Biogas Production from Thin Stillage

Exploring the microbial response to sulphate and ammonia

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Cover: Inside of the first digester before start-up of Norrköping Biogas plant 2007 (photo: L. Hejdenberg)

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Abstract

The biogas plant in Norrköping (Tekniska verken i Linköping AB, publ.), Sweden, operates with thin stillage, a residue from bio-ethanol fermentation, as the main feedstock. Thin stillage is energy-rich due to its high protein content, but due to its high nitrogen and sulphate content is a somewhat complicated feedstock. The high nitrogen concentration results in inhibition of the microbial process and also selects for nitrogen-tolerant, but slow-growing, syntrophic acetate-oxidising bacteria (SAOB). The high sulphate concentration in the feedstock results in production of toxic and inhibitory sulphides through the activity of sulphate-reducing bacteria (SRB). Measures currently applied at Norrköping biogas plant to optimise the degradation of thin stillage include: i) use of mesophilic temperature and addition of hydrochloric acid, ii) use of long hydraulic retention time and iii) addition of iron and trace elements.

This thesis investigated how to obtain a more efficient biogas process treating thin stillage, with Norrköping biogas plant as the model plant. It also explored the role of SRB in the anaerobic process at high nitrogen content and sought to identify optimal conditions for ammonia-tolerant methane-producing microorganisms. This was done by measuring SRB abundance in several large-scale biogas processes to identify conditions resulting in reduced numbers. In parallel, the effects of increasing temperature and organic load, calcium addition and a two-stage strategy were evaluated in laboratory studies. The results showed a correlation between high ammonia level and temperature with decreased abundance of SRB, but none of the operating strategies tested proved successful in repressing sulphate reduction. However, increasing ammonia and/or organic loading rate influenced both the acetogenic and methanogenic community, including potential SAOB. Moreover, increasing the temperature to 44 ºC resulted in increased abundance of thermotolerant SAOB and their partner methanogen and higher biogas yield (+22%). A maximum ammonia threshold concentration of approximately 1.1 g L^{-1} was identified.

Application of the findings reported in this thesis has resulted in increased process stability in biogas plants in Sweden.

Keywords: anaerobic digestion, biogas, sulphate, sulphate-reducing bacteria, acetogens, methanogens, syntrophic acetate-oxidising bacteria, hydrogen sulphide, thin stillage, ammonia inhibition

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Till min kära familj

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Moestedt, J., Nordell, E., Nilsson Påledal, S. (2013). Biogas production from thin stillage on an industrial scale – experience and optimisation *Energies* 6 (11), 5642-5655

II Moestedt, J., Nilsson Påledal, S., Schnürer, A. (2013). The effect of substrate and operational parameters on the abundance of sulphate-reducing bacteria in industrial anaerobic digesters. *Bioresource Technology* 132, 327-332.

III Moestedt, J., Nordell, E., Schnürer, A. (2014). Comparison of operational strategies for increased biogas production from thin stillage. *Journal of Biotechnology* 175 (1), 22-30.

IV Moestedt, J., Müller, B., Westerholm, M., Schnürer, A. (2015). Ammonia threshold for inhibition of anaerobic digestion of thin stillage independently of loading rate and responses in the methanogenic and acetogenic community (submitted)

V Moestedt, J., Nordell, E., Hallin, S., Schnürer, A. (2015). Attempts to separate sulphide and methane production with two-stage anaerobic digestion for industrial application (submitted)

The contribution of Jan Moestedt to the papers included in this thesis was as follows:

I Participated in planning the study and analysing the results. Main writer of the manuscript.

II Participated in planning the study and analysing the results. Performed all molecular work. Main writer of the manuscript

III Participated in planning the study and analysing the results. Performed all molecular work and monitored the reactors. Main writer of the manuscript.

IV Participated in planning the study and analysing the results. Performed part of molecular work and monitored the reactors. Main writer of the manuscript.

V Planned the study and participated in analysing the results. Monitored the reactors. Main writer of the manuscript.

Abbreviations

BMP	Bio-methane potential
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
DNA	Deoxyribonucleic acid
HRT	Hydraulic retention time
LCFA	Long-chain fatty acids
MSW	Municipal solid waste
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate-oxidising bacteria
SRB	Sulphate-reducing bacteria
TRFLP	Terminal restriction fragment length pattern
T-RF	Terminal restriction fragment
VFA	Volatile fatty acid
WWTP	Wastewater treatment plant

1 Introduction

The Swedish public company Tekniska verken i Linköping AB carries out biogas production at three sites; Linköping co-digestion plant, Linköping wastewater treatment plant (WWTP) and Norrköping co-digestion plant. Linköping co-digestion plant is currently one of the largest biogas plants in Sweden, treating slaughterhouse waste, the organic fraction of municipal solid waste (OFMSW) and industrial waste. The Norrköping biogas plant is smaller and is designed to treat mainly thin stillage, a residue from bio-ethanol production.

The gas produced at these two biogas plants, together with gas produced at Linköping WWTP, is upgraded to vehicle fuel quality and distributed to regional buses, taxis and private cars. The Linköping co-digestion plant was established in 1997 to solve the waste disposal problems of the local slaughterhouse industry. Biogas was also recommended for use in city buses at that time to reduce emissions from public transport and improve air quality in Linköping city. Slaughterhouse waste was hence the major substrate for anaerobic digestion. Since then, biogas production has expanded and at present (2015) Tekniska verken i Linköping AB owns several dedicated fuelling stations for biogas cars in and around the region of Östergötland. The gas produced by the different plants is used by the vast majority of buses operating within public transport in the region (in the cities of Linköping, Norrköping and Motala) and by private cars. The digestate, *i.e.* the liquid residue from anaerobic digestion, is valuable due to its high nitrogen content and is used as bio-fertiliser on agricultural land. Depending on the original substrate, bio-fertiliser is permitted for use as an organic fertiliser and is an important alternative for organic farms in the region. The Linköping and Norrköping biogas plants are thus great examples of how anaerobic digestion can be used to convert waste to energy and fill an important role in the development of a sustainable society.

However, to meet the increasing demand for biogas and to obtain economically feasible production, biogas production processes in general need to be optimised. The economic performance of biogas plants depends on many different factors, such as subsidies, legislation, feedstock, the price of electricity and petrol *etc*. Swedish legislation promotes biogas upgrading to vehicle fuel quality. However, despite the availability of subsidies, it is difficult to achieve profitability, particularly if feedstock is costly and/or the biogas plant operates at low efficiency. For example, the conditions in the Linköping and Norrköping plants differ, since the Linköping plant receives a gate fee for a large part of its feedstock, such as OFMSW, while the Norrköping plant has to pay for its feedstock, which mainly consists of stillage from bio-ethanol production that has an alternative market value as animal feed.

Much effort has been devoted to achieving efficient processes at the Linköping and Norrköping biogas plants in terms of degradation efficiency, gas production and gas quality. However, the substrate characteristics result in production of large amounts of undesired hydrogen sulphide (H_2S) and the release of ammonia (NH_3), both representing complications at the plants (Ek *et al.*, 2011; Paper **I**). Hydrogen sulphide originates from sulphur-containing amino acids, while ammonia is released from organically bound nitrogen in all amino acids. Hydrogen sulphide may also be produced through the activity of sulphate-reducing bacteria (SRB) (Rabus *et al.*, 2006; Paper **II**). Hydrogen sulphide is very corrosive and odorous and removal of this component is necessary to achieve vehicle fuel quality and for public approval of biogas production. Ammonia is also an issue, since at elevated levels it inhibits microorganisms producing biogas and may lead to process instability and lower biogas production. Both compounds can thus affect the profitability and efficiency of biogas plants. In Linköping the sulphides mainly originate from organically bound sulphur in proteins, while in Norrköping the sulphide produced originates from sulphate and from proteins. The removal of hydrogen sulphide is currently achieved in both plants by addition of iron to the incoming substrate mixture, which precipitates sulphides. However, iron addition is associated with additional operating costs.

The production of high levels of ammonia and sulphide in both the Linköping and Norrköping co-digestion plants affects their possibilities to reach high biogas yield and consequently a profitable outcome. To obtain a more economical process, in the Norrköping plant in particular, operating strategies that reduce the activity of SRB and permit operation at elevated ammonia levels need to be identified.

1.1 Aims of the thesis

The overall aim of this thesis was to identify how to obtain a more efficient biogas process at the Norrköping biogas plant owned by Tekniska verken i Linköping AB. To achieve this goal, the specific objectives of the component studies were to:

1. Explore the role of sulphate and sulphate-reducing bacteria in the anaerobic digestion process (Papers **I-V**, thesis summary).
2. Identify optimal conditions for ammonia-tolerant methane-producing microorganisms during anaerobic digestion of thin stillage (Papers **III** and **IV**).
3. Decrease the competiveness of sulphate-reducing bacteria in the biogas process by changing the environmental conditions (*i.e.* temperature, pH or nitrogen concentration) or by addition of inhibitory compounds (Papers **III-V**, thesis summary).

1.2 Norrköping biogas plant

The Norrköping biogas plant is designed to treat thin stillage as its main substrate. The plant is located within one kilometre from a bio-ethanol plant (Lantmännen Agroetanol AB) and the substrate (*i.e.* thin or condensed stillage) can be transported by tanker between the plants. The annual amount of substrate treated to date has been about 4000 ton volatile solids (VS) and the capacity is approximately 2 MW (Paper **I**). The plant has two main digesters of 1800-2000 m^3 and a post-digester of 4000 m^3. The digestate is transported to local farms by tanker for further storage (Figure 1). One public fuelling station is located directly at the plant, while the gas is transported either by pipeline or by tanker to additional fuelling stations and the city bus depot.

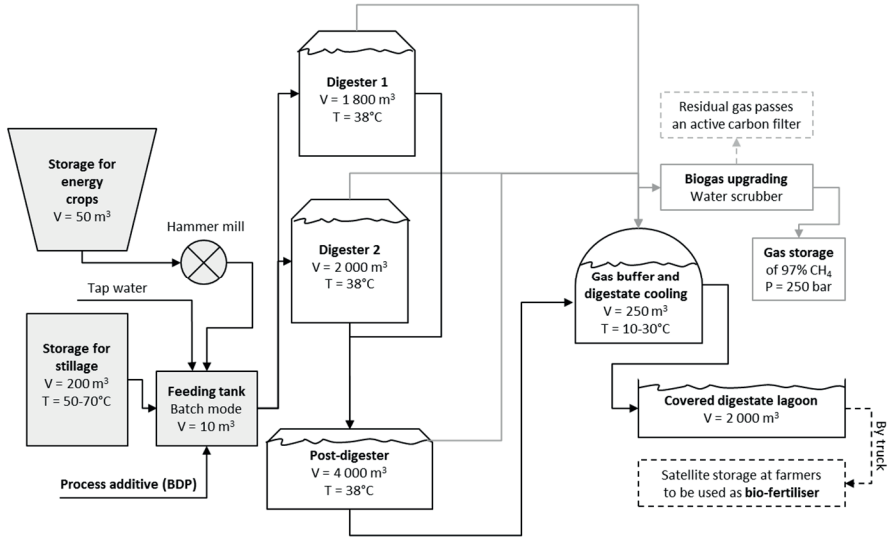

Figure 1. Schematic illustration of the Norrköping biogas plant. Black arrows illustrate organic flows and grey arrows gas flows.

