
PEEP PITK
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Declaration: Hereby I declare that this doctoral thesis, submitted for the doctoral degree at Tallinn University of Technology, is my original investigation and achievement and has not been submitted for the defence of any other academic degree elsewhere.

Peep Pitk

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Proteiini- ja liiendidirikaste tahkete tapamaja-jäätmete anaerobne kooskääritamine: ressursi analüüs ja protsessi optimeerimine

PEEP PITK
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INTRODUCTION

The quickly expanding welfare society of the 21st century has raised into spotlight many environmental issues and concerns about modern society's long-term sustainability. One of the problems is directly related to meat production, consumption and the increasing by-products generation. We are living in the world of limited resources and developing countries enormously expanding demand for meat products will lead to numerous global problems that have to be immediately dealt with or should have been dealt with “preferably already yesterday”. Livestock production requires a vast amount of fresh water and agricultural land for livestock feed production, is responsible for a considerable share of anthropogenic greenhouse gas emissions and has raised significant challenges related to manure management in intensive livestock regions. At the same time slaughterhouses are faced with challenges of liquid and solid wastes treatment in order to avoid environmental pollution, ensure maximum secondary resource recovery and minimise expenditures related to waste treatment and stabilisation. For decades or even centuries animal by-products (ABP) have been used for animal feed production, but due to the European bovine spongiform encephalopathy (BSE) epidemics, this utilization alternative has been significantly curtailed, leading to a demand for alternative methods for ABP valorization.

One of the most promising alternatives for both of the liquid and solid slaughterhouse wastes treatment and valorization is the anaerobic digestion (AD) process. AD of slaughterhouse wastes has been the focus of research for over 30 years, but still there are many questions to be answered in terms of process optimisation and inhibition. Slaughterhouses liquid and solid wastes are lipid- and protein-rich, i.e. they have high energy content, but at the same time lipids and proteins degradation intermediate compounds, long-chain fatty acids (LCFA) and ammonium (NH₄-N), are causing significant operational problems like sludge flotation and wash-out and decreased process efficiency called as “inhibited steady state”. Slaughterhouse wastewater AD has received somewhat more attention in terms of process operation and optimization, for example with the development of a specific lipid-rich wastewater treatment oriented AD technology of the Inverted Anaerobic Sludge Bed Reactor by Alves et al. (2007). Based on the current knowledge of solid slaughterhouse wastes AD, however it has to be admitted that there is a lot more work to be done. Most of the lipids and NH₄-N related toxicity experiments have been carried out with batch or fed-batch systems, with the addition of single substrates or substrate mixtures of LCFA, while NH₄-N concentrations have usually been artificially increased with NH₄Cl addition. This approach is reasonable for a basic process response and inhibition threshold values characterization, but experiments with “real” waste streams have to be carried out in order to understand complex substrate mixture
influence on the synergistic relations and inhibition mechanism of anaerobic microbial consortia. Another shortcoming is that detailed research has been conducted separately for LCFA inhibition and NH₄-N inhibition, meaning that there is a knowledge gap in understanding of the combined effect of high lipid and protein content substrate mixtures on AD process efficiency and inhibition. Another important aspect is related to current European biogas sector development. Biogas production has been over subsidised in many countries in Europe that has on a large extent resulted in development of irrationally expensive technological solutions and extra features, resulting in biogas production prices that are far from being competitive in the free market conditions. This was also one of the reasons, why in the framework of this thesis, it was decided to keep co-digestion process schemes as simple as possible and define the conditions at which process can be operated without sophisticated monitoring and control requirements.

Therefore, the main objective of the current thesis was first to conduct complex investigation of slaughterhouse solid wastes generation, Category 2 and 3 ABP rendering unit mass and energy balances and evaluate sterilization end-products potential for biogas production in AD, based on the example of cattle and swine processing slaughterhouse in Estonia. As sterilization procedure is from my point of view most reasonable treatment option for Category 2 and 3 ABP, the end-products of the sterilization process were chosen as substrates for anaerobic co-digestion experiments. Main aim of the experiments was to evaluate optimal co-substrates addition ratios, LCFA and NH₄-N related inhibition mechanisms and nutrients enriched digestate additional fertilizer value for sewage sludge or manure based co-digestion.
LIST OF PUBLICATIONS

The dissertation is based on the following publications, referred to in the text by the Roman numbers I – III and listed in the appendices with permission from the publishers.


AUTHOR’S CONTRIBUTION TO THE PUBLICATIONS

In *Publication I*, the author planned and performed the experimental work, collected, calculated and interpreted the data, and wrote the manuscript.

In *Publication II*, the author planned and performed the experimental work, collected, calculated and interpreted the data, and wrote the manuscript.

In *Publication III*, the author planned and performed the experimental work, collected, calculated and interpreted the data, and wrote the manuscript.

LIST OF CONFERENCE PRESENTATIONS

1. Pitk, P. Triple benefit of manure and solid slaughterhouse waste rendering products co-digestion: simple concept, increased volumetric biogas production and fertilizer value. *ManuREsource 2013 International conference on manure management and valorization, Bruges, Belgium, 5-6 December 2013 (oral presentation)*


**OTHER PUBLICATIONS & CONFERENCE PRESENTATIONS**


**ABBREVIATIONS**

ABP  animal by-products
AD  anaerobic digestion
BMP  biomethane potential
BOD  biological oxygen demand
BSE  bovine spongiform encephalopathy
CH$_4$  methane
CO$_2$  carbon dioxide
COD  chemical oxygen demand
CSTR  continuous stirred tank reactor
DAF  dissolved air flotation sludge
DS  decanter sludge
EPS  extracellular polymeric substances
FG  floating granules
GHG  greenhouse gas
HRT  hydraulic retention time
kD  kilo Dalton
kW  kilowatt
LCFA  long chain fatty acids
MBM  meat and bone meal
mcrA  the gene coding for the alpha subunit of methyl-coenzyme M reductase
MPR  methane production rate
NH$_4$-N  total ammonium
NH$_4^+$  ammonium ion
NH$_3$  free ammonia
OLR  organic loading rate
SAO  syntrophic acetate oxidation
SAO-HM  syntrophic acetate oxidation - hydrogenotrophic methanogenesis
SM  sterilized mass
SMA  specific methanogenic activity
SRM  specified risk material
SSHW  solid slaughterhouse waste
TAN  total ammonium nitrogen
TF  technical fat
TMP  theoretical methane potential
TS  total solids
VFA  volatile fatty acids
VS  volatile solids
1. LITERATURE REVIEW

1.1 Anaerobic digestion process and its global perspective

Anaerobic digestion (AD) is defined as the biological process that produces a gas mixture (biogas) that contains methane (CH$_4$) and carbon dioxide (CO$_2$) as its primary constituents through the concerted action of mixed microbial population under conditions of oxygen deficiency. Biological CH$_4$ production was first noticed by Volta in 1776, who described the release of CH$_4$ from a swamp (Lyberatos and Pullammanappalli, 2010). AD is a widespread biological process in nature and is an important part of the global organic matter mineralisation cycle. In nature, AD proceeds at wide range of environmental conditions (from psychrophilic to thermophilic temperature range; pH in the range of 4 to 10), utilising vast list of organic matter and proceeding at wide range of reaction rates (Angelidaki et al., 2011) providing a wide variety of options for organic matter valorization on industrial scale. AD has been the process most often used to stabilise wastewaters and biosolids since the early 20$^{th}$ century and is further developed at present (Amani et al., 2010). Nevertheless, just from the end of the 20$^{th}$ century humankind has finally acknowledged the true value of anaerobic fermentation with biogas production as one of the many alternatives of the final conversion of fermentation products contributing to the development of sustainable growth and the concept of circular bio-economy.

AD process has gained increasing importance within the last three decades, since biogas can be produced through biological treatment of wastes and wastewaters with different characteristics (Yenigün and Demirel, 2013). Currently, the technology is evolving from conventional waste treatment i.e. from the removal of pollutants to very intensive biogas production processes using concentrated wastes in the framework of bioenergy production. The production of renewable energy from organic waste streams is definitely one of the important aspects in the concept of sustainable development (de Vrieze et al., 2012). At the same time biogas is also an energy source that helps to reduce anthropogenic greenhouse gas emissions (GHG) if used as a replacement of fossil fuels and it also has many additional positive environmental aspects (Börjesson and Mattiasson, 2007; Weiland, 2010; Amani et al., 2010; Plugge et al., 2010) - for example, reduction of acid rains, global warming potential (Chynoweth et al. 2001) and waste volumes and inactivation of pathogens (Chen et al., 2008). Not less important aspect is the recirculation of stabilised digestate back into the soil as organic fertilizer and soil improver with concurrent reduction of requirement of mineral fertilizers for agriculture (Salminen and Rintala, 2002b; Demirel and Scherer, 2008; Luste et al., 2009). The undigested organic carbon of digestate can be used to enrich the humus content of soils.
(Verstraete, 2010) or be processed into a type of biochar and stored for longer periods of time in the biosphere (Lehmann, 2007).

Biogas formation in AD process is a complex microbiological process requiring combined activity of several groups of microorganisms with different metabolic capacities (Gerardi, 2003) (Figure 1). As performance of an AD system is primarily linked to the structure of the microbial community present in the digester, then operational and environmental parameters of the process affect the behaviour, performance and eventually the fate of the microbial community in anaerobic digesters (Demirel and Scherer, 2008).

**Figure 1. Key steps in the anaerobic digestion process. By Angelidaki et al., (2011).**
Methanogenic communities are generally divided into four different functional groups of bacteria and archaea. Primary fermenting bacteria hydrolyse complex material into substrates for a group of secondary fermenters, also known as syntrophs. The syntrophs obligately depend on two groups of methanogens, one that uses hydrogen, CO₂ and formate (hydrogenotrophic methanogens) and another that uses acetate (aceticlastic methanogens). The growth of the syntrophs is sustainable only through the removal of their waste products by the methanogens, because acetogenesis reactions under thermodynamic standard conditions are endergonic and naturally do not occur (Dolfing et al., 2008; Amani et al., 2010). As seen from Figure 1, the AD process proceeds in the following steps: the primary fermenting bacteria hydrolyse (depolymerize) polymers including proteins, polysaccharides, nucleic acids, and lipids by extracellular enzymes secreted by microorganisms to monomers (such as amino acids, sugars, purines, pyrimidines, and long-chain fatty acids). The same microbial group further ferments monomers to reduced compounds (alcohols, short-chain fatty acids, organic acids, and certain aromatics), hydrogen and CO₂. The reduced products are oxidized to acetate, hydrogen and CO₂ by the secondary fermenting bacteria. Hydrogen and CO₂ are converted to methane by the hydrogenotrophic methanogens whereas methanogenic acetate degradation is carried out by the aceticlastic methanogens (Hattori, 2008; Lyberatos and Pullammanappallil, 2010; Plugge et al., 2010). In the natural environment about two-thirds of the estimated one billion metric tonnes of methane produced each year in the Earth’s biosphere derives from the methyl group of acetate via the aceticlastic pathway (Ferry, 2010). Acetate degradation can proceed through two different pathways: direct cleavage of acetate by aceticlastic methanogens or syntrophic acetate oxidation (SAO) (Zinder and Koch, 1984, de Vrieze et al., 2012; Lü et al., 2013). The third methane formation pathway is methylotrophic methanogenesis where methylated C₁ compounds (methanol, methylamines, methylmercapto-propionate, dimethylsulfide, etc.) are converted to methane. Methylotrophic methanogenesis is limited to species from the order of Methanosarcinales with the exception of Methanosphaera sp. (Angelidaki et al., 2011). Hydrogenotrophic methanogenesis and aceticlastic methanogenesis are considered the key processes within AD as if these processes are inhibited the digestion is effectively blocked at acidogenesis. The organic matter is eventually mineralized to CH₄ and CO₂ through the interactions of all the above mentioned microbial groups. Organisms described as mediating hydrogenotrophic and aceticlastic methanogenesis are found within five phylogenetic orders (Karakashev et al., 2005) and one hyperthermophile Methanopyrales (Table 1). The order of Methanopyrales is represented by only one species Methanopyrus kandleri which is closely related to Methanobacteriales and Methanococcales, but possesses unusual features (Liu, 2010).
Table 1. Main characteristics of the methanogenic orders (Karakashev et al., 2005; Angelidaki et al., 2011).

<table>
<thead>
<tr>
<th>Order</th>
<th>Cell morphology</th>
<th>Physiology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methanobacteriales</em></td>
<td>Rods or filaments</td>
<td>Hydrogenotrophic; mesophilic or thermophilic</td>
</tr>
<tr>
<td><em>Methanococcales</em></td>
<td>Irregular cocci</td>
<td>Hydrogenotrophic; mesophilic or thermophilic</td>
</tr>
<tr>
<td><em>Methanomicrobiales</em></td>
<td>Small rods, irregular cocci, flat oval shaped cells</td>
<td>Hydrogenotrophic; mesophilic</td>
</tr>
<tr>
<td><em>Methanosarcinales</em></td>
<td>Rods or filaments (Methanosaetaceae), irregular cocci or Sarcina-like cells (Methanosarcinaceae)</td>
<td>Strict aceticlastic (Methanosaetaceae), aceticlastic or hydrogenotrophic (Methanosarcinaceae); mesophilic or thermophilic</td>
</tr>
<tr>
<td><em>Methanocellales</em></td>
<td>Rod-shaped</td>
<td>Hydrogenotrophic; mesophilic</td>
</tr>
<tr>
<td><em>Methanopyrales</em></td>
<td>Rod-shaped</td>
<td>Hydrogenotrophic; hyperthermophilic</td>
</tr>
</tbody>
</table>

1.2 Slaughterhouse waste anaerobic digestion

Increasing global demand for meat and dairy products has led to a concurrent increase in slaughterhouse wastest production worldwide. In the European Union slaughterhouse waste treatment and utilization is regulated by the Animal By-Product Regulation EC No 142/2011 (ABP Regulation). Animal by-products (ABP) are defined as bodies or parts of animals and products of animal origin not intended for human consumption, either because they are not fit for human consumption or there is no market for them as foodstuff (European Parliament and the Council, 2011). Slaughterhouses generate a huge amount of strong pollutant solid (e.g. stomachs, fat, viscera and intestines) and liquid (e.g. rust and excrements, purines, blood and wastewater) wastes, which may cause serious environmental problems if not treated properly (Cuadros et al., 2011). The organic wastes of meat industry may account to as much as over 50% of original animal mass (Buendia et al., 2008). SSHW are characterized by high solid content that are mainly composed of proteins and fats with different amounts of carbohydrates and inorganic compounds depending on the waste management and sorting technologies used (Rodríguez-Abalde et al., 2011). In general, slaughterhouse wastes can be divided into two main groups: a) wastewater of high organic content and dissolved air flotation sludge (DAF) from wastewater treatment units, and b) solid slaughterhouse wastes (SSHW) from meat products production.
During previous decades slaughterhouse wastewater treatment using AD has received much more attention than AD of SSHW (Salminen and Rintala, 2002), as SSHW were used to be treated and valorized by a rendering process and utilized for example as high-value animal feed. In recent years, because of epidemics of bovine spongiform encephalopathy (BSE) the utilization alternatives have been restricted and as a consequence the economic value of the SSHW rendering products have been substantially reduced (Kirchmayr et al., 2007; Luste et al., 2009; Palatsi et al., 2011). This has led to intensive research for alternative utilization pathways for SSHW valorization and AD has emerged as one of the possible technologies. AD has been considered one of the best alternatives for nutrient and energy recovery from the SSHW because of the theoretically high methane yields related to the high organics content, mainly composed of proteins and lipids (Hejnfelt and Angelidaki, 2009; Kaparaju et al., 2010; Palatsi et al., 2011). However, the high content of proteins and lipids may also cause inhibition of the digestion process due to high NH$_4$-N/free ammonia (NH$_3$) and long chain fatty acids (LCFA) concentrations accumulation at high loads (Salminen and Rintala, 2002; Edström et al., 2003; Bayr et al., 2012).

In regards to the use of SSHW for AD, the categorization and pre-treatment procedures have to be in accordance with the ABP Regulation. According to the ABP Regulation SSHW are categorized into three groups (Kirchmayr et al., 2007):

- **Category 1**: contains those materials with the highest risk for public health, animals, or the environment (hygienic risk, risk of BSE, etc.)

- **Category 2**: includes all animal by-products that can be allocated neither to category 1 nor to category 3 (e.g., manure or digestive tract content or animals not fit for human consumption)

- **Category 3**: comprises those animal by-products that would be fit for human consumption, but are not intended for human consumption due to the commercial reasons

Category 1 ABP cannot be treated by AD under any circumstances. AD of Category 2 and 3 ABP is acceptable after a) pressurized thermal pre-treatment by sterilization (133 °C, 3 bar and 20 min), or b) hygienization (70 °C for 60 min). In addition also pressurized alkaline pre-treatment is an option. Though manure and digestive tract content are included in Category 2 ABP, these can be used in biogas plants without sterilization (Luste et al., 2009).

In general there are three options for SSHW treatment by AD process:
1) High-rate AD of slaughterhouse wastewater (after primary treatment by dissolved air flotation units and fine screens)

2) On-site AD of all the liquid and solid waste fractions from a slaughterhouse

3) Using different fractions of SSHW (DAF, sterilized mass, meat and bone meal (MBM) etc.) as co-substrates for manure or sewage sludge based anaerobic digesters

High-rate AD of slaughterhouse wastewater is a complex issue with a myriad of alternatives for process optimization, but as the focus of the thesis is on SSHW anaerobic digestion then just brief introduction to the topic is presented here. For slaughterhouse wastewater treatment both aerobic and anaerobic systems can be used, but AD is preferred because significantly lower cost compared to aerobic systems is achieved and the produced biogas can be used as a fuel (Rodriguez-Matrinez et al., 2002). Treatment of lipid-rich wastewaters in anaerobic bioreactors may result in the production of large amounts of biogas as lipids hold a rather high energy potential - about 1 litre CH₄ can theoretically be produced from the complete oxidation of 1 gram of lipids (Sousa et al., 2009).

Slaughterhouse wastewater is usually loaded with solid and dissolved organic substances and characterized, in particular, by fats and proteins and their degradation products, such as volatile organic acids, amines and other organic nitrogen compounds (Tritt and Schuchardt, 1992). Wastewater from the meat industry is complicated to treat because of its specific characteristics, irregular discharge and considerable content of organic, mineral and biogenic matter (Arvanitoyannis and Ladas, 2008). The composition of slaughterhouse wastewater fluctuates in a wide range depending on slaughtered animals, water use and other aspects of daily slaughterhouse routines. Chemical oxygen demand (COD) for example, may vary daily in the wide range of 1.4 to 11.5 g/L (Johns, 1995; Massè and Masse, 2000; Rajeshwari et al., 2000). Usually, before directing slaughterhouse wastewater to high-rate anaerobic reactors, dissolved air flotation has been used to reduce wastewater load of fats, suspended solids and biochemical oxygen demand (BOD) (Mittal, 2006). Despite complicated nature of slaughterhouse wastewater, high-rate AD of slaughterhouse wastewater is in principle a feasible technology with high COD removal rates over 75% (Tritt and Schuchardt, 1992; Johns, 1995; Massè and Masse, 2000; Caixeta et al., 2002; Mittal, 2006; Plugge et al., 2010). However, there are several operational problems (even after application of dissolved air flotation unit) related to slaughterhouse wastewater high lipids, proteins and suspended solids content which can negatively affect process performance efficiency (Rajeshwari et al., 2000). Operational problems are related to sludge flotation, formation of fat scum layers at the surface of the reactors and inhibition/toxicity effects of the
intermediate compounds (LCFA, ammonium etc.) generated during AD of slaughterhouse wastewater (Mendes et al., 2006; Alves et al., 2009).

An interesting option for improving yields of AD of solid wastes is co-digestion. In most cases co-digestion improves the biogas production due to positive synergisms established in the digestion medium and the supply of missing nutrients by the co-substrates (Mata-Alvarez et al., 2000). Usually pretreated SSHW (hygienized or sterilized) have been used as co-substrates for manure or sewage sludge based biogas plants (Edström et al., 2003; Resch et al., 2006; Hejnfelt and Angelidaki, 2009; Luste and Luostarinen, 2010; Ek et al., 2011), but considerable effort has also been put into the optimization of mono-digestion of solely slaughterhouse waste fractions without addition of external substrates (Salminen and Rintala 2002b; Wang and Banks, 2003; Kirchmayr et al., 2007; Buendia et al., 2009; Palatsi et al., 2011; Resch et al., 2011; Bayr et al., 2012; Bayr et al., 2012b). For both of the described co-digestion options, there are similar main process limitations: increased NH₄-N and LCFA concentrations related process inhibition and biogas yield decrease.

Vavilin et al. (2008) have pointed out that for particulate materials, which are difficult to degrade (such as SSHW), hydrolysis must be coupled with the growth of hydrolytic bacteria and this factor can limit the overall degradation rate. AD of protein rich wastes such as animal manure, slaughterhouse wastes, fish processing residues, fermentation residues etc. leads to high NH₄-N concentrations in the fermentation sludge (Lü et al., 2013), while special requirements for NH₄-N tolerance of the microbiota of the AD is required (Ek et al., 2011). This could cause difficulties as AD at elevated NH₄-N concentrations leads to low substrate degradation, reduced CH₄ yields, unbalanced fermentation conditions and odour emissions at the biogas plants (Rajagopal et al., 2013). On the other hand lipids can cause biomass flotation and wash-out as during lipid hydrolysis by extracellular lipases LCFA are produced. These intermediate products have been described as inhibitory species mainly for acetogenic bacteria and aceticlastic methanogens (Rinzema et al., 1994). Attention is also required in terms of sulfide inhibition, as during proteinaceous waste AD sulfide is formed. The increased concentration of sulfides in the digester leads to higher concentrations of corrosive hydrogen sulfide (H₂S) in the biogas and can further lead to sulfide inhibition of the methanogens (Chen et al., 2008; Ek et al., 2011). Besides operational problems, reduced process efficiency means also economical drawback in the production of sellable biogas and AD feasibility (Karlsson and Ejlertsson, 2012).

Solely slaughterhouse waste mono-digestion at short hydraulic retention time (HRT) and increased organic loading rate (OLR) is definitely more challenging than SSHW co-digestion with manure or sewage sludge. For slaughterhouse waste mono-digestion surprisingly mainly NH₄-N related problems have been reported with much less pronounced LCFA related operational difficulties. NH₄-N inhibition review article by Rajagopal et al. (2013) reported that relatively
high N content often excludes the possibility of efficiently treating ABP in their original undiluted form. Karlsson and Ejlertsson (2012) investigated HCl addition for pH reduction of protein rich substrate fed laboratory reactor as a possible solution for the mitigation of the inhibition. They obtained 50% CH₄ yield increase by lowering pH by 0.2-0.4 units from initial value of pH 8, indicating a considerable increase of the microbial ability to utilise the organic material for biogas production. Fe, HCl and trace elements (TE) of Co, Ni, Se, W were used as process additives for pig SHW mono-digestion by Bayr et al. (2012b) and they observed the utmost importance of TE addition for stable and efficient biogas production at increased OLR while addition of Fe and HCl had no significant effect, contrary to results obtained by Karlsson and Ejlertsson (2012). TE addition during AD has been also shown to be important in order to maintain high process efficiency, particularly when digesting N-rich substrates (Gustavsson et al., 2011; Banks et al., 2012; Karlsson et al., 2012). The importance of the addition of the TE for AD process was thoroughly reviewed by Takashima and Speee (1991) and together with the above mentioned studies this clearly indicates importance of each substrate mixture macro- and micronutrients composition for high biogas yield and stability of the AD process. Resch et al. (2011) concluded that the main problem in slaughterhouse wastes mono-digestion was related to high NH₄-N concentration and concomitant process inhibition which could be mitigated by NH₄-N content reduction in the process by, for example, continuous NH₃ stripping or by membrane contactors with sulphuric acid recirculation (Lauterböck et al., 2012). Similar problems were reported by Kirchmayr et al. (2010) that high levels of NH₄-N led to high concentrations of volatile fatty acids (VFA), indicating poor microbiological activity and foaming problems. Proteins and lipids related process limitations and inhibition mechanism in SSHW AD are discussed in more detail in Sections 1.3 and 1.4.

Considerable effort has been made in the characterization and biogas potential measurements of separate fractions of fresh slaughterhouse waste streams (Tritt and Schuchardt, 1992; review by Kirchmayr et al., 2007; Hejnfelt and Angelidaki, 2009; Luste et al., 2009; Palatsi et al., 2011; Zhang and Banks, 2012; Yoon et al., 2014) and also many different pre-treatment alternatives have been applied to enhance the biodegradability of SSHW. Pre-treatment alternatives applied for SSHW have been thermal, ultrasound, base and acid treatments, enzyme and bacterial products addition and saponification (Masse et al., 2001; Masse et al., 2003; Mendes et al., 2006; Battimelli et al., 2009; Luste et al., 2009; Luste et al., 2011; Affes et al., 2013). Nevertheless, one of the most comfortable and highest hygienic risk minimizing treatment option for slaughterhouses generating Category 2 and 3 ABP is sterilization (133 °C, 3 bar and 20 min) that also fulfils all the requirements prescribed by ABP Regulation. Along possible treatment strategies for Category 2 and 3 ABP, the dry-rendering process via sterilization is one of the most established technologies. In the dry-
rendering process SSHW are pressurized and heated to temperature between 115 °C and 145 °C. As a result, moisture evaporates, freeing fat from protein, disintegrating the bones and adding an extra log reduction factor for pathogens and BSE control. The fat cells open due to changes in the cell walls of the tissue as moisture evaporates (Meeker, 2006). Mass of the sterilized products is reduced approximately by half with moisture evaporation, reducing significantly handling and transportation costs for slaughterhouse waste treatment facilities. At the same time it has to be acknowledged that high temperatures applied to different types of organic waste can produce compounds that are recalcitrant, toxic and/or inhibitory for anaerobic processes - these compounds are products of Maillard reactions, where carbohydrates react with amino acids to form melanoidines, which are difficult to degrade (Martins et al., 2001; Bougrier et al., 2008; Rodríguez-Abalde et al., 2011; Tampio et al., 2014).

Effect of thermal pre-treatment on SSHW has been investigated by many researchers. Edström et al. (2003) have reported that hygienization of SSHW led to a fourfold increase in the potential CH₄ yield compared to non-pasteurized SSHW, suggesting that this was due to an increased accessibility of lipids for microorganisms. Luste and Luostarinen (2010) evaluated the hygienization effect on slaughterhouse wastes co-digestion with sewage sludge and reported hygienized feed to be at higher extent hydrolysed and more easily degradable. In addition, 30% higher biogas production was obtained for hygienized substrate compared to non-hygienized substrate at exactly the same process conditions. Rodríguez-Abalde et al. (2011) have reported sterilization of SSHW mixture to result in higher biogas potential and increased CH₄ production rates compared to pasteurized and untreated piggery SSHW mixture, as sterilization produced significantly better thermal particles disintegration and hydrolysis, releasing more readily biodegradable compounds. At the same time poultry SSHW mixture (lower lipids and higher carbohydrates content) pasteurization resulted in decreased CH₄ production rate and marginally increased CH₄ yield, which was mainly proposed to be related to the presence of carbohydrates and the possible occurrence of Maillard reactions. To the contrary, Hejnfelt and Angelidakī (2009) have shown that thermal treatment at 70 °C, sterilization at 133 °C and treatment by addition of 50 or 100 g NaOH/L had no significant effect on the biodegradability and methane yields based on VS of mixed pork waste. It was assumed that pre-treatment had no effect primarily because the by-products were inherently easily degradable and inoculum was adapted to substrate composition. This was indicated by a short lag phase and a high biogas yield close to theoretical yield for untreated mixed pork waste.

