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CONTROL PARAMETERS FOR UNDERSTANDING AND PREVENTING PROCESS IMBALANCES IN BIOGAS PLANTS. EMPHAS IS ON VFA DYNAMICS

Bangsø Nielsen, Henrik; Ahring, Birgitte Kiær

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Control parameters for understanding and preventing process imbalances in biogas plants. Emphasis on VFA dynamics

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Diagnostiske tools til forståelse og afværgelse af procesproblemer i biogasanlæg

Ph.D. thesis

Henrik Bangsø Nielsen
Environmental Microbiology and Biotechnology Research Group
BioCentrum-DTU, Technical University of Denmark
Supervisor: Birgitte K. Ahring

Table of contents

Preface **3**
Acknowledgements **5**
Summary **6**
Resumé **8**
1. Introduction and aim of the study **10**
2. Anaerobic digestion in methanogenic environments **11**
2.1 Hydrolysis and fermentation 11
2.2 Acetogenesis 13
2.3 Methanogenesis 16
2.4 Anaerobic digestion versus aerobic digestion 16
2.5 Anaerobic reactors 18
2.6 Physical and chemical parameters affecting the anaerobic digestion process 19
2.6.1 Nutrient requirements 19
2.6.2 Temperature 20
2.6.3 pH 21
3. Process imbalances in CSTRs **23**
3.1 Organic and hydraulic overloading 23
3.2 Changes in operation parameters 24
3.2.1 temperature 24
3.3. Addition or production of toxic/inhibitory compounds 26
3.3.1 Antibiotics 28
3.3.2 Heavy metals 28
3.3.3 Long chain fatty acids 28
3.3.4 Ammonia 30
3.3.5 Sulphate and sulphide 32
3.3.6 VFA 33
4. Parameters for indication of process imbalances **35**
4.1 Biogas production rate and methane yield 35
4.1.1 Biogas production rate 36
4.1.2 Methane yield 36
4.2 Hydrogen 37
4.3 Carbon monoxide 39
4.4 VFA 39
4.5 pH and alkalinity 41
4.6 Organic matter reduction 41
4.7 Micobial and molecular methods 43
4.7.1 Microbial methods 43
4.7.2 Molecular methods 44
5. References **46**
Paper I **57**
Paper II **79**
Paper III **96**
Paper IV **111**
Paper V **134**

Preface

This thesis presents the results done at The Environmental Microbiology and Biotechnology Research Group, Biocentrum-DTU, The technical University of Denmark, in the period from 1/3-2002 to 30/4-2005. Throughout the period Professor Birgitte K. Ahring was the super-visor.

The thesis consists of a short summary and an introduction that 1) provides a background for understanding the most important aspects of anaerobic digestion and 2) gives an overview of the most important issues concerning process imbalances in bioreactors. The laboratory work during the project mainly focused on volatile fatty acids (VFA) dynamics during process imbalances in continuously stirred tank reactors and the use of VFA as indicators of process imbalances. The results are presented in following five papers:

Paper I Nielsen HB, Ahring BK (2005) Responses of the biogas process to pulses of oleate. Submitted to Biotechnology and Bioengineering.

Paper II Nielsen HB, Ahring BK (2005) Effect of protein and ammonia pulses on the biogas process. Prepared for submission to Water Research.
Part of the results was previously presented as:
Nielsen HB, Ahring BK (2004) Effect of protein and ammonia pulses on the biogas process. 10th World Congress on Anaerobic Digestion, AD-2004, Montreal, Canada, 3, 1790 –1794

Paper III Nielsen HB, Ahring BK (2005) Response of the biogas process to pulses of lipids and meat- and bone meal. Preliminary results.

Paper IV Nielsen HB, Hartmann H, Ahring BK (2005) Regulation and optimization of the biogas process: propionate as a key parameter. Prepared for submission to Water Research. Part of the results was previously presented as:
Hartmann H, Nielsen HB, Ahring BK (2004) Optimization of the biogas process using on-line VFA measurement. 10th World Congress on Anaerobic Digestion, AD-2004, Montreal, Canada, 3, 1285 –1289

Paper V Nielsen HB, Mladenovska Z, Hartmann H, Ahring BK (2005) Kinetics of propionate degradation in anaerobic continuously stirred tank reactors. Prepared for submission to Applied Microbiology and Biotechnology.

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I would like to thank Birgitte K. Ahring for her initiative to this project. I would also like to acknowledge former and present staff and students at The Environmental Microbiology and Biotechnology Research Group. Special thanks are given to Birgitte K. Ahring, Zuzana Mladenovska, Hinnerk Hartmann and Peter Westermann for sharing their expertise and to Thomas Andersen, Jinqquan Lu, Anette H. Løth and Gitte Hinz-Berg for invaluable technical assistance.

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Copenhagen, 11/1-2006

Summary

Anaerobic digestion is a widely used method for treatment of organic waste in bioreactors. The anaerobic digestion process depends on a finely balanced action of several microbial groups where the product of one group serves as the substrate for others. The growth rates and the sensitivity towards environmental changes differ widely between the different groups. As a consequence of this, an unrestrained reactor operation can lead to disturbances in the balance between the different microbial groups, which might lead to reactor failure. Therefore, reliable parameters and tools for efficient process control and understanding are necessary. The work of present study was directed towards this challenge.

Initially, the response of the anaerobic digestion process to various types of process imbalances was investigated with special focus on volatile fatty acid dynamics (VFA), methane production and pH. The experiments were carried out in lab-scale thermophilic continuously stirred tank reactors (CSTR) treating livestock waste. The imbalances included inhibition by long chain fatty acids (LCFA), inhibition by ammonia, organic overloading with proteins and organic overloading with industrial waste, i.e. meat and bone meal and lipids. During the main part of the experiments one reactor was connected to an online VFA sensor giving a detailed profile of the VFA dynamics during the process imbalances.

Based on the results it was concluded, that propionate was the most reliable single parameter for indication of process imbalances in biogas plants. At Danish full-scale biogas plants the biogas production is normally the only continuously measured parameter. In order to examine the usability of propionate as control parameter a reactor experiment was constructed in which the reactor operation either was carried out on the basis of the methane production or on the basis of fluctuations in the concentration of propionate. The experiment confirmed that propionate is a useful parameter for (1) indication of process imbalances and (2) for regulation and optimization of the anaerobic digestion process in CSTRs.

In order to gain a better insight in the activity of the propionate degraders under different operational conditions the kinetic parameters of propionate degradation by biomass from 10 CSTRs differing in temperature, hydraulic retention time (HRT) and substrates were estimated in batch substrate-depletion experiments. In general, a good relationship was observed between the maximum degradation rate (A_{max}) of propionate and the overall reactor performances while the half-saturation constant (K_m) was found to be in the same range (<1mM). However, when evaluating the accuracy of the substrate-depletion tests by use of radiotracer methodology a 14-

15% underestimation of A_{max} in the substrate-depletion was observed. This indicates a production of propionate during the experiments via degradation of higher organic compound. Therefore, when estimating the kinetic parameters of propionate degradation in substrate-depletion tests an important and difficult challenge, is to achieve a correct balance between the propionate concentration and biomass concentration.

Resumé

Anaerob udrådning er en vidt udbredt metode til behandling af organisk affald. Udrådningen er en kompleks proces, der kræver forekomsten af et fint afbalanceret mikrobielt fødenet, hvor een mikroorganismes nedbrydningsprodukt tjener som substrat for de efterfølgende mikroorganismer. De forskellige mikroorganismegrupper, der er aktive i den anaerobe nedbrydning, har forskellige vækstrater og udviser forskellig affinitet og følsomhed overfor ændringer i omgivelserne. Som følge af dette kan en ukontrolleret reaktordrift forårsage en ubalance mellem de forskellige mikroorganismer, hvilket kan føre til et sammenbrud af processen. Derfor er der behov for forskellige parametre og værktøjer for at opnå en bedre proceskontrol og for at forøge forståelsen af den anaerobe nedbrydningsproces. Nærværende arbejde har derfor været rettet mod dette mål.

Den anaerobe nedbrydningsproces' reaktionsmønster på en række procesforstyrrelser blev først undersøgt i laboratorie-skala reaktorer. Et særligt blik var rettet mod dynamikken af flygtige fede syrer (VFA), metanproduktionen og pH. Typen af procesforstyrrelser inkluderede: inhibering med langkædede fede syrer (LCFA), ammoniak-inhibering, organisk overbelastning med protein og organisk overbelastning med industriaffald i form af kød- og benmel samt fedt. Under de fleste forsøg var en af reaktorerne forbundet med en online VFA- sensor, hvis brug gav en detaljeret profil af VFA-dynamikken i reaktorerne under forstyrrelserne.

Udfra de opnåede resultater blev det konkluderet, at propionatkoncentrationen var den bedste enkeltparameter til indikation af procesubalance i biogasanlæg. Derfor blev der opstillet endnu et forsøg, hvor propionats brugbarhed som kontrolparameter blev testet. Dette eksperiment bekræftede at 1) propionat giver en god indikation på procesubalance og, at 2) propionat er velegnet til regulering og optimering af den anaerobe udrådningsproces.

Den sidste del af studiet var et screeningsforsøg, hvor de kinetiske parametre for nedbrydningen af propionat i biomasse fra 10 reaktorer blev bestemt. Reaktorerne varierede i temperatur, opholdstid og substrat. De kinetiske parametre blev bestemt i batchflasker ved direkte måling af propionatkoncentrationen. Generelt blev der observeret en god korrelation mellem den maximale nedbrydningsrate (A_{max}) af propionat og reaktorernes ydeevne mens halvmætnings-konstanterne for de enkelte reaktorer (K_m) var på samme niveau (<1mM). En evaluering af den anvendte metode ved hjælp af tilsætning af radioaktivt mærket propionat afslørede imidlertidigt en 14–15% underestimering af A_{max}. Dette resultat indikerede et input af propionat fra nedbrydningen af andet organisk materiale under forsøget. Derfor er det vigtigt, at der i forsøg, hvor de kinetiske parametre for VFA nedbrydning estimeres ved direkte måling af VFA

koncentrationen, opnås en korrekt afstemning af forholdet mellem VFA koncentration og biomasse koncentration.

1. Introduction and aim of the study

Anaerobic digestion of organic matter with a simultaneous production of biogas is an environmental attractive way for treatment of organic waste from livestock holdings, industries, wastewater treatment plants and households. The degradation process is complex and depends on a balanced action of several microbial groups consisting of hydrolytic/fermentative bacteria, acetogenic bacteria and methanogenic archaea. During the process biopolymers are initially hydrolyzed and fermented to volatile fatty acids (VFA) H_2 and CO_2, by the hydrolytic/fermentative bacteria. VFA such as propionate, butyrate and isobutyrate are subsequently oxidized by acetogenic bacteria producing acetate, H_2 and CO_2, and finally these products are converted to CH_4, CO_2 and H_2O by methanogens.

Today 20 full-scale centralized biogas plants are in operation treating approximately 1.2 million tons of manure per year together with approximately 300.000 tons organic industrial waste per year. Several of these biogas plants have been exposed to process imbalances mainly associated with the composition of the substrate that is treated by the plants. To avoid such imbalances and hinder suboptimal reactor performances reliable parameters for indication of process disturbances and process control are necessary. During a process imbalance intermediates, especially in the form of VFA, will accumulate in the reactor and it has for a long time been recognized that VFA concentration is one of the most important control parameters. The inhomogeneous nature of the organic waste makes VFA measurement time consuming and sampling is normally only done on a daily to weekly basis if any at all. However, a recently developed *in-situ* microfiltration system at BioCentrum-DTU has now made online VFA measurement in reactors treating complex waste possible. This system provides the opportunity to get a more profound insight in the complex dynamic interactions between the different microorganisms involved in VFA production and consumption.

The aim of the presented study was to examine some of the most commonly observed process imbalances in biogas plants with special focus on VFA dynamics. This was done in lab-scale CSTRs connected to an online VFA sensor. Based on the results from the experiments and published literature the succeeding task was to evaluate which single VFA that would make the most reliable parameter for indication of process imbalances in biogas plants and to examine the usability of this parameter in a lab-scale reactor experiment.

2. Anaerobic digestion in methanogenic environments

Biogas, which mainly consists of CH_4 and CO_2, is produced during anaerobic degradation of organic matter in the absence of inorganic electron acceptors (O_2, NO_3^-, SO_4^{2-}, Fe^{3+}, Mn^{4+}). Thus, only fermentation and respiration with protons and HCO_3^- as electron acceptors is possible (Stams 1994). A well-organized community consisting of several microbial groups, in which the product of one group is the substrate of the following groups, carries out the degradation (Gujer and Zehnder 1983). Examples of natural methanogenic environments are freshwaters such as paddy fields, sediments and swamps, the intestinal tract of ruminants and landfills. Furthermore, the anaerobic digestion process is utilized by man for treatment of organic waste in anaerobic bioreactors. 20 full-scale centralized biogas plants are in operation in Denmark. The main substrates of these plants are manure and other organic waste such as industrial waste, household waste and sewage sludge (Angelidaki and Ellegaard 2003). The main organic components of these complex wastes are biological polymers, e.g. carbohydrates, lipids and proteins and mono- and oligomers, e.g. sugars, long chain fatty acids, amino acids. During the degradation the main end products are CH_4, CO_2 and H_2O. As illustrated in figure 1, the way of degradation is divided into several steps.

2.1 Hydrolysis and fermentation

During the first step biological polymers are hydrolyzed to mono- and oligomers by extracellular enzymes, e.g. cellulases, xylanases, proteases, lipases, excreted by hydrolytic/fermentative bacteria. Mono- and oligomers are subsequently fermented by the same groups of bacteria to a broad range of reduced organic compounds such as succinate, lactate, alcohols and VFA along with CO_2 and H_2. The large variation in fermentation products is due to the ability of the fermentative bacteria to change their metabolism towards the most energetically favorable reactions, dependent on the external conditions (Thauer et al. 1977). One important parameter for regulation of this branched metabolism is the H_2 partial pressure of the environment. During glycolysis and betaoxidation glucose and fatty acids are oxidized to pyrovate and acetyl-CoA, respectively, while NAD^+ and FAD are reduced to NADH and $FADH_2$. During respiration NAD^+ and FAD is regenerated by oxidation of NADH and $FADH_2$ in a stepwise manner involving an electron transport chain where electrons are transported from one protein to another to a final external electron acceptor such as O_2, NO_3^- or SO_4^{2-}. In contrast to this fermentative bacteria

often lack complete electron transport chains and instead the regeneration of NAD^+ and FAD is carried out in processes where pyruvate and

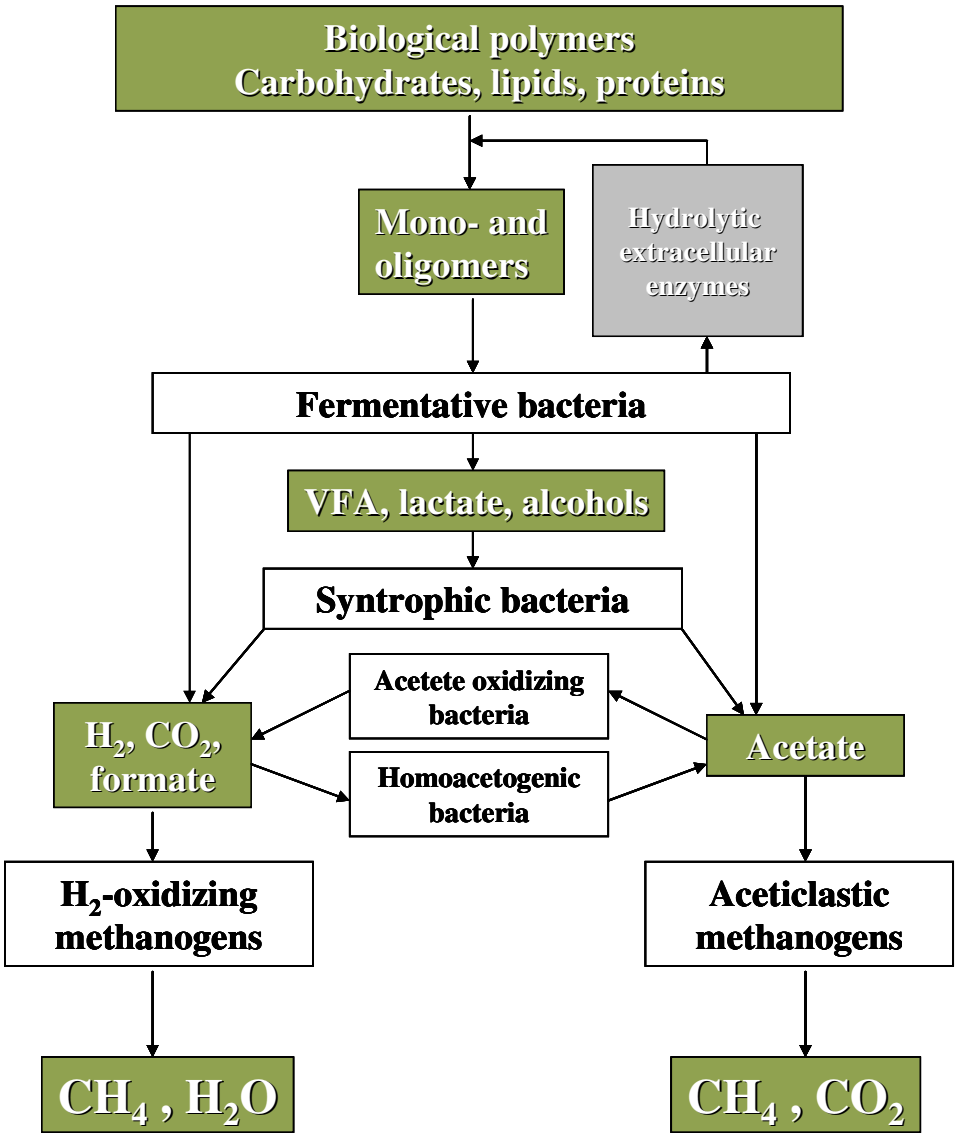

Figure 1 Illustration of the major pathways during anaerobic digestion of organic matter.

acetyl-CoA act as electron acceptors and are reduced to various acids and alcohols. These fermentation products are then subsequently excreted by the bacteria. However, at a H_2 partial pressure < 10^{-4} atm. NADH oxidation coupled to H_2 formation is possible (Wolin 1982):

$$NADH + H^+ \rightarrow H_2 + NAD^+$$

As a consequence of this reaction pyrovate/acetyl-CoA is retained inside the bacteria, which allows the bacteria to form acetate from acetyl-CoA resulting in a further energy yield. Therefore, at low H_2 partial pressure, which is obtained by hydrogen oxidizing methanogens, the fermentation pattern changes towards H_2, CO_2 and acetate at the expense of other fermentation products (Schink 1988).

2.2 Acetogenesis

Acetate, H_2, CO_2, formate and other C-1 carbons produced by the fermentative bacteria can be directly converted into methane, CO_2 and H_2O by methanogens. Due to a restricted metabolism of the methanogens VFAs longer than two carbons and alcohols longer than one carbon needs to by oxidized to acetate, H_2, formate and CO_2 by acetogenic bacteria before methanogenesis is possible. The two most important acetogenic reactions are the oxidation of propionate and butyrate. The quantitative contribution of these VFAs to methanogenesis (via H_2 or acetate) in cattle waste at 40°C and 60°C has been estimated to be approximately 13–17% for propionate and 8–9% for butyrate (Mackie and Bryant 1981). The oxidation of the compounds is under standard conditions endothermic (energy demanding) and energetically unfeasible (table 1). However, at a low H_2 partial pressure the processes becomes possible (Gibbs free energy, $\Delta G^{o`}$ becomes negative) and the energy yield increases with decreasing H_2 partial pressure (Westermann 1996). A low H_2 partial pressure is maintained by the H_2-oxidizing methanogens gaining energy (exothermic process) by converting H_2 and CO_2 to methane. Thus, acetogenesis is achieved in a syntrophic cooperation between acetogenic bacteria and H_2-consuming methanogens where each microbial group benefits from the existence of the other group. However, at decreasing H_2 partial pressure the energy yield from H_2-oxidaxion is lowered and oxidation of fatty acids combined with methanogenesis is only possible in a certain narrow H_2 partial pressure range (figure 2) (Stams 1994; Westermann 1996; Zinder 1993). Furthermore, the borders of the H_2 partial pressure are affected by temperature. At increasing temperature the energy yield of methanogenesis via oxidation of H_2 decreases while the energy yield of fatty acid oxidation increases, resulting in a shift of the H_2 partial pressure to a higher range (Westermann 1996).

During acetogenesis protons or bicarbonate is reduced and both H_2 and formate have been proposed as electron carriers in syntrophic degradation (Schink 1997, Zinder 1993). The above described syntrophic relationship deals with interspecies hydrogen transfer. Investigations have presented evidence of H_2 transfer by demonstrating syntrophic growth with methanogens that oxidize only H_2. Studies of interspecies electron transfer in mesophilic and thermophilic granules

Table 1 Equations and free energy changes $\Delta G^{o`}$ of some important reactions of acetogenesis and methanogenesis under standard conditions (H_2 and CH_4 in gaseous state; all compounds in aqueous solution at 1 M; 25°C, pH 7) (Thauer et al. 1977; Dolfing 1988; Zinder 1993).

	Equation		$\Delta G^{o`}$ (kJ/mol)
Acetogenesis			
Butyrate → Acetate	$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2$		+ 48.1
Propionate → Acetate	$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + 3H_2 + HCO_3^-$		+ 76.1
Ethanol → Acetate	$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$		+ 9.6
$H_2 + CO_2$ → Acetate	$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$		- 105
Methanogenesis			
$H_2 + CO_2$ → Methane	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$		- 135.6
Formate → Methane	$4HCOO^- + H^+ + H_2O \rightarrow CH_4 + 3HCO_3^-$		- 145
Acetate → Methane[1]	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$		- 31.0
Acetate → Methane [2]	a) $CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	a)	+ 104.6
	b) $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	b)	- 135.6
	a+b) $CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	a+b)	- 31.0

indicated that formate transfer was of no importance during oxidation of propionate and butyrate and indicated that the major electron transfer occurred via interspecies hydrogen transfer (Schmidt and Ahring 1993, 1995). Ahring and Westermann (1987) found that addition of H_2 to the gas phase immediately stopped growth and butyrate degradation in an anaerobic triculture. In contrast to this, Inanc et al. (1999) concluded that H_2 pressure had no effect on the degradation of propionate and butyrate in anaerobic sludge, and other studies of suspended cultures and flocs have indicated that formate is the major interspecies electron carrier in syntrophic associations (Boone et al. 1989; De Bok et al. 2002, Thiele and Zeikus 1988). The issue of interspecies electron transfer is, therefore, complex and seems to vary in different anaerobic systems. Probably, H_2 transfer becomes more important with shorter interbacterial distances, in for instance granular sludge, while formate transfer is more favourable in suspended cultures (Stams 1994); although results disagreeing with this consideration have been reported (Ahring and Westermann 1987). Irrespective of which component that acts as electron carrier in syntrophic degradations it is obviously that the hydrogen- and formate utilizing methanogens play an important role in the overall regulation of the anaerobic conversion (Schink 1997).

The role of homoacetogenic bacteria converting H_2 and HCO_3^- to acetate is unclear. As seen in table 1 this reaction is less energetically favorable than methanogenesis from H_2 and HCO_3^- and it seems reasonably to assume that homoacetogenic bacteria have little chance to compete with the hydrogenotrophic methanogens at low H_2 concentrations (Schink 1997). However, homoacetogens have an advantage of a more flexible metabolism, which allows them to consume two or more substrates simultaneously, e.g. sugar fermentation (Schink 1994). Some investigations indicate that in natural habitats with low temperatures (≈ 5°C), low acetate concentration (≈ 10 µM) and low H_2 partial pressure ($<10^{-4}$ atm.) homoacetogens may out compete H_2-consuming methanogens (Conrad and Wetter 1990; Kotsyurbenko et al. 2001; Schink 1997). For anaerobic digesters operated at mesophilic and thermophilic conditions it is generally accepted that the affinity of homoacetogens towards hydrogen is too low to obtain a H_2 partial pressure that allows a simultaneous growth of propionate and butyrate oxidizing bacteria (Stams 1994; Schink 1997).

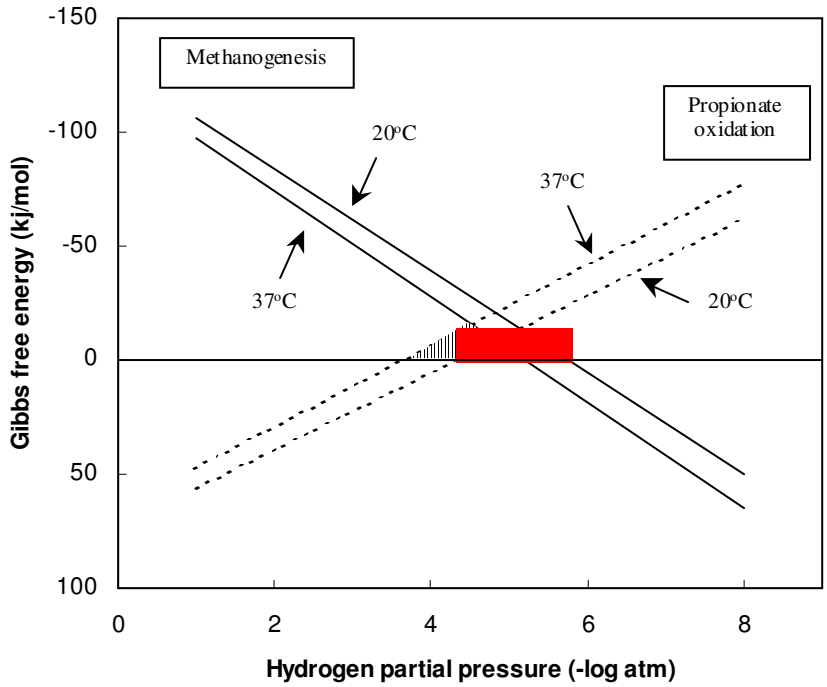

Figure 2 Free energy changes of propionate oxidation and methanogenesis from H_2 as a function of the H_2 partial pressure. The free energy change was calculated for both 20°C and 37°C. The following concentrations were used for calculations: propionate 0.1 mM; acetate 1 mM; HCO_3^- 10 mM; H^+ 10^{-4} mM, methane 0.6 atm. The red area indicates the H_2 partial pressure range where propionate mineralization is possible at 20°C, while the striped area indicates the H_2 partial pressure range where propionate mineralization is possible at 37°C. From Westermann (1996).

2.3 Methanogenesis

Methanogens belongs to the domain Archaea, and can be divided into two major groups: the hydrogenotrophic methanogens and the acetotrophic methanogens. The most important methane precursor in anaerobic digesters is acetate, which constitutes of approximately 70% of the total methane produced. The remaining 30% is mainly formed from H_2 or formate and to a minor extent other C-1 carbons (Mackie and Bryant 1981). Methanogenesis from acetate may proceed in two ways: 1) Aceticlastic methanogenesis where acetate is cleaved into methane and HCO_3^- and 2) methanogenesis where acetate-oxidation is coupled to the conversion of H_2 and HCO_3 to CH_4. While aceteticlastic methanogenesis is carried out by a single organism is acetate oxidation carried out in a two-step mechanism in which the methyl group of acetate is oxidized to HCO_3^- and H_2, followed by a reduction of HCO_3^- with hydrogen to methane (Mladenovska 1997; Zinder 1988; Zinder and Koch 1984). The aceticlastic reaction is in general the most dominant in anaerobic bioreactors treating complex organic material such as manure and operated at conventional temperatures up to 60°C (Mladenovska 1997). However, the dominating aceticlastic methanogens from moderate thermophilic biogas digesters have been found to have an upper limit at 60–65°C (Touzel et al. 1985; Zinder 1988; Zinder and Mah 1979) and it is reasonably to assume that the two-step process of acetate oxidation becomes more dominating at temperatures above these limits. Studies with radiolabeled acetate have also showed that the two-step process becomes dominant at acetate concentrations lower than 1mM (Ahring 1995). Furthermore, Schnürer et al. (1999) found a close correlation between the degree of acetate oxidation and the concentration of ammonia and potassium at mesophilic temperatures.

2.4 Anaerobic degradation versus aerobic degradation

The amount of energy available for the microorganisms during anaerobic methanogenic degradation of organic matter is small compared to aerobic degradation. Under aerobic conditions oxygen is used as the final electron acceptors and the main part of the energy is made available to the organisms. During anaerobic degradation most of the energy is recovered in methane (figure 3). This conservation of energy is the energetic background for commercial biogas production. Aerobic respiration of glucose to CO_2 and H_2O yields approximately 2822 kj/mol, equivalent to an amount of 38 mol ATP/per mol glucose. During the first anaerobic degradation step (fermentation) a maximum of 10% of the potential energy is available for the fermentative bacteria while the remaining 90% is retained in the fermentation products, mainly VFA. Of these

90% only 4% is available for the syntrophic bacteria and the methanogens while the last 86% is conserved as methane. The total energy yield under anaerobic conditions is approximately 390 kj/mol, which correspond to 5 mol ATP/mol glucose. Compared to aerobic conditions where the released energy is available to only one organism, the energy released under anaerobic conditions is shared between the fermentative bacteria, the syntrophic bacteria and the methanogens. The exact distribution of energy between the different trophic levels is dependent of the H_2 partial pressure. If the pressure is kept low by the methanogens the fermentative bacteria produces mainly H_2, CO_2 and acetate, which allow them to a maximum of 4 mol ATP/mol glucose. As a result of this only 1 mol ATP needs to be shared by the syntrophic bacteria and the methanogens. The synthesis of 1 mol ATP from ADP requires 60–70 kj. During electron transport phosphorylation 3 or 4 protons are required for synthesis of one ATP and the minimum amount of energy which living cells can make use of is 15–20 kj (Schink 2002). This amount of energy is exactly what's available to the individual microbial groups during many syntrophic degradation processes (Stams 1994 and Schink 1997).

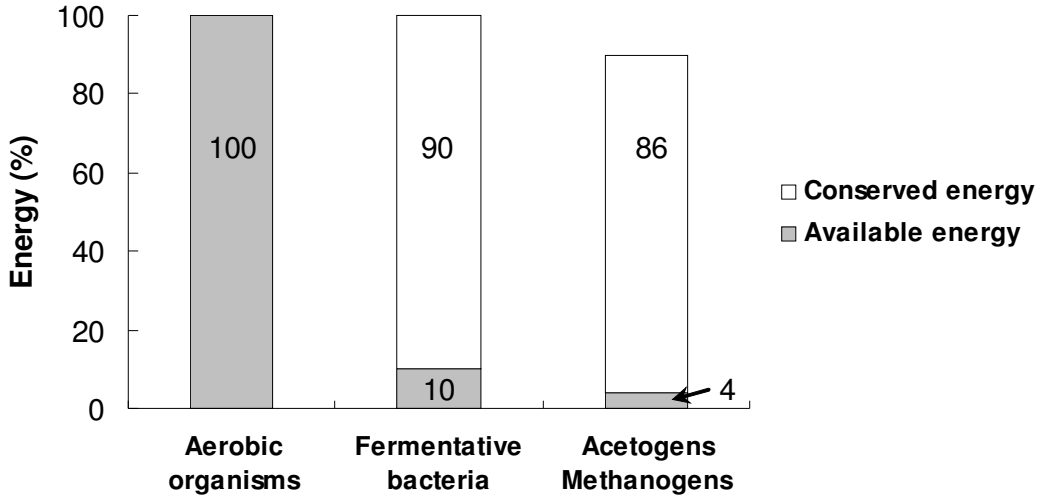

Figure 3. Available energy under aerobic and anaero bic degradation of glucose. From Westermann (1996).

The different energetics of aerobic and anaerobic degradation implies different advantages and disadvantage for aerobic and anaerobic treatment of waste. As a consequence of a higher energy yield and corresponding high growth rates aerobic treatment of waste/wastewater will result in a large production of biomass (sludge). This might generate disposal problems. Furthermore requires stirring and aeration of waste/wastewater and biomass a high amount of energy. In contrast to that, are the low biomass yield and the recover of energy as methane regarded as being a major advantage of anaerobic waste treatment. However, the low growth rates of anaerobic microorganisms and the strong interdependence of the different trophic levels make anaerobic digestion a more susceptible process towards a broad range of changes. Examples of this are given in chapter 3.

2.5 Anaerobic reactors

Anaerobic reactors can in general be divided into two main groups (Pind et al. 2003a):

- High-rate reactors, with a hydraulic retention time (HRT) of less than 5 days.
- Low-rate reactors with a retention time of more than 5 days and usually more then 10 days.

Often the substrate composition decides the choice of reactor. High-rate reactors are often used to treat dilute wastes, such as industrial wastewater, containing easily degradable soluble organic material. Since this Ph.D.-thesis focus on process imbalances in CSTRs (low-rate reactor) only a brief description of these reactor systems will be given here. The most used high-rate reactors are the upflow anaerobic sludge blanket reactor (UASB), the anaerobic filter reactors and the expanded/fluidized bed reactor. The essence of the UASB reactor (Lettinga et al. 1980) is the development of high-density microbial granules. The wastewater enters the reactor from the bottom and the granules are thereby mixed with the up streaming water and the gas bubbles being produced. In the upper reactor settler screens separates gas and particulate matter. This allows the granules to sediment back to the bottom while suspended bacteria leaves the reactor with effluent (Zinder 1993, Schink 1988). In anaerobic filter reactors a support matrix is provided for the microorganisms to adhere and perform degradation. The filter material, which for instance may consists of glass or Plexiglas beads, is fixed and in contrast to the UASB and the expanded/fluidized bed reactor is the biomass almost completely entrapped inside the reactor (Alves et al. 1998). This ensures a lower risk of biomass flotation and washout (Pind et al.

2003a). Unfortunately anaerobic filters are prone to clogging due to low flow rates and dense packed material (Zinder 1993, Pind et al. 2003a). In an expanded/fluidized bed reactor the microorganisms is attach to a carrier material consisting of small particles. The wastewater is pumped fast enough (often by a recycle pump) to expand or fluidize the carrier material inside the reactor. The main advantage of expanded/fluidized bed reactor is the extremely large surface area of the carrier material, which allows a high concentration of microorganisms in the reactor and a corresponding low HRT.

For treatment of inhomogeneous complex organic wastes such as manure, slaughterhouse waste and source sorted household waste the most commonly used reactor system is the CSTR (low-rate). In this system the microbes is completely suspended in the waste and are washed out with the effluent. Therefore, the HRT of the system needs to be higher than the generation time of the microbes in order to obtain a stable biogas process. In 2001 the average HRT of the 20 centralized full-scale biogas plants in Denmark was 23 ± 4 days for mesophilic plants (36–38°C) and 17 ± 4 days for thermophilic plants (51–53°C) (PlanEnergi, Midtjylland 2001). The major advantage of CSTRs is their ability of treating substrates containing high concentrations of suspended matter, for instance biofibers. The inhomogeneous composition of the substrates is also one of the main problems associated with the CSTRs since the nature of the substrate makes it difficult to obtain representative samples of the reactor content, when compared to the described high-rate reactors. Furthermore, the sample preparation is often time consuming hindering online sampling. As a consequence of this is the control of full-scale CSTRs often based on manual sampling and human intuition rather than sophisticated control systems (Ahring and Angelidaki 1997; Pind et al. 2003a). This problem will be further discussed in chapter 4.

2.6 Physical and chemical parameters affecting the anaerobic digestion process

2.6.1 Nutrient requirements. For a stable and efficient degradation it is required that nutrients are available to the microorganisms in sufficient amounts. The nutrients can generally be categorized as macro- or micronutrients. Nitrogen and phosphorus are the two most important nutrients. Nitrogen is essential for protein synthesis and the most important nitrogen source in reactors treating animal manure is ammonia. However, if ammonia is presented in high concentration it will be inhibitory to the degradation process (discussed later).

The requirement for phosphorus, which is found in nucleic acids, phospholipids, ATP, GTP, NAD and FAD, is less than nitrogen. Carbon, oxygen and H_2 are of cause also important elements since it is the main building blocks of cell material, but since the organic waste is rich in

these elements they will not be limiting. Instead the ratio of carbon to nitrogen and phosphorus may define the requirements and. A C:N:P composition of 100:28:6 for bacterial matter in general and C:P ratios for methanogens varying from 16:1 to 75:1 have been suggested (Alphenaar et al. 1993). Kayhanian and Rich (1995) found the C:N:P ratio for a optimal process in a thermophilic (55°C) CSTR treating municipal solid waste, sludge and manure to be approximately 180:25:1.

The sulfur requirement of the anaerobic digestion process is complex. Sulfur is used for synthesis of some amino acids but the methanogenes are unable to use sulfur in the oxidized forms, for instance SO_4^-. The reduced form of sulfur (sulphide) has been shown to have a stimulating growth effect of various methanogens (Kayhanian and Rich 1995) but may also inhibit methanogenesis in even small amounts (Hansen et al. 1999). Other essential nutrients that are required are calcium, iron, copper, magnesium, nickel, cobalt, and potassium (Kayhanian and Rich 1995). The cellular role of these elements varies from being cofactors for enzyme activity and components in metal complexes, to supporting cellular transport of nutrients and cations by increasing cell wall permeability (potassium). However, some elements like nickel, calcium, magnesium, sodium and potassium can in too high concentrations be inhibitory while sulphide and phosphate can influence the concentrations and bioavailability of some elements via precipitation.