2 Biogas production

Anaerobic digestion is the microbiological degradation of organic material in the absence of oxygen. The end product of anaerobic digestion is biogas, typically consisting of mainly methane (CH_4) and carbon dioxide (CO_2) (Angelidaki *et al.*, 2011). The process occurs in natural environments, but has historically also been exploited by humans for centuries. Anaerobic digestion is generally used at WWTP in Europe to stabilise and reduce the final amount of sludge, as well as producing biogas (Appels *et al.*, 2008).

In 2013, total biogas production in Sweden reached almost 1700 GWh, an increase of 37% from the 2006 level. The main feedstock for biogas production still originates from WWTP, but an increasing amount of biogas, corresponding to 34% of the total amount of biogas produced in Sweden in 2013, is being produced in co-digestion plants (Swedish Energy Agency, 2013). These co-digestion plants typically do not use sludge from WWTP, but aim for feedstocks with comparatively high gas potential and high availability. For European co-digestion plants, these feedstocks are typically energy crops, manure, industrial wastes, slaughterhouse waste, harvest residues and OFMSW (Weiland, 2010). These materials contain much more readily available energy and anaerobic digestion than WWTP sludge. Biogas production can thus fill a key function in recycling waste to energy, since various organic residues from fermentation and bio-refinery processes can be treated. It has even been suggested that biogas can supply the energy (methane) and nutrients (digestate) needed for growing the feedstocks and hence create an energy-efficient and cyclic industrial bio-industry (Martin *et al.*, 2014). The Norrköping co-digestion plant, with its industrial symbiosis with the city's bio-ethanol and district heating plant, has been mentioned as an example of this (Martin & Eklund, 2011). Co-digestion by combining substrates of different origins in a well-considered mix is also positive, as it can be used to achieve optimal conditions for the microorganisms involved in anaerobic digestion. Feedstock should hence be selected to obtain a complete and balanced nutrient content as

regards carbon:nitrogen (C:N) ratio, proteins, fats, carbohydrates, trace metals *etc*. Co-digestion can also be used to dilute inhibitory substances, such as sulphate, sulphur, nitrogen, long-chain fatty acids (LCFA) or salts. The volume of biogas produced from a specific amount of feedstock depends on: i) the structure of the material, which dictates the available fraction of organic material that can be converted into gas and ii) its content of fat, proteins and carbohydrates (Weiland, 2010). As a consequence, materials rich in protein and fat, such as slaughterhouse waste, residues from fermentation processes and OFMSW, are of great interest for biogas production due to the high gas yields and/or high degradability (Ek *et al.*, 2011).

2.1 Microbiology of anaerobic digestion

The anaerobic digestion process is traditionally separated into four different microbiological steps: i) hydrolysis, ii) acidogenesis, iii) acetogenesis and iv) methanogenesis. These steps are performed by diverse microbial groups, in close dependence on each other (Figure 2).

2.1.1 Hydrolysis and acidogenesis

During hydrolysis, extracellular enzymes are excreted by primary fermentative bacteria to convert polymeric organics (fat, proteins and carbohydrates) to monomers. The monomers (fatty acids or glycerides, amino acids and sugars) are then accessible for uptake. The primary fermentative bacteria degrade these monomers during the step called acidogenesis and produce volatile fatty acids (VFAs), succinate, lactate, alcohols, carbon dioxide and hydrogen as fermentation products (Angelidaki *et al.*, 2011; Schink, 1997; Zinder, 1984). At the same time, organically bound nitrogen and sulphides are released from amino acids and/or nucleic acids as ammonium (NH_4^+), ammonia (NH_3) and hydrogen sulphide (H_2S) (Figure 2). The hydrogen and acetate can be used directly in methanogenesis, while the residual organic products from acidogenesis need additional degradation before methanisation (Figure 2). If the hydrogen and acetate concentrations are kept low by consumption further along the anaerobic digestion chain, the products of acidogenesis are mainly acetate and hydrogen (Worm *et al.*, 2010).

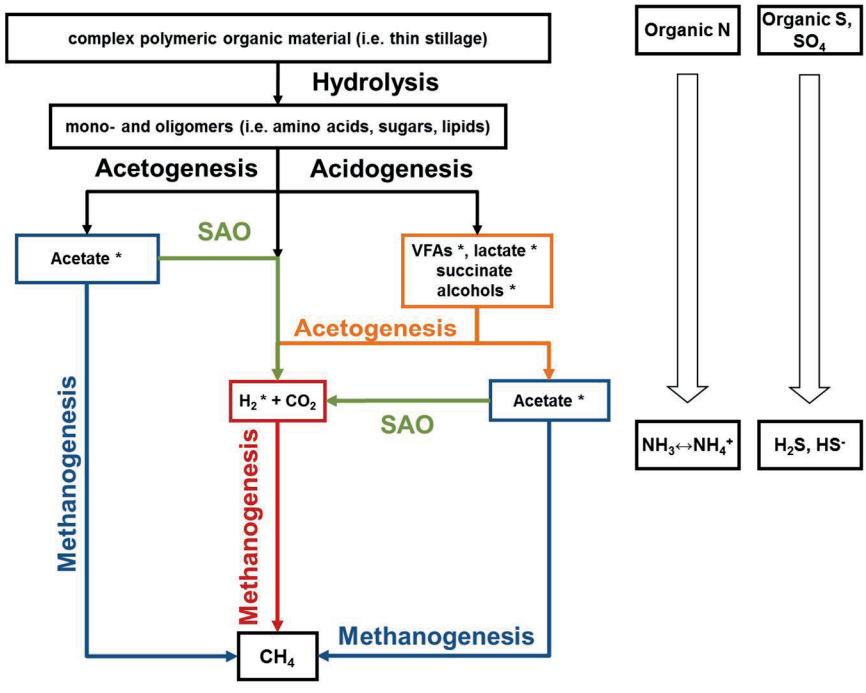

Figure 2. The different degradation pathways of the anaerobic digestion process. * indicates a substrate that can be utilised by SRB (■ Syntrophic degradation, ■ Syntrophic acetate oxidation, ■ Acetotrophic methanogenesis, ■ Hydrogenotrophic methanogenesis).

2.1.2 Acetogenesis

The products from acidogenesis need to be consumed to avoid accumulation of VFA and hence loss of methane. Moreover, accumulation of acids can result in decreasing pH and potentially even in disruption of the whole anaerobic digestion process (Ahring *et al.*, 1995; Zinder, 1984). During acetogenesis, the VFAs, sugars and alcohols resulting from acidogenesis therefore need to be further degraded by secondary fermentative (syntrophic) bacteria to hydrogen and acetate (Angelidaki *et al.*, 2011; Madigan *et al.*, 2006). The reactions performed by these syntrophic bacteria produce acetate, hydrogen and carbon dioxide in different ratios and are typically thermodynamically unfavourable under standard conditions, *i.e.* positive standard energy (Table 1). For these reactions to proceed, the hydrogen pressure needs to be reduced to low levels and the acetate concentration can also be of importance (Schink, 1997). The hydrogen pressure needed for the degradation to proceed depends on the type of VFA present and the prevailing environmental conditions. For example, at pH 7.0 and 25 ºC, the hydrogen level required is approximately 10^{-3} atm. for

butyrate and even lower for acetate (about 10^{-4} atm.) (Worm *et al.*, 2010; Lee & Zinder, 1988a; Zinder & Koch, 1984). If hydrogen is not removed, the reversed reaction to acetate can be thermodynamically favoured and is performed by homoacetogens (Saady, 2013; Paper **V**).

Table 1. *Free energy requirement for oxidation of products from acidogenesis (Schink, 1997; Zinder, 1984). ΔG' values based on Zinder (1984) with typical conditions for anaerobic digestion: 37 °C; pH 7; [acetate$^-$]=[propionate$^-$]=[butyrate$^-$]=1mM; [HCO_3^-] =20mM; CH4 = 0.6 atm; $H_2 = 10^{-4}$ atm.*

Intermediate	Reaction	Standard free energy (ΔG°', kJ mol^{-1})	Free energy (ΔG', kJ mol^{-1})
Propionate$^-$ + $3H_2O$ →	Acetate$^-$ + HCO_3^- + $3H_2$+ H^+	+ 76	- 5
Butyrate$^-$ + $2H_2O$ →	2Acetate$^-$ + $2H_2$ + $2H^+$	+ 48	- 17
[1]Acetate$^-$ + $2H_2O$ →	$2HCO_3^-$ + $4H_2$ + H^+	+ 105	+ 7
[2]Acetate$^-$ + H_2O →	CH_4 + HCO_3^- + H^+	- 31	- 24
[3]$2HCO_3^-$ + $4H_2$ + H^+ →	CH_4 + $3H_2O$	- 136	- 32

[1]Acetate oxidation
[2] Acetotrophic methanogenesis
[3] Hydrogenotrophic methanogenesis

2.1.3 Methanogenesis

Methanogenesis is performed by organisms called methanogens belonging to the domain *Archaea*. The most commonly occurring methanogens in a biogas process are typically divided into two functionally different groups. The first group, hydrogenotrophic methanogens, typically consume hydrogen together with carbon dioxide or formate produced during acidogenesis and acetogenesis (Table 1). These hydrogen-consuming methanogens are represented by four different orders: *Methanomicrobiales*, *Methanococcales*, *Methanobacteriales* and hyperthermophilic *Methanopyrales* (Angelidaki *et al.,* 2011). The relatively newly discovered *Methanomassiliicoccales* also use hydrogen, but reduce methyl compounds (Dridi *et al.*, 2012).

The other group, acetotrophic methanogens, can cleave acetate directly into one methyl and one carboxyl group, and the methyl group is converted to methane (Zinder, 1984). Acetotrophic methanogens are only represented by one order, *Methanosarcinales*, which includes the two methanogenic families *Methanosarcinacea* and *Methanosaetaceae*. *Methanosaetaceae* sp. only use acetate, while *Methanosarcinacea* sp. are metabolically more diverse and can also use hydrogen, carbon monoxide, carbon dioxide, methanol and methylated C_1 compounds as their carbon source and energy source (De Vrieze *et al.*, 2012; Angelidaki *et al.*, 2011). *Methanosarcinacea* sp. are also relatively fast-growing, with doubling times shorter than one day, while *Methanosaetaceae*

sp. are more slow-growing (doubling time 4-6 days) (Angelidaki *et al.*, 2011; Zinder, 1984).

Acetate can thus be directly degraded by acetotrophic methanogenesis or, alternatively, as has been suggested for environments sub-optimal for the methanogens performing acetotrophic methanogenesis, through syntrophic acetate oxidisation (SAO), where oxidation of acetate is combined with hydrogen consumption (Figure 2, see also section 2.2.3).

The degradation of acetate is important since in mesophilic conditions approximately two-thirds of the methane produced is derived from acetate, through acetogenesis, rather than directly from hydrogen and carbon dioxide (Worm *et al.*, 2010).

