished. During those 2 months the reactors were incubated at 55 °C as batches. Thereafter, semi-continuous feeding of the reactors started with one daily feeding with diluted fresh manure at an HRT of 20 days until constant production from diluted manure. Opposite to E1 feeding with manure was maintained during the entire experiment, except for $R_{no\text{-}feed}$, to simulate full scale co-digestion operation where feeding is rarely stopped.

As in E1, the $R_{no\text{-}feed}$ was the slowest to recover in experiment E2, although the recovery time was reduced to 10 days compared to 40 days in E1, and with lower accumulated VFA levels (Fig. 3 and Table 4). Daily feeding of the reactor with manure (R_{feed}), improved the process performance, due to dilution and washing effect, in accordance with experiment E1. However, discontinuation of the feeding is the most common action, to recover inhibition in full scale biogas plants. It is broadly accepted that when a process is inhibited and stressed, continuing reactor loading would lead to further VFA accumulation and maybe acidification. However, in our study, where LCFA inhibition was the cause of imbalance, waiting for process self-recovery was the worse strategy.

The effect of the dilution strategies in experiment E2 was similar to experiment E1 (Figs. 2 and 3) and was confirmed by the total LCFA degradation profiles of $R_{inocula}$ and R_{water}. The concentration of total LCFA (C18:1, C18:0 and C16:0 in Fig. 4) was reduced immediately after the dilution action with inoculum or water, to 60%, of the original LCFA concentration. The main difference between $R_{inocula}$ and R_{water} was the higher content of microbial biomass in $R_{inocula}$, resulting in a faster LCFA degradation rate (slopes in Fig. 4) and consequently in shorter recovery time and lower VFA accumulation levels compared to R_{water}, both in RUN3 and in RUN4 (Table 3). In R_{water} dilution strategy in E2 a clear improvement compared to E1 was observed (Tables 3 and 4), reducing the differences with the other dilution strategies (R_{water} compared to $R_{inocula}$ or R_{manure} in Table 4) by new biomass and organic matter introduced during daily feeding with manure. Dilution by manure still showed faster recovery compared to dilution with water (Table 4), which might be due to the higher biomass/LCFA ratio in R_{manure} compared to R_{water}. Similar results, where increasing the biomass/LCFA ratio by e.g. recirculation, could successfully recover LCFA inhibited process, have previously been reported (Hwu et al., 1997; Mladenovska et al., 2003; Salminen and Rintala, 2002b). In industrial facilities is not always easy to obtain new uninhibited inoculum, therefore, in such cases, dilution by fresh manure might be more practical.

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

Table 4
Process parameters obtained during E2 (RUN3 and RUN4).

RUN	Max Prod. Rate (L CH4/L day)		Max VFA (mM)		Recovery time (days)	
	3	4	3	4	3	4
$R_{control}$	0.37	0.39	04.8	04.3		
$R_{no\text{-}feed}$	0.77		60.4		10	
R_{feed}	1.14	1.64	56.3	65.3	4	5
$R_{inocula}$	0.82	0.90	26.4	37.7	3	3
R_{manure}	0.96	1.07	39.3	36.6	4	2
R_{water}	0.83	0.76	26.6	31.4	5	7
$R_{bentonite}$	1.47	1.88	51.2	41.0	2	3
R_{fiber}	1.13	1.58	56.1	49.3	3	3

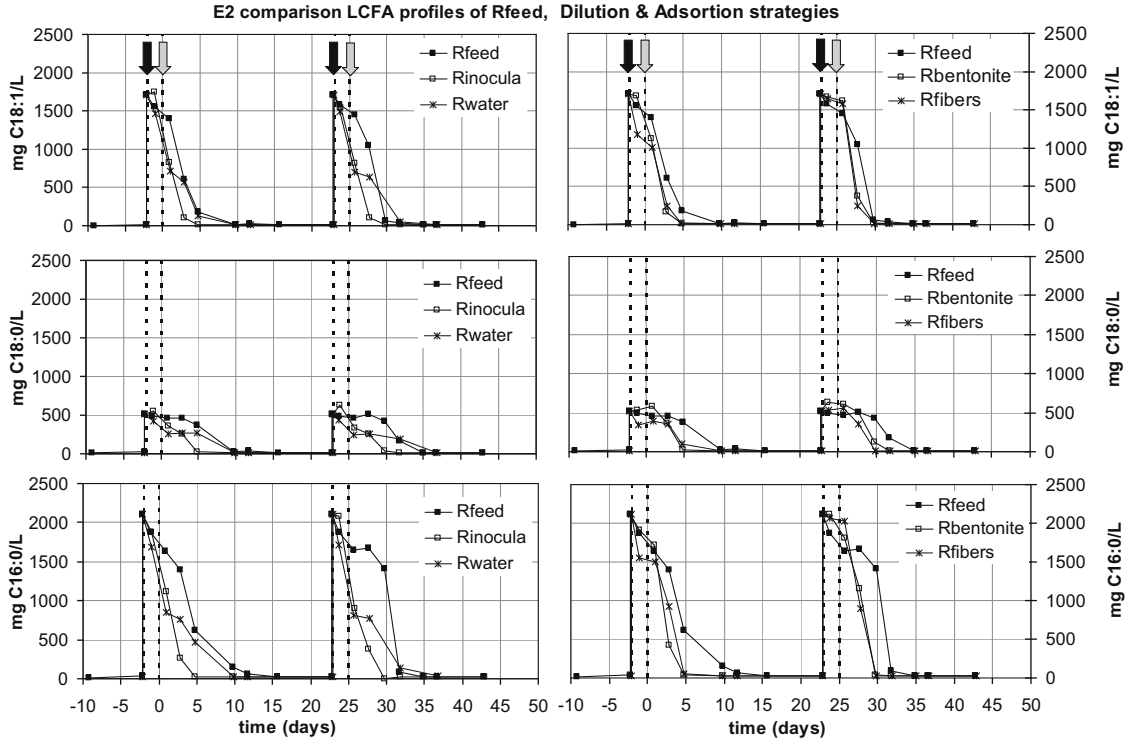

Fig. 4. LCFA degradation profiles during E2 (semi-continuous feeding with manure after recovery action was applied) for control (R_{feed}), dilution and adsorption strategies. Arrows indicate the LCFA pulse (4 g/L) and the time of recovery action application.

sult in recovery of the process, by binding LCFA and thus removing the cause of inhibition.

In E1 a reduced (R_{fiber}) or negative ($R_{bentonite}$) effect of recovery action in the RUN2 was observed (RUN1 compared to RUN2 in Fig. 2 and Table 3). This could be explained with the assumption that the residual absorbents from RUN1 may not possess the same absorbent capacity as "un-used" adsorbents. Active adsorption sites of remaining adsorbents might have been occupied by biomass or remaining organic matter. Adsorbents, like bentonite, have been described as support matrices for immobilization of anaerobic consortia, due to their adsorption capacity over microorganism (Chauhan and Ogram, 2005; She et al., 2006). During the E2, in both runs of adsorption strategies (RUN3 and RUN4), the same quantity of adsorbents was used (5 g VS L^{-1}), resulting in a very similar behaviour of the system for both runs (Fig. 3 and Table 4).

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

3.4. Adaptation of the system to LCFA pulses

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

From the $R_{no\text{-}feed}$, the process adaptation after the repeated LCFA pulses can be clearly seen as a reduction of the recovery time from 40 to 10 days and as a lower VFA accumulation, 92.8 mM compared to 60.4 mM for the RUN1 and RUN3, respectively (Figs. 2 and 3, and Tables 3 and 4). The observed adaptation is in agreement with previously reported by Cavaleiro et al. (2008), Nadais et al. (2006), and Sousa et al. (2007), where is it proposed that discontinuous treatment of LCFA, or LCFA pulses, would promote the development of an active anaerobic community, able to efficiently degrade LCFA. It is important to mention that, during the time between experiment E1 and E2, the reactors have been incubated without feeding, as batches, for a period of 2 months. In the literature, periods of non-feeding have been related with an improvement of the capacity for degradation of fatty wastes in terms of production, adsorption capacity and system stability (Coelho et al., 2006).

The other strategy that had identical set-up for all the runs and can easily be used for elucidation of any adaptation of the process was the strategy applied in R_{feed}. In Fig. 5 all R_{feed} experiments (E1 and E2) are shown together, with overlapping time axis, in order to be able to visually compare the time needed for process recovery (days), the maximum specific methane production rate (g COD_CH_4 g^{-1} VS day^{-1}) and acetate maximum consumption rate (g COD_Ac g^{-1} VS day^{-1}) as process parameters. The process seemed to adapt to the LCFA, with subsequent LCFA pulses. Only in RUN4 similar recovery time was achieved but, for all subsequent runs, higher maximum methane production or acetate maximum degradation rates were observed (Fig. 5). Nielsen and Ahring (2006) have similarly shown that a system submitted to previous oleate pulses, induced an increase in the tolerance level of aceto-clastic methanogens towards oleate. The adaptation or increased resistance to LCFA detected in R_{feed} and $R_{no\text{-}feed}$, can possibly be attributed to an increase in microbial biomass (higher biomass/LCFA ratio), or to changes in the microbial populations (selection of more LCFA resistant species), or changes in population structure (aggregate formation or more resistant structures).

