Degradation of proteinaceous material, ammonium accumulation and microbial dynamics in anaerobic digesters

Nedbrytning av proteinrikt substrat, ammonium-akkumulering og mikrobiell dynamikk i anaerobe reaktorer

Philosophiae Doctor (PhD) Thesis

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Table of contents

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Linn

Summary

Anaerobic digestion (AD) of organic material from Norwegian farms can contribute to the agricultural value chain. Agricultural waste, including animal manure, is in large supply in Norway and AD of these fractions can reduce carbon emission, provide energy in the form of methane, and increase recovery of nutrients. To make AD cost effective in Norwegian agriculture, process optimization and high methane yields are necessary. One way to increase energy yields from agricultural wastes is by co digesting them with other more nutrient rich substrates like fish residues. Such substrates contains considerable amounts of energy rich fat and protein, and thus represent high methane potentials. In addition, coordination of organic wastes from agriculture and aquaculture could improve the sustainability and value chains in both these sectors. Moreover, co-digestion of substrates with different properties may enhance the nutrient balance and buffer capacity. During AD of fat and protein rich substrates, there is a risk for elevated concentrations of ammonium-nitrogen and fatty acids, which inhibits the methane producing microbial community, and might cause instability and failure of the AD process. Proper adjustment of operational parameters and adaptation of the microbial community are crucial to avoid process failure during AD of energy rich substrates. The focus of this thesis was to evaluate AD process stability and methane production from energy rich organic fractions with animal manure as a co-substrate. In co-digestion trials using ensiled fish waste and cow manure, a mixing ratio of 13 % fish waste and 87 % cow manure (volume based) operated with a retention time of 30 days seemed promising with regard to stable AD process and high methane yields. The experiments also showed that the biogas process maintained stable at a retention time of 20 days, which implies that the amount of fish waste in the substrate can be increased if the amount of manure is simultaneously increased. The microbial communities involved in the AD processes were monitored and analyzed, particularly focusing on the development and

establishment of ammonium tolerant microorganisms. The experiments showed that the microbial communities adapted to an increased load of proteins and fats, and that the presence of some key functioning microorganisms seemed to play important roles in these systems.

Sammendrag

Anaerob nedbrytning (AD) av organisk material fra norske gårder kan bidra til å bedre verdikjeden i landbruket. Avfall fra landbrukssektoren, inkludert husdyrgjødsel, er i stor tilførsel i Norge og anaerob nedbrytning av disse fraksjonene vil føre til redusert karbon-utslipp, energiproduksjon i form av metan og en økt resirkulering av næringsstoffer. For å gjøre AD kostnadseffektivt i norsk landbruk er det nødvendig med prosessoptimalisering og økt metan -utbytte. En måte å øke energiutbyttet fra organisk materiale i landbruket er ved sambehandling med andre mer næringsrike substrater, for eksempel avskjær og slakteavfall fra akvakulturnæringen. Slike substrater inneholder betydelige mengder energi i form av fett og protein, og representerer derfor høye metan - potensialer. Koordinering av organisk avfall fra landbruk og akvakultur kan også bidra til å øke både bærekraft og verdikjede i de to sektorene. I tillegg vil substrater med ulike egenskaper forbedre næringsbalansen og bufferkapasiteten og dermed gi et økt metan - utbytte. Når protein og fett brytes ned anaerobt er det en risiko for opphopning av ammonium-nitrogen og fettsyrer som kan hemme metan - produserende mikrobielle samfunn og ustabilitet og kollaps av biogassprosessen kan inntreffe. Riktig justering av driftsparametere og tilpasning av mikrobielle samfunn er avgjørende for å unngå prosesskollaps under nedbrytning av energirike substrater. Fokuset i denne oppgaven var å undersøke biogassprosess-stabilitet og metan - produksjon fra energirike organiske fraksjoner i sambehandling med husdyrgjødsel. I sambehandlingsforsøk med ensilert fiskeavfall og storfegjødsel var et blandingsforhold på 13 % fiskeavfall og 87 % gjødsel (volum basert) og oppholdstid på 30 dager lovende med tanke på stabil biogassprosess og høyt metanutbytte. Forsøkene viste også at biogassprosessen forble stabil med en oppholdstid på 20 dager, og dette viser at andelen fiskeavfall i substratet kan økes hvis andelen gjødsel økes samtidig. De mikrobielle samfunnene som er involvert i nedbrytningsprosessen ble monitorert og analysert med

hovedfokus på utvikling og etablering av ammonium -tolerante mikroorganismer. Forsøkene viste at det mikrobielle samfunnet tilpasset seg økt belastning av proteiner og fett, og at enkelte nøkkel-organismer spilte en viktige roller i disse systemene.