It has been reported that addition of specific nutrient might improve the anaerobic digestion process but in general the basic nutrient requirements for a stable process are fulfilled in biogas plants treating animal manure (Kayhanian and Rich 1995).

2.6.2 Temperature. One of the most important physical parameters affecting anaerobic degradation is temperature. Conventional biogas plants are often operated as one-stage systems under constant mesophilic (30°C–37°C) or moderate thermophilic (50°C–55°C) conditions. In Denmark the first plants were operated at mesophilic temperatures but today the thermophilic process has gained the dominating position. Using thermophilic temperatures in preference to mesophilic temperatures has various advantages including higher degradation rates, enabling a shorter treatment times, and a better sanitation effect (Buhr and Andrews 1977; Hashimoto et al. 1981; Varel et al. 1980). Experiments have demonstrated that the microorganisms, involved in the anaerobic degradation process at thermophilic conditions, have different demands with respect to optimum growth temperature. Thermophilic hydrolytic and fermentative bacteria exhibit growth optimum in a broad temperature range between 55°C–75°C (Wiegel 1992; Zinder

1986). Thermophilic H_2-oxidizing methanogens exhibit optimal growth at temperatures between 55°C–70°C (Zinder et al. 1984; Zinder 1988; Wasserfallen et al. 2000), and some species are able to grow at temperatures above 90°C (Amend and Shock 2001). The majority of the thermophilic aceticlastic methanogens have lower growth optima in the range between 50°C to 65°C (Mladenovska 1997). Ahring (1994) showed that the conversion of acetate and butyrate to methane in cattle manure digested at 55°C is strongly reduced above 60°C, which is the optimum temperature for these processes, while the conversion of propionate to methane proceeds fastest at 55°C. According to these results it is obvious, that the total degradation potential of the microorganisms involved in the digestion process is not fully utilized when a reactor is operated at a constant thermophilic temperature. This fact has increased the interest for implementing two-phase systems with different temperatures and HRT to obtain optimal conditions for the different microbial consortia involved in the degradation process. In that context it has been demonstrated that the implementation of an extremely thermophilic pretreatment CSTR, operated at 68°C and 3 days HRT, before a thermophilic CSTR, operated at 55°C and 12 days HRT, resulted in a 6-8% higher methane yield, when compared to a conventional reactor operated at 55°C and 15 days HRT (Nielsen et al. 2004).

2.6.3 pH. As with temperature, the various microbial consortia involved in the anaerobic digestion process show different optima with regard to pH. Methane formation is limited to a pH range of approximately 5.5–8.5 although most methanogens have a pH optimum between 6.5 and 8.0. Comprehensive studies show that aceticlastic methanogens tends to have slightly lower pH optima than the hydrogenotrophic methanogens (Lowe et al. 1993; Mladenovska 1997). The fermentative bacteria often have lower pH optima (5–7) than the methanogens (Zinder 1986; Pind et al.2003a). The complexity of different pH optima makes the balancing of pH in some reactor systems an important operation issue. However, in biogas plants, where the main feedstock normally is manure the pH is often controlled by strong buffer systems. The bicarbonate system ($CO_2/HCO_3^-/CO_3^{2-}$) is normally the strongest buffer but also ammonia (NH_4^+/NH_3) and acetate (CH_3COOH/CH_3COO^-) influences the pH (Sommer and Husted 1995). As a consequence of these buffer systems the pH of plants treating manure is often stable and in the range from 7.5–8.0 (Pind et al. 2003a). Since the solubility of CO_2 becomes smaller at increasing temperature the pH is often higher in thermophilic reactors than in mesophilic reactors.

A part from the direct impact of pH and temperature on the growth of microorganisms, these parameters also affect other parameters such as the dissociation of important compounds, e.g. ammonia and sulphide.

3. Process imbalances in CSTRs

Most likely process imbalances in anaerobic CSTRs are characterized by an increase in H_2 partial pressures or acetate, for example during overload of fermentable substrate or inhibition of the methanogens. Under such conditions the pool of intermediates (VFA, alcohols etc) will increase and in case of methanogenic inhibition a lowering in methane production is observed. If the imbalance is allowed to proceed the increasing VFA levels may lower pH resulting in a further inhibition of the methanogens. In worst case scenarios this reaction pattern continuous and results in a complete inhibition of the entire process. Basically the causes leading to process imbalances in CSTRs can be divided into 4 groups:

- Organic overloading
- Hydraulic overloading
- Changes in operation parameters
- Addition or production of toxic/inhibitory material

The boundaries between these groups can be rather indistinct since a process imbalance often is caused by an interaction of various factors; for instance ammonia concentration, temperature and pH.

3.1 Organic and hydraulic overloading

Process imbalances caused by hydraulic overloading arises when the retention time of the substrate/biomass becomes lower than the generation time of some of the important microbial groups involved in the digestion process and these are washed out of the reactor. The specific growth rate of fermentative bacteria is higher than the growth rates of the VFA oxidizing bacteria and the methanogens (Angelidaki et al. 1993; Angelidaki et al. 1999) and normally the slow-growing fatty acid oxidizers (especially the propionate oxidizers) and the aceticlastic methanogens are most affected during a hydraulic overloading. Hydraulic overloading might occur as a consequence of a diminished reactor volume, for example due to bad stirring resulting in a floating layer of suspended matter. Furthermore the hydraulic retention time of a reactor can decrease if the feeding volume is increased. During such circumstances a combined hydraulic/organic overloading might occur. During an organic overloading more substrate is

Table 2 Methane production and VFA concentrations (acetate and propionate) in a mesophilic and thermophilic reactor treating cattle manure at different loading rates. HRT was decreased from 13 to 10, 9 and 5 days while the organic loading rate was increased from 3 to 6, 9 and 12 g VS/l. From Mackie and Bryant (1990, 1995).

HRT	VS in feed	Organic loading rate	Reactor temperature	CH_4 production		VFA	
						Acet.	Prop.
(days)	(%)	(g VS/l x d)	(°C)	ml/(gVS)	ml/(l reactor vol)	mM	mM
13	4	3	40	210	620	0.9	0.2
13	4	3	60	240	700	1.3	0.6
10	6	6	40	180	1090	1.6	11.5
10	6	6	60	220	1280	1.6	3.0
9	8	9	40	130	1180	2.3	51.9
9	8	9	60	210	1910	3.0	13.9
5	6	12	40	110	1270	3.3	47.7
5	6	12	60	200	2340	2.7	11.7

added to the reactor than the microorganisms can degrade. As mentioned this might happen if the feeding volume is increased but also if the concentration/degradability of organic material in the feedstock is increased. During such conditions the slowest processes of the overall degradation acts as bottlenecks and the substrates will accumulate in the reactor.

Due to differences in the growth rates of the microbial groups at various temperatures the outcome of an overload is dependent of the operation temperature. Mackie and Bryant (1995) exemplified this by studying combined hydraulic/organic overloading in a mesophilic (40°C) and a thermphilic (60°C) reactor treating cattle manure. By a stepwise increase in loading rate the HRT was decreased from 13 to 10, 9 and finally 5 days while the organic loading rate was increased from 3 to 6, 9 and 12 gVS/(l x d). During each change the methane production was more affected in the mesophilic reactor than in the thermophilic reactor and the accumulation of VFA was most pronounced in the mesophilic reactor (table 2).

3.2 Changes in operation parameters

3.2.1 Temperature. The different temperature optima of the microorganisms involved in the anaerobic degradation process makes anaerobic digesters sensitive to changes in temperature. Many investigations on this issue have been carried out and the present section only presents some of the more significant studies.

The effect of temperature changes is very much dependent on the original temperature of the reactor (psychrophilic, mesophilic, thermophilic) and at the type of temperature change, e.g. upward or downward, long-term or short-term. Ahring et al. (2001) investigated the effect

of a long-term increase of the operation temperature from 55°C to 65°C of a CSTR treating cattle manure (figure 4). The result of the increase was an immediate process imbalance characterized by a decrease in methane production and a significant increase in VFA concentration. However, approximately 30 days (two HRTs) after the changes the process stabilized. The overall process was still inhibited, e.g. methane yield was lower and VFA levels higher than before the temperature change, but the overall acidification yield was in the same range as before the temperature shift. The study also revealed a pronounced change in the microbial composition (rRNA contribution) illustrated by a significant decease in the level of the domain *Bacteria* from 74–79% at 55°C to 57–62% at 65°C and a corresponding increase in the domain *Archaea* from 18–23% at 55°C to 34–36 at 65°C. El-Mashed (2004) studied short-term (5–10 hours) upward and downward temperature fluctuations in CSTRs at 50°C and 60°C treating cattle manure. Here the upward temperature fluctuations affected the acidogenesis and methanogenesis activity more severely than the downward temperature fluctuations. In contrast to Ahring et al. (2001), found El-Mashad et al. the acidification yield to be smaller at 60°C than at 50°C, although hydrolysis was in the same range.

The importance of the original reactor temperature was studied by Ahn and Forster (2002), who imposed both upwards and downwards temperature changes to a mesophilic (35°C) and a thermophilic (55°C) upflow anaerobic filter treating simulated paper mill wastewater. Both reactors were first lowered by approximately 10°C for 11 days, returned to the original temperature to reestablish and then increased 10°C for approximately 6 days. The response of the reactors was different. The downward temperature changes affected the mesophilic reactor (lower biogas production, and accumulation of VFA) while the thermophilic reactor was unaffected. The upward temperature change did not show any harmful effect on the mesophilic reactor while, the treatment efficiency of the thermophilic digester immediately dropped.

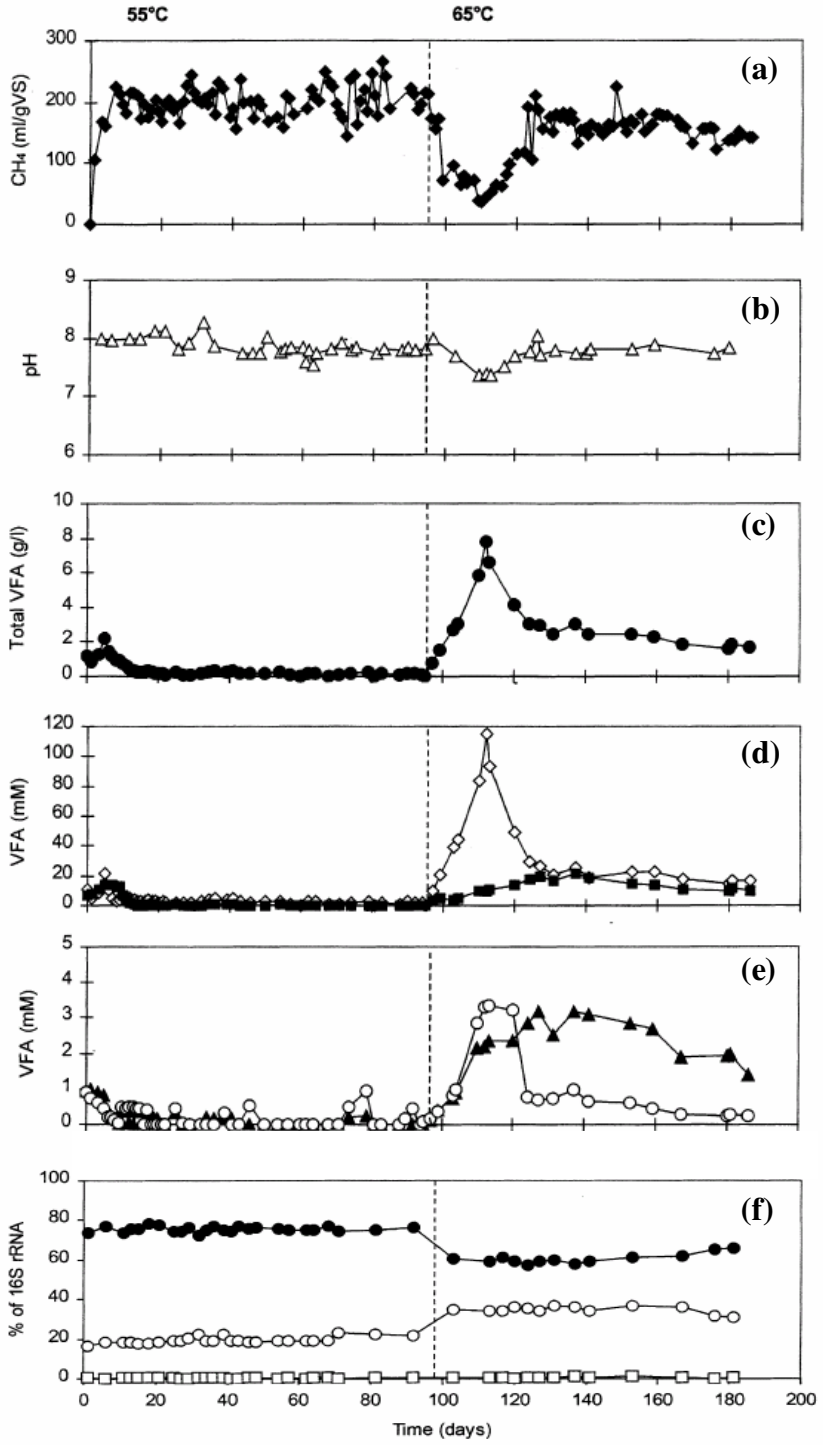

Figure 4 Effect of long-term temperature increase (55°C to 65°C) in a CSTR treating cattle manure. **a)** ◆: methane yield. **b)** △: pH. **c)** ●: total VFA. **d)** ◇: acetate; ■: propionate. **e)** ○: butyrate; ▲: isobutyrate. **f)** 16S rRNA amounts of ●: *Bacteria;* ○: *Archaea* and □: *Eucarya*. From Ahring et al. (2001).

3.3 Addition or production of toxic/inhibitory compounds

The most commonly observed process imbalances in biogas plants are related to the substrate composition. The main feedstock of Danish centralized biogas plants is manure but the plants are difficult to run with an economically profitable result if the process is solely based on this substrate (Hjort-Gregersen, 1999). In order to increase the economical feasibility of the plants manure is, therefore, often co-digested with organic wastes from industries and municipalities. These wastes are often characterized by high concentrations of easy degradable organics such as lipids and proteins (Ahring et al. 1992; Angelidaki and Ellegaard 2003). Besides organic overloading caused by addition of the substrates various compounds associated with the substrates may inhibit the degradation process. These compounds may be directly present in the feedstock (exogenic inhibtion) or may be produced during the degradation (endogenic inhibition).

Table 3 Effect of antibiotics on the anaerobic digestion process. [a]Levels of VFA removal after exposure of anaerobic sludge to antibiotics. –: VFA removed ≤ 10%; +: 10% < VFA removed < 50%; ++: 50% < VFA removed < 90%; +++: VFA removed ≥ 90%. The values were compared to unexposed controls. [b]Concentrations of various antibiotics that resulted in a 20, 50 and 80% inhibition of methane production. – No inhibition at the highest concentration tested (500–1000 mg/l). From Sanz et al. (1996).

Antiobiotic	Mode of action	[a]Extent of VFA removal			[b]Concentration (mg/l) inhibiting methane production by:		
		C_2	C_3	C_4	20%	50%	80%
Rifampicin	RNA polymerase	+++	++	+	100	>250	–
B-Lactamic							
Ampicillin	Cell wall	+++	++	++	10	–	–
Novobiocin	Cell wall	+++	++	++	10	–	–
Penicillin	Cell wall	+++	++	++	10	–	–
Aminoglycosides							
Streptomycin	Protein synthesis	+++	+	+	18	–	–
Kanamycin	Protein synthesis	+++	++	++	100	–	–
Gentamicin	Protein synthesis	+++	++	++	35	–	–
Spectinomycin	Protein synthesis	+++	++	++	>20	–	–
Neomycin	Protein synthesis	++	++	+	20	>500	–
Tetracyclines							
Chlortetracycline	Protein synthesis	–	+	–	5	40	152
Doxycycline	Protein synthesis	+++	+++	–	8	–	–
Macrolides							
Tylosin	Protein synthesis	+++	+/++	+/++	15	–	–
Erythromycin	Protein synthesis	+++	+++	+++	–	–	–
Chloramphinicol	Protein synthesis	–	–	–	11	26	41

3.3.1 Antibiotics. Antibiotics are used as feed additives and can be found in manure, but also in the wastewater of pharmaceutical industries. The purpose of antibiotics is to inhibit microbial growth and for that reason antibiotics play a potential role as process inhibitors in biogas plants. Sanz et al. (1996) studied the effect of 15 different antibiotics on both acetogenesis and methanogenesis at 30°C and found that most antibiotics did not or only partly inhibit the digestion process (table 3). Furthermore, the majority of the antibiotics lacked activity against acetoclastic methanogens and was only active on acetogenic bacteria. Only chlortetracycline and chloramphinicol were powerful inhibitors of the process. The authors concluded that most of the antibiotics normally used in livestock farms will not drastically affect biogas production if used at recommended concentrations. A similar conclusion was also drawn by Massé et al. (2000) who studied the effect of antibiotics on psychrophillic anaerobic digestion of swine manure.

3.3.2 Heavy metals. Heavy metals may be present in wastewater and accumulate in the sludge during treatment and finally end up in a biogas plant. Some heavy metals affect the microbial activity by being essential microelements necessary for microbial growth but in higher concentrations the same elements may be inhibitory to the microorganisms (for instance Nickel). The toxicity of heavy metals depends upon the concentration of the various chemical forms that they may take under anaerobic conditions at the present temperature and pH (Ahring and Westermann 1983). Codina et al. (1998) calculated the EC50 values (concentration exhibiting a 50% reduction in microbial activity) of different heavy metals on methane formation from anaerobic domestic sludge at 30°C and found the relative toxicity to be Zn>Cr>Cu>Cd>Ni>Pb. The EC50 values of Zn, Cr, Cu, Cd and Ni were between 50–350 mg/l while the EC50 for Pb was higher than 1000 mg/l. Normally the concentration of dissolved heavy metals will be kept below toxicity level via precipitation. This together with the fact that biogas plants mainly are based on agricultural wastes makes process imbalances caused by heavy metals a minor problem (PlanEnergi Midtjylland, 2001).

3.3.3 Long Chain Fatty Acids (LCFA). LCFA such as oleate and stearate may be present in high concentrations in for example vegetable oil and is also produced during degradation of lipids. It is well documented that these compounds may inhibit anaerobic microbial activity at even low concentration (Alves et al. 2001; Angelidaki and Ahring 1992; Cavaleiro et al. 2001; Hanaki et al. 1981; Hwu et al. 1996; Koster and Cramer 1987; Lalman and Bagley 2000; Lalman and Bagley 2001; Lalman and Bagley 2002; Rinzema et al. 1989; Rinzema et al. 1994; Shin et al.

2003). LCFA inhibits acetotrophic and hydrogenotrophic methanogens as well as syntrophic acetogens. Acetotrophic methanogens are found to be more severely affected than hydrogenotrophic methanogens (Hanaki et al. 1981, Lalman and Bagley 2000). The adsorption of LCFA to the surface of microbial cell membranes has been indicated as the reason for inhibition (Henderson 1973), which suggests that the inhibitory effect of LCFA is dependent on the LCFA:biomass ratio. Other reports indicate that the inhibitory effect of LCFA is concentration dependent (Angelidaki and Ahring 1992, Koster and Cramer 1987; Rinzema et. al 1994). For many years the mechanism of inhibition by LCFA has been ascribed to mechanisms of cell wall damage and bactericidal effects (Rinzema et al. 1994) but recent research suggest that the inhibition is due to transport limitation such as product diffusion limitation, e.g . biogas release (Pereira et al. 2003; Pereira et al. 2004).

Controversy exists whether microorganisms involved in the anaerobic degradation process can adapt to LCFA. Based on results from batch experiments Rinzema et al. (1994) concluded that the acetotrophic methanogens from granular sludge are unable to adapt to capric acid, neither after repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic concentrations. These results were in good agreement with those of Angelidaki and Ahring (1992), who found that no adaptation to oleate and stearate occurred, when biomass from a digester fed with cattle manure was exposed to non-inhibitory concentrations of the acids. However, continuous experiments with fixed-bed reactors have shown that pre-exposition of acetotrophic methanogens to lipids benefits the development of resistance to oleate (Alves et al. 2001). Cavaleiro et al. (2001) found that a hydraulic shock (reduction of the hydraulic retention time) of oleate induced an increase in the resistance of acetotrophic methanogens to oleate; whereas an organic shock of oleate (increase in substrate concentration) resulted in a lower resistance towards oleate.

Of the different LCFAs oleate is normally considered to be one of the more toxic. Several issues associated with inhibition of oleate, including VFA dynamics during inhibition (example given in figure 5) and adaptation, was investigated during this Ph.D. The results are presented in paper I of the thesis.

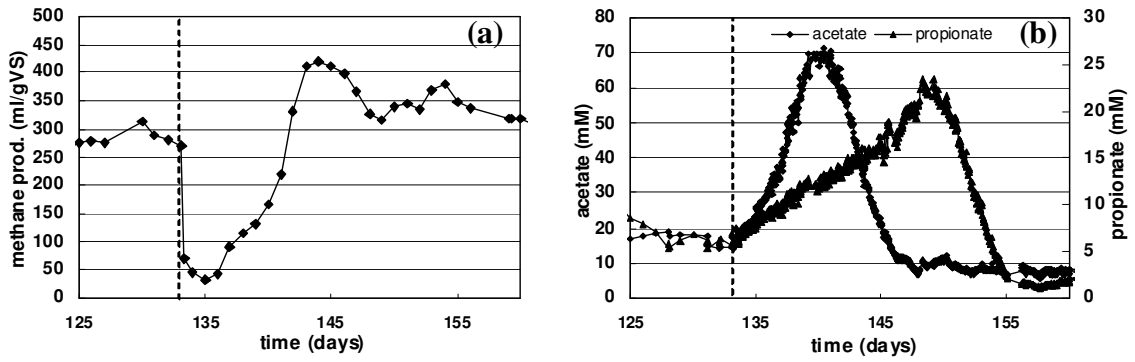

Figure 5 Effect of LCFA on the biogas process. A thermophilic (55°C) CSTR treating a 1:1 mixture of cattle and pig manure was added 2.0 g/l oleate at day 133. A severe inhibition of the process was observed immediately after oleate was added, illustrated by an instant drop in methane production and a significant increase in VFA concentration. **a)** ◆: methane production. **b)** ◆: acetate; ▲: propionate (Nielsen 2005, paper I of the thesis).

3.3.4 Ammonia (NH_4^+/NH_3). Ammonia nitrogen exists in aqueous solution as either ammonium ion or ammonia, according to the following equilibrium reaction:

$$NH_4^+ \rightleftharpoons NH_3 + H^+$$

The effect of ammonia nitrogen on the anaerobic digestion process has for many years been subject to intense studies. Ammonia is essential for bacterial growth but may – if present in high concentration –also inhibit the process. For unadapted methanogens ammonia inhibition has been observed to begin at 1.5–2.0 g-N/l (Van Velsen 1979; Hashimoto 1986) while for an adapted biogas process an ammonia tolerance of 3–4 g-N/l has been reported (Angelidaki and Ahring 1993). An inhibition of the biogas process by ammonia does not necessarily stop the process but may lead to suboptimal performance of the reactor. In that context Hansen et al. (1998 and 1999) obtained a stable thermophilic degradation of swine manure at an ammonia concentration of 6 g-N/l, characterized by a low methane yield and high concentrations of VFA. Manure normally has an ammonia concentration of 2–4 g-N/l but especially pig manure and chicken manure may contain ammonia in concentrations often higher than 4 g-N/l. Furthermore, manure contains compounds, e.g. protein and urea, that releases ammonia when degraded. In addition to this,

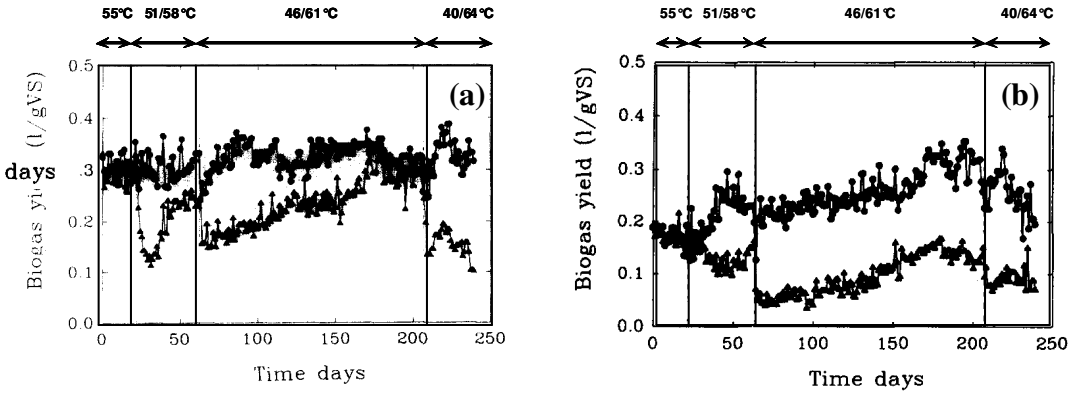

Figure 6 Effect of temperature on the biogas yield during thermophilic digestion of cattle manure at different ammonia concentrations. CSTRs (55°C) were operated at an ammonia concentration of **a)** 2.5 g-N/l or **b)** 6.0 g-N/l. After steady state was reached the temperature was either decreased (●) to 51, 46 and 40°C or increased (▲) to 58, 61 and 64°C. As illustrated, the biogas yield was not influenced by temperature in the range from 40–55°C when the ammonia concentration was 2.5 g-N/l. In contrast, increasing temperature from 55–64°C resulted in a decrease in methane yield in both low and high ammonia reactors. However the process seemed to adapt to the higher temperature when the ammonia concentration was only 2.5 g-N/l. The reduction in temperature in the reactor operated at 6.0 g-N/l resulted in an increase in methane yield and a better process stability, indicated by a lowering in VFA concentrations (data not shown). From Angelidaki and Ahring (1994).

industrial co-substrates are often rich in proteins. From these facts it is evident that biogas plants treating manure in co-digestion with industrial waste often are operated close to the inhibitory level of ammonia. However, it should be kept in mind that a potential inhibition by ammonia should not be related directly to the total ammonia nitrogen concentration ($NH_4^+ + NH_3$) but to the concentration of free ammonia (NH_3) which has been suggested to be the active component during inhibition (Hashimoto 1986; Koster and Lettinga 1984). The free ammonia concentration increases with temperature and pH and can be calculated by the following equation:

$$[NH_3] = \frac{[T\text{–}NH_3]}{1 + \frac{[H^+]}{K_a}}$$

where $[NH_3]$ is the free ammonia concentration, $[T\text{–}NH_3]$ is the total ammonia concentration and K_a is the dissociation constant. The temperature dependency is the reason why thermophilic reactors are more easily inhibited by ammonia and that reactors operated at high ammonia concentrations are more sensitive towards increases in temperature (figure 6) (Angelidaki and Ahring 1994). Angelidaki and Ahring (1994) reported that anaerobic digesters treating cattle manure was inhibited when the concentration of free ammonia exceeded 700 mg-N/l while Hansen et al. (1998) found, in a batch experiment, the inhibition level to be approximately 1100

mg-N/l. A level of 80 mg-N/l was reported for initial inhibition of an unadapted process (Koster and Lettinga 1984; De Baere et al. 1984).

During the present study the effect of protein and ammonia pulses to CSTRs treating cattle manure was investigated. Examples are given in figure 7 and are further discussed in paper II of the thesis.

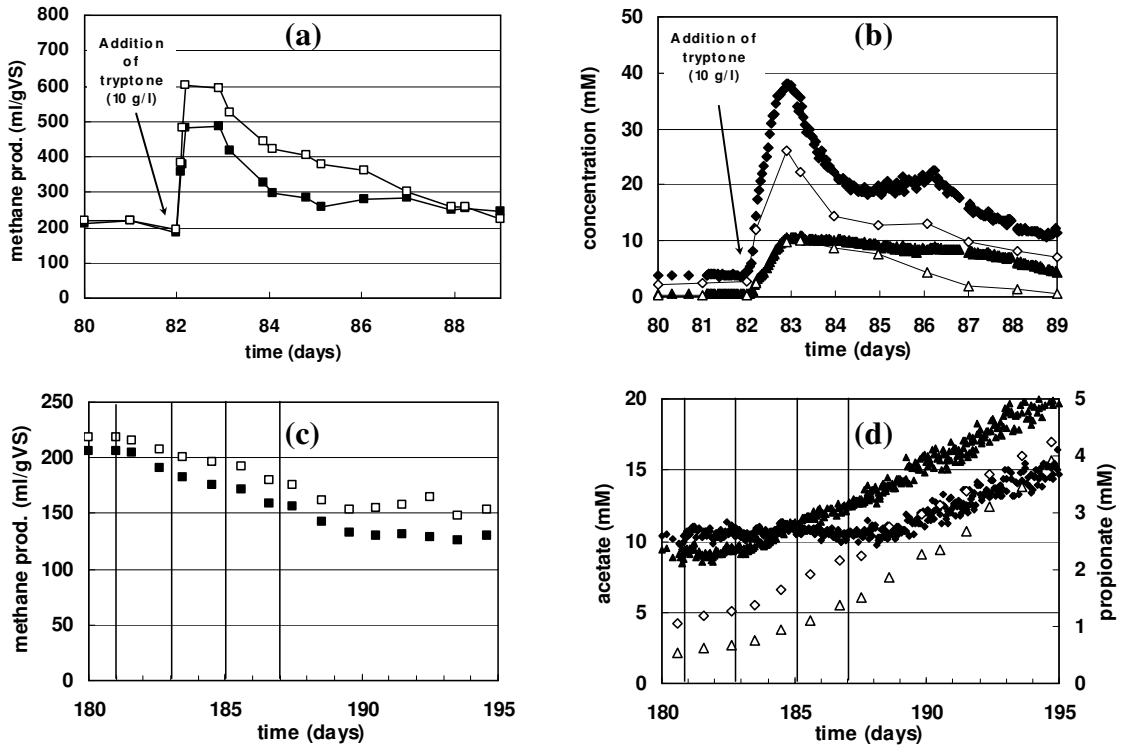

Figure 7 Effect of protein (tryptone) and ammonia pulses on thermophilic digestion of cattle manure at different ammonia loads. Two CSTRs (53°C) were operated at an ammonia concentration of 3.0 g-N/l (R1) and 1.7 g-N/l (R2). a + b) 10 g/l tryptone was added at day 82. c + d) 3.0 g/l NH_4Cl (0.79 g ammonia-N/l) was added at day 181, 183, 185 and 187. □: methane production R1; ■: methane production R2; ◆: acetate concentration in R1; ◇: acetate concentration in R2; ▲: propionate concentration in R1; △: propionate concentration in R2. (Nielsen 2005, paper II of the thesis).

3.3.5 Sulphate and sulfide. In addition to ammonia swine manure contains a high concentration of sulphate resulting from a protein rich diet. Sulphate reducing bacteria can utilize sulphate as electron acceptor and thereby compete with acetogens and methanogens for important substrates. At the same time sulphate is reduced to sulphide (S^{2-}, HS^-, H_2S) which can inhibit the biogas process at even low concentrations (≈ 23 mg/l, figure 8) (Hansen et al. 1999). The most toxic agent of sulphide is assumed to be H_2S. At decreasing pH a higher percentage of the total sulphide will be in form of H_2S and therefore more toxic. The toxicity of H_2S itself increases with

increasing pH, but at pH >7.6 the H_2S concentration is low due to the dissociation characteristics of sulphide and it seems likely that the levels of total sulphide is responsible for the inhibition during these conditions (O'Flaherty et al. 1998).

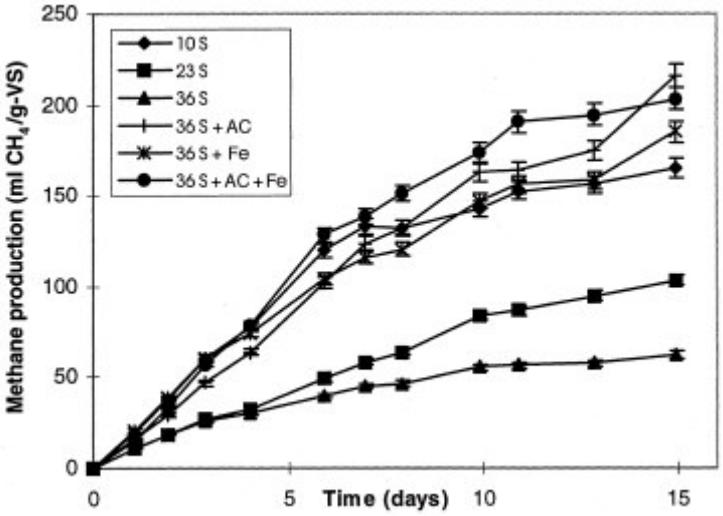

Figure 8 Methane production in thermophilic (55°C) batch vials containing pig manure as substrate and different concentrations (mg/l) of H_2S (S), activated carbon (AC) and Fe^{2+}. All vials had an ammonia concentration of 4.6 g-N/l and pH of 7.7. Addition of sulphide to a total of 23 mg S^{2-}/l or higher resulted in n inhibition. The methane production decreased from 165 ml/gVS in vials with 10 mg S^{2-}/l to 100 and 62 ml/gVS in voals with 23 and 36 mg S^{2-}/l, respectively. The sulphide inhibition could be counteracted by adding activated carbon or $Fe^{2+.}$ From Hansen et al. (1999).

3.3.6 VFA. Inhibition of the anaerobic digestion process by VFA is a complex issue. It is generally considered that high concentrations of VFA may inhibit the process. However, the inhibition is associated with the undissociated form of the VFAs, which are thought to freely permeate the cellular membrane of the microorganisms, and will be highest in systems with low pH (Switzenbaum et al. 1990; Mösche and Jördening 1999). Ahring et al. (1995) studied the effect of high concentration of individual VFA (acetate, propionate, butyrate and valerate) on methane production from manure at 55°C and found that the methane production rate increased for all VFA up to 50 mM (figure 9). A slight decrease was observed at propionate and valerate concentrations of 100 mM while an inhibition of acetate and butyrate not was observed until above 100 mM. A comparable experiment was performed by Mechichi and Sayadi (2005) with olive mill wastewater at 37°C. Here the maximum methane production rates were obtained at concentrations of 125 mM for acetate, 100 mM for propionate and butyrate and 50 mM for valerate. Above these concentrations the methane production decreased immediately. Both experiments concluded that concentrations of all VFA up to 50 mM mainly are a sign of process

inhibition rather than a cause of inhibition. However, the experiments did not examine the co-inhibitory effects of the various VFA. Taking this and the pH dependency into account no general assumptions on inhibitory levels of VFA is possible (Pind et al. 2003b).

Product inhibition by acetate has demonstrated by several authors (Ahring and Westermann 1988; Mösche and Jördening 1999; Pind et al 2003b). Ahring and Westermann (1988) found that butyrate degradation was inhibited at acetate concentrations of 25 mM, while Pind et al (2003b) observed a product inhibition of propionate and isovalerate degradation in a thermophilic CSTR following a shock load of approximately 35 mM acetate.

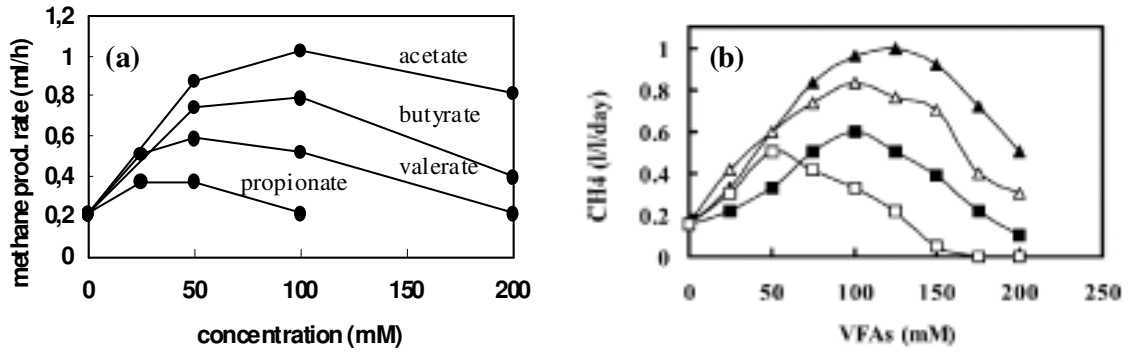

Figure 9 Effect of single VFA concentrations on methane production rate from cattle manure (Ahring et al. 1995) and olive mill wastewater (Mechichi and Sayadi 2005). **a)** Cattle manure at 55°C. **b)** Olive mill wastewater at 37°C. ▲: acetate; △: propionate; ■: butyrate; □: valerate.