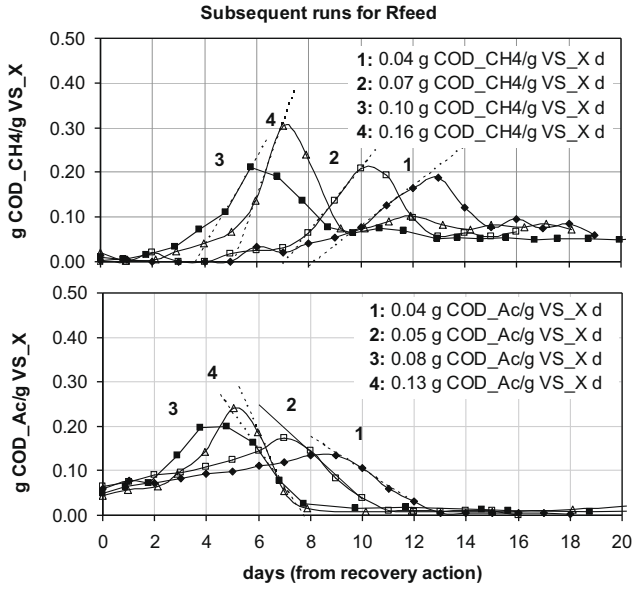

Fig. 5. Methane production rate (g COD_CH_4/g VS day) (A), and acetate consumption rate (g COD_Ac/g VS day) (B), for all semi-continuous-feeding runs (R_{feed}). Numbers indicate the subsequent runs. Discontinuous-lines are the calculated maximum slopes of methane and acetate rates.

4. Conclusions

Among the seven recovery strategies tested and evaluated, dilution of the reactors content with inoculum, thus increasing the biomass/LCFA ratio, or the addition of adsorbents, were found to be the best strategies to recover thermophilic manure reactors submitted to LCFA inhibition. The use of adsorbents seems to be the most reliable strategy for application on industrial facilities, where it is not easy to introduce dilution, emerging as a simple, feasible and cost-effective solution. The effect of adsorbents was related with competition with biomass in adsorbing LCFA, thus reducing their inhibitory effect, mainly due to the surface adsorption and transport sites saturation. On the other hand, broadly accepted practice, in real plants, to stop the feeding when an inhibition/imbalance of the process is detected revealed to be the worst approach to face LCFA inhibition in terms of recovery time and process stability.

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

Acknowledgements

The authors thank Hector Garcia from DTU (Denmark) and Dr. Montse Lloveras from Universitat de Lleida (Spain), for Lab supervision and assistance in LCFA-FAME determination method development. This work was supported by the Spanish Ministry of Education and Science (CAD/CRAI – Project REN 2004-00724) and Danish Energy Council (EFP-05 Journal No. 33031-0029).

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

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ABSTRACT: Batch activity tests and molecular profiling of the microbial community structure were performed on biomass samples taken at different times during continuous operation of biogas reactors, operated at 55C, with manure as a basis substrate, and submitted to successive inhibitory pulses with long-chain fatty acids (LCFA). Experimental data of the activity tests and microbial composition analysis were integrated into a mathematical model.
Improvement of hydrogenotrophic and acidogenic (ß-oxidation) activity rates was detected upon successive LCFA pulses, while a diferential toxicity effect over anaerobic trophic groups was observed. Recovery capacity was measured, while an adaptation process to LCFA inhibition was confirmed.
DGGE population profiles of 16S rDNA eubacterial and archaeal genes revealed that no significant changes on microbial community composition took place upon loading with LCFA. The sequencing of DGGE predominant bands showed close phylogenetic affinity to ribotypes from specific ß-oxidation bacteria families (*Syntrophomonas* and *Clostridiaceae*), while the main syntrophic archaeae domain was related with the genus *Methanosarcina.*
To explain the reported adaptation process, the hypothesis of an increase in the population of specific LCFA degrading microorganism, was further supported by mathematical modeling. From experimental and simulation results, the need to introduce modifications in the *IWA ADM1 Model*, related to LCFA degradation, was evidenced. New kinetics considering the relation between inhibitory substrate and specific biomass, as an approximation to the adsorption process, improved the model fiting and provided a better insight on the physical nature of the LCFA inhibition process.

KEYWORDS: *thermophilic anaerobic digestion, LCFA inhibition-adaptation, 16S rDNA profiling, ADM1 model*.

INTRODUCTION

Lipid containing wastes are interesting substrates for biogas production because due to their high methane yield potential. Lipids are initially hydrolyzed to glycerol and long chain fatty acids (LCFA), which are further converted, by syntrophic acetogenic bacteria, to hydrogen (H_2) and acetate (Ac), and finally to methane (CH_4) by methanogenic archaea. The degradation pathway of LCFA, are through ß-oxidation, has been reported as the rate-limiting step of the whole anaerobic digestion process (Lalman and Bagley, 2002). Recent advances in molecular microbial ecology techniques have brought new insights on the microorganisms that are involved in the ß-oxidation process. LCFA-degrading bacteria were found to be closely related to the *Syntrophomonadaceae* or *Clostridiaceae* families (Hatamoto et al., 2007; Sousa et al., 2007). These microorganisms are commonly proton-reducing acetogenic bacteria that

require the syntrophic interaction with H_2-utilizing methanogens and acetoclastic methanogens (Schink, 1997; Sousa et al., 2007).

LCFA are known to inhibit the methanogenic activity and its accumulation in anaerobic reactors is commonly reported as a major operational problem. These effects were initially attributed to permanent toxicity resulting from cell damage and are known to affect both syntrophic acetogens and methanogens (Rinzema et al., 1994; Hwu et al., 1998). Further studies have demonstrated that LCFA inhibition is reversible and that microorganisms, after a lag phase, are able to efficiently methanise the accumulated LCFA (Pereira et al., 2004). Physical adsorption of LCFA and their accumulation on the cell wall has recently been proposed as the main inhibitory mechanism. LCFA accumulation on the cell walls appears to create a physical barrier that hinders the transfer of substrates and metabolites (Pereira et al., 2005).

Despite the fact that LCFA inhibition is well documented and has a significant impact on the anaerobic digestion process, this phenomenon has neither been included in IWA ADM1 reference model (Batstone et al., 2002). Some models have been developed so far, in which the LCFA inhibition is mainly modeled as a non-competitive process on the lipolytic, acetogenic or methanogenic activities (Angelidaki et al., 1999; Salminen et al., 2000; Lokshina et al., 2003). However, the microbial aspects of the LCFA adaptation process remain poorly characterized, and further modelling developments are still required in order to link the results from physiological activity test and the characterization of microbial population dynamics throughout whole process.

Adaptation to inhibitory levels of LCFA has recently been reported in the thermophilic co-digestion with manure (Palatsi et al., 2009). In that particular study, biomass was successively exposed to inhibitory pulses of LCFA and the acclimatization process was characterized by monitoring the decreasing duration of the recovery time, the increasing methane yield, and the higher degradation rates measured. The outflow of the described reactors was used in the present study as source of LCFA inhibited and non-inhibited biomass. A similar behavior on inhibition and adaptation processes has also been reported in other recent studies on anaerobic co-digestion of lipids (Nielsen and Ahring, 2006; Cavaleiro et al., 2009). Currently, there is a poor evidence on whether this adaptation process is the result of a microbial population shift towards the enrichment of specific and better adapted LCFA-degraders (population adaptation or microbial community structure changes), or to the phenotypic adaptation of the existing microrganisms to high LCFA concentrations (physiological acclimatation).

The aim of the present study is to gain a deeper insight on the LCFA inhibition and adaptation process of the anaerobic consortium, by analyzing and comparing biomass samples obtained from reactors exposed to LCFA pulses (Palatsi et al., 2009). These samples were characterized in terms of specific physiological activity rates and of the microbial structure, by means of anaerobic batch activity test and by culture-independent molecular profiling, respectively. The results were used in the development and testing of a new mathematical model for the simulation of inhibition and adaptation process.

MATERIAL AND METHODS

Analytical Methods

Total solids (TS), volatile solids (VS), total Kjeldhal nitrogen (TKN), ammonia nitrogen (NH_4^+-N) and pH were determined according to Standard Methods (APHA, AWA,WEF, 1995).

Methane content in the biogas (%CH_4) and volatile fatty acids concentration in the liquid media (VFA), corresponding to Acetate (Ac), Propionate (Pr), *iso*-Butyrate (iso-Bu), *n*-Butyrate (n-Bu), *iso*-Valerate (iso-Va), *n*-Valerate (n-Va) and Hexanoate (Hex), were measured in a gas chromatograph fitted with a flame ionization detection, GC-FID (GC 20100, Shimatzu, Japan). Two different capillary columns: Porapak 60/80 Molsieve (6ft 3mm) and ZEBRON Phase ZB-FFAP (30mx0.53mmx1.00 μm), were used for CH_4 or VFA determination, respectively, as described elswere (Angelidaki et al., 2007).