Abbreviations and terms

AD	Anaerobic digestion
CSTR	Continuously stirred tank reactor
HRT	Hydraulic retention time
OLR	Organic loading rate
VFA	Volatile fatty acid
LCFA	Long chained fatty acids
C/N	Carbon / Nitrogen
FWS	Fish waste silage
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate oxidizing bacteria
q-PCR	Quantitative polymerase chain reaction
GHG	Greenhouse gas
NH_4^+	Total ammonium
CH_4	Methane
CO_2	Carbon dioxide
H_2	Hydrogen

List of papers

Paper I:

Solli, Linn., Bergersen, Ove., Sørheim, Roald., Briseid, Tormod. 2014. Effects of a gradually increased load of fish waste silage in co-digestion with cow manure on methane production. Waste Management, Volume 34, pages 1553 - 1559.

Paper II:

Solli, Linn., Haavelsrud, Othilde Elise., Rike, Anne Gunn., Horn, Svein Jarle. 2014. A metagenomic study of the microbial communities in four parallel biogas reactors. Biotechnology for Biofuels, Volume 7: 146.

Paper III:

Solli, Linn., Schnurer, Anna., Horn, Svein Jarle. 2017. Process stability and dynamics of ammonium-tolerant microorganisms during co-digestion of fish waste silage and manure; submitted manuscript.

1. INTRODUCTION

1.1 Anaerobic digestion as part of the circular bio economy

During recent years, the focus on sustainable production and exploitation of biological resources has increased. At the same time we increasingly face global challenges such as depletion of natural resources, climate change and the need for more energy. To cope with these issues, changes of routines for production, consumption, recycling and disposal of biological resources are needed. Aiming to secure healthy food, feedstuffs and energy sources, many countries are developing strategies for establishment of a sustainable bio economy. Energy production from renewable sources has gained increased attention during recent years, and microbiological AD of organic waste is in this context an applicable technique. AD of organic material will enhance nutrient recovery and provide energy in the form of CH_4 (Weiland, 2010). Moreover, by including AD and biogas production as part of the energy system, and possibly decrease the exploitation and emission from fossil energy sources, GHG emissions can be reduced. Compared to CO_2, CH_4 is a 20 times more potent GHG, and microbial degradation is the primary source of global CH_4 emissions (Liu and Whitman, 2008). Thus, AD operated in closed systems followed by combustion of CH_4 to CO_2 can significantly reduce carbon emissions.

Primary industries such as farming, livestock producers, fishery and aquaculture have a large impact on essential and limited resources such as land area and sea space, soil nutrients, water, and energy. Collection and AD of organic materials from these industries can reduce the emission of pollutants to land and sea. Moreover, essential nutrients can be recovered during the AD process, which in turn will contribute to secure production safety and sustainability in the agricultural sector. During AD, a large variety of microorganisms are involved in several

degradation steps in which the final products are CH_4 (50-70%), CO_2 (30-50%) and small amounts of other trace gases. CH_4 is an energy carrier that can be used for heating and production of electricity and as a vehicle fuel after upgrading (e.g. removal of trace gases like hydrogen sulfide, H_2S).

1.1.1 Anaerobic digestion in Norway and Europe – exploitations and potentials

The history of AD and biogas production for energy use might go as long back as 10 centuries before current era, and household scale self-sufficient biogas production has been, and is, widely used in developing countries (Surendra et al., 2014). The first documented attempt of up scaled utilization of AD for energy purposes in Europe was a sewage sludge digester constructed in India and built in England in the 1890s (Bond and Templeton, 2011). The number of biogas power plants in Europe has increased since then, and the development of biogas infrastructure has risen sharply in the past 10 to 15 years.