4. Parameters for indication of process imbalances

As described in chapter 2 the anaerobic degradation process consists of a series of metabolic reactions catalyzed by a well-organized community including several microbial populations. Normally during a process imbalance intermediates such as H_2, VFA and alcohols will accumulate accompanied by fluctuations in the gas production. In theory this provides several parameters, which can be used as indicators of process instability. However, the complexity of the process has made it difficult to find a simple and suitable control parameter reflecting the metabolic state of the entire process. Furthermore, the hunt for reliable control parameters is often impeded by the complexity of the substrate that is fed the biogas plants. Examples of this is 1) the high alkalinity which makes it possible to withstand high concentrations of VFA without significant drops in pH; 2) the inhomogeneous material which hinders online sampling and 3) the relatively low degradation rates which results in a slow response to changes in loading rate. These are just some obstacles that are associated with the selection of control parameters for biogas plants (Pind et al. 2003a). During the selection of process parameters it should also be considered which requirements are necessary for obtaining an adequate process control. An optimal process parameter would fulfil all of the following demands:

- It should give a significant early warning of process imbalance but should not give an unnecessary warning.
- It should give an indication of suboptimal reactor performances and not just a warning of a potential break down of the process.
- It should give a good indication of when the process has stabilized following a process imbalance.
- It should be simple to monitor on a real-time or online basis with a reliable, robust instrument that is easy to handle.

The present chapter discusses the usefulness of different process parameters that has been suggested as the most obvious stress indicators in CSTRs.

4.1 Biogas production rate and methane yield

4.1.1 Biogas production rate. At Danish full-scale biogas plants monitoring of the biogas production rate ((l biogas/(l reactor x day)) is normally the only continuously measured parameter. Unfortunately, this parameter cannot be used as a single parameter for direct indication of process imbalance since the methane production rate of an anaerobic digester not only reflects the state of the process but also reflects the actual loading of the reactor (Ahring et al. 1995). Thus, an organic overloading may first give rise to an increased biogas production followed by a drop when VFA or other inhibitory compounds have accumulated in the reactor. Indication of an organic overloading by the gas production rate will, therefore, often be too late. This problem is illustrated in paper III and IV of the thesis. Nevertheless, for biogas plants monitoring of biogas production is a vital indicator of process imbalance since the response to fast changes is instantaneous. However, an increase or decrease in methane production should always be evaluated in close relationship with other parameters, preferably VFA. Steyer et al. (1999) successfully used the biogas production rate for control purposes of fluidized bed reactors (figure 10). However, their control strategy was based on a more or less uniform substrate composition and included pH as a key parameter. Therefore, this strategy can hardly be transferred directly to biogas plants treating complex organic wastes, since the substrate composition is more inconsistent.

4.1.2 Methane yield. Methane yield (ml/g volatile solids) is another parameter that has been tested as process indicator (Ahring et al 1995). It was found that the change in methane yield following various process imbalances was too small. In addition to this, the methane yield parameter requires a precise and time-consuming estimation of the organic content of the substrate.

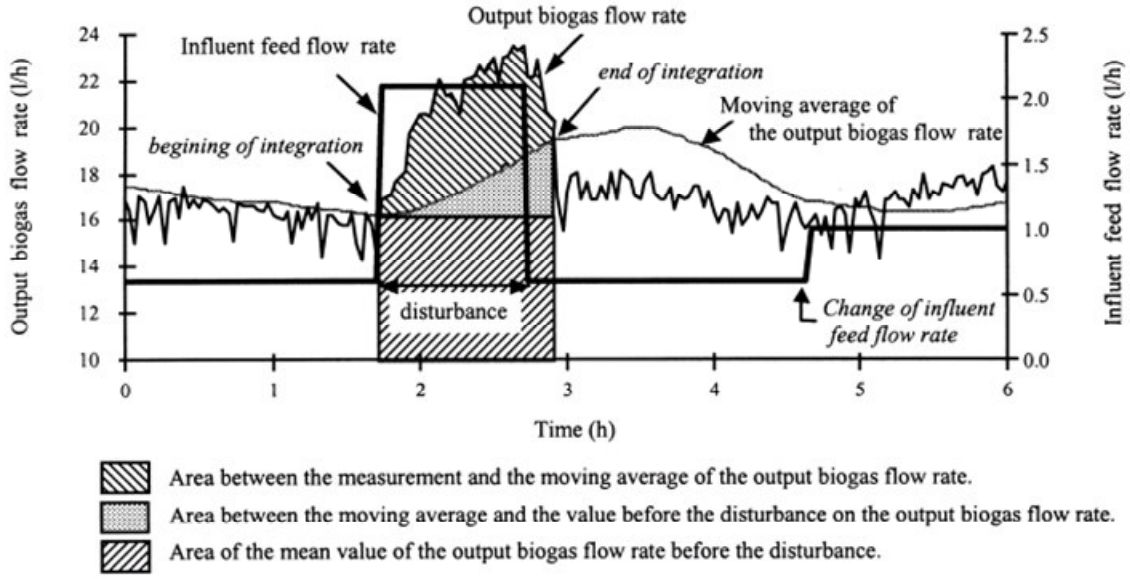

Figure 10 Regulation of reactor operation by using gas production and pH as control parameters. By increasing the influent of a fluidized bed reactor treating wine distillery wastewater for a short period the extra biogas volume was compared with the expected biogas volume. From the results the authors concluded that three scenarios was possible: 1) If the measured extra gas volume was almost as the expected then the microbial population was able to manage an increase of the loading rate and the input flow rate was then increased by a certain amount. 2) If the measured extra gas volume was lower than the expected then the microbial population had reached its maximum treatment capacity and the input flow rate was kept constant at the value applied before the disturbance. 3) If the measured extra gas volume was much lower than the expected one then the reactor was overloaded and the input flow rate was decreased by a certain amount. When the process had reached a new stable state after the change in influent flow rate the methodology was applied again. pH was used as an alarm to stop the strategy if the value fell below a certain level. Experiments demonstrated a good capability of the control strategy. The figure illustrates the control strategy when the measured extra gas volume was almost as the expected (scenario 1). From Steyer et al. (1999).

4.2 Hydrogen

The transfer of H_2 plays an important role in the overall regulation of the anaerobic digestion process. This key position makes H_2 an interesting control parameter. Due to its low solubility and ease of measurement, H_2 concentration in the gas phase has been suggested as the most obvious control parameter by several investigators. Unfortunately, the results from experiments concerning H_2 concentration in the gas phase have been incompatible. In some investigations a fast response of H_2 to operational changes such as increasing loading rates has been observed, while other comparable investigations were unable to find any correlation between reactor performance and the H_2 concentration (Archer et al. 1986; Hickey and Switzenbaum 1991; Kidby and Nedwell 1991). In a review by Switzenbaum et al. (1990) it was concluded that the complicated dynamics of H_2 in anaerobic ecosystems and variability for given reactors and substrates limit the possibility of using H_2 as a stand-alone factor. This complex behavior was

exemplified by Guwy et al. (1997) in a series of experiments with a fluidized bed reactor treating bakers yeast wastewater (figure 11). In one experiment sharp peaks in the H_2-partial pressure were observed following step-wise increases in the flow-rate while in another experiment two similar overloads gave a very different response in H_2-partial pressure. Furthermore, a switch in batch feed from an old batch to a new batch resulted in a remarked increase in H_2 partial pressure without any remarkable changes in other control parameters, i.e. gas production and VFA.

The use of H_2 partial pressure as a process parameter depends on a stable transfer of H_2 from liquid to gas phase. This is not always the case and it has been suggested that dissolved hydrogen in the liquid phase is more reliable for process monitoring. Frigon and Guiot (1995) found dissolved H_2 to be a more attractive parameter than gaseous hydrogen due to a better correlation with propionate concentration. Although no control experiment was applied, Cord-Ruwisch et al. (1997) found the use of a critical set-point level of the dissolved H_2 concentration to be an effective tool for a stable operation of a lab-scale CSTR continuously loaded with glucose media. In comparison, a semi-continuous loaded industrial digester gave a much more complex and temporary behavior of the H_2 concentration in the gas phase. In contrast to these results, Voolapalli and Stuckey (2001) observed that severe organic shock loads of a CSTR gave no significant accumulation of dissolved hydrogen. Instead VFAs accumulated in the reactor and the removal of these took at least 5 times longer than hydrogen.

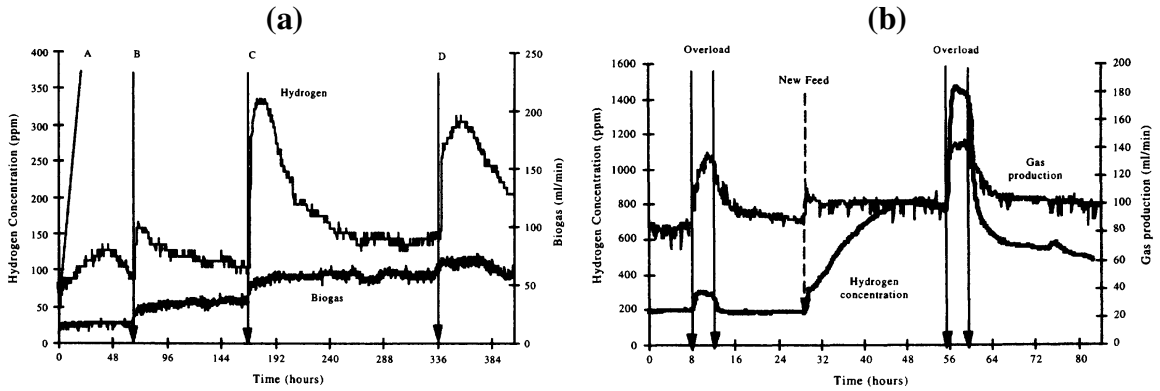

Figure 11 H_2-concentration in the gas phase and biogas production in a high rate fluidized bed reactor (37°C) treating synthetic bakers yeast wastewater. During steady state the reactor was operated at a HRT of 8.7–10.2 h and a loading rate of 27–33 kg COD/(m^3 x d). **a)** After a two week shut-down period the flow-rate of the influent was increased at points A: from 0 to 12.3 kg COD/(m^3 x d); B: from 12.3 to 24.8 kg COD/(m^3 x d); C: from 24.8 to 30.8 kg COD/(m^3 x d) and D: from 30.8 to 38.5 kg COD/(m^3 x d). The figure shows that the H_2-concentration gave a distinct peak following each increase in flow rate but no linear relationship between the H_2-concentration and the flow-rate could be seen. The biogas production increased approximately proportional to each increase in flow-rate. **b)** Two organic overloads lasting for 4 h each were performed by first increasing the flow rate from 27.1 to 57.6 kg COD/(m^3 x d) and secondly from 27.1 to 64.5 kg COD/(m^3 x d). At time 29 h a fresh feed was used. The first overload resulted in an increase in H_2-concentration from 200 to 300 ppm while the change of feedstock resulted in a more pronounced increase from 200 to 800 ppm. Following the second overload the H_2-concentration increased from 800 to 1450 ppm. From Guwy et al. (1997).

The inconsistent results involving H_2 measurement excludes H_2 as a sole parameter for indication of process instability in biogas plants since these often are subject to frequent changes in organic loading rate and substrate composition. The turn-over rate of hydrogen in anaerobic digesters is on the other hand very low and variations in H_2 concentration are fast and may occur in response to some disturbances. This justifies the use of H_2 as a control parameter although it should always be during simultaneous measurement of other process indicators.

4.3 Carbon monoxide

CO is a trace gas that evolves during methanonogenesis of acetate and monitoring of CO may give insight into the aceticlastic reaction. Hickey et al. (1991) found a fast response of CO to organic overloads of mesophilic (35°C) reactors treating waste-activated sludge and observed a strong correlation between acetate concentration in the liquid phase and CO concentration in the gas phase. Due to the low solubility, CO in the gas phase is easy to measure. However, to my knowledge CO monitoring for process control has not yet been reported.

4.4 VFA

During process imbalance a build up of catabolic intermediates such as VFA and alcohols is normally observed. The rate of accumulation depends on a wide range of factors such as operation temperature, hydraulic retention time (HRT), loading rate, substrate and the type of process disturbance. It is well recognized that VFA concentration is one of the most important parameter for accurate process control (Ahring and Angelidaki 1997; Ahring et al. 1995; Hill and Holmberg 1988; Hill et al. 1987). Traditionally, measurement of VFA concentration in CSTRs treating livestock waste has been based on manual sampling, due to the inhomogeneous nature of the substrate. However, lately improved technology has made online measurement of VFA in CSTRs treating complex organic wastes possible. Hansson et al. (2002, 2003) tested the utility of online near-infrared (NIR) spectroscopy for measurement of VFA dynamics in a lab-scale mesophilic CSTR treating the organic fraction of municipal solid waste. Regression analysis showed a good correlation for propionate in the range of 4–40 mM but acetate failed to give any correlation. The response to increases in the loading rate was reproducible and could be detected within 5 minutes indicating the possibility of developing an early warning biogas system based on NIR monitoring of propionate. Another VFA online system was developed by Pind et al. (2002). This method is based on an *in-situ* filtration technique that makes it possible to perform microfiltration inside the reactor system (Pind et al. 2002). The system which was tested in lab-

scale reactors and at a full-scale biogas plant could perform automatic VFA analysis on manure at a frequency of 15 minutes in a measuring range from 0.1 to 50 mM. The system was also used during the present study (paper I and II) and is illustrated in figure 12. As described, the prepared sample was analyzed on a GC, which provides full information on all individual VFAs. Unfortunately, GC´s are expensive and often only available for research institutions. Alternatively, analytical methods based on titration principles can be used. These methods provide a cheap alternative to GC´s and several online titration techniques for determination of VFA concentration in wastewaters have been published (Bouvier et al. 1999; Feitkenhauer et al. 2002; Lahav et al. 2002). However, all these methods can only be used for total VFA analysis.

The two most abundant VFAs during digestion of animal manure are acetate and propionate. According to literature, an unbalanced digestion often results in a relatively high concentration of propionate and variations in the propionate:acetate ratio has been suggested as a reliable indicator of process imbalance (Hill et al. 1987; Marchaim and Krause 1993). Hill et al. (1987) examined the literature and proposed that a propionate:acetate ratio higher than 1.4 indicated impending process failure. Later, other results have clearly contradicted this statement and found that the acetate:propionate ratio is useless for detection of process imbalance (Hill and Bolte 1989; Ahring et al. 1995; Pullammanappallil et al. 2001). Instead Ahring et al. (1995) suggested that a combined parameter reflecting the concentrations of both butyrate and isobutyrate could be a reliable tool for indication of process instability. Various associations between individual VFA concentrations and process imbalance have also been suggested. Hill et al. (1987) concluded that an acetate concentration higher than 13 mM would indicate process imbalance and Hill and Holmberg (1988) concluded that concentrations of isobutyrate and isovalerate higher than 0.06 mM (as acetate) was an indication of process instability and could provide as much as a week´s notice of failure. However, reports shows that stable reactor performance can occur at VFA concentrations well above these limits (Angelidaki and Ahring 1994; Ahring et al. 2001; Nielsen et al. 2004) and no general assumptions of the dependency between VFA concentration levels and process instability can be made.

From literature it appears that propionate oxidizers often are the slowest growing (Öztürk 1991) and energetically most sensitive (Stams 1994; Schink 1997) microorganisms in the overall anaerobic degradation process. Accumulation of propionate is often observed following changes such as pulses, overloading, and feed composition due to short-term increases in H_2 levels (Ahring et al. 1995; Pind et al. 2003b). At the same time propionate is often one of the last VFAs to decrease following a process imbalance (paper I, II, IV of this thesis). Therefore, for an

optimal performance of CSTRs treating complex organic wastes special attention should be given to fluctuations in propionate concentration. A successful lab-scale experiment using propionate as a sole control parameter was carried out during the present study. The result of this experiment is presented in paper IV of the thesis.

4.5 pH and alkalinity

pH measurement is easy but application of pH as control parameter in CSTRs treating livestock waste is not recommendable. The high alkalinity and strong buffer system of manure makes it possible for the system to resist even high increases in VFA without any pronounced drops in pH. A drop in pH will, therefore, often be too late for an early indication of a process imbalance. Since pH not necessarily reflects the metabolic activity in the reactor, due to the strong buffer systems, alkalinity measurement is a way to estimate the buffer capacity in the liquid. Especially the bicarbonate buffer system is interesting and bicarbonate alkalinity has received the most attention (Pind et al. 2003a). Furthermore, the ratio between VFA and alkalinity has been suggested as a control parameter (Switzenbaum et al. 1990).

4.6 Organic matter reduction

The main purpose of anaerobic digestion is the reduction of organic matter. The difference in organic matter content in the influent and effluent of a digester, therefore, reflects the efficiency of the process. For a high-rate reactor receiving a uniform substrate this method can be useful during steady state. However, due to long HRTs and relative low degradation rates in CSTRs the detection time of an acute process imbalance is too slow and the method is only useful for monitoring gradual changes in reactor performance. Furthermore, large fluctuations in volume and organic matter content of the influent of full-scale biogas plants impede the utility and reduce the reliability of this method.

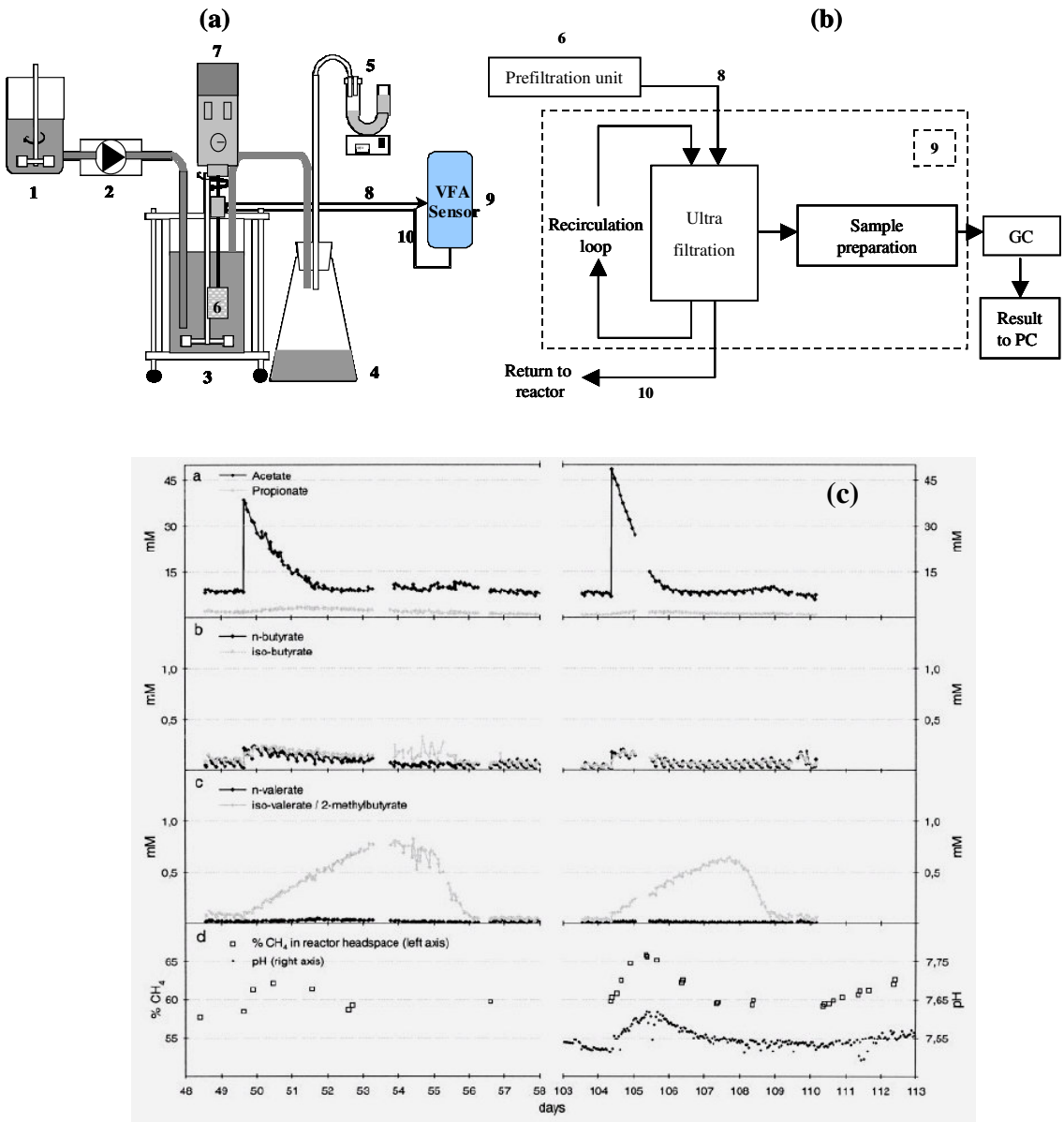

Figure 12 a) Lab-scale CSTR set-up connected to an online VFA sensor (Pind et al. 2002). 1: feedstock storage; 2: feeding pump; 3: 4.5 litre CSTR with a working volume of 3.0 liters. The reactor was stirred for one minute every third minute at 100 rpm and a stable operational temperature was obtained by circulating heated water from a water bath through the reactor jacket (not shown); 4: effluent storage; 5: gas meter; 6: rotating prefiltration unit placed inside the reactor and connected to an online VFA sensor; 7: stirrer for prefiltration; 8: sample outlet via peristaltic pumping; 9: VFA-sensor; 10: excess sample return to reactor. **b)** Principle description of the VFA sensor (9). The prefiltration unit (6) removed particles of 0.1 mm and the liquid sample was driven out of the reactor by a peristaltic pump (8). A recirculation pump then pumped the sample through an ultrafilter producing a clear permeate. Preparation of the sample was done by mixing equal amount of permeate and 1% $H_3PO_4^-$ by a peristaltic pump. The prepared sample was then transferred by an autoinjector to a GC for analysis. The excess permeate from the ultrafiltration was returned to the reactor (10) ensuring a high recirculation of sample material. The entire procedure (sampling and GC-analysis) took approximately 15 minutes. **c)** VFA dynamics in a thermophilic (55°C) lab-scale CSTR treating cattle manure following pulses of acetate (Pind et al. 2003b). VFA monitoring was performed using the online sensor and revealed a complex and fast response of the anaerobic process.

4.7 Microbial and molecular methods

Classical microbiological and modern molecular-based methods are powerful tools for studying the microbial composition and activity in complex environments. However, all these methods are time-consuming and for that reason not applicable for early indication of changes in process stability in anaerobic reactors. So far these methods are mostly used for obtaining a better process understanding. The methods are numerous and in reference to some of the published reviews and articles concerning these techniques, the following sections provide a brief overview of some of the most important ones (Ahring and Angelidaki 1997; Amann et al. 1998; Muyzer 1999; Oude Elferink 1998; Switzenbaum et al. 1990; Sørensen and Ahring 1993; Sørensen and Ahring 1997; Wilderer et al. 2002).

4.7.1 Microbial methods. Traditional quantification and activity measurement of microorganisms are mainly based on selective growth media. Two of the most commonly used methods for estimating the number of viable microorganisms in a sample is the most probable number (MPN) method and the spread plate method. During estimation of MPN a dilution series of specific sample are inoculated in a selective liquid media and the identification of viable cells is then related to development of gasses, depletion of the substrate or increase in turbidity. The method is often used and can give useful information on the number of microorganisms that are able to grow in an artificial media. However, the method is far from perfect and will underestimate the number of organisms that 1) are attached to solid substrates, 2) are associated to each other like threaded microorganism (for example acetoclastic *Methanosaeta*) or 3) grow in syntrophic consortia. In the spread plate method the sample is spread on a solid media and the quantification of viable cells is based on the number of colony forming units (CFU). As with the MPN this method is based on the microbes' ability to grow on an artificial media and will also lead to an underestimation of the number of cells.

For estimation of microbial activity specific methanogenic activity (SMA) tests are widely used. In these tests the activity of various physiological groups involved in the terminal anaerobic process during degradation of specific substrates in an artificial media is determined by following the rate of accumulation of methane. The test is excellent for measuring the degradation rate of substrates which are directly converted to methane, for instance H_2/CO_2 and acetate, but gives only an indirect indication of the conversion rate of more complex compounds such as propionate and butyrate. The measurement of the conversion of these compounds should be based on the depletion of the compound itself and not on the methane production.

Furthermore, for high background levels of substrates in a reactor the SMA method was found to be useless. The SMA test can, therefore, only be used for monitoring the state of the methanogenesis during steady state or during initial process imbalances.

Traditional microscopic analyses can be used for characterization and direct identification of micoorganisms, but this technique is limited by the fact that cell morphology of most microorganisms is very similar. However, many methanogens can be identified by epifluorescence microscopy by detection of coenzyme factor F-420. Attempts have been made to relate the methanogenic activity in an ecosystem and the F-420 content of the ecosystem biomass. Results show that the methanogenic activity cannot be clearly related to F-420.

4.7.2 Molecular methods. Since approximately 99% of all microorganisms in nature cannot be cultivated and isolated in pure cultures alternative methods are necessary. Over the last decades many molecular techniques have been developed which now allow a much better direct identification of microorganisms in bioreactors. These techniques include the use of biomarkers such as membrane lipids (PLFA, PLEL), immunological tests such as the enzyme-linked immunosorbent assay (ELISA) (identification of methanogens), and, most important, nucleic acid based methods.

A flow diagram showing the different steps in the analysis of microbial diversity and activity from nucleic acid based methods is given in figure 13. Following DNA extraction and amplification (PCR) and cloning of 16s rDNA genes (or 23s rDNA), DNA fragments can be sequenced to reveal the identity of the corresponding microorganism by comparative analysis with 16s rRNA sequences kept in public databases. This technique is a powerful tool to explore microbial diversity in anaerobic reactors but is very time-consuming and not suitable for studying population changes over time. Instead, from the knowledge of 16S rDNA sequences gene probes can be designed. The probes are short single stranded DNA sequences that are complementary for the rRNA of specific bacteria and will hybridize to them. For identification of the microorganisms the probes are either radioactively labelled by ^{32}P or chemically linked to fluorescent dyes. Both types of probes have successfully been used for identification and quantification of methanogens in anaerobic bioreactors. In comparison to ^{32}P labelled probes which are used for ex situ hybridisation (dot blot) of extracted nucleic acids, an advantage of fluorescently labelled probes is that they can be used for *in situ* hybridization to whole cells, a method known as FISH. The probe-targeted cells can be visualised either by traditional epifluorescence microscopy or confocal laser scanning microscopy (CLSM). In contrast to

epifluorescence microscopy, CLSM allows depth-resolved scanning making it possible to study the spacial organization of the microorganisms. For that reason this method has become an important tool for analysis and quantification of microorganisms living in flocs and biofilms. Besides being quantitative FISH will also, in general, confirm cellular activity since rRNA is degraded in inactive or dead cells.

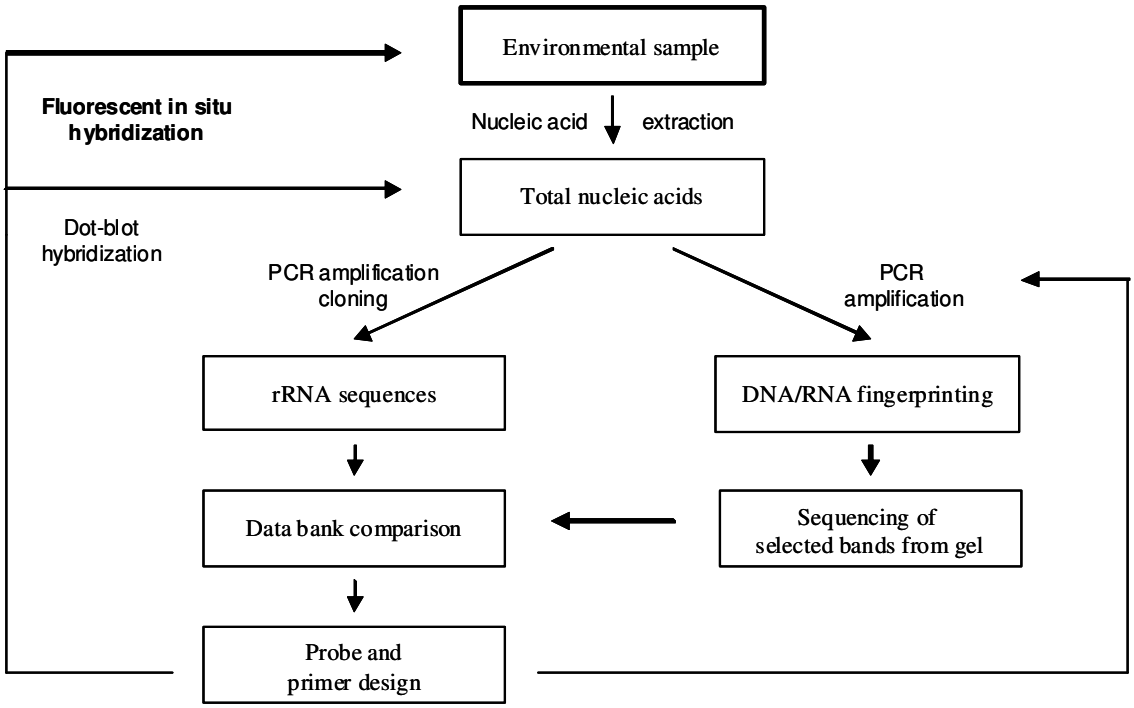

Figure 13 Flow chart for full cycle rRNAanalysis. FISH shown in boldface is recommended for routine analysis by testing laboratories and plant operators. The other steps in the cycle are necessary to obtain a complete picture of the microbial populations in a reactor. They can be performed by testing laboratories to obtain supplementary information regarding the diversity of bacteria present (using fingerprinting methods) and to construct nucleic acid probes (cloning of rRNAsequences, construction of a gene library and design of new gene probes) for more specific FISH analysis as needed. From Wilderer et al. (2002).

Another important molecular method for determination of cellular activity is microautoradiography (MAR). This method is based on *in situ* uptake of specific radioactively labeled (^{14}C, ^{32}P, ^{3}H) compounds by the microbes, which then is detected by a photographic emulsion and CLSM. Combining MAR and FISH gives the opportunity of identifying specific microorganisms present in a sample and at the same time give a clear confirmation of their activity. This combination is one of the best methods today for providing information about the ecophysiology of single bacteria in complex microbial communities.

Genetic fingerprinting techniques can be used to obtain a profile of the microbial diversity in complex microbial systems. These techniques can be used to compare the microbial community in different samples or to follow the population dynamics of one community over time. Classical examples of fingerprinting techniques are denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), which has been routinely used for some years. These methods are based on the melting behavior of the 16S rRNA gene on a polyacrylamide gel containing a gradient of DNA denaturants or a temperature gradient. Due to melting the mobility of the DNA fragments in the gel is significantly reduced. The melting behavior is dependent on the G+C content and the nucleotide sequence of the DNA. As a consequence of this, different DNA fragments will stop migrating at different positions in the gel resulting in a unique pattern in which each band corresponds to a single species (figure 14). A band of interest can subsequently be eluted from the gel and sequenced to identify the corresponding microbial species. From the sequence information an oligonucleotide probe for FISH can be designed and the actual abundance of a gel band that appears to be dominant after DGGE/TGGE analysis can be verified *in situ*.

Terminal Restriction Fragment Length Polymorphism (TRFLP) is another fingerprinting technique. By this technique DNA is amplified with a primer set where one primer is fluorescently end labelled. The product is subsequently cut by restriction enzymes into smaller fragments. Since the restriction site of each species is different this procedure provides fragments diverging in size. The fluorescence pattern of the fragments is subsequently digitally analysed providing information of the size of the different fragments (= different species) and the fluorescence intensity of the individual fragments (= abundance of species).

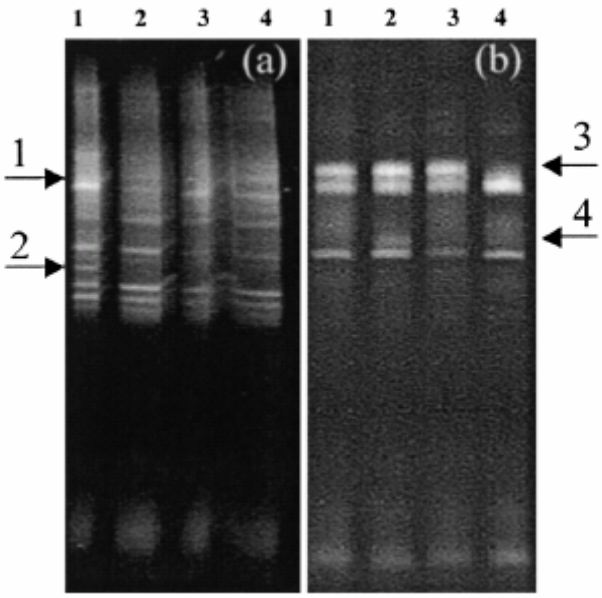

Figure 14 TTGE profiles showing the diversity of (a) bacteria and (b) archaea in samples from a thermophilic (68°C) CSTR treating cattle manure. The reactor was inoculated with biomass from a reactor operated with cattle manure at 65°C and fed with cattle manure in gradually increasing volumes, corresponding 4 days HRT from day 10 to 45, 3 days HRT from day 46 to 172. Two different batches of cattle manure were used as feedstock. Batch 1 was used from day 1 to 113, and batch 2 from day 113 to 172. Arrows indicate changes in banding pattern (species composition). Lane 1: day 42 and 4-days HRT; lane 2: day 63 and 3-day HRT; lane 3: day 104 and 3-day HRT; lane 4: day 154, 41 days after change of feedstock. From Nielsen et al. (2004).

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Paper I

Responses of the biogas process to pulses of oleate

H.B. Nielsen and B.K. Ahring

The Environmental Microbiology and Biotechnology Group, BioCentrum-DTU, Building 227, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45 45 25 61 83; fax: + 45 45 88 32 76; e-mail: bka@biocentrum.dtu.dk

Abstract

The effect of oleate on the anaerobic digestion process was investigated. Two thermophilic continuously stirred tank reactors (CSTR) were fed with mixtures of cattle and pig manure with different total solid (TS) and volatile solid (VS) content. The reactors were subject to increasing pulses of oleate. Following pulses of 0.5 and 1.0 g oleate/l, the most distinct increase in volatile fatty acid (VFA) concentrations were observed in the reactor with the lowest TS/VS content. This suggests a higher adsorption of oleate on the surfaces of biofibers in the reactor with the highest TS/VS and a less pronounced inhibition of the anaerobic digestion process. On the other hand, addition of 2.0 g oleate/l severely inhibited the process in both reactors, and a significant increase in all VFA concentrations combined with an immediate drop in methane production was noticed. However, 20 days after the reactors had been exposed to oleate both reactors showed a lower VFA concentration along with a higher methane production than before the pulses. This indicates that oleate had a stimulating effect on the overall process. The improved acetogenic and methanogenic activity in the reactors was confirmed in batch activity tests. In addition to this, toxicity tests revealed that the oleate pulses induced an increase in the tolerance level of acetotrophic methanogens towards oleate. When evaluating the usability of different process parameters (i.e. VFA and methane production) as indicators of process recovery, following the inhibition by oleate, propionate was found to be most suitable.
Key words Biogas process, VFA dynamics, process imbalance, long chain fatty acids.

Introduction

Anaerobic digestion is a technology widely used for treatment of organic waste. In Denmark, several joint large-scale biogas plants combine the treatment of manure together with organic waste from slaughterhouses and food processing industries. Some of these codigestion mixtures contain a high concentration of easily degradable lipids, which have a large biogas potential. During the digestion process, lipids are initially hydrolysed to long chain fatty acids (LCFA) (e.g. oleate, stearate) and glycerol by hydrolytic/fermentative bacteria. Glycerol is subsequently fermented to various types of alcohols, VFA and formate (Jarvis et al. 1997; Biebl 2001) and LCFA are converted to acetate and H_2 through β-oxidation by syntrophic acetogenic bacteria (Weng and Jeris 1976). Finally, acetate and H_2 are converted to CH_4, CO_2 and H_2O by methanogens (Schink 1988). During the anaerobic degradation of lipids, the oxidation of LCFA

is known to be the rate limiting step (Rinzema et al. 1994) and it is well documented that LCFA inhibit the anaerobic microbial activity at even low concentrations (Alves et al. 2001; Angelidaki and Ahring 1992; Cavaleiro et al. 2001; Hanaki et al. 1981; Hwu et al. 1996; Koster and Cramer 1987; Lalman and Bagley 2000; Lalman and Bagley 2001; Lalman and Bagley 2002; Rinzema et al. 1989; Rinzema et al. 1994; Shin et al. 2003). In fact, LCFA inhibits both acetotrophic and hydrogenotrophic methanogens as well as syntrophic acetogens. Acetotrophic methanogens are found to be more severely affected than hydrogenotrophic methanogens (Hanaki et al. 1981; Lalman and Bagley 2000). The adsorption of LCFA to the surface of microbial cell membranes has been indicated as the reason for inhibition (Henderson 1973), which suggests that the inhibitory effect of LCFA is dependent on the LCFA:biomass ratio. Other reports indicate that the inhibitory effect of LCFA is concentration dependent (Angelidaki and Ahring 1992, Koster and Cramer 1987; Rinzema et. al 1994). For many years, the mechanism of inhibition by LCFA has been ascribed to mechanisms of cell wall damage and bactericidal effects (Rinzema et al. 1994) but recent research suggest that the inhibition is due to transport limitation such as product diffusion limitation, e.g. biogas release (Pereira et al. 2003; Pereira et al. 2004a). Due to inhibition of acetogenic bacteria and methanogens, process inhibition in anaerobic digesters caused by LCFA will lead to both a decrease in methane production and an increase in VFA concentration (Cavaleiro et al. 2001).