Biomass and specific batch test

Samples from the outflow of semi-continuous thermophilic (55ºC) laboratory reactors, fed with manure, and exposed to two successive LCFA pulses (4 g L^{-1}), were used in subsequent anaerobic batch assays as inoculum, and as biomass to run molecular microbiology analysis. Manure inflow, used as the basic substrate, maintained reactor hydraulic retention time (HRT) at 20 days, with a corresponding organic loading rate (OLR) of 1.0 g VS L^{-1} d^{-1}. Fresh manure was diluted with distilled water in order to decrease the ammonia level (1.41±0.25 g TNK L^{-1}; 0.92±0.03 g NH_4^+-N L^{-1}) and ensure that the pulse of LCFA was the only inhibitory compound throughout the experiments. Samples were withdrawn from the reactors at different stages; before each LCFA pulse

(samples I and III), when the process was clearly inhibited (samples II and IV), and when it recovered and reached a new steady state (sample III and V). The sampling program is shown in Table 1. The time between sampled biomass I and III was 25 days, and between samples III and V was 24 days. So, in all cases, more than one HRT was allowed before it was assumed that a new state was established.

Analysis of LCFA concentrations in reactors showed that the concentration of LCFA at sampling time II and IV was ≈4 g L^{-1}, while LCFA were not detected at samples III and V. More detailed experimental set-up and results from reactors operation can be found in Palatsi et al. (2009).

Specific batch activity tests of non-inhibited (samples I, II and V) and LCFA inhibited biomass (samples II and IV) were performed in anaerobic batch assays with specific substrates, according to Table 1. Glass bottles (118 mL total volume) were inoculated with 2.5 g VS L^{-1} of sampled biomass, resuspended in basic anaerobic medium (Angelidaki et al., 2007), previously amended with 31mM $NaHCO_3$. A reducing solution of sodium sulfide (3.20 mM Na_2SO_3) was also added, to fill up the glass bottles up to a final total liquid volume of 50 mL. pH was also adjusted (7.5-8.0). The flasks were stirred and bubbled with N_2 gas in order to remove O_2, before closing them with rubber stoppers and aluminium crimps. In order to measure the aceticlastic methanogenesis and acetogenic activity rates, the bottles were amended with 20mM and 10mM of acetate (Ac) and butyrate (Bu), respectively, while the hydrogenotrophic methanogenesis was assayed by injecting 70 mL H_2 and 40 mL CO_2 in flasks headspace (1atm, 20ºC), according to the assay described in Angelidaki et al.(2007). Inhibited and non-inhibited biomass, without the addition of any synthetic substrate, were included as controls to determine the methane production derived from the depletion of the LCFA adsorbed onto the biomass (for samples II and IV) and from from utilization of residual organic matter present in the samples (for samples I, III and V). The activity tests (for all series) were conducted in quadruplicate (3 vials for CH_4 analysis + 1 vial for VFA determination). CH_4 and VFA were monitored in the headspace and in the liquid medium, respectively. Batch tests set-up and monitored variables are presented in Table 1.

The specific biomass activity rate was determined by linear regression on the initial slope of the accumulated methane production curve per VS content of biomass (mg COD_{CH4} g VS^{-1} d^{-1}). For substrates that are not directly converted into methane, like butyrate or LCFA, the methane production rate is only a valid measure of syntrophic

activity, when the aceticlastic and hydrogenophilic steps are not the rate limiting process (Dolfing and Bloemen, 1985). Consequently, the maximum specific substrate utilization rate in the assays with butyrate was also calculated from the steepest linear decline in substrate concentration (mg COD_{Bu} g VS^{-1} d^{-1}), as described by Nielsen and Ahring (2006). In control vials with the inhibited sampled biomass (control +LCFA) in Table 1, the LCFA maximum specific utilization rate was estimated from the initial maximum slope of Ac production (mg COD_{Ac} g VS^{-1} d^{-1}), assuming that Ac was the main product from LCFA β-oxidation (Batstone et al., 2002).

Table 1. Summary of batch tests set-up and monitored variables in assays.

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

Note: Roman numbers indicate biomass samplings from reactors. LCFA pulses were introduced in reactors on day 0 and day 25. Days in parenthesis indicates time from the second LCFA pulse. (A) Gas substrate units kmol m^{-3}. Sfa(0) is LCFA remaining concentration from reactors pulse (4 g L^{-1}) adsorbed onto biomass introduced in vials.

Molecular analysis of microbial community structure

The effect of LCFA pulses, and subsequent biomass inhibition and adaptation response, on the anaerobic microbial community composition was analyzed at beginning and at the end of reactor operation (samples I and V, according to Table 1). Since the main LCFA-degrading microorganisms are proton-reducing bacteria, it is important to monitor the dynamics not only of the syntrophic eubacteria but also of the archaeal community in microbial studies related to LCFA inhibition and adaptation processes.

Reactor samples of 2 mL were fixed in 1 mL of guanidine thyocyanate (4M-Tris-Cl pH 7.5:0.1M, autoclaved) and 0.5 mL of N-lauroyl sarcosine (10% N-LS autoclaved) as decribed previously. Fixated samples were immediatly frozen and stored at -20ºC until further processed.

The total sample DNA was extracted by using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., USA), according to the instructions of the manufacturer. The V3-V5 variable regions of the eubacterial 16S rDNA gene was amplified by the polymerase chain reaction (PCR) using the F341 and R907 primers (Yu and Morrison, 2004). A nested approach was used to amplify archaeal 16S rDNA, based on Raskin et al. (1994).

The primer pairs ARCH0025F-RCH151R and F344-R915 were used respectively for the first and the nested PCR reactions. The products of the nested PCR were used in subsequent DGGE analysis. The forward primer used in the generation of the DGGE amplicons included a GC clamp at the 5' in order to stabilize the melting behavious of the DNA fragments during DGGE. All PCR reactions were performed in a Gradient Mastercycler (Eppendorff, Germany).

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

Relevant DGGE bands were excised with a sterile filter tip, resuspended in 50 µl sterilized Milli-Q water, and stored at 4°C overnight. These extracts were subsequently reamplified by PCR and sequenced. Sequencing was accomplished using the ABI prism BigDye Terminator v. 3.1 cycle sequencing kit (Perkin-Elmer Applied Biosystems, USA) and an ABI 3700 DNA sequencer (Perkin-Elmer Applied Biosystems, USA), according to instructions of manufacturer. Sequences were edited using the BioEdit software package v. 7.0.9 (Ibis Biosciences, USA) and compared against the NCBI genomic database with the BLAST search alignment tool (Altschul et al., 1990). Nucleotides sequences obtained in the present study have been deposited in the GenBank database under accession numbers XXXX-XXXX expected 01/08/09 GenBank.

Mathematical modeling and parameter estimation

Processes related monitored variables (Table 1) were modeled with IWA ADM1 as basis model, implemented in MatLab (The Mathworks, USA), applying same structure, nomenclature and units (Batstone et al., 2002). Data obtained from activity batch test were used to estimate several unknown parameters and the initial biomass concentrations, as explained below. The default values for kinetic parameters and stochiometric coefficients suggested by Batstone et al.(2002), for thermophilic operation, were adopted, with the following exceptions: a) The value of LCFA inhibition of hydrogenoctrophic methanogenesis ($K_{I,h2\ fa}$), which is not given for

thermophilic range by Batstone et al.(2002), was assumed to be the same as for mesophilic, $K_{I,h2\ fa}=5\ 10^{-6}\ kg\ COD\ m^{-3}$; b) The adopted value for the liquid-gas mass transfer coefficient was $k_La= 45\ d^{-1}$; c) The pH was assumed to be constant, since a buffering solution was added to each vial and no significant pH change was detected. In all simulations, the initial value for inorganic nitrogen was $S_{in}(0)=10^{-2}\ kmol\text{-}N\ m^{-3}$. Initial specific substrates concentrations in each vial, used as model initial vector, are described in Material and Methods section and in Table 1.

Time course of monitored variables obtained from batch vials with non-inhibited biomass (samples I, III and V), with H_2/CO_2, Ac and Bu as substrates (Table 1), were used to estimate by sequential optimization procedure (step-by-step, where values found were then used as fixed parameters in next step) the initial H_2, Ac and Bu degraders populations, $X_i(0)$ (kg COD_X m^{-3}), using ADM1 and its default biochemical parameters values (Batstone et al., 2002), as indicated in Table 2.

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

The second approach, named as *Inhibition Model*, considered the uptake of LCFA to be described by the Haldane's inhibition kinetics and both methanogenic processes (uptake of acetate and hydrogen) to be affected by a non-competitive term with a common LCFA inhibition constant, K_I (kgCOD_S_I m^{-3}), as shown in Table 2. Such inhibition kinetics have already been proposed by other authors. Angelidaki et al. (1999), studying manure codigestion with glycerol trioleate or bentonite bound oil degradation, considered a non-competitive LCFA inhibition to the lipolitic, acetogenic and methanogenic steps and the *Haldane* inhibition kinetics on the ß-oxidation process. Salminen et al.(2000) and Lokshina et al.(2003), using solid slaughterhouse waste,

considered a non-competitive inhibition kinetics due to LCFA, affecting acetogenesis and methanogenesis. With those assumptions, new initial values for $X_{fa}(0)$, $k_{m,fa}$ and K_I, were estimated by multiple parameter optimization (Table 2).