In 2014 there was more than 17.000 biogas plants operated in Europe, with a total capacity of over 8.000 MW_{el} (European Biogas Association, 2016). However, the total number of biogas plants in Norway is only 48, of which 26 are sewage treatment plants (ENOVA, 2014). The Norwegian Government has set the ambitious goal that within 2020, 30 % of the animal manure should be treated by AD (Landbruks- og matdepartementet, 2009). Moreover, the Ministry of Climate and Environment recently presented a 'New Norwegian strategy for biogas' (Klima- og Miljødepartementet, 2014), with an aim of stimulating the production of biogas in Norway. Major objectives with the new strategy is increased nutrient recovery and reduction of GHG emissions, especially from the agricultural sector. Total production of biogas in Norway in

2010 was approx. 0.5 TWh. Of this, 0.27 TWh originated from landfills, 0.16 TWh from sewage sludge and 0.06 TWh from biogas plants based on organic waste and manure (Sletten and Maas, 2013). The biogas production in Norway is relatively low compared to neighboring countries, e.g., Sweden produced 1.6 TWh from AD in 2012 (Klima- og Miljødepartementet, 2014). Low production of biogas in Norway is largely due to the high proportion of affordable electricity produced from hydropower. Although the utilization of organic material for biogas production in Norway is currently low, the total unexploited potential is estimated to be 1.7 TWh (0.7 and 1 TWh from animal manure and wet organic waste, respectively) (Sletten and Maas, 2013).

The agricultural sector produces the largest fraction of potential biogas substrates in Norway, i.e. more than 50 % of the total potential. Manure and byproducts from crop- and oilseeds are estimated to constitute about 40 and 10 % of the methane potential, respectively (Raadal et al., 2008). In addition to agriculture and farming, a large potential for biogas production in Norway is found within the aquaculture sector. Fishery and fish farming (aquaculture) produces significant amounts of organic waste, both from processing fish for food (offals), fish that dies (dead-fish), and sludge from farming (feces and excess feed) (Arvanitoyannis and Kassaveti, 2008). The total methane energy potential from Norwegian industry is estimated to be approx. 23 % of the total, and fish offal and dead fish from fish industry constitute 46 % of this (Raadal et al., 2008). Although a portion of this potential is utilized for other purposes (e.g. animal feed and extraction of oils), there is a considerable biogas potential from Norwegian aquaculture. In addition to agriculture and industry, mainly sewage sludge and household waste covers the rest of the total potential.

1.1.2 Anaerobic digestion as a part of the aqua- and agricultural value chains

Due to low energy prices, and the fact that Norwegian agriculture is dominated by small and scattered farms, only a few farm biogas plants have been built so far. To get Norwegian farmers to adopt biogas production, the technology must be easy to use and economically viable, and hence there is a need to make farm scale biogas production robust and cost effective. Smaller biogas plants will normally demand a higher investment per energy unit produced than for a larger plant. Moreover, compared to large plants, a small on-farm plant will often represent a greater challenge in terms of operating capacity, since large industrial plants usually have dedicated personnel. Nevertheless, there is potential for more biogas production on farms.

In addition to the energy yields from AD, there are several other significant driving forces for establishment of AD on, or close to farms. By adopting AD, essential nutrients for plant production can be managed in a more sustainable way. Most fertilizers commonly used in agriculture contain the three basic plant nutrients nitrogen, phosphorus, and potassium of which nitrogen is required in the largest quantity (Masclaux-Daubresse et al., 2010). Plants cannot readily utilize organic nitrogen, and during AD a share of 10 – 30 % of the total nitrogen is mineralized to plant available NH_4^+ (Möller and Müller, 2012). Phosphorous is essential for plant growth, and the global phosphorous reservoirs are limited and unequally spread around the world (Cordell et al., 2009). It has been estimated that the world's phosphorous reservoir could be depleted within the next 50 to 100 years (Cordell et al., 2009; Smil, 2000). It will therefore be crucial in the future to recover nutrients from organic streams in order to provide the fertilizers needed for food production. Technologies for nutrient recycling from organic waste streams are established and under development (Batstone et al., 2015; Jensen et al., 2014), and such technologies can be used within a sustainable bio economy strategy to increase