Controversy exists whether microorganisms involved in the anaerobic degradation process can adapt to LCFA. Based on results from batch experiments, Rinzema et al. (1994) concluded that the acetotrophic methanogens from granular sludge are unable to adapt to capric acid, neither after repeated exposure to toxic concentrations, nor after prolonged exposure to non-toxic concentrations. These results were in good agreement with those of Angelidaki and Ahring (1992), who found in batch that no adaptation to oleate and stearate occurred, when biomass from a digester fed with cattle manure was exposed to non-inhibitory concentrations of the acids. However, continuous experiments with fixed-bed reactors have showed that pre-exposure of acetotrophic methanogens to lipids benefits the development of resistance to oleate (Alves et al. 2001). Cavaleiro et al. (2001) found that a hydraulic shock (reduction of HRT) of oleate induced an increase in the resistance of acetotrophic methanogens towards oleate; whereas an organic shock of oleate (increase in substrate concentration) resulted in a lower resistance towards oleate.

In the present paper, we describe the effect of various pulses of oleate on the biogas process in thermophilic CSTR´s. The objectives of the study was:

1) to evaluate the effect of increasing concentrations of particulate organic matter, in the form of biofibers, on the process stability; and
2) to examine the tolerance of acetotrophic methanogens to oleate, before and after pulses of oleate.
3) to give a precise description of the complex VFA dynamics and fluctuations in methane production following pulses of oleate.

Materials and methods

Substrates

Two different mixtures of substrates were used. Feedstock 1 consisted of a 1:1 mixture of cattle and swine manure that was blended and diluted with tap water to a TS concentration of 5.5–5.6% (w/vol) and a VS concentration of 4.3–4.4%. Feedstock 2 was prepared the same way as feedstock 1 with the exception that biofibers (particular lignocellulosic material in the form of digested straw) were added obtaining a final concentration of 7.2–7.5% TS and 5.3–5.6% VS.

Reactor set-up

Two 4.5 litre CSTRs with a working volume of 3.0 litre (Angelidaki and Ahring 1993) were inoculated with effluent from a stable lab-scale reactor at 55°C operating on cattle manure. One reactor, R1, was fed with feedstock 1 and the other reactor, R2, was fed with feedstock 2 resulting in organic loading rates of 2.5–2.6 and 3.1–3.3 gVS/(l reactor vol. x d), respectively. Due to technical problems (stirrer capacity) R2 was fed with feedstock 1 from day 150 to 160. Both reactors were stirred by a propeller every third minute for one minute at 100 rpm and operated at 55°C with a hydraulic retention time (HRT) of 17 days. Prior to initiation of experiments, the reactors were operated for a period of 50 days at stable reactor performance. Pulses of sodium oleate were added R1 on day 104 (0.5 g/l), 111 (1.0 g/l), 133 (2.0 g/l) and 161 (2.0 g/l), while sodium oleate were added R2 on day 104 (0.5 g/l), 112 (1.0 g/l) and 132 (2.0 g/l). A recently developed online VFA sensor (Pind et al. 2002) was connected to the reactor on day 132 to obtain detailed information about the fluctuations of the VFA levels in R1, caused by the most intense pulses.

Specific Methanogenic Activity (SMA)

The SMA of the reactor biomass in R1 was tested on day 100 and 160 by the method of Sørensen and Ahring (1993). 50-ml serum bottles were added 15 ml of anaerobic basal medium (Mladenovska and Ahring 2000), which had been adjusted to a pH of 7.9 and flushed with 100% N_2. The basal medium was supplemented with substrates in the following final concentrations: 200 mM sodium formate or 50 mM sodium acetate. Substrate was omitted from control series. Media with or without substrates were autoclaved at 141°C for 40 min and 0.5 g/l Na_2S and 10 ml/l vitamin solution were added (DSMZ medium no. 141, DSMZ 1989). H_2/CO_2 was added to the autoclaved medium (without other substrate) by pressurizing the vials with H_2/CO_2 (80%/20%) to 101kPa overpressure. Finally, all bottles were inoculated with 5 ml of reactor content, flushed with N_2, closed with butyl rubber stoppers and aluminium crimps, and incubated in a shaking water bath at 55°C. After inoculation, the dilution rate of the biomass was 5:16.4. Methane production was measured every second hour for the first 12 hours and 3 times per day for 5 days. All experiments were conducted in triplicates.

Kinetics of VFA-degradation

Kinetics of VFA-degradation in reactor R1 were determined on day 100 and 160. Media was prepared as in the SMA experiments and substrate was added in concentrations of 50 mM sodium acetate, or 29 mM sodium propionate or 20 mM sodium butyrate. All bottles were inoculated with 5 ml reactor content and incubated in a shaking water bath at 55°C. Progress curves of acetate, propionate and butyrate degradation was made by withdrawing 0.3 ml of media/biomass mixture every 8–12 hours for 4–7 day and kinetics were determined by applying an integrated solution to the Michaelis-Menten equation (Ahring and Westermann 1987):

$$\frac{\ln S_0/S_t}{t} = \left(\frac{-1}{K_m} \times \frac{S_0 - S_t}{t} \right) + \frac{V_{\max}}{K_m}$$

Where S_0 is the initial substrate concentration, S_t is the substrate concentration at time t and K_m is the half-saturation constant. $V_{\max}$ is the maximum substrate utilization in the vials and was calculated from the steepest linear decline in substrate concentration. The biomass was considered to be constant during the entire experiment since the theoretical increase in VS was too low compared to the initial VS concentration in the vials. Therefore, the maximum specific

substrate utilization (A_{max}) of the reactor biomass was derived directly from V_{max}. All experiments were conducted in triplicates.

Toxicity tests

The effect of sodium oleate on methane production from acetate was tested in batch experiments before and after R1 was spiked with oleate on day 100 and 160, respectively. 50-ml serum bottles containing 15 ml anaerobic basal medium were inoculated with 5 ml reactor content and added sodium acetate to a final concentration of 50 mM. The bottles were flushed with N_2/CO_2 (80%/20%), closed with butyl rubber stoppers and aluminium crimps and incubated in a shaking water bath at 55°C. When the methane production from acetate was increasing exponentially, sodium oleate in concentrations of 0.2 g/l, 0.4 g/l, 0.6 g/l, 0.8 g/l and 1.0 g/l were added. All experiments were conducted in triplicates.

Batch experiments

The effect of sodium oleate (0.2–1.0 g/l) on VFA-dynamics in manure diverging in TS/VS-contents was tested in batch experiments. 25 ml of manure from feedstock 1 and feedstock 2 were distributed in 116 ml serum bottles and inoculated with 15 ml of reactor content from R1 and R2, respectively. The bottles were flushed with N_2/CO_2 (80%/20%), closed with butyl rubber stoppers and aluminium crimps and incubated at 55°C. Sodium oleate was added on day 8 when the methane production was increasing exponentially. 1 ml samples for determination of the VFA concentrations were withdrawn each bottle on day 0, 8 (before addition of oleate) and 21. All experiments were conducted in triplicates.

Analytical methods

TS, VS, pH and ammonia content were determined using standard methods (Greenberg et al., 1998). CH_4 production from the SMA tests and 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 2 x 1 ml of the reactor content was acidified with 30 µl 17% phosphoric acid, centrifuged at 10500 rpm for 20 min, and analyzed on a GC equipped with flame ionisation detector. Samples for ammonia determination was frozen at -20°C and measured at the end of the experiment.

Results

Reactor experiments

From day 50–100 a stable process (i.e. stable methane production and stable VFA levels) was observed in both reactors. The TS and VS content of the reactor biomass were 2.9/2.1% (w/vol) and 5.4/4.1% (w/vol) in R1 and R2, respectively. pH was stable and between 7.9–7.93 for both reactors. The total nitrogen content (Kjeldahl-N) of the feedstock was in average 3.7 g-N/l while the ammonia-N was in average 2.5 g/l. The performance of R1 from day 100–190 and R2 from day 100–160 are illustrated in figure 1. Before and after addition of oleate a stable performance was obtained in both reactors but during the experiment an increase in the methane production occurred. R1 produced between 253 and 268 ml CH_4/gVS before oleate addition and between 314 and 329 ml CH_4/gVS after oleate addition (day 177–189) while R2 produced between 230 and 254 ml CH_4/gVS before oleate addition and between 292 and 319 ml CH_4/gVS after oleate addition (day 154–160). Both reactors were also subject to a lowering of all VFA levels at the end of the experiment.

Addition of oleate in concentrations of 0.5 and 1.0 g/l affected the process stability in both reactors illustrated by increasing VFA concentrations. The increase in all individual VFAs, except acetate, was more distinct in R1 than R2 (table 1). At the same time both pulses had a positive effect on the methane production in R2 while the pulse of 1.0 g oleate/l had a negative effect on the methane production in R1.

A pulse of 2.0 g oleate/l resulted in a pronounced increase in all VFA concentrations and an instant drop in the methane production in both reactors. As during the pulses of 0.5 and 1.0 g oleate/l the increase in isobutyrate, isovalerate and valerate were higher in R1 than in R2 and the methane production in R2 (2.4 times drop) was not as clearly affected as the methane production in R1 (4 times drop). However, in contrast to the pulses of 0.5 and 1.0 g oleate/l the reactors showed approximately the same increase in acetate and propionate concentration while the increase in butyrate concentration was highest in R2. The decrease in methane production after the first pulse of 2.0 g oleate/l was followed by a simultaneous drop in pH with a minimum of 7.54 in R1 (day 141) and 7.81 in R2 (day 135).

The response to a second pulse of 2.0 g oleate/l in R1 was similar but more moderate for all parameters compared to the first pulse of 2.0 g oleate/l. All VFAs and methane production values

showed a faster return to a stable level after the second pulse of 2.0 g oleate/l (table 2) and all VFA peaks were lower following the second pulse (table 1).

SMA and VFA-kinetics

Table 3 shows the results of the SMA tests and kinetics of VFA degradation obtained on day 100 and 160. The consumption profile of acetate is shown in the figure 2. An increased methanogenic activity from H_2/CO_2, formate and acetate was observed on day 160. The initial degradation rate of acetate, propionate and butyrate increased as well but the estimated A_{max} of acetate and propionate was lowest at day 160. A_{max} of butyrate remained constant. The half-saturation constant, K_m, of all VFA showed no significant changes, from day 100 to 160, although a small decrease was observed for acetate.

Toxicity tests

The tolerance of the acetotrophic methanogens to oleate increased during the experiment (figure 3). On day 100 the methane production from acetate was inhibited at oleate concentrations between 0.2 and 0.4 g/l while the inhibitory level of oleate on day 160 was between 0.6 and 0.8 g/l.

Batch experiments

The TS/VS content of the manure/inoculum mixtures used in the batch experiments was 4.6/3.60% (w/vol) and 6.7/5.2% (w/vol), respectively. When adding oleate to the different substrates a pattern similar to the one in the reactor experiments was observed (figure 4). In the beginning of the experiment equal VFA concentrations were found in the different substrate mixtures but on day 21, after various amounts of oleate had been added on day 8, higher concentrations of all measured VFAs were found in the substrate with the lowest TS/VS content. One exception was propionate, which was found in the same concentrations when 1.0 g oleate/l had been added.

Discussion

Overall reactor performance before and after oleate addition

The organic loading was during the entire experiment 25–27% higher in R2 than in R1. This resulted in an increase in the methane production rate (l/(l reactor volume x day)) in R2 compared

to R1 of approximately 13% before the oleate addition and 14% after the oleate addition (data not shown). However, as illustrated in fig 1.a and 1.b the methane yield (ml CH_4/gVS) was highest in R1 corresponding to approximately 5–10% before the oleate addition and 6–7% higher after the oleate addition. These dissimilarities between organic loading, total methane production and relative methane production were probably due to the complex structure of the biofibers that were added to the feedstock of R2. Biofibers consist of lignocellulosic material that is only slowly degraded under anaerobic conditions (Colberg 1988) and are to some extent undegradable. Therefore, a relatively higher percentage of the biogas potential of feedstock 2 compared to feedstock 1 was unexploited resulting in a higher methane yield in R1.

Effect of oleate pulses on the overall biogas process

Addition of oleate to the reactors resulted in temporary inhibitions of the anaerobic digestion process but, interestingly, oleate had a stimulating effect on the overall process in both reactors. This was demonstrated by the higher methane yields and lower VFA concentrations at the end of the experiment. Furthermore, the SMA-tests and substrate-depletion tests of R1 revealed a higher initial degradation of all substrates from day 100 to day 160. These results indicate that the oleate pulses influenced the microbial biomass in the reactors. Mass balance analysis shows that the lowering in VFA concentration at the end of the experiment could only count for approximately 30% of the increase in methane production. This suggests that the oleate pulses not only affected VFA degradation but also induced an increase in the hydrolysis.

The addition of 2.0 g oleate/l resulted in an instant drop in methane production and a steep increase in all measured VFAs for both reactors. Oleate is known to inhibit acetotrophic and hydrogenotrophic methanogens as well as syntrophic acetogens (Hanaki et al. 1981), which is the most reasonable explanation for these observations. Rinzema et al. (1994) concluded that syntrophic acetogens and hydrogenotrophic methanogens recover before acetotrophic methanogens when exposed to oleate, and according to this the recovery time[1] of acetate should be longer than the other measured VFAs. However, in the present experiment acetate together with butyrate was the first VFA that recovered to a stable level after the pulses of 2.0 g oleate/l. With acetate being a degradation product of other VFAs it is possible that the high concentrations of acetate in the period following the oleate addition could have caused a product inhibition resulting in a further increase in concentration and recovery time of other VFAs. Acetate is

[1] The recovery time of the different VFAs is defined as the period of time from when a VFA started to increase, following an oleate pulse, and until the VFA had declined and re-established at a stable level.

known to inhibit butyrate degradation at a concentration of 25 mM (Ahring and Westermann 1988) and product inhibition of isovalerate and propionate degradation by acetate has been demonstrated as well (Mösche and Jördening 1999; Pind et al. 2003). On the other hand propionate began to decrease 1–3 days after acetate had decreased and reached a stable level, which indicates that the syntrophic acetogens degrading propionate was more affected by the oleate pulses than the acetotrophic methanogens.

Effect of TS/VS content

The variations of the VFA concentrations in the two reactors after addition of 0.5 and 1.0 g oleate/l indicate that the process stability was more affected in R1 than in R2 (fig 1). These observations were confirmed in the batch experiments where the highest concentrations of acetate, propionate, isobutyrate and butyrate after oleate addition were found in the substrate with the lowest TS/VS content (fig 4). LCFA disappear from the aqueous phase and accumulate in the solid phase within the first 24 hours of incubation (Hanaki et al. 1981), and the ability of LCFA to adsorb on surfaces of active and inactive granular sludge has been reported (Hwu et al. 1998). Therefore, a higher adsorption of oleate or intermediates of its degradation, for example palmiate (Pereira et al. 2002; Pereira et al. 2004b), on the surfaces of the biofibers in R2 with a subsequent lower adsorption of LCFA onto the surfaces of the microbial cells, could have minimized the inhibition of the microbes in R2 compared to R1. This could be the explanation for the observed pattern after the first two pulses of oleate. The severe process failure in both reactors after the pulse of 2.0 g oleate/l shows that the adsorption capacity of the biofibers in R2 was too small to prevent an inhibitory oleate concentration in the reactor.

Adaptation of microorganisms to oleate

The toxicity test on day 100 with inoculum from R1, showed that the methane production from acetate was inhibited at oleate concentrations between 0.2 and 0.4 g/l. In a similar experiment Angelidaki and Ahring (1992) found the inhibitory level of oleate to be between 0.1 and 0.2 g/l. After depletion of the biomass/substrate mixtures and a new addition of oleate and acetate, the authors found the inhibitory level of oleate to be the same. In the present experiment, the inhibitory level of oleate to acetate degradation increased from day 100 to day 160 to a concentration of 0.6–0.8 g/l (fig 3). This reflects an adaptation of the acetotrophic methanogens to oleate during the experiment. Following the inhibition period, the higher methane production in vials with oleate than in the control vials showed that oleate was consumed. In the reactor

experiments, an adaptation of the methanogenic communities to oleate was indicated by the faster recovery time of methane production, following the second pulse of 2.0 g oleate/l to R1.

The inhibition level of oleate towards the acetotrophic methanogens in the reactors was higher than in the toxicity test. An explanation for these differences might be that in a continuously fed reactor, the inhibition level is only observed when the reduction of growth rate of the microorganisms approaches the dilution rate of the reactor (Angelidaki and Ahring 1993). In the toxicity test (batch conditions), oleate was added when the methane production from acetate was increasing exponentially and a decrease in microbial activity would be directly reflected in the methane production. Furthermore, the dilution of biomass in the toxicity test increased the oleate:biomass ratio and lowered the relative adsorption capacity of oleate on biofibers, which could have resulted in an increased inhibition in the toxicity test.

Adaptations of acetogenic bacteria to oleate was not tested in batch experiments but in the reactors the maximum degradation rate of all VFAs, with the exception of propionate, were higher after the second pulse of 2.0 g oleate/l than after the first pulse of 2.0 g oleate/l (table 2). At the same time the peaks in all VFA concentrations were lower and the recovery time of all VFA levels faster after the second pulse of 2.0 g oleate/l. This indicates an increase in the tolerance towards oleate for most of the acetogenic microorganisms in the reactor. The observed pattern may also have been a consequence of an increase in the population of acetogenic bacteria effective in degrading oleate, reducing the concentration of oleate and moderating the exposure time of the acetogens to inhibitory levels of oleate. Finally, another explanation could be that the microbes had adapted to resist sudden increases in VFA concentrations. An explanation that was supported by the SMA tests and VFA kinetics, where the initial degradation of all substrate increased significantly from day 100 to day 160 (table 3).

According to table 2, the maximum degradation rate of propionate in R1 was lowered following the second pulse of 2.0 g oleate/l when compared to the first pulse. This was supported by the kinetic calculations where A_{max} of the propionate degradation decreased from day 100 to day 160. The increased initial degradation of propionate in the batch experiments and the faster recovery of propionate after the second pulse of 2.0 g oleate/l contradict these results. However, the kinetic calculations were based on batch experiments where no oleate was present and do not reflect the conditions in the reactor following the oleate pulses. Therefore, it is plausible to suggest that the syntrophs degrading propionate did not adapt to oleate. The faster recovery time of propionate following the second pulse of 2.0 g oleate/l was probably a result of the faster recovery of acetate, resulting in a shorter product inhibition period of the propionate degradation.

Evaluation of process stability

Control of the anaerobic biogas process and suitable indicators of process imbalance have received much focus the last decades. Changes in the VFA levels in anaerobic digesters treating cattle manure was demonstrated to be a good parameter for prediction of process instability such as hydraulic overloading, organic overloading and temperature increases while an evaluation of the biogas process based only on methane production was found to be doubtful (Ahring et al. 1995). In the present study, process imbalance following addition of 0.5 and 1.0 g oleate/l to R1 was only indicated by the increasing VFA concentrations. R1 showed no changes in the methane production after the addition of 0.5 g oleate/l and only a brief decrease after the addition of 1.0 g oleate/l was observed. Process inhibition following the pulses of 2.0 g oleate/l was in both reactors clearly indicated by a step increase in the various VFA concentrations and an instant drop in the methane production. For operational purposes, this could justify the use of VFA concentrations as well as methane production for indication of process inhibition. However, when using methane production as a process parameter, it should be kept in mind that lipid-containing organic wastes are added biogas plants with the purpose of improving the methane production. A higher methane production as a consequence of lipid addition may, therefore, be misleading when evaluating process stability. This problem was also illustrated in the present experiment were the addition of 0.5 and 1.0 g oleate/l to R2 resulted in an increase in the methane production while the increases in the VFA levels, especially propionate, indicated a slight inhibition of the biogas process.

Ahring et al. (1995) evaluated the responses of specific VFAs to changes in the biogas process and the changes of isobutyrate and isovalerate were found to be the fastest and most pronounced. In the present experiment all VFAs, with the exception of valerate, showed a fast increase following the pulses of oleate and no conclusions could be drawn whether, which specific VFA gave the best indication of the beginning of the process imbalances. However, following all pulses of oleate, propionate showed the longest recovery time and gave the best indication of when the process had stabilized after the various imbalances. In fact, propionate did not decrease before 2 days after acetate had dropped and stabilized.

The fluctuation pattern of total VFA followed the pattern of acetate and the significant changes in propionate concentration were not reflected through that parameter. This stresses out that total VFA concentration is unusable for indication of process stabilization after process imbalances caused by oleate inhibition.

Conclusions

The addition of oleate to CSTRs treating mixtures of cattle and pig manure resulted in temporary inhibitions of the anaerobic digestion process. However, the increased methane yield and the lowered VFA concentrations after the process had recovered showed that oleate had a stimulating effect on the overall process. Following pulses of 0.5 and 1.0 g oleate/l only slight inhibitions were observed but the fluctuations in the VFA concentrations indicated that the process was more affected in R1 than in R2. Higher adsorption of oleate, or intermediates of its degradation, on the biofibers in R2 possibly minimized the adsorption of LCFA onto the surfaces of the microbial cells in the reactor. Addition of 2.0 g oleate/l led to an inhibition of the acetotrophic and hydrogenotrophic methanogens as well as the syntrophic acetogens illustrated by an instant drop in methane production and a steep increase in all measured VFA. Following the inhibitions, propionate showed the longest recovery time and gave the best indication of process stabilization. Toxicity tests before and after the pulses of oleate revealed that acetotrophic methanogens do adapt to oleate.

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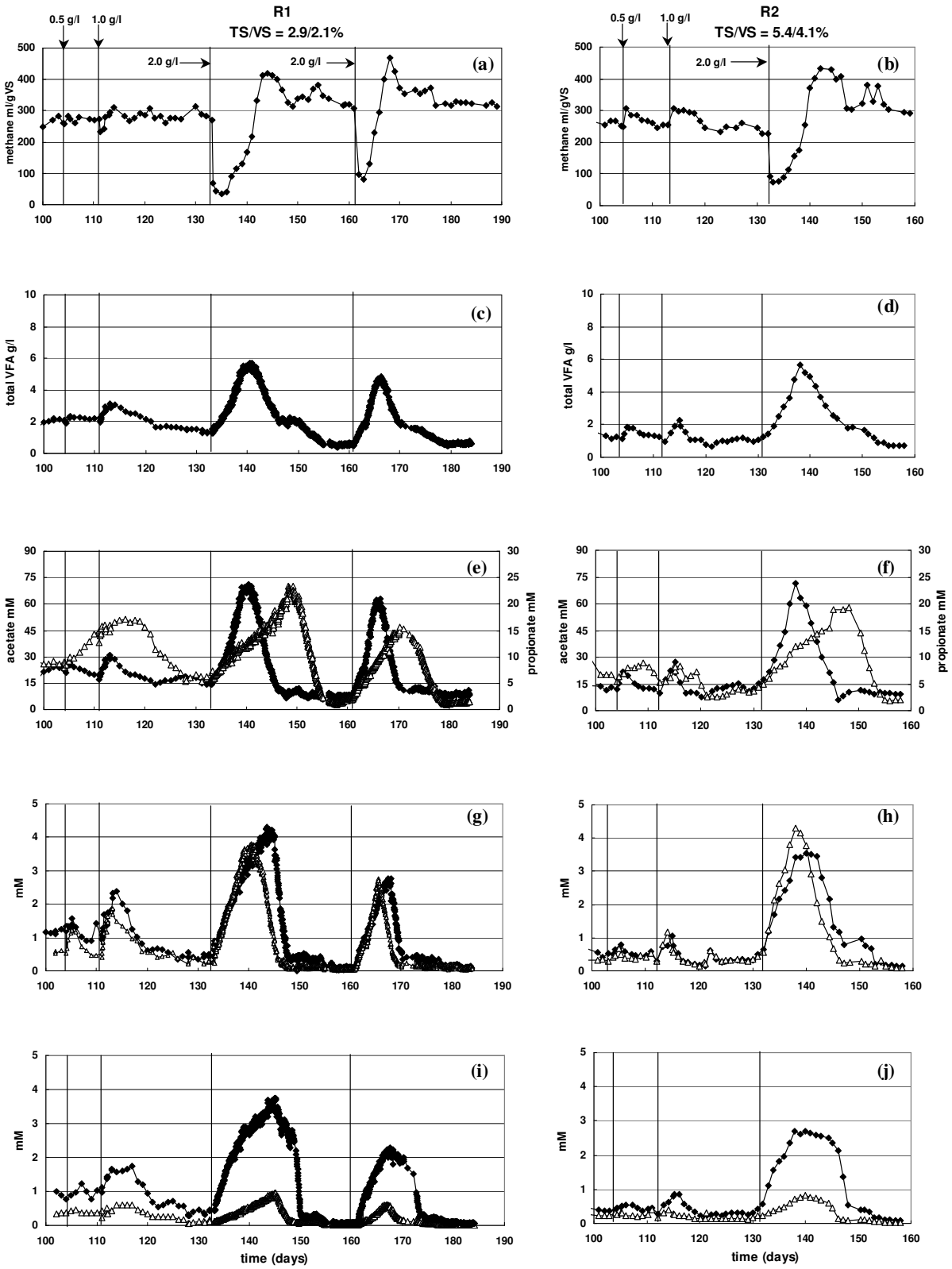

Figure 1. Reactor experiments. Methane yield and VFA concentrations in R1 and R2 during increasing pulses of oleate. **a + b**) ◆: methane yield; **c + d**) ◆: total VFA; **e + f**) ◆: acetate, △: propionate; **g + h**) ◆: isobutyrate, △: butyrate, **i + j**) ◆: isovalerate, △: valerate.

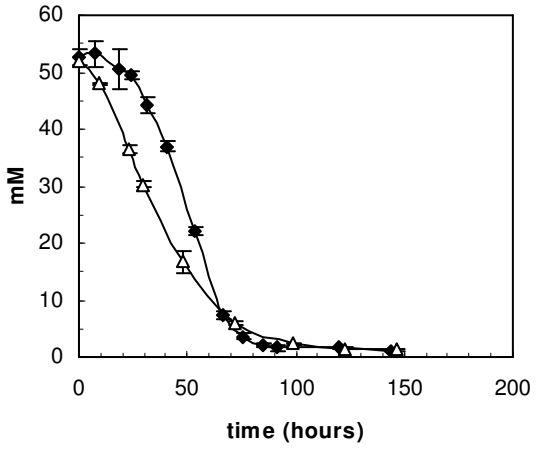

Figure 2. Acetate consumption profile. Biomass from R1 was diluted (5:16.4) in BA media and added acetate in a final concentration of 50 mM. ◆: day 100; △: day 160.

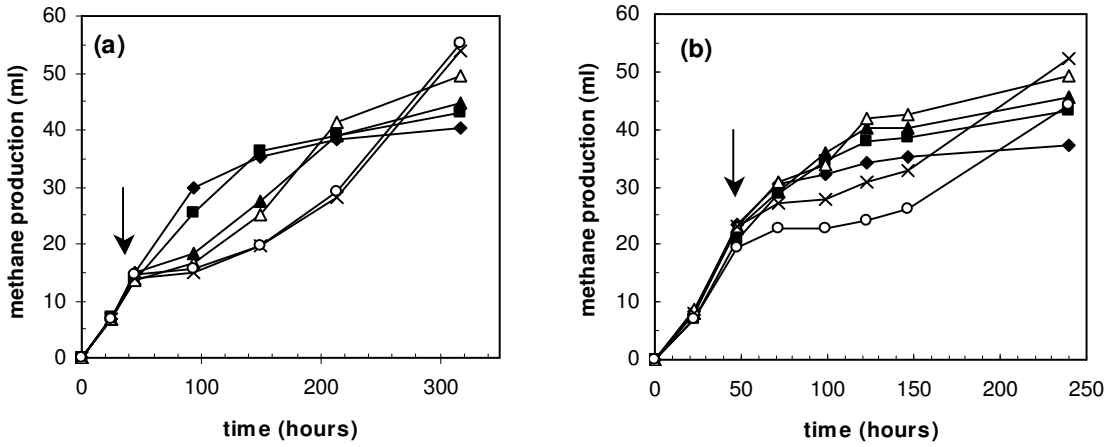

Figure 3. Toxicity tests. Methane production from acetate under batch conditions. Biomass from R1 was diluted (5:16.4) in BA media and added acetate in a final concentration of 50 mM at **a**) day 100 and **b**) day 160. Oleate was added in various amounts at time marked with an arrow; ◆: 0.0 g/l; ■: 0.2 g/l; ▲: 0.4 g/l; △: 0.6 g/l; ○: 0.8 g/l; ×: 1.0 g/l. All experiments were conducted in triplicates.

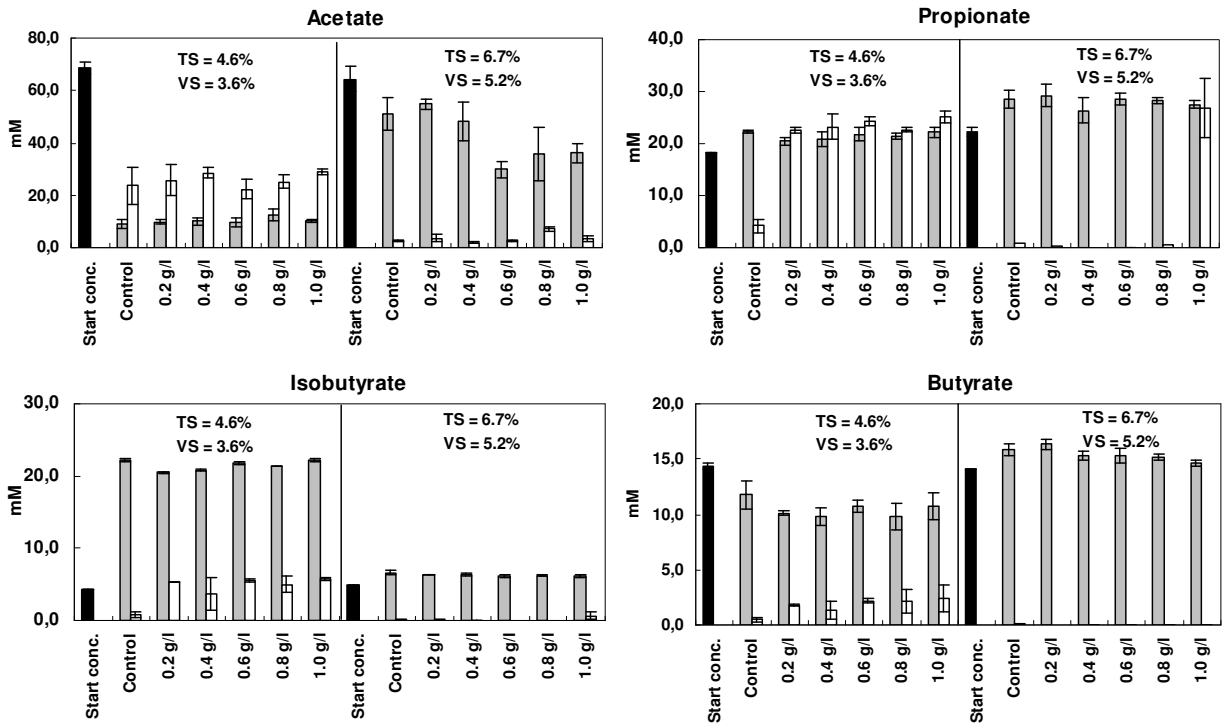

Figure 4. Batch experiments. The effect of various concentrations of sodium oleate (0.2–1.0 g/l) on VFA-dynamics in manure diverging in TS/VS-contents. Oleate was added on day 8 of the experiment. Black columns: start concentration in manure/inoculum mixture; grey columns: concentration on day 8 before addition of oleate; white columns concentration on day 21. Results are given as means of triplicates with standard deviations.

Table 1. Approximate increases in VFA levels in R1 and R2 caused by various pulses of oleate. ND: not determined.

	Acetate increase (mM)		Propionate increase (mM)		Isobutyrate increase (mM)		Butyrate increase (mM)		Isovalerate increase (mM)		Valerate increase (mM)	
Oleate pulse	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
0.5 g/l	5.3	9.7	6.5	3.3	0.38	0.30	0.71	0.21	0.45	0.17	ND	ND
1.0 g/l	14.0	17.2	ND	3.0	1.36	0.75	1.46	0.88	0.75	0.44	0.17	0.12
2.0 g/l no. 1	56.4	58.5	17.0	15.2	3.75	3.10	3.56	3.86	3.30	2.29	0.80	0.59
2.0 g/l no. 2	55.1		13.1		2.72		2.60		2.17		0.53	

Table 2 Maximum VFA degradation rates in reactor R1 and recovery time of the different VFAs after pulses of 2.0 g/l oleate. The recovery time of the different VFAs represents the period of time from when a VFA started to increase, following an oleate pulse, and until the VFA had declined and re-established at a stable level.

	Pulse 1		Pulse 2	
	VFA-degradation (mmol/h)	Recovery time (days)	VFA-degradation (mmol/h)	Recovery time (days)
Acetate	0,597	≈ 13,50	0,705	≈ 10,00
Propionate	0,178	≈ 22,00	0,103	≈ 18,00
Isobutyrate	0,062	≈ 15,00	0,047	≈ 9,25
Butyrate	0,040	≈ 15,00	0,034	≈ 9,25
Isovalerate	0,092	≈ 17,25	0,045	≈ 12,50
Valerate	0,017	≈ 15,75	0,008	≈ 9,25

Table 3. The specific methanogenic activity (SMA) and VFA-degradation activity of biomass from R1. Initial activity: activity within the first 12-14 hours; maximum activity: activity during exponential growth. Results are given as means of triplicates with standard deviations

	SMA		VFA-degradation		
	Initial	**Maximum**	Initial	A_{max}	K_m
	µmol/(gVS x h)	µmol/(gVS x h)	µmol/(gVS x h)	µmol/(gVS x h)	mM
Before oleate addition (day 100)					
Control	15.7 ± 1.13	15.7 ± 1.13			
H_2/CO_2	56.8 ± 3.25	56.8 ± 3.25			
Formate	88.1 ± 2.12	88.1 ± 2.12			
Acetate	50.8 ± 1.95	101.1 ± 11.58	16.6 ± 2.46	106.6 ± 5.56	13.81 ± 2.569
Propionate			No degradation	29.6 ± 1.04	1.16 ± 0.677
Butyrate			28.6 ± 1.01	38.5 ± 2.65	0.67 ± 0.306
After oleate addition (day 160)					
Control	18.3 ± 2.60	18.3 ± 2.60			
H_2/CO_2	68.6 ± 5.68	68.6 ± 5.68			
Formate	145.1 ± 12.61	145.1 ± 12.61			
Acetate	68.8 ± 3.03	108.1 ± 18.94	46.2 ± 3.18	87.1 ± 1.42	8.13 ± 4.219
Propionate			13.7 ± 3.90	25.1 ± 2.77	1.96 ± 0.877
Butyrate			39.5 ± 1.16	39.5 ± 1.16	1.11 ± 0.120

Paper II

Effect of protein and ammonia pulses on the biogas process

H.B. Nielsen and B.K. Ahring
The Environmental Microbiology and Biotechnology Group, BioCentrum-DTU, Building 227, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45 45 25 61 83; fax: + 45 45 88 32 76; e-mail: bka@biocentrum.dtu.dk

Abstract

Two thermophilic continuously stirred tank reactors (CSTR), R1 and R2, were subject to pulses of tryptone and ammonia. R1 was operated at an ammonia concentration of 3.0 g-N/l, corresponding to the average ammonia concentration in Danish centralized biogas plants. R2 was operated at an ammonia concentration of 1.7 g-N/l. Shock loads of tryptone (10 g/l, 10 g/l, 15 g/l) had an immediate stimulating effect on methanogenesis for both reactors illustrated by significant peaks in methane production but also lead to a hydraulic overloading illustrated by a steep increase in volatile fatty acids (VFA) concentration. Three days after the pulses a second peak in acetate concentration and a decrease in methane production indicated an ammonia-inhibition of the acetoclastic methanogens. During the pulses of tryptone the performance of R1 was slightly more affected than R2. Pulses of ammonia (0.79 g-N/l) resulted in a decrease in methane production of both reactors but no immediate increases in VFA concentrations was observed illustrating that the ammonia inhibition during this experiment was an overall inhibition of the biogas process and not only an inhibition of the methanogens.
Key words Biogas process, VFA dynamics, ammonia inhibition, protein degradation, process imbalance.