Table 2. Process rates modifications used in different model approaches

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

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

The last approach, was named as *Inhibition-Adsorption Model*, and included an approximation of the physical adsorption of LCFA onto biomass, as an inhibition mechanism. Adsorption is considered as a rapid physico-chemical phenomenon, while desorption (degradation) is a biologically mediated process by LCFA-degraders (Hwu et al., 1998). Pereira et al. (2004) proposed a modification of the *Haldane* equation for the LCFA inhibition process, which consider ths *biomass-associated substrate* per VS unit, S_{ba} ($M_{substrate}$ $M_{biomass}^{-1}$), instead of the total substrate concentration (S_{fa}). Consequently, by adopting this concept, the proposed *Inhibition-Adsorption Model* assumes the following hypothesis: a) Inhibition of LCFA uptake process can be expressed by the Haldane kinetics; b) A Non-competitive reversible inhibition term can be used on acetogenesis and methanogenesis; c) In the the previous inhibition processes, the inhibitory constant (K_I) is replaced by a new inhibitory term, $K_{IFA}=K_I' \cdot X_{fa}/S_{fa}$, proportional to the specific ratio between the LCFA degrading population and the substrate (X_{fa}/S_{fa}), being higher (less inhibition) when this ratio value increases (Table 2).

The objective function to be minimized in sequential optimization procedures, for each step or specific substrate, *k*, was calculated according to Eq 1;

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

Where, y^*_{kji} represents the measured value of variable *j*, in vial *k*, at time *i*, and y_{kji} is the corresponding simulated value. Variable *j* from vials *k* has n_{kj} measured values at successive different times *i*. The weight factor, w_{kj}, used in optimization was defined as Eq 2;

$$w_{kj} = \left[n_{kj} (\max(y^*_{kji}) - \min(y^*_{kji}))^2 \right]^{-1} \qquad Eq.2$$

with $\max(y^*_{kji})$ and $\min(y^*_{kji})$, being the maximum and minimum measured value of varaible *j* in vial (step) *k*. The objective function used in the multiparameter estimation with datasets II and IV was calculated according to Eq.3, and the optimization routine followed the downhill simplex method (Nelder and Mead, 1965) as implemented in the MatLab package.

$$fobj = \sum_k fobj_k \qquad Eq.3$$

Model data fitting accuracy was measured by the coefficients of determination *R2* defined in Eq.4;

$$R2_{kj} = 1 - \frac{\sum_{i=1}^{nkj} (y^*_{kji} - y_{kji})^2}{\sum_{i=1}^{nkj} (y^*_{kji} - \bar{y}^*_{kji})^2} \qquad Eq.4$$

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

RESULTS AND DISCUSSION

Specific batch tests

First set of batch tests analyzed were the ones with biomass taken from reactors just before the application of LCFA pulses (samples I and III in Table 1), or when system had recovered from previous inhibition stage (samples V in Table 1). Table 3 summarizes the results of activity batch tests on specific substrates; H_2/CO_2, Ac and Bu, respectively, as model substrates for the main trophic groups. Mean separation was

performed on the calculated rates by Multiple Range Test (MRT) with a significance level $\alpha = 0.05$ (Sheskin 2000).

Table 3. Substrate utilization rates of non-inhibited biomass (I. III and V).

Substrare	Unit	I	III	V
H_2/CO_2	mg COD_{CH4}/g VS^{-1} d^{-1}	91.13±5.89 a	131.69±6.61 b	147.24±3.70 c
Ac	mg COD_{CH4}/g VS^{-1} d^{-1}	127.75±6.53 a	122.92±8.25 a	135.02±10.70 a
Bu	mg COD_{CH4}/g VS^{-1} d^{-1}	183.40±18.82 a	181.77±2.57 a	183.87±37.40 a
	mg COD_{Bu}/g VS^{-1} d^{-1}	-263.80	-285.83	-230.86

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

From the results on net methanogenic activities (Table 3), a significant increase on the hydrogenotrophic methanogenic activity rate was observed (from 91.13 to 147.24 mg COD_{CH4} g VS^{-1} d^{-1}), while the net acetoclastic methanogenic activity remained at relatively similar level along time (127.75, 122.92 and 135.02 mg COD_{CH4} g VS^{-1} d^{-1}). These results are in agreement with previous findings on suspended sludge and fixed bed reactors subjected to LCFA inhibition, which concluded that hydrogenotrophic methanogens appeared to be more resistant to LCFA inhibition than acetoclastic methanogens (Templer et al., 2006). It may thus be expected an improvement on the hydrogenotrophic activity, compared to acetoclastic methanogens, upon successive inhibition-recovery stages, a trend that has been observed in the present study (Table 3).

Concerning the the acetogenic activity, the n-butyrate (Bu) uptake rate remained fairly constant (263.80, 285.83 and 230.86 mg COD_{Bu} gVS^{-1} d^{-1} respectively for samples I, III and V) and no significant statistical differences were found in terms of methane production rate (COD_{CH4}) (Table 3). Similary, Nielsen and Ahring (2006) found that, the maximum substrate utilization rate for Ac and Bu, in biomass from thermophilic reactors fed with a mixture cattle and pig manure, subjected to oleate pulses (2 g L^{-1}), decreased or remained constant, while the methanogenic activity rate from H_2/CO_2, but also from formate and Ac, experienced an increase.

To analyse the inhibitory effect of LCFA pulses on specific activities of representative trophic groups, a second sets of batch tests were run with biomass, sampled 2-3 days after each LCFA pulse, and when biogas production in the reactor evidenced a clear inhibition behavior (samples II and IV, according to Table 1). Tests were performed with H_2/CO_2, Ac, and Bu as methanogenic and acetogenic model substrates, respectively. The sampled biomass (II and IV) presented remaining adsorbed LCFA concentration. Additionally, one vial was included as control, (*Control + LCFA) which* was incubated with only this sample, without any substrate supplementation, in order to

follow the ß-oxidation process. The specific activities of inhibited biomass are summarized in Table 4.

Table 4. Substrate utilization rates of LCFA inhibited biomass (II and IV).

Substrate	Units	II	IV
H_2/CO_2(+LCFA)	mg COD_{CH4}/g VS^{-1} d^{-1}	67.57±7.88 a	90.76±2.67 b
Ac(+LCFA)	mg COD_{CH4}/g VS^{-1} d^{-1}	44.63±1.30 a	56.67±4.43 a
Bu(+LCFA)	mg COD_{CH4}/g VS^{-1} d^{-1}	183.95±3.80 a	174.05±15.39 a
	mg COD_{Bu}/g VS^{-1} d^{-1}	-183.24	-161.81
Control(+LCFA)	mg COD_{CH4}/g VS^{-1} d^{-1}	163.35±8.75 a	218.77±16.08 b
	mg COD_{Ac}/g VS^{-1} d^{-1}	104.93	153.62

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

In general, a clear reduction in all monitored metabolic activities was observed upon the application of a LCFA pulse (Table 4 compared to Table 3). During activity batch tests of the LCFA inhibited biomass, the remaining LCFA concentration (from the reactor pulse and adsorbed onto the biomass) was completely consumed and methane production reached a maximum plateau close to the theoretical value. These results confirm that LCFA inhibition is a reversible phenomenom, since neither syntrophic acetogenic nor methanogenic activities were irreversibly damaged, in accordance to what has previously been reported (Pereira et al., 2004). Acetoclastic methanogenesis was the most affected by LCFA activity with 44.63-56.67 mg COD_{CH4} gVS^{-1} d^{-1} compared to 127.75-122.92 mg COD_{CH4} g VS^{-1} d^{-1} for the LCFA-inhibited and uninhibited biomass respectively (Table 3). Those vials exhibited not only lower methane production rates but also a longer lag-phase, compared to the activities before the LCFA pulse. It has been proposed that accumulation of LCFA, adsorbed onto the biomass, can hinder the transfer of substrates and products throught the membrane, causing a delay in the initial methane production (Pereira et al., 2005). The hypothesis of LCFA-induced transport limitation phenomena, was reinforced by the fact that H_2, the smallest methanogenic substrate molecule, was reported to be the first to be transformed into methane, suggesting a faster transport of this molecule through the adsorbed LCFA layer, in relation to other substrates of higher molecular weight, in LCFA inhibited systems (Pereira et al., 2005). In agreement with this, the measured hydrogenotrophic methanogenic activity was less affected by the LCFA inhibitory pulse in the present assays, with rate values of 90.76 mg COD_{CH4} gVS^{-1} d^{-1} (Table 4), similar to initial system hydrogenotrophic activity, prior to the LCFA inhibitory pulse (91.13 mg COD_{CH4} g VS^{-1} d^{-1} on Table 3).

It has also been described in literature that methanogens are more susceptible to LCFA inhibition than acidogens (Lalman and Bagley, 2002; Mykhaylovin et al., 2005), which is also in agreement with the lower differences in acetogenic activities detected on Bu vials, before and after LCFA inhibition (I-II on Table 3 and III-IV on Table 4).