recycling of essential nutrients such as nitrogen and phosphorous. The agriculture is Norway's fifth largest contributor to GHG emission (8.3 % of total emissions), mainly in the form of CH_4 and nitrous oxides from animals and fertilizers (Pettersen et al., 2017). In addition to GHG emission to air, the use of animal manure and chemical fertilizers for soil quality improvement results in considerable nutrient runoff. Excess plant nutrients like nitrogen and phosphorus flows into streams and lakes, and cause eutrophication and overgrowing in waters and seas. Currently, the total runoff of phosphorous and nitrogen from Norwegian agriculture is 790 and 27.000 tons per year, respectively (Miljødirektoratet, 2016). By adopting AD and biogas production, energy can be produced on the farm, a larger portion of nutrients can be recovered, and the pollution from both GHG emissions and nutrient runoff will be reduced. A potential large source for both reduction of pollution, and biogas production in Norway is the waste fractions from aquaculture; sludges and slaughterwaste. Fish farming at sea is carried out using open cages, and the excess organic fractions of sludge accumulating under the cages is a tremendous source of pollution and nutrient leachate. Fish farming is the largest source of anthropogenic emissions of nutrients along the Norwegian coast, and approx. 10.000 and 60.000 tons of phosphorous and nitrogen, respectively, are discharged from fish farming every year (Miljødirektoratet, 2016).

Norway is the world's largest producer of Atlantic salmon, and the fish industry experiences great loss, as many fish dies in fish farms. The current annual loss of Atlantic salmon in Norwegian aquaculture is approx. 50 million individuals (Statistics Norway, 2016), where one salmon can weigh up to 5 kg (Rosten et al., 2013). Moreover, during evisceration and processing of fish for food production, 40 % of the fish is disposed as waste (Liaset and Espe, 2008). The total rest raw material (e.g. guts, heads, liver, fins) from industrial fishing and fish

farming of cod fish, herring fish and shell fish is more than 800.000 tons (RUBIN, 2012). Out of this, approx. 200.000 tons are disposed of as waste, and dumped either at sea or on land. Disposing of this nutrient rich organic fraction have a potentially large environmental impact, and the cost for disposing fish offals and dead fish represents a large expense for the fish industry.

Sludge, fish offals and dead fish are rich in protein and fat, which make them potentially energy rich biogas substrates. However, anaerobic degradation of such energy rich substrates stands a great risk of process disturbances from accumulation of inhibiting nitrogen compounds and fatty acids (Gebauer, 2004; Gebauer and Eikebrokk, 2006; Kafle et al., 2013). Nevertheless, a possible strategy to reduce problems with such process instability would be to mix organic fractions with different properties.

In areas where it is logistically reasonable, combining waste streams from agriculture and aquaculture could lead to more robust biogas processes, and thus increased CH_4 yields. Agricultural wastes such as animal manure are usually energy poor, but provides advantageous buffering capacity and nutrient composition (Angelidaki and Ellegaard, 2003; Weiland, 2010, 2003; Whalen et al., 2000) and are thus commonly used in biogas production as co-substrates. Aquacultural wastes are energy rich, and thus represents high CH_4 potentials which can more easily be achieved by dilution and co-digestion with animal manure (Callaghan et al., 1999; Lanari and Franci, 1998). The economy of biogas operations can be enhanced by achieving more stable processes and increased CH_4 yields, and the overall bio economy for both agriculture and aquaculture could be improved by a closer co-operation between these sectors.

1.2 Reactions and microorganisms involved in anaerobic digestion

Production of biogas is a microbial process that takes place in the absence of oxygen (or at low oxygen levels), and the final products from AD are mainly CH_4 and CO_2. Essentially, the AD process consists of four steps, namely hydrolysis, fermentation (acidogenesis), acetogenesis and methanogenesis (Figure 1). The degradation process is not always complete, and in addition to biogas and traces of other gases, final products may include reduced compounds such as fatty acids and nitrogen-compounds. To maintain a stable and robust biogas production process, numerous different groups of microorganisms need to be active and properly adjusted to the environment, and each other. Unsuccessful maintenance of the microbial interactions will increase the risk of biogas production process failure and breakdown.