Introduction

Anaerobic digestion is widely used for treatment of organic waste with a simultaneous production of energy (biogas). In Denmark 22 large-scale centralized biogas plants are in operation treating more than 1.2 mill tons of manure. Due to a high water content combined with a large fraction of lignocellulosic material – recalcitrant to degradation – the methane production of manure is relatively low (10–20 m^3 CH_4/tons manure) (Angelidaki and Ellegaard 2003). Biogas plants are, therefore, difficult to run with an economically profitable result if the process is solely based on manure (Hjort-Gregersen, 1999). To increase the methane production several plants combine the treatment of manure together with organic wastes rich in protein and fat (e.g. slaughterhouses and food processing industries). Complete mineralization of proteins to methane

and carbon dioxide under anaerobic conditions is a multi-step process consisting of four major steps (Ramsay and Pullammanappallil 2001): 1) hydrolysis of the protein by extracellular enzymes into large organic molecules in the form of polypeptides and amino acids 2) fermentation of amino acids into organic acids, ammonia, carbon dioxide, and small amounts of hydrogen and sulphur-containing compounds 3) degradation of organic acids (acetogenesis) into acetate, hydrogen and carbon dioxide and finally 4) formation of methane (methanogenesis) from acetate or hydrogen and carbon dioxide. Ammonia, which is produced during the degradation of proteins, is essential for bacterial growth but may – if present in high concentration –also inhibit the anaerobic digestion process. For unadapted methanogens ammonia inhibition has been observed to begin at 1.5–2.0 g-N/l (van Velsen 1979; Hashimoto 1986) while for an adapted biogas process an ammonia tolerance of 3–4 g-N/l was reported (Angelidaki and Ahring 1993). An inhibition of the biogas process by ammonia does not necessarily stop the process but may just leave to suboptimal performance of the reactor. Thus, obtained Hansen et al. (1998 and 1999) a stable thermophilic degradation of swine manure at an ammonia concentration of 6 g-N/l with a low methane yield and high concentrations of VFA. The inhibition of the biogas process is caused by free ammonia (NH_3) (Hashimoto 1986) which concentration besides the total ammonia concentration (NH_4^+ and NH_3) also depends on temperature and pH. Thus, will an increase in temperature or pH lead to an increase in the free ammonia concentration. Angelidaki and Ahring (1994) reported that anaerobic digestors treating cattle manure was inhibited when the concentration of free ammonia exceeded 700 mg-N/l while a level of 80 mg-N/l was reported for initial inhibition of an unadapted process (Koster and Lettinga 1984; De Baere et al. 1984).

The main feedstock of Danish centralised biogas plants is livestock waste, which normally has an ammonia concentration of 2–4 g-N/l. In the present study we visited a number of thermophilic Danish centralized biogas plants and found the ammonia concentration in the reactors to be in the same range (table 1). Although dependent on the exact temperature and pH, it is evident that the plants are operated closely to the inhibitory level of ammonia. As mentioned earlier is the treatment of manure often combined with the treatment of organic wastes containing high amounts of proteins, which easily releases further amounts of ammonia when degraded. A sudden addition of large amounts of waste rich in proteins may, therefore, cause a temporary increase in ammonia concentration, which could leave to an inhibition of the process. In that context we investigated the effect of sudden additions of protein and ammonia on methane

production and VFA dynamics in two CSTRs operated at different ammonia levels.

Materials and methods

Substrates

Cattle manure was obtained from the full-scale biogas plant in Lintrup, Denmark. The manure was mixed with water to a total solid (TS) content of 5.9% and a volatile solid content of 4.5%. The total nitrogen content (Kjeldahl-N) of the diluted manure was 2.2–2.4 g-N/l while the ammonia-N content was 1.3–1.4 g-N/l. The diluted manure was blended 1–2 min and kept at 2°C until used. Tryptone, an enzymatic hydrolysate of casein, rich in peptones and amino acids was used as protein source. The tryptone contained 13% total-N and 4.9% amino-N. The VS content was 96%. The manufacturer gave no information about the sulphur content in the product.

Characterisation of full-scale biogas plants

Before setting up lab-experiments random samples were taken from 7 thermophilic Danish centralised biogas plants and analysed with regard to total-N content, ammonia-N content, temperature and hydraulic retention time (HRT). The results are given in table 1.

Reactor experiments

Two continuously stirred tank reactors (CSTR), R1 and R2, with a working volume of 3.0 l were inoculated with digested cattle manure from a 55°C steady-state lab-scale reactor. The reactors were operated at the same average conditions as the examined full-scale biogas plants, i.e. 53°C and a HRT of 16 days (table 1). A propeller stirred the reactors for one minute every third minute at 100 rpm. Circulating the heated water from a water bath through the reactor jackets ensured stable operational temperature. Both reactors were fed with cattle manure at an organic loading rate of 2.8 g VS/(l reactor vol. x d). As mentioned, was the natural ammonia concentration of the cattle manure between 1.3 and 1.4 g-N/l. After the reactors had been in operation for a period of 32 days (two HRT) stable operation indicated that steady state was obtained. Subsequently, to obtain the same ammonia concentration in R1 as in the average biogas plant the ammonia concentration in the reactor was gradually raised by adding NH_4Cl to the feedstock. The feedstock of R2 was not added NH_4Cl. At day 67 the ammonia concentration was 3.0 g-N/l and 1.7 g-N/l in R1 and R2, respectively, and the reactors were subsequently operated at these conditions for one HRT. Pulses of 10 g/l tryptone were added the reactors at day 82 and 89 while a pulse of 15 g/l tryptone were added at day 103. At day 181, 183, 185 and 187 pulses of 3.0 g

NH_4Cl/l (0.79 g ammonia-N/l) were added the reactors and the concentration of the feedstock was raised with the same amount. VFA concentrations, CH_4 production, ammonia concentration and pH were monitored during the entire experiment. During pulses of tryptone and ammonia the VFA dynamics in R1 were closely monitored by online VFA measurement (Pind et al. 2002).

Batch experiments

Specific Methanogenic Activity (SMA). SMA of the reactor biomass was measured at day 80 according to the method of Sørensen and Ahring (1993). 50-ml serum bottles were added 15 ml of anaerobic basal medium (Mladenovska and Ahring 2000), which had been flushed with 100% N_2 and pH had been set to 7.7. Substrates were added in the following final concentrations: 200 mM sodium formate or 50 mM sodium acetate. Substrate was omitted from control series. Media with or without substrates were autoclaved at 141°C for 40 min and 0.5 g/l Na_2S and 10 ml/l vitamin solution were added (DSMZ medium no. 141, DSMZ 1989). H_2/CO_2 was added to the autoclaved medium (without other substrate) by pressurizing the vials with H_2/CO_2 (80%/20%) to 101kPa overpressure. Finally, all bottles were inoculated with 5 ml of reactor content and incubated in a shaking water bath at 53°C. After inoculation the dilution rate of the biomass was 5:16.4. Methane production was measured every second hour for 14 hours. All experiments were conducted in triplicates.

Effect of ammonia on methanogenesis. The effect of elevated ammonia concentrations on the methane production was tested in batch experiments at day 75. Effluents from the reactors were distributed in 116 ml vials in amounts of 50 ml and added extra ammonia in concentrations of: R1) 0.5, 1.0, 1.5 and 2.0 g-N/l; R2) 0.5, 1.0, 1.5, 2.0 and 3.0 g-N/l. No ammonia was added control vials. The vials were flushed with N_2/CO_2 (80/20%), closed with butyl rubber stoppers and aluminium crimps and incubated at 53°C for 72 hours. The methane production was measured frequently and the relative inhibition of the methane production in the test vials compared to the control vials was calculated.

Effect of ammonia on tryptone deamination. The effect of ammonia concentration on the deamination capacity of the reactors was tested in batch experiments at day 81. According to the method of Gallert et al. (1997) effluents from the reactors were colleted over a period of 1 week from day 74 to day 81. At day 81 the effluents were added 10 g/l tryptone and distributed in portions of 100 ml in 500 ml vials. Subsequently, ammonia in the form of NH_4Cl was added in concentrations of 0.5–3.0 g-N/l and the vials were flushed with N_2/CO_2 (80/20%) and closed with butyl rubber stoppers and aluminium crimps. Control vials were not added ammonia. All vials

were incubated at 53°C and samples of 3 ml were taken at the beginning of the experiment and after 17 h, 44 h and 120 h. The ammonia concentration of the samples was determined and the deamination capacity was calculated indirectly by subtracting the initial ammonia content of samples from the final ammonia content after incubation. All experiments were conducted in duplicates.

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 was determined by gas chromatography using thermal conductivity detection. For determination of pH, ammonia content and VFA (when not measured by online sensor) samples of approximately 15-20 ml of digested manure were withdrawn the reactors. pH was determined immediately. For VFA determination 2 x 1 ml of the reactor content was acidified with 30 µl 17% phosphoric acid, centrifuged at 10500 rpm for 20 min, and analyzed on a gas chromatograph equipped with flame ionisation detector. Samples for ammonia determination was frozen at -20°C and measured at the end of the experiment.

Results and Discussion

Batch experiments

SMA. Although the overall methane production rate in R1 and R2 was in the same range (figure 3) when the SMA experiments were carried out the results of the experiments revealed a higher SMA from hydrogen and acetate in R2 compared to R1, while the activity from formate was highest in R1 (table 2). The reason for these dissimilarities is unknown. However, in continuously fed digestors an inhibition of the process is only detected when the reduction of the growth rates of the active biomass approaches the dilution rate of the digestor, while in batch experiments the reduction in growth rate of a specific trophic level will be directly reflected by the outcome of the experiment. When comparing the SMA of R1 to R2 the decrease in the activity of the hydrogenotrophic methanogens (34%) was higher than the decrease in activity of the acetotrophic methanogens (18%). This result is in agreement with Wiegant and Zeeman (1986) who suggested that hydrogen-consuming methanogens is more vulnerable towards ammonia than acetate consuming methanogens under thermophilic conditions but contradicts the results of Angelidaki and Ahring (1993) who found the opposite.

Effect of ammonia on methanogenesis. When increasing the ammonia concentration in the effluents of R1 and R2 an inhibition of the methane production was observed in both reactors (figure 1, a,b). The inhibition was most pronounced for R1 showing that the higher ammonia concentration in R1 compared to R2 led to an increased sensitivity of the biogas process to sudden increases in ammonia concentration. (figure 1, a). However, when relating the magnitude of inhibition to the absolute ammonia concentration in the vials, the process in the effluent from R2 was most affected (figure 1, b). This illustrates an adaptation of the biogas process in R1 to the elevated ammonia concentration in the reactor.

Effect of ammonia on tryptone deamination. The effect of elevated ammonia concentrations on tryptone deamination in R1 and R2 is illustrated in figure 2. Apparently the deamination process was most effective in R1 despite the higher ammonia concentration of the reactor. During the first 17 hours the deamination rate of R1 was between 0.72 g-N/(l x d) for vials added 2.0 g-N/l and 1.20 g-N/(l x d) for the control vials, while the rate for R2 was between –0.31 g-N/(l x d) for vials added 3.0 g-N/l and 0.42 g-N/(l x d) for vial added 0.5 g-N/l. Gallert et al. (1997) observed deamination rates of 0.55 g/(l x d) for a mesophilic (37°C) reactor and 0.32 g/(l x d) for a thermophilic (55°C) reactor treating source-sorted biowaste. The ammonia content of the thermophilic reactor in that experiment was 0.43 g-N/l.

The total-N content of the tryptone used in the present experiment was 13%, which could result in a maximum ammonia release of 0.13 g-N/g tryptone. The total amount of ammonia released in the control vials after 7 days of incubation was 0.123 g-N/g tryptone for R1 and 0.122 g-N/g tryptone for R2. This shows an almost complete tryptone deamination for both reactors.

Reactor experiments

Day 0–80, gradual increase of ammonia concentration. The gradual increase of ammonia concentration in R1, from day 32 to 67, had no impact on the overall reactor performance when compared to R2 (figure 3). This is in agreement with Angelidaki and Ahring (1993) who reported that during a gradual increase in ammonia concentration in a thermophilic (55°C) CSTR treating cattle manure, a concentration of 3 g-N/l did not affect the process. pH was slightly higher in R2 than R1, despite the higher ammonia concentration in R1. This resulted in a relatively smaller difference in the concentration of free ammonia between the reactors. From day 70–80 the free ammonia concentration was between 0.38–0.40 g-N/l in R1 and 0.26–0.35 g-N/l in R2.

Day 80–130. Effect of tryptone pulses on the biogas process.

The addition of easily degradable tryptone had a stimulating effect on methanogenesis in both

reactors illustrated by the significant peaks in methane production but also lead to a hydraulic overloading illustrated by the increasing VFA concentrations immediately after tryptone was added. (figure 4). Ramsay and Pullammanappallil (2001) investigated the catabolic reaction of casein degradation in an anaerobic mesophilic CSTRs and found that VFA was formed in the following relative amounts: acetate 53–59%; propionate 3–8%; isobutyrate 6–7%; butyrate 14–16%; isovalerate 12–15% and valerate 5–8%. In the present experiment the ratio of the VFA increase following pulse no. 1 and 2 was for both reactors in the same range with the exception of propionate and valerate (acetate 53–59%; propionate 11–18%; isobutyrate 8–9%, butyrate 4–14%, isovalerate 12–13% and valerate 0.1–0.7%). The lower increase in valerate and higher increase in propionate could be explained by 1) a fast conversion of valerate to propionate combined with 2) an inhibition of the propionate oxidizing syntrophic bacteria from an elevated H_2 partial pressure resulting from the increased fermentation. Pind et al (2003) observed a complete removal of valerate within 36 hours with a simultaneous increase in propionate concentration when increasing the valerate concentration from 0.05 mM to 25 mM in a thermophilic CSTR treating cattle manure.

Approximately three days after tryptone was added a second peak in acetate concentration was observed, with the exception of the first pulse in R2. In addition to this R1 exhibited a decrease in methane production three days after pulse no. 2 and 3 and R2 exhibited a decrease in methane production three days after pulse no. 3. These patterns indicate an imbalance between the acetate-producing and acetate-consuming microorganisms, which might be due to a periodical ammonia inhibition of the aceticlastic methanogens. Following each pulse of tryptone the ammonia concentration in the reactors increased with the following peaks: R1) 3.64 g-N/l at day 84, 4.42 g-N/l at day 91 and 5.06 g-N/l at day 105; R2) 2.48 g-N/l at day 84, 3.16 g-N/l at day 93 and 3.90 g-N/l at day 107. These peaks in ammonia concentration and the subsequent periodical decrease in methane production is in agreement with Angelidaki et al. (1993) who found the thermophilic (55°C) digestion of cattle manure to be inhibited at an ammonia concentration of approximately 4 g-N/l. The free ammonia concentration in reactors increased as well exhibiting the following peaks: R1) 0.84 g-N/l at day 84, 1.12 g-N/l at day 91 and 0.89 g-N/l at day 105; R2) 0.67 g-N/l at day 84, 0.811 g-N/l at day 93 and 0.78 g-N/l at day 107. For both reactors the highest concentration of free ammonia was observed following the second pulse of tryptone although the highest concentration of total ammonia was observed following the third pulse. The reason for this observation is that no increase in pH was observed following the third pulse possibly due to the high VFA concentrations in the reactors. No clear pattern could be observed between the free

ammonia concentrations and the methane production in the reactors. The free ammonia concentration was highest following the second pulse of tryptone but the inhibition of the methanogenesis was highest following the third pulse. An explanation for this observation could be that other compounds released through tryptone degradation could have inhibited the biogas process. Casein contains besides a high concentration of nitrogen some sulphate, present in cysteine and methionine (Ramsay and Pullammanappallil 2001), which act as electron acceptor of sulphate reducing bacteria. Sulphate reduction is more energetically favourable than methanogenesis (Zinder 1993) and high concentrations of sulphate might have resulted in a competition between sulphate reducing bacteria and methanogens, which could have contributed to the observed decrease in methane production following the third pulse of tryptone. Further more is sulphate metabolised to sulphide (S^{2-}, HS^{-}, H_2S) that might inhibit the biogas process at concentrations around 23–50 mg S^{2-}/l (Hansen et al. 1999; Karhadkar et al. 1987).

Following all pulses of tryptone the increases in the VFA levels were more distinct in R1 than R2 (table 3) with the exception of propionate following pulse no. 2 and butyrate and valerate following pulse no. 3. At the same time was the methane yield between 15–30 % higher in R2 than in R1 following the first two pulses. An explanation of these differences could be that although the overall biogas process of R1 was adapted to an ammonia tolerance of 3.0 g-N/l were the methanogens under a "higher daily stress" and, therefore, had less capacity to withstand a sudden pulse of tryptone. This explanation was supported by the paradox observed in the batch experiments where 1) the inhibition of methane production by ammonia was more pronounced in the effluent from R1 than in R2 and 2) the production rate of ammonia from tryptone was higher in R1 than in R2.

Day 180–195. Effect of ammonia pulses on the biogas process. Before addition of ammonia (figure 5) a stable reactor performances was re-established for both reactors although the ammonia concentrations had increased from day 80 to 180. Thus, were the total ammonia/free ammonia concentrations at day 180 3.4/0.37 g-N/l and 1.9/0.26 g-N/l in R1 and R2, respectively. The methane yield in R2 was 4-6% higher than in R1, although the total ammonia concentration and free ammonia concentration in R1 didn't exceed the inhibitory levels of 4.0 and /0.7 g-N/l, respectively. (Angelidaki et al 1993 and Angelidaki et al 1994). Following each pulse of ammonia the free ammonia concentration in both reactors was below the inhibitory level of 0.7 g-N/l suggested by Angelidaki and Ahring (1994): R1) 0.462 g-N/l at day 183, 0.606 g-N/l at day 187 and 0.519 g-N/l at day 194; R2) 0.362 g-N/l at day 183, 0.500 g-N/l at day 187 and 0.450 g-N/l at day 194. Despite that, the reactors showed a decrease in methane yield from day 180 to day

195 corresponding to 38% and 30% for R1 and R2, respectively. The general response of the biogas process to the pulses of ammonia was, however, more moderate than to the pulses of tryptone. The decrease in the methane yields was observed immediately after pulse no. 1 but no remarkable increases in the VFA concentrations was observed before pulse no. 3–4. These lacking/moderate increases in VFA illustrates that the ammonia inhibition was an overall inhibition of the biogas process and not only an inhibition of the methanogens. At the same time revealed the precise online measurement in R1 that the degradation of propionate was the most sensitive step in the conversion of VFA to methane since propionate was the first VFA to increase following the ammonia pulses.

Conclusions

Addition of tryptone had an immediate stimulating effect on the biogas process in both reactors despite the differences in ammonia concentration. However, following each pulse of tryptone the increase in the VFA levels was most distinct in the reactor operated at an ammonia concentration of 3.0 g-N/l whereas the methane yield was 15–30 % higher in the reactor operated at an ammonia concentration of 1.7 g-N/l following pulse no. 1 and 2. Although not significant, these variations illustrates that a reactor operated at an ammonia concentration corresponding to the ammonia concentration in the average joint biogas plants in Denmark, has less capacity to withstand a sudden pulse of protein when compared to a reactor operated at a lower ammonia concentration. Batch experiments supported this observation since the inhibition by ammonia was most pronounced in the effluent of the reactor operated at 3.0 g-N/l, but also revealed an adaptation of the process to the elevated ammonia concentration.

Pulses of ammonia resulted in a decrease in methane production of both reactors although the free ammonia concentration was well below the inhibitory level previously suggested by Angelidaki and Ahring (1994). No immediate increases in VFA concentrations was observed following the ammonia pulses, which illustrates that ammonia inhibition during that experiment was an overall inhibition of the biogas process and not only an inhibition of the methanogens.

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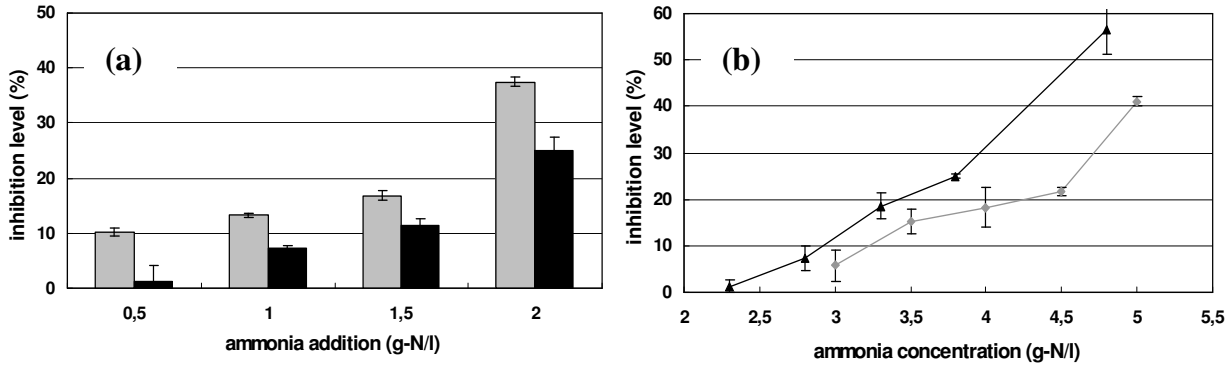

Figure 1 Batch experiments. Effect of ammonia on methanogenesis. Effluents from R1 (3.0 g ammonia-N/l) and R2 (1.8 g ammonia-N/l) were distributed in vials and added various amounts of extra ammonia. No ammonia was added control vials. The vials were incubated at 55°C for 72 hours and the methane production was measured frequently. **a)** Relative inhibition of the methane production in test vials compared to control vials in relation to increased ammonia concentration in the vials. Grey bars: R1; Black bars: R2. **b)** Relative inhibition of the methane production in test vials compared to the control vials of R2 (1.8 g-N/l) in relation to the absolute ammonia concentration in the vials ▲: R1, ◆: R2.

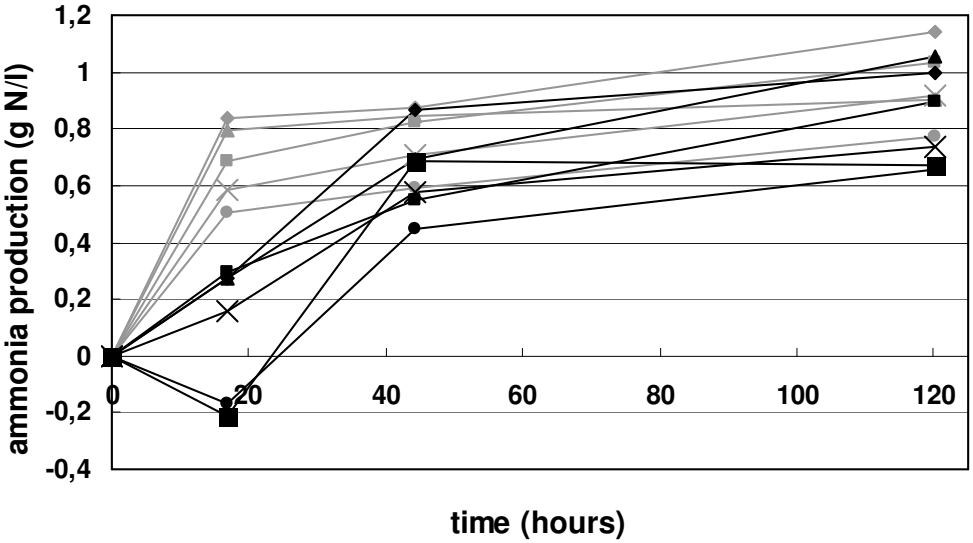

Figure 2 Batch experiments. Effect of ammonia on tryptone deamination. Effluents from R1 (3.0 g ammonia-N/l) and R2 (1.8 g ammonia-N/l) were distributed in vials, added 10 g/l tryptone and various amounts of extra ammonia. No ammonia was added control vials. The vials were incubated at 55°C and the ammonia production was calculated by subtracting the initial ammonia content of samples from the final ammonia content after incubation. ◆: R1 control; ■: R1 + 0.5 g ammonia-N/l; ▲: R1 + 1.0 g ammonia-N/l; ×: R1 + 1.5 g ammonia-N/l; ●: R1 + 2.0 g ammonia-N/l; ◆: R2 control; ■: R2 + 0.5 g ammonia-N/l; ▲: R2 + 1.0 g ammonia-N/l; ×: R2 + 1.5 g ammonia-N/l; ●: R2 + 2.0 g ammonia-N/l; ■: R2 + 3.0 g ammonia-N/l.

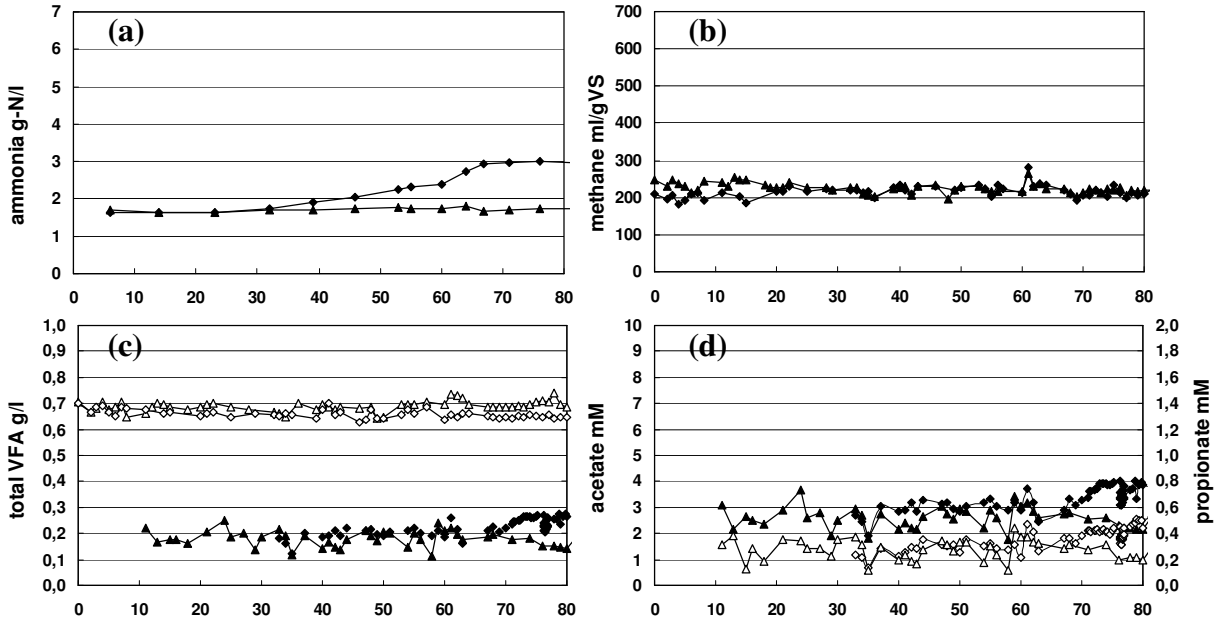

Figure 3 Reactor experiments. The effect of gradual increasing ammonia concentration in R1. **a)** ◆: ammonia concentration R1, ▲: ammonia concentration R2; **b)** ◆: methane yield in R1, ▲: methane yield in R2; **c)** ◆: total VFA concentration in R1, ▲: total VFA concentration in R2, ◇: pH of R1, △: pH of R2; **d)** ◆: acetate concentration in R1, ▲: acetate concentration in R2, ◇: propionate concentration in R1, △: propionate concentration in R2.

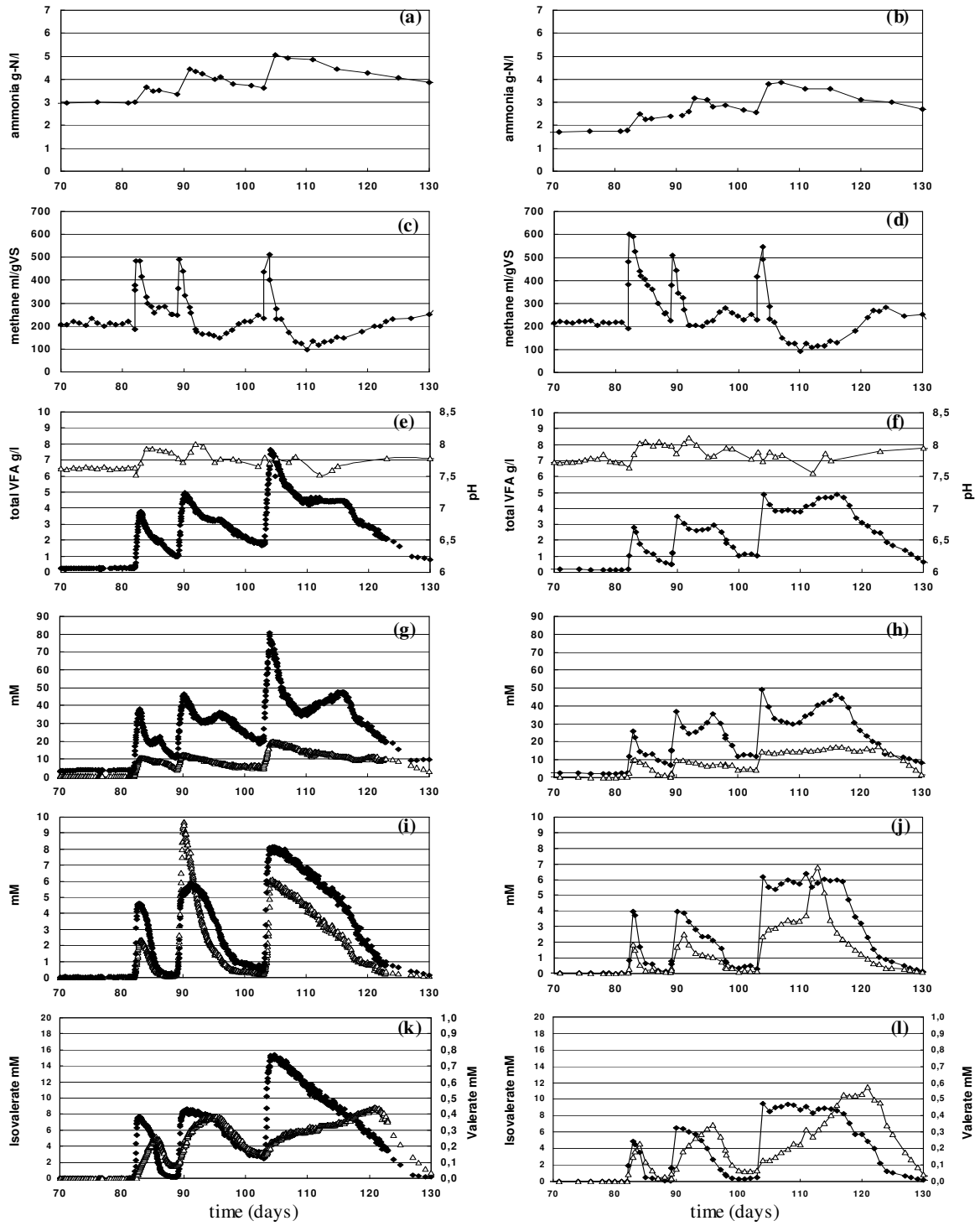

Figure 4 Reactor experiments. The effect of tryptone pulses on the anaerobic digestion process in thermophilic CSTRs treating cattle manure. R1 and R2 were operated at an ammonia level of 3.0 g-N/l and 1.8 g-N/l, respectively. Pulses of 10 g/l tryptone were added day 82 and 89 while 15 g/l tryptone were added at day 103. **a + b)** ◆: ammonia concentration; **c + d)** ◆: methane yield; **e + f)** ◆: total VFA, △: pH; **g + h)** ◆: acetate, △: propionate; **i + j)** ◆: isobutyrate, △: butyrate; **k + l)** ◆: isovalerate, △: valerate.

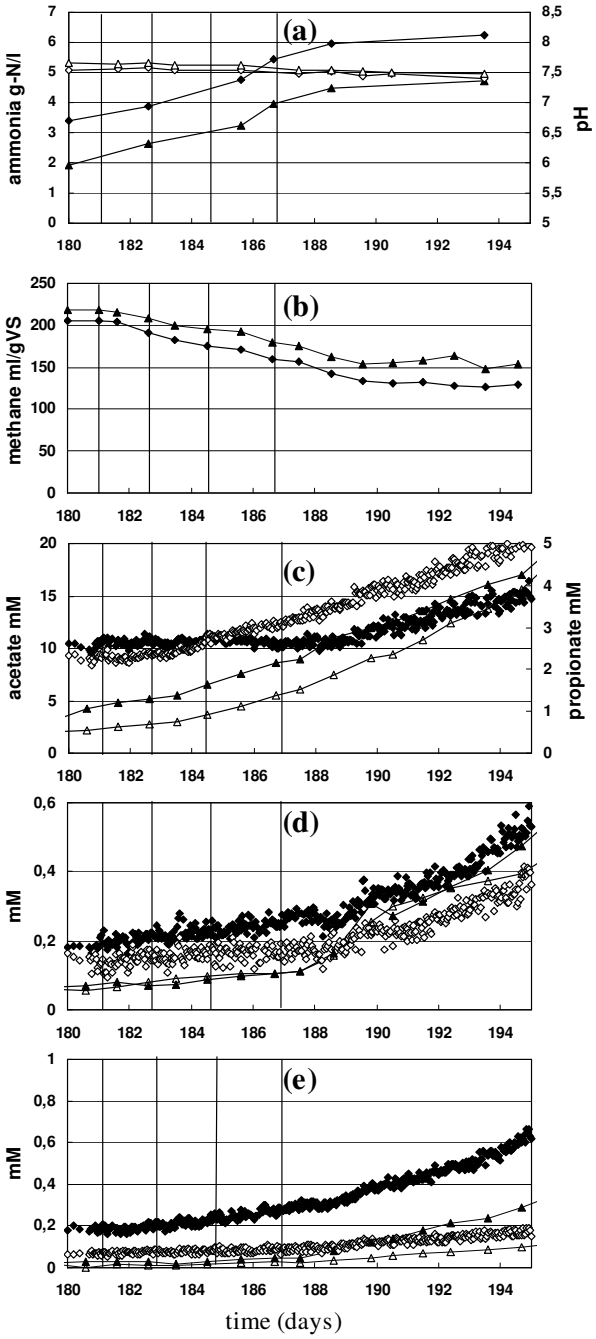

Figure 5 Effect of ammonia pulses on the anaerobic digestion process in thermophilic CSTRs treating cattle manure. R1 and R2 were operated at an ammonia level of 3.0 g-N/l and 1.8 g-N/l, respectively. Pulses of 0.79 g ammonia-N/l were added on days marked with vertical lines. **a)** ◆: ammonia concentration R1, ▲: ammonia concentration R2, ◇: pH R1, △: pH R2; **b)** ◆: methane yield R1, ▲: methane yield R2; **c)** ◆: acetate R1, ▲: acetate R2, ◇: propionate R1, △: propionate R2; **d)** ◆:isobutyrate R1, ▲: isobutuyrate R2, ◇: buyrate R1, △: butyrate R2; **e)** ◆:isovalerate R1, ▲: isovalerate R2, ◇: valerate R1, △: valerate R2.

Table 1 Total-N and ammonia-N content, temperature and hydraulic retention time of 7 randomly selected thermophilic biogas plants in Denmark.

Biogas plant	Total-N (g/l)	Ammonia-N (g/l)	Temperature[a] (°C)	HRT[a] (days)
Filskov	5.2	3.6	53	11 ± 1.5
Lintrup	4.7	3.5	53	19 ± 3.0
Studsgård	4.0	3.0	52	22 ± 3.5
Lemvig	4.5	3.1	52.5	17 ± 1.0
Thorsø	4.1	2.7	53	14 ± 2.0
Sinding	3.4	2.6	51	17 ± 2.5
Vaarst Fjellerad	4.3	3.1	53	14 ± 2.5
Average	4.3	3.1	52.5	16.3

[a]Data from the biogas plants and Planenergi Midtjylland, 2001.

Table 2 Specific Methanogenic Activity of biomass taken from R1 and R2 as µmol/(ml biomass x h). Results are given as means of triplicates with standard deviations.

Sample	R1	R2
Control	0.52 ± 0.060	0.52 ± 0.028
H_2/CO_2	0.68 ± 0.131	1.03 ± 0.026
Formate	3.09 ± 0.645	2.33 ± 0.268
Acetate	2.27 ± 0.021	2.78 ± 0.182

Table 3 Approximate increase in VFA concentrations in R1 and R2 caused by pulses of tryptone.

	VFA-tot. (g/l)		Acetate (mM)		Propionate (mM)		Isobutyrate (mM)		Butyrate (mM)		Isovalerate (mM)		Valerate (mM)	
Tryptone pulse	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
10 g/l no. 1	3.4	2.6	34.0	23.3	10.0	9.7	4.6	3.9	2.2	1.8	7.5	4.9	0.26	0.23
10 g/l no. 2	3.8	3.0	35.5	29.5	7.4	9.0	5.6	3.9	9.5	2.4	8.3	6.5	0.31	0.33
15 g/l no. 1	5.8	3.9	61.0	37.4	14.5	9.9	7.6	5.9	5.7	6.7	12.4	9.0	0.28	0.51

Paper III

Responses of the biogas process to pulses of lipids and meat- and bone meal

H.B. Nielsen* and B.K. Ahring

The Environmental Microbiology and Biotechnology Group, BioCentrum-DTU, Building 227, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45 45 25 61 83; fax: + 45 45 88 32 76; e-mail: bka@biocentrum.dtu.dk

Abstract

The anaerobic degradation of lipids and meat and bone meal (MBM) was studied. In batch experiments various concentrations (1 g/l–10 g/l) of the substrates were digested and the methane potential was determined. Subsequently, two thermophilic continuously stirred tank reactors (CSTR) fed with a mixture of cattle and pig manure was subject to consecutive pulses of lipids and MBM (5 gVS/l). Following two pulses of lipids only 20.1% and 18.8% of the methane potential was utilized possibly because of flotation and washout of the lipids from the reactor. The utilization rate of MBM following the first two pulses was higher corresponding to 43.9% and 31.9% of the methane potential, but at the same time all volatile fatty acid (VFA) concentrations increased significantly indicating the beginning of a process imbalance. Consequently, a third pulse of MBM lead to a 30% decrease in methane production lasting for more than 20 days. The results show that MBM could be an attractive substrate for increasing the methane production at Danish centralized biogas plants, but that accurate process control including VFA measurement is essential when adding MBM to a biogas reactor.