2.2 Anaerobic digestion of thin stillage

Residues from ethanol fermentation are very abundant organic sources that can be used for biogas production. The annual production of ethanol for vehicle fuel in Europe is 4000 million litres (EurObserv'er, 2012). For each litre of ethanol produced, approximately 5-10 litres of stillage are created as a residue (Börjesson & Tufvesson, 2011). Whole stillage is the organic fraction separated from ethanol during the distillation process and large volumes of whole stillage with high water content (87-90%) are produced. At present, the most common use for stillage is as animal feed. To increase its suitability for animal feed applications and to reduce waste volumes, the stillage is dried at the ethanol plant by energy-intense centrifugation. After centrifugation of whole stillage, thin stillage with high water content (90%) is obtained as the supernatant fraction. For animal feed applications, this fraction is further vaporised by heat to decrease the water content to about 70%. This treatment of whole stillage poses a threat to the economic viability of ethanol plants due to the costly procedures involved, which can require up to one-third of the total energy consumption at the plant (Drosg *et al.*, 2013). A potential way of circumventing this problem is to use whole stillage or thin stillage directly, without drying, for biogas production (Drosg *et al.*, 2013; Wood *et al.*, 2013). This would eliminate the energy requirement for drying stillage and instead result in the production of renewable energy (in the form of biogas), which could be used to meet the internal energy demand and thus greatly improve the energy balance at bio-ethanol plants (Martin *et al.*, 2014; Drosg *et al.*, 2013; Wood *et al.*, 2013). Against this background, anaerobic digestion of thin stillage is an interesting proposition. The Norrköping co-digestion plant is one such plant, built in 2007 to treat a small part of the stillage fractions produced at a nearby bio-ethanol plant (Paper **I**). Thin stillage consists of soluble

organics which are readably available for degradation. The chemical composition is mainly high levels of proteins together with fibre and sugars which have not been converted to ethanol during fermentation (Kim *et al.*, 2008; Wilkie *et al.*, 2000). However, the gas yield is quite low, about 0.3 m^3 CH_4 kg^{-1} VS (2.9 kWh kg^{-1} VS) with a methane content of 50-55% (Schmidt *et al.*, 2013; Westerholm, 2012b; Alkan-Ozkaynak & Karthikeyan, 2011; Dererie *et al.*, 2011; Gustavsson *et al.*, 2011; Paper **III**). Degradation of stillage fractions can also be problematic due to the high protein content, leading to elevated ammonia concentration. Moreover, addition of sulphuric acid is common during the ethanol fermentation process, which leads to sulphate reduction during the anaerobic process (Paper **I**). If sulphate reduction occurs in the process, the resulting H_2S needs to be taken care of in order to maintain air quality in the vicinity of the biogas plants and prevent public complaints about the biogas industry. Hydrogen sulphide is not only odorous, but also very reactive, corrosive and even toxic to humans and to the microorganisms involved in anaerobic digestion. In Sweden, the sulphide content in commercial biogas is strictly regulated to <28 ppmv (Swedish Standard 15 54 38, SP Technical Research Institute of Sweden), in order to control odour and corrosive effects.

2.2.1 The effect of sulphate

The presence of alternative electron acceptors in anaerobic digestion can lead to disruption of methanogenesis. Oxygen is detrimental to anaerobic digestion for many reasons, *e.g.* it is a superior electron acceptor (E_0' for O_2/H_2O: + 0.82) and can cause oxygen toxicity in methanogens. Other plausible electron acceptors such as nitrate (E_0' NO_3^-/N_2: + 0.75) and sulphate (E_0' for SO_4^{2-} /SO_3^{2-} and SO_3^{2-}/HS^-: - 0.52 and - 0.22 resp.) also affect methanogenesis, as both are more favourable than carbon dioxide (E_0' for CO_2/CH_4: - 0.25).

Table 2. *Standard free energy for oxidation of products from acidogenesis with SO_4^{2-} as electron acceptor (Thauer et al., 1977).*

Intermediate	Reaction	Standard free energy ($\Delta G^{\circ\prime}$, kJ mol^{-1})
$Propionate^- + ¾SO_4^{2-} + H^+ \rightarrow$	$Acetate^- + HCO_3^- + ¾HS^- + ¼H^+$	- 38
$Butyrate^- + ½\ SO_4^{2-} \rightarrow$	$2Acetate^- + ½\ HS^- + ½\ H^+$	- 28
$Acetate^- + \ SO_4^{2-} \rightarrow$	$2\ HCO_3^- + HS^-$	- 48
$SO_4^{2-} + 4H_2 + H^+ \rightarrow$	$HS^- + 4H_2O$	- 152

By comparing the standard free energy for methanogenesis (Table 1) with that for sulphate reduction (Table 2), it is obvious that for each mole of electron

donor oxidised, bacteria utilising sulphate will yield more energy than acetogens and/or methanogens. This also applies for competition with acetogens for other electron donors such as alcohols and lactate. Using thin stillage, rich in sulphate, will thus result in competition for electron donors between SRB and acetogens on the one hand, and methanogens on the other. It is apparent that even in standard conditions, SRB will be able to oxidise longer VFA to acetate, and acetate to carbon dioxide (Table 2; Figure 2).

The thermodynamic advantage automatically leads to SRB being able to grow more successfully than methanogens at lower electron donor concentrations. This means that SRB may consume electron donors to the level where methanogenesis is no longer favourable (Kristjansson *et al.*, 1982). This phenomenon of SRB out-competition by lowering electron donor concentration has been observed for hydrogen in *e.g.* lake sediment (Lovley *et al.* (1982). Growth rate is also critical for competition and, as reviewed by Stams *et al.* (2003), SRB generally have higher growth rates that methanogens.

According to the ΔG' value (Table 2), sulphate reduction will thermodynamically always out-compete methanogenesis or acetogenesis, except when the sulphate concentration is too low. Therefore the effect of chemical oxygen demand (COD) (electron donor) to sulphate ratio has been evaluated in numerous studies seeking to identify the lower boundary for sulphate limitation (COD:sulphate ratio) and thus methanogenic dominance. Theoretically, a COD:sulphate of ratio of 0.67 g g^{-1} is required for complete reduction of all sulphate present and a larger ratio will result in excess electron donors which cannot be oxidised through sulphate reduction. Different COD:sulphate ratio values (1, 1.97, 2, 2.7, 3.7, 2, 4, 20.9 *etc*.) have been reported as critical for methanogenic dominance (Jing *et al.*, 2013; Dar *et al.*, 2008; O'Reilly & Colleran, 2006; Stams *et al.*, 2003; Oude Elferink *et al.*, 1994; McCartney & Oleszkiewicz, 1993). This broad range of ratios may be due to several factors, such as source of inoculum (which may or may not have been exposed to sulphate previously), type of substrate (complex or defined medium), reactor configuration (immobilised biomass or not), experimental set-up (batch or continuous), hydraulic retention time (HRT) and other process parameters. Nevertheless, there is consensus in the literature that a higher COD:sulphate ratio allows methanogens to successfully compete with SRB (Dar *et al.*, 2008; Stams *et al.*, 2003; Weijma *et al.*, 2002; Oude Elferink *et al.*, 1994; Visser *et al.*, 1993; Isa *et al.*, 1986). Studies on full-scale biogas production plants treating thin stillage to evaluate the optimal COD:sulphate ratio are scare. A noteworthy finding in this thesis is that a ratio of 28-34, much higher than the threshold values reported, still resulted in complete reduction of

sulphate and vast amounts of sulphides being produced in the Norrköping biogas plant (Paper **I**).

Paper **II** studied the abundance of SRB in 19 different full-scale biogas plants in Sweden. These plants included processes with low sulphate concentration and different levels of potential inhibitory factors such as temperature, ammonium, ammonia and VFA and operating parameters such as substrates, HRT and organic loading rate (OLR). Our hypothesis was that environmental factors can be identified and used to yield a favourable methanogenic competition, as a consequence of SRB limitation. However, the results showed that SRB prevailed in equal abundance in all processes studied, even at low sulphate concentration (Paper **II**). The SRB abundance in anaerobic digesters (10^5-10^7 gene copies) was similar to that in the other environments studied. However, the levels were still lower at higher nitrogen levels (>200 mg NH_3 L^{-1}) and a trend for lower abundance at higher temperatures (>45 ºC) was also observed (Paper **II**). The function of SRB at low sulphate levels is described elsewhere, in studies which report that SRB are metabolically flexible and that certain species can grow fermentatively on various electron donors (Plugge *et al.*, 2011; Oude Elferink *et al.*, 1994). Furthermore, SRB may grow as acetogens by syntrophic degradation of propionate, lactate or ethanol in association with hydrogenotrophic methanogens (Stams & Plugge, 2009). This probably explains how SRB persist in most biogas processes at low sulphate levels, as indicated by the relatively high SRB abundance in digesters with sulphate-depleted substrates (Paper **II**).

2.2.2 Sulphate-reducing bacteria

The SRB are capable of dissimilatory sulphate reduction and use the energy from this reduction for cell synthesis and growth. The ability for dissimilatory sulphate reduction is distinguished from the widespread ability for assimilatory sulphate reduction by excretion of H_2S instead of incorporation of sulphur for biosynthesis (Rabus *et al.*, 2006). The majority of SRB belong to the subclass δ-proteobacteria, but in addition SRB are represented in the Gram-positive genera *Desulfotomaculum* and *Desulfosporosinus* and in the separate branches of *Thermodesulfobacterium* and *Thermodesulfovibrio* (Rabus *et al.*, 2006). Archeal strains (*Archaeoglobus*, *Thermocladium* and *Cladivigra*) are also capable of dissimilatory reduction of sulphate, as summarised by Barton and Fauque (2009). Natural habitats for SRB are anoxic environments such as marine sediments, flooded soil, rice paddies, hot springs *etc*. (Barton & Fauque, 2009). We also observed them in different types of full-scale biogas processes (Paper **II**). Different groups of SRB can use different electron donors

such as H_2, ethanol, methanol, lactate, acetate, propionate, butyrate, succinate, fructose, glucose *etc*. for sulphate reduction (Plugge *et al.*, 2011; Rabus *et al*., 2006). The electron donor can be oxidised completely to CO_2 (complete oxidation) or incompletely to acetate due to the inability for terminal oxidation of acetyl-CoA (Rabus *et al.*, 2006). Typical incomplete oxidisers are bacteria belonging to the genera *Desulfovibrio*, *Desulfomicrobium* and *Desulfobulbus* and, to some extent, *Desulfotomaculum* (Devereux *et al*., 1989).

SRB have been analysed and studied in several different environments using different strategies. In anaerobic digesters, these bacteria have been studied mainly by culture-based techniques such as isolation (Suzuki *et al*., 2010; Zellner *et al*., 1989) and most probable number (MPN) counting (Harada *et al*., 1994), but also to some extent by molecular tools such as fluorescence *in situ* hybridisation (FISH) (Zahedi *et al*., 2013; Boonapatcharoen *et al.*, 2007) and polymerase chain reaction (PCR) (van den Brand *et al.*, 2014; Paper **II**).