In order to obtain complex understanding of slaughterhouse waste treatment sustainability, also environmental and economic aspects have to be considered: materials flow, energy balance and resource efficiency of the slaughterhouses needs to be investigated. It has to be emphasised that the composition of slaughterhouse waste varies considerably. In some regions there are centralized
and specialized slaughterhouse facilities processing only one or two animal species, whereas in other regions small slaughterhouses handle several animal species (Alvarez and Linden, 2008). The first detailed analysis of liquid and solid wastes compositions and flows in modern slaughterhouses was carried out by Tritt and Schuchardt (1992). Detailed description of slaughterhouse waste flows is presented in Figure 2. Even though AD was considered as an alternative for variety of slaughterhouse waste streams treatment, AD of end-products of rendering unit (animal carcass plant on Figure 2) was not considered as an option. This is mainly because the rendering end-products like meat and bone meal (MBM) were generally used at that time as high value animal feed. Following the epidemics of BSE, the use of MBM as a low-cost food for fish and animals has been progressively prohibited within and outside of the European Union (Wu et al., 2009) and this has urged the industry to look at other valuable valorization possibilities - one of them being AD. Edström et al. (2003) have evaluated slaughtered animal based ABP production and calculated total biogas derived energy potential from waste generated during slaughter of one cattle and pig to be about 360 kWh and 39 kWh, respectively. Recently, Yoon et al. (2014) carried out a material and energy recovery investigation for poultry slaughterhouse. They reported high amount of nitrogen recoverable as plant nutrients and 35.4 m³ CH₄ per 1000 slaughtered heads as recoverable energy through AD. The described examples confirm the valorization of SSHW wastes using AD to be an interesting option for slaughterhouses not only from a sanitation point of view, but also to increase degradability and energy recovery.
Figure 2. Materials flow of liquid and solid residues and wastes in slaughterhouses, and possible disposal system by Tritt and Schuchardt (1992).
1.3 Anaerobic digestion of lipids and proteins

1.3.1 Degradation of lipids and long chain fatty acids inhibition

Lipids are extremely hydrophobic organic molecules that do not dissolve in water (Sanders, 2001; Gerardi, 2003) and are composed of LCFA bonded to glycerol, alcohols or other groups by an ester or ether linkage (Affes, 2013). Lipids, natural oils, and fats are abundantly present in nature. Lipids are constituents of membranes of bacteria, archaea, and eukaryotes, while oils and fats are storage compounds for carbon and energy in all kinds of living organisms (Sousa et al., 2010). Fats and oils are a subgroup of lipids that have the alcohol groups esterified with fatty acids, predominantly in the form of triglycerides (glycerol backbone with three LCFA). Fats contain saturated LCFA, and oils are normally composed of unsaturated fatty acids, which confer lower melting temperatures (Alves et al., 2009). LCFA are carboxylic acids that normally contain an even number of carbon atoms – usually from 12 to 24. The carboxylic acid contains an aliphatic tail (chain), which is either saturated or unsaturated, depending on the absence or presence of double bonds. LCFA are responsible for approximately 90-95% of the original lipid COD (Hanaki et al., 1981; Sousa et al., 2009; Affes, 2013). The qualitative composition of LCFA in fats is variable and highly dependent on the origin of the lipid source. However, palmitic acid, stearic acid, and oleic acid are the most abundant saturated and unsaturated LCFA present in lipid-rich wastes and wastewaters (Hwu et al., 1998; Alves et al., 2009; Sousa et al., 2009; Battimelli 2010; Affes, 2013).

During AD lipids are initially hydrolysed by extracellular lipases produced by fermentative bacteria, meanwhile LCFA and glycerol are released into the fermentation media. LCFA and glycerol are further biodegraded by syntrophic acetogenic bacteria into VFA and H2, which are further converted into biogas (Schink, 1997; Cavaleiro et al., 2001; Lalman and Bagley, 2002; Gerardi, 2003; Battimelli et al., 2009; Palatsi et al., 2010; Hunter et al., 2011). LCFA are converted to acetate and H2 by obligate hydrogen-producing acetogens (Schink, 1997). Glycerol is specifically fermented to various types of alcohols, VFA, and formate (Nielsen and Ahring, 2006). LCFA biodegradation occurs through sequential steps: (1) LCFA adsorption to the cell surface, (2) LCFA uptake, and (3) LCFA conversion to lower molecular weight components via β-oxidation (Sousa et al., 2010). The degradation of LCFA takes place through the β-oxidation pathway, but as these reactions are at standard conditions (1 M concentration, or 10^5 atm for gases) thermodynamically unfavorable (Table 2), then β-oxidation is highly dependent on syntrophic cooperation with hydrogenotrophic archaea or sulphate-reducing bacteria during fatty-acid degradation (Hanaki et al., 1981; Lalman and Bagley, 2002; Sousa et al., 2009). Therefore the function of methanogens is to consume H2 and decrease its steady-state pressure to (10^-4–10^-5 atm), in order to make the overall substrate conversion thermodynamically favorable. Another
important factor in syntrophic interactions is the distance between the H\textsubscript{2} producing acetogens and the H\textsubscript{2} consuming methanogens - the shorter the better (Plugge et al., 2010). To date, 14 syntrophic fatty acid degrading bacteria have been obtained in pure culture or in coculture with H\textsubscript{2} consuming microorganisms, but LCFA higher than lauric acid (i.e. with more than 12 carbon atoms) are utilized only by seven syntrophic bacteria: *Syntrophomonas sapovorans, S. saponavida, S. curvata, S. zehnderi, S. palmitatica, Thermosyntrophus lipolytica*, and *Syntrophus aciditrophicus* (Sousa et al., 2009; Sousa et al., 2010).

Although lipids have high biogas yield and are attractive substrates for anaerobic co-digestion, there are many operational concerns related to lipids use and in practice the AD of lipids is often hampered as the theoretical methane production is not easily achieved (Neves et al., 2009). AD of high lipid content wastes and wastewaters have been reported to cause inhibition of aceticlastic bacteria and methanogens, substrate and product transport limitation, sludge flotation and biomass washout due to the adsorption of lipids/LCFA onto the biomass. This all leads to digester foaming, blockages of pipes and pumps, and clogging of gas collection and handling systems (Hanaki et al., 1981; Rinzema et al., 1994; Alves et al., 2009; Hunter et al., 2011; Zhang et al., 2013; Salvador et al., 2013; Sousa et al., 2013). The sensitivity of bacteria to LCFA is related to their cell wall structure with gram-positive species and methanogens being more easily inhibited than gram-negative organisms (Roy et al., 1985). Hanaki et al. (1981) reported that LCFA produced by the hydrolysis of neutral fats inhibited both the $\beta$-oxidation of LCFA themselves and CH\textsubscript{4} production, although neutral fats itself were not inhibitory. As oleic acid has a good solubility, it is recognized as one of the most toxic LCFA (Cavailero et al., 2001). Later on, LCFA $\beta$-oxidation has been confirmed as the rate-limiting step of the whole lipids AD process (Lalman and Bagley, 2002; Shin et al., 2003; Kim et al., 2004).

For many years the mechanism of inhibition by LCFA was ascribed to permanent cell wall damage and bactericidal effects (Angelidaki and Ahring 1992; Rinzema et al., 1994), but more recent research has suggested that LCFA toxicity is not permanent and that LCFA do not exert a bactericidal effect on methanogens (Pereira et al., 2004; Alves et al., 2009; Palatsi et al., 2010). Although metabolic inhibition of LCFA may also occur, the important feature is that the metabolic or physical effect that is behind a temporary decrease in the methanogenic activity is a reversible phenomenon, which is eliminated after the mineralization of the biomass-associated LCFA (Pereira et al., 2005). Studies by the same group have also shown that inhibition is most probably linked to transport limitation, such as product diffusion limitation, for example biogas release (Pereira et al., 2004; Pereira et al., 2005). In addition, Pereira et al. (2005) have also proposed three mechanisms for biomass-associated LCFA accumulation: (1) adsorption of LCFA onto the sludge that can affect transport and the protective functions of the bacteria cell wall, and
Table 2. Gibbs free energy changes for some of the acetogenic and methanogenic reactions (presumably) involved in syntrophic conversion of different fatty acids (Sousa et al., 2009).

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Equation</th>
<th>( \Delta G^0 ) (kJ per reaction)*</th>
<th>( \Delta G' ) (kJ per reaction)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acids oxidation reactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleate (C18:2)</td>
<td>Linoleate(^-) + 16H(_2)O (\rightarrow) 9acetate(^-) + 14H(_2) + 8H(^+)</td>
<td>+272</td>
<td>-215</td>
</tr>
<tr>
<td>Oleate (C18:1)</td>
<td>Oleate(^-) + 16H(_2)O (\rightarrow) 9acetate(^-) + 15H(_2) + 8H(^+)</td>
<td>+338</td>
<td>-177</td>
</tr>
<tr>
<td>Stearate (C18:0)</td>
<td>Stearate(^-) + 16H(_2)O (\rightarrow) 9acetate(^-) + 16H(_2) + 8H(^+)</td>
<td>+404</td>
<td>-139</td>
</tr>
<tr>
<td>Palmitate (C16:0)</td>
<td>Palmitate(^-) + 14H(_2)O (\rightarrow) 8acetate(^-) + 14H(_2) + 7H(^+)</td>
<td>+353</td>
<td>-124</td>
</tr>
<tr>
<td>Butyrate (C4:0)</td>
<td>Butyrate(^-) + 2H(_2)O (\rightarrow) 2acetate(^-) + 2H(_2) + H(^+)</td>
<td>+48</td>
<td>-22</td>
</tr>
<tr>
<td><strong>Methanogenic reactions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogen</td>
<td>H(_2) + 1/4HCO(_3)(^-) + 1/4H(^+) (\rightarrow) 1/4CH(_4) + 3/4H(_2)O</td>
<td>-34</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>Acetate(^-) + H(_2)O (\rightarrow) HCO(_3)(^-) + CH(_4)</td>
<td>-31</td>
<td>-</td>
</tr>
</tbody>
</table>

*Gibbs free energies (at 25°C) calculated at standard conditions (solute concentrations of 1M and gas partial pressure of 10\(^5\) Pa).

**Gibbs free energies (at 25°C) for fatty acids concentrations of 1mM, considering acetate stoichiometric accumulation (9mM, 8mM or 2mM for linoleate/oleate/stearate, palmitate and butyrate degradation, respectively) and H\(_2\) depletion to a partial pressure of 1 Pa.

form a hydrophobic layer of LCFA around biomass aggregates. This phenomenon considerably reduces exchange between the media and the “encapsulated” bacteria; (2) entrapment of LCFA in biomass aggregates, that can lead to biomass flotation in the reactor and, as a consequence, to biomass leakage; (3) precipitation of the LCFA with divalent ions such as Ca\(_{2+}\) or Mg\(_{2+}\) makes them inaccessible to anaerobic biomass and hence reduces their biodegradability (Girault et al., 2012). These findings have been approved by scientific community and main research focus has been directed to detailed investigation of LCFA inhibition mechanisms, to find possible countermeasures and stimulate prevailing syntrophic acetogens and methanogens at different process conditions and lipids loadings. For example, Palatsi et al. (2010) used mixture of oleate, sterate and palmitate (40:10:50 on weight base) as a feed for LCFA pulse loads for manure based AD in order to investigate anaerobic biomass response to increased LCFA loadings, reporting recovery capacity of \(\beta\)-oxidizing bacteria and syntrophic methanogens after initial LCFA accumulation, while no significant microbial community shift occurred.
Sousa et al. (2007) have reported an oleate-degrading community being able to rapidly degrade palmitate, which is obvious as palmitate is a key intermediate in oleate degradation. However, the consortium enriched with palmitate, degraded oleate only poorly. All bacteria that degraded unsaturated fatty acids also degraded saturated fatty acids, but the opposite was not the case. In addition, Sousa et al. (2008) have confirmed that oleate/palmitate-degrading cultures showed different microbial composition, concluding that the community structure in a reactor might depend on the saturation degree of the LCFA-feed and that not all the β-oxidative degraders have the ability to degrade both saturated and unsaturated LCFA. Palatsi et al. (2012) have also confirmed palmitate to be the main intermediate of oleate degradation in non-adapted sludge, while for LCFA adapted biomass palmitate as intermediate was detectable in marginal amounts, which was further confirmed by similar behavior observed by Cavaleiro et al. (2009).

Hwu et al. (1998) have described LCFA inhibition mechanism in four steps: first, after a LCFA-pulse or biomass exposure, the LCFA rapidly disappears from the aqueous phase and is adsorbed onto the solid phase. Second, depending on the initial LCFA-pulse concentration, the LCFA-concentration could increase in the aqueous phase as a consequence of desorption mediated by the initial methane produced. Third, the LCFA concentration decreases in the aqueous phase as a consequence of the biological degradation of the adsorbed LCFA. Finally, methane is ultimately recovered once the remaining adsorbed LCFA concentration is reduced. Palatsi et al. (2010) have proposed LCFA inhibition kinetics within the IWA ADM1 model framework, suggesting that adsorption plays an important role in the overall LCFA inhibition adaptation process and that there is a need to introduce modifications in IWA ADM1 model (Batstone et al., 2002) if dealing with the degradation of lipids. Integrating all of the previous knowledge, Zonta et al. (2013) have developed a model for LCFA inhibition mechanism for granular sludge based process regarding the adsorptive nature and transport limitations of LCFA, the new insights on microorganisms involved in β-oxidation process and the possible membrane damage caused by LCFA exposure. Results from the application of the two proposed models confirmed that the aceticlastic methanogens population is more sensitive to the LCFA inhibition than the acidogenic population. In addition it was evident that distribution of saturated/unsaturated LCFA degraders play an important role in the system evolution.

From the viewpoint of LCFA inhibition of methanogens both aceticlastic and hydrogenotrophic methanogens have been reported to be affected by LCFA, but aceticlastic methanogens have been found to be more severely affected than hydrogenotrophic methanogens (Hanaki et al., 1981; Alves et al., 2001; Pereira et al., 2005; Palatsi et al., 2010; Palatsi et al., 2011; Baserba et al., 2012; Sousa et al., 2013). Sousa et al. (2013) have reported hydrogenotrophic methanogens to be more resistant to LCFA inhibition than aceticlastic methanogens, but also different resistance to LCFA toxicity was observed in comparison between Methanobacterium formicicum and Methanospirillum hungatei related to their cell
membrane composition. For aceticlastic and hydrogenotrophic methanogens unsaturated LCFA oleate was reported to be more toxic than saturated LCFA of palmitate and stearate, but toxic effect of oleate was definitely more severe for aceticlastic methanogens. It is important to note that these results were obtained in pure culture experiments. In many of the studies anaerobic methane producing consortia have been reported to be able to adapt to lipids/LCFA rich feed as both aceticlastic and hydrogenotrophic methanogenic activities were increasing during continuous exposure to LCFA-rich feed. Based on the results of Cavaleiro et al. (2009) and Salvador et al. (2013) methanogens were able to adapt to oleate rich substrate mixture within few oleate feeding-reaction cycles and achieved up to 98% of the CH\textsubscript{4} yield (based on COD balance), with OLR as high as 11.5 kg COD/m\textsuperscript{3} day. The dominant methanogens evolved during the reactor operation were hydrogenotrophic Methanobacterium formicicum and aceticlastic Methanosaeta concilii. At the same time dominance of aceticlastic methanogen Methanosaeta concilii can be the result of the artificial oleate based substrate used and low N loading of process, because conversion from Methanosaeta concilii dominated inocula to Methanosarcina siciliae dominated consortia after pulse loadings of lipid- and protein-rich slaughterhouse wastes was reported by Palatsi et al. (2011). This is also one of the studies focused on real lipid-rich wastes inhibitory mechanism clarification, concluding that during batch AD tests of SSHW lipids had a limiting effect on the global process kinetics. It was concluded that hydrolytic-acidogenic bacteria did not limit the substrate degradation and the process was held at the acetogenic and methanogenic stage. Despite the severe inhibition at the highest substrate doses (15 g COD/L), the system was able to recover methanogenic activity and finally to degrade the substrate by an adaptation phenomenon related to an enrichment of both specific eubacterial (proteolytic and \(\beta\)-oxidative) and archaeal populations.

1.3.2 Protein degradation and ammonium/ammonia inhibition

Proteins are complex compounds of high molecular weight that can vary from 14.6 kD to 250 kD. In sludge they are found in solution as soluble microbial products, but also attached to the solid particles as extracellular polymeric substances (EPS). Due to the size of proteins microorganisms produce exoenzymes (proteases or peptidases) to break down the proteins into amino acids and subsequently absorb them into their cells to utilize the carbon source. Amino acids are converted to organic acids, H\textsubscript{2} and CO\textsubscript{2} once inside the cells, which are then released along with NH\textsubscript{4}-N into the bulk phase (Gerardi, 2003; Shanmugam and Horan, 2009). Even though NH\textsubscript{4}-N is an essential nutrient for bacterial growth it also has a significant role in the performance and stability of AD of N-rich organic feedstock - at optimal concentrations it ensures sufficient buffering capacity of methanogenic media in AD by increasing the stability of the digestion process and counteracting VFA accumulation. However, on the other hand NH\textsubscript{4}-N has regularly been reported as the primary cause of digester failure because of its direct inhibition
of microbial activity (Sung and Liu, 2003; Calli et al., 2005; Chen et al., 2008; Hejnfelt and Angelidaki, 2009; Prochazka et al., 2011; Rajagopal et al., 2013; Yenigün and Demirel, 2013).

Many pathways for NH$_4$-N inhibition have been proposed such as change in intracellular pH of methanogens, an increase of maintenance energy requirements and inhibition of the specific enzyme reactions (Wittmann et al., 1985). NH$_4^+$ and NH$_3$ are the two principal forms of inorganic ammonium nitrogen (NH$_4$-N) in aqueous anaerobic process (Rajagopala et al., 2013). The NH$_3$ fraction of NH$_4$-N is positively correlated with increased pH and temperature, is a toxic component and has been shown to be inhibitory to most microorganisms involved in the biogas process (Hansen et al., 1998; Chen et al., 2008). Since the NH$_3$ has been suggested to be the actual toxic agent, an increase in pH would result in increased toxicity (Borja et al., 1996). The NH$_3$ fraction of NH$_4$-N can be calculated by the following equations as described by Körner et al. (2010):

$$\text{NH}_3\, (\%\, \text{of} \, \text{NH}_4\text{-N}) = \frac{100}{1+10^{(pK_a-pH)}} \quad \text{Eq. 1}$$

$$pK_a = 0.09108 + \frac{272.92}{(273.2+T)} \quad \text{Eq. 2}$$

Beside pH, temperature and the initial concentration of NH$_4$-N, the important factors determining the extent of NH$_3$ inhibition are organic loading rate and acclimatization of inoculum, which both have a direct or indirect effect on inhibitory concentrations threshold values for different AD systems (Yenigün et al., 2013). NH$_3$ and NH$_4$-N toxicity on AD has been of specific interest for more than half a century with pioneering studies published in 1960s. The beneficial effect of NH$_4$-N at concentrations of 50-200 mg/L and toxic effect of NH$_4$-N over 3000 mg/L was already reported by McCarty (1964). Comprehensive overviews of ammonium/ammonia inhibitory effect on AD in batch incubations, laboratory co-digestion experiments and full-scale process monitoring trials have been published in recent years (Kirchmayr et al., 2007; Chen et al., 2008; Kirchmayr et al., 2010; Rajagopal et al., 2013; Yenigün et al., 2013). NH$_4$-N inhibition threshold values have been reported to be in a wide range of 0.8 to 14 g/L depending on the process conditions, origin of inocula and previous adaption of inocula to elevated NH$_4$-N concentration etc.

Inhibition of the AD process has usually been indicated by a decrease in the steady state CH$_4$ production rates and an increase in the intermediate digestion products, such as VFA concentrations (Sung and Liu, 2003; Calli et al., 2005; Chen et al., 2008; Moestedt et al., 2013). Depending on the origin of the inocula 150-770 mg NH$_3$/L have been reported to be inhibition inducing NH$_3$ threshold concentrations, with syntrophic propionate oxidizers activity inhibition and accumulation of propionate in the effluent as the best indication of process disturbance (Calli et al., 2005; Nielsen et al., 2007). Angelidaki and Ahring (1993) investigated NH$_4$-N/NH$_3$ effect on manure based CSTR systems at thermophilic
temperature with NH$_4$Cl used for NH$_4$-N concentration increase at stable OLR. It was concluded that it was possible to obtain a stable digestion of manure with NH$_4$-N concentrations exceeding 4 g/L after an initial adaptation period. However, the CH$_4$ yield was lower (approx. 25% lower than for uninhibited reactors) and the VFA concentrations were higher than in reactors with a lower NH$_4$-N load. This phenomenon is called “inhibitory steady state” and extent of the process performance decline is dependent on the reactor setup and process conditions.

Sprott and Patel (1986) claimed that adaptation to elevated NH$_4$-N concentrations results in the selection of resistant methanogens already present in seed sludge rather than in adaptation through genetic changes of initially sensitive methanogens. In regard to methanogens, aceticlastic methanogens higher sensitivity to NH$_4$-N toxicity has been confirmed by Angelidaki and Ahring (1993) and Hansen et al. (1998). In addition, Borja et al. (1996) reported a higher sensitivity of the aceticlastic methanogens compared to the hydrogenotrophic methanogens. The specific growth rate for the aceticlastic methanogens was halved at NH$_4$-N concentrations of 4 g/L compared to 7.5 g/L for the hydrogenotrophic methanogens. In comparison of aceticlastic methanogens, Methanosarcinaceae has been reported to be more resistant to higher NH$_4$-N concentrations than Methanosaetaceae (Sprott and Patel, 1986). A more detailed overview about methanogens resistance and sensitivity to NH$_4$-N inhibition and related dominant methanogenic pathway shifts is given in the next section in connection with syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis dominance in anaerobic digesters at elevated NH$_4$-N concentrations.

Regarding NH$_3$ concentration and inhibition threshold value calculations Hafner and Bisogni (2009) have concluded that in anaerobic digesters NH$_3$(aq) exists in equilibrium also with other forms of nitrogen besides NH$_4^+$, including carbamate (NH$_2$COO$^-$) and the mineral struvite (MgNH$_4$PO$_4$·6H$_2$O) which indicates that simple equilibrium calculation (Eq. 2) (without corrections for non-ideal behavior) substantially overestimates NH$_3$(aq) concentration for all but dilute digesters. Inclusion of an estimate of the activity coefficient for NH$_4^+$ in the simple equilibrium calculation results in much more accurate estimates of NH$_3$(aq) concentration. This explains why most of the manure based co-digestion experiments have reported significantly higher NH$_3$ inhibition threshold values compared to sewage sludge digestion experiments and NH$_3$ toxicity incubation assays. Nielsen et al. (2008) have suggested that in non-ideal solutions such as manure, the concentration of hydrogen ions [H$^+$] is not the same as the activity of hydrogen ions H$. In order to calculate ionic strength corrected concentration dependent ionization constants, the same study recommended that individual ion activities in each acid/base and buffer equilibrium must be calculated to correct the thermodynamic ionization constants. On the other hand it has to be accepted that the analytical capacity required for continuous estimation of NH$_4^+$ activity coefficient for manure based CSTR experiments is laborious, expensive and in most cases irrational. Nevertheless, effect of the NH$_4^+$ activity coefficient has to be acknowledged and considered in the interpretation of the experimental results. In
addition, Kirchmayr et al. (2010) have concluded that NH₃ is a function of NH₄-N and VFA concentrations, pH, CO₂ and the digester temperature, suggesting that high concentrations of NH₃ should be accompanied by high concentrations of VFA, which act as acid counter ions to the NH₄⁺ and therefore are necessary to keep values of NH₃ low. Thus high concentrations of VFA are not an indicator for severe process instabilities in the context of high NH₃ concentrations, but the process of AD of high nitrogen containing substrates may be described as equilibrium between the alkaline ions of NH₄⁺ and the acid groups of VFA. Based on above statements NH₃ calculations according to Eq. 1 are in manure based reactors overestimated and the related NH₃ concentrations cannot be used for assessment of exact inhibitory threshold values of NH₃. However, it is still definitive that increased NH₄-N concentrations over optimal levels for methanogens induce VFA accumulation leading to biogas yield decrease, malodorous effluent composition (restricting its land application) and increased GHG emissions if proper post-treatment is not applied.

1.3.3 Syntrophic acetate oxidation and aceticlastic methanogenesis competition

Syntrophic acetate oxidation (SAO) is the metabolic pathway of methanogenesis from acetate by the coculture in which acetate is first oxidized to CO₂ and H₂ by one organism, while H₂ is subsequently used by a second organism to reduce CO₂ to CH₄ (Zinder and Koch, 1984). The existence of SAO pathway was first hypothetically proposed by Barker in 1936, but, it was first experimentally reported to be possible by Zinder and Koch in 1984 (Hattori, 2008). As acetic acid is associated with the lowest levels of energy released from the fatty acids degraded in syntrophic co-operations, the partial pressure of H₂ has to be maintained below 10⁻⁴ atm for this reaction to proceed. Under standard conditions, acetate oxidation coupled to CH₄ formation releases only small amount of energy (ΔG°' = -31.0 kJ/mol) (Zinder and Koch., 1984; Schnürer et al., 1996; Hattori, 2008) (Table 3).

Table 3. Reactions involved in acetate and hydrogen metabolism (Hattori et al., 2008).

<table>
<thead>
<tr>
<th>Process</th>
<th>Reaction</th>
<th>ΔG°' (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Aceticlastic methanogenesis</td>
<td>*CH₃COO⁻ + H₂O → *CH₄ + HCO₃⁻</td>
<td>-31.0</td>
</tr>
<tr>
<td>(2) Syntrophic acetate oxidation</td>
<td><em>CH₃COO⁻ + 4H₂O → H</em>CO₃⁻ + 4H₂ + HCO₃⁻ + H⁺</td>
<td>+104.6</td>
</tr>
<tr>
<td>(3) H₂- consuming methanogenesis</td>
<td>4H₂ + HCO₃⁻ + H⁺ → CH₄ + 3H₂O</td>
<td>-135.6</td>
</tr>
<tr>
<td>(4) sum of (2) + (3)</td>
<td><em>CH₃COO⁻ + H₂O → H</em>CO₃⁻ + CH₄</td>
<td>-31.0</td>
</tr>
<tr>
<td>(5) H₂ consuming acetogenesis</td>
<td>4H₂ + 2HCO₃⁻ + H⁺ → CH₃COO⁻ + 4H₂O</td>
<td>-104.6</td>
</tr>
</tbody>
</table>

(*) represent the fate of the methyl group carbon of acetate. It was assumed that 100% of the labeled carbon was converted to CH₄ (reaction 1) or HCO₃⁻ (reaction 4).
So far only six SAO bacteria have been reported, three mesophilic: *Clostridium ultunense* strain BST (Schnürer et al., 1996), *Syntrophaceticus schinkii* (Westerholm et al., 2010) and *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011a) and three thermophilic: *Thermacetogenium phaeum* strain PB, *Thermotoga lettingae* strain TMO and strain AOR (Fotidis et al., 2013; Lü et al., 2014). Syntrophic associated partners to SAO bacteria are hydrogenotrophic methanogens, for example *Methanocucllus* (Schnürer et al., 2013), *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* (Karakashev et al., 2006) or aceticlastic methanogens belonging to family *Methanosarcinaceae* with ability to utilize different substrates under various conditions (Plugge et al., 2010).