Keywords: Lipids, meat and bone meal, regulation of the biogas process, methane production, VFA.

Introduction

Today 22 centralized biogas plants are in operation in Denmark. Each plant is shared by several farms and the main purpose of the plants is to treat livestock manure and reuse the material as fertilizer (Ahring et al., 1992). The plants range in size from 550 m^3 to 8500 m^3 with a conversion capacity of 25 to 500 tons biomass per day. In 2001 the centralized biogas plants treated

approximately 1.2 million tons of manure (Angelidaki and Ellegaard, 2003). Unfortunately, the plants are difficult to run with an economically profitable result if the process is based on manure alone (Hjort-Gregersen, 1999). To increase the economical feasibility of the plants the manure is often co-digested with organic waste from food industries and municipalities. Slaughterhouse waste contains a high biogas potential, due to high concentrations of lipids and proteins and waste in the form of stomach and intestinal content has for many years been successfully applied to increase the methane production of the biogas plants. Recently, MBM has also received a lot of attention. MBM is the dried and rendered product from mammal tissues and can be fed as a protein source for cattle, pigs and chickens. However, the product has diminished in value due to the spread of bovine spongiform encephalopathy (BSE) in cattle. Besides good storage stability MBM contains a considerable amount of energy and could be an attractive substrate for biogas plants. The methane potential of MBM is approximately 570 ml/g Volatile Solids (VS) (Angelidaki and Ellegaard, 2003) but there is still some uncertainty about the potential of obtainable gas from anaerobic digestion of MBM in continuously operated reactors. To our knowledge there has been no investigations evaluating the usability of MBM in biogas plants.

Drawbacks of adding waste products containing large amounts of lipids and proteins are the formation of inhibitory degradation products such as long chain fatty acids (LCFA) and ammonia. These compounds may destabilize the anaerobic digestion process and accurate process control of the biogas plants against mal-function is essential to prevent sub-optimal performance and process failure. Although it is well known that VFA concentration is an important parameter for accurate process control (Ahring et al., 1995) measurement of the biogas production is normally the only continuously measured parameter used at large-scale biogas plants. However, changes in methane yield (ml/g volatile solids) following process imbalances such as changes in hydraulic retention time (HRT), organic loading and temperature might be relatively small while measurement of the methane production rate ((l/(l reactor x day)) not only reflects the state of the process but also reflects the actual loading of the biogas reactor (Ahring et al. 1995). Consequently, detection of a change in gas production due to minor process imbalances may be too late for preventing complete break down of the process.

In the present paper we present the results of experiments with two types of industrial waste products, i.e. lipids and MBM. The purpose of the study was to determine the methane potential of a lipid mixture and MBM following shock loads of the substrates to CSTRs and to relate these

results to the ultimate methane yield of the substrates determined in batch digestion. Furthermore, we evaluate the potential of adding MBM to biogas plants and discuss the usability of methane production and VFA as indicators of process instability in CSTRs following pulses of lipids and MBM.

Materials and methods

Substrates

Raw cattle manure and pig manure was obtained from the full-scale biogas plant in Hashøj, Denmark. The manure was mixed in a ratio of 1:1, blended 1–2 min and kept at 2°C until used. Green Farm Energy, Randers, Denmark, delivered the industrial organic waste. The fat, which was solid at room temperature, originated from a food processing industry producing margarine from palm oil. Palm oil has a high content of saturated fatty acids (≈ 50%). For practical reasons the fat was mixed with rapeseed oil in a 1:1 ratio resulting in a thick fluid. Daka Bio-industries, Ringsted, Denmark, produced the MBM. The dry and finely ground product was produced from discarded animal parts from slaughterhouses and fallen stocks.

Batch experiments

The methane potential of the lipid mixture and MBM and the effect of these complex organic wastes on the anaerobic digestion process were determined in batch experiments. Effluents from two thermophilic CSTRs (described below) were collected over a period of one week. The effluents were mixed, inoculated at 55°C for 16 hours and subsequently distributed in 116-ml vials in portions of 50 ml. The vials were supplied with the lipid mixture, MBM and a 1:1 mixture (w/w) of the two substrates in final concentrations of 1.0 gVS/l, 2.0 gVS/l, 5.0 gVS/l and 10.0 gVS/l. Control vials were only added effluent. To obtain anaerobic conditions all vials were flushed with N_2/CO_2 (80/20%) and closed with butyl rubber stoppers and aluminium crimps. The vials were incubated at 55°C for 62 days. Methane production was frequently measured by gas chromatography using flame-ionization detection. The methane production of the test vials was corrected for the methane produced in the control vials. All experiments were conducted in triplicates.

Reactor set-up

Two 4.5 litre CSTRs, R1 and R2, with a working volume of 3.0 litre (Angelidaki and Ahring, 1993) were inoculated with cattle manure that had been digested in a stable lab-scale reactor at 55°C. The reactors were fed every six-hour with the manure mixture. The organic loading rate of the reactors was 2.35 gVS/(l reactor vol. x d). Both reactors were stirred by a propeller for one minute every third minute at 100 rpm and operated at 55°C with a HRT of 15 days. Circulating the heated water from a water bath through the reactor jackets ensured stable operational temperature.

Analytical methods.

The biogas production was measured four times a day and the CH_4 content of the biogas was determined 1–2 times a week by gas chromatography using thermal conductivity detection. VFA concentrations and pH of the reactors was determined on a daily basis. 15–20 ml reactor content was withdrawn and pH was determined immediately. For VFA determination 2 x 1 ml of the reactor content was acidified with 30 µl 17% phosphoric acid, centrifuged at 10500 rpm for 20 min, and analyzed on a GC equipped with flame ionisation detector. Total Solids (TS), VS, Total-N (Kjeldahl) and ammonia concentrations was determined using standard methods (Greenberg et al., 1998).

Pulses of lipids and MBM

After the reactors had been in operation for a period of two HRTs a stable operation, i.e. stable biogas production and stable VFA concentrations, indicated that steady state had been obtained. At day 170 a lipid pulse of 5 gVS/l were added R1 while a MBM pulse of 5 gVS/l were added R2. Following these pulses the methane production was used as the only parameter for indication of process stabilization. The VFA concentrations were not used as a control parameter of the reactors, although the effect of the consecutive pulses of lipid and MBM on the VFA dynamics was continuously evaluated. Thus, when the biogas production had returned to a level similar to the level before the pulses the reactors were subject to additional pulses. Consequently, R1 was added lipid (5 gVS/l) at day 175 while R2 was added MBM (5 gVS/l) at day 177 and 181.

Results

Substrates

The composition of the manure mixture is shown in table 1. The TS/VS content of the lipid mixture was 99.6% and 99.6%, respectively, while the TS/VS content the MBM was 95.9% and

66.8%, respectively. Total-N and ammonia-N of the MBM was 95.4 g/l and 5.7 g/l, respectively. The protein and fat composition of the MBM was not determined but according to the producer the MBM contained 50–60% protein and 8–14% fat. Phosphorus and Calcium content of the MBM was 4.0–4.4% and 7.8–12.4%, respectively.

Batch experiments

The degradation profile of the lipid mixture, MBM and the lipid:MBM mixture is shown in figure 1. An inhibition of the methane production in the beginning of the experiment was observed for all vials containing lipids. For all vials containing pure samples of lipid the inhibition period lasted for approximately 6–8 days while the inhibition period for vials containing lipid:MBM mixture varied more and lasted for 2–13 days. No inhibition was observed for vials only added MBM. The final methane potential of the organic wastes was 1145 ± 22.9 ml/gVS for the lipid mixture, 534 ± 10.8 ml/gVS for MBM and 853 ± 57.8 ml/gVS for the lipid:MBM mixture.

Reactor experiments

From day 30 following start-up a stable process was observed for both reactors. From day 150–170 the methane yield of the reactors was 306.4 ± 10.8 ml/gVS for R1 and 294 ± 10.9 ml/gVS for R2. pH was stable and between 7.77–7.90 for both reactors. The ammonia concentration in the reactors was 2.8 ± 0.08 g/l. The reactor performances following pulses of lipid and MBM are illustrated in figure 2. The first pulse of lipids to R1 led to an immediate but short-term increase in methane production from 0.69 l/(l reactor volume x day) at day 170 to 1.09 l/(l reactor volume x day) at day 171. The second pulse of lipids at day 175 led to a more moderate increase in methane production from 0.77 l/(l reactor volume x day) at day 175 to 1.04 l/(l reactor volume x day) at day 175.5. At day 186 the methane production had stabilized at a level corresponding to the level before the pulses. Following the first pulse the acetate concentration of the reactor increased from 10.0 mM at day 170 to 26.7 mM at day 172 while the propionate concentration increased from 1.0 mM at day 170 to 7.8 mM at day 175. Only small increases (<1.0 mM) in the concentration of isobutyrate, butyrate, isovalerate and valerate was observed. No significant increases were observed in the VFA concentrations following the second pulse and no changes in pH and ammonia concentration in R1 were observed following any of the lipid pulses.

The first pulse of MBM showed the same but more significant trends as during the first pulse of lipids. The methane production increased from 0.72 l/(reactor volume x day) at day 170 to 1.16 l/(l reactor volume x day) at day 170.5 but five days after the pulse the methane production had stabilized at its original level. Following the second pulse of MBM the methane production increased from 0.73 l/(l reactor volume x day) at day 177 to 1.12 l/(l reactor volume x day) at day 177.5. This time the return of the methane production to the original level only lasted four days. After the third pulse of MBM the change in methane production was more moderate and increased from 0.75 l/(l reactor volume x day) at day 181 to 1.06 l/(l reactor volume x day) at day 181.5. Two days after the pulse the methane production rate was at its original level and, subsequently, the production decreased until day 190 where it constituted only half of its original level. The inhibition continued for several days and at the end of the experiment the methane production had not fully recovered and constituted only for ¾ of its original level. Following the MBM pulses significant changes in all VFA concentrations in R2 was observed. After each pulse the acetate concentration increased steeply with peaks of 34 mM at day 178, 39 mM at day 173 and 42 mM at day 183. Although the acetate concentration also tended to show a fast decrease following each peak, the acetate concentration never returned to its original level. The changes in the other measured VFA´s showed a different pattern than acetate. The increases in concentration were not as steep but more constant reaching a peak at day 196 of 31 mM for propionate, 7 mM for isobutyrate, 7 mM for butyrate, 10 mM for isovalerate and 1.9 mM for valerate. At the end of the experiment only butyrate had recovered to a level close to the level before MBM was added. Following the MBM pulses a steady increase in the ammonia concentration in R2 was observed from 2.8 g-N/l (0.61 g-N/l as free ammonia) at day 170 to a maximum of 4.11 and 4.15 g-N/l at day 183 and 197, corresponding to a free ammonia concentration of 0.85 and 0.61 g-N/l, respectively. pH in R2 remained constant following the first two pulses but showed a slight drop after the third pulse with a minimum of 7.65 at day 197. This was also the reason for the lowering of the free ammonia concentration from day 183 to 197.

Discussion

Batch experiments

A good proportion between the final methane yields of the lipid mixture, the MBM and the MBM:lipid mixture was observed. The methane yield of MBM:lipid mixture (853 ml/gVS) constituted approximately the average of the lipid + MBM methane yield (1145 ml/gVS + 534 ml/gVS = 840 ml/gVS). The estimated methane potential of the lipid mixture was however 13%

higher than the theoretical methane yield (1014 stp.[2]ml/gVS) of lipids (Angelidaki and Ellegaard, 2003). The methane potential of the MBM, where the VS fraction mainly consists of proteins, was slightly higher than the theoretical yield of proteins (496 stp.ml/gVS). MBM might contain up to 14% fat, which easily accounts for these differences. Angelidaki and Ellegaard, (2003) reported a methane yield of 570 ml/gVS from MBM. These high methane potentials make MBM an attractive substrate for increasing the methane production of Danish centralized biogas plants.

During anaerobic degradation lipids are initially hydrolysed to LCFA, which can inhibit anaerobic microbial activity at even low concentrations (Hanaki et al., 1981, Koster and Cramer, 1987, Angelidaki and Ahring, 1992). In the present experiments the microbial growth in all vials containing lipid was inhibited in the beginning of the experiment illustrated by the lag phase in the methane production. The lag phase in vials containing the lipid mixture showed only a slight variation, lasting 6–8 days, while the lag phase in vials added the MBM:lipid mixture gradually increased with the concentration of the mixture. At 1% concentration the duration of the lag phase in vials containing the mixture (0.5% lipid + 0.5% MBM) exceeded the lag phase of vials containing 1% lipid. These observations indicate a co-inhibition of the anaerobic digestion process induced by the MBM and the lipid mixture. Co-inhibition of the biogas process was also reflected by the final methane yields where the production in vials containing 1% of the MBM:lipid mixture at day 62 was 766 ± 47.0 ml/gVS compared to an average of 881 ± 7.8 ml/gVS in vials containing 0.1%, 0.2% and 0.5% of the MBM:lipid mixture. However, at the end of the experiment a production of methane was still observed in vials containing 1% MBM:lipid mixture and further incubation of the vials might have counterbalanced the dissimilarities in the final methane yield of the different series. Therefore, it cannot be concluded whether the MBM:lipid mixture exerted a irreversible inhibitory effect of the biogas process.

Reactor experiments

When relating the extra amount of methane produced by the reactors following the pulses to the methane potential of the lipid mixture (from literature) and the MBM (this experiment) the utilization rate of the two substrates can be estimated. Calculations show that only 20.1% and 18.8% of the biogas potential of the lipid mixture was utilized during the first 5 days after the two pulses, and that the total utilization rate of the second pulse, calculated from day 175–189, was 29.8%. The utilization rate of the two first MBM pulses was higher corresponding to 43.9% and

[2] Standard temperature and pressure (0°C and 1 atm.)

31.9% of the biogas potential. The lower utilization of the lipid mixture was probably due to the adsorptive character of the lipids. In UASB reactors fatty matter (LCFA) has been reported to adsorb on surfaces of active and inactive granular sludge (Hwu et al., 1998), which might leave to flotation and washout of the granules (Lettinga and Hulshoff Pol, 1992). In the present study the feedstock of the reactors contained a significant amount of biofibers (particular lignocellulosic material) and a whitish flotation layer in the effluent of R2 less than 24 hours after the pulses visualized a washout of lipid adsorbed onto the biofibers. This coating of the biofibers and subsequent flotation and washout of the lipid from the reactor also explains why no inhibition of the biogas process in R2 was observed (in contrast to the batch experiments) following the lipid pulses. In that context Hwu et al. (1998) found the critical LCFA-specific loading causing sludge bed washout to be far below the toxicity level of the LCFA.

The inhibition of the process in R2 following the third pulse of MBM resulted in an average decrease in methane production of 30% from day 181–225, when compared to the original level. The reason for the inhibition is unknown but might be due to an increase in ammonia concentration. Angelidaki and Ahring (1994) found a free ammonia concentration of approximately 0.7 g-N/l to be inhibitory during digestion of cattle manure and it seems likely that the increase in ammonia concentration, from to 0.61 g-N/l at day 170 to 0.85 g-N/l at day 183, induced the inhibition. Furthermore, increasing concentrations of phosphate and calcium might have resulted in a precipitation of important elements which could have deteriorated the inhibition.

In a previous experiment it was found that pulses of tryptone (partly digested protein) to a CSTR fed with cattle manure had an immediate effect on the biogas process, illustrated by a significant increase in methane production and VFA concentrations (Nielsen, 2005). Approximately three days after tryptone was added a second peak in acetate concentration was observed and the reactor showed a decrease in methane production three days after pulse no. 2 and 3. In the present study the methane production also increased significantly immediately after the pulses, but the inhibition following the third pulse of MBM was more severe than the inhibitions following the tryptone pulses. Acetate showed almost the same pattern by having a significant but short peak following each pulse as a result of an increased fermentation. The second peak of acetate 10–14 days after the third pulse of MBM and the decrease in methane production indicated an inhibition of the acetoclastic methanogens. The constant increase of the other measured VFAs during the present experiment was different from the reaction pattern following pulses of tryptone. In that

experiment isobutyrate, butyrate, isovalerate and valerate showed a steep increase with a subsequent decrease following each pulse of tryptone.

Evaluation of process stability

The results of the present study clearly simplify that the methane production rate and the methane yield is unsuitable as sole parameters for indication of process instability in anaerobic digestors. The increased methane production following the pulses of MBM was only a reflection of an increased loading and the return of the methane production to the original level following pulse no. 1 and 2 excluded any indication of process imbalance. As a consequence of the insufficient process control R2 was severely inhibited following the third pulse of MBM. This could have been avoided by including the VFA-dynamics as a control parameter.

When evaluating the usability of the individual VFAs as indicators of process stabilisation following the MBM pulses the significance value (z) of the change in the VFA concentrations can be calculated as (Ahring et al., 1995):

Equation (1)

where x is the VFA concentration at a specific day following the MBM pulses, y is the average VFA concentration during a period of one HRT before the first MBM pulse and SD is the standard deviation of the VFA concentrations one HRT before the first MBM pulse. The z values were determined at day 177 and 181 when the methane production had returned to its original level following pulse no. 1 and 2, and at day 196 when the methane production had stabilised following the third pulse and all VFA´s at the same time showed a maximum in concentration (table 2). All individual VFAs exhibited significant changes within a 5% confidence level ($z > 1.96$) At day 177 and 181 the most significant changes was provided by isovalerate while butyrate showed the most significant change at day 196. However the selection of parameters for indication of process stabilisation cannot only be based on the relative changes in concentration but also the fluctuation pattern of the parameter should be considered. In that context, acetate gave a poor indication due to the fast decrease following each peak. Also butyrate seemed as a problematic indicator due to the fast decrease at the end of the experiment, when the biogas process was still inhibited.

To summarize, the high methane potential of MBM obtained in the batch experiments makes MBM an attractive substrate for increasing the methane production at Danish centralized biogas plants. However, continuous pulses of MBM to anaerobic digestors may leave to process imbalance and accurate process control including VFA measurement is essential when adding MBM to a biogas reactor.

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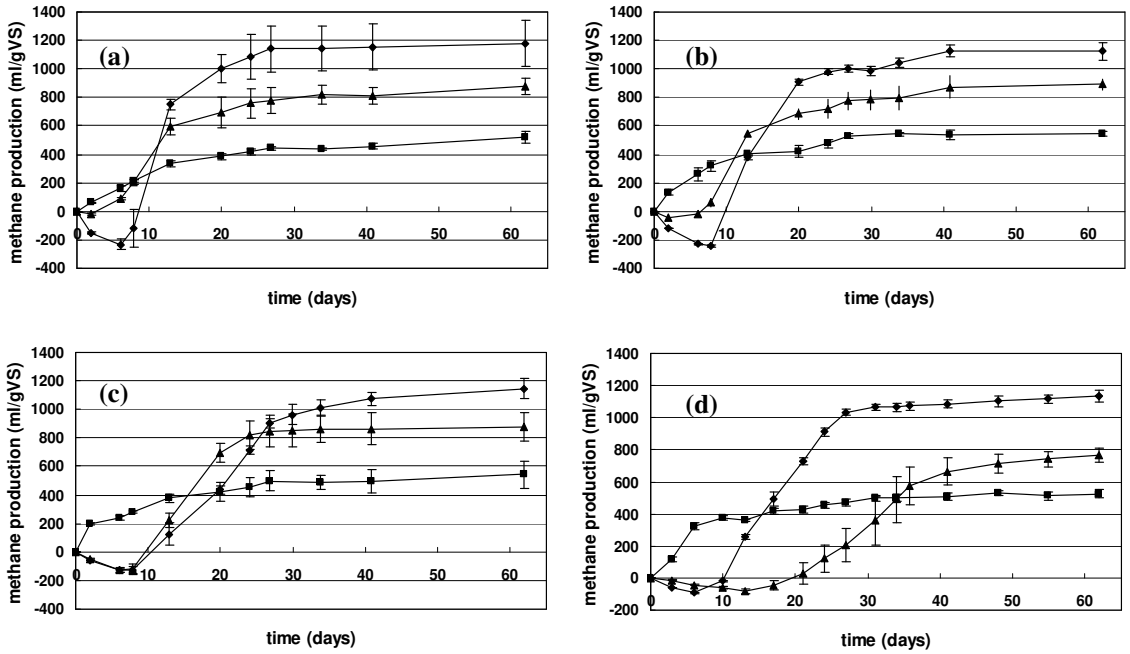
(a)
(b)
(c)
(d)
methane production (ml/gVS)
time (days)
1400
1200
1000
800
600
400
200
0
-200
-400
0
10
20
30
40
50
60

Figure 1

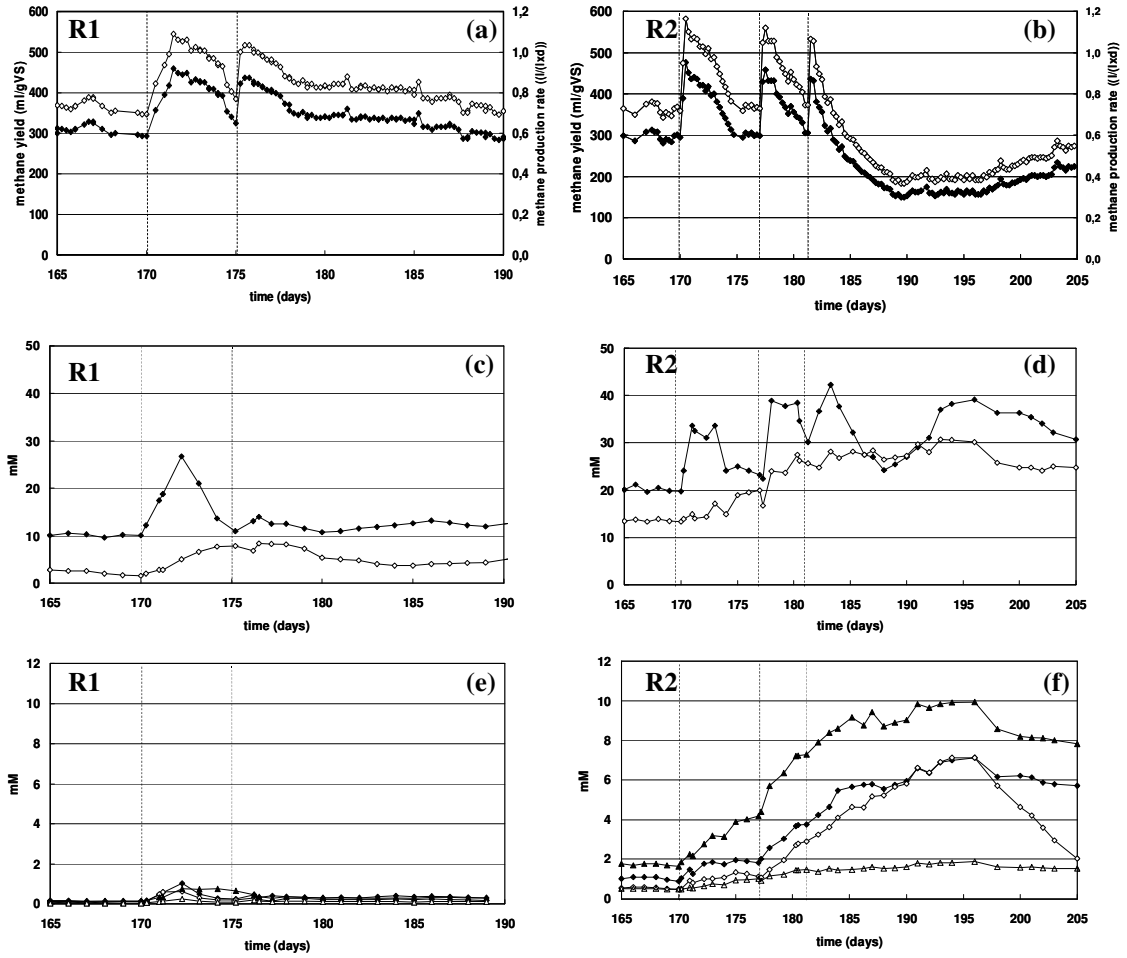
R1
(a)
methane yield (ml/gVS)
methane production rate ((l/(lxd))
time (days)
R2
(b)
methane yield (ml/gVS)
methane production rate ((l/(lxd))
time (days)
R1
(c)
mM
time (days)
R2
(d)
mM
time (days)
R1
(e)
mM
time (days)
R2
(f)
mM
time (days)

Figure 2

Captions to Figures

Figure 1. Batch experiments. Degradation profile of various concentrations (w/vol) of ◆: lipid mixture, ■: MBM and ▲: MBM:lipid mixture at 55°C. **a)** 0.1%; **b)** 0.2%; **c)** 0.5%; **d)** 1.0%.

Figure 2. Reactor experiments. Methane production and VFA concentrations in R1 and R2 during pulses of lipids and MBM, respectively. **a + b**) ◆: methane yield, ◇: methane production rate; **c + d**) ◆: acetate, ◇: propionate; **e + f**) ◆: isobutyrate, ◇: butyrate, ▲: isovalerate, △ valerate. The horizontal dotted lines indicate when lipid and MBM were added. Calulations of the methane yield was only based on the VS content of the manure and did not include the shock loads of lipid and MBM.

Equation 1.

$$z = \frac{(x - y)}{\mathrm{SD}}$$

Table 1 Components of the cattle and pig manure mixture used as feedstock.

Component	Content
Total-N (g/l)	4.45 ± 0.20
Ammonia-N (g/l)	2.73 ± 0.05
TS (g/l)	45.4 ± 1.34
VS (g/l)	35.2 ± 1.25
Total VFA (g/l)	8.2 ± 0.69
Acetate (mM)	90.2 ± 7.52
Propionate (mM)	26.6 ± 1.79
Isobutyrate (mM)	4.3 ± 0.38
Butyrate (mM)	10.2 ± 1.89
Isovalerate (mM)	4.0 ± 0.23
Valerate (mM)	1.6 ± 0.25

Table 2 Significance test values (z) at day 177, 181 and 196 in R2. The z values are significant within a 5% confidence level (z > 1.96).

	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	VFA-tot.
Day 177	5	25	9	14	44	26	15
Day 181	17	47	29	57	99	48	33
Day 196	33	64	65	160	147	68	63

Paper IV

Regulation and optimization of the biogas process: propionate as a key parameter

H.B. Nielsen, H. Hartmann and B.K. Ahring

The Environmental Microbiology and Biotechnology Group, BioCentrum-DTU, Building 227, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45 45 25 61 83; fax: + 45 45 88 32 76; e-mail: bka@biocentrum.dtu.dk

Abstract

An online volatile fatty acid (VFA) sensor was installed at a full-scale centralized biogas plant in Denmark. The monitoring gave a detailed profile of the VFA dynamics during steady state operation. During the installation of the VFA sensor, the plant was exposed to two incidents of process disturbances caused by accidental overloading with industrial waste. Both incidents were characterized by a significant increase in all individual VFA concentrations. Following the disturbances, the level and dynamics of the propionate concentration could best describe the normalizing of the process. Subsequently, in a lab-scale reactor experiment, we compared the prospective of using either propionate or methane production as single control parameters for regulation of the anaerobic digestion process. Two thermophilic continuously stirred tank reactors (CSTR) were fed with a mixture of cattle and pig manure together with various concentrations of meat and bone meal (MBM) and lipids. One reactor suffered from a severe process imbalance due to inadequate process control based on methane production. The performance of the other reactor showed that propionate could serve as an efficient indicator of process imbalance.

Key words Biogas process, regulation, process imbalance, propionate, VFA.

Introduction

The control of full-scale biogas plants against mal-function and overloading is crucial to prevent sub-optimal performance and process imbalance causing economical losses. Therefore, development of reliable tools for evaluation and control of the biogas process is necessary. The biogas process is a highly complex process where organic material is degraded under anaerobic conditions with a simultaneous production of methane. The degradation is carried out by microbial consortia, consisting of bacteria and archaea, linked in a complex food web (Gujer and Zehnder 1983). Initially, biopolymers are hydrolyzed and fermented to short-chain VFA (e.g. acetate, propionate, butyrate and isobutyrate), alcohols, H_2 and CO_2, by hydrolytic and fermentative bacteria. VFA such as propionate, butyrate and isobutyrate are subsequently oxidized by syntrophic bacteria producing acetate, H_2 and CO_2, and finally these products are converted to CH_4, CO_2 and H_2O by methanogens (Schink, 1988). The complexity of the process has made it difficult to find a simple and suitable control parameter reflecting the metabolic state of the entire process. At Danish full-scale biogas plants the biogas production is normally the only continuously measured parameter. However, this parameter does not directly indicate

process imbalance because the methane production rate (l/(l reactor vol. x d)) of an anaerobic digester not only reflects the state of the process but also reflects the actual loading of the digester (Ahring et al. 1995). In addition to that, changes in methane yield (ml/g volatile solids) following process imbalance might be relatively small (Ahring et al. 1995). During process imbalance, a build up of catabolic intermediates such as VFA and alcohols is normally observed. The rate of accumulation depends on a wide range of factors such as operation temperature, hydraulic retention time (HRT), loading rate, substrate and the type of process disturbance. It is well recognized that VFA concentration an important parameter for accurate process control (Ahring and Angelidaki 1997; Ahring et al. 1995; Hill and Holmberg 1988; Hill et al. 1987; Pind 2001) but controversy exist whether how and which VFAs that should be applied for indication of process imbalances. Hill et al. (1987) suggested that a propionate/acetate ratio higher than 1.4 indicated impending digester failure but other results have clearly contradicted this statement (Ahring et al. 1995; Pullammanappallil et al. 2001). Ahring et al. (1995) suggested that a combined parameter reflecting the concentrations of both butyrate and isobutyrate could be a reliable tool for indication of process instability. Various associations between the actual concentration of individual VFA concentrations and process imbalance have also been suggested. Hill et al. (1987) concluded that an acetate concentration higher than 13 mM would indicate process imbalance and Hill and Holmberg (1988) showed that concentrations of isobutyrate and isovalerate higher than 0.06 mM was an indication of process instability. However, several experiments in our laboratory shows that stable reactor performance can occur at VFA concentrations well above these limits (Angelidaki and Ahring 1994; Ahring et al. 2001; Nielsen et al. 2004).

Traditionally measurement of VFA concentrations in anaerobic bioreactors treating livestock waste has been done by manual sampling methods performed on a daily basis. However, our recently developed *in-situ* microfiltration system has made on-line VFA monitoring of biogas reactors treating livestock waste possible (Pind et al. 2002). With the application of this new technique we have previously studied the VFA dynamics in thermophilic reactors during periods of organic overloading (addition of protein) and inhibition by long chain fatty acids (LCFA) (Nielsen and Ahring 2005, paper II and I). In both studies all VFAs, except valerate, increased rapidly following each perturbation but propionate showed the slowest recovery back to the original concentration and, therefore, gave the best indication of when the biogas process had reestablished at a stable level. This is in good agreement with kinetic studies showing that

propionate degraders are the slowest growing and most sensitive VFA degrading microorganisms in the anaerobic digestion process (Öztürk 1991, Ahring et al. 2001). Furthermore, we studied the effect of sudden additions of MBM to a thermophilic CSTR and concluded that acetate and butyrate gave a poor indication of process recovery (Nielsen and Ahring 2005, paper III).

In the present study we present the results from on-line VFA monitoring at a large-scale Danish biogas plant during periods of stable and unstable reactor performances. On the basis of these and our previous results (Nielsen and Ahring 2005, paper I–III) we investigated the prospective of using either methane production or propionate concentration as single parameters for regulation of the anaerobic digestion process in thermophilic lab-scale CSTRs treating pig and cattle manure together with complex organic industrial waste.

Materials and methods

A) Characterization of process parameters at different full-scale biogas plants

Before selecting a full-scale biogas plant for installation of the online VFA sensor random samples were taken from 8 different centralized Danish biogas plants for characterization of the state of the biogas process at these plants. All samples were taken within the same week during a tour visit in February 2002. The samples were kept on ice for maximum 5 days and subsequently analyzed with regard to VFA levels, specific methanogenic activity (SMA) and biogas potential of the reactor effluent.

Measurement of SMA was based on the method by Sørensen and Ahring (1993). 30-ml serum bottles were added 9 ml anaerobic basal medium (Mladenovska and Ahring 2000), which had been flushed with 100% N_2 to achive the same pH as the various samples. The media were autoclaved at 141°C for 40 min and 0.5 g/l Na_2S and 10 ml/l vitamin solution were added (DSMZ medium no. 141, DSMZ 1989). Test vials were either supplemented with sterile-filtrated sodium acetate (0.2-µm minisart filter, Sartorius AG, Goettingen, Germany) in a final concentration of 50 mM or added H_2/CO_2 by pressurizing the vials with H_2/CO_2 (80%/20%) to 101kPa overpressure. Substrate was omitted from control series. Finally, all bottles were inoculated with 1 ml reactor content and incubated at the same temperature as the corresponding reactor. All reactor samples had been pre-incubated for approximately 16 hours before inoculation to ensure an active biomass. After inoculation the dilution rate of the biomass was 1:10.2 in vials containing acetate

and 1:9.2 in control vials and vials with H_2/CO_2. Methane production was measured every second hour for 14 hours. All experiments were conducted in triplicates.

The biogas potential of the reactor effluents were determined by distributing portions of 50 ml reactor effluents into 116-ml vials. The vials were flushed with N_2/CO_2 (80%/20%), closed with butyl rubber stoppers and aluminium crimps and incubated at the same temperature as the corresponding reactor. The methane production was measured every 4–5 day for a period of 60 days.

B) Online VFA measurement at a full-scale biogas plant

Based on the results from the random samples taken at the various biogas plants, which is discussed later, it was decided to install the on-line VFA sensor at Lintrup biogas plant in order to observe the VFA dynamics of the plant during continuous operation. The sensor was installed at the plant from December 2002 to October 2003. A detailed description of the sensor is given by Pind et al. (2002).

C) Regulation an optimization of the biogas process in lab-scale reactors

Substrates. The main feedstock of the reactors was raw cattle manure and pig manure, which had been mixed in a ratio of 1:1 and blended 1–2 min (table 1). The manure was obtained from the full-scale biogas plant in Hashøj, Denmark, and kept at 2°C until used. MBM and fat was obtained from Green Farm Energy, Randers, Denmark (table 1). The fat, which was solid at room temperature, originated from a food processing industry producing margarine from palm oil. Palm oil has a high content of saturated fatty acids (≈ 50%). For practical reasons the fat was mixed with rapeseed oil in a 1:1 ratio resulting in a thick fluid. Daka Bio-industries, Ringsted, Denmark, produced the MBM. The dry and finely ground product was produced from discarded animal parts from slaughterhouses and fallen stock.

Two reactor experiments were performed:

Experiment one. During this experiment, methane production was the only parameter used for indication of process stability. Still, samples for determination of the VFA concentrations in the reactors were taken on a daily basis. The samples were acidified with 30 µl 17% phosphoric acid per ml, frozen at -20°C and not measured until the end of the experiment. Two 4.5 litre CSTRs with a working volume of 3.0 litre (Angelidaki and Ahring 1993) were used (figure 1). One reactor functioned as a control reactor and the other, R1, as a test reactor. Both reactors were stirred by a propeller every third minute for one minute at 100 rpm and operated at 53°C with a

HRT of 15 days. The reactors were inoculated with cattle manure that had been digested in a stable lab-scale reactor at 53°C and 17 days HRT. During start-up, the reactors were fed with 100 ml manure mixture per day. Full loading was applied from day 17 corresponding to 200 ml/d and 2.3–2.5 gVS/(l reactor vol. x d). 1% (w/vol) MBM and 1% lipid mixture were added the feedstock of R1 when the methane production had stabilized (day 29). At day 49 the addition of MBM and lipid mixture was increased to 2% of each substrate and at day 69 both substrates were omitted from the feedstock.

Experiment two. Experiment one functioned as a template for experiment two. Thus, experiment two was a repetition of experiment one with the exception that propionate was used as the only parameter for indication of process stability. Samples for determination of VFA were taken and analysed on a daily basis. Before start-up, the content of R1 was discarded and the reactor was re-inoculated with the same material as used in experiment one (figure 1). The reactor was named R2. The operation of the control reactor was continued as previously. Start-up, initiation of full loading and addition of 1% MBM and 1% lipid mixture was performed at the same days as during experiment one. However, at day 50 MBM was omitted from the feedstock of R2 and at day 63 the lipid mixture was also omitted. At day 68 0.5% MBM and 0.5% lipid were added the feedstock. The motivations for the decisions concerning the loading of the reactor are discussed later.

Analytical methods

Total solids (TS), Volatile solids (VS), pH and ammonium 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 and VFA were determined by gas chromatography using thermal conductivity detection. For manual VFA determination samples of approximately 15–20 ml of digested manure were withdrawn the reactors. 2 x 1 ml of the reactor content was acidified with 30 µl 17% phosphoric acid, centrifuged at 10500 rpm for 20 min, and analyzed on a GC equipped with flame ionisation detector.