In relation to the control vials, LCFA batchs (Control+LCFA), a clear improvement on the ß-oxidation process along time was observed (from 163.35 to 218.77 mg COD_{CH4} g VS^{-1} d^{-1} or from 104.93 to 153.62 mg COD_{Ac} g VS^{-1} d^{-1} in terms of substrate production rate, Table 4). Mladenovska et al. (2003) described the biomass of digested manure and lipids to be more active and with higher initial rates of methane production than the biomass of solely digested manure (not exposed to lipids). These results were related to the importance of the interaction microorganism-substrate-particle size and, in particular, on the effect of lipids over aggregation and cell density. Pereira et al.(2004) reported an enhancement on the microbial activity upon depletion of adsorbed LCFA, by an increase or development of specific degrading populations, while Nielsen and Ahring (2006) also reported increasing oleate tolerance (from 0.3 to 0.7 g L^{-1}), in manure thermophilic systems exposed to oleate pulses, suggesting different explanations for this behavior, like the induction of higher hydrolysis rates, an increase on biomass concentration or changes in the microbial composition.

The observed differential LCFA inhibition effect on distinct trophic groups might, in principle, to be related to an enrichment of specific populations involved on LCFA degradation process. Nevertheless, a shift in bacterial and archaea communities could not be excluded. Those hypotheses will be discussed further, by means of molecular biology techniques and mathematical modelling tools.

Microbial community structure

The DGGE molecular profiling of PCR amplified eubacterial and archaeal ribotypes was performed on biomass samples taken at the beginning and at the end of reactors operation (Samples I and V, according to Table 1). Despite the fact that both sampling events were separated in time by more than 40 days (equivalent to two HRT intervals), and the biomass suffered two inhibitory LCFA pulses and subsequent recoveries stages during this period, no significant differences were observed in the microbial community structure of eubacteria and archaeae (Figure 1).

Up to 12 bands from the DGGE profiles were successfully excised, reamplified and subsequently sequenced. BLAST sequences comparison against NCBI genomic

database resulted mainly in close maches with sequences of uncultured microorganism related to families of *Clostridiaceae, Syntrophomonadaceaae, Bacillaceae* and *Synergites,* all from the *Firmicutes* eubacterial phylum (Figure 2). Members from these taxa are well known as LCFA degrading anaerobic syntrophic bacteria (Sousa et al., 2007; Hatamoto et al., 2007).

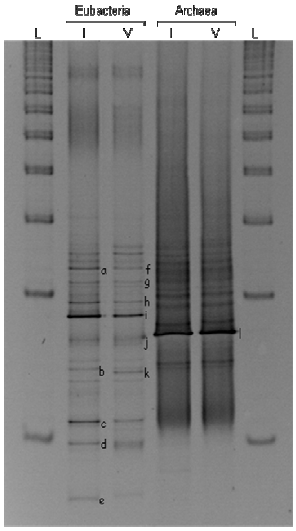

Figure 1. *DGGE profiles on eubacterial and archaeal 16S rDNA amplified from samples I and V. A standard ladder (L) has been used at both gel ends in order to check the DNA migration homogeneity. Successfully excised and sequenced bands have been named with lower-case letters.*

One of the DGGE depicted ribotypes (band *e*) was relatively homologous (95%) to sequences from uncultured bacteria in different anaerobic digesters and, more distantly (93%), to *Syntrophomonas wolfei* strain, as the closest phylogenetically defined match. *Syntrophomonas* genus has been described previously as specific syntrophic LCFA degrading bacteria (Lorowitz et al., 1989; Sousa et al., 2008).

The *Clostridiaceae* appears to be one of the most represented bacterial families in the microbial community of anaerobic digesters. In our study, band *f* is closely related to uncultured bacteria previously found in different solid waste-thermophilic anaerobic bioreactors (96-97% of sequence homology) and to strains of *Clostridium stercolarium* and *Clostridium thermocellum* (95%). This later species is also the closest cultivated microorganism to the sequence of band *g* (93%). Despite the fact that band *a* is clustered with other sequences from the *Clostridiaceae* family, it has a poor homology with database sequences from uncultured and cultured species related to clostridia. Besides the β-oxidation process, *Clostridium* species are related to peptide and polysaccharides degradation in thermophilic anaerobic systems and, have been found to

be particulary resistant to the environmental changes experienced during the anaerobic storage and management of pig slurry (Peu et al., 2006).

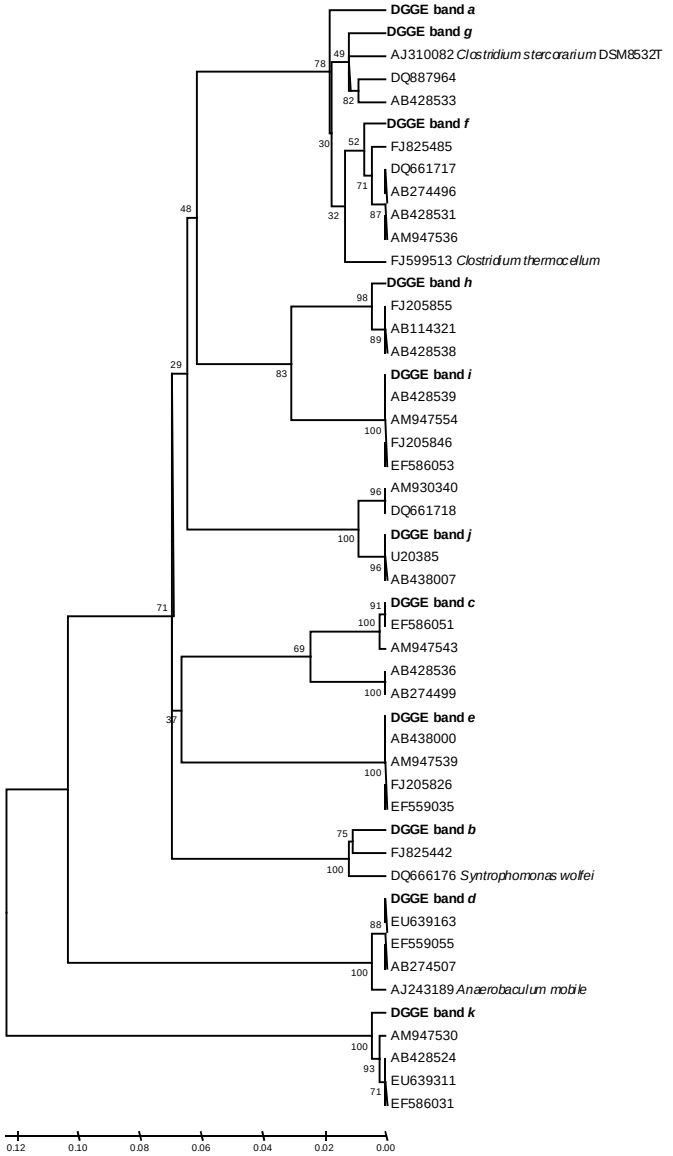

Figure 2*. Phylogenic eubacteria tree of 16S rDNA from DGGE excised bands and from closely related sequences deposited at the GenBAnk database (accession numbers are given between box brackets). The tree was generated using the Neighbour-joining algorithm and the Kimura 2-parameter correction, and was bootstrapped 1000 times. still some revision is needed*

Band *j* was identical to the type strain sequence of the described *Bacillus infernus*. This halotolerant and thermophilic bacterium is the only strictly anaerobic species in the genus *Bacillus* and is able to grow on formate and lactate with Fe(III), MnO_2, trimethylamine oxide, or nitrate as an electron acceptor (Boone et al., 1995). This microorganism has been isolated from deep terrestrial subsurface areas and, to our

knowledge, there are no previous records of its occurrence in anaerobic digesters. Yet, a very similar uncultured ribotype (99% sequence homology) was obtained during the composting of hyperthermophilically pre-treated cow dung wastes (Figure 2).

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

No reference strains were found to be sufficiently related to the sequences from bands *h, i, k* and *e* for its phylogenetic assignment. The sequence from band *i* was highly homologous, or ever identical, to a number of uncultured ribotypes obtained from different mesophilic and thermophilic anaerobic digesters degrading organic solid wastes (Goberna et al., 2009; Kröber et al., 2009; Tang et al., 2004). Interestingly, these environments also contained ribotypes that were closest related to the band *h*, though sequence homology was relatively low in this case (90%). On the other hand, bands *h* and *k* were both related to uncultured bacteria from thermophilic microbial fuel cell (Wrighton et al., 2008), with a sequence homology of 90% and 96%, respectively. Similary, the sequence of band *e* is also highly homologous (99%) to other uncultured ribotypes obtained from the same composting sample that has previously been reported from band *j* (Figure 2).

In relation to the archaeal domain, a single predominant band was observed in the DGGE profiles (band *l*). The associated sequence was 97% homologous to that of the *Methanosarcina thermophila* type strain. This thermophilic archeon is a methanogen that has been found in a wide variety of thermophilic anaerobic digesters treating organic wastes. Sequence homology of band *l* was higher in relation to another strain of the same species that was enriched in thermophilic anaerobic digester operated at high concentration of volatile fatty acids (Hori et al., 2006). Mladenovska et al.(2003) compared a digestion of cattle manure at mesophilic conditions to digestion of a mixture of manure with glycerol trioleate (2% w/w). They did not find any differences in the diversity of archaea species, although the reactors showed different performance. The vast majority of clones they detected were phylogenetically close to *Methanosarcina siliciae.* In our study, the origin of the inoculum and the daily manure feeding system might have exherted a strong influence on enrichement of specific methanogenic

populations. Karakashev et al. (2005) studied the influence of environmental conditions and feeding on methanogenic populations in a real scale biogas plants, reporting a dominance of *Methanosarcinaceae* members on manure digesters. Kaparaju et al.(2009) also reported the predominance of *Methanosarcinaceae* on the pilot plant (Kogens-Lyngby, Denmark), which was used as source of inoculum for semi-continuous reactors sampled in the present study (Palatsi et al., 2009).