Despite the phylogenetic distance between SRB, they have a common gene encoding the key enzyme for dissimilatory sulphate reduction, dissimilatory sulphite reductase (dsrAB) (Wagner *et al.*, 1998). This gene can be used for detecting SRB and has the advantage of targeting the whole SRB group, which contains phylogenetically distantly related species. The conserved characteristics of the dsrAB functional gene allowed Wagner *et al.* (1998) to amplify a 1.9 kbp sequence appropriate for phylogenic analyses. However, for quantification using environmental samples, alternative primers generating a shorter product have been designed (Geets *et al.*, 2006). This primer pair yields a 350 bp conserved sequence and has been used in several studies on environmental samples (He *et al.*, 2010; Gittel *et al.*, 2009; Dar *et al.*, 2007; Foti *et al.*, 2007; Leloup *et al.*, 2007; Kondo *et al.*, 2004), and in biogas processes in this thesis (Paper **II**). For quantification, it is assumed that the gene is present as single gene copies, despite several dsrAB gene copies having been observed for *Desulfovibrio* species, which may bias the results if this group dominates in the sample (Kondo *et al.*, 2004). In order to perform phylogenetic analyses on biogas samples in addition to quantification, in preliminary analyses we targeted the original, longer sequence of 1.9 kbp. However, despite PCR protocol optimisation and evaluation of alternative primers, no product of the correct length could be obtained from these environmental samples (unpublished data).

An alternative approach for phylogenetic analysis of SRB is to target the 16S rRNA gene of different bacteria known to have the capacity to perform sulphate reduction. However, primers targeting the 16S rRNA gene would be too degenerated and general, and would amplify several other microbial groups if designed to target all known SRB. Thus to target the phylogenetically

distantly related SRB, Daly *et al.* (2000) designed primers targeting six sub-groups of SRB: 1) *Desulfotomaculum*, 2) *Desulfobulbus*, 3) *Desulfobacterium*, 4) *Desulfobacter*, 5) *Desulfococcus-Desulfonema-Desulfosarcina* and 6) *Desulfovibrio-Desulfomicrobium*.

The existence of different SRB in anaerobic digesters has been observed in several studies, but no complete mapping of SRB has been performed for biogas reactor material. Therefore the primers designed by Daly *et al.* (2000) were used for this purpose on reactor material from the Norrköping biogas plant. Nested PCR, with a general initial amplification to increase the product yield and sensitivity of the analysis, was applied. Amplification of all groups except *Desulfobacterium* (group 3) was obtained, indicating that this group was not present in the inoculum. The sequences obtained were analysed using clone libraries, MAFFT v7.017 multiple alignment (Katoh *et al*., 2002) and subsequently with a PHYML tree-building algorithm (Guidon & Gascuel, 2003) with Geneiuos R6 (Biomatters Ltd.). Clones obtained with clone libraries of each group are indicated with blue font in the phylogenetic tree (Figure 3). Coverage for the clone library of group 1 was 87.5%, group (2) 88.6%, group 4 87.6%, group 5 64.7% and group 6 84.0% (>96% similarity).

Operational taxonomic units (OTUs) from group 2 grouped together with *Desulfobulbus* (corresponding to 47% of colonies from group 2). The highest similarity for these OTUs was obtained for *Desulfobulbus elongates* (96.0%) and *Desulfobulbus alkaliphilus* (95.7%). *Desulfobulbus* species such as *D. propionicus* and *D. alkaliphilius* primarily use propionate (other electron donors are also utilised) as an electron donor and typically oxidise this incompletely to acetate (Sorokin *et al.*, 2012).

Other group 2 OTUs grouped with *Clostridia* species and thermophilic sulphate-reducing *Thermodesulfobium narugense* and *Thermodesulfovibrio yellowstonii*. Furthermore, OTUs identified as *Stenotrophomonas maltophilia* (99.7%) and *Lactobacillus ultunensis CCUG 48460* (98.9%), which do not utilise sulphate, were present (Figure 3). Group 1 and group 4 OTUs grouped with *Desulfotomaculum*, but with low similarity to known organisms. The highest similarity was to two thermophilic *Desulfotomaculum* species. G6 OTU3 (8% of group 6) was identified as *Syntrophaceticus schinkii strain Sp3* (99.9 %) and grouped together in a cluster composed of *Desulfotomaculum* species and *Thermacetogenium pheum* (Figure 3). *Syntrophaceticus schinkii* is not capable of dissimilatory reduction of sulphate, but *Thermacetogenium phaeum* has been suggested to reduce sulphate during acetate oxidation (Hattori *et al.*, 2000). Similarly to *Desulfobulbus, Desulfotomaculum* is an incomplete propionate oxidiser (Devereux *et al.*, 1989). The fact that all SRB identified belong to the incomplete oxidisers indicates that this pathway

dominated over complete oxidation and suggests that acetate was still available for methane production. The energetic for propionate oxidation to acetate is quite favourable in the presence of sulphate, -38 kJ mol^{-1} (Colleran *et al.*, 1995). However syntrophic oxidation of propionate without sulphate reduction is rather unfavourable (Table 1), and thus it is likely that propionate oxidation via sulphate reduction is an important pathway and that SRB could help to avoid propionate accumulation in sulphate-containing anaerobic digesters.

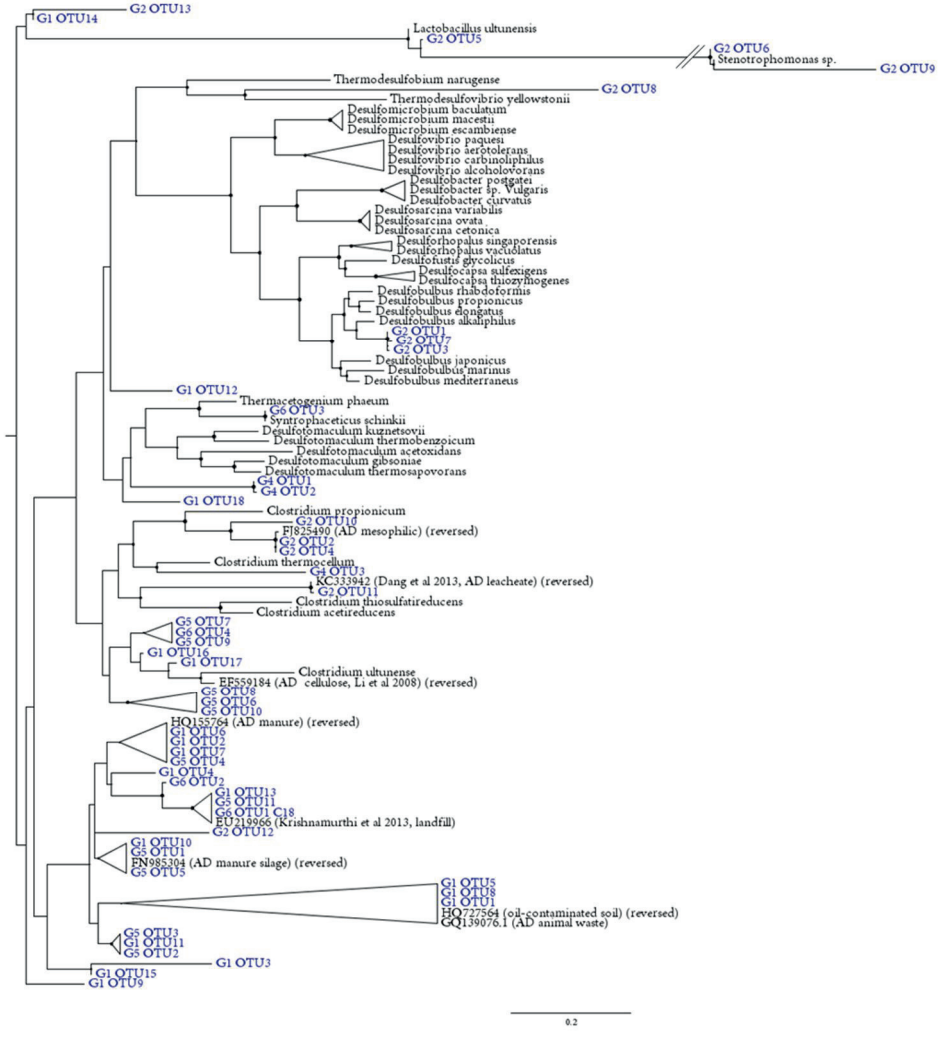

Figure 3. Clone libraries of SRB-subgroups 1, 2, 4-6. Blue font indicates OTUs from the reactor sample. Nodes with bootstrap proportion > 50 are indicated by circular node shapes

Most OTUs of group 1, 4 and 5 and a few OTUs of group 2 and 6 did not cluster with known SRB, however. Instead, a large cluster contained *Clostridium* species such as *C. propionicum* (89.2% G2 OTU2), *C. thermocellum* (90.3% G5 OTU8), *C. acetireducens* (90.7% G6 OTU4) and *C. thiosulfatireducens* (88.1% G6 OTU4). *Clostridium propionicum* can convert alanine to lactate and further to propionate (Kuchta & Abeles, 1985), while *C. thermocellum* is a thermophilic bacteria capable of cellulose conversion into ethanol, acetate and hydrogen (Weimer & Zeikus, 1977). *Clostridium acetireducens* converts several amino acids while using acetate as an electron acceptor (Orlygsson *et al.*, 1996) and *C. thiosulfatireducens* reduces thiosulphate and elemental sulphur and can use various amino acids (Hernandez-Eugenio *et al.*, 2002). In addition to clostridia species, *Stenotrophomonas maltophilia* (99.7%) and *Lactobacillus ultunensis* (98.9%) were also identified. *Stenotrophomonas maltophilia* is a non-fermentative bacterium which belongs to the *g*-proteobacteria *Xanthomonadles*. It has been observed to degrade aromatic compounds in the presence of nitrate as an electron acceptor (Su & Kafkewitz, 1994). *Lactobacillus ultunensis* converts several sugars to lactic acid (Roos *et al.*, 2005).

However, none of these bacteria can reduce sulphate and it is most probable that the detection of these was a result of poor primer selectivity. The primers designed by Daly *et al*. (2000) were created using a large number of SRB isolates, but only a limited number of negative controls. Hence, our theoretical analysis of primer specificity showed that group 2, 5 and 6 primers have low selectivity and could theoretically amplify species belonging to *Clostridia*, *Thermotoga* and *Enterobacter*. This explains the clones obtained clustering with Clostridia species and hits of *e.g. Syntrophaceticus schinkii*, *Stenotrophomonas* and *Lactobacillus*.

2.2.3 The effect of ammonia

Thin stillage contains a large amount of proteins. During anaerobic digestion of thin stillage, ammonia (NH_3) and ammonium are thus released (Figure 2), sometimes in very high quantities (Eskicioglu *et al.*, 2011; Gustavsson *et al.*, 2011; Paper **IV**). Ammonia-nitrogen (NH_3-N) has been identified as an inhibitory compound for methanogens, the proposed mechanism being that the small, neutral ammonia molecule can pass over the cell membrane. Once inside the cell, the lower internal cell pH causes a shift towards ammonium-nitrogen (NH_4^+-N), which affects both the cell pH and the trans-membrane potential (Sprott & Patel, 1986). As a consequence of this inhibitory effect of ammonia on the microorganisms involved in methane production, it has been suggested that a high NH_4^+-N concentration cause a

shift in acetate degradation from acetotrophic methanogenesis to SAO (Fotidis *et al.*, 2014; Werner *et al.*, 2014; Schnürer & Nordberg, 2008; Schnürer *et al.*, 1994). Moreover, increasing NH_4^+-N or NH_3-N has been observed to promote increasing abundance of SAOB, while the ammonia-sensitive acetotrophic *Methanosaetaceae* simultaneously decrease or are undetected (Sun *et al.*, 2014; Westerholm *et al.*, 2011a). This has also been observed during anaerobic digestion of thin stillage (Westerholm *et al.*, 2012; Paper **III** and **IV**). In addition to being ammonia-sensitive, *Methanosaetaceae* are also sensitive to the high pH often observed in ammonia-rich processes and are thus most likely replaced by SAOB in degradation of ammonia-rich substrates such as thin stillage. The SAO pathway has been suggested to always dominate if *Methanosaetaceae* are absent (De Vrieze *et al.*, 2012; Karakashev *et al.*, 2006). The abundance and the community structure of acetogens/SAOB are both affected by increasing NH_3-N and specific genotypes of potential SAOB have been observed to increase in abundance in response to different ammonia levels (Müller *et al.*, 2015; Paper **IV**).