In general terms SAO can be considered as a competitive pathway to the generally recognized prevailing acetate cleavage pathway to CH₄ and CO₂ by aceticlastic methanogenes. Aceticlastic methanogenesis is an exergonic reaction (ΔGo° = -31.0 kJ/mol), thus theoretically it proceeds in the absence of other reactions such as hydrogenotrophic methanogenesis. Until now only two aceticlastic methanogens are known - *Methanosarcinaceae* and *Methanosaetaceae*. *Methanosarcinaceae* has a higher growth rate (0.6 d⁻¹) and yield coefficient, high half-saturation coefficient and is dominating at higher acetate concentrations (<15 g/L), while *Methanosaetaceae* has lower growth rate (0.2 d⁻¹) and lower yield coefficient, low half-saturation coefficient and is dominating at lower acetate concentrations (< 3 g/L) (Jetten et al., 1992; Ferry, 1993; Conklin et al., 2006; Hattori, 2008; De Vrieze et al., 2012). As there are distinct differences between *Methanosarcinaceae* and *Methanosaetaceae* – the former being more resistant to changes of process conditions (e.g. NH₄-N concentration increase) with the ability to consume variety of substrates and change metabolism in accordance, while *Methanosaetaceae* is able to consume solely acetate in the narrow concentration range and at optimal process conditions. Karakashev et al. (2005) monitored methanogenic communities in 15 biogas plants and concluded that *Methanosaetaceae* was dominant in sewage sludge digesters at low VFA and NH₄-N concentrations, while *Methanosarcinaceae* was dominating in manure digesters at higher level of NH₄-N (above 1.5 g/L) and VFA concentrations. The same was documented also by Sprott and Patel (1986) - CH₄ formation by *Methanosaeta concilii* was completely inhibited at NH₄-N level of 560 mg/L while methane formation by *Methanosarcina barkeri* was not inhibited even at NH₄-N level of 2800 mg/L. *Methanosarcina sp.* has been reported to be tolerant to NH₄-N concentrations up to 7 g/L, salt concentrations up to 18 g Na⁺/L, a pH shock of 0.8–1.0 units and acetate concentrations up to 15 g COD/L (de Vrieze et al., 2012). The reason for *Methanosarcinaceae* higher resistance to VFA inhibition is related to its ability to form multicellular aggregates. *Methanosarcinaceae* resists inhibition by VFA, NH₃ and H₂S because of a slower diffusion rate of the ionic compounds inside the *Methanosarcinaceae* aggregates. This may also explain why the inhibiting NH₃ concentrations of aceticlastic methanogens differ significantly (Demirel and Scherer, 2008; Vavilin et al., 2008). Single coccus shaped
*Methanosarcina* cells were shown to form large multicellular clusters at NH$_3$ concentrations over 150 mg/L providing resistance against NH$_3$ inhibition up to 700 mg/L. At higher NH$_3$ concentrations inhibitory effect was detected via VFA accumulation, simultaneously with *Methanosarcina* aggregates disintegration (Calli et al., 2005).

In regard to the robustness of *Methanosarcinaceae* compared to delicate *Methanosaetacea*, the competition between aceticlastic methanogenesis and the syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) pathway is in principal limited to competition between multitalent *Methanosarcinaceae* and bacteria carrying out SAO-HM. As methanogenesis from acetate (aceticlastic methanogenesis or SAO-HM) in general is the most important step for the biogas production process (Fotidis et al., 2013) considered to be a precursor of two-thirds of the CH$_4$ produced in anaerobic bioreactors (Sprott and Patel, 1986; Jetten et al., 1992), the detailed understanding of environmental factors influencing dominance of each of the pathways is of the utmost importance. The main parameters influencing aceticlastic methanogenesis competition with the SAO pathway have been reported to be concentration of NH$_4$-N, NH$_3$, pH, temperature, dilution rate, acetate concentration, VFA and synergetic stress of VFA and NH$_4$-N (Schnürer et al., 1999; Fotidis et al., 2013; Lü et al., 2013; Sun et al., 2014).

Numerous batch incubations, laboratory fed-batch experiments and full-scale anaerobic digesters monitoring campaigns have been carried out and reported in order to investigate process conditions inducing changes from aceticlastic methanogenesis to the SAO-HM pathway in the anaerobic reactors. Schnürer et al. (1999) have reported SAO pathway dominance in the full-scale reactors containing high levels of salts (mainly NH$_4$-N and potassium) and VFA, but with the closest correlation to high NH$_4$-N concentrations. In the same study, the main synergistic hydrogenotrophic methanogenic partner identified for SAO pathway was belonging to genus *Methanocuellus*. In addition to high NH$_4$-N (up to 4 g/L) and VFA (up to 50 mmol/L) concentrations, Karakashev et al. (2006) reported that the absence of *Methanosaetacea* in the digesters correlated with SAO occurrence in full-scale anaerobic digesters. The variety of hydrogenotrophic methanogens was much wider in the anaerobic reactors with the presence of SAO pathway: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinacea* and uncultured archaea were found in the same study. Schnürer and Nordberg (2008) conducted semi-continuous lab-scale experiments with the results indicating shift from aceticlastic methanogenesis to SAO-HM pathway at NH$_4$-N concentrations > 3 g/L and it was claimed to be induced by increasing NH$_4$-N concentration that resulted in consecutive VFA accumulation. In agreement with the idea that the NH$_4$-N inhibitory effect is associated with NH$_3$ content (Chen et al., 2008), the pathway switch occurred at NH$_3$ concentrations above 128-330 mg/L. At the same time, in the enrichment culture at 7g NH$_4$-N/L methane was produced solely via SAO-HM, while at 6 g NH$_4$-N/L also aceticlastic methanogenesis was still present (although in minor extent). Similar results have been obtained also by Westerholm et al. (2012)
in semi-continuous laboratory experiments with increasing protein load. Their results confirmed switch from aceticlastic methanogenesis to SAO-HM pathway at NH4-N concentrations > 3 g/L and determined at least 40-45 days of HRT as a necessity for maintaining stable process in SAO-HM dominated process conditions. Enrichment with SAO bacteria was carried out in the same experimental reactors in order to increase acetate conversion capacity, but it had no noticeable effect on the process performance. Results in line with the above conclusions were also obtained by Zhang et al. (2013) who conducted batch incubation tests at increased NH4-N concentrations. They concluded that methanogens in anaerobic sludge have a strong mcrA transcriptional response to NH4-N stress without a change in the community structure. At inhibitory NH4-N conditions even though mcrA composition analysis showed that the structure of the methanogen community remained highly stable, with Methanosetaecae dominating the methanogen community in all incubations, then the composition of mcrA transcripts, however, showed a substantial response to the addition of ammonium. The relative abundance of Methanosetaecae transcripts declined with increasing amounts of ammonium, whereas the transcript level of Methanobacteriales mcrA was relatively resistant.

Compared to the studies described above a step further was made by Fotidis et al. (2013) who conducted batch incubation experiments to compare stepwise and sudden acetate and NH4-N concentrations increase (up to 9 g/L acetate and 7 g/L of NH4-N) inhibitory effect on thermophilic and mesophilic suspended sludge inocula from full-scale operated biogas plants. The stepwise acetate concentration increased acclimatization process developed in all the cultures favorable conditions for aceticlastic Methanosarcinaceae sp. to entirely outcompete other acetate consumers such as SAO bacteria and Methanosetaecae sp. and the same happened with stepwise increased NH4-N concentration cultures, with completely inhibited thermophilic cultures at NH4-N concentrations > 5 g/L. Sudden increase of NH4-N concentration in incubation experiments resulted in a change from aceticlastic methanogenesis to SAO-HM pathway with more diverse community of methanogens. In addition, batch incubation series at 55°C for investigation of VFA and NH4-N synergistic effects on aceticlastic methanogenesis versus SAO-HM pathway dominance was carried out by Lü et al. (2013) with granular inocula. They concluded that the total dominance of SAO-HM pathway was achieved only if NH4-N concentration was 6-7 g/L and acetate at the value of 15 g/L, but concurrently both SAO and hydrogenotrophic methanogens activities were also inhibited with more severe effect on SAO. At lower acetate concentrations there was also presence of aceticlastic methanogenesis, but at significantly reduced rate. In the other way round, there was dominance of aceticlastic methanogenesis at 1-4 g NH4-N/L, even at acetate concentration of 15 g/L. From both of the studies one general conclusion was made that Methanosarcinacea is really a multitalented methanogen being able to shift its methanogenic pathway from aceticlastic methanogenesis to hydrogenotrophic methanogenesis as the levels of VFA or NH4-N increased. However, the results reported by Lü et al. (2013) showed that there were also limits
for survival of *Methanosarcinacea* – it could not survive at higher concentrations than 6-7 g NH₄-N/L and acetate of 250 mmol/L, even if they were functioning via hydrogenotrophic methanogenesis pathway. In these conditions they would be eventually substituted by hydrogenotrophic methanogens.

Most comprehensive study has been published recently by Sun et al. (2014), who carried out full-scale biogas plants process monitoring and correlation to SAO bacteria abundance. SAO-HM pathway was dominant in all co-digestion plants with NH₃ concentrations between 0.16 and 0.82 g/L, which is in line with most of the results described above. The multivariate approach supported the findings in the individual analysis and showed a tendency for the abundance of *C. ultunense*, *S. schinkii* and *T. acetatoxydans* to be positively correlated with $^{14}$CO₂/$^{14}$CH₄ ratio, NH₄-N and VFA. Contrary to previous results, a wide range of HRT (20-110 days) in the reactors did not allow to accept HRT as a factor influencing SAO. This is partly in conflict with the conclusions made by Moestedt et al. (2013) and Westerholm et al. (2012) claiming at least 40-60 days were required for stable digestion process and 40-45 days HRT were necessary for stable lab-scale semi-continuous reactor operation, respectively.

From a somewhat different viewpoint Hao et al. (2011) and Hao et al., (2012) studied the role of SAO-HM pathway in acetate stressed (6 g/L) batch experiments at thermophilic temperature without ammonium inhibition. At slightly acidic conditions (initial pH 6.0-6.5) acetate conversion was primarily initiated via aceticlastic pathway, while at pH=5.5 SAO-HM was the dominant pathway initiating methanogenesis during aceticlastic methanogens severe inhibition. These results further confirmed SAO-HM pathways utmost importance in AD systems operation and viability at non-optimal conditions. As SAO-HM pathway is dominating methane production from acetate under inhibitory conditions, the more in depth understanding of different process conditions effect is required in order to improve biogas plants volumetric production and guarantee stable digestion process at increased organic loading rates.
2. AIMS OF THE DISSERTATION

The main aim of the dissertation research was to investigate slaughterhouse rendering facility (treating Category 2 and 3 ABP-s) sterilization unit energy and mass balance in relation to sterilization process end-products valorization possibilities and limitations via AD. In terms of AD process optimization and efficiency increase, rendering facility lipid- and protein-rich end-products were used as real waste streams representing co-substrates for sewage sludge or cattle manure based AD processes.

The specific aims of the study were the following:

- evaluate cattle and swine slaughterhouse waste production quantities, rendering facility energy consumption and mass balance; analyse rendering process end-products composition and biogas potential (Publication I)
- calculate rendering process end-products energy content in relation to raw slaughterhouse waste quantities treated (Publication I)
- evaluate sterilized mass advantages and disadvantages as co-substrate for anaerobic digestion process: sewage sludge and sterilized mass co-digestion positive synergistic effects and process limitations/inhibition (Publication II)
- study effect of lipids rich technical fat and lipids + proteins rich decanter sludge addition to dairy manure based anaerobic digestion process-volumetric biogas production increase versus LCFA and ammonium inhibition (Publication III)
3. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used are available in Publications I to III.

3.1 Slaughterhouse waste rendering unit description and sterilized SSHW sampling (Publication I)

Rendering process in Rakvere Meat Processing Plant included crushing, dry rendering, pressing, decanter centrifuging, separation and milling for MBM mixture. As a first step, Category 2 and 3 SSHW with TS content of 40-50% were disaggregated (particle size < 50 mm) in a crusher and transported to the cookers where it was treated at 133 °C, 3 bar for 20 minutes. Condensation water from dry rendering was directed to wastewater treatment unit buffer/mixing tank. During dry-rendering approximately 45% of the raw material was converted to sterilized mass and 55% of it was evaporated and directed to wastewater treatment. Sterilized mass samples were taken from dry-rendering reactor right after batch cycle, were cooled down to room temperature and kept at 4 °C until use. Sterilized mass was further separated to MBM, technical fat and decanter sludge. Samples of separated fractions were stored under the same conditions as sterilized mass. Primary wastewater treatment in rendering facility was flocculation with NaOH + Fe₃SO₄ and subsequent flotation in order to remove fat rich suspended solids from the wastewater.

Dairy manure samples were collected from Rahinge dairy free barn farm (Ilmatsalu, Estonia) and stored at 4 °C during the whole experiment. In order to avoid mixing problems in laboratory reactors long pieces of silage and fibres in manure were separated by using a screw press. Inoculum used as seed for the lab reactors was obtained from pilot scale (200 L) CSTR operated with the manure from the same origin at mesophilic conditions.

3.2 Biomethane potential and specific methanogenic activity measurements (Publications I and II)

BMP and methane production rate (MPR) measurements were carried out in duplicate or triplicate and in accordance with the protocol proposed by the International Water Association Task Group for the Anaerobic Biodegradation, Activity and Inhibition Assays (Angelidaki et al., 2009), with 1.2 L OxiTop-C (WTW, Weilheim, RFA) respirometric system. The working volume of the prepared batch assays were 200 ml. Substrate and inoculum were added to each assay at substrate to inoculum ratio (S/I) of 0.25-0.5 (on VS basis). Bottles were flushed with N₂ gas for 1.5 minutes and closed with airtight stoppers to maintain
anaerobic conditions. Prepared assays were incubated at 37.5 °C for 35-42 days and maintained under continuous mixing conditions.

For specific methanogenic activity (SMA) measurement acetate and H₂/CO₂ (80:20) gas mixture were used as substrates to monitor reactors aceticlastic and hydrogenotrophic methanogenic activities. SMA measurements were carried out at mesophilic temperature (37 °C) with S/I ratio of 0.25 and inocula concentration of 2.5 g/L. Automatic Methane Potential Test System (Bioprocess Control Sweden AB) was used for aceticlastic SMA measurements. Hydrogenotrophic SMA was carried out with batch anaerobic vials (120 mL total volume vials with 50 mL of media working volume).

Biogas composition was analysed with gas chromatograph (Model 3700 with thermal conductivity detector and PorapakQ column 1.8 m x 3.17 mm) and operation conditions were as follows: oven temperature 60 °C and detector temperature 130 °C.

3.3 Fed-batch reactor experiments (Publication II and III)

Process performance and CH₄ yield monitoring experiments were carried out in anaerobic fed-batch reactor systems. Reactors were made of plexiglas (5 L total capacity) with a working volume of 4.5 L. Reactors were operated at 37 ± 1°C, by hot water circulating through the reactors water jacket using intermittent mixing conditions (160 rpm, 15 min on/off) by magnetic stirrer (MAG MS7, IKA, Germany). Substrate feeding was performed once per day. Biogas production in reactors was continuously measured with an on-line milligascounter (MGC-1 V3, Ritter®, Germany). Temperature in the reactors was monitored daily, while pH and methane content in produced biogas (%CH₄) was measured weekly. Effluent characteristics were analysed at the end of each loading period, according to Analytical methods section in Publications II and III.

3.4 VFA and LCFA analysis (Publications II and III)

VFA analysis was performed using a GC 2014 ATF/SPL (Shimadzu, Japan) gas chromatograph equipped with a Zebron ZB–WAXplus capillary column (35m x 0.25 mm x0.25 μm) and flame ionization (FID) detector.

Total LCFA (C12 to C24) were determined according to the method described by Palatsi et al. (2009) based on direct methylation-extraction procedure. LCFA were identified and quantified by a GC CP-3800 gas chromatograph (Varian, USA), fitted with a CP7489:CP-Sil 88 FAME capillary column (50m * 0.25mm * 0.2µm) and FID detection (Publication II).

Total LCFA in reactor effluent samples of Publication III were analysed according to the following procedure: total lipids were extracted from each sample by a modified Folch procedure (Folch et al., 1957), using
dichloromethane/methanol mixtures (2:1, v/v). Lipids were esterified with acetyl chloride/methanol. Fatty acid methyl-esters (FAME) were analysed using an Agilent 6890A chromatograph (Agilent Technologies Inc, USA), equipped with a FID detector and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 μm film thickness).

3.5 Floating granules sonication (Publication II)

A Model S-250 D sonicator (Branson Sonic Power Co., USA) equipped with a titanium horn disruptor working at a constant operational frequency of 20 kHz was used for sonication of floating granules (FG). Aliquots (45.8 mL) of FG solution (0.13 g FG and 44.29 g deionised water) were treated in the lab-scale sonicator in glass vials of 120 mL. The treatment was done in duplicate at ambient temperature (20 ºC). Sonication was performed at three levels: low (10 x10³ kJ/kgTS), medium (100 x10³ kJ/kgTS), and high (200 x10³ kJ/kgTS). The energy was applied with power amplitude (β) of 12-25-50% and time exposure between 5-190 seconds, which led to three energy densities (0.5; 1.1; 2.2 W/mL), respectively.

3.6 Calculations

The theoretical CH₄ potential at standard conditions (STP, 0 ºC and 1bar) was estimated according to the following equation (Eq. 3), based on the protein, lipid and carbohydrate contents of the substrates, as suggested by Angelidaki and Sanders (2004):

\[
CH_4 \text{ yield (L CH}_4 \text{ kg/VS)} = 496*X + 1,014*Y + 415*Z \quad \text{Eq. 3}
\]

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

The unionised fraction of the ammonia nitrogen (NH₃) was calculated according to the following equation (Eq. 4), as described by Körner et al. (2001):

\[
NH_3 \text{ (% of TAN)} = 100/(1+10^{pKa-pH}) \quad \text{Eq. 4}
\]

\[
pKa =0.09108+272.92/(273.2+T) \quad \text{Eq. 5}
\]

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

Ecological footprint of meat industry is very high due to the high water and feed consumption for livestock production. Remarkable GHG emissions and eutrophication problems are aggravated by excessive leaching of nutrients from inefficient manure management systems and wastewater and solid waste treatment systems of slaughterhouses. Considering predictions of continuous global human population increase and the quickly growing developing countries desire to reach the European and North American copious lifestyle, then resource capacious meat production and derived by-products efficient and environmentally friendly treatment, reuse and valorization are the main challenges to be faced by the humankind. Acknowledging these global problems, the current thesis was focused on characterization of solid slaughterhouse waste (SSHW) composition and detailed evaluation of mass and energy balances of SSHW conventional dry rendering technology with special emphasize on optimization of the anaerobic co-digestion processes as a highly efficient valorization pathway for the treatment of SSHW.

The thesis can be divided into three parts:

1) Detailed investigation of conventional industrial slaughterhouse (processing cattle and swine) waste production, with a specific focus on the analysis of rendering facility (treating Category 2 and 3 ABP) sterilization process mass and energy balance.

2) Characterization and analysis of the sterilization process and slaughterhouse wastewater treatment solid end-products (sterilized mass (SM); fractionated products of technical fat, decanter sludge, meat and bone meal (MBM) and (DAF)) composition and measurement of their biomethane potentials. An assessment of Estonian meat industry waste products energetic potential via AD was carried out.

3) Study and optimization of AD of slaughterhouse rendering facility sterilization process end-products. Investigation of the potential increase of volumetric biogas production in the co-digestion processes of lipid- and protein-rich substrates with sewage sludge or manure and evaluation of the limiting and inhibitory mechanisms. Specifically NH₄-N and LCFA related AD process operational constraints were studied using conventional low-rate laboratory CSTR at mesophilic temperature.
4.1 Evaluation of solid slaughterhouse waste resource and energy production potential (Publication I)

4.1.1 Quantities, composition and fertilizer value of slaughterhouse wastes

The first objective of the study was to collect data for the general overview of Estonian meat industry market, on slaughterhouses by-products production and applied ABP treatment technologies. In 2009, slaughterhouses in Estonia produced 22,709 tons of by-products from which the Category 1 wastes share was only 7.8% of the total amount (Table 4). Sterilization as the rendering procedure was used in all of the three ABP treatment facilities in Estonia and the similar structure of the ABP treatment sector has been maintained until the year 2014. Two of the largest meat processing companies have been rendering their own Category 2 and 3 slaughterhouse wastes on-site and the remaining ABP waste stream collected from all over Estonia is being treated in governmental ABP treatment facility mixed together with Category 1 ABP-s. The referred solution causes significant problems because if Category 1 ABP are mixed with the other category waste streams, then

Table 4. Animal by-products from Estonian slaughterhouses in 2009.

<table>
<thead>
<tr>
<th>Amount, t/year</th>
<th>Cattle</th>
<th>Pig</th>
<th>Poultry</th>
<th>Total by-products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 3</td>
<td>7,055</td>
<td>5,906</td>
<td>4,350</td>
<td>17,311</td>
</tr>
<tr>
<td>Category 2</td>
<td>2,049</td>
<td>1,184</td>
<td>400</td>
<td>3,633</td>
</tr>
<tr>
<td>Manure/digestive</td>
<td>1,965</td>
<td>1,143</td>
<td>0</td>
<td>3,108</td>
</tr>
<tr>
<td>tract content</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Category 1</td>
<td>1,765</td>
<td>0</td>
<td>0</td>
<td>1,765</td>
</tr>
<tr>
<td>Total</td>
<td>10,869</td>
<td>7,090</td>
<td>4,750</td>
<td>22,709</td>
</tr>
</tbody>
</table>

the whole stream has to be treated as Category 1 specified risk material (SRM). As only 25% of the wastes originating from slaughterhouses, treated at the governmental ABP treatment facility are classified as Category 1 ABP, then remaining 75% of the waste stream is also converted into SRM. SRM has to be burned in special incinerators that are currently not present in Estonia. Instead of the efficient regional solution for energy and resource recovery, the handling of ABP is turned into additional expenditure. Finding efficient solutions for SSHW valorization is of utmost importance, because after processing more than half (57%) of the slaughtered cattle bodyweight is considered as slaughterhouse by-product. The corresponding value for pigs is 19.8%, meaning that significant proportion of potentially edible (proteinaceous and high energy content) feed or raw materials are just considered and treated as wastes.
SM and DAF are the main solid waste streams from the slaughterhouse rendering unit and wastewater treatment dissolved air flotation unit, respectively. SM is especially interesting co-substrates for AD, because of its high TS and nutrient content (Table 5), which enables it to be transported for much longer distances than substrates like liquid manure (low energy content, large volumes). Of equal importance is also the high macro- and micronutrients content of the digestate, end-product of SM anaerobic co-digestion, which can be recirculated back to the land as organic fertilizers. One ton of SM has 14 times higher fertilizer value compared to the dairy manure with 11.5% TS, 35.7 kgN/ton TS and 12.9 kgP/ton TS (Table 5). Thus, co-digestion of dairy manure and SM increases nutrient concentrations in digestate and can significantly reduce digestate requirements per hectare of agricultural land. Obviously, digestate additional fertilizer value related to the use of sterilized SSHW is depending on the peculiarities of the specific regions – distribution of nitrate sensitive areas and agricultural land availability etc., so the feasibility of the use of sterilized SSHW in AD has to be carefully analysed taking into account the regional circumstances.

Table 5. Characteristics of the sterilized solid slaughterhouse waste, flotation sludge and dairy manure.

<table>
<thead>
<tr>
<th>Parameter (mg/kgTS)</th>
<th>Sterilized mass</th>
<th>Decanter sludge</th>
<th>MBM</th>
<th>Technical Fat</th>
<th>DAF</th>
<th>Dairy manure</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS (%)</td>
<td>96</td>
<td>99</td>
<td>99</td>
<td>99</td>
<td>22</td>
<td>11.5</td>
</tr>
<tr>
<td>VS (%)</td>
<td>87</td>
<td>76</td>
<td>66</td>
<td>100</td>
<td>86</td>
<td>82.9</td>
</tr>
<tr>
<td>TN</td>
<td>59,800</td>
<td>67,600</td>
<td>78,600</td>
<td>2,080</td>
<td>41,800</td>
<td>35,700</td>
</tr>
<tr>
<td>P</td>
<td>22,250</td>
<td>43,725</td>
<td>61,410</td>
<td>40</td>
<td>12,875</td>
<td>12,900</td>
</tr>
<tr>
<td>TOC</td>
<td>553,000</td>
<td>484,000</td>
<td>416,000</td>
<td>773,000</td>
<td>675,000</td>
<td>450,000</td>
</tr>
<tr>
<td>S</td>
<td>2,400</td>
<td>2,800</td>
<td>3,100</td>
<td>&lt;25</td>
<td>6,300</td>
<td>4,900</td>
</tr>
<tr>
<td>K</td>
<td>3,793</td>
<td>4,531</td>
<td>5,153</td>
<td>15</td>
<td>1,386</td>
<td>22,490</td>
</tr>
<tr>
<td>Ca</td>
<td>34,567</td>
<td>60,000</td>
<td>92,398</td>
<td>54</td>
<td>25,435</td>
<td>19,915</td>
</tr>
<tr>
<td>Mg</td>
<td>1,133</td>
<td>2,116</td>
<td>2,527</td>
<td>7</td>
<td>862</td>
<td>10,875</td>
</tr>
<tr>
<td>Na</td>
<td>4,543</td>
<td>7,194</td>
<td>8,444</td>
<td>51</td>
<td>3,391</td>
<td>5,370</td>
</tr>
<tr>
<td>Ni</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>4.6</td>
<td>1.25</td>
</tr>
<tr>
<td>Zn</td>
<td>68.3</td>
<td>107</td>
<td>99</td>
<td>&lt;1</td>
<td>152</td>
<td>173</td>
</tr>
<tr>
<td>Cu</td>
<td>21.2</td>
<td>14</td>
<td>15</td>
<td>3</td>
<td>21.4</td>
<td>27.2</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>9.3</td>
<td>7.2</td>
<td>5.3</td>
<td>371.6</td>
<td>16.2</td>
<td>12.6</td>
</tr>
<tr>
<td>C:P ratio</td>
<td>2.7</td>
<td>1.5</td>
<td>1.3</td>
<td>52</td>
<td>3.3</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Technical fat is solely energy source co-substrate for AD with negligible nutrients content and theoretically it should be nearly 100% converted to biogas and on a marginal extent to microbial biomass, because estimated biomass yield on lipids is significantly lower than that on proteins and carbohydrates (for fat 0.038 g
VSS/g COD; protein 0.2 g VSS/g COD; carbohydrates 0.35 g VSS/g COD) (Alves et al., 2009).