Results and discussion

A) Characterization of process parameters at different full-scale biogas plants

The VFA concentrations in the eight different full-scale biogas plants varied from 1.4–22.8 mM

for acetate, 0.7–12.6 mM for propionate, 0.1–3.5 mM for isobutyrate, and 0.2–0.6 mM for butyrate (table 2). The range of the measured SMA was 4.9–204.3 µmol/(gVS x h) for acetate, 9.3–28.6 µmol/(gVS x h) for H_2/CO_2 and 0.5–12.8 µmol/(gVS x h) for the control vials. In a similar experiment Ahring (1995) found the SMA in three other centralized thermophilic biogas plants in Denmark to be 42–625 µmol/(gVS x h) for acetate, 103–199 for H_2/CO_2 µmol/(gVS x h) and 54–169 µmol/(gVS x h) for the control series. The VFA concentrations were in the same range as in the present experiment. The samples in the experiment of Ahring (1995) had been handled in the same manner as in the present experiment and the data shows that the biomass activity of different biogas plants differs significantly. The methane potential of the reactor effluents was in the range from 1.7 to 11.9 m^3/m^3. The high biogas potential of the effluent of the reactors in Lintrup and Studsgaard was a sign of low efficiency of the process. However, no correlation between the VFA level, the biomass activity and the rest potential could be found. This shows that random samples only give a limited characterization of the state of the biogas process.

B) Online VFA measurement at a full-scale biogas plant

According to specifications from Lintrup biogas plant, the biogas potential of the reactor effluent represented approximately 38% of the total methane production of the plant at the time when the sample was taken (February 2002). Due to the high biogas potential of the effluent together with the low SMA and the relatively high VFA concentrations in the reactors it was decided to install the online VFA sensor at Lintrup biogas plant. In contrast to the random samples, which only gave a momentary value of the VFA level, the online measurement gave a detailed profile of the VFA dynamics in the biogas reactor.

VFA levels at normal operation. The dynamics of the VFA during normal process operation with a reactor feeding every 12 h for 6 h are given in figure 2. The figure shows that the acetate concentration changed significantly depending on the feeding periods. During each feeding period a significant increase could be seen for the acetate concentration from 2-4 mM to 12-17 mM. After the feeding of the reactor had stopped, the acetate concentration could still be high for up to 2 h, but the concentration generally dropped, during the 12 h when the reactor was not fed, to about the same level as before feeding. The top acetate concentration rose from 15.5 mM to 16.5 mM during the week (0 – 96 h) when “fresh” substrate was fed to the reactor while stored substrate was fed during the weekend and at the beginning of the week.

The fluctuations in the propionate concentration before and after the feeding were more moderate than for acetate. The average level, however, rose after each feeding from 0.6 mM to 2.2 mM and finally to 2.9 mM. This was obviously also correlated to feeding of "fresh" substrate throughout the week.

The concentration of butyrate and valerate were except for the isoform of valerate below detection limits for the non-disturbed process. The concentration of iso-valerate was generally much lower than acetate and propionate, but showed the same dynamics as acetate according to the feeding periods. During each feeding the iso-valerate concentration more than doubled from 0.1 mM to 0.23 mM.

From the VFA pattern mainly five key values could be identified for characterization of the process balance:

- The peak acetate concentration at the end of the feeding period (12 – 15mM).
- The slope of the acetate concentration after stop of feeding (-0.84 – -0.74 mM/h).
- The lowest acetate concentration before start of a new feeding period (2 – 4 mM).
- The average propionate concentration over the whole period (0.6 – 2.9 mM).
- The slope of the propionate concentration after stop of feeding (-0.08 – -0.05 mM/h)

VFA levels following process imbalances. The VFA concentrations following two incidents of process disturbances due to accidental overload of industrial waste are shown in figure 3. Both incidents were characterized by a significant increase in all VFA concentrations. The biogas production decreased as well but no data were delivered by the plant. The first process disturbance (figure 3 a, b) recovered after 4 days of not feeding the reactor and was classified as a minor imbalance. The second (figure 3 c, d) was a severe process inhibition. Here the reactor had not recovered after 8 days and had to be restarted by inoculation from another reactor.

The minor disturbance was reflected in the following VFA concentrations: acetate > 45 mM, propionate > 15 mM, Iso-valerate > 2.0 mM, iso-butyrate > 1.0 mM, butyrate > 0.75 mM, valerate > 0.5 mM. Due to technical problems no VFA measurement was performed from hour 36 to 72 but still a rather clear pattern of the VFA dynamics following the process imbalance was observed. While the levels of acetate, iso- valerate, iso-butyrate, butyrate and valerate decreased following the first 12 hours of not feeding the reactor, the propionate concentration stayed almost unchanged at 15 mM for more than 3 days. When regular feeding of the reactor was restarted

after 3 days, the acetate concentration followed the same "steady state" dynamics and the concentration of butyrate, iso-butyrate, valerate and iso-valerate fell below detection limits. However, the decrease of the propionate concentration was much slower and rather independent of the feeding periods and it took 2 more days until the propionate concentration was below 5 mM. Therefore, the level and dynamics of the propionate concentration could best describe the normalizing of the process.

The severe process inhibition was exemplified by the following VFA concentrations: acetate > 55 mM, propionate > 15 mM, iso-valerate > 3.5 mM, iso-butyrate > 3.0 mM, butyrate > 8.0 mM, valerate > 1.5 mM. While the acetate concentration dropped below 30 mM during 24 h of not feeding the reactor, the propionate concentration stayed at a level between 10 mM and 15 mM for 8 days. The severe process inhibition was particularly reflected by a high concentration of butyrate, iso-butyrate, iso-valerate and valerate. The concentration levels were 10 times, 3 times, 1.75 times and 3 times higher than after the minor process disturbance, respectively. The concentration stayed unchanged at these high levels for 8 days. It lowered during feeding of the reactor with effluent from a second reactor for inoculation at day 8, but the levels were still high for 6 further days. No VFA measurement was performed from hour 30 to 120.

C) Regulation an optimization of the biogas process in lab-scale reactors

The composition of the various substrates with regard to TS and VS, total-N and ammonia-N content and methane potential is given in table 2. The methane potential of the substrates was determined in another experiment than the present (Nielsen and Ahring 2005, paper III). The reactor performances, pH and VFA-dynamics of the lab-scale reactors are illustrated in figure 4 and 5.

Experiment one. In experiment one, the regulation of the reactor operation was based only on the methane production of the reactor. Following start-up, a steady increase in methane production was observed for both R1 and the control reactor. The increase continued until day 10 where it stabilized at a level of 0.33–0.35 l/(l x d) corresponding to 280–300 ml/gVS. When full loading was applied, the methane production rate increased further until day 22 where it reached a level of 0.62–0.67 l/(l x d) while the methane yield decreased and ended at a level of 255–272 ml/gVS. The acetate concentration increased in both reactors to a level of approximately 15 mM in R1 and 12 mM in the control reactor. The propionate concentration remained more or less the same for both reactors corresponding to 4–5 mM. During the rest of the experiment the methane production of the control reactor stayed between 243–284 ml/gVS while the acetate concentration

stayed between 15–20 mM. The propionate concentration showed a slow increase from day 36 with a peak of 11mM at day 50. pH was stable and between 7.77 and 7.91. When the methane production in R1 had been stable for 7 days, the feedstock was supplemented with 1% MBM and 1% lipid mixture (day 29). This resulted in an immediate steep increase in methane production with a maximum of 1.29 l/(l x d) and 363 ml/gVS at day 38. A minor decrease was subsequently observed and the methane production stabilized from day 43 at a level of 1.17–1.18 l/(l x d) and 322–327 ml/gVS. The acetate concentration increased until it reached a level of 21–23 mM while the propionate concentration showed a peak of 10 mM at day 36. However, during the period from day 43–49 where the methane production stabilized both acids showed a further increase in concentration. To obtain a further increase in the methane production in R1, the feedstock was supplemented with 2% MBM and 2% lipid mixture from day 49. As a consequence of this, the methane production rate increased to a maximum of 1.43 l/(l x d) at day 58. However, following this peak a rapid decrease was observed and at day 66 the methane production in R1 was lower than the methane production in the control reactor. MBM and lipid mixture was, therefore, excluded from the feedstock from day 66. The concentration of acetate and propionate, which had started to increase at day 43, was at that point 60 mM and 31 mM, respectively. Following day 66, the acetate concentration continued to increase and peaked at day 72 with 74 mM, while the propionate stabilized and began a slow decrease from day 72. The methane production rate continued to decrease until day 69 where it stabilized at 0.25 l/(l x d). From day 76, the methane production began to improve and at day 90, it reached the same level as the control reactor.

The reason for the process disturbance in R1 is unknown but is in all probability due to the addition of MBM or the lipid mixture. During the period where MBM was added the ammonia concentration in R1 increased from 2.7 g-N/l at day 29 to 3.81 g-N/l at day 66, corresponding to a free ammonia concentration of 0.53 and 0.57 g-N/l, respectively. Angelidaki and Ahring (1994) found the inhibition level of free ammonia to be approximately 0.7 g-N/l during digestion of cattle manure. According to these results, an ammonia inhibition was not the reason for the breakdown of the process in R1. The production of long chain fatty acids (LCFA) from the degradation of the lipid mixture could be another explanation. LCFA might inhibit anaerobic microbial activity at even low concentrations (Angelidaki and Ahring 1992).

Experiment two. In experiment two, the regulation of the reactor operation was only based on the fluctuations in propionate concentration in the reactor. The performance of the control reactor is not illustrated but during the entire experiment the process was stable with a methane production of 250–280 ml/gVS, with acetate and propionate concentrations of 13–20 mM and 4.1–6.2 mM,

respectively. During start-up and following the initiation of full loading the performance of R2 was very similar to the performances of R1 and the control reactor in experiment one. Only exceptions were a slightly higher methane production in R2 from day 1–8 and a higher acetate concentration in R2 during start-up. The reaction pattern of R2 following the addition of 1% MBM and 1% lipid mixture to the feedstock was the same as in R1; i.e. increasing methane production and increasing acetate and propionate concentrations. The expected increase in the VFA concentration reflected an imbalance between the acid-producing and acid-consuming microorganisms as a result of an increased hydrolysis/fermentation and possibly a short-term increase in H_2 partial pressure. A pattern that previously has been observed when the organic loading rate of a reactor has been suddenly increased (Ahring et al. 1995). Therefore, no changes in the operation procedure were made. The acetate concentration dropped from day 21 when the propionate was still increasing and showed a slight stabilization from day 38. However, the acetate concentration continued to vary from day to day. As in experiment one, the propionate concentration exhibited a decrease from day 37–38, possibly because of an increased syntrophic activity. The decrease continued until day 45 where the concentration stabilized. However, from day 47 the propionate concentration suddenly started to increase again and at day 50 the concentration had doubled (2.5 mM to 5.1 mM). This increase was interpreted as an indication of a potential process imbalance. At day 50 the ammonia concentration of the reactor was 3.3 g-N/l corresponding to a free ammonia concentration of 0.66 g-N/l. In order to avoid a further increase, MBM was omitted from the feedstock from day 50. As expected, the lower organic loading rate resulted in a lower methane production rate, but the methane yield and utilization rate stayed in the same range. The acetate concentration that had increased from 11 mM at day 47 to 18 mM at day 50 stabilized at a level between 16 mM and 21 mM from day 50–62. However, the propionate concentration continued to increase and at day 63 the concentration was 16.5 mM. In order to avoid a breakdown of the process, as seen in experiment one, the lipid mixture was also excluded from the feedstock. This terminated the increase in propionate concentration and at day 68 the concentration had dropped to about 12.5 mM. The acetate concentration dropped from 19 mM at day 63 to 11 mM at day 66. The methane production rate decreased further and the methane yield decreased as well due to the exclusion of the easy degradable lipid mixture. The utilization rate of the feedstock showed a significant peak because the reactor still contained some lipid, which couldn't be excluded from the calculations. From day 68 the feedstock was added 0.5% MBM and 0.5% lipid mixture. This resulted in an immediate increase in acetate concentration with a peak of 27 mM at day 72. However, no changes were observed in the

propionate concentration and from day 69–90 the concentration stayed between 9.3 mM and 12.1 mM. The methane production of the reactor increased and from day 72–90 the methane production rate and methane yield was in the same as during the period when the feedstock was added 1% lipid. However, the utilization rate of the substrate was considerably higher than during any other period where MBM or lipid mixture was added.

The biogas production has previously been successfully used for control purposes of anaerobic reactor systems. Steyer et al. (1999) used the biogas production and pH as control parameters in a high-rate fluidized bed reactor treating a more or less uniform wine distillery waste. Following a short-term increase in organic loading, it was concluded whether the microbial population was able to manage a permanent increase of the loading rate, by comparing the extra biogas output of the reactor to the expected output. Subsequently, the input flow rate of the reactor was adjusted. pH was used as an alarm to stop the strategy if the value fell below a certain level. The control strategy was suitable for safe operation of the studied system but can hardly be transferred to biogas plants treating manure in combination with complex organic waste because of the buffering capacity of the manure, the high HRT of the reactor and the unknown substrate composition. At Danish biogas plants the operation of the plants are normally based on a "trial and error" strategy and the experience of the plant operator. The biogas production is normally the only control parameter and no characterization of the substrate is made. In practice, this means that a random amount of organic matter is added the reactors and the biogas production is subsequently followed. The results of the present experiments clearly demonstrate that this strategy is unreliable and that methane production cannot be used as a single parameter for indication of process imbalance in reactors treating complex organic waste. This was exemplified from day 43–49 in experiment one. During that period the methane production of R1 showed a stabilization following a minor peak giving no indication of a process imbalance, while the increase in VFA concentration indicated the initiation of a process disturbance. As a consequence of the inadequate process control a further increase in the loading of the reactor was performed, resulting in a breakdown of the process. Any indication of process imbalance by the methane production was not observed until approximately day 61–62, which was too late to prevent the process failure. Experiment one also demonstrated that pH is unsuitable as a single parameter for indication of process imbalance in reactors treating manure. Before the breakdown pH was stable and between 7.82–7.92 but during the breakdown no changes was observed until day 65 where pH started to drop until it reached a level of 7.57 at day 76. Furthermore, it was demonstrated that

the propionate/acetate relationship cannot be used for indication of process imbalances since the propionate/acetate ratio during the breakdown was well below the limit of 1.4 suggested by Hill et al. (1987) (figure 5c).

In contrast to experiment one, a process imbalance was effectively avoided in experiment two because of the precise warning by propionate from day 47–63. Furthermore, when comparing the reactor performances of R1 and R2 the results show that propionate in general proved to be a good parameter for regulation of the biogas process. During the first 50 days of the experiment, only small differences were observed in the methane production rate between R1 and R2 (figure 2, table 3). The higher methane production rate in R1 from day 38–50 was only due to a higher VS content in the feedstock of R1 during that period. From day 50 to day 64, the methane production rate was significantly higher in R1 than in R2 because of the higher organic loading rate. Nevertheless, the process in R1 was very ineffective and the methane yield and utilization rate was significantly higher in R2. The methane production rate, the methane yield and the utilization rate were highest in R2 from day 64–90 because of the breakdown of the process in R1. The total methane production from day 50–90 was 85.7 and 110.2 liters in R1 and R2, respectively, corresponding to a difference of 29% (table 3). Therefore, we conclude that the overall biogas process was most efficient in R2 because of a more accurate regulation of the reactor operation.

In order to obtain a simple strategy for safe operation of Danish biogas plants, the potential of using either methane or propionate as single control parameters was examined in the present study. For an improvement of the strategy, other parameters, especially hydrogen, should be considered. The transfer of hydrogen plays an important role in the overall regulation of the anaerobic digestion process and especially in the oxidation of propionate. For that reason hydrogen could seem as an obvious control parameter. However, complicated dynamics of hydrogen in anaerobic ecosystems and variability for given reactors and substrates makes hydrogen inadequate as a single control parameter (Switzenbaum et al. 1990). The use of hydrogen as a control parameter should always be during simultaneous measurement of other process parameters. By combining measurement of hydrogen with measurement of the biogas production (CH_4 and CO_2), VFA and pH, the thermodynamically conditions for the conversion of VFA can be calculated. Such analysis would result in an improved understanding of the biogas process during process imbalance. With respect to the results of the present study, thermodynamically analysis would have shown whether the increase in propionate concentration,

in experiment two from day 47–63, was caused by a thermodynamically inhibition of the propionate oxidation (caused by an increase in hydrogen partial pressure) or a kinetic inhibition of the propionate oxidizing bacteria. To our knowledge no control strategy including thermodynamic considerations has been presented for manure based CSTRs.

Conclusions

Online VFA monitoring at Lintrup biogas plant enabled characterization of the specific process dynamics during normal operation. Following imbalances caused by accidental overload of the reactors with industrial waste, the concentration level and dynamics of propionate could best describe the renormalization of the process. In the lab-scale experiment, where two CSTRs were fed with a mixture of cattle and pig manure together with various concentrations of MBM and lipids, it was demonstrated that propionate is a key parameter for (1) indication of process imbalances in biogas plants treating complex organic waste and (2) for regulation and optimization of the biogas process. The results also showed that the methane production cannot be used as a single reliable parameter for indication of process imbalances.

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Figure legends

Figure 1 *Lab-scale reactor setup and operation.*

Figure 2 *VFA online monitoring at Lintrup biogas plant. VFA dynamics during normal operation.* ***a)*** *◆: acetate, ◇: propionate;* ***b)*** *◆: isobutyrate, ◇: butyrate, ▲: isovalerate, △: valerate.*

Figure 3 *VFA online monitoring at Lintrup biogas plant. VFA dynamics during minor (a + b) and severe (c + d) process imbalances.* ***a)*** *◆: acetate, ◇: propionate;* ***b)*** *◆: isobutyrate, ◇: butyrate, ▲: isovalerate, △: valerate;* ***c)*** *◆: acetate, ◇: propionate;* ***d)*** *◆: isobutyrate, ◇: butyrate, ▲: isovalerate, △: valerate.*

Figure 4 *Lab-scale reactor experiments. Reactor performances during start-up, full loading and addition of MBM and lipids. ◆: R1, ◆: R2 and ◆: the control reactor* ***a)*** *methane yield;* ***b)*** *methane production rate;* ***c)*** *utilization rate of the biogas potential of the substrate. For calculation of the utilization rate during periods where lipid was added the theoretical methane potential of lipids (1014 ml/gVS) was applied.*

Figure 5 *Lab-scale reactor experiments. pH and dynamics of acetate and propionate during start-up, full loading and addition of MBM and lipids.* ***a)*** *◆: pH in R1, ◆: pH in R2 and ◆: pH in the control reactor;* ***b)*** *◆: acetate concentration in R1, ▲: propionate concentration in R1, ◆: acetate concentration in R2, ▲: propionate concentration in R2, ◆: acetate concentration in the control reactor, ▲: propionate concentration in the control reactor;* ***c)*** *◆: propionate:acetate ratio in R1, ◆: propionate:acetate ratio in R2.*

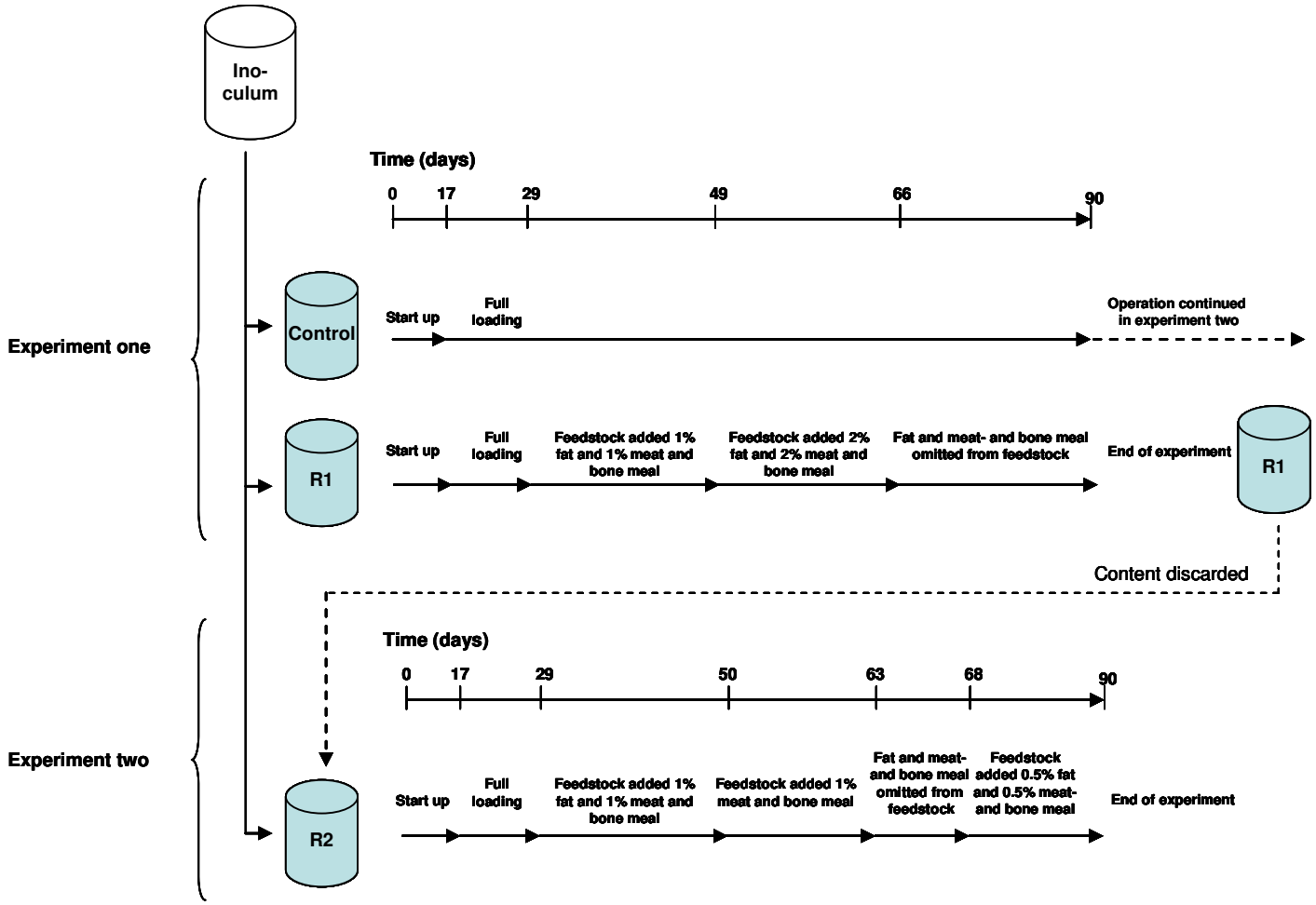
Ino-
culum
Time (days)
0
17
29
49
66
90
Experiment one
Control
Start up
Full
loading
Operation continued
in experiment two
R1
Start up
Full
loading
Feedstock added 1%
fat and 1% meat and
bone meal
Feedstock added 2%
fat and 2% meat and
bone meal
Fat and meat- and bone meal
omitted from feedstock
End of experiment
R1
Content discarded
Time (days)
0
17
29
50
63
68
90
Experiment two
R2
Start up
Full
loading
Feedstock added 1%
fat and 1% meat and
bone meal
Feedstock added 1%
meat and bone meal
Fat and meat-
and bone meal
omitted from
feedstock
Feedstock
added 0.5% fat
and 0.5% meat-
and bone meal
End of experiment

Figure 1

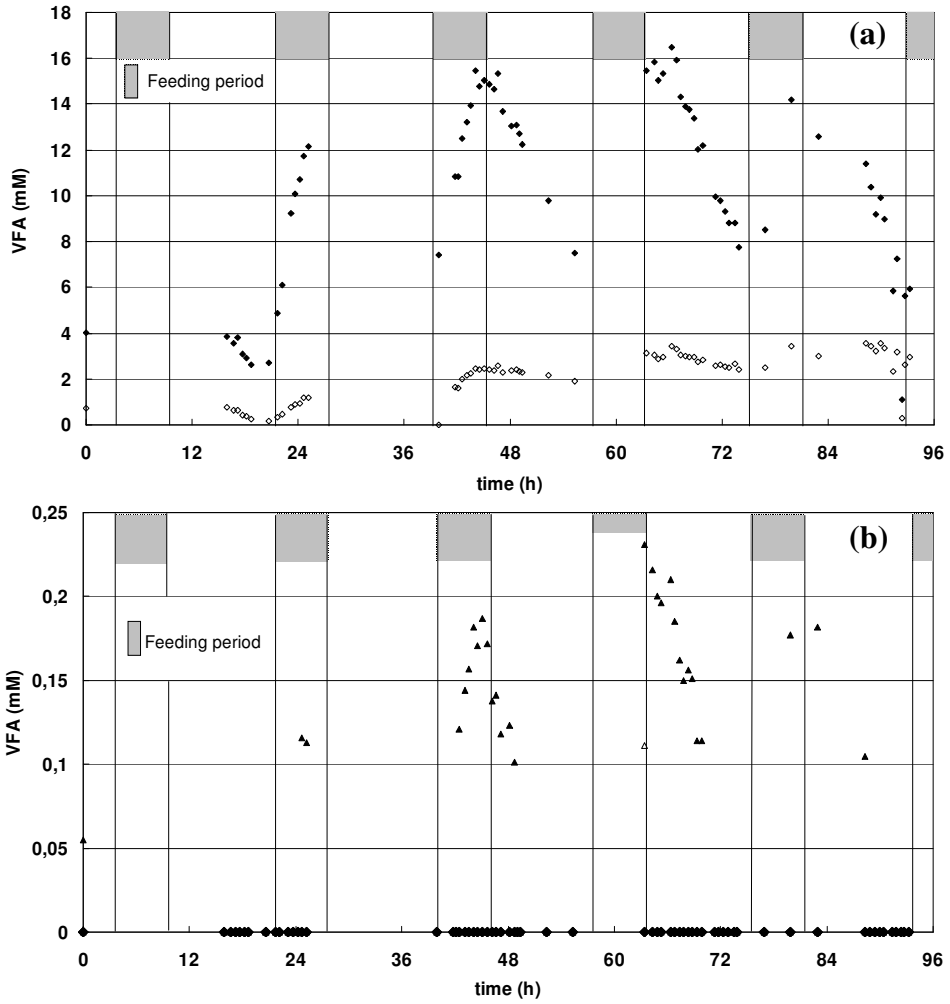

Figure 2

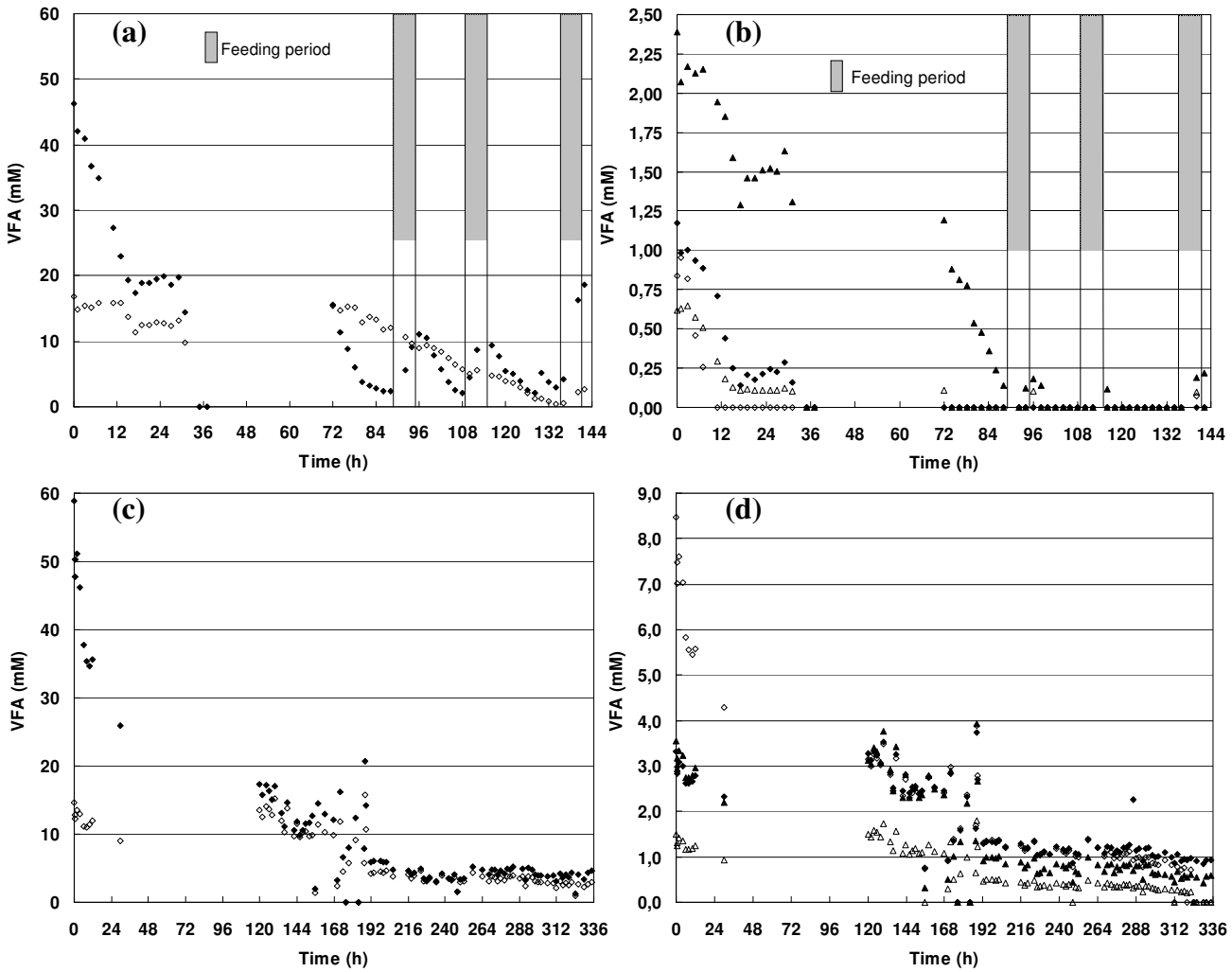
(a)
Feeding period
VFA (mM)
Time (h)
(b)
Feeding period
VFA (mM)
Time (h)
(c)
VFA (mM)
Time (h)
(d)
VFA (mM)
Time (h)

Figure 3

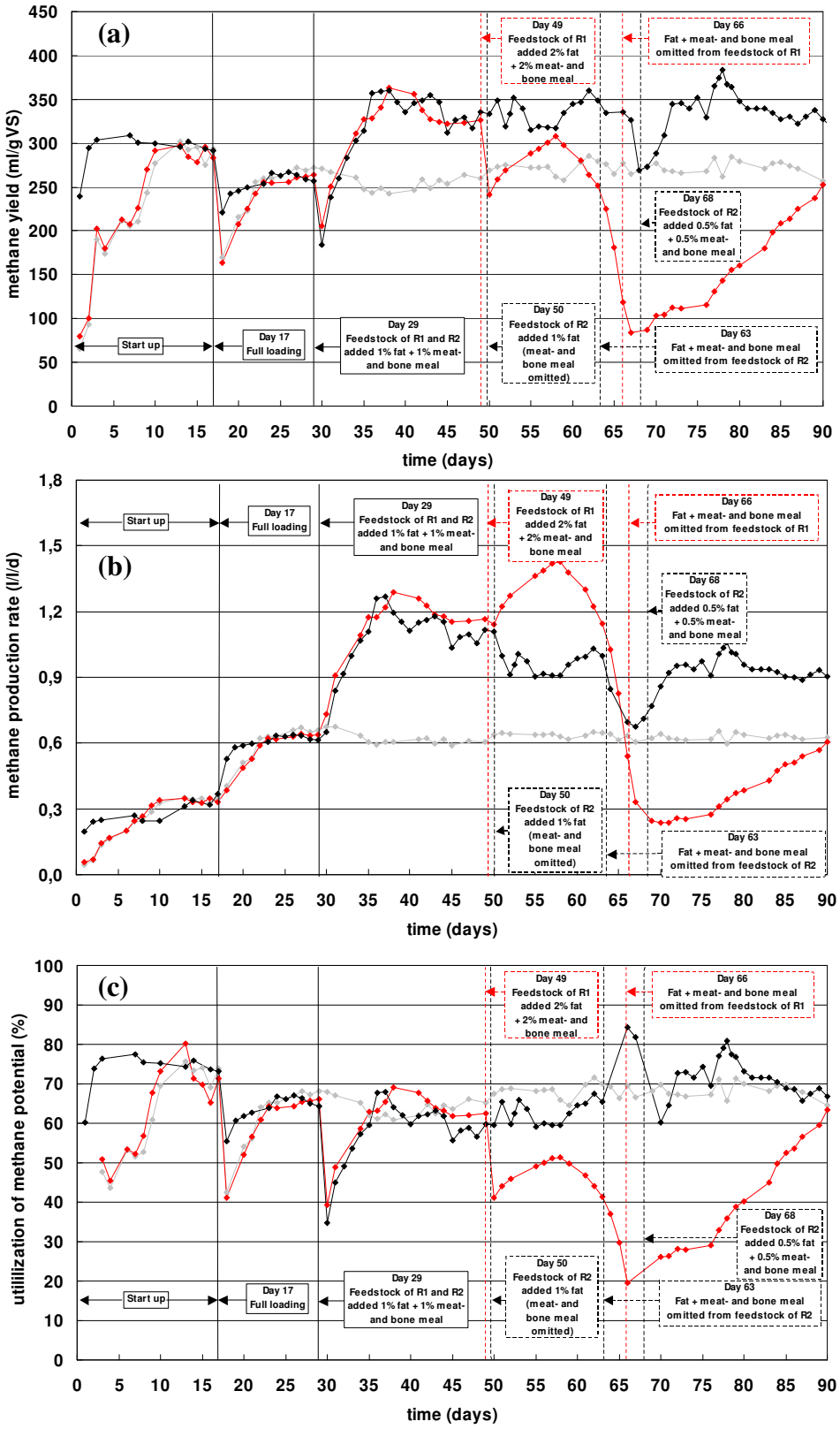
(a)
methane yield (ml/gVS)
time (days)
Start up
Day 17
Full loading
Day 29
Feedstock of R1 and R2 added 1% fat + 1% meat- and bone meal
Day 49
Feedstock of R1 added 2% fat + 2% meat- and bone meal
Day 50
Feedstock of R2 added 1% fat (meat- and bone meal omitted)
Day 63
Fat + meat- and bone meal omitted from feedstock of R2
Day 66
Fat + meat- and bone meal omitted from feedstock of R1
Day 68
Feedstock of R2 added 0.5% fat + 0.5% meat- and bone meal
(b)
methane production rate (l/l/d)
time (days)
(c)
utililization of methane potential (%)
time (days)

Figure 4

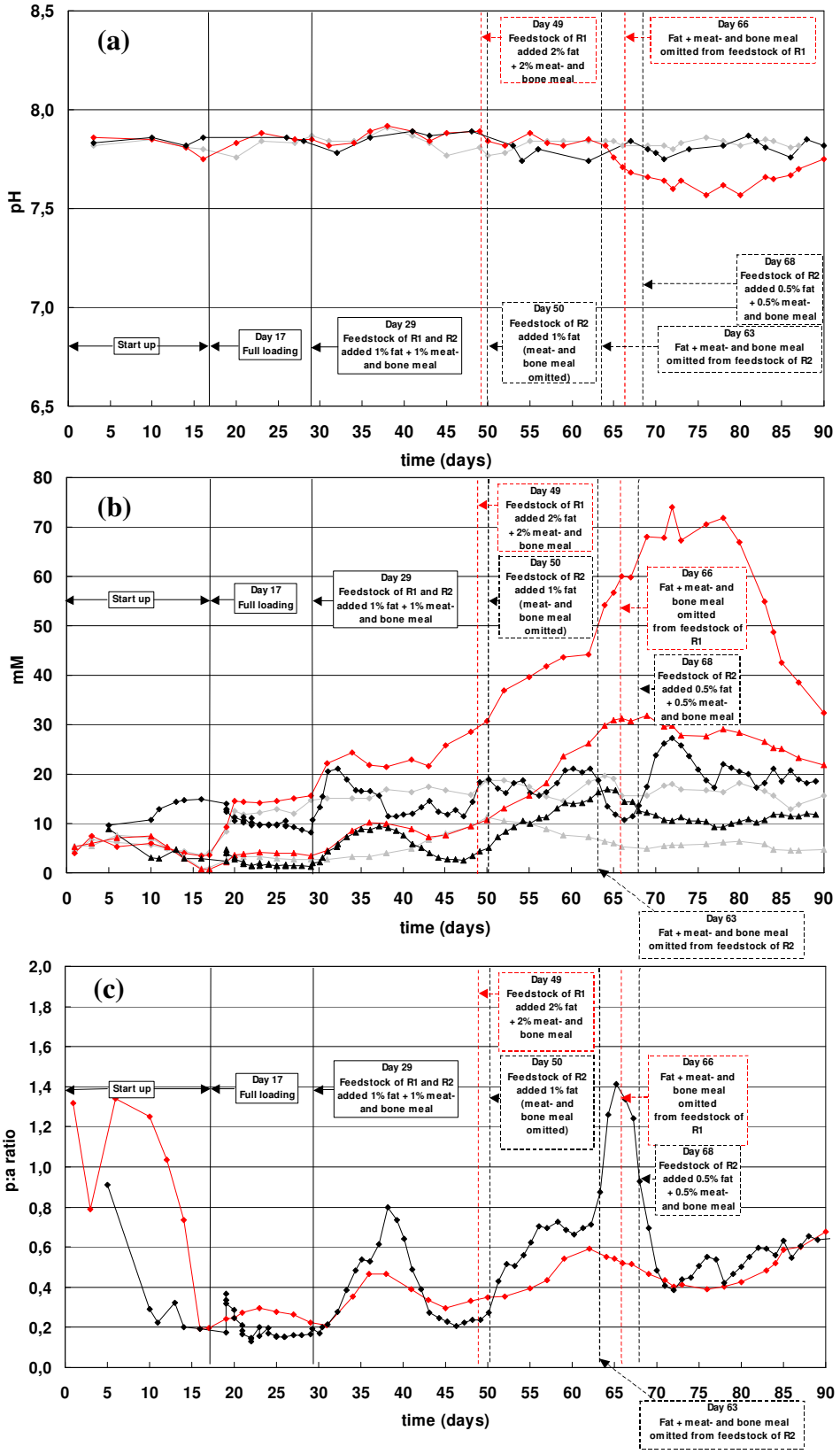
(a)
pH
8,5
8,0
7,5
7,0
6,5
Day 49
Feedstock of R1
added 2% fat
+ 2% meat- and
bone meal
Day 66
Fat + meat- and bone meal
omitted from feedstock of R1
Day 68
Feedstock of R2
added 0.5% fat
+ 0.5% meat-
and bone meal
Start up
Day 17
Full loading
Day 29
Feedstock of R1 and R2
added 1% fat + 1% meat-
and bone meal
Day 50
Feedstock of R2
added 1% fat
(meat- and
bone meal
omitted)
Day 63
Fat + meat- and bone meal
omitted from feedstock of R2
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
time (days)
(b)
mM
80
70
60
50
40
30
20
10
0
Day 49
Feedstock of R1
added 2% fat
+ 2% meat- and
bone meal
Day 50
Feedstock of R2
added 1% fat
(meat- and
bone meal
omitted)
Day 66
Fat + meat- and
bone meal
omitted
from feedstock of
R1
Day 68
Feedstock of R2
added 0.5% fat
+ 0.5% meat-
and bone meal
Start up
Day 17
Full loading
Day 29
Feedstock of R1 and R2
added 1% fat + 1% meat-
and bone meal
Day 63
Fat + meat- and bone meal
omitted from feedstock of R2
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
time (days)
(c)
p:a ratio
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Day 49
Feedstock of R1
added 2% fat
+ 2% meat- and
bone meal
Day 50
Feedstock of R2
added 1% fat
(meat- and
bone meal
omitted)
Day 66
Fat + meat- and
bone meal
omitted
from feedstock of
R1
Day 68
Feedstock of R2
added 0.5% fat
+ 0.5% meat-
and bone meal
Start up
Day 17
Full loading
Day 29
Feedstock of R1 and R2
added 1% fat + 1% meat-
and bone meal
Day 63
Fat + meat- and bone meal
omitted from feedstock of R2
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
time (days)

Figure 5

Table 1 Components of the manure mixture, lipid mixture and MBM used as feedstock in the lab-scale reactor experiments.