The $\Delta G'$ of acetate oxidation is only negative if the hydrogen pressure is kept low and this can be performed by hydrogenotrophic methanogens or by other hydrogen scavengers (such as SRB) if alternative electron acceptors are present (Table 1). The energy requirement for ATP synthesis is assumed to be -60 kJ, while SAO yields $\Delta G'$ -25 kJ mol^{-1} acetate, and hence SAO is correlated with slow growth rates (Westerholm *et al.*, 2012; Hattori, 2008; Schnürer & Nordberg, 2008). *Methanomicrobiales* and *Methanobacteriales* are considered tentative partners to SAOB during SAO (Fotidis *et al.*, 2013; De Vrieze *et al.*, 2012; Westerholm *et al.*, 2011a; Schnürer *et al.*, 1999; Paper **III** and **IV**). In addition to hydrogenotrophic methanogens, *Methanosarcinacea* sp. have been detected in several SAO-dominated processes and are considered to be comparatively tolerant to typical stresses, *e.g.* NH_4^+-N, pH and temperature changes (De Vrieze *et al.*, 2012; Karlsson *et al.*, 2012; Westerholm *et al.*, 2012; Hao *et al.*, 2011; Sasaki *et al.*, 2011; Shimada *et al.*, 2011; Westerholm *et al.*, 2011a; Karakashev *et al.*, 2005; Paper **III**). *Methanosarcinacea* have also been suggested to operate as the hydrogen-consuming partner organism during SAO (De Vrieze *et al.*, 2012; Karlsson *et al.*, 2012).

Only a few SAOB have been isolated to date. These are the thermophilic *AOR* (Lee & Zinder, 1988b), *Thermacetogenium phaeum* (Hattori *et al.*, 2000), and *Thermotoga lettingae* (Balk *et al.*, 2002), the thermotolerant *Tepidanaerobacter acetatoxydans* (Westerholm, 2011) and the mesophilic *Clostridium ultunense* (Schnürer *et al.*, 1996) and *Syntrophaceticus schinkii* (Westerholm *et al.*, 2010). Doubling times are generally longer for SAOB when oxidising acetate with a hydrogen-consuming partner than for the

acetotrophic methanogens, especially for mesophilic environments: up to 28 days for *C. ultunense* and 78 days for *Syntrophaceticus schinkii* compared with less than 9 days for different methanogens (Westerholm, 2012a; Demirel & Scherer, 2008). In addition to ammonia, temperature has been identified as being selective for SAO. This is most likely because of the increased standard free energy at elevated temperature during SAO ($\Delta G^{\circ\prime}$ = -31 kJ at 37 ºC (Table 1) and -36 kJ at 60 ºC), but also because the relative fraction of NH_3-N compared with NH_4^+-N increases at higher temperatures and thus increases the inhibitory effect of ammonia on *Methanosaetaceae* (Hansen *et al.*, 1998).

2.3 Optimisation of anaerobic digestion of thin stillage

The overall biogas yield from an industrial production plant is considered to be correlated to the microbial population structure and activity. Two steps, hydrolysis and methanogenesis (Figure 2), are generally identified as bottlenecks that ultimately affect the biogas yield of a digestion plant (Angelidaki *et al*., 2011; Appels *et al*., 2008; Ahring *et al*., 1995). The degradation of substrates with complex structures, *e.g.* lignocellulolytic materials such as straw or materials that have already been microbiologically degraded, such as sewage sludge, is typically rate-limited by the hydrolysis step. On the other hand, methanogenesis is usually the bottleneck for easily accessible and energy-rich substrates such as proteins, soluble carbohydrates and fat. In this case, the build-up of VFA is a common indicator of too slow methanogenesis, leading to elevated hydrogen pressure and product inhibition of the syntrophic oxidation of acids (Angelidaki *et al.*, 2011; Ahring *et al.*, 1995; Zinder, 1984). The aim in any industrial biogas plant should therefore be to increase the rate of the step acting as the bottleneck. In the case of hydrolysis, this is commonly achieved by pretreatment of the feedstock, as reviewed by Carrere *et al.* (2010). Optimisation of the methanogenesis step is more complicated. The hydrogen pressure is, as already described, of the utmost importance for successful anaerobic digestion, so the OLR is often kept at a suboptimal level to avoid the risk of too much hydrogen being produced in the fermentative steps, leading to VFA build-up. However, the suboptimal OLR reduces the amount of gas produced and can thereby compromise the economic performance of the plant. Optimisation of methanogenesis thus involves providing optimal conditions for the methanogens, including temperature, concentration of inhibitory substances, availability of nutrients *etc*.

The bottleneck during anaerobic digestion of thin stillage, which contains readily available organic matter, is most likely methanogenesis. Moreover, the high content of sulphate and incoming nitrogen affect the possibilities to optimise the process, since these factors need to be considered in order to avoid VFA accumulation.

2.3.1 Optimisation using laboratory-scale experiments

Full-scale biogas processes are often not suitable for evaluation of strategies for process optimisation. The organic load, HRT and substrate composition are often variable or difficult to control. In addition, biogas production cannot be risked, in the case the evaluated strategy is unsuccessful. Hence laboratory experiments are generally used, as they typically result in similar gas yields and process performance as in full-scale processes (Grimm *et al.*, 2014; Paper **I**, **III** and **IV**). The experiments are often performed in down-scaled reactors, for example 12 L reactors (active volume 9 L) (Nordell *et al.*, 2011). With laboratory experiments, it is possible to gain better control over factors such as substrate composition, HRT and other environmental factors than at full-scale level. It is also possible to use several reactors in parallel and change single factors and compare the effects. However, there are several factors that compromise the value of the results. First, there is a risk of obtaining too favourable and over-specialised processes compared with full scale when using large batches of the same substrate, resulting in low variation in composition (giving lower uncertainty in the results) together with strictly controlled environmental and operating conditions (*i.e*. HRT, OLR, temperature *etc*.). In the long run, this can lead to overestimation of the optimal OLR and gas production and can create a specialised, even less diverse and perhaps more sensitive microbial population due to low variability of the substrate, operation *etc*. Second, semi-continuous feeding in 24-hour cycles, as commonly applied for practical reasons in laboratory-scale studies when treating feedstock with a high dry solids content, results in occasional (directly after feeding) very high OLR. This in turn results in a higher VFA concentration (and most likely higher hydrogen pressure) and a pH drop during the beginning of the feeding cycle compared with continuous operation. Semi-continuous feeding is hence typically reported to have negative effects on process stability and to affect the methanogenic population, because of unnatural variations in OLR (Conklin *et al.*, 2006). However, recent results suggest the opposite, *i.e.* that higher biogas production can be obtained during semi-continuous feeding regimes, indicating an separation of methanogenic pathways throughout the feeding cycle (Schmidt, 2015; Mulat, 2014; Polag, 2014).

In order to simulate the process accurately in laboratory-scale reactors, selection of inoculum and experiments needs to be carried out carefully. Factors of importance in obtaining accurate results include for example: i) maintaining anoxic conditions to avoid oxidation of H_2S and inhibition of anaerobic microorganisms, ii) leaving at least three HRT before changing experimental conditions, since this is the time needed to reach chemical (and biological) equilibrium in the reactors, iii) using accurate temperature control to avoid ambiguous effects on microbial composition, iv) controlling the active volume in the reactor in order to keep the correct HRT and OLR, and v) analysing the organic content in substrate and digestate correctly (taking VFA levels into account) to obtain correct OLR, VS destruction *etc*.

Accurate volumetric gas production and methane gas measurements are also important for evaluating the performance of the biogas process and should preferably be determined by online measurements. The measurement data may allow kinetic analyses in a semi-continuous mode (Nordell *et al.*, 2013). The kinetic analyses can be used both as an early warning indicator and for evaluation of process stability (Paper **III**). In addition to gas kinetics, process stability can be determined by combining several factors, the most important being methane content, specific methane production, gas kinetics, VFA concentration, alkalinity, trace element concentration, pH, NH_4^+-N, NH_3-N, H_2S and degree of degradation (Boe, 2010; Paper **III** and **V**).

In addition to semi-continuous experiments, biochemical methane potential (BMP) tests can be used as an evaluation tool. BMP tests are mainly used to determine the methane potential of organic materials (Raposo *et al.*, 2011; Angelidaki *et al.*, 2009). In brief, inoculum and substrate are added together with buffer, metals and vitamins to N_2-flushed flasks. The excess pressure as a result of anaerobic degradation and the methane content are measured to calculate the total amount of methane produced from a specific amount of organic matter. BMP tests also indicate if a material is quickly transformed into gas or not. It should be emphasised that the results from BMP tests are restricted in their applicability for continuous biogas processes. For instance, the methane potential from BMP tests is maximum yield. In continuous operation, a fraction (1/HRT) of all material in the digester is removed each day (including fresh substrate), so a part of the substrate added is always washed out. However, this inconsistency is not always the case, and the BMP for thin stillage varies between 0.29-0.32 m^3 CH_4 kg^{-1} VS depending on batch from the bio-ethanol plant *etc*. This can be compared with the specific biogas production of 0.28-0.32 m^3 CH_4 kg^{-1} VS obtained in semi-continuous operation (Paper **III** and **IV**). However, results regarding pretreatment methods, degradation rates or inhibition studies may be used indicatively, since positive

or negative results obtained during BMP tests are also likely to occur during continuous operation, but not necessarily to the same extent.

2.3.2 Optimisation with maintained sulphate reduction

The sulphide produced during sulphate reduction is inhibitory to several anaerobic microorganisms. Neutral H_2S can pass over cell membranes and is therefore the most toxic form. The toxicity of H_2S is also dependent on the pH and temperature. High pH and high temperature force the equilibrium of $H_2S \leftrightarrow HS^- + H^+$ to the right and to the less toxic form of the sulphide (Stams *et al.*, 2003). McCartney and Oleszkiewicz (1993) reported that 50% inhibition of SRB occurred at 85 mg S (as H_2S) L^{-1}, while methanogens have been reported to be inhibited between 50 and 270 mg S (as H_2S) L^{-1}, independent of pH, while lower levels apply for SRB (Oude Elferink *et al.*, 1994).