4.1.2 Mass and energy balance of solid slaughterhouse waste rendering process

Detailed investigation of Category 2 and 3 cattle and swine processing slaughterhouse wastes dry-rendering process and generated wastewater mass balance was carried out in collaboration with Rakvere Meat Processing Plant (RMPP), utilizing 16,000 cows and 240,000 pigs annually. Dry-rendering process of SSHW and wastewater treatment flow scheme in RMPP is presented on Figure 3. During slaughtering and meat processing in RMPP 5,000 tons/year of Category 2 and 3 SSHW were generated and directed for treatment to on-site rendering facility. As raw SSHW on average composed of 55% of water, then from 1 ton of raw SSHW 550 L of wastewater and 450 kg of sterilized mass (SM) was produced. SM was further directed to decanter centrifuge and in the end three different fractions

![Figure 3. Solid slaughterhouse waste dry-rendering process flow scheme and its fractionation into various rendering process end-products.](image)
were produced - 135 kg of technical fat, 275 kg of MBM and 40 kg of decanter sludge. From the wastewater treatment unit DAF was removed at a rate of 6.45 kg/m³ wastewater. Approximately 20 m³ of wastewater and 206 kg of Category 2 and 3 ABP are produced per ton of food products produced in RMPP.

From energy balance point of view, firstly biomethane potential of sterilized SSHW and DAF were measured (Table 6). Although lipid and protein rich wastes are generally considered as problematic substrates for AD, then over 90% of final BMP value was achieved already for the 10th day (Figure 4), confirming the attractiveness of sterilized SSHW as a co-substrate for AD. Also Rodriguez-Abalde et al. (2011) have reported a 211.6% increase of degradation rate of sterilized piggery SSHW in comparison to untreated SSHW, confirming the positive effect of sterilization process on the biodegradability of SSHW. The highest methane yield of sterilized SSHW of 978 m³ CH₄/tVS was obtained for technical fat, while lowest yield of 390 m³ CH₄/tVS was obtained for MBM in BMP measurements. Wu et al. (2009) have reported BMP values for MBM in the similar range between 389 to 503 m³ CH₄/tVS, while BMP values obtained for sterilized SSHW in current thesis were higher than results of 287-515 m³ CH₄/tVS reported by Bayr et al. (2012) and maximum methane potential of Category 2 sterilized ABP of 317 ± 7 m³ CH₄/tVS reported by Pozdniakova et al. (2012). Variable range of Category 2 and 3 sterilized SSHW BMP values is mainly dependent on raw material composition differences. Also adaption of inocula for the substrates used for BMP measurements is highly important - this has been specially emphasized by Pozdniakova et al. (2012).

### Table 6. Solid slaughterhouse wastes dry-rendering process products mass balance and methane production potentials measured in batch assays at 37.5 °C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methane production (CH₄, m³/t VSₚₒₜ₆)</th>
<th>Methane production (CH₄, m³/t raw material)</th>
<th>Mass, kg/t raw material</th>
<th>Methane production (CH₄, m³/t raw material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterilized mass</td>
<td>834</td>
<td>685</td>
<td>450</td>
<td>308.3</td>
</tr>
<tr>
<td>Decanter sludge</td>
<td>607</td>
<td>459</td>
<td>40</td>
<td>18.4</td>
</tr>
<tr>
<td>MBM</td>
<td>390</td>
<td>259</td>
<td>275</td>
<td>71.2</td>
</tr>
<tr>
<td>Technical fat</td>
<td>978</td>
<td>966</td>
<td>135</td>
<td>130.4</td>
</tr>
<tr>
<td>Flotation sludge</td>
<td>650</td>
<td>131</td>
<td>6.45 m³ wastewater</td>
<td>0.85</td>
</tr>
</tbody>
</table>


Based on the BMP measurement values primary energy balance for RMPP rendering facility was calculated. The average energy consumption (process heat + electrical energy) for sterilization of 1 ton of Category 2 and 3 SSHW at the rendering facility has been calculated to be 639.6 kWh. If SM and DAF from wastewater treatment unit were used as co-substrates in AD, then theoretically 2,950 kWh of primary energy could be produced from 1 ton of raw Category 2 and 3 SSHW. Thus, the energy produced through AD of SM and DAF obtained from 1 ton of raw Category 2 and 3 SSHW was calculated to be 4.6 times higher than the energy consumed during the rendering process. Although encouraging numbers were obtained from primary energy input-output comparison, the technological energy conversion coefficients, transportation fuel requirements and final utilization pathways of produced biogas have to be taken into account in the economic feasibility assessment.

![Figure 4. Cumulative methane production from sterilized solid slaughterhouse waste at 37.5 °C.](image)

Numerous alternatives for lipid and protein rich solid slaughterhouse wastes treatment and biodegradability enhancement have been investigated during last decades [e.g. slaughterhouse waste streams mono-digestion (Salminen and Rintala 2002b; Wang and Banks, 2003; Kirchmayr et al., 2007; Buendia et al., 2009; Resch et al., 2011; Bayr et al., 2012; Bayr et al., 2012b), different pre-treatment alternatives as thermal, ultrasound, base and acid treatments, enzyme and bacterial products addition and saponification (Masse et al., 2001; Masse et al., 2003; Mendes et al., 2006; Battimelli et al., 2009; Luste et al., 2009; Luste et al., 2011; Affes et al., 2013)]. Nevertheless, based on the obtained results from this study probably the most reasonable option for Category 2 and 3 SSHW treatment and
valorization would still be sterilization and use of SM or its fractionated end products as high energy and nutrient value co-substrates for sewage sludge or manure based biogas plants. At the same time it is important to note that technical fat has been used as a fuel for heat production also in RMPP. Depending on the rendering facility energy production technological solutions, technical fat might not be available for AD as granted and definitely it could have a rather high market value.

In a more general perspective, if all the Category 2 and 3 SSHW from Estonian slaughterhouses were dry-rendered and the obtained sterilized products used for biogas production, approximately 55 GWh of primary energy from currently unused renewable resource could be produced annually. That is equal to the amount of 5.5 million m$^3$ of biomethane for transportation fuel (m$^3$ biomethane is equal to 1 L of gasoline). From macro- and micronutrients viewpoint proposed co-digestion pathway would allow recycling of 500 tons of N and 178 tons of P back to the agricultural land as organic fertilizer. Taking into account restriction of digestate application of 25 kg P/ha annually, the mineral fertilizers could be partly replaced on a 7120 ha of agricultural lands. On the other hand it has to be acknowledged that biogas production of such a high nutritional value resource as is SSHW should not be the first option for valorization, but considered as “plan B” if other alternatives are hard to implement and are economically not feasible. More preferred options for valorization would be protein extraction, animal feed production etc.

Abovementioned conclusions set Category 2 and 3 SSHW into totally different perspective. Based on the obtained values in terms of resource recovery and energetic valorization potential, the following suggestions for restructuring of the Estonian governmental ABP treatment facility operations are proposed:

1) Separate reception lines for Category 1 SRM and Category 2 and 3 ABP should be established to avoid converting of all the material into expensive to utilize Category 1 specified risk material

2) Carry out technical and operational aspects related feasibility study of cattle carcasses separation into Category 1 SRM and Category 2 and 3 ABP fractions on-site in the farms or mobile fractionation units, in order to evaluate options for cattle carcasses sustainable valorization via sterilization and AD

3) Full-scale pilot studies of manure and sterilized SSHW co-digestion should be carried out, in order to assess the increased digestate nutrient concentration effect on plant nutrients uptake, nutrient losses through NH$_3$ emissions and leaching during land application and the optimal level of N and P concentrations in the digestate
4.2 Sterilized solid slaughterhouse waste utilization as co-substrates for anaerobic digestion (Publication II and III)

4.2.1 Sterilized mass and its fractionated products suitability for AD as co-substrates (Publication II)

On the basis of the characterization and analysis of SSHW resources in Publication I, sterilized mass (SM) was chosen as co-substrate for anaerobic co-digestion laboratory experiments with sewage sludge (SS). SM was the first choice of possible co-substrates, because it is the primary end-product of dry-rendering unit and if further fractionation of SM to MBM, technical fat and decanter sludge could be skipped, then it would allow considerable cost reduction for the rendering process. SS was chosen as the base substrate because of the interest of local wastewater treatment plant to build anaerobic digesters for co-digestion of sewage sludge and SSHW. The main problem associated with SM direct use as co-substrate is related to the presence of bone particles (although smaller than < 50 mm), which could have detrimental impact on different parts of AD technology (e.g. abrasion and clogging of pumps, teared sealings, accumulation of bone particles in the bottom of the digester etc.). Nevertheless, it was decided to manually separate bone particles from SM and to use it as a co-substrate for co-digestion with SS in our experiments.

The main purpose of the laboratory co-digestion experiments with SS and SM was to determine optimal amount of SM addition, related increase in the volumetric biogas production and to monitor organic overload induced process inhibition mechanisms. As inocula for co-digestion experiments were taken from wastewater treatment plant anaerobic digester that had been operating under low organic load conditions, the prevalence of aceticlastic methanogens species of *Methanosaetacea* was assumed. Sundberg et al. (2013) and Sun et al. (2014) have shown that aceticlastic Methanosaetaceae were dominating in SS based anaerobic digesters in similar operational conditions as in the current study at up to 5% of SM addition (at 68.5% of VS load). Co-digestion process was maintained stable at this loading without any indication of inhibition and with 5.7 times increased volumetric biogas production (Figure 5). The nutrient composition of the effluent was considerably enriched in comparison to SS mono-digestion. Although the total N was not measured in the effluent samples, the NH₄-N concentration was increased by 2.47 and P concentration by 2.25 times. Further increase of the share of SM up to 7.5% of the SM in the input mixture initiated process efficiency decrease - methane production and yield were reduced, effluent COD concentration was significantly increased indicating reduced substrate degradability and also an increase in the total LCFA concentration up to 1330 ± 63.4 mg/L was noticed. Palatsi et al. (2009) have shown that LCFA concentrations over 2.8 gCOD-LCFA g/L were necessary to inhibit thermophilic digestion of manure in batch and semi-continuous experiments,
Figure 5. SS and SM co-digestion process parameters: 1a) cumulative methane production, mL/day (-), organic loading rate, kgVS m\(^3\)/day (-); 1b) SM share of input VS, % (♦), VS removal efficiency (●), %; 1c) pH (○), ammonium, mg/L (▲) and free ammonia, mg/L (■) concentration patterns. Dashed vertical lines indicate the different feeding periods of sterilized mass co-digestion.
indicating that LCFA concentration in the current study was operated at the LCFA inhibition threshold concentration level (g LCFA = 2.83-2.92 g COD). Despite the fact that nice correlation between increased ammonium concentrations in the range of 2.8-2.9 g NH₄-N/L and methane yield reduction was found (Figure 6), the VFA concentrations during the daily feeding cycle did not show remarkable accumulation, although VFA accumulation has usually been reported to be the first indication of ammonium inhibition (Nielsen et al., 2007; Boe et al., 2008). It should also be mentioned that there are few studies where acetate and propionate accumulation have not been shown to be the best indicator of ammonia or LCFA inhibition of the AD (Nakakubo et al. 2008; Bruni et al., 2013; Girault et al., 2014), confirming that there is still considerable uncertainty regarding ammonium and LCFA inhibition mechanisms on AD. Based on the limited analytical capacity of the present work, as only few random VFA profile measurement series were analysed, the definite conclusions could not be drawn. However, one possible explanation could be that fermentative bacteria were more inhibited than methanogens as VS removal efficiency decreased and 40% of COD concentration increase in the effluent was noticed compared to 5%SM loading period.

![Figure 6. Correlation between methane yield and accumulated ammonium concentrations at stable process conditions of different sterilized mass loading periods.](image)

Figure 6. Correlation between methane yield and accumulated ammonium concentrations at stable process conditions of different sterilized mass loading periods.

Final increase of SM addition to 10% of the input mixture resulted in the complete process failure due to severe LCFA accumulation and foaming. The reactor content was divided into two distinctive layers - normal liquid phase and liquid surface layer filled with tight foam that clogged also gas outlets and led to the
halt of the experiment. Both layers were sampled for LCFA analyses. LCFA concentration in the foam layer was as high as 9,172 ± 701.2 mgCOD-LCFA/gsample, mainly formed by palmitate of 44.4% and stearate 38.5%, while the total LCFA measured value in the liquid phase of the reactor was in comparison 2,001 ± 309.2 mgCOD-LCFA/L. The results of the experiment confirm LCFA related process inhibition to have more severe consequences than increased NH₄-N concentrations related process efficiency decrease, as the formed LCFA-rich foam is thick, hard to disperse of and if it clogged the gas collection line then serious technological maintenance problems occur.

In conclusion it can be said that SS and SM co-digestion at 5%SM addition was optimal co-substrate addition ratio with concurrent 5.7 times increased volumetric methane production. In addition, usefulness of SS and SM co-digestion in the context of nutrients accumulation in the effluent of the AD process has to be seriously considered, as it can cause significant operational problems for conventional wastewater treatment plants with activated sludge processes. Additional 2.46 times higher NH₄-N and 2.25 times higher P load in the effluent require extra readily available C source for efficient operation of anaerobic P removal and also denitrification processes. As activated sludge processes are often operated at their maximum system capacity and at readily available carbon source deficiency, which means that additional N and P load would require external addition of methanol or some other C-sources. This is all accompanied with increased expenditures and the energy requirement for aeration of the activated sludge unit. Possible solution could be application of anaerobic ammonium oxidation (ANAMMOX) or completely autotrophic nitrogen removal over nitrite (CANON) process in the digestate reject water recirculation line (Ahn, 2006) that would decrease the ammonium concentration load on waste activated sludge process. Most of the P could be removed by efficient solid-liquid separation unit.

Publication II experiments can be considered as an important step for the improvement of the laboratory experiments design mainly in terms of the reactors operational and analysis plan preparation. As SM bone particles separation and crushing on a real-scale without the use of decanter centrifuge is rather complicated, it was decided that the technical fat, MBM and decanter sludge would be used as co-substrates in the next experiments.

4.2.2 Sterilized mass and its fractionated products as co-substrates for AD (Publication III)

Experiments for Publication III were decided to be carried out with dairy manure as the base substrate, mainly because of the complications with increased nutrient concentration of effluent that co-digestion of SS and SM could cause. Biogas production subsidies are low in Estonia meaning that biogas plants have to be of simple design and with non-sophisticated operation procedures, but at the same time provide high volumetric biogas production. Slogan for Estonian agricultural
biogas sector could be “Waste based and keep it as simple as possible biogas production”. In the intensive livestock production regions in Europe manure and digestate accumulation are serious problems that have raised a considerable interest in the digestate down-stream processing technologies like separation, digestate drying, ammonia recovery etc. As Estonian agricultural sector in general does not have problems with manure surplus, then also digestate down-stream processing (separation, drying etc.) is not mandatory necessity. On the contrary, farms supplying the biogas plants with manure are interested in concentrating nutrients into the digestate, but at the same time maintaining the TS content of digestate below 6-7% to retain good flowability and ease of land application with injection techniques. Interesting alternative for meeting these requirements could be SSHW co-digestion with manure. Main purpose of the Publication III was to carry out technical fat and decanter sludge co-digestion experiments with manure to evaluate the following aspects:

- determine optimal technical fat and decanter sludge loadings for stable process and simple operation
- compare process performance and efficiency of solely lipid-rich co-substrate addition with balanced lipids and proteins content co-substrate addition
- elucidate process behaviour at increasing lipids concentration with specific attention on the LCFA degradation and inhibition mechanism
- monitor the increased ammonium concentration effect on process efficiency and specific methanogenic activity

4.2.2.1 Technical fat and decanter sludge co-digestion with manure: process efficiency, limitations and floating granules formation (Publication III)

Reactor1 (R1) with decanter sludge (DS) addition and Reactor2 (R2) with technical fat (TF) addition to manure were both started up with inocula from dairy manure based 200 L pilot reactor and. For the pilot reactor, R1 and R2 similar process parameters for manure mono-digestion period were achieved. Average methane yield of 232.8 ± 4.8 m³ CH₄ tVS⁻¹ corresponding to VS removal efficiency of 28.4 ± 1.5% was achieved at process conditions of OLR of 1.36 kgVS m⁻³ d⁻¹ and NH₄-N concentration of 2.4 ± 0.02 g L⁻¹. Similar manure based AD process parameters have been reported by Nielsen et al. (2007) with 243-248 m³ CH₄ tVS⁻¹.

R1 was further operated with DS addition as co-substrate. Co-digestion process was maintained stable up to 5% of DS addition ratio corresponding to maximum of 57.2% of VS load and lipids concentration of 23.0 ± 1.2 g L⁻¹, that has been reported to be in the same range as the concentration of Na-oleate addition to induce light bubbly foam formation in manure based AD (Kougias et al., 2013). Nevertheless, no remarkable LCFA accumulation or foaming was observed in R1 that is most
probably related to balanced lipids and proteins content of DS and process conditions that were not forced to extreme conditions. OLR was at maximum 3.76 kgVS m⁻³ d⁻¹ and NH₄-N raised maximum to 4.5 g L⁻¹. Although NH₄-N value was 1.5 times higher than 3.0 g L⁻¹ reported by Schnürer and Nordberg (2008) as a threshold value for switch to SAO pathway, the SMA results did not indicate signifi-

![Figure 7. Aceticlastic and hydrogenotrophic specific methanogenic activity tests results for R1 and R2.](figure7)

Figure 7. Aceticlastic and hydrogenotrophic specific methanogenic activity tests results for R1 and R2.

Figure 7. Aceticlastic and hydrogenotrophic specific methanogenic activity tests results for R1 and R2.

Figure 7. Aceticlastic and hydrogenotrophic specific methanogenic activity tests results for R1 and R2.

Figure 7. Aceticlastic and hydrogenotrophic specific methanogenic activity tests results for R1 and R2.

significant changes in methanogenic activity values (Figure 7). Compared to dairy manure mono-digestion, addition of 5%DS to manure increased volumetric CH₄ production 3.44 times from 7.2 to 24.8 m³ CH₄ t⁻¹. Despite the process being stable
during 5%DS loading period the effluent TS content and residual biomethane potential gradually increased in time with simultaneous decrease in CH₄ production efficiency. This indicated that single stage AD with HRT of 22.5 days was not sufficient for maintaining high organic material conversion efficiency and it is necessary to apply post-digester with HRT of approximately 10-15 days as a “polishing” step to get maximum added value out of the DS addition.

The fertilizer value of the R1 digestate was also significantly improved compared to manure nutrient composition - N content increased by 63%; NH₄-N by 72%, P by 176%. In addition, the whole spectra of macro- and micronutrients in the digestate were considerably increased compared to manure mono-digestion. As the content of the main plant nutrients was significantly increased it clearly showed the additional value of SSHW co-digestion for agricultural biogas plants if the best available technology for land application could be applied (digestate direct land injection etc.).

R2 was operated with TF as a co-substrate added to manure. Experiment was planned based on the results of the preliminary study where 1.5%TF addition guaranteed stable process performance at input mixture lipids concentration of 17.16 g L⁻¹, while 3%TF addition caused clear lipids overload with intensive foam formation and LCFA inhibition. Accordingly, TF addition to R2 was initiated at 2%TF addition (representing 39.4% of VS load). The load of 2%TF was maintained for 3 HRT which allowed to maintain stable process operation at methane yield of 338.5 ± 19.8 m³ CH₄ tVS⁻¹ – surprisingly the latter value corresponded only to 62.5% of the theoretical methane potential (TMP) of TF. As no accumulation of intermediate compounds or foaming occurred, then there was considerable confusion about the reasons behind the low TMP. Soon it was found that instead of foaming white floating granules (FG) (Figure 8 and 9) were formed that were floating separately or in agglomerates on the top of the liquid layer. This was also clear explanation of the low TMP of R2.

FG cross-section revealed that they were partly hollow inside, being probably one of the reasons leading to their flotation. Similar phenomenon of formation of floating granules has only been described by Varin et al. (2013), but mainly in acidic conditions at pH between 5 and 6. It was reported that at neutral or higher pH values LCFA were by-passed with suspended sludge from the reactors without formation of granules, being in contradiction with FG formation at pH over 7.5 in the current experiments.

FG had the following composition: 38.3%TS, 79.2%VS of TS and all of the VS was composed of salts of LCFA or lipids. Calcium salts of LCFA were the dominant salts forming FG. It should be noted that precipitation of LCFA by CaCl₂ have been reported in the previous studies instead of the formation of FG during AD (Ahn et al., 2006; Boe et al., 2012; Hanaki et al., 1981; Koster, 1987; Roy et al., 1985; Zhang et al., 2010). Calcium salts of LCFA are relatively insoluble compared to, for example sodium salts (Koster, 1987), explaining the reason for calcium salts of LCFA dominance in formation of FG in R2. The main mineral compound of FG
was calcium at 41.17 g kg\(^{-1}\), being at considerably higher concentrations than phosphorous, potassium or sodium. Palmitate had the highest share of 42.6% of the LCFA in the FG, even though palmitate contributed only 20.2% of the LCFA fraction in TF (Table 7).

After removal of the floating granules from R2, the TF addition was reduced to 1%TF load, which resulted in the recovery from previous limited state and in stable process achieving 83.5% of TF conversion efficiency and methane yield increase by 22% up to 336.1 ± 10.3 m\(^3\) CH\(_4\) t\(^{-1}\) VS\(^{-1}\). At the same time concentrations of intermediate metabolic compounds were similar to the manure mono-digestion period levels and no formation of FG was observed. Mladenovska et al. (2003) have reported similar CH\(_4\) yield increase of 22% by addition of 2% (w/w) glycerol trioleate to cattle manure. During TF addition periods hydrogenotrophic activity in

![Figure 8. Floating granules (on the right) and floating granules and technical fat agglomerates collected from the R2 surface layer (on the left).](image)

**Table 7. Long chain fatty acids spectra and their proportional share in technical fat and floating granules from R2.**

<table>
<thead>
<tr>
<th>LCFA</th>
<th>Technical Fat g kg(^{-1})TS</th>
<th>Floating granules Lipids + (%) LCFA g kg(^{-1})</th>
<th>Lipids (%) g kg(^{-1})</th>
<th>LCFA salts (%) g kg(^{-1})</th>
<th>Salts (%)</th>
<th>Lipids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>146.1</td>
<td>14.8</td>
<td>114.8</td>
<td>105.6</td>
<td>42.6</td>
<td>9.2</td>
</tr>
<tr>
<td>18:0</td>
<td>89.8</td>
<td>12.4</td>
<td>61.1</td>
<td>54.3</td>
<td>21.9</td>
<td>6.8</td>
</tr>
<tr>
<td>18:1</td>
<td>322.4</td>
<td>44.6</td>
<td>101.3</td>
<td>69.2</td>
<td>27.9</td>
<td>32.1</td>
</tr>
<tr>
<td>Other</td>
<td>165.0</td>
<td>22.8</td>
<td>25.9</td>
<td>19.0</td>
<td>7.7</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>723.3</td>
<td>-</td>
<td>303.1</td>
<td>248.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

22% up to 336.1 ± 10.3 m\(^3\) CH\(_4\) t\(^{-1}\) VS\(^{-1}\). At the same time concentrations of intermediate metabolic compounds were similar to the manure mono-digestion period levels and no formation of FG was observed. Mladenovska et al. (2003) have reported similar CH\(_4\) yield increase of 22% by addition of 2% (w/w) glycerol trioleate to cattle manure. During TF addition periods hydrogenotrophic activity in
R2 remained stable, but it was about 25% lower than hydrogenotrophic methanogenic activity in R1. After removal of FG from the system and reduction of TF loading to 1%TF the hydrogenotrophic activity increased up to 0.35 g\textsubscript{COD-CH\textsubscript{4}} g\textsubscript{VS}\textsuperscript{-1} day\textsuperscript{-1} (Figure 9). Aceticlastic methanogenic activity after adaptation to increased LCFA concentration increased more than 2.2 times being consistent with the results obtained by Silvestre et al. (2014) during co-digestion of sewage sludge and grease trap waste.

Pereira et al. (2005) have previously proposed three mechanisms for biomass-associated LCFA accumulation: precipitation, adsorption and entrapment. Based on the results of the current study the mechanism of floating non-biomass associated LCFA “granules” should be added to the list and considered as a possible alternative pathway of lipids degradation and LCFA accumulation in suspended...
sludge based AD. In addition, results of the R2 operation indicated that solely lipids overloaded manure based AD does not lead to sudden process failure, but the system has the ability to neutralize surplus LCFA to “storage compounds” of Ca-salts of LCFA.

### 4.2.2.2 Biodegradability and possible benefits of floating granules formation

Even though FG can be considered as beneficial inhibition alleviating “storage compounds” it was necessary to evaluate their biodegradability by BMP tests, because FG contain high energy potential that could be lost if FG were discarded from the reactors with effluent. It was found that the inherent biodegradability of

*Table 8. Sonication pre-treatment conditions and its effect on anaerobic biodegradability (AB) and methane production rate (MPR) of FG from R2. Final values after 45 days batch test. (β)- power amplitude*

<table>
<thead>
<tr>
<th>Sonication</th>
<th>Anaerobic biodegradability (AB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔT, (°C)</td>
</tr>
<tr>
<td>NO</td>
<td>-</td>
</tr>
<tr>
<td>L1</td>
<td>0.7</td>
</tr>
<tr>
<td>L2</td>
<td>0.4</td>
</tr>
<tr>
<td>L3</td>
<td>21.7</td>
</tr>
<tr>
<td>M1</td>
<td>8.6</td>
</tr>
<tr>
<td>M2</td>
<td>8.9</td>
</tr>
<tr>
<td>H1</td>
<td>17.4</td>
</tr>
<tr>
<td>H2</td>
<td>18.1</td>
</tr>
<tr>
<td>OPT</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*Inocula activity control tests*

<table>
<thead>
<tr>
<th></th>
<th>AB (%COD)</th>
<th>MPR, L CH₄ kg⁻¹COD d⁻¹</th>
<th>Lag phase, day</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>25±5</td>
<td>2.7 ± 1.46</td>
<td>16</td>
</tr>
<tr>
<td>C18:1</td>
<td>60±1</td>
<td>17.9 ± 0</td>
<td>12</td>
</tr>
<tr>
<td>VFADa</td>
<td>90±7</td>
<td>39.1 ± 7.0</td>
<td>0</td>
</tr>
</tbody>
</table>

FG was very low, resulting in long lag phase and low degradation rate – the lag phase with minor methane production lasted for 19 days. After 30 days of measurement only 31.2 ± 9.4% and after 45 days 56.0 ± 13.0 % of FG were biodegraded based on COD balance (Table 8 and Figure 10). For enhancing FG biodegradability sonication as a pre-treatment technology was used, as accessibility
for methane producing consortia seemed to be the main reason behind low biodegradability of FG.