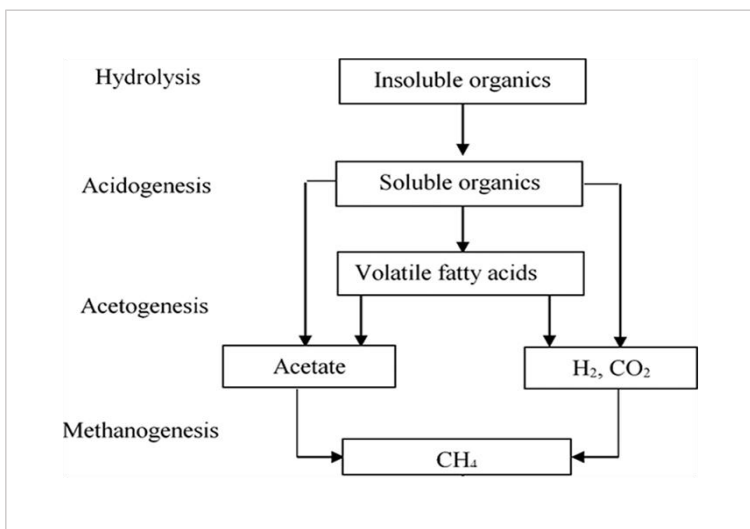

Figure 1: overwiev of anaerobic digestion and CH_4 – production. Adopted from (Beschkov, 2017).

1.2.1 Hydrolysis, fermentation and acetogenesis

The current knowledge about basic metabolism during AD is quite firm, but there are still gaps in the information about the responsible microorganisms. The bacteria involved in AD can be divided in two major groups: the hydrolyzing and fermenting bacteria (primary fermentative bacteria) and the acetogenic bacteria (secondary fermentative bacteria) (Weiland, 2010). Microbes that are directly involved in CH_4 production belong to the phylum Euryarchaeota and are generally called methanogenic Archaea, or methanogens. In both number and complexity, the bacterial groups involved in the first steps of AD are superior to the methanogenic group that carries out the final reaction (Rajagopal et al., 2013).

Most of the bacteria involved in the initial steps of AD typically belongs to the phyla Firmicutes, Bacteriodetes and Proteobacteria, where the classes of Clostridia and Bacilli are usually the most abundant (Klocke et al., 2007; Krause et al., 2008; Kröber et al., 2009; Schlüter et al., 2008; Wirth et al., 2012), and these microbes play crucial roles in the initial steps of degrading organic material. CH_4 is produced by methanogens during the last step of AD, and the complete process of breaking down organic material to biogas is comprised of numerous interdependent biochemical reactions. Hydrolytic and fermentative bacteria initiate the first reaction steps of AD by secretion of extracellular enzymes that degrade complex organic polymeric material (carbohydrates, lipids and proteins) into smaller monomeric compounds. Carbohydrates are degraded mainly to simple sugars such as glucose, and proteins are degraded to amino acids by hydrolytic peptidases and proteases. Lipids are initially hydrolyzed by lipases to glycerol and long chained fatty acids (LCFA). Following hydrolysis, fermentative bacteria converts the hydrolyzed monomers to alcohols, short-chained volatile fatty acids (VFA, e.g. acetic, propionic and butyric acid), NH_4^+ and H_2-gas. Only a few of the

products from fermentation can directly be utilized by methanogens, including acetate, H_2 and CO_2, while other more complex products such as propionate, butyrate and alcohols need to be further oxidized to acetate, H_2 and CO_2 by acetogenic bacteria (Liu and Whitman, 2008). During this step, a large and complex group of fermentative H_2-producing acetogenic bacteria carries out various reactions to degrade the hydrolyzed compounds. Accumulation of H_2 can inhibit the acetogenic bacteria, and for maintenance of their metabolism, a low concentration of H_2 is essential, and such conditions are provided by H_2 scavenging microorganisms (Weiland, 2010). The last step of AD is methanogenesis, and involves conversion of mainly H_2 and acetate to CH_4 and CO_2.