In addition to the toxicity, metal sulphides have extremely low Ks values, causing large amounts of trace elements such as cobalt (Co), nickel (Ni) and zinc (Zn) to precipitate and thereby become unavailable to trace element-requiring microorganisms (Jansen *et al.*, 2007). This is somewhat problematic in a biogas process, as methanogens are typically dependent on the trace metal level in the feedstock. Several key enzymes and co-factors such as vitamin B12 and F430 require specific metals to function. The most commonly reported trace metals used to optimise biogas processes are Co, Ni, selenium (Se), tungsten (W), Zn and molybdenum (Mo) (Gustavsson *et al.*, 2013; Karlsson *et al.*, 2012; Gustavsson *et al.*, 2011; Feng *et al.*, 2010; Jansen *et al.*, 2007; van der Veen *et al.*, 2007; Zandvoort *et al.*, 2006; Paper **I**).

To avoid these negative effects of H_2S, iron chloride addition is commonly used in full-scale processes (Ek *et al.*, 2011; Paper **I**). The iron (Fe) does not affect the activity of SRB *per se*, but at high iron concentrations most sulphides produced are precipitated as iron sulphide and the cell toxicity and precipitation of trace elements is reduced. Another options to reduce the level of sulphide is to micro-aerate the headspace of the digester in order to obtain oxidation to elemental sulphur or to use other downstream processes to clean the gas, such as ozone treatment, active carbon, microbial sulphide oxidation by *Thiobacillus etc*., but this does not affect the sulphide toxicity in the digester (Ramirez *et al.*, 2011; Van der Zee *et al.*, 2007). Another strategy is to add a combination of iron chloride, hydrochloric acid (HCl) and trace elements in order to optimise anaerobic digestion of sulphur-rich substrates such as thin stillage (Ejlertsson, 2006). When iron precipitates sulphides, it is possible to add necessary trace elements without risking direct precipitation and thus positive effects can be obtained, as indicated in Paper **I**.

However, the use of iron chloride to reduce the effects of hydrogen sulphide allows sulphate reduction to proceed, which leads to: i) the SRB remaining active and consuming electron donors, *i.e*. potential substrate for methanogens, and hence reduced gas yields and ii) the need for a large amount of iron solution to precipitate the H_2S produced, severely affecting the profitability of the biogas plant.

2.3.3 Optimisation at high ammonia concentration

During full-scale operation of the Norrköping plant with thin stillage substrate, the total ammonia level has reached almost 6 g NH_4^+-N L^{-1} on several occasions, with correlated ammonia concentrations of about 0.5 g NH_3-N L^{-1} (Paper **I**). With few exceptions, ammonia inhibition has not caused process disturbance at the plant, despite ammonia levels previously reported to be inhibitory being observed (Rajagopal *et al.*, 2013; Paper **I**). One factor which may explain this is the combined addition of iron chloride and HCl, both of which are acidic, to the full-scale process in the Norrköping plant. By lowering the pH, the fraction of NH_3-N can be reduced and hence combined addition of acidic compounds can be used to deliberately reduce inhibition. In addition, the optimal pH for methanogens is well below what has been observed during anaerobic digestion of thin stillage (typically about pH 8) and enhanced process performance can thus also be expected from reducing the pH (Karlsson & Ejlertsson, 2012; Ek *et al.*, 2011; Paper **I**). Ammonia inhibition can also be restricted by the use of low temperatures (Eskicioglu *et al.*, 2011; Hansen *et al.*, 1998). Eskicioglu *et al.* (2011) showed reduced process stability during thermophilic digestion of whole stillage. Thus it is wise to avoid thermophilic process temperatures when treating nitrous feedstock such as stillage (Paper **I**).

Additional adjustments for high nitrogen levels include applying long HRT in order to reduce the risk of washing out the slow-growing SAOB (Westerholm, 2012a; Hattori, 2008). However, the need for long HRT may be counteracted by addition of necessary trace elements such as Co and Ni to obtain a more efficient SAO-dominated anaerobic process (Karlsson *et al.*, 2012; Paper **I**).

In order to avoid suboptimal operation (low OLR and long HRT), it is of particular interest to find additional alternatives to optimise the methanogenic process in this environment. One possible way to do this is to increase the OLR by concentrating the incoming nitrogen-rich feedstock. In stillage, nitrogen is bound to organic matter as proteins and directly depends on the incoming TS content (Wilkie *et al.*, 2000). SAOB are known to be slow-growing and thus a shortening of HRT might involve an elevated risk of washout (Schnürer *et al.*, 1999). However, by increasing the incoming TS content, the HRT can be kept

long enough for slow-growing SAOB, while at the same time the OLR can be increased and hence potentially result in increased gas production. The effect of increasing the incoming substrate concentration was thus evaluated for thin stillage in (Paper **IV**). The ammonia was successively increased from 0.3 to 1.1 g L^{-1} NH_3-N and this correlated with an increase of OLR from 3.2 to 6.0 g VS L^{-1} d. A control reactor in which only ammonia was increased by external nitrogen addition (urea) was also established. At 1.1 g NH_3-N L^{-1}, process instability was observed irrespective of the OLR applied and was identified as the ammonia threshold for degradation of thin stillage (Paper **IV**). However, until this level was reached, the specific biogas production was maintained, illustrating that elevated ammonia levels combined with high OLR are feasible. Other studies have reported a wide range of maximum ammonia (NH_3) concentrations for anaerobic digestion, but with a highest value of about 1 g L^{-1} (Lauterböck *et al.*, 2012; Hansen *et al.*, 1998; Angelidaki & Ahring, 1993). Additional full-scale benefits of concentrating the ingoing feedstock are: reduced use of fresh water, reduced amount of digestate, *i.e.* bio-fertiliser, to be deposited and also increased bio-fertiliser value by higher outgoing NH_4^+-N concentration in the digestate. In Paper **IV**, changes in the microbial population were observed for both methanogens and acetogens in response to increasing ammonia level, as well as OLR. At all ammonia and OLR levels tested, the methanogens were dominated by *Methanoculleus* within the *Methanomicrobiales*, which has been determined to be a potential SAO partner at high ammonia concentrations (Westerholm *et al.*, 2012; Westerholm *et al.*, 2011a; Schnürer *et al.*, 1999). However, within this genus, different species were found to dominate depending on OLR and ammonia concentration (Paper **IV**). This further underlines the important role of hydrogen consumption by *Methanoculleus* species in ammonia-stressed SAO processes. The acetogenic populations showed clear shifts in response to both increasing ammonia and OLR (Paper **IV**), as reported previously (Westerholm *et al.*, 2011b). The acetogenic group dominating at start-up (*i.e.* lower ammonia concentration) was completely replaced by other groups, including previously observed potential SAOB, with increasing ammonia concentration depending on OLR (Paper **IV**). The clear shifts in both methanogenic and acetogenic populations in response to increasing ammonia levels and maintained methane yields indicate that optimised microbial populations for such processes may be obtained if sufficient adaptation time is allowed. However, the shifts in methanogenic and acetogenic population did not affect the threshold of ammonia inhibition (Paper **IV**), indicating a general inhibition level of ammonia irrespective of the specific microorganisms performing the degradation.

In order to obtain optimal conditions for methanogenesis at elevated ammonia conditions, an alternative temperature to the commonly used mesophilic (35-38 ºC) or thermophilic (50-60 ºC) temperatures could be considered. The known SAOB *T. acetatoxydans* has its highest growth rates at around 42-44 ºC (Westerholm, 2011), and this organism has been observed in processes operating with stillage (Sun *et al.*, 2014; Westerholm *et al.*, 2012). In line with this, Paper **III** showed that the abundance of *T. acetatoxydans* and its potential partner methanogen, *Methanomicrobiales*, increased significantly during a slow increase in temperature from 38 to 44 ºC during anaerobic digestion of thin stillage. This increase also had beneficial effects on biogas production (up to 22% increase), indicating the importance of this particular SAO couple (Paper **III**). Furthermore, this temperature is now being applied in full-scale processes with satisfactory results, including very low VFA levels and high process stability.

The ammonia and ammonium tolerance of SRB has been only sparsely studied, but in Paper **II** we found decreased abundance at elevated concentrations. At ammonia concentrations >200 mg NH_3-N L^{-1}, the SRB abundance (log 5.5 ± 0.2 copies mL^{-1}) was slightly lower than in low-ammonia processes (log 6.0 ± 0.2 copies mL^{-1}). This indicates that one alternative parameter that affects the competition between methanogens, acetogens and/or SAOB on the one hand and SRB on the other could be ammonia. Paper **II** showed that the abundance of SRB was lower at higher temperatures, although thermophilic SRB also exist, such as strains within *Desulfomicrobium, Desulfotomaculum, Thermodesulfobacterium, Thermodesulfovibrio* and archaea *Archaeoglobus* (Rabus *et al.*, 2006; Pender *et al.*, 2004). It is of course still very difficult to obtain out-competition of SRB given the higher growth rates, substrate affinity and diversity among SRB compared with methanogens (Stams *et al.*, 2003; Kristjansson *et al.*, 1982). However, at higher temperatures and/or ammonia concentrations, the methanogenic pathways are indicated to shift towards SAO and hydrogenotrophic methanogenesis (Westerholm, 2012a; Schnürer & Nordberg, 2008). SAOB and SRB do not necessarily compete with each other, since potential SRB could either produce substrate for SAOB (incomplete sulphate reduction) or remove the hydrogen produced by SAOB (complete sulphate reduction). Hydrogenotrophic SRB could possibly be outcompeted by the hydrogenotrophic methanogens and the SAOB *T. acetatoxydans* at elevated temperature and ammonia levels. This competition has not been studied elsewhere and was evaluated in Paper **III** by increasing the temperature from 38 to 44 ºC while treating thin stillage. The abundance of *T. acetatoxydans* and the plausible hydrogenotrophic methanogens surpassed the SRB abundance and the SAOB abundance increased by a factor of 100,

while SRB abundance remained stable (Paper **III**). Despite the expected proliferation of the SAOB/methanogenic couple, sulphate reduction remained high throughout the experiment.

One possible explanation for this result could be the dominance of incomplete oxidising SRB bacteria, as found in the inoculum used for the start-up of the reactors used in Paper **III** (see section 2.2.2). The incomplete oxidising SRB would produce acetate and thus no competition with SAOB or methanogens would occur, with maintained sulphate reduction as a result. Furthermore, SRB could still out-compete the hydrogenotrophic methanogens for hydrogen, resulting in complete sulphate reduction combined with SAO. In addition, on reaching the ammonia inhibition threshold (1.0-1.1 g NH_3-N L^{-1}) for maintained biogas production using thin stillage, sulphate reduction still remained efficient, indicating that there are ammonia-tolerant SRB (Paper **IV**).