Mathematical modeling and parameter estimation

Data from batch activity assays were used to test the three model approaches summarized in Table 2, in order to determine whether the detected improvement or adaptation process can be explained by an increase of specific degrading populations (X_i), and/or a modification of the adsorption-inhibition process, assuming that microbial composition shift was not the reason for the observed adaptation.

The *IWA ADM1 Model* simulations and the experimental data from batch activity test with not inhibited biomass (batch with samples I, III and V), are shown in Figure 3. The coefficient *R2* of the experimental data fit ranging from *0.78 to 0.99*, for H_2/CO_2, Ac and Bu vials, respectively. Estimates of the initial biomass content of specific trophic groups, $X_i(0)$, are summarized in Table 5a. When the initial population concentration, $X_i(0)$, and the maximum uptake rate, $k_{m,i}$, were simultaneously estimated at each step, the obtained $k_{m,i}$ values were relatively close to those suggested by Batstone et al.(2002) and no significant differences in the coefficients of determination were found. Moreover, at the tested initial substrate concentrations (in activity assays, $S_i(0)>>>Ks_i$), the effect of changing the half saturation constants (Ks_i) resulted in small variations in the final results. For that reason, the sequential parameter estimation of initial X_i values, by adopting suggested biochemical parameters from IWA ADM1 (Batstone et al., 2002), was considered adecuate.

Table 5a. Estimated parameter values for non inhibited batch tests data sets I, III and V

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

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

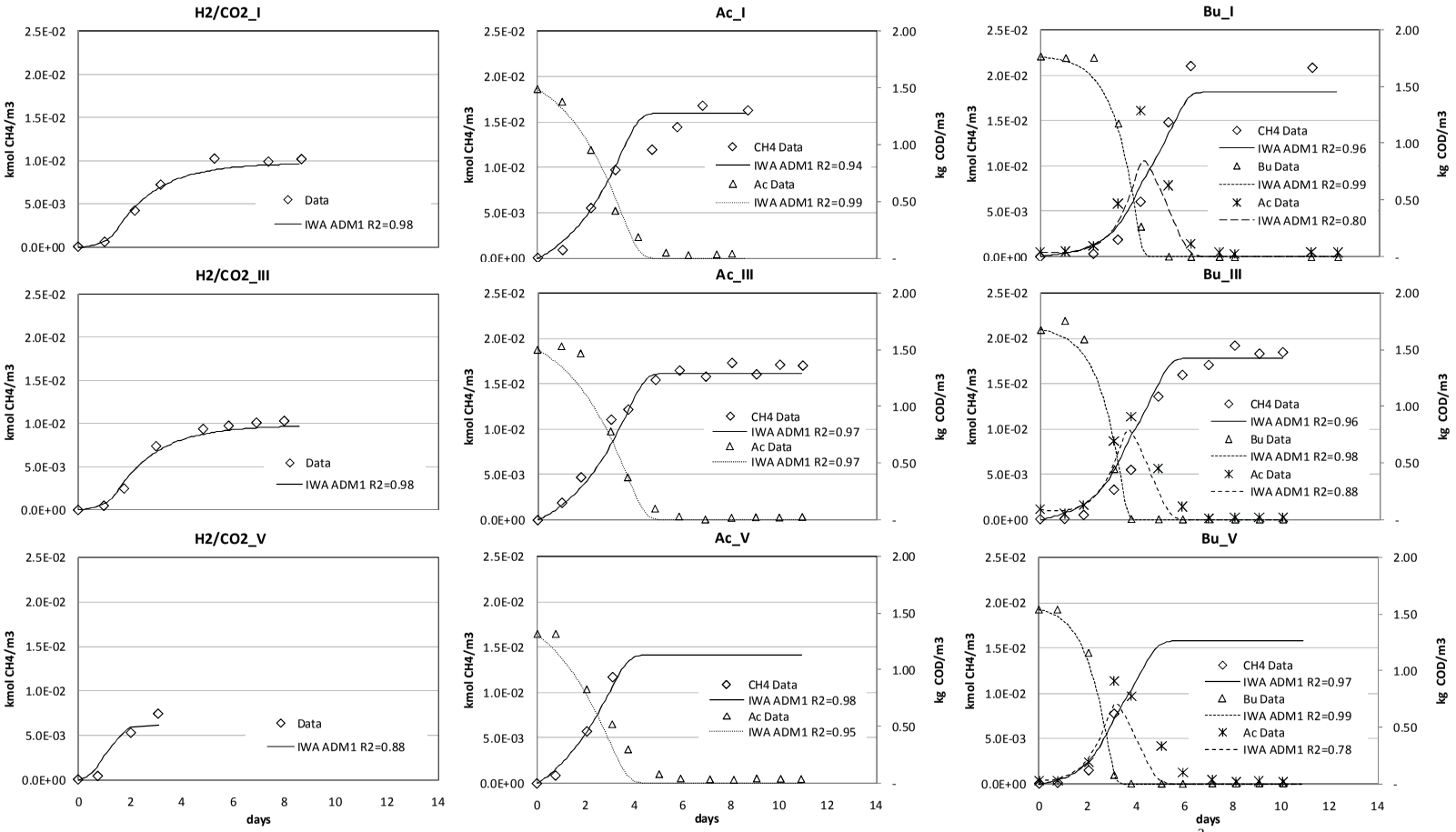

Figure 3*. Experimental data (n_{kj} point markers) and IWA ADM1Model results (lines) for activities (k) to H_2/CO_2, Ac, Bu in terms of CH_4 (kmol CH_4 m^{-3}) production and substrate degradation (kg COD m^{-3}) for non-inhibitedbiomass I, III and V. Coefficients of determination (R2) for model fitting are introduced in figure.*

Due to technical difficulties on the measurement of the CH_4 production in batch V, caused by an operational problem on the GC-FID, model fitting in this batch, was based mainly on the VFA production-degradation profile (Figure 3). Based on the estimated initial biomass content of specific microorganisms, $X_i(0)$, results, an initial acetoclastic methanogenic population stability can be outlined (Table 5a). However, the initial hydrogenotrophic methanogenic population $X_{h2}(0)$ increased along sampling time, which could explain the improvement of the hydrogenotrophic activity (H_2/CO_2) observed (Table 3). From the microbial community analysis, it was not possible to differentiate between methanogenic populations, because the most abundant isolated archaeae was affiliated with the group *Methanosarcina* (Figure 2), which are able to produce CH_4 via Ac and also via H_2/CO_2 (Balch et al., 1979).

In the analysis of batch data-sets II and IV, the initial amount of hydrogenotrophic methanogens $X_{h2}(0)$, aceticlastic methanogens $X_{ac}(0)$, and butyrate acetogens $X_{bu}(0)$, were assumed to be the same as in tests I and III respectively (Table 5a), as explained in the Material and Methods section. The initial content of LCFA in batch tests II and IV ($S_{fa}(0)$, *2.23* and *2.64 kg COD* m^{-3}) was the remaining concentration from the previous LCFA pulse in the reactor, adsorbed on the biomass. As general procedure, in each tested approach with inhibited batch tests data, a multiple parameter estimation ($X_{fa}(0)$, $k_{m,fa}$ and K_I) was performed for batch test II and the obtained kinetic parameter values were then used in the estimation of the initial LCFA degraders population, $X_{fa}(0)$, as sole parameter optimized in batch test IV (Table 5b).

The first approach to estimate $X_{fa}(0)$ and $k_{m,fa}$ parameters was the *IWA ADM1 Model* (Table 2). Figure 4 shows the predicted values for each modeled variable versus the experimental data. Although the predicted CH_4 production curve and S_{ac} or S_{bu} evolution values are acceptable in some cases, it was not possible to find an unique set of parameters ($X_{fa}(0)$ and $k_{m,fa}$) able to fit all experimental data together, with sufficiently high coefficients of determination (Figure 4). Hence, the need to introduce modifications in IWA ADM1 model, in order to express adequately the LCFA inhibition process is justified.

The second tested approach, named *Inhibition Model*, introduced the *Haldane* inhibition kinetics for the ß-oxidation and the reversible non-competitive inhibition kinetics for acetate or hydrogen methanogenesis (Table 2), as previously reported (Angelidaki et al., 1999; Salminen et al., 2000; Lokshina et al., 2003). The optimized parameter values for batch test II are shown in Table 5b. An increase in the initial LCFA degrading

population, $X_{fa}(0)$, from 2.40 10^{-3} to 4.45 10^{-2} kgCOD_X m^{-3}, in batch test IV was detected, maintaining a maximum degradation rate and inhibition constant of $k_{m,fa}$=*21.69 kg COD_S kg COD_X*$^{-1}$ *d*$^{-1}$ and K_I=*3.35 kg COD m*$^{-3}$, respectively. Coefficients of determination and model fitting are shown in Figure 4.