From the wide range of energy densities used for FG sonication, the medium level sonication density at $101.4 \times 10^3 \text{ kJ kg}^{-1}\text{TS}$ and $2.2 \text{ W mL}^{-1}$ was sufficient to achieve maximum FG accessibility, the fastest degradability and the highest methane production rate – see Table 8. Although sonication pre-treatment experiment confirmed that biodegradability of the FG can be enhanced, its technical and economical reasonability in the full-scale remains highly questionable. The formation of the so called LCFA “storage compounds” should be avoided because they strongly reduce process efficiency. However, formation of FG provides a flexible control and monitoring mechanism for high lipids loaded AD process operation optimization, without constant threat of foam formation and clogging of the gas collection systems etc. As the concrete mechanism of FG formation remained still unclear, further research is required to elucidate triggering mechanism and important process parameters influencing this phenomenon. Definitely LCFA/Ca ratio and concentrations are the most important parameters to be considered, but also reactor hydrodynamic conditions, feeding regime and frequency could be proposed to have the significant importance.
Figure 10. Anaerobic biodegradability and methane production rate of fat granules (a,b). Increment of anaerobic biodegradability and methane production rate (in comparison to untreated sample) of sonicated fat granules as a function of supplied energy (c,d). Figure illustrates values after 30 days of batch test, at the end of the methane production rate active phase. AB – anaerobic biodegradability (% initial COD); MPR – methane production rate (L CH4 kg⁻¹ COD day⁻¹); sE – supplied energy; L – low energy, M – medium energy; H – high energy; OPT – optimum energy.
5. CONCLUSIONS

In the first part of the thesis, a detailed analysis of the characteristics and end-product composition of a cattle and swine processing slaughterhouse Category 2 and 3 ABP rendering facility sterilization unit was carried out, with the following main conclusions:

1) Sterilized solid slaughterhouse wastes are attractive co-substrates for AD because of their high energy and nutrients content, ease of access for anaerobic consortia, fast biodegradability and low costs of longer distances transportation.

2) Theoretically 4.6 times more primary energy can be produced from 1 ton of raw ABP sterilization end-products compared to the energy required for the sterilization procedure.

3) The maximum valorization of Category 2 and 3 solid slaughterhouse wastes using AD in Estonia would allow annual production of 5.5 million m³ of biomethane for the replacement of fossil transportation fuels, while nitrogen and other nutrients enriched digestate could be used as organic fertilizer on 7120 ha of agricultural land in replacement of mineral fertilizers.

The co-digestion of sterilization end-products with sewage sludge or manure based AD was studied in the second part of the thesis. Initial focus was on the optimal co-substrate share in the input to achieve maximum volumetric biogas production, while maintaining high biodegradability rate and ease of operation. Not less important was LCFA and NH₄-N induced inhibition mechanism characterization and description:

4) Sewage sludge and sterilized mass (SM) co-digestion at 5%SM addition increased volumetric biogas production 5.7 times with over 2 times increased NH₄-N and phosphorous content in the digestate. The increased nutrient content remarkably improved fertilizer value of the digestate, but increased NH₄-N content in the digestate reject water stream could cause considerable reduction of the conventional waste activated sludge process nutrient removal efficiency.

5) Process efficiency decrease was noticeable at LCFA and NH₄-N concentrations of 1.3 g/L and 2.8-2.9 g/L, respectively. Process termination at 10%SM addition was caused by intensive LCFA accumulated foam formation.
6) Technical fat (TF) addition to cattle manure at 2% TF addition (39.4% VS load) resulted in a surprising process response with formation of floating granules of Ca-salts of LCFA.

7) New mechanism of floating non-biomass associated LCFA “granules” was proposed as an alternative pathway for degradation of lipids and LCFA accumulation in suspended manure based AD.

8) Decanter sludge (DS) addition at 5% DS load maintained stable process with 3.44 times increased volumetric CH₄ production from 7.2 to 24.8 m³ CH₄/t, at equal lipids load with TF overloaded reactor and without any sign of foaming or floating granules formation, indicating the importance of a “balanced diet” of proteins and lipids for the AD processes.
BIBLIOGRAPHY


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There are not enough words to thank my supervisor and “academic dad” professor Raivo Vilu for giving me an opportunity to do research in the field of environmental biotechnology and at the same time 100% believing in and trusting the younger generation as equal partners. Also the way of teaching to “swim” has been highly efficient and has motivated me continuously to work outside of the comfort zone, search for new challenges and to be actively involved in the public discussions and political debates related to environmental issues.

Special gratitude goes also to Ahto Vili from Torma POÜ. Thanks to Ahto I have understood what anaerobic digestion in the context of sustainable agriculture really means and the insight that he has given me to conventional agriculture is for me priceless! Great thanks also to Mr. Maarek Jõhve, technical manager in Rakvere Meat Processing Plant, for introducing me in detail the everyday life of the slaughterhouse, its waste management and related problems. Our discussions were interesting and with valuable input for the thesis Publication I.

My sincere gratitude goes also to adjunct Professor Prasad (L.N) Kaparaju from Department of Biological and Environmental Sciences, University of Jyväskylä. Accepting my request to be my PhD thesis co-supervisor just based on my enthusiastic presentation of my ideas and interests to deal with AD related research was rather “crazy”, but for me highly encouraging and motivating.

The same kind of words and gratitude are also directed to PhD Jordi Palatsi for accepting my request and kindly welcoming me in IRTA, GIRO Joint Research Unit IRTA-UPC in Caldes de Montbui, Barcelona for a several research stays related to slaughterhouse wastes AD and for providing me invaluable insight to the topic. Thank you my dear friend!

The international gratitude list could be continued ad infinitum, so I tribute to all the international colleagues who have been kindly ready to welcome me all over the world and have had an open discussions on whatever topics I have raised!

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Of course the last and the most important ones- my family, thanks to whom I am who I am today and I am really happy with it 😊. My PhD adventure would have been limitlessly harder without the warm and understanding support from my
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ABSTRACT

Slaughterhouses have stringent standards for their liquid and solid wastes treatment procedures. While liquid slaughterhouse wastes can quite efficiently and reliably be treated by modified high-rate AD systems, then solid wastes treatment is still in search of the best solution. Accordingly, the main aim of the thesis was to carry out a complex investigation of Category 2 and 3 ABP sterilization process (133 °C, 3 bar and 20 min) mass and energy balance and its end-products valorization potential via anaerobic co-digestion. Based on the investigation results, sterilization was confirmed to be an attractive solution regarding many important aspects: firstly, the water content of the sterilization end-products was reduced almost to zero, which enables transportation of these products for longer distances at reduced costs; secondly, energy content of the end-products is up to dozens of times higher than for the usual substrates like manures and sewage sludge; thirdly, one ton of raw solid slaughterhouse waste sterilization consumes 4.6 times less primary energy than can be produced from sterilized mass and dissolved air flotation sludge derived from it; fourthly, if sterilized mass, technical fat, decanter sludge or meat and bone meal would be used as co-substrates for AD with sewage sludge or manure, a remarkable volumetric biogas production increase already at 1% of co-substrate addition could be achieved; fifthly, macro- and micronutrients rich sterilization end-products co-digestion enriches digestate nutrient composition and fertilizer value, but increased attention has to be paid to the mitigation of nutrients loss during storage and also on the nutrients bio-availability; sixthly, although not in the focus of current thesis, sterilization is also an effective solution in terms of ABP hygienic safety.

Sterilization end-products co-digestion experiments operation below inhibitory threshold concentrations resulted in the above described benefits, but more importantly for lipids overloaded AD processes a new long-chain fatty acids (LCFA) accumulation mechanism was observed and characterized. As stated by Pereira et al. (2005), then commonly accepted mechanism for biomass-associated LCFA accumulation have been precipitation, adsorption and entrapment, but from now on the mechanism of floating non-biomass associated LCFA “granules” should also be added to the list and considered as a possible alternative pathway of lipids degradation and LCFA accumulation in suspended sludge based AD. In addition, a balanced addition of protein- and lipid-rich co-substrate to manure based AD ensured a stable process at equivalent lipids loading rate which did not allow efficient process operation with solely lipid-rich co-substrate, confirming importance of “balanced diet” and the synergistically higher resistance of anaerobic consortia to potentially inhibitory process disturbances.

Based on the results of the thesis, also concrete suggestions for Estonian governmental ABP treatment facility operation and management were worked out, that would lead to more efficient highly valuable resource recovery and valorization
via AD or other alternative pathways. To further promote the increase of stakeholders interest, an evaluation of the primary energy production potential for annually produced Category 2 and 3 solid slaughterhouse wastes in Estonia was carried out, with the conclusion that maximum of 5.5 million litre of gasoline equivalent of biomethane could be produced and used as local renewable resources derived transportation fuel.
KOKKUVÕTE

Tapamajades tekkivate loomsete kõrvalsaaduste käitlemine on Euroopas äärmiselt rangelt reguleeritud ja peab vastama kõrgetele hügieeninõuetele, et vältida võimalikke ohte ja riske inimeste ja loomade tervisele. Vedelate tapamajajäätmete anaerobseeks käitlemiseks on tänapäeval olemas mitmeid spetsialiseeritud reaktoritehnoloogiaid, mis võimaldavad saavutada kõrgete orgaanilise efektiivust ning samal ajal säilitada stabiilne kääritamisprotsess. Tahkete tapamajajäätmete anaerobse kääritamiseks on olukord keerulisem ning siiani alles otsitakse parimaid tehnoloogilisi lahendusi. Üheks võimalikuks lahenduseks on 2. ja 3. kategooria loomsete kõrvalsaaduste steriliseerimine (133 °C, 3 bar ja 20 min) ning steriliseerimisprotsessi lõpp-produktide (steriliseeritud mass, tehniline rasv, dekantermuda ja lihakondjahu) kasutamine anaerobse kooskääritamise toorainena. Sellest tulenevalt oli käesoleva doktoritöö eesmärgiks uurida kompleksset steriliseerimisprotsesside massi- ja energiabilanssi, steriliseerimisprotsesside lõpp-produktide kooskääritamise potentsiaali ja piiranguid. Doktoritöös saadud tulemuste alusel saab kitsendada steriliseeritud tapamajajäätmete sobivust anaerobse kääritamise lisatoorainena, lähtudes järgnevatest asjaoludest: 1) steriliseeritud tapamajajäätmete lõpp-produktide vee sisaldus on minimaalne, mis võimaldab majanduslikult tasuvalt transportida oluliselt pikemaik vahevald, kui seda on võimalik teha vedelsooni või teiste sarnaste toorainetega; 2) steriliseerimisprotsessi lõpp-produktide energiasisaldus on kümme kord kõrgeeratud anaerobse kääritamise baastoorainetega; 3) ühe tonni tahkete tapamajajäätmete steriliseerimiseks kulub 4,6 korda vähem primaarenergiat, kui on ühe tonni tahkete tapamajajäätmete käitlemisel tekkinud steriliseeritud massist ja tapama ja reoveepuhasti flotatsioonimudast võimalik primaarenergiat toota; 4) protsessi biogaasi tootlikkus on reaktorite hauhuhi kuht kohta võimalik kordades suurendada vaid 1% steriliseeritud massi, tehnilise rasva, lihakondjahu või dekantermuda lisamisel vedelsoonnikul või reoveestsetel baseeruvatele anaerobse kooskääritamise protsessidele; 5) steriliseerimisse lõpp-produktid on makro- ja mikrotoitainete rikkad ning nende lisamine anaerobsele kääritamise protsessile suurendab oluliselt kääritusjäägi väetusväärtust. Maksimaalse väetamisefekti saavutamiseks on vaja olulist tähelepanu pööramažaima teistainete kadude vähendamisele hoiustamise ajaal; 6) tapamajajäätmete steriliseerimise võimaldab oluliselt vähendada ka tapamajajäätmete käitlemisega seotud hügieeniriske.

Eelpool kirjeldatud eelised saavutakse tapamajajäätmete steriliseerimise lõpp-produktide optimaalse kasutamisel anaerobse kääritamise lisatoorainena. Doktoritöö käigus olulisilisaks tulemuseks tuleb siiski pidada uue pika-ahelaga rasvhapete inhibitiooni mehanismi tuvastamist ning kirjeldamist. Pereira jt (2005) on analüüsides kääritamisprotsessi põhjustatud kolme võimaliku biomassiga seotud pika-ahelaga rasvhapete akumulaatsiooni mehanismi: nende sadestamine, adsorpsioon ja spetsiifiliste komplekside teke tänuses biomassi “kinnijäämisele” pika-

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ahelaga rasvhapete vahele. Käesoleva doktoritöö tulemuste alusel saab siia nimetirja lisada **pika-ahelaga rasvhapete granulatsiooni ja sellest tuleneva flotatsiooni.** Samas on oluline rõhutada, et proteiini- ja liipiïdirikaste lisasubstraatide kasutamine vedelsõnnikul baseeruvatel kääritamisprotsessides on eelistatav, kui vaid rasvarikaste lisaturnainete kasutamine, sest see võimaldab samaväärsset rasva kontsentratsioonide juures säilitada protsesside stabillise ning ära hoida protsessi efektiivsust vähendavat pika-ahelaga rasvhapete granulatsiooni ja flotatsiooni. See võimaldab järeltada, et kuigi liipiïid on teoreetiliselt parim võimalik lisaturnain biogaasi toodangu suurendamiseks reaktori mahuühiku kohta, siis “tasakaalustatud dieet” on eelduseks ka anaeroolsete elektrofikusustel, säilitamaks suuremat vastupanuvõimet erinevate potentsiaalsete protsessi inhibeerivate ühendite mõju suhtes.

Doktoritöö tulemuste alusel töötati täiendavalt välja ka konkretset settepanekud Eesti loomsete kõrvalsaaduste riikliku käitluskeskuse töö ümberkorraldamiseks, mis võimaldaks oluliselt efektiivsemalt väärib nada riigis tekkivate loomsete kõrvalsaaduste jäätmevoogu anaeroolse kääritamise kaudu. Teostatud analüüs näitas, et loomsete kõrvalsaaduste sektori ümberkorraldamine võimaldaks anaeroolset kooskääritamist kasutades toota kuni 5,5 miljonit m³ biomaani aastas, mida oleks võimalik kasutada kohaliku taastuva kütuseallikana transpordisektoris-biomaani m³ on kütteväärtselt sisuliselt võrdne bensiini liitriga.
CURRICULUM VITAE

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3. Education

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4. Professional Employment

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2011- present Tallinn University of Technology, Department of
Chemistry, project manager/researcher; research and
management of the Estonian Energy Technology program project on anaerobic digestion processes

2010-2012 Competence Center of Food and Fermentation Technologies, a scientist; slaughterhouse waste biogas production potential and co-digestion experiments

2006–2010 Tallinn University of Technology, Faculty of Biotechnology, a research assistant; wastewater treatment, different substrate biogas potential measurement, chemical analysis, gas analysis, measurement data processing etc.

2006-2009 Competence Center of Food and Fermentation Technologies, a scientist; yeast factory wastewater AD.

2004-2008 Player at Estonian Basketball Championship (Teams: BC Nybit, Rapla BC, TTÜ BC)

2003-2004 Military service as a paramedic

5. Administrative responsibilities

2012- present Individual member of Estonian Renewable Energy Association

2013 Co-author of roadmap “100% renewable energy- transition to clean energy” by Estonian Renewable Energy Association

2011- present Estonian Biogas Association member of the Board

6. Additional education

2013 13th World Congress on Anaerobic Digestion, Santiago de Compostela, Spain

2013 2 months research stay at IRTA research center in Barcelona, Spain. Participation in ADAW project activities related to solid slaughterhouse waste anaerobic digestion

2012 Participation at Biotechnology Studies Delft Leiden BSDL-EDU PhD course „Advanced course on environmental biotechnology“

2012 2 week laboratory knowledge exchange in GIRO Technological Center, Barcelona, Spain. Topic of slaughterhouse waste anaerobic digestion
UNESCO-IHE on-line course "Biological Wastewater Treatment: Principles, Modelling and Design"

12th World Congress on Anaerobic Digestion, Guadalajara, Mexico

Participation on 2 week course „Environmental Technology for Treatment and Management of the Bio-waste Manure“ at University of Southern Denmark

International Workshop on Anaerobic Digestion of Slaughterhouse Waste, Barcelona, Spain

3 weeks research stay on Microbial Fuel Cell process design in Arizona State University, The Biodesign Institute

3 months research stay in Institute for Bio-processing and Analytical Measurement Techniques (IBA), Heilbad Heiligenstadt, Germany. Acquiring process data from biogas plants; processing data; writing master thesis based on the collected data and materials about biogas sector R&D in Germany

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Grete Raba bachelor thesis: “Characterization of manure based AD start-up without addition of external inoculum”

Käťlin Lužkov bachelor thesis: “AS Estonian Cell aspen pulp mill wastewater anaerobic digestion with HydroThane ECSB (External Circulation Sludge Bed) pilot reactor”

Juhan Pürjer master thesis: “Energetic potential and possible problems of waste activated sludge and biowastes co-digestion on Kuressaaare region example”

Pitk, P., Kaparaju, P., Vilu, R.

**Methane potential of sterilized solid slaughterhouse wastes.**

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Methane potential of sterilized solid slaughterhouse wastes

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ABSTRACT

The aim of the current study was to determine chemical composition and methane potential of Category 2 and 3 solid slaughterhouse wastes rendering products (SSHWRP) viz. meat, decanter sludge, meat and bone meal (MBM), technical fat and flotation sludge from wastewater treatment. Chemical analyses showed that SSHWRP were high in protein and lipids with total solids (TS) content of 96–99%. Methane yields of the SSHWRP were between 390 and 978 m³ CH₄/t volatile solids (VS) decreased. Based on batch experiments, anaerobic digestion of SSHWRP from the dry rendering process could recover 4.6 times more primary energy than the energy required for the rendering process. Estonia has technological capacity to sterilize all the produced Category 2 and 3 solid slaughterhouse wastes (SSHWH) and if separated from Category 1 animal by-products (ABP), it could be further utilized as energy rich input material for anaerobic digestion.

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1. Introduction

Treatment and utilization of different Category ABPs are strictly defined for EU member states by the Animal By-Product Regulation ABPR 1069/2009/EC — replacing the ABPR 1774/2002/EC (Kirchmayr et al., 2003; European Parliament and Council, 2009). Utilization of ABPs is a mandatory expenditure for animal processing facilities with increasing costs related to energy and disposal prices. In 2009, Estonian meat processing sector produced 22,709 tons of slaughterhouse waste. Two largest meat processing plants in Estonia sterilized 10,273 tons of Category 2 and 3 SSHW in their own rendering plants (ESO, 2010). The rest of the SSHW was mixed with the Category 1 ABPs, sterilized in the governmental rendering facility and produced sterilized meat (melt) was incinerated in special incineration plants in Estonia or neighbouring countries.

An interesting option for reorganizing the current scheme of functioning of the ABPs treatment sector is to collect and treat the Category 1 ABPs separately in a specialized treatment plant. The rest of the waste stream can be used as co-substrates for agro-industrial or municipal biogas plants in order to enhance energy and fertilizer recovery. Design of technologically and economically efficient anaerobic digestion processes is the key aspect for sustainable ABP's treatment.

Anaerobic digestion has been considered one of the best alternatives for nutrient and energy recovery from the SSHW because of the high content of protein and lipids (Heinfelt and Angelidaki, 2009; Karapaju et al., 2010). However, the high content of proteins and lipids may cause inhibition of the digestion process due to high ammonia and long chain fatty acids concentrations accumulating at high loads (Salminen and Rintala, 2002; Edström et al., 2003; Bayr et al., 2012).

As there was technological capacity to sterilize all Category 2 and 3 SSHW produced in Estonia, the current study was focused on determining chemical composition and methane production potential of SSHWRP viz. meat, decanter sludge, meat and bone meal (MBM), technical fat and flotation sludge from the wastewater treatment unit. Sterilization in the studied facility (Rakvere Meat Processing Plant (RMPP)) was based on widely used dry rendering technology. In dry rendering process the raw material is heated to temperature between 115 and 145 °C, evaporating moisture and freeing fat from protein. The fat cells open due to changes in the cell walls of the tissue as moisture evaporates (Meeker, 2006).

Energy production potential of Category 2 and 3 raw SSHW via sterilization and anaerobic digestion was compared against Category 2 and 3 raw SSHW rendering process energy requirements. In addition, co-digestion of melt with manure was evaluated in batch assays in order to assess increase in methane yield per mass unit of input.

Sterilized slaughterhouse waste methane potential and characteristics have previously been reported only in few studies (Heinfelt and Angelidaki, 2009; Rodriguez-Abalde et al., 2011; Bayr et al., 2012), but to our knowledge there is no data published about Category 2 and 3 SSHW rendering process mass and energy balance, considering energy recovery potential by anaerobic digestion.
2. Methods

2.1 Solid slaughterhouse wastes

Substrates were collected from RMPP rendering facility, Estonia. RMPP utilizes 16,000 cows and 240,000 pigs annually. During slaughtering and meat processing, 5000 ton/year of Category 2 and 3 SSHW were generated and directed to their own rendering facility. From animal reception area, manure was collected together with paunch content and tumbling mesh waste and sent for composting to avoid excessive loading of the wastewater treatment unit. Rendering process included crushing, drying, rendering, pressing, milling for meat and bone mixture, decanter centrifuging and separation. As a first step, Category 2 and 3 SSHW with TS content 40–50% were disaggregated (particle size < 50 mm) in a crushe and transported to the cookers where it was treated at 133 °C, 3 bar for 20 min. Condensation water from dry rendering was directed to wastewater treatment unit buffer/mixing tank. During drying rendering, approximately 45% of the raw material was converted to melt and 55% of it was evaporated and directed to wastewater treatment. Melt samples were taken from dry rendering reactor right after batch cycle, then it was cooled down to room temperature and kept at 4 °C until use. Melt was further separated to MBM, technical fat and decanter sludge. Samples of separated fractions were stored under the same conditions as melt.

Primary wastewater treatment in rendering facility was based on flocculation with NaOH + Fe₂SO₄ and subsequent flotation in order to remove fat rich suspended solids from the wastewater. As a final step, treated wastewater was sent for final treatment to municipal wastewater treatment plant. During wastewater treatment, approximately 13,050 m³ of flotation sludge was generated annually. The produced sludge was further compacted with a belt-filter press to achieve TS content of around 20% and eventually it was sent to a composting facility. As a result, 6.45 kg of flotation sludge was generated for every m³ of wastewater treated and 640 kg of flotation sludge was produced for every rendered ton of Category 2 and 3 raw SSHW. Flotation sludge samples were collected from belt-filter press outlet storage tank and stored at 4 °C until use.

2.2 Manure

Manure was collected from Torna dairy farm. Torna dairy farm had 570 dairy cows, 260 bovine animals and 120 calves. Manure was collected by scraping system and TS content was around 11.5%. Torna dairy farm was selected as it was located near the RMPP facility and can provide an opportunity for co-digestion of SHWWRP with manure.

2.3 Inoculum

Inocula for biomethane potential (BMP) measurements were obtained from Tallinn wastewater treatment plant (WWTP) anaerobic digester which treated a mixture of primary and secondary sludge at 37–38 °C. Influent to the WWTP originated from Tallinn city centre, residential areas and different industries – this ensured sufficiently diverse community of the inoculum for BMP measurements. Chemical composition of inoculum was 2.4% TS, 57.6% VS, 1.5 g/l total nitrogen (TN), 1.02 g/l ammonium nitrogen (NH₄), 0.6 g/l total phosphorus (TP), 18.1 g/l chemical oxygen demand (COD) and pH of 7.5.

2.4 BMP measurements

Batch tests of BMP were carried out in duplicate with 1200 ml Oxidex-C (WTW, Weilheim, RFA) respirometric system in accordance with the protocol proposed by the International Water Association Task Group for the Anaerobic Biodegradation. Activity and Inhibition Assays (Angelidaki et al., 2009). Working volume of the prepared batch assays were 200 ml. To each assay, substrate and inoculum were added at substrate to inoculum ratio (S/I) of 0.25–0.5. Prepared assays were incubated at 37.5 °C and maintained under continuous mixing conditions. Bottles were flushed with N₂ gas for 1.5 min and closed with airtight stoppers to maintain anaerobic conditions. Methane content was analysed with gas chromatograph (Model 3700 with thermal conductivity detector and PorapakQ column 1.8 m x 3.17 mm) and operation conditions were: oven temperature 60 °C and detector temperature 130 °C. Sampling intervals were dependant on biogas production intensity. Biogas production in control assays of inocula were subtracted from the sample assays. All BMP measurements were operated for 35–42 days.

2.5 Analytical methods

Substrate analysis was carried out by accredited laboratory Estonian Environmental Research Centre (EERCC). The following parameters were determined according to EVS-EN 150 and ISO standard methods: TS, VS, pH, total Kjeldahl nitrogen (TKN), TP, total organic carbon (TOC), sulphur (S), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and heavy metals cadmium (Cd), chromium (Cr), nickel (Ni), lead (Pb), zinc (Zn) and copper (Cu). HACH-LANGE spectrophotometer DR 2800 and HACH-LANGE cuvette tests were used for the determination of COD, TN, TP, and NH₄ of the inoculum. Inoculum TS and VS content were measured according to Standard Methods (APHA, 1998).

3. Results and discussion

3.1 Solid slaughterhouse waste resource

Overview of the slaughterhouses waste generation in Estonia is presented in Table 1. Estonia has long tradition with animal husbandry and meat production. In 2009, approximately 585,000 pigs and 60,000 cattle were raised and 2/3 of the animals were used as a raw material for meat industry. During the same year, slaughterhouses produced 22,709 tons of by-products with Category 1 slaughterhouse waste contributing only 7.8% (ESO, 2010). Manure and digestive tract content, which do not require pre-treatment before anaerobic digestion, contributed more than 85% of Category 2 slaughterhouse waste.

Food grade meat and by-products distribution per slaughtered animal were evaluated for cattle, pig and poultry and results are presented in Table 2. After processing, more than half (57%) of the slaughtered cattle bodyweight was considered as slaughterhouse by-product. The corresponding value for pigs was 19.8%. Results presented here were comparable with the data provided by Marcos et al. (2010).

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal by-products from Estonian slaughterhouses in 2009 (ESO, 2010).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Category</th>
<th>Animal by-products (ton/year)</th>
<th>Total by-products (mt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category 1</td>
<td>1765</td>
<td>1765</td>
</tr>
<tr>
<td>Total</td>
<td>10,869</td>
<td>22,709</td>
</tr>
<tr>
<td>Pig</td>
<td>1164</td>
<td>40</td>
</tr>
<tr>
<td>Cattle</td>
<td>5906</td>
<td>3450</td>
</tr>
<tr>
<td>Manure digestive tract content</td>
<td>1143</td>
<td>3108</td>
</tr>
</tbody>
</table>
3.2. Substrate composition

Characteristics of the SSHWWRP are listed in Table 3. All SSHWWRP had high TS content of 96–99%. Decanter sludge, MBM and melt had high nitrogen and phosphorous content and low C:N ratio. Flotation sludge had TS content of 22% and 57% of TS was crude fat. It is clear from the composition and physical characteristics that SSHWWRP can only be used as co-substrates and in relatively small amounts in order to avoid organic overload and potential ammonia or LCFR inhibition of anaerobic digestion. Co-digestion of different types of agricultural wastes can be a measure for mitigating the deficiencies of a particular feedstock for a mono-digestion. Co-digestion is beneficial in balancing the C:N ratio of the digester feed (Creamer et al., 2010).