2.3.4 Inhibition of sulphate-reducing bacteria

Instead of manipulating the environmental conditions in the biogas process, more direct tools can be used to remove SRB. Undesired sulphide production is not only an issue for biogas production processes, but also for manure storage, sewage systems or other sanitation facilities (Zhang *et al.*, 2008). Consequently, specific inhibition, directed to limit the sulphate reduction, has been interesting for decades and has been well studied. Tekniska verken i Linköping AB adds nitrate, which is an alternative electron acceptor to sulphate and very oxidative, to the sewer system of Linköping city to reduce sulphide production. However, nitrate cannot be used in anaerobic digestion due to negative effects on methane production. Other strategies to decrease the activity of SRB include *e.g.* addition of molybdate (MoO_4^{2-}), which has been evaluated in numerous studies (Peu *et al.*, 2011; Isa & Anderson, 2005; Patidar & Tare, 2005; Nemati *et al.*, 2001; Fukui *et al.*, 2000; Liu & Fang, 1997; Singh & Singh, 1995). Molybdate works as a steric analogue to sulphate, binding to active sites of proteins used for sulphate uptake (Peck, 1959). Most of these experiments have resulted in total inhibition of sulphate-reducing bacteria, but the effect on methanogens has been contradictory, ranging from positive effects to total inhibition, probably depending on differences in inoculum, sulphate:molybdate ratio and molybdate concentration. Evaluation of the effect of molybdate on the Norrköping plant revealed that sulphide production was reduced by 27% at 1.2 mM molybdate and >70% at 2.4 mM or higher molybdate concentrations (Figure 4).

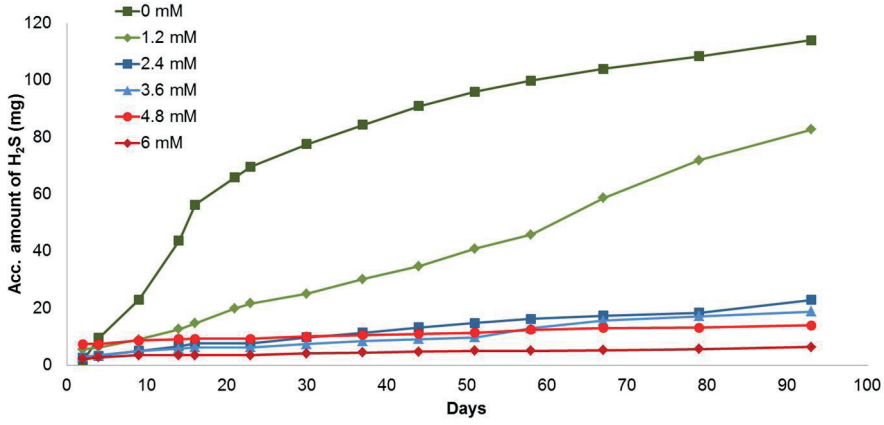

Figure 4. Accumulated amount of hydrogen sulphide (H_2S) during a bio-methane potential (BMP) test investigating the effect of adding different concentrations (mM) of molybdate (MoO_4^{2}) on inoculum from the Norrköping co-digestion plant.

This is in agreement with several other studies on sediments, manure and pure culture, which identified the necessary concentration for successful inhibition of sulphate reduction as 2-5 mM molybdate (Zahedi *et al.*, 2014; Biswas *et al.*, 2009; Predicala *et al.*, 2008; Patidar & Tare, 2005; Scholten *et al.*, 2000). For sulphide production, the IC_{50} was determined to be 1.5 mM according to the one phase exponential decay function (Figure 5).

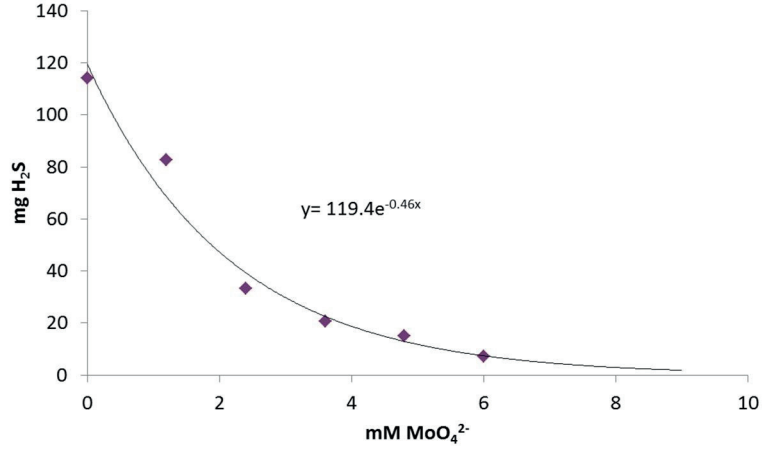

Figure 5. Correlation between hydrogen sulphide (H_2S) production and molybdate (MoO_4^{2-}) concentration in bio-methane potential (BMP) tests. The trend line indicates the one-phase exponential decay of sulphide production.

Increasing molybdate concentration also resulted in decreasing methane production. At 1.2 mM MoO_4^{2-}, the methane yield was reduced by 9.6% compared with the untreated control and this difference increased to 19.9% at 2.4 mM (Figure 6). Isa and Anderson (2005) observed almost complete inhibition of both methane and sulphide production at 2.5 mM, while Scholten *et al.* (2000) observed a stimulation effect by molybdate as the electron flow shifted from SRB to methanogens. The reduced methane yield shown in Figure 6 was most likely due to a biocide effect, as suggested by Isa and Anderson (2005). An alternative explanation is that SRB, which are critical for fatty acid fermentation also in the absence of sulphate, were inhibited and that this led to accumulation of VFAs and reduced methane yield. Contradicting this is the fact that any accumulated VFA should have been converted to methane within the 90-day experiment. The complete reduction of sulphide production, but also negative effects on methanogens, observed in this study is in agreement with Zahedi *et al.* (2014), who studied the abundance of different microbial groups. For full-scale application, a reduction of 10-20% in methane yield is of course not feasible and therefore molybdate addition is not applied in full-scale operations in Sweden or elsewhere.

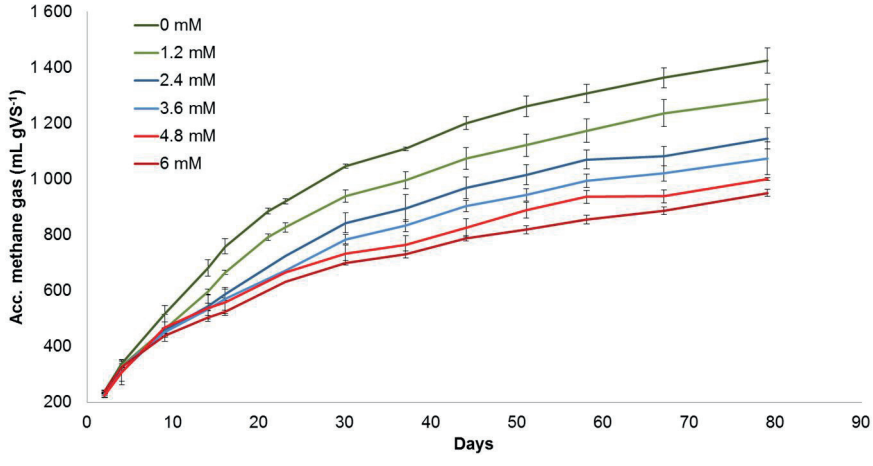

Figure 6. Accumulated methane production in triplicate inhibition bio-methane potential (BMP) tests. Each line corresponds to a different molybdate concentration indicated in the colour key.

2.3.5 Reducing the bioavailability of sulphate

Another approach to avoid the activity of SRB could be to decrease the bioavailability of sulphate. To our knowledge, no studies have previously been carried out on this aspect of anaerobic digestion. The theory is that addition of a precipitating agent could reduce the bioavailable sulphate for SRB, and thus

the amount of sulphate being reduced would decrease. There are a few sulphate salts with relatively low bioavailability. Among those with the lowest solubility in water is barium sulphate ($BaSO_4$; 0.002 g L^{-1} at 20 ºC), but for anaerobic digestion applications calcium sulphate (gypsum) ($CaSO_4$; 2.1 g L^{-1} at 20 ºC) could be considered. Calcium is administered in anaerobic digestion processes for several other reasons, such as for reduction of LCFA inhibition (Kleyböcker *et al.*, 2012; Ahn *et al.*, 2006; Koster, 1987), granulation in UASB reactors (Tiwari *et al.*, 2006; Yu *et al.*, 2001) and stabilisation of extracellular hydrolytic enzymes (Harris *et al.*, 2010; Ramani *et al.*, 2010; Swamy *et al.*, 1994). Addition of $CaSO_4$ would allow for a maximum reduction in free SO_4^{2-} of about 50-70% compared with the incoming concentration in the thin stillage. Hence, the iron addition and electrons going to SRB could theoretically be reduced by the same proportion.

Calcium addition was evaluated using two laboratory reactors as used in (Paper **III**). After 100 days of start-up, iron was replaced by calcium (Figure 7). By adding approximately 1 g L^{-1} calcium to the substrate between days 100 and 300, it was possible to reduce the dose of iron by 40% without increasing the H_2S concentration (Figure 7). However, when the iron dosage was reduced to below 50%, the H_2S concentration started to increase. This was expected according to the solubility discussion above predicting about 50-70% reduced iron requirement. Simultaneously, the alkalinity decreased in the reactor, indicating that precipitation of $CaCO_3$ had occurred. Following the increase in H_2S, iron addition was increased to correspond to a reduction of 40%. The calcium addition was additionally increased in order to regain the low sulphide level and further shift the reaction equilibrium towards $CaSO_4$, but without success. When the calcium addition was increased the free phosphate concentration also decreased, indicating precipitation of ions other than sulphate. It could also be possible that SRB with the ability to utilise precipitated sulphate proliferated, but to confirm this further studies are needed. As Figure 8 illustrates, $CaSO_4$ precipitation in digester material was clearly visible after centrifugation and salts adhered to reactor parts after the experiment.

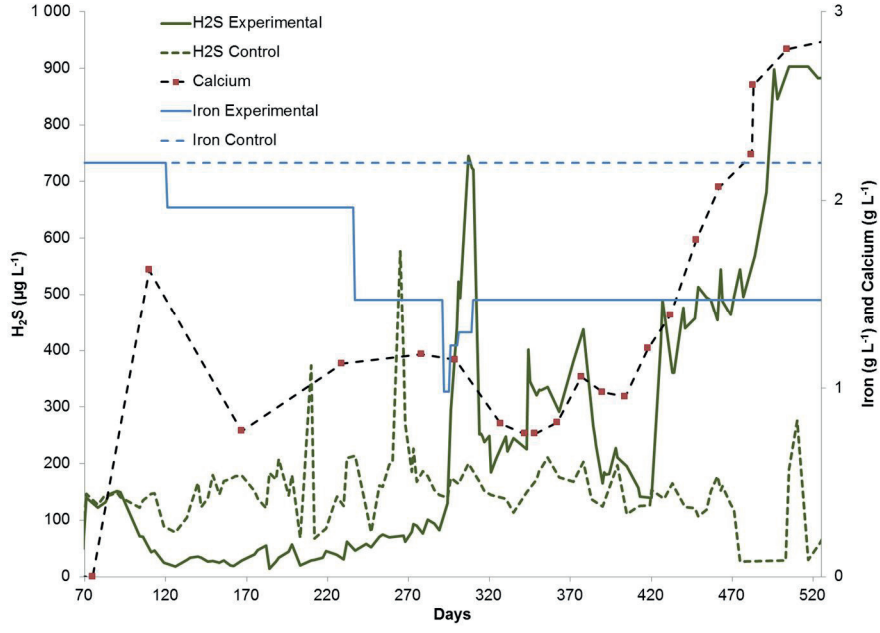

Figure 7. Hydrogen sulphide (H_2S) production in response to iron and calcium addition between days 70 and 520 of digestion.