1.2.2 Methanogenesis

While a large consortia of bacteria are involved in the first degradation steps, a more narrow and specialized group of methanogenic archaea (methanogens) carries out the last step. The conversion of final products from hydrolysis, fermentation and acetogenesis to CH_4 mainly proceeds through transformation of acetate, or CO_2 combined with H_2 or formate as electron carriers (Thauer, 1998). The reactions involving CH_4 formation are carried out by the two functional groups called acetoclastic and hydrogenotrophic methanogens (Karakashev et al., 2005). CH_4 formation from acetate can proceed through two pathways, and the most commonly described is acetoclastic methanogenesis (Zinder, 1993). During acetoclastic methanogenesis, acetate is directly cleaved into methyl- and carboxyl groups by acetoclastic methanogens, followed by conversion of the methyl- and carboxyl groups to CH_4 and CO_2, respectively (Whitman et al., 2006). Among the methanogenic archaea, only members of the order

Methanosarcinales use acetate cleavage as a mechanism for CH_4 formation (Dworkin et al., 2006). The methanogenic population in biogas reactors with acetate cleavage as the main pathway for CH_4 production is typically dominated by members of the genera *Methanosaeta* and *Methanosarcina* (Demirel and Scherer, 2008). *Methanosaeta* is commonly detected in biogas reactors with low acetate concentrations (Griffin et al., 1998; Zheng and Raskin, 2000), while the abundance of *Methanosarcina* typically increase when acetate concentrations are elevated (Griffin et al., 1998; McMahon et al., 2001; Stroot et al., 2001).

During hydrogenotrophic methanogenesis, H_2 or formate is utilized to convert CO_2 to CH_4, and contrary to the narrow ability for methanogenic acetate utilization, all methanogenic orders are able to use H_2 for CH_4 production (Weiland, 2010). Members of the orders *Methanosarcinales*, *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales* are typically detected in biogas reactors with hydrogenotrophic methanogenesis as the main pathway for CH_4 production (Karakashev et al., 2005). Acetate has traditionally been considered to be the most important substrate for methanogenesis (Hatti-Kaul et al., 2016). However, the focus on H_2 as a precursor for methanogenesis has increased, and a dominance of hydrogenotrophic methanogenesis is common in many biogas reactors (Kampmann et al., 2012; Klocke et al., 2008; Zhu et al., 2011). In general, hydrogenotrophic methanogens are described to have a higher tolerance for extreme conditions, e.g. increased temperatures and high concentrations of fatty acids and nitrogen (Chen et al., 2008; Hanaki et al., 1981; Rajagopal et al., 2013; Ziganshin et al., 2016).

1.2.3 Syntrophic interactions between bacteria and methanogens in biogas reactors

Since the energy generated during AD is low compared to aerobic degradation, the conversion of complex organic material to biogas in anaerobic environments is dependent upon efficient cooperation between several types of microorganisms with various metabolic demands. The degree of interdependency in anaerobic microbial communities varies. Some microbes benefits from utilizing metabolic products from other organisms, while other microbes are completely dependent on other organisms' behavior and metabolic pathways. In syntrophic relations in biogas reactors, cooperation along the food chain is obligate between metabolically different microorganisms. Syntrophic microbes rely on each other for carbon and nutrient supply, and at the same, they keep the concentration of intermediate products at a minimum. The syntrophic reactions often involves, and are completely dependent on interspecies transfer of H_2 (McInerney et al., 2008), and such transfer involves the production and utilization of H_2 by bacteria and methanogens, respectively. When an oxidative fermentation process yields H_2 (e.g. during conversion of ethanol to CH_4) the net free energy change (ΔG) will be endergonic, i.e. thermodynamically impossible, if H_2 is not scavenged at the same rate as it is produced. Due to the syntrophic relations between microorganisms and their transfer of H_2, the H_2 concentration is kept low and the fermentation process becomes thermodynamically favorable.

Syntrophic acetogenic bacteria converts metabolites produced during hydrolysis and fermentation (e.g. ethanol, propionate, butyrate) into acetate and H_2, and these reactions are of crucial importance during methanogenesis, due to the accumulation of unfavorable metabolites that will occur if these reactions are stagnated. Methanogens are inhibited when concentrations of products from earlier steps are elevated, and acetogenic activity is thus essential for the CH_4 production process to proceed. Since the metabolic efficiency of the syntrophic acetogenic

bacteria strongly depend on the H_2 removal by a methanogen, the microorganisms involved are interdependent on each other to maintain methane production. Several factors involving syntrophic operations in biogas reactors has been investigated, such as impact from increased H_2 formation on methane yields (Bagi et al., 2007) and reactions involving syntrophic conversions of propionate and butyrate to acetate and H_2 (Li et al., 2012; Müller et al., 2010; Schmidt et al., 2013).