3.3. Methane potential and mass balance of solid slaughterhouse wastes

BMP measurement results for studied substrates and mass balance for SSHWWRP are presented in Table 4. Cumulative methane production curves are shown in Fig. 1. Methane production started immediately for all the samples without noticeable lag phase. Category 2 and 3 SS HW degradation was improved by sterilization as approximately 90% of methane potential was produced within 10 days. A 211.6% increase in degradation rate between untreated and sterilized piggery SS HW was also reported by Rodriguez-Abalde et al. (2011). BMP experiments showed that SS HWWRP have high energy potential with methane yields of 390–978 m^3 CH₄/TVS_{added}. Highest methane yield of 978 m^3 CH₄/TVS_{added} was obtained for technical fat while lowest yield of 390 m^3 CH₄/TVS_{added} was obtained for MBM. Methane production potentials obtained for SS HWWRP in this study were higher than the potentials of 287–515 m^3 CH₄/TVS_{added} reported by Bayr et al. (2012). For untreated Category 2 and 3 SS HW methane potential of 308.3 m^3 CH₄/t was calculated based on the melt methane yield and rendering process mass balance.

Solid slaughterhouse waste rendering process flow scheme is presented in Fig. 2. Mass balance for rendering process showed that 450 kg of melt was produced for each ton of raw material processed. Water in the raw material was evaporated, condensed and directed to wastewater treatment. Melt was further fractionated to 40 kg of decanter sludge, 275 kg of MBM and 135 kg of technical fat. Approximately 496,000 m^3 of wastewater was produced annually at RMPP. During treatment, 3,200 ton of thickened flotation sludge was removed from the wastewater. Based on methane potential obtained for flotation sludge, 0.85 m^3 of methane can be produced per 1 m^3 of treated wastewater.

3.4. Energy balance and co-digestion of melt with manure

3.4.1. Rendering facility primary energy balance

Only fat was used as energy resource and it was burned in a gas boiler at RMPP. Calorific value of fat has been measured to be 39,200 MJ/t. This is equivalent to 1165 m^3 of natural gas (33.66 MJ/m^3). Fat is suitable for combustion as it has low concentration of nutrients that are lost into atmosphere or remain unusable in the ash. The average energy consumption (process heat + electrical energy) for sterilization of 1 t of Category 2 and 3 SS HW at the rendering facility has been calculated to be 639.6 kWh. However, with 135 kg of fat produced from 1 t of raw material, approx. 1470 kWh of energy can be recovered through combustion. For the same amount of fat, 1394 kWh (CH₄ calorific value = 10 kWh/m^3) of primary energy can be produced through

---

Table 2

<table>
<thead>
<tr>
<th>Food and by products</th>
<th>Cattle</th>
<th>Pig</th>
<th>Poultry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average weight of animal, kg</td>
<td>%</td>
<td>Mass, kg</td>
<td>%</td>
</tr>
<tr>
<td>Food products</td>
<td>43.0</td>
<td>239.2</td>
<td>80.2</td>
</tr>
<tr>
<td>Category 3 by-products</td>
<td>39.7</td>
<td>218.4</td>
<td>16.8</td>
</tr>
<tr>
<td>Category 2 by-products</td>
<td>9.3</td>
<td>51.2</td>
<td>3.0</td>
</tr>
<tr>
<td>Manure/digestive tract content*</td>
<td>8.9</td>
<td>40.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Category 1 by-products</td>
<td>8.0</td>
<td>44.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Total by-products</td>
<td>57.0</td>
<td>313.7</td>
<td>19.8</td>
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</table>

* Elucidates how large part of Category 2 by-products are manure and digestive tract content.

---

Table 3

<table>
<thead>
<tr>
<th>Parameter (mg/kg TS)</th>
<th>Melt</th>
<th>Decanter sludge</th>
<th>Meat and bone meal</th>
<th>Fat</th>
<th>Flotation sludge</th>
<th>Manure</th>
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<td>99</td>
<td>99</td>
<td>99</td>
<td>22</td>
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<tr>
<td>VS (%)</td>
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<td>79</td>
<td>66</td>
<td>100</td>
<td>86</td>
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<td>78,600</td>
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<td>2800</td>
<td>3100</td>
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<td>4900</td>
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<td>4531</td>
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<td>Zn</td>
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<td>15</td>
<td>3</td>
<td>21.4</td>
<td>27.2</td>
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### Table 4
Solid slaughterhouse wastes rendering process products mass balance and methane production potentials measured in batch assays at 37.5 °C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methane production (mL CH₄/VS added)</th>
<th>Methane production (mL CH₄/VS)</th>
<th>Mass, kg/t raw material</th>
<th>Methane production (mL CH₄/VS added)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melt</td>
<td>814</td>
<td>685</td>
<td>450</td>
<td>308.3</td>
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<tr>
<td>Decanter sludge</td>
<td>607</td>
<td>459</td>
<td>40</td>
<td>18.4</td>
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<tr>
<td>Meat and bone meal</td>
<td>290</td>
<td>259</td>
<td>275</td>
<td>71.2</td>
</tr>
<tr>
<td>Fat</td>
<td>978</td>
<td>966</td>
<td>135</td>
<td>130.4</td>
</tr>
<tr>
<td>Flotation sludge</td>
<td>650</td>
<td>131</td>
<td>6.45</td>
<td>0.85</td>
</tr>
</tbody>
</table>

![Graph](image1.png)

**Fig. 1.** Cumulative methane production from solid slaughterhouse waste rendering products at 37.5 °C.

anaerobic digestion i.e., ~11.3% lower energy than that produced through combustion.

If melt and flotation sludge from wastewater treatment were directly used as co-substrates in biogas plants, then theoretically 2950 kWh of primary energy can be produced from 1 ton of raw Category 2 and 3 SSHW. Thus, the energy obtained through anaerobic digestion of SSHWRP that had been generated from 1 ton of raw Category 2 and 3 SSHW was 4.6 times higher than the energy consumed during the rendering process. In a more general perspective, if all the Category 2 and 3 SSHW from Estonian slaughterhouses were dry-rendered and the obtained SSHWRP used for biogas production, then approx. 55 GWh of primary energy from unused renewable resource can be produced.

### 3.4.2. Effect of co-digestion of melt with manure
Addition of melt to manure in proportions of 2.5% and 5% per wet weight increased the methane production by 1.75 and 2.70 times respectively, compared to manure digestion alone – see Fig. 3. Methane production was delayed for both additional loadings. For example, sample with 2.5% melt addition reached 85% of total BMP yield on day 20, while it took 25 days for sample with 5% melt addition. Increasing melt proportions indicated a longer adaption time requirements for microbial biomass and need for continuous experiments to be carried out in order to assess possible ammonia or LCF inhibition at the proposed loadings.

From nutrient management point of view, N and P nutrients in melt (TKN content of 59.8 kg/t TS and TP content of 22.25 kg/t TS) can be recycled as plant nutrients and thus replace utilization of inorganic fertilizers. High TS content allows SSHWRP to be feasibly transported for longer distances to areas that are in nutrient deficiency, providing flexibility in logistics and optimized nutrient recycle. One ton of melt had 14 times higher fertilizer value compared to analysed dairy manure with 11.5% TS, 35.7 kgN/t TS and 12.9 kgP/t TS. Thus, co-digestion of dairy manure and melt

![Diagram](image2.png)

**Fig. 2.** Solid slaughterhouse waste rendering process flow scheme and its fractionation into various rendering process products (MBM: meat and bone meal).
Meat Processing Plant is acknowledged for providing the waste products of the rendering process and valuable data.

References


Fig. 3. Effect of melt addition to manure in proportions of 2.5% and 5% per wet weight.

increases nutrient concentrations in digestate and can significantly reduce digestate requirements per hectare of agricultural land. Advantages or disadvantages of the higher nutrient concentrations in digestate depend on pre-treatment and anaerobic digestion process conditions, manure/digestate availability in the region and properties of agricultural land area where it can be utilized.

4. Conclusions

In this study, five different Category 2 and 3 SSWRPs were characterised with high content of lipids and proteins and methane potentials of 390–678 m³ CH₄/kg VS. Besides high methane yield of melt 685 m³ CH₄/t, it was also considered as a valuable substrate for anaerobic co-digestion due to 14 times higher fertilizer value than manure. Anaerobic digestion of SSWRPs from dry rendering facility could recover 4.6 times more primary energy than the energy required for the rendering process.

Acknowledgements

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

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

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Co-digestion of sewage sludge and sterilized solid slaughterhouse waste: Methane production efficiency and process limitations

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HIGHLIGHTS

► Sterilized mass (SM) is an attractive substrate for biogas production.
► High TS, fat and proteins content of SM give methane yield of 590.5 m³·t⁻¹.
► SM addition of 5% (w/w) to sewage sludge increased methane production 5.7 times.
► Free ammonia inhibition threshold value was determined at 596.5 ± 68.6 gN/NS⁻¹ L⁻¹.
► SM addition of 10% (w/w) was detrimental to AD process due to intensive foaming.

ABSTRACT

The rendering product of Category 2 and 3 Animal By-Products is known as sterilized mass (SM) and it is mainly composed of fat and proteins, making it an interesting substrate for anaerobic digestion. Batch and semi-continuous laboratory experiments were carried out to investigate the effect of SM addition in co-digestion with sewage sludge on methane production and possible process limitations. Results showed that SM addition in the feed mixture up to 5% (w/w), corresponding to 68.1% of the organic loading, increased methane production 5.7 times, without any indication of process inhibition. Further increase of SM addition at 7.5% (w/w) caused methane production decrease and volatile solids removal reduction, that was mainly related to remarkably increased free ammonia concentration in the digester of 596.5 ± 68.6 gN/NS⁻¹ L⁻¹. Sterilized mass addition of 10% (w/w) caused intensive foaming. LCFA accumulation of 91.7 ± 70.2 mgCOD·L⁻¹·g⁻¹ sample and termination of the experiment.

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1. Introduction

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

Anaerobic digestion (AD) of sewage sludge (SS) has been largely applied at industrial and municipal wastewater treatment plants (WWTP) for decades. It is a well-known, efficient and environmentally sustainable technology which enables simultaneous energy recovery as biogas, as well as stabilization and volume reduction of sludge (Luostarinen et al., 2009). AD has also been considered as one of the best alternatives for energy recovery from ABP and slaughterhouse wastes (Heinjfelt and Angelidaki, 2009; Martinez-Sosa et al., 2009; Palatsi et al., 2011). According to the ABP Regulation EC No. 142/2011 the product of the rendering process, sterilized mass (SM), can be used as substrate for AD. SM is considered as an attractive substrate for AD due to its high organic content (mainly in the form of proteins and fats) and due to its high methane potential (Pitk et al., 2012). However, slow hydrolysis rates, operational problems and possible process inhibition have been reported when treating ABP. One of the major problems associated with the anaerobic treatment of ABP is the high fat and lipid content, that can cause sludge flotation and biomass washout, as
well as the potential inhibition of microbial activity due to the produced-accumulated long chain fatty acids (LCFA) (Alves et al., 2009; Edström et al., 2003). Floating LCFA could affect substrate bioavailability and biomass activity, thus causing common plant operation obstacles such as fouling-sccm overflow and process inhibition (Salminen and Rintala, 2002). In addition, ammonia is also produced during the anaerobic degradation of nitrogen containing compounds, such as proteins contained in ABP (Resch et al., 2011). Free (un-ionized) ammonia (NH3) has been suggested to be the main cause of inhibition in high nitrogen loaded AD processes (Chen et al., 2008). The hydrophobic ammonia molecule may diffuse passively into the cell, causing proton imbalance, and/or potassium deficiency in microorganisms, particularly in methanogens (Salminen and Rintala, 2002). Free NH3 concentration increases with increase in pH and temperature. Thus, the inhibitory effect of ammonia nitrogen on AD process depends on ammonium concentration, pH and temperature (Angelidaki and Ahring, 1994). A wide range of inhibiting ammonia concentrations have been reported in the literature. The total ammonia nitrogen (TAN, NH4+ and NH3) concentrations that can cause a 50% reduction in methane production range have been reported to be from 1.7 to 14 g L−1 (Chen et al., 2008; Nakashima et al., 2008).

Only few studies have focused on the use of ABP rendering products as co-substrates for biogas production (Bayr et al., 2012; Heijnfelt and Angelidaki, 2009; Pikt et al., 2012). On the other hand, several studies have successfully demonstrated the use of dissolved air flotation sludge and grease trap sludge also as feedstock for biogas production in co-digestion processes (Creamer et al., 2010; Davidson et al., 2008; Luostarinen et al., 2009; Silvestre et al., 2011). The present study was therefore focused on evaluating the process performance and methane production during anaerobic co-digestion of SS with SM. The effect of organic loading rate, with respect to the dosage of SM in the feed, on maximum methane production was also determined. In addition, potential process limitations due to free NH3 concentration, organic overloads (volatile fatty acids (VFA) accumulation) and lipids (or LCFA) inhibition were also evaluated.

2. Methods

2.1. Substrates and inoculum

Sewage sludge (SS) was collected from a full scale WWTP facility (Tallin, Estonia). SS used as substrate in the present experiment, was obtained from a primary clarifier and it mainly contains primary sludge and small proportion of waste activated sludge, that is recycled to the primary clarifier. To consider the variations in sludge characteristics in the sampled WWTP facility, up to five SS sampling campaigns were performed (SS1−SS5). Inoculum (I), used as anaerobic seed sludge in batch test and lab reactors, was also obtained from the full-scale anaerobic digester of the WWTP, treating a mixture of primary and secondary sludge.

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

2.2. Batch experiments

Biomethane potential (BMP) tests were carried out with OxITop-C (WTW, Weilheim, Germany) respirometric system in accordance with the protocol proposed by Angelidaki et al. (2009). BMP experiments were performed with SM and SS samples to determine substrates methane potential and assess biodegradability. Details of the batch experimental set-up are described elsewhere (Pikt et al., 2012). Briefly, to each assay, substrate (SS or SM) and inoculum (I) were added in a volatile solids (VS) ratio (VSsubstrate/VSinoculum) of 0.50 and 0.25 for SS/I and SM/I, respectively. Vials (in duplicate for each assay) were flushed with N2 gas, sealed immediately with air-tight stoppers and incubated at 37.5 °C under continuous mixing condition. Methane production from I vials (controls) was subtracted from the SM and SS tests to determine net methane potentials.

2.3. Reactor experiments

Process performance and CH4 yields during the anaerobic co-digestion of SS with SM was conducted in 2 plexiglas lab reactors (5 L total capacity) with a working volume of 4.5 L. Reactors were operated at 37 ± 1 °C by hot water circulating through the reactors waterjacket, and under intermittent mixing conditions (160 rpm, 15 min on/off) by magnetic stirrer (MAG M57.1KA, Germany). Substrate feeding was performed once per day (including weekends) during the whole experimental period (220 days). SS was fed using a sterile plastic 100 mL syringe, while SM was manually fed through an inlet pipe at the top of the reactor.

Reactor 1 (R1) was inoculated with 4.5 L of inoculum and maintained in batch conditions (day 1 to 15) for initial inoculum starvation (removal of residual organic matter). After this initial step, reactor was exclusively fed with SS (from day 15 to 37) with a loading rate of 2.25 mLg−1day−1, resulting in an hydraulic retention time (HRT) of 20 days. Thereafter, co-digestion of SM with SS was initiated (day 37 onwards). HRT was fixed in 22.5 days (for feeding convenience), while the proportion of SM in the feed was gradually increased from 0 to 10% (w/w), in 2.5% steps along the experimental period.

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

Biogas production in reactors was continuously measured with a gas,se-milligascounter (MGC-1 V3, Ritter, Germany). Reactors temperature and pH were monitored daily, while methane content in produced biogas (%CH4) was measured weekly. Effluent characteristics were analysed at the end of each loading period, according to Analytical methods section.

2.4. Analytical methods and calculations

Total (TS) and volatile (VS) solids, total chemical oxygen demand (CODTOD), total nitrogen (TN), total phosphorous (TP), and ammonium (TAN) were analysed according to the process conditions and OLR regime changes. Analyses were conducted by accredited laboratories Estonian Environmental Research Centre and Agricultural Research Centre, pH, TN, TP, total organic carbon (TOC), sulphur, potassium, magnesium, raw fat and raw protein in SM were determined according to EVS-EN ISO and ISO standard methods. HACH-LANGE spectrophotometer DR 2800 and cuvette tests were used for the determination of CODTOD, TP, and NH4+ in both SS and Inoculum. TS and VS contents were determined according to Standard Methods (APHA, 1998).

Methane content in the biogas was analysed with gas chromatograph (Model 3700, with thermal conductivity detector (TCD) and PorapakQ column 1.8 m × 3.2 mm) and N2 was used as carrier gas. VFA analysis was performed using GC 2014 AT/
SPL (Shimadzu, Japan) gas chromatograph equipped with a Zebron ZB-WAXplus capillary column (35 m × 0.25 mm × 0.25 µm) and flame ionization (FID) detector. Total LCFA from C12 to C24, including LCFA forming part of glycerides, were determined according to the method described by Palatsi et al. (2009), based on direct methylation-extraction procedure. LCFA were identified and quantified by GC CP-3800 gas chromatograph (Varian, USA), fitted with CP7489/CP-Sil 88 FAME capillary column (50 m × 0.25 mm × 0.25 µm) and FID detection.

Theoretical CH₄ potential at standard conditions (STP, 0 °C and 1 bar) was estimated according to the following Eq. (1), based on the protein, lipid and carbohydrate contents of the substrates, as suggested by Angelidaki and Sanders (2004):

\[ \text{CH}_4 \text{ yield (dm}^3\text{CH}_4 \text{kg}^{-1} \text{VS}) = 496 + X + 1014 \times Y + 415 \times Z \]  

where \(X\%\) of proteins \(Y\%\) of lipids and \(Z\%\) of carbohydrates. The unionized fraction of the ammonia nitrogen (NH₃) was calculated according to the Eq. (2), as described by Körner et al. (2001):

\[ \text{NH}_3 \% \text{ of TAN} = 100/(1 + 10^{(\text{pH} - \text{pK}_a)}) \]

\[ \text{pK}_a = 0.09108 + 272.92/(273.2 + T) \]

where \(\text{pK}_a\) is the dissociation constant dependent of temperature \(T\) and the temperature in Celsius degrees (°C).

3. Results and discussion

3.1. Substrates characterization

Fresh SS from WWTP was sampled five times (SS₁-SS₅) during the experimental period and the variations in samples composition are shown in Table 1. TS content fluctuated around 3.3 ± 0.4 while VS content remained more or less stable (65.6 ± 1.8% of TS). The variations in SS characteristics were related to sampling time (seasonal variation) and to the settling efficiency in primary settler (daily basis variation). On the other hand, variation in COD, TN and TP concentrations among the five SS samples were lower than 30% (Table 1).

Chemical composition of SM is also presented in Table 1. After the dry-rendering process, SM contained only 4% water. The organic matter present in SM was mainly composed of fats and proteins (55.8% and 31.1% of the VS content, respectively) reaching a high TOC value of 53 ± 13.5 gTOC kg⁻¹ VS and a C/N ratio of 9.3 ± 0.7 (Table 1).

3.2. Batch experiments

BMP experiments were conducted with only one of the five SS samples. This sample (SS₅) had a 3.1% of TS and a 65.8% of VS/TS. The methane potential of SS₅ estimated by the BMP test was

\[ 239.9 \text{ m}^3 \text{CH}_4 \text{ m}^{-3} \text{VS} (\text{or } 5.0 \text{ m}^3 \text{CH}_4 \text{ t}^{-1}) \]

As expected, due to the high organic content, SM had a higher methane yield of 719.25 m³ CH₄ t⁻¹ VS (or 590.5 m³ CH₄ t⁻¹). The obtained methane yield correspond to 99% of the theoretically estimated SM methane potential (721.1 m³ CH₄ t⁻¹ VS, according to Eq. (1), indicating a high biodegradability and energetic potential of the SM.

The experimental methane yield obtained for SM in the BMP test was a 14% lower than the previously reported values by Pitk et al. (2012) with samples obtained in the same rendering facility. This is expected due to the daily variations of the ratio of slaughtered cattle/pigs in the facility, and consequently characteristics of the SM differ in time. If different facilities are sampled, this heterogeneity could be higher. For example, Bayr et al. (2012) reported a significantly lower methane yields (515 m³ CH₄ t⁻¹ VS or 343 m³ CH₄ t⁻¹) for similar substrates.

3.3. Reactor experiment

3.3.1. Process performance

Reactors operational conditions (HRT, ORL and nitrogen loading rate (NLR)) and efficiencies (VSeffective methane production and yields) are summarized in Fig. 1 and Table 2. After the described reactor start-up (first 15 days), R1 was fed with SS alone with a HRT of 20 days and at an equivalent ORL of 1.4 kgVS m⁻³ day⁻¹, obtaining a stable methane production of 1.5 ± 0.2 LCH₄ day⁻¹ (Fig. 1). Methane yield during the mono-digestion of SS₅ (days 15-37) was 233.9 ± 37.4 m³ CH₄ t⁻¹ VS added (65.9 ± 1.1 m³ CH₄ t⁻¹). This value is quite similar to the obtained value in the BMP test. Mean VS removal efficiency during this period was 46.8% (Table 2 and Fig. 1).

Co-digestion of SS with SM was initiated on day 38 and HRT was regulated to 22.5 days. Initially, SM was introduced to feeding mixture in a proportion of 2.5%. This small fraction represents an important organic content of the reactors input, representing a 42.9% in terms of VS, increasing the reactor ORL up to value of 2.13 kgVS m⁻³ day⁻¹. Methane production was rapidly increased up to values of 3.8 ± 0.3 LCH₄ day⁻¹ (Fig. 1), corresponding to a mean methane yield of 396.1 ± 29.3 m³ CH₄ t⁻¹ VSs. On day 63, the SM proportion was increased up to a 5% of the input, increasing reactor ORL and methane yield up to 2.68 kgVS m⁻³ day⁻¹ and 618.9 ± 70.7 m³ CH₄ t⁻¹ VS, respectively, without any sign of process imbalance. Contrary, when the SM was added at an amount of 7.5% (3.55 kgVS m⁻³ day⁻¹), the process efficiency started to decrease. Process limitations were detected by a gradual decrease in methane production and by the stabilization of the VS removal rates (Table 2). Reactor operation at 7.5% of SM was divided into two separate periods of 43 days (P₁ and P₂). During P₁ (days 116-159), methane yield was 644.8 ± 63.6 m³ CH₄ t⁻¹ VS with a VS removal efficiency similar to the one obtained with the addition of a 5% of SM (Table 2). This indicated process imbalance, as ratio of highly biodegradable SM in input mixture had increased. Definite

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SS₁</th>
<th>SS₂</th>
<th>SS₃</th>
<th>SS₄</th>
<th>SS₅</th>
<th>SS_MEAN</th>
</tr>
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<tbody>
<tr>
<td>TS (%)</td>
<td>3.1</td>
<td>4.1</td>
<td>3.2</td>
<td>3.1</td>
<td>3.1</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>VS (%)</td>
<td>65.9</td>
<td>68.2</td>
<td>68.9</td>
<td>65.8</td>
<td>65.6± 1.8</td>
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<tr>
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<td>42.8</td>
<td>30.3</td>
<td>32.1</td>
<td>35.6</td>
<td>36.7± 5.9</td>
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<tr>
<td>TN (mg L⁻¹)</td>
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<td>1346</td>
<td>1165</td>
<td>1449</td>
<td>1259</td>
<td>1349± 144</td>
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<tr>
<td>N₂O (%)</td>
<td>184</td>
<td>224</td>
<td>139</td>
<td>197</td>
<td>215</td>
<td>195± 31.8</td>
</tr>
<tr>
<td>TP (mg L⁻¹)</td>
<td>568</td>
<td>666</td>
<td>857</td>
<td>708</td>
<td>543</td>
<td>614.4± 60.7</td>
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<tr>
<td>TOC (g L⁻¹)</td>
<td>nd</td>
<td>nd</td>
<td>9.3</td>
<td>nd</td>
<td>8.1</td>
<td>8.7± 0.9</td>
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</table>

n.d not determined.
implications of process imbalance appeared during P2 (days 160–202), in which methane production started to decrease in conjunction with a reduction in VS removal rates (Table 2). Finally, increase in SM amount in the feed ratio up to 10% w/w (representing 81.92% in terms of VS daily load) resulted in a clear decrease in methane production (Fig. 1). After two weeks of operation at 10% of SM addition, process started to show symptoms of failure with intensive foaming and clogging of gas pipes that led to termination of the experiment.

R2 was used as a control reactor and was started on day 122 of the experiment, at the same time when 7% SM loading was initiated in R1. R2 was also operated with a HRT of 22.5 days and with OLR of 0.9 ± 0.04 kgVS m⁻³ day⁻¹, producing 92.9 ± 17.0 m³CH₄ t⁻¹ VS (19.9 ± 0.4 m³CH₄ t⁻¹) with a VS removal efficiency of 41.6. These values are lower than the ones reported during the SS mono-digestion in R1, due to the lower COD content of SS–S; and probably to a lower biomass activity of R2 compared with R1. Still, those values can be considered “normal” for anaerobic digestion of SS (Davidsson et al., 2008; Luostarinen et al., 2009).

Effluent concentration was analysed at the end of each loading regime and the results are presented in Table 3. Results showed that organic concentrations increased in accordance with the increasing OLR, nitrogen load and influent TS content increase. To conclude, the addition of 2.5% and 5% of SM increased CH₄ production by 2.9 and 5.7 times (respectively to R1 SS mono-digestion) without detecting any process instability. First instability was detected with SM addition at 7.5% (representing an OLR of 3.55 kgVS m⁻³ day⁻¹, and a nitrogen loading rate of 0.25 kgN m⁻³ day⁻¹). Further SM addition of 10% resulted in nitrogen load increase up to maximum load of 0.32 kgN m⁻³ day⁻¹. This increase in N was 5.3 times higher compared to initial SS mono-digestion value causing a clear TAN accumulation in the reactor over the optimal values required for stable reactor operation.

In the present study, optimal OLR and methane production obtained during 5% SM addition (2.68 kgVS m⁻³ day⁻¹ and 618.9 ± 70.7 m³CH₄ t⁻¹ VS, respectively) were higher than those reported in the literature. Salminen and Rintala (2002) have reported methane yields of 520–550 m³CH₄ t⁻¹ VS under mesophilic conditions for solid slaughterhouse waste operated at considerably lower OLR of 0.8 kgVS m⁻³ day⁻¹ with 50 days HRT. Hejnefelt and Angelidaki (2009) reported only 40% higher methane production during co-digestion of 5% pork by-products mixed with pig manure at 37°C compared to digestion of manure alone. On the other hand, Bayr et al. (2012) reported higher methane yields of 720 m³CH₄ t⁻¹ at OLR of 1.0 and 1.5 kgVS m⁻³ day⁻¹ in mesophilic CSTR experiments. However, the above authors used only energy rich rendering and slaughterhouse wastes for co-digestion.