The last approach, called *Inhibition-Adsorption Model*, replaced the constant inhibitory factor, K_I, by a term K_{IFA} proportional to the ratio X_{fa}/S_{fa} (Table 2) to model the adsorption effect of LCFA on the cell walls. Estimated parameter values for test II were presented in Table 5b. An increase in the initial LCFA degrading population, $X_{fa}(0)$, from *9.89 10*$^{-4}$ to *1.30 10*$^{-3}$ *kg COD m*$^{-3}$, was also detected at sample IV (Table 5b), while initial $K_{IFA}(0)$ value remained between *1.15-1.15 kg COD m*$^{-3}$. The obtained coefficients of determination and model fitting are shown in Figure 4. The best model fittings were obtained with the *Inhibition-Adsorption Model*, although the obtained parameter set is not probable unique and these results could be considered as a first approach to express the importance of the LCFA/biomass ratio in the adsorption-inhibition process. The *Inhibition-Adsorption Model* was able to reproduce not only the lower production rates when system was inhibited but also the longer lag-phase during system inhibition.

Table 5b. *Estimated parameters values for inhibited batch tests data sets II and IV.*

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

Units; Xi (kg COD m^{-3}*); $K_{m,fa}$ (kg COD_S kg COD_X*$^{-1}$ *d*$^{-1}$*); K_I and K_I' (kg COD m*$^{-3}$*)*

Modelling results suggest that adsorption plays a key role in the overall LCFA inhibition-adaptation process, and that there is a need to introduce modifications in IWA ADM1 model when dealing with degradation of lipids. The proposed *Inhibition-Adsorption Model*, which produces a better fit of the experimental results than classical models, and provides a better representation of the physical nature of the overall LCFA

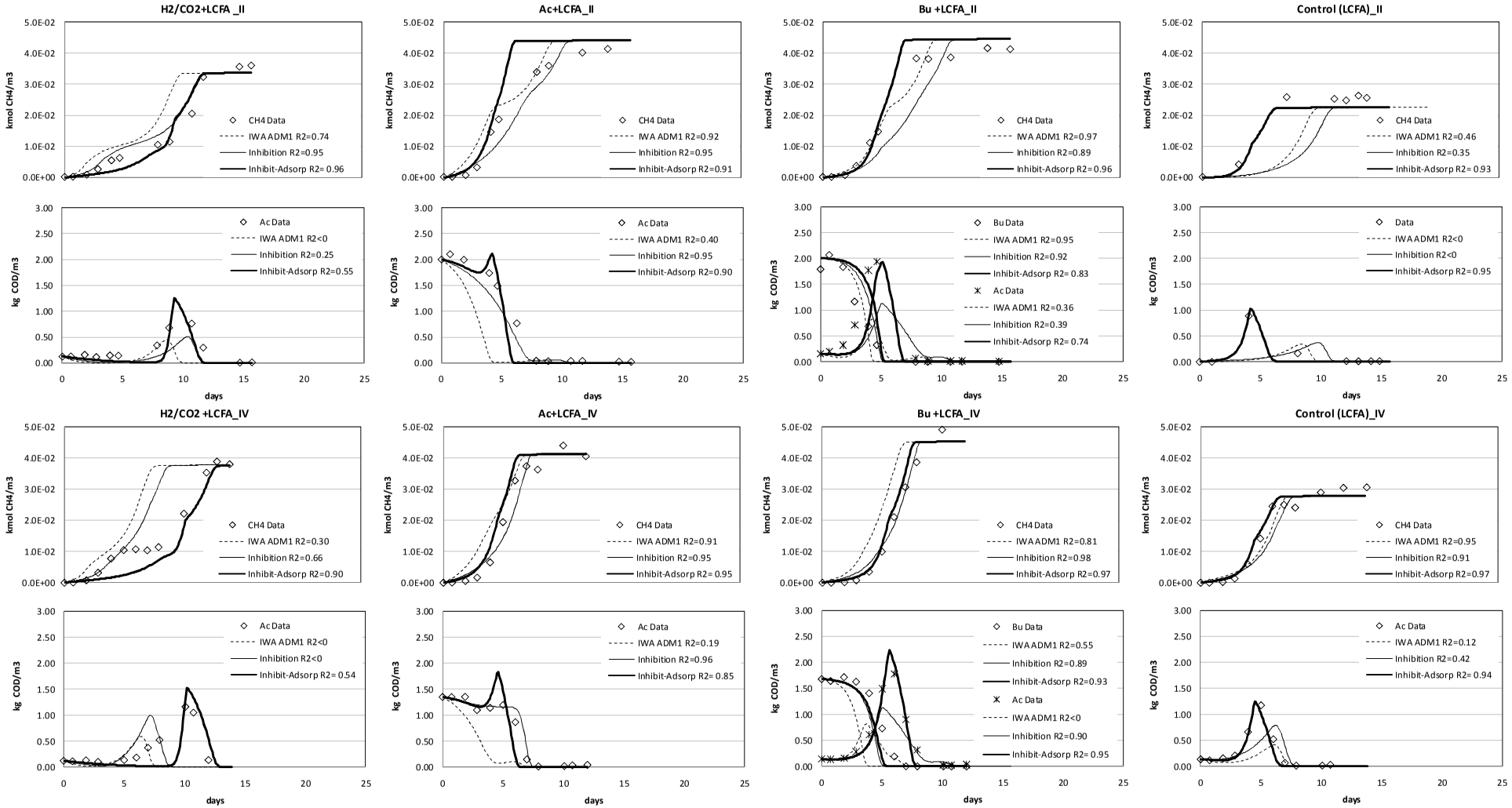

Figure 4 *Experimental data (n_{kj} point markers) and different Models results (lines) for activities (k) to H_2/CO_2, Ac, Bu and LCFA (Controls) in terms of CH_4 (kmol CH_4 m^{-3}) production and substrate degradation (kg COD m^{-3}) for inhibited biomass II and IV. Coefficients of determination (R2) for model fitting are introduced in figure.*

inhibition process. More work and new experimental data, designed specifically to study biosorption, are needed to mathematically express the adsorption-inhibition process. It is important to notice that for all tested models, or approaches, an increase in the initial hydrogenotrophic methanogens and LCFA degrading population occurred along time. This phenomenon, together with the stability of the microbial community composition found previously, might explain the observed LCFA adaptation process, as the result of the physiological acclimatation of existing populations.

CONCLUSIONS

In a multidisciplinary approach, LCFA inhibition and adaptation processes have been investigated using biomass exposed to successive inhibition-recovery stages by means of specific activity batch test, the characterization of the microbial populations by culture-independent molecular biology tools, and the mathematical modeling of the involved biochemical and physical processes.

Specific acivity test evidenced: the differential sensitivity of LCFA over major microbial trophic groups, the system recovery capacity and the adaptation of the ß-oxidazing bacteria and syntrophic methanogens upon exposition to succesives LCFA inhibitory pulses.

On the other hand, the application of LCFA pulses had little effect on the microbial community structure of eubacteria and archaeae by DGGE 16S rDNA, and many of the identified microorganisms were closely related to previously identified microorganisms found in anaerobic digesters where relatively high concentrations of LCFA are likely to occur, like *Clostridiaceae* and *Syntrophomonadae* for eubacteria and *Methanosarcina* for archaeae domains

Modeling results suggest that there is a need to introduce modifications on IWA ADM1 model when dealing with lipids degradation, and that the adsorption process plays a key role in the overall LCFA inhibition-adaptation process. The proposed *Inhibition-Adsorption Model* produces a better fit of results than classical inhibition models, and better expresses the physical nature of the overall LCFA inhibition process, was just a preliminary approximation and should be submitted to further improvements. The obtained increase in initial hydrogenotrophic methanogens and LCFA degradering population concentration along time, together with microbial community structure

analysis, could explain the reported adaptation process on reactors by physiological process.

Acknowledgements

The authors would like to thank Miriam Guivernau from GIRO (Barcelona, Spain) for Lab assistance in PCR-DGGE and ribotypes sequencing. This work was supported from the Danish Energy Agencies research program EFP-05 Journalnr.:33031-0029.

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Genetablering af biogasprocessen

Biogasanlæggene kan spare betydelige beløb, hvis der gribes ind i tide, når den biologiske proces viser tegn på ubalance. I den forbindelse kan fortynding af biomassen og tilsætning af frisk eller afgasset biomasse være et vigtigt redskab, men det er vanskeligt at give en præcis opskrift på den bedste strategi. Det viser en række forsøg, som forskere på Danmarks Tekniske Universitet har udført.

foto: torben skøtt/biopress

Tilsætning af frisk biomasse kan være et vigtigt redskab, hvis den biologiske proces viser tegn på ubalance. Billedet er fra fællesanlægget i Nysted på Lolland.