2.3.6 Optimisation by utilising the sulphate-reducing bacteria

An alternative method, instead of trying to minimise the activity of SRB, is to utilise the superior abilities of SRB in the anaerobic process as suggested by (Paper **V**). Hydrolysis/acidogenesis and acetogenesis/methanogenesis can be separated by using a two-stage process (Park *et al.*, 2008; Yilmaz & Demirer, 2008; Schober *et al.*, 1999). Therefore, SRB could be utilised to ferment VFA and produce sulphides in the first stage, and later methanogenesis would continue in the second stage. The lower pH in the first stage process would allow most sulphides to dissociate with the gas in this stage, and thereby separation of sulphides and methane can be accomplished and the need for iron addition reduced. Moreover, sulphate reduction at low pH (5.5-6.0) has been reported previously (Lopes *et al.*, 2010; Ren *et al.*, 2007; Mizuno *et al.*, 1998). Further positive effects would be that the first stage acidogenesis produces CO_2 (Figure 2), and hence the methane content in the second-stage reactor could increase and the upgrading costs could be reduced. A combined reduction in iron addition and reduced upgrading costs could potentially improve the profitability of sulphate-treating co-digestion plants.

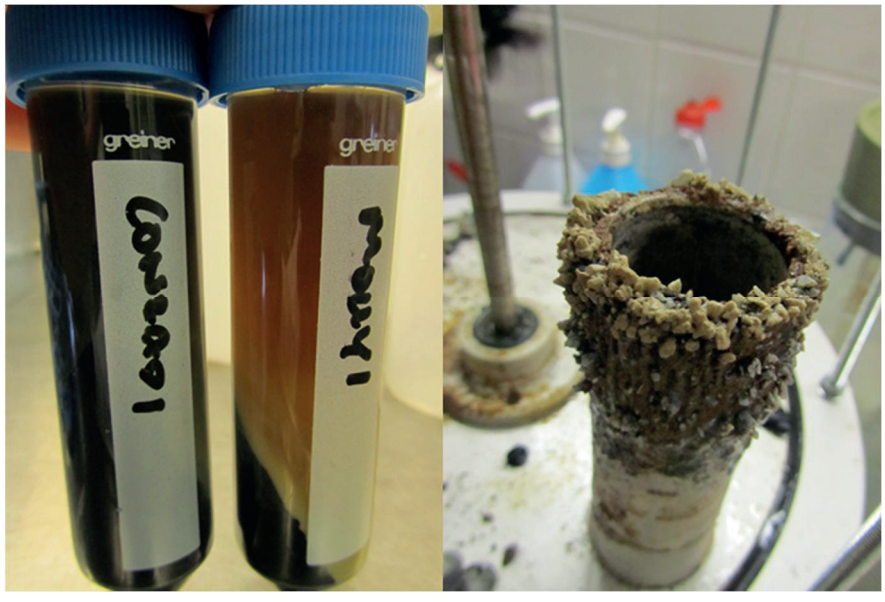

Figure 8. (Left) Tubes of centrifuged reactor materials, where white precipitation after calcium addition can be seen in the right-hand tube. (Right) Precipitate adhering to reactor parts after termination of the experiment.

In the acidogenic stage SRB are efficient VFA degraders (Ren *et al.*, 2007) and less sensitive to H_2 according to their thermodynamic energies, so incomplete oxidisation would result in efficient acetate production and possibly an even more stable acidogenic process. The advantage of incomplete oxidisers compared with complete oxidisers is that acetate could still be converted into methane after sulphate reduction. Separation of the single-stage process of the Linköping and Norrköping biogas plants, which treat OFMSW and a mixture of thin stillage and OFMSW, respectively, was thus evaluated in laboratory-scale reactors in Paper **V**. The substrate mixtures used in the full-scale plants resulted in too low pH (3.8-4.5), lower than observed for maintained sulphate reduction. Consequently, sulphate reduction only occurred in the second stage. The low pH in the first stage was a result of the high OLR following the short HRT needed for separation of the stages and the inherent high VS load (46.9-14.1 g VS L^{-1} d^{-1}). In order to maintain sulphate reduction in the first stage reactor, dilution of the substrate mixture and possibly also chemical addition for pH buffering would be necessary. However, dilution increases the volume of digestate to be transported and chemical addition would reduce the profitability of replacing iron. Hydrogen production in the first stage was low and instead acetate was the major product (Paper **V**), which was most likely caused by homoacetogenesis (Saady, 2013). Using only OFMSW, acetate

production persisted and was an appropriate energy carrier from the first to the second stage reactor, allowing very low energy losses and high methane content (+6%) in the second stage and increased methane yield overall (+12%) (Paper **V**).

3 Conclusions

This thesis confirmed that SRB are present in anaerobic biogas processes. It also revealed that SRB are present in biogas processes irrespective of sulphate concentration and that they can act as fermenters in sulphate-depleted environments. If sulphate is added to such processes, hydrogen sulphide is immediately produced. The sulphides produced by SRB in digestion processes treating thin stillage can be successfully treated by combined addition of iron and trace elements in order to obtain high process stability and clean gas. However, this is a costly measure and, moreover, it does not overcome the loss of biogas following SRB consumption of electron donors.

Studies to identify the conditions needed to decrease the competiveness of SRB compared with methanogens indicated decreased SRB abundance at elevated nitrogen levels and temperatures, but experiments at high ammonia concentrations did not result in decreased SRB activity. Instead, the increased ammonia concentration led to process failure of the methanogenic biogas process beyond an ammonia threshold for maintained process stability of 1-1.1 g L^{-1}. The ammonia threshold was independent of OLR, indicating that high OLR (*i.e.* concentrated feedstock) of thin stillage can be applied in ammonia-stressed processes as long as the ammonia is kept below the threshold value. Applying a high OLR by concentrating the feedstock resulted in higher gas production and is also desirable as comparatively lower amounts of tap water have to be used and a smaller volume of digestate needs to be disposed of. The increasing ammonia concentration resulted in evolution of different acetogenic and methanogenic populations. From the results presented in Papers **III** and **IV**, it is clear that *Methanoculleus* species in cooperation with SAOB proliferate in ammonia-stressed systems treating thin stillage. Interestingly, different species within *Methanoculleus* and SAOB dominate depending on OLR and ammonia level.

However, the possibilities to influence the competition between SRB and methanogens while treating thin stillage proved to be limited. SRB were

resilient to increased temperature and high ammonia concentration and still out-competed the microorganisms responsible for methanogenesis in these environments. Precipitation of sulphate in the form of calcium sulphate (gypsum) to reduce its bioavailability was also unsuccessful.

The methods which proved successful in reducing SRB activity, such as molybdate addition or low pH in the biogas process, had similar effects on methane production. Hence, molybdate or low pH could be used to reduce sulphate reduction in a two-stage process, but not in a one-stage biogas process.

Despite the unsuccessful out-competition of SRB, some of the results from this thesis are already being applied in the full-scale biogas plants operated by Tekniska verken i Linköping AB, fulfilling the aim of identifying optimal operating conditions for methane-producing microorganisms at elevated ammonia concentrations. Anaerobic digestion at 42-44 ºC was found to be a successful method to optimise the SAOB pathway and is now applied at the co-digestion plants with very satisfactory results. After the change of temperature and a consequent adjustment of trace element addition, high process stability has been observed. Another possible approach for commercial application is the two-phase process, which led to elevated methane content and increased biogas production for the Linköping case and could reduce the cost of gas upgrading. The stability of acetogenesis in the first stage of the process will hence be further evaluated at laboratory scale for possible full-scale application.

Overall, this thesis showed that VFA oxidation is important in high ammonia processes to avoid process instability. It is possible that the SRB fill an important function as propionate degraders in the presence of sulphate and thus should not be inhibited.

4 Perspectives

For future full-scale treatment of thin stillage, the most likely alternative is to dilute the stillage with other, sulphur-poor substrates. In this way, the fraction of sulphides being produced per unit of feedstock would decrease and thereby also the amount of iron required per unit volume of clean biogas produced. Moreover, if the sulphur-limited substrate were also nitrogen-depleted, it would be possible to reduce the ammonia inhibition.

Another alternative, not thoroughly evaluated in this thesis, is the separation of calcium sulphate (gypsum) from thin stillage. Sulphate is easily precipitated in pure substrate, but on mixing this with the anaerobic digestion material, several other ions would compete with sulphate for the calcium ion. The operation might also result in dilution of the material, increasing the solubility of gypsum. It would therefore be interesting to evaluate the possibility to fraction out the precipitated gypsum within stillage fractions subsequent to calcium addition. This could possibly be done by centrifugation. Calcium could perhaps be added prior to the conventional centrifugation of whole stillage, resulting in a gypsum-rich stillage and sulphate-depleted thin stillage.

Furthermore, the role of trace elements in the competition between SRB and methanogens has not yet been determined. Since mono-digestion of thin stillage requires trace element addition, addition of particular elements to favour methanogens over SRB would be interesting. Known effective trace elements for methanogenesis, such as cobalt and nickel, did not show such discriminating effects in this thesis. Other possibilities are use of adhesive materials selecting for active methanogenic biomass, for example nylon fibres. However, this would most likely be problematic in high solids degradation as applied in Linköping and Norrköping, due to the risk of clogging *etc*.

Applied research was the main focus in this thesis, so the emphasis on development of new molecular techniques was limited. Therefore, future research evaluating the role of SRB in biogas processes should focus on this subject. If longer PCR products could be obtained from digester samples when

targeting the specific functional gene, more information about the SRB community in anaerobic biogas processes could be obtained. If, for example, the metabolic role of SRB could be mapped with this information together with the physiological characteristics of the SRB detected, it is possible that clues on how to select for methanogens would emerge. Alternatively, there are groups of SRB that mainly oxidise longer VFA to acetate without being sensitive to hydrogen pressure. These SRB could perhaps be desirable in an anaerobic biogas process for process stability (*i.e.* avoiding VFA accumulation) if the sulphides produced could be treated efficiently. In that sense, addition of sulphate could be a way of avoiding cases of process failure due to acid accumulation and imminent pH decrease. A corresponding addition of iron would be needed to avoid negative effects from the sulphides produced. However this would be more efficient if acetate-oxidising SRB were present, leading to complete neutralisation of the acids.

The selection of incomplete oxidising SRB in the hydrolysis/acidogenesis stage of a two-stage process should also be further evaluated. The incomplete oxidising SRB tolerate high hydrogen pressure, so the amount of VFA produced ought to be favoured if these are active. Separation of sulphide and methane formation is still of interest for thin stillage, perhaps in co-digestion with *e.g.* cattle manure to obtain buffered acidic process and avoid too low pH. The sulphides could be oxidised and distributed as elemental sulphur, while the methane produced (with higher methane content and without sulphide inhibition) in the second stage would be cheaper to upgrade and the iron consumption would decrease.

A first-stage process without sulphate reduction could also be beneficial for certain substrates. There are examples of sulphate-rich materials in which hydrolysis is the rate-limiting step, for example lignocellulose-rich materials from the pulp and paper industry. It is possible that hydrolysis of these substrates could be enhanced if the toxic sulphides were removed, as is actually the case for a two-stage process with low pH in the first stage.

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