3.3.2 Process limitations

To elucidate the possible causes of the detected process imbalance in R1, when the SM addition was >5%, the reactor content composition was analysed in the end of the different loading periods. TAN and NH₃ concentration evolution in conjunction with OLR and pH are shown in Fig. 1c and summarized in Table 3. TAN concentration during mono-digestion of SS in R2 was 585 ± 51.1 mgN-NH₄ L⁻¹. At 5% of SM loading in R1, TAN concentration was increased up to values of 1931 mgN-NH₄ L⁻¹, although the process remained stable (Fig. 1). These NH₄⁺ values were similar to the values of 2.1–3.1 gN-NH₄ L⁻¹, reported to be non-inhibitory for anaerobic digestion (Procházka et al., 2012). Ammonium accumulation increased with the SM loading at 7.5%. Moreover, the estimated free NH₃ concentration reached values of

![Fig. 1. Reactor (R1) process parameters: (a) cumulative methane production, ml day⁻¹ (%); (b) organic loading rate, kgVS m⁻³ day⁻¹ (%); (c) SM share of input VS, %; (d) VS removal efficiency; (e) pH, (f) ammonium, mg L⁻¹ (%); and free ammonia, mg L⁻¹ (%); concentration patterns. Dashed vertical lines indicate the different feeding periods of sterilized mass co-digestion.](image-url)

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SS</th>
<th>SM 2.5%</th>
<th>SM5%</th>
<th>SM7.5%S</th>
<th>SM7.5%C</th>
<th>SM10%</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT (days)</td>
<td>20</td>
<td>22.5</td>
<td>22.5</td>
<td>22.5</td>
<td>22.5</td>
<td>22.5</td>
<td>22.5</td>
<td></td>
</tr>
<tr>
<td>OLR (kgVS m⁻³ day⁻¹)</td>
<td>1.4</td>
<td>2.13</td>
<td>2.68</td>
<td>3.55</td>
<td>3.55</td>
<td>4.54</td>
<td>0.9 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>NLR (kgN m⁻³ day⁻¹)</td>
<td>0.06</td>
<td>0.13</td>
<td>0.2</td>
<td>0.25</td>
<td>0.25</td>
<td>0.32</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Methane production (LCH₄ day⁻¹)</td>
<td>1.5 ± 0.2</td>
<td>3.8 ± 0.3</td>
<td>7.5 ± 0.9</td>
<td>10.4 ± 1.0</td>
<td>9.4 ± 0.8</td>
<td>11.5 ± 0.8</td>
<td>0.4 ± 0.1</td>
<td>41.6</td>
</tr>
<tr>
<td>Methane yield (m³CH₄ t⁻¹ VS)</td>
<td>2.13 ± 3.74</td>
<td>396.1 ± 29.3</td>
<td>618.9 ± 70.7</td>
<td>644.8 ± 63.6</td>
<td>584.8 ± 50.2</td>
<td>551.3 ± 36.0</td>
<td>92.9 ± 17.0</td>
<td>31.8 ± 3.5</td>
</tr>
</tbody>
</table>

n.d. not determined.
Table 3
Mean values of reactors (R1 and R2) outflow characteristics at the different operational periods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SS</th>
<th>SM2.5%</th>
<th>SM5%</th>
<th>SM7.5%</th>
<th>SM7.5%N1</th>
<th>SM7.5%N2</th>
<th>SM10%</th>
<th>R2</th>
<th>SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.3 ± 0.1</td>
<td>7.5 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>8.2 ± 0.1</td>
<td>8.4 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COD_{TS} (mg L^{-1})</td>
<td>193</td>
<td>30.9</td>
<td>34.6</td>
<td>48.4</td>
<td>47.5</td>
<td>52.9</td>
<td>20.2 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH_4^+ (mg L^{-1})</td>
<td>783</td>
<td>1226</td>
<td>1931</td>
<td>2795</td>
<td>2993</td>
<td>3700</td>
<td>585 ± 51.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free NH_3 (mg L^{-1})</td>
<td>28 ± 4.2</td>
<td>85.5 ± 6.4</td>
<td>246.5 ± 34.6</td>
<td>548 ± 33.9</td>
<td>645 ± 41</td>
<td>1035 ± 115.3</td>
<td>21 ± 6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P_{PO_4} (mg L^{-1})</td>
<td>621</td>
<td>1227</td>
<td>1396</td>
<td>1946</td>
<td>2000</td>
<td>2443</td>
<td>572.5 ± 55.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCFA (mgCOD/LCFA g^{-1})</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>1170 ± 6.4</td>
<td>1330 ± 63.4</td>
<td>(2001 ± 309.2/9172 ± 7012)^a</td>
<td>744 ± 198.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Fig. 2. Relation between TAN (mg L^{-1}) or OLR (kgdrym^{-1} d^{-1}) and methane yield at stable process conditions of different sterilized mass loading periods.

Table 4
VFA production-consumption pattern before and after feeding at SM7.5%N1 loading rate in R1.

<table>
<thead>
<tr>
<th>SM7.5%N1</th>
<th>mgCOD/VFA g^{-1} L^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>Propanoic</td>
</tr>
<tr>
<td>Before feeding: 10 am</td>
<td>118</td>
</tr>
<tr>
<td>11 am</td>
<td>368</td>
</tr>
<tr>
<td>1 pm</td>
<td>393</td>
</tr>
<tr>
<td>3 pm</td>
<td>314</td>
</tr>
<tr>
<td>4.30 pm</td>
<td>296</td>
</tr>
<tr>
<td>6 pm</td>
<td>245</td>
</tr>
<tr>
<td>7.30 pm</td>
<td>212</td>
</tr>
<tr>
<td>Before feeding: 10 am</td>
<td>89</td>
</tr>
</tbody>
</table>

596.5 ± 68.6 mgNO_3 N L^{-1} during the 7.5% of SM addition (days 116-202), according to Fig. 2 and Table 3. This NH_3 level detected in R1 were within the inhibitory range reported in literature. Galler and Winter (1997) indicated that NH_3 concentrations of 560-680 mgNO_3 N L^{-1} caused a 50% inhibition of methanogenesis at pH of 7.6 under thermophilic condition. Similarly, Angelidaki and Ahring (1994) reported a decrease in biogas yield during anaerobic digestion of manure where NH_3 levels reached a concentration of 700 mgNO_3 N L^{-1}. The over 2-fold increase in NH_3 concentrations at SM7.5% (596.5 ± 68.6 mgNO_3 N L^{-1}) period compared to SM5% loading (246.5 ± 34.6 mgNO_3 N L^{-1}) was produced compared to the high NLR and the high pH values. The direct relation between the free ammonia concentration (causing process inhibition) and OLR to methane yield is graphically reported in Fig. 2. As most of the organic loading during co-digestion came from nitrogen rich SM, then methane yield relation to OLR had similar pattern to ammonia inhibition curve. Methane yield increased concurrently with NH_3 concentration up to value of 548 ± 33.9 mgNO_3 N L^{-1} (period P1 of 7.5% SM addition). Higher NH_3 concentrations (during period P2 of 7.5% SM addition) caused process imbalance and methane yields decrease (Fig. 2). Finally, NLR in R1 continued increasing along SM addition (up to SM10%) causing a clear NH_3 accumulation (1035.5 ± 115.3 mgNO_3 N L^{-1}) in the reactor over the optimal required values for stable operation, concluding in an inhibitory process.

VFA monitoring along experimental time did not indicate an organic overload or other process imbalance in R1. As example, in Table 4 are summarized the VFA production and consumption after a feeding pulse during SM7.5%N1 addition period, when VS removal efficiency and methane production started to decrease. After feeding, VFA concentration reached a maximum concentration of 505 mgCOD/VFA L^{-1}, within few hours, and then started to decrease. Acetate and iso-butyrate were the dominant identified VFAs, while the propionate concentration remained stable throughout feeding cycle between 12 and 22 mgCOD/VFA L^{-1}. At the end of the daily feeding cycle (24 h), total VFA concentration reached the similar level of 108 mgCOD/VFA L^{-1} as before the feeding cycle. Nakakubo et al. (2008) studying process indicators of ammonia inhibition during thermophilic animal manure digestion, concluded that acetate and propionate were not always accumulated in ammonia inhibited systems, and consequently not always used as process indicators.

Therefore, some LCFA measurements were carried out in the reactor effluents in order to assess the possible inhibition due to fats or LCFA accumulation. One LCFA sample was taken from R2, as control, while three samples from R1, during SM7.5% and SM10% loading regimes were selected (Table 3). Total LCFA concentration in R2 was 744.4 ± 198.24 mgCOD/LCFA g^{-1} sample. On the other hand, total LCFA concentration in R1 was between 1170 ± 6 and 1330 ± 63 mgCOD/LCFA g^{-1} sample during SM7.5% loading. Palasti et al. (2009) reported that LCFA concentrations over 2.8 mgCOD/LCFA g^{-1} sample were necessary to inhibit thermophilic digestion of manure in batch and semi-continuous experiments (resulting in a temporary cease of the methane production). Thus, the imbalance or inhibition detected in R1 during SM7.5% addition seems to be more related with ammonia inhibition than with organic overload or LCFA inhibitory effect. Contrary, when R1 was submitted to SM10% loads, reactor resulted in intense foaming and clogging of gas outlets and thus the experiment had to be terminated. Two samples were collected for LCFA analyses, one from the top foamy layer and the other one from the lower part of the reactor. LCFA concentration in the top layer was up to 9172 ± 7012 mgCOD/LCFA g^{-1} sample, mainly formed by palmitic and oleic acids, while the detected value in the lower part of the reactor was of only 2001 ± 309.2 mgCOD/LCFA g^{-1} sample. Thus, complete process failure at SM10% loading was apparently due to an accumulation of LCFA. Liu et al. (2007) demonstrated that high ammonia concentrations could also affect the lipid hydrolysis rates and the LCFA accumulation-inhibitory process.

4. Conclusion
Results of semi-continuous reactor experiments confirmed the suitability and attractiveness of sewage sludge co-digestion with...
SM. Based on the obtained data, optimal process conditions were established at loading rate of 5% of SM, with an equivalent OLR below 2.68 kgVSS m$^{-3}$ day$^{-1}$. These conditions enhanced methane production by 5.7 times compared to SS mono-digestion. During this period NH$_4^+$ concentration remained below inhibitory levels (<500 mgN/L$^{-1}$). Higher SM addition (up to 10% w/w) caused process imbalance, initiated by NH$_4^+$ accumulation and finalizing with lipids-LCFA accumulation, foaming, methane yield reduction and termination of the experiment.

Acknowledgements

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References


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Mesophlic co-digestion of dairy manure and lipid rich solid slaughterhouse wastes: process efficiency, limitations and floating granules formation.

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Mesophilic co-digestion of dairy manure and lipid rich solid slaughterhouse wastes: Process efficiency, limitations and floating granules formation

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HIGHLIGHTS

- 5% decanter sludge addition to dairy manure increased biogas production 3.5-fold.
- 1% pure fat addition allows efficient and stable co-digestion process with manure.
- 2% pure fat addition to dairy manure induced formation of floating granules.
- Floating granules were agglomerates of calcium salts of LCFA.
- Floating LCFA "granules" is a new proposed mechanism for LCFA accumulation.

ABSTRACT

Lipid and protein rich solid slaughterhouse wastes are attractive co-substrates to increase volumetric biogas production in co-digestion with dairy manure. Addition of decanter sludge (DS), containing 42.2% of lipids and 35.8% of proteins (total solids basis), up to 5% of feed mixture resulted in a stable process without any indication of long chain fatty acids (LCFA) or free ammonia (NH3) inhibition and in 3.5-fold increase of volumetric biogas production. Contrary, only lipids addition as technical fat (TF) at over 2% of feed mixture resulted in formation of floating granules (FG) and process efficiency decrease. Formed FG had low biodegradability and its organic part was composed of lipids and calcium salts of LCFA. Anaerobic digestion process intentionally directed to FG formation, could be a viable option for mitigation and control of lipids overload and derived LCFA inhibition.

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1. Introduction

Biogas production in European agricultural sector has gained more importance and recognition as an efficient and sustainable manure treatment technology. The main drawbacks in use of dairy manure are that reactor volumes are large and its energy content is low, so high energy content co-substrates are required for improved economic feasibility of the biogas plants (Neves et al., 2008), especially in the countries with low renewable energy subsidies. Solid slaughterhouse wastes (SSHW), which are rich in proteins and lipids, are interesting co-substrates to improve volumetric biogas production of manure based biogas plants (Edström et al., 2003; Bayr et al., 2012), especially after sterilization (133 °C, 3 bars for 20 min) and fractionation to high energy content technical fat, meat and bone meal and decanter sludge (Pitk et al., 2012). However, lipids and proteins are compounds reported to cause process imbalance at high loading rates due to long chain fatty acids (LCFA) and free ammonia (NH3) accumulation leading to decreased process performance and inhibition (Edström et al., 2003; Nielsen et al., 2007; Heijniet and Angelidaki, 2009; Palatsi et al., 2011). As manure based suspended sludge anaerobic co-digestion processes have high buffering capacity, then VFA build-up due to degradation of proteins and lipids at higher loading rates does not lead to immediate process acidification. Instead, process imbalances and efficiency reductions are mostly related to LCFA accumulation and NH3 toxicity. Decrease of process efficiency during anaerobic digestion of lipids is mainly attributed to accumulation and by-pass of LCFA through the reactor or precipitation with divalent ions (Pereira et al., 2005). For
LCFA degradation β-oxidation has been reported as a rate-limiting step, which also has been reported to be inhibited by LCFA accumulation itself (Hanaki et al., 1981; Kim et al., 2004). Regarding LCFA inhibition, suspended sludge has been reported to be more advantageous than granular sludge as it has higher capacity of LCFA adsorption and degradation (Perreira et al., 2002). For suspended sludge oleic acid has been shown to be more toxic than palmitic acid, as oleic acid fed sludge was “encapsulated” by LCFA (85% of it as palmitic acid) that created physical barrier around microbial biomass and hindered the transfer of substrate and products. This has been confirmed by specific methanogenic activity tests, clearly showing that oleic acid fed and LCFA encapsulated suspended sludge being able to transform only H2/CO2 and ethanol to methane, while palmitic acid fed suspended “non-encapsulated” sludge was able to consume in addition acetate and propionate (Perreira et al., 2004). In terms of toxicity limit, a sixteen-fold higher IC0 value was found for palmitic (1100 ± 50 mg/l), than for oleic acid (70 ± 10 mg/l) revealing a higher tolerance of aceticlastic methanogens to palmitic acid, than to oleic acid (Perreira et al., 2005; Nielsen and Ahring, 2006) and Palatii et al. (2010) fed manure based continuous stirred reactors (CSTR) with oleate pulses, concluding that process performance and inhibition severity by oleate was related to oleate: biomass ratio and biotemplates content in the reactor, that can be used as adsorption media for LCFA. In addition, pulse loadings induced increase in the tolerance level of aceticrophic methanogens towards oleate. Basirba et al. (2012) have emphasized the importance of continuous LCFA exposure for development of a stable and active reactor for anaerobic digestion of lipids containing wastes, based on continuous oleate and manure fed CSTR experiments.

With respect to proteins degradation, increase of ammonium and NH3 concentrations in the digester may lead to so-called “inhibited steady state” due to VFA accumulation, pH decrease and biogas production stabilization at lower rate (Angelidaki and Ahring, 1993). This “inhibited steady state” is related to switch from generally prevailing aceticlastic methanogenic consortia, which are more sensitive to NH3 and VFA build-up, to syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. That was experimentally demonstrated by Schnürer and Nordberg (2008) in pilot-scale studies on ammonium concentrations >3 g L−1. However, contradictory results regarding ammonium concentrations and ammonium acetate interactions have been obtained in batch experiments regarding the conditions that induce the switch from aceticlastic methanogenesis to SAO pathway (Fotidis et al., 2013; Lü et al., 2013). In addition, presence of high concentrations of lipids and proteins in the feed mixture promote foaming at increased loading rates, causing clogging of gas pipes and blockages of gas mixing devices (Ganidi et al., 2009).

Kougias et al. (2013) reported lipids and proteins related foam characteristics to be different, with gelatine fed reactor foam being tight and compact, while the foam created in the Na-Oleate fed reactor being light bubbly with colourfull refection. According to present knowledge there are only a few studies about anaerobic co-digestion of manure with concentrated lipids or lipids and proteins rich SSW in suspended sludge CSTR systems (Nielsen et al., 2007; Heijnfelt and Angelidaki, 2009; Bayr et al., 2012). Most of the studies have reported results on the effect of organic loading rate (OLR), biogas yield, VFA profiles, ammonium and free ammonia concentrations interactions at mesophilic and thermophilic temperatures. However, no data about lipids degradation efficiency influenced by LCFA accumulation, precipitation or by-pass through the reactor has been reported. Main limitation of LCFA inhibition studies has usually been that single LCFA substrate (oleate or palmitate) have been used (Perreira et al., 2004, 2005; Nielsen and Ahring, 2006; Palatii et al. 2010; Basirba et al., 2012), which definitely has somewhat different effect than real waste with mixture of variety of LCFAs in it. In the present study co-digestion of liquid dairy manure with lipid rich technical fat or lipid and protein rich decanter sludge was investigated to elucidate LCFA inhibition in manure based suspended CSTR systems. High lipids concentration substrate mixture effect on the process performance and biogas production efficiency with special attention on lipids conversion mechanism, LCFA accumulation and occurrence of foaming was studied.

2. Methods

2.1 Substrates and inoculum

Samples of liquid dairy manure (LM1 and LM2) were collected from Rahinge free barn farm (Ilmastu, Estonia) and stored at 4 °C during the whole experiment. In order to avoid mixing problems in laboratory reactors long silage pieces and fibers in LM were separated by using a screw press. Inoculum used as seed for the lab reactors was obtained from pilot scale (201L) CSTR operated with the LM from the same origin at mesophilic conditions.

Lipid rich co-substrates, decanter sludge (DS) and technical fat (TF) were obtained from cattle and bovine slaughterhouse Category 2 and 3 animal by-products (ABP) rendering facility sterilization unit (Rakvere, Estonia). Sterilization procedure at the slaughterhouse was carried out in accordance with. European Parliament and the Council. 2011. Regulation (EC) No. 142/2011, at 133 °C, 3 bar for 20 min. More detailed description of sterilization end-products can be found in Pitk et al. (2012).

2.2 Experimental setup

Co-digestion experiments of LM with DS and TF were conducted in 2 plexiglas CSTR laboratory reactors (5 L total capacity) with a working volume of 4.5 L. Temperature in the reactors was maintained at ±1 °C by hot water circulating through the reactors waterjackets. Reactors content were mixed intermittently at 160 rpm (15 min on/off) by magnetic stirrer (MAG M57, IKA, Germany). Substrate feeding was performed daily during the whole experimental period of 200 days in a fed-batch feeding regime. The start-up procedure was the same for both reactors (R1 and R2), with the addition of 4.5 L of inoculum and maintaining batch conditions during 2 days for temperature stabilization and initiation of biogas production. After the start-up both reactors were exclusively fed with LM for 45 days with a loading rate of 200 mL day−1, resulting in an hydraulic retention time (HRT) of 22.5 days. This 45 days of LM, mono-digestion period was considered as control phase for both of the reactors. After control phase, co-digestion of LM with DS was initiated in R1 and co-digestion of LM1 and TF was initiated in R2. R2 was operated for 200 days but R1 had to be stopped due to reactor technical failure on day 170. Biogas production in reactors was continuously measured with an on-line milligascounter (MGC-1 V3, Ritter, Germany), while methane content in produced biogas (%CH4) was measured weekly by gas chromatography. Temperature in the reactors was checked daily while different process parameters and intermediate compounds were analysed weekly. Complete characterization of effluents was carried out at the end of each loading period according to methods described in Section 2.3.

Samples of floating granules (FG) formed in R2 were removed from the reactor to analyse its morphology and composition. Model 5-250 D sonifier (Branson Sonic Power Co., USA) equipped with a titanium horn disruptor, working at a constant operational frequency of 20 kHz was used for sonication of FG. Aliquots (45.8 mL) of FG solution (0.13 g FG and 44.29 g demineralized water)
were treated in the lab-scale sonifier in glass vials of 120 mL. The treatment was done in duplicate at ambient temperature (20.1 °C). Sonication was performed at three levels: low (10 × 10^5 kJ kg⁻¹), medium (100 × 10^5 kJ kg⁻¹), and high (200 × 10^5 kJ kg⁻¹). The energy was applied with power amplitude (β) of 12.5-25% and a time exposure between 3 and 190 s, which led to three energy densities (0.5; 1.1; 2.2 W mL⁻¹).

2.3. Analytical methods

Total solids (TS), volatile solids (VS), crude fat content (lipids), total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC), ammonium (NH₄) and macro- and micro-nutrients were determined in samples of raw substrates and effluents of the reactors. Effluent samples were collected at the end of different OLR periods. TOC, TN, TP, sulphur, potassium, magnesium, sodium and calcium in TF and DS samples were determined according to EVS-EN ISO and ISO standard methods in the Estonian Environmental Research Centre (Tallinn, Estonia) and in the laboratory of Plant Biochemistry of the Estonian University of Life Science (Tartu, Estonia).

Different parameters were used for process monitoring. Ammonium was measured by colorimetric method using DR2800 spectrophotometer (Hach-Lange, Germany). A HACH HQ30d (Hach-Lange, Germany) portable pH-meter was used for measuring pH inside the reactors. HACH BIOGAS Titrator station (Hach-Lange, Germany) was used for volatile fatty acids/alkalinity (VFA/ALK) ratio titration. VFA's (C2–C6) were analysed by using a gas chromatograph (GC 2014 ATFi/SPL, Shimadzu, Japan) equipped with a Zebrom ZB-WAXplus capillary column (35 m × 0.25 mm × 0.25 μm) and flame ionization detector (FID) (Pihk et al., 2013). Total LCPA in effluents (LCPAS) was analysed according to the following procedure: total lipids were extracted from each sample by a modified Fohk procedure using dichloromethane:methanol (2:1, v/v). Lipids were esterified with acetyl chloride/methanol. Fatty acid methyl-esters (FAEM) were analyzed using an Agilent 6890A chromatograph (Agilent Technologies Inc. USA), equipped with a FID detector and fused silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.20 μm film thickness). Hydrogen was the carrier gas, and the injector split ratio was 1:10. Methane content in the biogas was analysed with gas chromatograph (Model 3700, with thermal conductivity detector and Parapak Q column (1.8 m × 3.17 mm)) and N₂ was used as carrier gas.

Total macronutrients content of dairy manure, effluent samples and floating granules were measured by X-ray fluorescence (XRF) method in the Tallinn University of Technology (TUT) Institute of Geology (Tallinn, Estonia). Loss on ignition (LOI) was determined from 1 g of sample material at 920 °C. S4 Pioneer spectrometer (Bruker AXS GmbH) equipped with X-ray tube with a rhodium anode was used. The samples were measured with a manufacturer's standard as MultiRes modification (pre-calibrated standardless method). For oxidizable macro- and micronutrients spectra, Thermo Scientific XSeries 2 Quadrupole Inductively Coupled Plasma Mass Spectrometer (ICP-MS) was used. ICP-MS analysis was carried out at the TUT Institute of Geology (Tallinn, Estonia). Micronutrients and trace elements were determined from solutions which were prepared following the nitric and hydrochloric acid digestion of a 0.25 g pulverized sample in Anton Paar MW3000 microwave oven.

Samples of formed floating granules (FG) were fixed, embedded and sectioned in a cryostat as described in Palati et al. (2012). Morphology of the FG formed in R2, were examined by Scanning Electron Microscope-Energy Dispersive X-ray Spectrometry (SEM-EDS) JEOL-6510 at CGIT-UB at the University of Barcelona (Barcelona, Spain).

2.4. Specific batch tests and calculations

Biomethane potential (BMP) and methane production rate (MPR) measurements for sonicated FG were performed in batch anaerobic vials in accordance with the protocol proposed by Angeldaki et al. (2009). Residual biomethane potential tests (rBMP) were performed with reactors effluent samples at the end of the different OLR periods. rBMP experiments were made as BMP with the exception that no seed sludge was used as inocula (as it was already contained in reactor samples). OxTop-C (WTW, Weilheim, Germany) respirometers were used for rBMP tests.

For specific methanogenic activity (SMA) measurement acetate and H₂/CO₂ (80:20) gas mixture were used as substrates to monitor reactors aceticlastic and hydrogenotrophic methanogenic activity. SMA measurements were carried out at mesophilic temperature (37 °C), with substrate to inocula ratio (S/I) of 0.25 (VS basis) and inocula concentration of 2.5 g L⁻¹. Automatic Methane Potential Test System (Bioprocess Control Sweden AB) was used for aceticlastic SMA measurements. While hydrogenotrophic SMA was carried out with batch anaerobic vials (120 mL total volume vials with 50 mL of media working volume).

Theoretical methane potential (TMP) at standard conditions (STP, 0 °C and 1 bar) was estimated according to the following equation (Eq. (1)), based on the protein, lipid and carbohydrate contents of the substrates, as suggested by Angeldaki and Sanders (2004):

\[ CH_4 \text{yield} \left( \text{mg} \, \text{CH}_4 \, \text{g}^{-1} \text{VS} \right) = 496 \times X + 0.014 \times Y + 415 \times Z \]

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

The un-ionised fraction of the ammonia nitrogen (NH₃) was calculated according to the equation (Eq. (2)), as described by Körner et al. (2001):

\[ NH_3 (\% \text{ of } NH_3^+) = 100 / \left( 1 + 10^{pK_a - \phi(\text{pH})} \right) \]

where pKa = 0.09108 + 272.92/273.2 + T (°C)

3. Results and discussion

3.1. Substrate characterization

Two different samples of LM (LM₁ and LM₂), DS (DS₁ and DS₂) and one sample of TF were used in the experiments. Composition of the substrates used is shown in Table 1. Changes in LM samples composition were related to the variations in water use and feed ration of the dairy farm. LM₃ had approximately 1.5 times higher TS and lipids content than LM₁ (Table 1). The main difference of DS samples was in lipids concentration decreasing from 42.2% to 35.1% in DS₁ to DS₂. Analysis of TF indicated that this co-substrate is principally pure fat with minor traces of macronutrients (Table 1). DS and TF composition in the present study was similar to the composition reported by Bayr et al. (2012) with the exception of volatile solids content of DS being 13% higher in the present study, which was probably related to the raw SSWH samples composition differences.