	TS (%)	VS (%)	Total-N (g/kg)	Ammonia-N (g/kg)	Methane potential (ml/gVS)
Manure mixture	4.54 ± 0.145	3.52 ± 0.125	4.5 ± 0.20	2.73 ± 0.05	398 ± 20.3
Lipid mixture	99.6 ± 0.034	99.6 ± 0.030	-	-	1145 ± 22.9*
Meat- and bone meal	95.9 ± 0.040	66.8 ± 0.271	95.4 ± 4.77	5.7 ± 0.22	534 ± 10.8

Table 2 VFA levels, SMA and rest potential of reactor samples from 8 full-scale biogas plants in Denmark.

	Acet (mM)	Prop (mM)	Isobut (mM)	But (mM)	Total (VFA g/l)	SMA Acetate (µmol/(gVSxh))	SMA H_2/CO_2 (µmol/(gVSxh))	SMA Control (µmol/(gVSxh))	CH_4 potential (m^3/m^3)
Lintrup	22.8	3.7	0.5	0.4	1.64	10.5 ± 1.65	10.2 ± 3.81	5.0 ± 1.44	11.9 ± 0.22
Studsgaard	12.3	1.1	0.3	0.3	0.84	7.8	17.2 ± 7.37	3.1 ± 2.45	9.6 ± 0.11
Lemvig	8.4	1.6	0.2	0.2	0.62	60.6 ± 6.25	9.3 ± 3.76	3.4 ± 1.30	5.7 ± 0.29
Thorsø	6.9	2.5	0.2	0.2	0.59	7.9 ± 1.07	28.6 ± 2.65	3.1 ± 0.63	3.8 ± 0.06
Sinding	5.6	1.1	0.1	0.2	0.42	204.3 ± 7.40	16.2 ± 5.66	8.45	6.1 ± 0.28
Århus	1.4	0.7	0.1	0.2	0.14	4.9 ± 1.01	17.2	2.4 ± 0.82	1.7 ± 0.05
Vaarst	9.7	12.6	0.5	0.6	1.40	11.6 ± 3.82	13.4	0.5 ± 0.30	5.4 ± 0.11
Filskov	9.1	9.7	3.5	0.2	1.35	102.1 ± 3.30	25.6 ± 1.16	12.8 ± 2.97	-

Table 3 Total methane production (l) in R1 and R2 during the different operation periods.

	Reactor	
Period	R1	R2
Day 0–17	13.2	14.4
Day 17–29	18.8	20.1
Day 29–49	68.8	64.8
Day 50–65	56.8	42.5
Day 65–90	28.9	67.7

Paper V

Kinetics of propionate degradation in anaerobic continuously stirred tank reactors

H.B. Nielsen, Z. Mladenovska, H. Hartmann and B.K. Ahring
The Environmental Microbiology and Biotechnology Group, BioCentrum-DTU, Building 227, Technical University of Denmark, DK-2800 Lyngby, Denmark; telephone: +45 45 25 61 83; fax: + 45 45 88 32 76; e-mail: bka@biocentrum.dtu.dk

Abstract

The kinetic parameters of anaerobic propionate degradation by biomass from 10 continuously stirred tank reactors (CSTR) operated at different temperatures, hydraulic retention times (HRT) and substrate conditions were investigated in substrate-depletion experiments. The maximum propionate degradation rate, A_{max}, was in the range from 18.5 to 30.8 µmol/(gVS x h) and the half saturation constant, K_m, was in the range from 0.46–6.60 mM. The accuracy of the substrate-depletion method was subsequently evaluated by use of radiotracer methodology. A_{max} was found to be 14–15% higher in the radioisotope experiment. This indicates an input of propionate via degradation of higher organic compounds, which in the substrate-depletion experiments resulted in an underestimation of A_{max}. K_m was found to be 4–6 times higher in the radiotracer experiment, which indicates a lowered affinity of the syntrophs towards labeled propionate at low concentrations.

Introduction

Anaerobic digestion is an environmental attractive way of treating organic waste from agriculture, households and industry. In Denmark alone, more than 20 full-scale centralized biogas plants have been built along with a number of farm-scale plants. The anaerobic digestion process is highly complex and carried out by different microbial consortia linked in a complex food web (Gujer and Zehnder 1983). The interdependence of the different consortia is the keystone of the process and an exact balance between the different trophic levels is essential for obtaining a stable digestion process. During periods of unbalanced operation intermediates in the form of volatile fatty acids (VFA) will accumulate, making VFA a good parameter for early indication of process disturbances (Ahring et al. 1995). A change in VFA concentrations in an anaerobic digestor is an indirect indication of changes in the activity of the different trophic groups involved in the overall digestion process. Simple tests for direct measurement of the

activity of the different microbial groups are, therefore, important tools for efficient process understanding (Switzenbaum et al. 1990; Sørensen et al. 1993). Some activity tests focuses on the methane production from specific substrates and is excellent for measuring the activity of methanogens (Sørensen et al. 1993, Colleran et al. 1992; Coates et al. 1996). However, for measurement of VFA degradation these tests are not useful since the conversion of most VFA to methane is through several steps. The measurement of methane production from VFA is , therefore, only an indirect indication of the substrate conversion activity and may lead to wrong estimations of the degradation rates. Nevertheless, literature often reveals studies where these methods have not been used correctly. The problem of using the methane production as indication of syntrophic activity is exemplified in figure 1. The figure illustrates the degradation of butyrate and propionate to acetate and methane in batch vials at 55°C. For butyrate the maximum methane production rate was observed in the time period from hour 50–72. However, during that period butyrate had already been depleted and the concentration was below the half saturation constant, K_m. The observed maximum methane production rate was, therefore, more a reflection of the maximum methane production rate from acetate and not the degradation rate of butyrate. A similar pattern was found for propionate. Therefore, the measurement of VFA degradation should be based on the depletion of the substrate itself and not the methane production.

We have previously demonstrated that process control of thermophilic anaerobic CSTRs treating livestock waste can be based on the measurement of propionate concentration alone, and it is our believe that propionate is a key factor for developing simple and reliable methods for evaluation and control of the anaerobic digestion process. (Nielsen 2005).

The aim of the present study was to estimate the kinetics parameters; $A_{\max}$ and K_m, of propionate degradation in biomass taken from 10 CSTR operated at different temperatures, HRT and substrate conditions. This was done by substrate-depletion tests in batch vials. The results were related to the overall performance of the reactors. Furthermore, we verified the accuracy of the substrate-depletion test by measuring the conversion of radioactive labeled propionate during batch incubation of the biomass.

Materials and methods

Reactor experiments

Reactor set-up one. The effect of industrial waste, containing high concentrations of lipid and protein, on thermophilic anaerobic digestion was studied. Four CSTRs (figure 2a) with an operation temperature of 53°C and a HRT of 15 days were inoculated with cattle manure that had been digested in a stable lab-scale reactor at 55°C. The reactors were named R1-HBN (control reactor), R2-HBN, R3-HBN and R4-HBN. All reactors were fed with a 1:1 mixture of cattle and pig manure at an organic loading rate of 2.3–2.5 gVS/(litre reactor volume x day). R1-HBN, R2-HBN and R3-HBN was fed four times a day while R4-HBN was fed only once a day. The feedstock of R2-HBN was supplemented with 1% (w/vol) lipids and 1% (w/vol) meat and bone meal (MBM) resulting in an organic loading rate of 3.4–3.6 gVS/(litre reactor volume x day). The feedstock of R3-HBN was added 0.5% lipids corresponding to 2.6–2.8 gVS/(litre reactor volume x day).

Reactor set-up two. In this experiment we investigated the effect of mesophilic and thermophilic temperatures on anaerobic digestion. Furthermore, we studied a two-stage digestion model (73°C/55°C) for treatment of manure and sludge. Four different CSTRs were used. R1-ZM (figure 2a) was operated at 55°C and 15 days HRT. The reactor was inoculated with a mixture of cattle manure and pig manure that had been digested in a stable lab-scale reactor at 53°C. The reactor was fed with cow manure, pig manure and sludge in a ratio of 4:4:2 three times a day at an organic loading rate of 2.6 gVS/(litre reactor volume x day). R2-ZM (figure 1a) was operated at 37°C and 18 days HRT with an organic loading rate of 2.2 gVS/(litre reactor volume x day). The reactor was inoculated with reactor content from the mesophilic biogas plant in Hashøj, Denmark. The substrate was the same as for R1-ZM. The two-stage set-up consisted of two reactors with different volume and temperature (figure 2b). The pre-treatment reactor, R3-ZM, was operated at 73°C and 2 days HRT. The reactor was fed with the same substrate and the same volume as R1-ZM and R2-ZM but due to the small reactor volume the organic loading rate was 19.5 gVS/(litre reactor volume x day). R3-ZM was connected to R4-ZM. This reactor was operated at 55°C and 13 days HRT. The reactor was fed by pumping the effluent of R3-ZM directly into the reactor. The organic loading rate of R4-ZM was, therefore, unknown. The total volume and HRT of the two-stage system was the same as for R1-ZM and R2-ZM.

Reactor set-up three. This study intended to improve the anaerobic digestion of cattle manure by thermal pre-treatment of the solid fraction (biofibers) of the manure. Two CSTRs (figure 2a), R1-HWH and R2-HWH, with an operation temperature of 55°C and HRT of 17 days were inoculated with a 1:1 mixture of cattle manure an pig manure that had been digested in a stable lab-scale

reactor at 55°C. The reactors were fed with cattle manure three times a day at an organic loading rate of 3.0 gVS/l. The solid fraction in the feedstock of R1-HWH (obtained by centrifugation of the raw manure mixture at 2000 rpm for 10 min) had been autoclaved at 140°C for 40 minutes before feeding.

Substrate-depletion experiments

50-ml serum bottles were added 15 ml of anaerobic basal medium (Mladenovska and Ahring 2000), which had been flushed with 100% N_2 and pH had been set to the same pH as that of the different reactor biomass. The vials were sterilized by autoclavation at 141°C for 40 min. Subsequently the vials were added 0.5 g/l Na_2S and 10 ml/l vitamin solution (DSMZ medium no. 141, DSMZ 1989) and inoculated with 5 ml reactor content. The vials were flushed with N_2, closed with butyl rubber stoppers and aluminium crimps, and pre-incubated in a shaking water bath at 100 rpm and the same temperature as the corresponding reactor. After 16 hours of pre-incubation the vials were added sterile sodium propionate to a final concentration of 20 mM and re-incubated in the water bath. After addition of sodium propionate the dilution ratio of the reactor content was 5:16.4. Progress curves of propionate degradation (figure 1) were made, by withdrawing 0.3 ml media/reactor content mixture every 8–12 hours for 4–7 days. The samples was diluted with 0.7 ml BA media in Eppendorpf tubes, acidified with 30 µl 17% $H_3PO_4^-$ and centrifuged at 10500 rpm for 20 min. The samples were analyzed on a gas chromatograph equipped with flame ionisation detector. When the propionate concentration was below 8 mM samples of 1 ml samples were analysed and the dilution step was omitted. K_m were determined by applying an integrated solution to the Michaelis-Menten equation (Ahring and Westermann 1987):

$$\frac{\ln S_0/S_t}{t} = \left(\frac{-1}{K_m} \times \frac{S_0 - S_t}{t} \right) + \frac{V_{\max}}{K_m}$$

Where S_0 is the initial substrate concentration, S_t is the substrate concentration at time t and K_m is the half-saturation constant. $V_{\max}$ is the maximum substrate utilization in the vials and was calculated from the steepest linear decline in substrate concentration (figure 3 phase 3). The maximum specific substrate utilization ($A_{\max}$) of the reactor biomass was calculated from the steepest linear decline in substrate concentration, which represented at the minimum 50% of the

initial substrate concentration (Rebac et al. 1999). All experiments were conducted in at least triplicates.

Radioisotope experiments

The degradation rates of propionate in reactor R1-ZM, R2-ZM and R3-ZM were also determined by radioisotope experiments. For each reactor eight vials were prepared with the same medium and substrate conditions as described earlier in the substrate-depletion experiments. Vial 1–3 served in each experiment as controls. During the entire experiment the propionate concentration in these vials was measured every 8–12 hour and the kinetic parameters were determined as during the substrate-depletion experiments. When the concentration was approximately 75% of the initial concentration (≈ 15 mM) the 1-[^{14}C]-sodium propionate ($CH_3CH_2{}^{14}COOH$) was added to vial 4–8 as 1 ml stock solution giving a final concentration of approximately 2.17 kBq/ml. The addition of propionate did not significantly affect the pool size. Vial 4 was subsequently autoclaved and used as killed control. Just before addition of radio labelled propionate, the propionate concentration in vial 5–8 was determined and immediately after addition of labelled propionate the radioactivity was counted. In this way the initial radioactivity could be related to the propionate concentration. Subsequently, the radioactivity was measured every 5–8 hours.

For determination of labeled propionate samples of 0.3 ml were diluted with 0.7 ml BA media in Eppendorpf tubes. In order to remove $^{14}CO_2$ from the liquid, the tubes supplemented with 44 µl 17% $H_3PO_4^-$ and allowed to equilibrate for one hour. Subsequently, the tubes were centrifuged at 10500 rpm for 20 min and 250 µl of the supernatants were transferred to scintillation vials containing 4 ml BCS scintillation liquid (Amersham). The radioactivity was counted in a LKB 1217 Rackbeta liquid scintillation counter.

Results

Reactor experiments

The reactor operation and performance parameters of the various set-ups during steady state conditions are summarized in table 1.

Substrate-depletion experiments

The calculated kinetic parameters of the propionate degradation are presented in table 1. A_{max} was in the range 18.5–30.8 µmol/(gVS x h) corresponding to 0.53 µmol/(ml ino. x h) for R2-HWH to 1.24 µmol/(ml ino. x h) for R4-HBN. No degradation was observed for R3-ZM. The half-

saturation constant, K_m, was for all reactors below 1 mM with the exception of R1-HWH. K_m of R2-HWH could not be calculated due to a limited number of experimental points in the K_m area.

Radioisotope experiments

The kinetic parameters of the propionate degradation in the radioisotope experiment and the second substrate-depletion experiments are presented in table 2. In all radioisotope experiments A_{max} tended to be higher than the corresponding A_{max} in the substrate-depletion experiments. A_{max} in the substrate-depletion experiments was in the same range as during the first substrate-depletion experiments (table 1). K_m in the radioisotope experiment was in the range from 3.96 mM to 4.21 mM, which was higher than the K_m values in the substrate-depletion experiments.

Discussion

Significant variations in the kinetic parameters of propionate degradation have been reported, depending on biomass composition, reactor type, substrate, temperature and HRT (table 3). In the present study we examined the propionate degradation in 10 different CSTRs. In the substrate-depletion tests K_m was estimated to be in the range between 0.46–0.95 mM, with the exception of HWH-1, and seemed only little affected by the differences in temperature and substrate composition. A_{max} showed slightly higher variation, but not as pronounced as in the literature and gave in general a good reflection of the reactor performances. The affinity of the biomass to propionate showed a pronounced variation and did not reflect the performance of the reactors (table 1).

For all reactors the maximum degradation rate was lower than the degradation rate observed by Gavala et al. (2003) in anaerobic sludge at mesophilic and thermophilic temperatures (table 3). The estimation of A_{max} was based on the VS-concentration of the reactor content and A_{max} might have been underestimated in the present study, due to high concentrations of particulate organic material (dead VS) in the reactors treating manure. However, from data listed in the publication by Gavala et al. (2003) calculations show that the maximum degradation rate of propionate in that experiment was approximately 0.66 µmol/(ml ino. x h) for the mesophilic sludge and 1.18 µmol/(ml ino. x h) for the thermophilic sludge. These values are in the same range as found in the present study

The performances of the different reactor setups will be discussed in the following sections.

Reactor set-up one. The only differences in the operation parameters between R1-HBN and R4-HBN were the feeding cycle. Nevertheless, R4-HBN showed a better reactor performance, illustrated by the higher methane yield and the lower levels in acetate and propionate concentrations. The increased reactor performance was also reflected in the substrate-depletion experiments where A_{max} of the propionate degradation was higher in R4-HBN than in R1-HBN. The reason for these differences is unknown. The addition of easy degradable organic waste to the feedstock of R2-HBN and R3-HBN increased the methane yield of these reactors, when compared to R1-HBN. The substrate-depletion experiments did also reflect the improved performances of these reactors. Thus was A_{max} slightly higher in R2-HBN and R3-HBN when compared to R1-HBN.

Reactor set-up two. The difference in the degradation rate of propionate and affinity observed between R1-ZM (55°C) and R2-ZM (37°C) is in agreement with Gavala et al. (2003) who found a higher ability to utilize propionate in thermophilic anaerobic sludge than in mesophilic sludge (table 3). The lacking degradation of propionate at 73°C (R4-ZM) was in good agreement with other investigations. (Ahring 1994; Van Lier et al. 1996, Ahring et al. 2001; Nielsen et al. 2004). Ahring (1994) demonstrated that the conversion of propionate to methane in cattle manure digested at 55°C was strongly reduced above 55°C, which was the optimum temperature for the process. Van Lier et al. (1996) observed the same tendencies for thermophilic granular sludge although the maximum degradation rate of propionate was at 60°C (table 3). Ahring et al. (2001) and Nielsen et al. (2004) found the conversion of propionate to methane to be completely inhibited in digestors operated at 65°C and 15 days HRT or 68°C and 3 days HRT, respectively.
When comparing the kinetic parameters of propionate degradation in R1-ZM to R4-ZM a higher A_{max} was observed in R4-ZM. The reason for this result is unknown but might be a result of an increased hydrolysis caused by the hyperthermophilc pretreatment step or the lower HRT in R4-ZM. A similar result was reported by Nielsen et al. (2004) who found the specific methanogenic activity from a broad range of substrates to be higher in a CSTR operated at 55°C and 12 days HRT and fed with manure digested at 68°C and 3 days HRT, compared to a CSTR operated at 55°C and 15 days HRT. Despite the higher A_{max}, the propionate affinity in R4-ZM was lower than in R1-ZM due to the lower K_m. This indicates the existence of different propionate oxidizing bacteria in the two reactors.

Reactor set-up three. The less efficient digestion process in R2-HWH compared to R1-HWH, were directly reflected by A_{max}, which was significantly lower in R2-HWH. The results of the experiment also demonstrated a clear improvement of the methane yield from a mixture of cattle and pig manure as a consequence of the thermal pre-treatment of the solid fraction. The hydrolysis of this particulate matter is commonly recognized as being the rate-limiting step of the biogas process and based on the presented results thermal pretreatment of this fraction seems to be a promising method for improving the methane yield of manure.
The affinity in R1-HWH was significant lower than the affinity in the other reactor set-ups. This could be the reason for the high propionate concentration in the reactor.

When summarizing the results of the substrate-depletion experiments it can be concluded that the maximum degradation of propionate, A_{max}, in general gave a good reflection of the overall state of the biogas process in the reactors. Although the kinetic parameters of other VFAs were not estimated, the result is in good agreement with other results (Nielsen 2005) where we concluded that propionate is a good parameter for indication of the state of the biogas process. However, it should be noted that the missing syntrophic activity at temperatures above 60°C makes propionate useless as parameter for indication of process stability at these temperatures.

Radioisotope experiments vs. substrate-depletion experiments

The method for determination of A_{max} by radioactive labeled propionate was in the present investigation based on fact that during degradation of propionate the carboxyl group (C-1) is directly liberated as CO_2 (Koch et al. 1983; Krylova et al. 1997). Some investigations have observed a conversion of propionate into higher fatty acids. For mesophilic anaerobic sludge Tholozan et al. (1988) estimated that about 20% of propionate was converted into butyrate while Lens et al. (1996) found that propionate could be converted into valerate and 2-methylbutyrate. Bok et al. (2001) observed for a mesophilic syntrophic co-culture that propionate was dismutated to acetate and butyrate via a six-carbon intermediate. Based on these observations is it possible that A_{max} in the radioisotope experiment could be underestimated since the conversion of ^{14}C-1 in propionate into for example ^{14}C-2 in butyrate (Tholozan et al. 1988) would not be registered by the method applied. However, during the present experiment no net production of butyrate was observed at any time in any vials and the concentration of butyrate decreased during the entire experiment showing that the consumption rate was higher than the production rate (data not shown). The possible underestimation of A_{max} was, therefore, probably of immaterial

significance. In the three examined biomasses A_{max} was found to be 14–15% higher in the radioisotope experiment than in the substrate-depletion experiments. This observation gives an evidence of an input of propionate via degradation of higher organic compounds during the experiment, which in the substrate-depletion experiments resulted in an underestimation of the degradation rate. Substrate-depletion experiments of reactor effluent from different reactors (R1-HBN, R1-ZM, R2-ZM, R4-ZM, data not shown) showed that that an average of 4 ml methane was produced per ml effluent during a period of 30–60 days. Mackie and Bryant (1981) estimated that approximately 17% and 13% of the produced methane is formed through propionate at thermophilic and mesophilic conditions, respectively, and theoretically 1.75 mol methane is formed from 1 mol of propionate. Relating these results to the present results, calculations shows a theoretical background input of propionate corresponding to approximately 14–19% of the initial concentration in the batch vials. The half saturation constant was also found to be highest in the radioisotope experiments, which could be a result of lowered affinity of the syntrophic bacteria towards labeled propionate at low concentrations or be a result of a change in the degradation pathway of propionate. These results make it obvious that both examined methods have limitations. Therefore, when estimating the kinetic parameters of propionate degradation different aspects should be taken into account. 1) The inoculum concentration should be as low as possible in order to reduce the input of propionate from higher organic background compounds, but should at the same time be a precise representative of the reactor content. 2) The propionate concentration should be well above the K_m value of the inoculum, which according to literature is in the range from 0.04–4.47 mM. However, a relatively low inoculum concentration compared to propionate concentration would give an overestimation of A_{max} since significant microbial growth would occur before the propionate is fully degraded. In the present experiment the biomass was considered to be constant during the entire experiment since the theoretical increase in VS was too low compared to the initial VS concentration in the vials (Gavala et al. 2003). This was also seen in practice where no changes in the degradation rate were observed during the entire phase three. In case of microbial growth an incorrect estimation of A_{max} can be avoided by continuous measurement of the VSS concentration in the vials during the experiment. Unfortunately, this would reduce the simplicity of the method especially for reactors containing a high amount of particulate organic material such as biofibers. A prolonged pre-incubation of the vials before addition of propionate would reduce the problems concerning high background input of propionate, but could possibly also change the microbial composition and environmental

conditions, for example nutrient concentrations and pH. Therefore, the most important and difficult factor is to achieve an exact balance between the propionate concentration and inoculum concentration. To ensure that no significant microbial growth or adaptation has occurred during the experiments, the maximum degradation rate of propionate should be linear (figure 2). It might be argued that the optimal estimation of A_{max} would be in the beginning of the experiment when no growth at all has occurred. However, experiments in our lab generally shows a very low if any degradation of propionate during the first 24 of the experiments, possibly because of a high sensitivity of the syntrophs towards changes in the environment.

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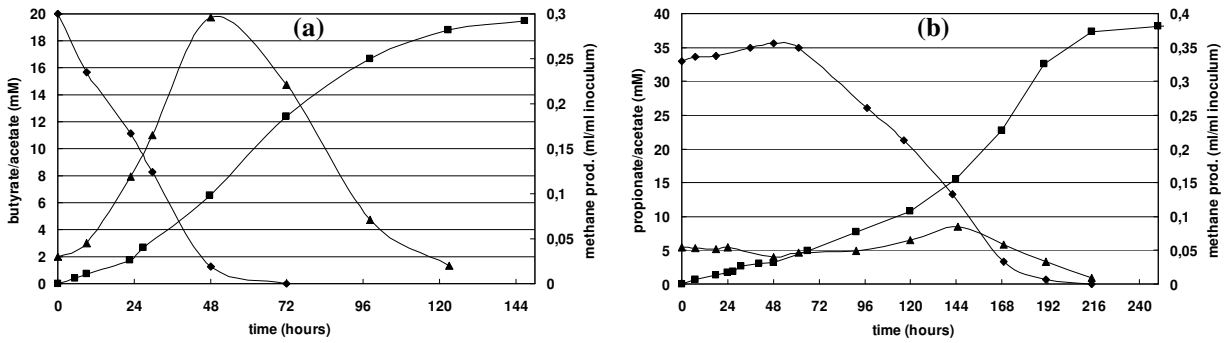

Figure 1 Example of anaerobic butyrate and propionate degradation in batch vials. The vials contained anaerobic basal medium and inoculated inoculated with digested cattle manure from a thermophilic (55°C) CSTR and supplemented with **a)** sodium butyrate to a final concentration of 20 mM and **b)** sodium propionate to a final concentration of 33 mM. The dilution ratio of the inoculum:basal medium was 5:16.4. **a)** ◆: butyrate; ▲: acetate; ■: methane; **b)** ◆: propionate; ▲: acetate; ■: methane.

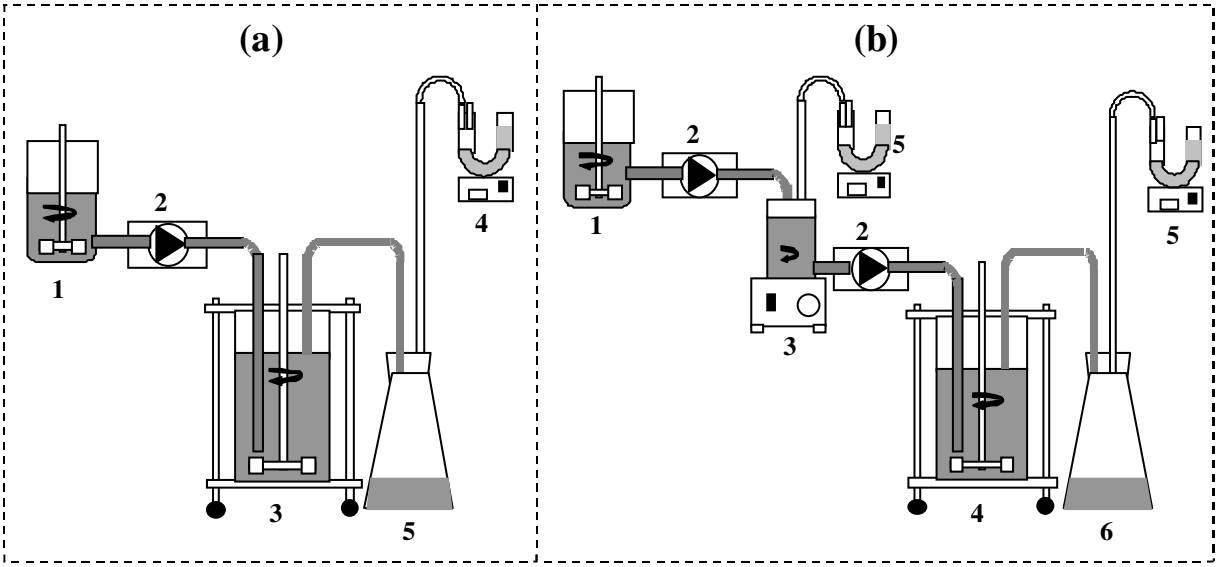

Figure 2 a) Reactor set-up used for R1-HBN, R2-HBN, R3-HBN, R4-HBN, R1-ZM, R2-ZM, R1-HWH and R2-HWH. 1: feedstock storage; 2: feeding pump; 3: 4.5 litre CSTR with a working volume of 3.0 litres. The reactor was stirred for one minute every third minute at 100 rpm and a stable operational temperature was obtained by circulating heated water from a water bath through the reactor jacket (not shown); 4: gas meter; 5: effluent storage. **b)** Two-stage system. 1: feedstock storage; 2: feeding pumps; 3: 0.9 litre 73°C CSTR with a working volume of 0.6 litres. The reactor was a glass beaker with a double wall that was closed with a butyl rubber stopper. The reactor was stirred constantly at 250 rpm by a magnet. The stable operational temperature was obtained by circulating heated water from a water bath through the double wall; 4: 4.5 litre CSTR with a working volume of 2.6 litres. The reactor was similar to the reactor described previously; 5: gas meter; 6: effluent storage.

Table 1 Reactor operation parameters, reactor performance and kinetic parameters of propionate degradation during steady state conditions.

Reactor	Reactor parameters		Substrate	Loading	Methane yield	VFA conc. Ac./Prop./Isobut/But	Kinetics of propionate degradation		
	HRT (days)	Temp (°C)		gVS(l x d)	ml/gVS	mM	A_{max} µmol/(gVS x h)	K_m mM	Affinity A_{max} / K_m
R1-HBN	15	53	Cow manure:pig manure (1:1)	2.3–2.5	304 ± 15.1	11.2/3.1/0.1/0.1	27.0 ± 0.95	0.64 ± 0.167	42.2 ± 0.26
R2-HBN	15	53	Cow manure:pig manure (1:1) + 1% fat + 1% meat and bone meal (w/vol)	3.4–3.6	340 ± 14.6	12.7/2.6/0.3/0.1	28.5 ± 2.48	0.64 ± 0.022	44.5 ± 0.09
R3-HBN	15	53	Cow manure:pig manure (1:1) + 0.5% fat (w/vol)	2.6–2.8	394 ± 21.9	12.6/3.3/0.3/0.2	29.0 ± 1.63	0.61 ± 0.124	47.5 ± 0.21
R4-HBN	15	53	Cow manure:pig manure (1:1)	2.3–2.5	341 ± 17.6	3.6/0.7/0.170.1	30.8 ± 1.12	0.56 ± 0.121	55.0 ± 0.22
R1-ZM	15	55	Cow manure : pig manure : sludge (4:4:2)	2.6	240 ± 21.0	5.4/1.0/0.2/0.0	25.9 ± 4.04	0.46 ± n.d.	56.3 ± n.d
R2-ZM	18	37	Cow manure : pig manure : sludge (4:4:2)	2.2	229 ± 32.1	2.4/0.3/0.0/0.0	22.8 ± 0.24	0.95 ± 0.081	24.0 ± 0.09
R3-ZM	2	73	Cow manure : pig manure : sludge (4:4:2)	19.5	3.5 ± 1.05	113/30.2/6.8/10.0	No degradation	No degradation	No degradation
R4-ZM	13	55	Cow manure : pig manure : sludge (4:4:2). Pretreated 2 days at 73°C	n.d.	n.d.	6.2/1.0/0.1/0.0	29.1 ± 3.78	0.79 ± 0.371	36.8 ± 0.49
R3+R4-ZM	2+13 = 15	73/55	Cow manure : pig manure : sludge (4:4:2)	2.6	271 ± 43.9	-	-	-	-
R1-HWH	17	55	Cow manure : pig manure (1:1) with autoclaved solids (140°C, 40 min)	3.0	250	27.1/18.4/0.6/0.3	30.7 ± 0.83	6.60 ± n.d.	4.7 ± n.d.
R2-HWH	17	55	Cow manure : pig manure (1:1). Solids	3.0	220	27.4/21.8/1.1/0.3	18.5 ± 0.80	n.d.	-

Table 2 Kinetic parameters of propionate degradation in batch vials estimated from substrate-depletion experiments and radioisotope experiments

Reactor	Substrate-depletion experiments		Radioisotope experiments	
	A_{max} µmol/(ml ino. x h)	K_m mM	A_{max} µmol/(ml ino. x h)	K_m mM
R1-ZM	25.7 ± 4.45	0.89 ± 0.465	29.2 ± 4.79	4.10 ± 0.383
R2-ZM	22.6 ± 2.17	0.78 ± 0.276	25.9 ± 0.57	4.21 ± 0.254
R4-ZM	32.5 ± 1.44	0.61 ± 0.064	37.3 ± 1.54	3.96 ± 1.420

Table 3 Kinetic parameters of anaerobic propionate degradation found in literature.

System and conditions	K_m or K_S mM	Degradation rate	References
Mesophilic sludge, 37°C	0.84	50 µmol/(gVS x h)	Gavala et al. (2003)
Thermophilc sludge, 55°C	0.99	87 µmol/(gVS x h)	Gavala et al. (2003)
Psychrophillic granular sludge, 10°C	0.06–0.13	26 µmol/(gVSS x h)*	Rebac et al. (1999)
Granular sludge, 40°C	0.98	≈ 37µmol/(gVSS x h)*	Van Lier et al. (1996)
Granular sludge, 45°C	1.16	≈ 89µmol/(gVSS x h)*	Van Lier et al. (1996)
Granular sludge, 50°C	0.63	≈ 138 µmol/(gVSS x h)*	Van Lier et al. (1996)
Granular sludge, 55°C	2.51	≈ 186 µmol/(gVSS x h)*	Van Lier et al. (1996)
Granular sludge, 60°C	2.86	≈ 224 µmol/(gVSS x h)*	Van Lier et al. (1996)
Mesophilic sludge, 35°C	2.8	13–25 µmol/(gVSS x h)*	Lens et al. (1996)
Adapted granules, 35°C	0.04–0.06	920–1300 µmol/(gVSS x h)	Wu et al. (1993)
Adapted granules, 35°C	0.04–0.06	32–75 µmol/(ml x h)	Wu et al. (1993)
Thermophilic granules, 55°C	-	70 µmol/(gVS x h)	Schmidt and Ahring (1993)
Adapted granular sludge	2.00	-	Fukuzaki et al. (1990)
Continuous enrichment culture	0.15–4.46	-	Smith and McCarty (1989)
Continuous-flow mixed cultures, 33°C	2.20	-	Gujer and Zehnder (1983)
Continuous-flow mixed cultures, 35°C, 14.5 d HRT	0.15	-	Heyes and Hall (1983)
Continuous-flow mixed cultures, 35°C, 8.2 d HRT	4.47	-	Heyes and Hall (1983)
Mesophilic sludge, 40°C, 10 d HRT	-	0.09–0.31 µmol/(ml x h)	Mackie and Bryant (1981)
Thermophilic sludge, 60°C, 10 d HRT	-	0.12–0.31 µmol/(ml x h)	Mackie and Bryant (1981)
Mesophilic sludge, 33°C, 40 d HRT	0.04–0.19	0.16–0.30 µmol/(ml x h)	Kaspar and Wuhrmann (1977)

*Value is a rough estimated subtracted from illustrated data in the publication.