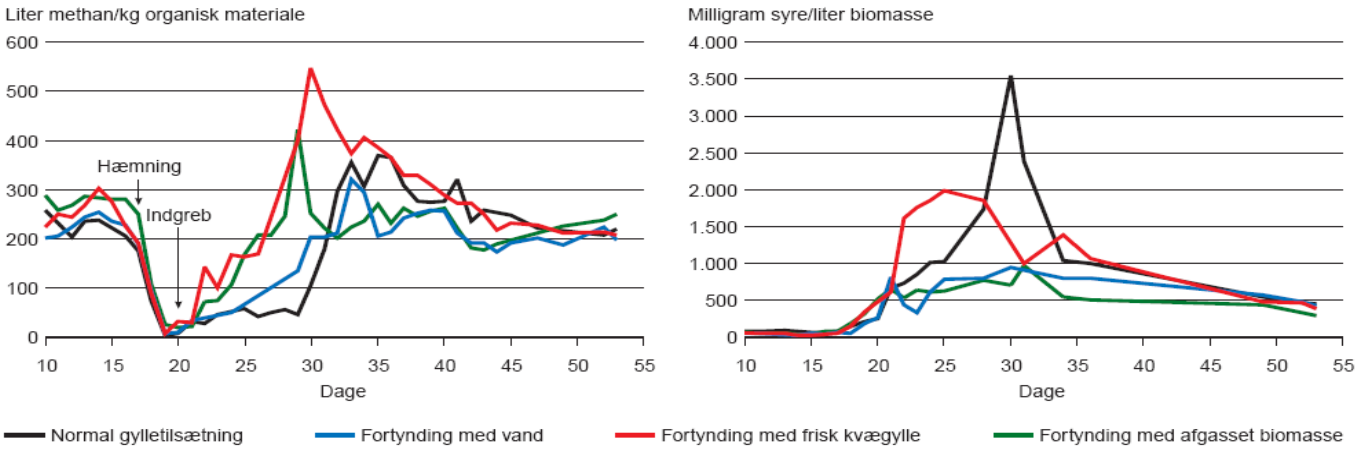
Af Henrik Bangsø Nielsen og Rena Angelidaki

De fleste biogasanlæg har i større eller mindre omfang været udsat for driftsforstyrrelser, ligesom flere anlæg har oplevet, at processen i værste fald kan bryde helt sammen. Sådanne sammenbrud kan have alvorlige økonomiske konsekvenser for anlægget, og det er et emne, som der er meget fokus på blandt driftsledere.

Driftsforstyrrelser og sammenbrud bliver ofte kædet sammen med de typer biomasse, anlæggene får tilført, og det har desværre vist sig, at en kombination af gylle og organisk affald kan være en farlig cocktail. Er der meget protein og fedt i blandingen, kan det medføre høje koncentrationer af ammonium og langkædede fede syrer (LCFA), som kan hæmme processen.

Den bedste måde at undgå sådanne hæmninger er ved at have en indgående viden om den type biomasse, anlægget får tilført – både hvad angår den kemiske sammensætning, og hvordan biomassen nedbrydes i anlægget. Derudover er det vigtigt at kunne foretage en præcis dosering af de forskellige typer affald, ligesom en detaljeret procesovervågning er meget vigtig.

Desværre er det langt fra altid, at anlæggene har mulighed for at opfylde disse betingelser. Fortankene er begrænset i antal og størrelse, og driftslederne vil derfor ofte være tvunget til at blande affaldet sam-



Figur 1. Forsøg med genopretning af biogasprocessen i forskellige reaktorer, hvor ammonium-koncentrationen efter 17 dage blev hævet fra 1 til 9-11 gram N/liter biomasse. I den første reaktor blev der ikke grebet ind, og der blev kun tilsat den normale gyllemængde. I de tre andre reaktorer blev halvdelen af reaktorindholdet, tre dage efter hæmningen, erstattet med enten vand, afgasset biomasse eller frisk gylle. Disse reaktorer blev ligeledes tilsat den samme daglige mængde gylle, som før hæmningen indtraf. Reaktortemperaturen var under hele forsøget 52° C, og den gennemsnitlige opholdstid var på 15 dage.

men. Variationer i sammensætningen, mængden og leveringsfrekvensen af de forskellige typer affald er ligeledes et problem. Det betyder nemlig, at anlæggene kan være tvunget til at tilføre reaktorerne en bestemt type affald på et tidspunkt, hvor det kan give problemer med stabiliteten i den biologiske proces. Endelig er en komplet overvågning af samtlige procesparametre[1] en både tidskrævende og dyr løsning, hvilket gør, at overvågningen på de fleste anlæg er mangelfuld.

Som en konsekvens af disse begrænsninger sker der jævnligt forskellige driftsforstyrrelser på biogasanlæggene. Spørgsmålet er derfor: Hvad kan driftslederne gøre for hurtigt at genetablere processen, når de registrerer et fald i gasproduktionen?

På Institut for Miljø og Ressourcer på Danmarks Tekniske Universitet har vi gennemført en række forsøg med genopretning af biogasprocessen i laboratorie-reaktorer. Forsøgene, der blev støttet af Energiforskningsprogrammet, var primært baseret på fortynding af biomassen med enten vand, gylle eller afgasset biomasse. Processen blev hæmmet ved at tilsætte enten ammonium eller LCFA til fire reaktorer med kvæggylle. Udfaldet af de forskellige strategier samt en beskrivelse af forsøgene er vist i figur 1 og 2.

foto: torben skøtt/biopress

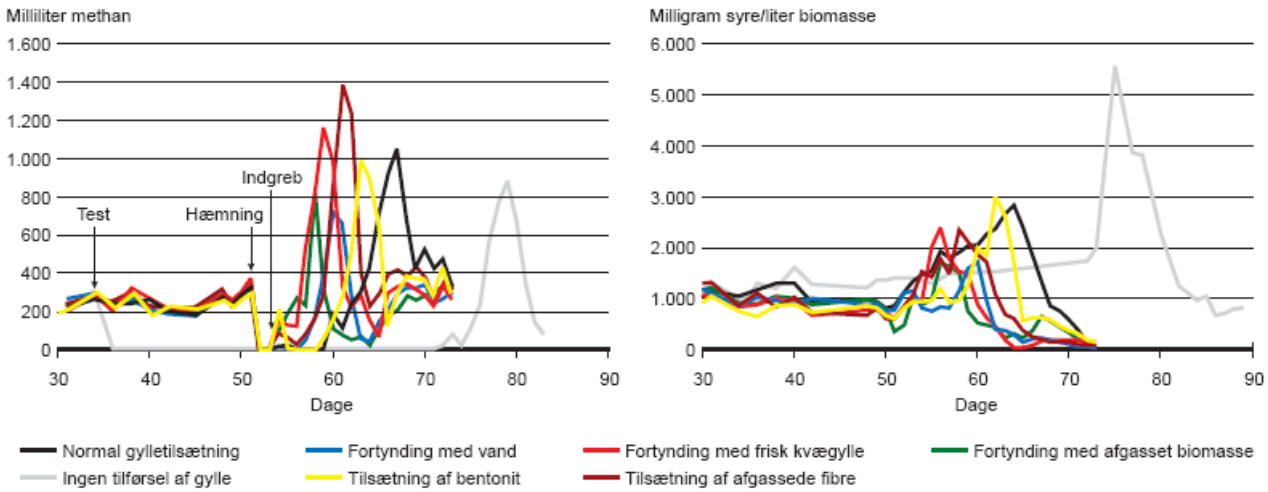
Kombinationen af gylle og organisk affald giver masser af gas, men det kan også være en farlig cocktail, der i værste fald kan sætte den biologiske proces i stå. Her er det aflæsning af storkøkkenaffald på biogasanlægget i Hashøj ved Slagelse.

Ammonium-hæmning

Efter hæmning med ammonium viste det sig, at den mest effektive metode til genopretning af processen var at udskifte halvdelen af reaktorindholdet med afgasset biomasse eller frisk kvæggylle (se figur 1). Med den strategi tog det cirka seks dage at komme tilbage til den oprindelige gasproduktion, mens det tog 10 – 11 dage, hvis der i stedet blev tilført 50 procent vand, eller tilførslen af den daglige mængde gylle blev halveret.

Efter knap seks dage blev der registreret en markant forøgelse af gasproduktionen i den reaktor, der fik tilsat 50 procent frisk kvæggylle. Under hele genetableringsperioden producerede denne reaktor således mellem

fortsættes på side 13 ▶



Figur 2. Forsøg med genopretning af biogasprocessen efter tilsætning af 5 gram oleate (C18-fedtsyre) per liter biomasse. I den første reaktor blev der tilsat oleate efter 34 dage for at sikre, at der opstod en tydelig hæmning (hæmningstest). Reaktoren blev derefter overladt til sig selv, og der blev ikke tilsat nogen form for biomasse. De øvrige seks reaktorer (testreaktorer) blev hæmmet efter 50 dage. I en af disse reaktorer blev der kun tilsat den normale gyllemængde. I tre andre reaktorer blev halvdelen af reaktorindholdet, tre dage efter hæmningen, erstattet med enten vand, afgasset biomasse eller gylle. I de to sidste reaktorer blev der tilsat 8,7 gram afgassede gyllefibre per liter biomasse eller 5,0 gram bentonit per liter biomasse. De seks testreaktorer fik efter 63 dage tilført den normale mængde gylle. Reaktortemperaturen var under hele forsøget 52 °C, og den gennemsnitlige opholdstid var på 15 dage.

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ISBN.nr. : 978-87-91855-75-7