3.2. Reactor experiment

Reactors operational conditions (HRT, OLR, TN loading rate (NLK), lipids loading rate (LLR) and co-substrates input ratios) and efficiencies (VS and lipids removal efficiencies, methane production and yields) are summarized in Figs. 3 and 4 and Tables
Table 1
Characterization of dairy manure, technical fat, decanter sludge (used as substrates) and floating granules that were formed during R2 operation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Manure (L_M)</th>
<th>Manure (L_M)</th>
<th>Unit</th>
<th>Technical fat</th>
<th>Decanter sludge (DS1)</th>
<th>Decanter sludge (DS2)</th>
<th>Floating granules (FG)</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>43</td>
<td>6.1</td>
<td>%</td>
<td>99.7</td>
<td>95.1</td>
<td>97.2</td>
<td>38.3</td>
<td>%</td>
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<tr>
<td>VS</td>
<td>72.2</td>
<td>76.9</td>
<td>%</td>
<td>TS</td>
<td>100</td>
<td>83.5</td>
<td>79.5</td>
<td>79.2</td>
</tr>
<tr>
<td>Lipids</td>
<td>2.3</td>
<td>3.5</td>
<td>g L⁻¹</td>
<td>99.6</td>
<td>42.2</td>
<td>35.1</td>
<td>14.4</td>
<td>% of TS</td>
</tr>
<tr>
<td>Protein</td>
<td>nd</td>
<td>nd</td>
<td>–</td>
<td>35.8</td>
<td>37.8</td>
<td>nd</td>
<td>% of TS</td>
<td></td>
</tr>
<tr>
<td>TN</td>
<td>49</td>
<td>4.7</td>
<td>g L⁻¹</td>
<td>2.1</td>
<td>57.2</td>
<td>nd</td>
<td>g kg⁻¹</td>
<td></td>
</tr>
<tr>
<td>NH₄-N</td>
<td>2.5</td>
<td>2.9</td>
<td>g L⁻¹</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>TOC</td>
<td>nd</td>
<td>nd</td>
<td>g kg⁻¹</td>
<td>77.3</td>
<td>48.4</td>
<td>nd</td>
<td>g kg⁻¹</td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>14</td>
<td>18.5</td>
<td>g kg⁻¹</td>
<td>40</td>
<td>43.725</td>
<td>nd</td>
<td>1081</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>S</td>
<td>8.2</td>
<td>8.2</td>
<td>g kg⁻¹</td>
<td>25</td>
<td>2800</td>
<td>nd</td>
<td>940</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>K</td>
<td>37.8</td>
<td>44.2</td>
<td>g kg⁻¹</td>
<td>14.8</td>
<td>45.3</td>
<td>nd</td>
<td>1411</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>Ca</td>
<td>26.6</td>
<td>20.7</td>
<td>g kg⁻¹</td>
<td>53.7</td>
<td>60.000</td>
<td>nd</td>
<td>107.490</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>Mg</td>
<td>12.8</td>
<td>13.1</td>
<td>g kg⁻¹</td>
<td>6.8</td>
<td>21.16</td>
<td>nd</td>
<td>1326</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>Na</td>
<td>nd</td>
<td>13.2</td>
<td>g kg⁻¹</td>
<td>50.9</td>
<td>7194</td>
<td>nd</td>
<td>297</td>
<td>mg kg⁻¹</td>
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<tr>
<td>Cu</td>
<td>nd</td>
<td>0.05</td>
<td>g kg⁻¹</td>
<td>2.6</td>
<td>13.6</td>
<td>nd</td>
<td>4.8</td>
<td>mg kg⁻¹</td>
</tr>
</tbody>
</table>

Fig. 1. R1 process parameters and metabolic intermediate compounds concentration profiles. Dashed vertical lines indicate the different feeding periods of decanter sludge co-digestion.

2 and 3. For the initial 45 days R1 and R2 were both operated only with L_M at the stable OLR of 1.36 kgVS m⁻³ d⁻¹ and NH₄-N concentration of 2.4 ± 0.02 g L⁻¹. Reactors average methane yield was 232.8 ± 4.8 m³ CH₄ t⁻¹, corresponding to VS removal efficiency of 28.4 ± 1.5% (see Tables 2 and 3). R1 and R2 manure mono-digestion process conditions and efficiency are closely comparable to
experiments carried out by (Nielsen and Ahring, 2006). rBMP of reactors effluents were measured at the end of the LM₁ mono-digestion periods and it was 15.8 ± 1.3% of the methane production in average. LM₁ mono-digestion period of 45 days was considered as control phase and no other control reactor was further on operated.

3.2.1. Co-digestion of decanter sludge

Co-digestion of LM₁ and DS₁ was started in R₁ on day 46 at 3%DS₁ addition to the input mixture representing 44.0% of VSₜot. OLR and LLR were increased to 2.41 kgVS m⁻³ d⁻¹ and 0.63 kgDET m⁻³ d⁻¹, respectively (Table 2). The 3%DS₁ load was kept for 3 HRT-s reaching stable biogas production at methane production yields of 305.7 ± 19.8 m³CH₄⁻¹ VS⁻¹ that represents a 75.5% of TMP of DS₁ (Table 2). This degradation efficiency is slightly lower than 84.86% of TMP (calculated by Eq. (1)) obtained for DS₁ BMP test value of 532.8 ± 15.5 m³CH₄⁻¹ VS⁻¹. The mentioned difference is also related with TS% increase in R₁ effluent throughout the full experiment period. After an initial VFA and LCFAs accumulation in R₁, system adapted to DS₁ addition and most of intermediates concentrations were reduced to control period level, although acetate concentration remained around 0.56 ± 0.14 g L⁻¹ (Fig. 1). Also peak of accumulated LCFAs was reduced to the control levels in the end of 3%DS period (day 110). OLR was between 3.08 and 3.76 kgVS m⁻³ d⁻¹ at the highest loading rate of 5%DS, representing 47.4–57.2% of the VSₜot increasing also the load of the lipids to the level of 0.99–1.06 kgDET d⁻¹ (Table 2). These differences are due to manure (LM₁ to LM₃) and decanter sludge (DS₁ to DS₃) changes at day 143. Nevertheless, despite this increase of the loads of lipids and proteins no intensive foam generation, intermediate compounds accumulation or other inhibitory phenomena occurred during 5%DS operation period and methane production efficiency decreased only slightly from 75.5 to 72.3%, confirming stable process performance.

During 5%DS addition period R₁ feed mixture contained lipids at concentration of 23.0 ± 1.2 g L⁻¹. Kougias et al. (2013) reported a value of 24 g L⁻¹ Na-oleate addition (OLR of 5.2 kgVS m⁻³ d⁻¹) as a concentration to induce light bubbly foam formation just at slightly higher lipids concentration than in current study. At day 143, after manure and decanter sludge change, OLR increased from
Table 2

<table>
<thead>
<tr>
<th>R1 (HRT 22.5)</th>
<th>Days</th>
<th>OLR (kg VS m⁻³ day⁻¹)</th>
<th>NLR (kg m⁻³ d⁻¹)</th>
<th>LLR (kg VS m⁻³ d⁻¹)</th>
<th>Lipids input (g l⁻¹)</th>
<th>TN input (g l⁻¹)</th>
<th>DS (mg m⁻³)</th>
<th>DS (mg CH₄ kcal m⁻³)</th>
<th>CH₄ prod (m³ CH₄ kcal m⁻³)</th>
<th>CH₄ prod (m³ CH₄ kcal m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM₁ mono-dig</td>
<td>8–45</td>
<td>1.39</td>
<td>0.22</td>
<td>0.10</td>
<td>2.26</td>
<td>4.88</td>
<td>0</td>
<td>0</td>
<td>229.4 ± 17.2</td>
<td>72.0 ± 0.5</td>
</tr>
<tr>
<td>LM₁ + 3x DS₅</td>
<td>45–112 2.41</td>
<td>0.29</td>
<td>0.63</td>
<td>14.22</td>
<td>6.45</td>
<td>3.0</td>
<td>44.0</td>
<td>305.7 ± 19.8</td>
<td>166.2 ± 1.1</td>
<td>75.5</td>
</tr>
<tr>
<td>LM₁ + 5x DS₅</td>
<td>113–142 3.08</td>
<td>0.33</td>
<td>0.99</td>
<td>22.20</td>
<td>7.50</td>
<td>5.0</td>
<td>57.2</td>
<td>305.9 ± 11.8</td>
<td>212.2 ± 0.9</td>
<td>66.9</td>
</tr>
<tr>
<td>LM₂ + 5x DS₅</td>
<td>143–170 3.76</td>
<td>0.33</td>
<td>1.06</td>
<td>23.86</td>
<td>7.48</td>
<td>5.0</td>
<td>47.4</td>
<td>297.9 ± 19.0</td>
<td>248.1 ± 1.6</td>
<td>72.3</td>
</tr>
<tr>
<td>R2 (HRT 22.5)</td>
<td>Days</td>
<td>OLR (kg VS m⁻³ day⁻¹)</td>
<td>NLR (kg m⁻³ d⁻¹)</td>
<td>LLR (kg VS m⁻³ d⁻¹)</td>
<td>Lipids input (g l⁻¹)</td>
<td>TN input (g l⁻¹)</td>
<td>DS (mg m⁻³)</td>
<td>DS (mg CH₄ kcal m⁻³)</td>
<td>CH₄ prod (m³ CH₄ kcal m⁻³)</td>
<td>CH₄ prod (m³ CH₄ kcal m⁻³)</td>
</tr>
<tr>
<td>LM₁ mono-dig</td>
<td>8–45</td>
<td>1.39</td>
<td>0.22</td>
<td>0.10</td>
<td>2.26</td>
<td>4.88</td>
<td>0</td>
<td>0</td>
<td>226.2 ± 2.44</td>
<td>74.0 ± 0.5</td>
</tr>
<tr>
<td>LM₁ + 2x TF</td>
<td>46–112 2.25</td>
<td>0.22</td>
<td>0.98</td>
<td>22.13</td>
<td>4.85</td>
<td>2.0</td>
<td>39.4</td>
<td>318.5 ± 19.8</td>
<td>171.1 ± 1.0</td>
<td>62.5</td>
</tr>
<tr>
<td>LM₁ + 2.6x TF</td>
<td>113–142 2.50</td>
<td>0.22</td>
<td>1.25</td>
<td>29.09</td>
<td>4.65</td>
<td>2.6</td>
<td>46.0</td>
<td>320.8 ± 10.3</td>
<td>181.5 ± 0.6</td>
<td>54.1</td>
</tr>
<tr>
<td>LM₁ + 2.6x TF</td>
<td>142–154 3.16</td>
<td>0.21</td>
<td>1.30</td>
<td>29.32</td>
<td>4.67</td>
<td>2.6</td>
<td>36.4</td>
<td>321.9 ± 21.1</td>
<td>23.1 ± 0.9</td>
<td>60.0</td>
</tr>
<tr>
<td>LM₁ + 1x TFₜₜ</td>
<td>155–200 2.49</td>
<td>0.21</td>
<td>0.60</td>
<td>13.44</td>
<td>4.66</td>
<td>1.0</td>
<td>17.8</td>
<td>326.1 ± 18.8</td>
<td>188.8 ± 0.9</td>
<td>65.5</td>
</tr>
</tbody>
</table>

n.d. – not determined.

3.08 to 3.76 kg VS m⁻³ d⁻¹ and as a consequence VFA and LCFA started to accumulate (Fig. 1). Despite that, process remained stable as NH₃ concentration in R1 had raised to maximum of 4.35 g l⁻¹, providing sufficient additional buffering capacity to counteract VFA accumulation. At the same time estimated NH₃ concentration stayed below 415 mg l⁻¹ that has been stated to be below inhibiting concentrations for high N loading adapted reactor systems (Pitkä et al., 2013; Yenigün and Demir, 2013). Compared to dairy manure mono-digestion, addition of 5% DS to manure increased volumetric CH₄ production 3.44 times from 7.2 to 24.8 m³ CH₄ l⁻¹.

The SMA and rBMP measurements performed at the end of each loading periods are summarized in Table 3. SMA for acetoclastic and hydrogenotrophic methanogens in R1 remained constant throughout DS addition periods, indicating a stable process operation without limiting factors for activity of methanogens. Although at the end of the 5% DS period ammonium concentration in R1 was over 4.5 g l⁻¹ (Table 2) that is 1.5 times higher than 3.0 g l⁻¹ reported by Schnürer and Nordberg (2008) as a threshold value for switch to SAO pathway, the SMA results did not indicate changes in activity values. Increasing values of rBMP in R1 at higher OLR were in accordance with process performance reflecting TS, LCFA and also VFA accumulation in the system (Table 3).

Composition of reagents effluents were analysed for macro- and micronutrients at the end of the experiment (Table 4) in order to verify that process performances were not limited by nutrient deficiency or overload. As DS had considerably higher content of nutrients than TF (Table 1), the higher concentrations of nutrients in the effluent of R1 were expected. None of the macro- and micronutrients, neither for R1 nor R2 were in the critical range of deficiency or excess compared to values provided by Chen et al. (2008) and Schattauer et al. (2011), so process efficiencies or limitations were directly related to organic matter degradability and conversion efficiency of intermediate metabolic compounds. On the other hand manure co-digestion with DS increased digestate fertilizer value in terms of TN, TP and other macro- and micronutrients content, which has considerable additional value from the viewpoint of dairy farms manure management valorisation and sustainability.

3.2.2. Co-digestion of technical fat

Co-digestion of LM₁ and TF in R2 was started on day 46 with 2%TF addition to the input mixture, representing 39.4% of VSLoad (Table 2) and producing a direct increase in biogas production (Fig. 2). OLR and LKR were increased up to 2.25 kg VS m⁻³ d⁻¹ and 0.98 kg Kjøl m⁻³ day⁻¹, respectively (Table 2 and Fig. 2). 2% TF load was maintained for 3 HRT’s reaching stable methane production yield of 338.5 ± 19.8 m³ CH₄ m⁻³, which corresponded only to 62.5% of TOMP of TF(Table 2). After initial TS, VFA and LCFA accumulation (Fig. 2) in R2, process adapted to TF addition, recovering to initial values for these process intermediates (for day 100 VFA and LCFA concentrations had declined to control period levels). At the same time methane production remained only at 62.5% of theoretical TF methane yield. Although manure based CSTR reactors at high LKR have been reported to cause foaming or sludge flotation (Heinjelt and Angelidaki, 2009; Kougiass et al., 2013) then no foaming occurred in R2. Contrary, white floating granules (FG) were formed (Supplementary Fig. 1) floating on the top of the liquid layer, explaining the low theoretical methane yield of R2. Similar phenomenon of formation of floating granules has been described by Varin (2013), but mainly in acidic conditions at pH between 5 and 6. It was reported that at neutral or higher pH
values LCFA were by-passed with suspended sludge from the reactors without granules formation being in contradiction with occurrence of FG formation at pH over 7.5 in the current experiments. FG composition is presented in Table 5, but in short it had 38.3% TS, 79.2% VS of TS and all of the VS was composed of salts of LCFA or lipids. Dominant salt forming FG was calcium salt of LCFA, despite many previous articles (Alii et al., 2006; Hanaki et al., 1981; Koster, 1987; Roy et al., 1985; Zhang et al., 2010) referring to different LCFA’s precipitation by CaCl2 addition, rather than formation of FG in anaerobic digestion. Calcium salts of LCFA are relatively insoluble compared to, for example, sodium salts (Koster, 1987) being the reason for calcium salts of LCFA dominance in formed FG in R2. Other mineral compounds in FG were present in negligible concentrations compared to calcium (Table 1). Palmitate had the highest share of 42.6% of the LCFA in the FG, even though in TF palmitate contributed only 20.2% of the LCFA fraction. COD concentration of FG was determined to be 1083 gCOD kg⁻¹. Floating granules of Ca-salts of LCFA in high lipids loaded CSTR reactor indicate additional parameter of LCFA/Ca ratio that have to be considered in the process setup. LCFA/biomass ratio and bioflocs content was reported to be of importance by Nielsen and Ahring (2006), in order to maintain high process efficiency.

Based on the results of current study mechanism of floating non-biomass associated LCFA granules, in addition to previously reported biomass-associated LCFA accumulation mechanisms of precipitation, adsorption and entrapment by Pereira et al. (2005), have to be considered as alternative pathway of lipids degradation and LCFA accumulation.

As VFA and LCFA in the liquid phase at the end of 2%TF period remained at low levels (Fig. 2), then TF addition was increased to 2.6%TF. At the same time OLR increased to range of 2.5–3.16 kgVS m⁻³ d⁻¹ and share of TF in the input mixture varied during the same loading period between 36.4 and 46.0% of VSin (difference due to manure change) according to Table 2. Formation of FG continued at 2.6%TF load (after day 154) with simultaneous increase in VFA and LCFA concentrations. Considering that the conversion of lipids to methane was only 57.1 ± 4.1%, and the accumulation of metabolites (VFA and LCFA) was observed, it was a clear sign of overload of lipids in the process, which would have ended with definite process failure without FG formation. It was then decided to cease the operation of R2 to open the cover and remove all the FG in composition and biodegradability analysis. After FG removal from R2, process was continued with 1%TF addition at OLR of 2.49 kgVS m⁻³ d⁻¹ but with approximately 2 times lower lipids load of 0.6 kgAT m⁻³ d⁻¹ compared to the system loading when FG formation was started (Fig. 2). Process recovered from previous limited state, achieved 83.5% of TF conversion efficiency to methane (336.1 ± 10.3 m³ CH4 t⁻¹ VS⁻¹) with intermediate metabolic compounds concentrations similar to the control period level and no FG formation (Tables 2 and 3).

The results of SMA and rBMP measurements performed at the end of each loading periods for R2 are summarized in Table 3. During TF addition periods hydrogenotrophic activity in R2 remained at the same level, but it was about 25% lower than hydrogenotrophic methanogenic activity in R1. After removal of FG from the system and reduction of TF loading to 1%TF the hydrogenotrophic activity increased up to 0.35 μmol CH4 μmol⁻¹ VS⁻¹ day⁻¹. Acetoclastic methanogenic activity after adaption to increased LCFA concentration increased more than 2.2 times being consistent with the results obtained by Silvestre et al. (2014) with sewage sludge and grease trap waste co-digestion. Also Pereira et al. (2005) have described that during oleate/palmitate β-oxidation both butyrate and acetate should be formed, which may result in the enrichment of both acetoclastic methanogens and acetogenic butyrate degrading bacteria. On the other hand R2 methanogenic activity behaviour is partly contradictory to conclusion made in batch culture.
experiments by Hanaki et al. (1981) that LCFA addition inhibited mainly methane production from acetate while hydrogen was readily utilized without lag period even in the processes under inhibiting conditions. After FG removal and process stabilization at 1%TF loading, with reduced VFA and LCFA concentrations comparable to the manure mono-digestion levels, acetodlastic methanogenic activity was again decreased and hydrogenotrophic methane production favoured over it. This is in accordance with conclusions made by Palati et al. (2010) and Baserba et al. (2012) reporting that in manure based reactors with oleate addition hydrogenotrophic methanogenesis was an important pathway for methane formation, while low abundance of the strict acetodlastic methanogen Methanosetaeac was present (see Fig. 3).

R2 RBFM increasing values at higher OLR and decrease after removal of FG from system were reflecting the process efficiency in the liquid part of the system omitting the biogas potential contained in the FGs (Table 3). Results of the R2 operation indicate that solely lipid co-substrates overloaded manure based anaerobic digestion does not lead to sudden process failure, but the system has ability to neutralize surplus LCFA-s to “storage compounds” of Ca-salts of LCFA. In general perspective these “storage compounds” formation should be avoided as it reduces process efficiency, but on the other hand it could provide flexible and operational errors forgiving control and monitoring mechanism for anaerobic digestion process.

3.2.3. Floating granules formation, composition and morphology

Composition of formed FGs and its general composition and LCFA profile are presented in Table 1 and Table 4. FG had a TS content of 38.3% and 79.2% VS of TS. Main mineral part of FG was calcium at 41.17 g kg⁻¹, being at considerably higher concentrations than phosphorous, potassium or sodium (Table 5).

Organic part of FG agglomerates was nearly 100% composed of lipids and LCFA. LCFA/Ca ratio (g/g dry weight) for FG in this study was 6.05, that was at lower range of the ratios reported by Roy et al. (1985) between 8.8 and 15.5 for experiments with calcium salt precipitates of oleate and stearate, respectively. In TF and lipids adsorbed onto FG agglomerates oleate contributed almost half of the share of the LCFA spectra. In FG palmitate was the most dominant; LCFA contributing 42.6%, followed by oleate, stearate and minor part of other LCFA-s (Table 5). LCFA proportions in FG indicate oleate being the most efficiently degraded LCFA, while palmitate degradation was inefficient, being mainly used for conversion to calcium salts of LCFA, forming main share of the FG composition. This is in agreement with conclusions made by Pereira et al. (2005) stating that transformation of oleic acid to palmitic acid is dependent on the biological activity, is a fast and non-limiting step in oleic acid degradation and the accumulation of palmitic acid onto the sludge suggests that its further degradation is a rate-limiting step under continuous operation. FG cross-section and SEM pictures are presented in Supplementary data. FG were partly hollow inside, being probably one of the reasons leading to their flotation. As can be seen from Supplementary data, the FG were also used as growth surface for microorganisms.

Considering lipids concentrations in reactors input mixtures, then the process response of R1 and R2 in terms of lipids conversion and degradation was totally different. For IM-1 mono-digestion period in both of the reactors influent Lipids/Ca ratio (g/g) was around 2 and it did not induce FG formation. At the initiation of 2%TF addition period Lipids/Ca ratio in R2 influent was increased to 20 that initiated FG formation while for 1%TF addition period at Lipids/Ca ratio of 5.6 in the input mixture the process performed normally. Also for R1 there was no FG formation even at 5%DS loading period at Lipids/Ca ratios between 5.0 and 5.6, despite the LLR being equal to R2 2%TF addition period. Floating granules LCFA/Ca ratio was 6.05 that related more to R1 input mixture Lipids/Ca ratio, but still FG formation occurred only in R2 at high Lipids/Ca ratio of 20. In the framework of this study exact reasons for floating FG formation in R2, instead of previously reported precipitation of Ca salts of LCFA (Ahn et al., 2006; Hanaki et al., 1981; Koster, 1987; Roy et al., 1985; Zhang et al., 2010), remained unclear. Nevertheless, high lipids loaded digesters intentionally directed to favour FG formation could be a viable option for mitigation and control of lipids overload and LCFA accumulation, but it
Fig. 4. Anaerobic biodegradability and methane production rate of fat granules (a and b). Increment of anaerobic biodegradability and methane production rate (in comparison to untreated sample) of sonicated fat granules as a function of supplied energy (c and d). Figure illustrates values after 10 days of batch test, at the end of the methane production rate active phase. AB – anaerobic biodegradability (% initial COD); MPR – methane production rate (LCH₄ kg⁻¹d⁻¹); SE – supplied energy; L – low energy, M – medium energy; H – high energy; OPT – optimum energy.

Table 6
Sonication pre-treatment conditions and its effect on anaerobic biodegradability (AB) and methane production rate (MPR) of FG from R2. Final values after 45 days batch test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ΔT (°C)</th>
<th>β (%)</th>
<th>&gt;10³ kJ kg⁻¹</th>
<th>W mL⁻¹</th>
<th>AB (% SCOD)</th>
<th>MPR (L CH₄ kg⁻¹d⁻¹)</th>
<th>Lag phase (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>0.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>56 ± 13</td>
<td>7.4 ± 0.33</td>
<td>19</td>
</tr>
<tr>
<td>L1</td>
<td>0.7</td>
<td>12</td>
<td>1.01 × 10³</td>
<td>0.5</td>
<td>68 ± 5</td>
<td>13.4 ± 4.2</td>
<td>12</td>
</tr>
<tr>
<td>L2</td>
<td>0.4</td>
<td>25</td>
<td>1.06 × 10³</td>
<td>1.1</td>
<td>54 ± 1</td>
<td>9.9 ± 0.4</td>
<td>12</td>
</tr>
<tr>
<td>L3</td>
<td>2.17</td>
<td>50</td>
<td>1.07 × 10³</td>
<td>2.2</td>
<td>63 ± 17</td>
<td>19.1 ± 4.5</td>
<td>12</td>
</tr>
<tr>
<td>M1</td>
<td>1.85</td>
<td>25</td>
<td>1.13 × 10³</td>
<td>1.1</td>
<td>73 ± 4</td>
<td>17.9 ± 6.9</td>
<td>8</td>
</tr>
<tr>
<td>M2</td>
<td>1.90</td>
<td>50</td>
<td>1.14 × 10³</td>
<td>2.2</td>
<td>82 ± 3</td>
<td>26.3 ± 13.9</td>
<td>8</td>
</tr>
<tr>
<td>H1</td>
<td>1.74</td>
<td>25</td>
<td>2.016 × 10³</td>
<td>1.1</td>
<td>78 ± 7</td>
<td>19.9 ± 4.8</td>
<td>8</td>
</tr>
<tr>
<td>H2</td>
<td>1.87</td>
<td>50</td>
<td>2.025 × 10³</td>
<td>2.2</td>
<td>72 ± 4</td>
<td>13.5 ± 3.0</td>
<td>12</td>
</tr>
<tr>
<td>OPT</td>
<td>1.90</td>
<td></td>
<td>6.37 × 10³</td>
<td>2.2</td>
<td>70 ± 1</td>
<td>22.1 ± 8.4</td>
<td>12</td>
</tr>
</tbody>
</table>

| Inocula activity control tests | – | – | – | – | 25 ± 5 | 2.7 ± 1.46 | 16 |
| C16:0     | – | – | – | – | 60 ± 1 | 17.9 ± 0   | 12 |
| C18:1     | – | – | – | – | 50 ± 7 | 39.1 ± 7.0 | 0  |
| VFAmax 20 | – | – | – | – |        |             |    |

β – power amplitude.

requires further investigation of the mechanism and environmental conditions triggering FG formation.

3.2.4 Biodegradability of floating granules

Inherent biodegradability of FG was evaluated first with BMP test and it resulted in long lag phase and low degradability ratio. Lag phase with minor methane production lasted for 19 days. After 30 days of measurement only 31.2 ± 9.4% and after 45 days 56.0 ± 13.0% of FG were biodegraded based on COD balance (Fig. 4). For evaluation of FG biodegradability sonication as a pre-treatment technology was used. The higher the energy densities applied on kJ kg⁻¹ or W mL⁻¹ of FG aliquots the smaller the FG particles were obtained but no direct correlation between particle size and COD biodegradability or MPR were obtained (Table 6 and Fig. 4). Optimal (OPT) level sonication density was chosen according to sonication pre-studies (data not shown), based on FG physical degradation (Table 6). Nevertheless, medium level sonication density at 1014 × 10³ kJ kg⁻¹ and 2.2 W mL⁻¹ was sufficient to achieve maximum FG degradability, as high or OPT level sonication densities did not improve FG biodegradability or MPR any further. Sonication treatment results indicated that if the FG are destroyed and particle size reduced then the FG biodegradability and lipids conversion efficiency for CSTR systems could be increased. Floating surface layer of the digester through sonication appliance could be introduced, but economic rationality behind it is highly questionable.

4. Conclusion

Dairy manure co-digestion with lipid and protein rich solid slaughterhouse waste is attractive solution for obtaining up to 3.5-fold increased volumetric biogas production. Determination of lipids overload values and expected subsequent LGFA degradation efficiency reduction resulted in surprising process response
at 2% of lipids addition to dairy manure with formation of white floating granules of calcium salts of LCFA with low inherent biodegradability. Contrary, when balanced lipids and proteins mixture was used at similar load of lipids then stable and efficient process without any indication of formation of the floating granules was maintained.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.biortech.2014.05.033.

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