1.2.4 Syntrophic acetate oxidation

In addition to acetoclastic methanogenesis, an alternative reaction for CH_4 production from acetate is called syntrophic acetate oxidation (SAO). The reaction involves conversion of acetate by a syntrophic acetate oxidizing bacteria (SAOB) followed by hydrogenotrophic methanogenesis in which CO_2 is reduced to CH_4 (figure 2).

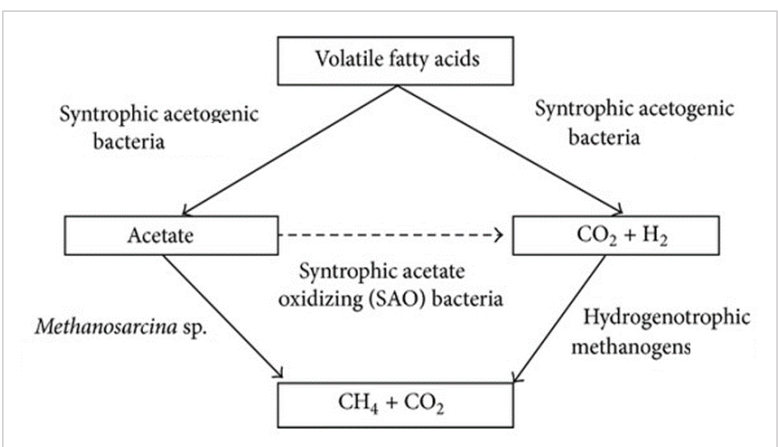

Figure 2. Syntrophic acetate oxidation and hydrogenotrophic methane formation. Adopted from (Ali Shah et al., 2014).

The reaction consists of two separate steps initiated by oxidation of acetate to CO_2, H_2 and formate by a SAOB, followed by conversion of H_2 and CO_2 to CH_4 by a hydrogenotrophic methanogen (Zinder and Koch, 1984).

a: [$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$] **b:** [$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$]

Energetically, the oxidation of acetate to CO_2 and H_2 is an unfavorable reaction at standard conditions [**(a)** $\Delta G° = +104$, 6 kJ / mol]. However, the subsequent reaction where CH_4 is produced from CO_2 and H_2 is thermodynamically favorable [**(b)** $\Delta G° = -135$,6 kJ / mol], and the sum of these reactions results in a final change in free energy of -31,0 kJ / mol.

SAO can only proceed if the H_2 pressure is kept low by the action of a hydrogenotrophic methanogen (Schink, 1997; Stams, 1994). However, if the H_2 levels becomes too low, the reaction will stagnate due to inactivation of hydrogenotrophic methanogenesis. These conflicting requirements results in a demand for a narrow range of H_2 concentration during AD (Stams, 1994). The H_2 concentration should be in the range of 10 – 50 Pa at thermophilic (55-60 ℃) conditions (Hattori et al., 2001). At mesophilic (~ 37 ℃) conditions, the H_2 concentrations for maintenance of syntrophic activity is found to be between 1.6 – 6.8 Pa (Schnürer et al., 1997). Hence, sustained and sufficient flow of H_2 is crucial to maintain the activity of SAOB and their partner methanogens.

Various factors have been suggested as driving forces for the different syntrophic relations occurring in biogas reactors, and NH_4^+ has proven to be a strong regulating factor for development of SAO (Schnürer et al., 1999; Schnürer and Nordberg, 2008; Westerholm et al., 2011a, 2016). The shift in reaction pathway from acetoclastic methanogenesis to SAO at elevated NH_4^+ levels is possibly a consequence of inhibition of acetoclastic methanogens,

which are known to be more sensitive to NH_4^+ than hydrogenotrophs (Angelidaki and Ahring, 1993). The contribution from SAO in biogas reactors is considered to be significant, and factors affecting the reaction other than NH_4^+ are described. Among these are acetate concentration (Ahring et al., 1993; Ahring, 1995; Hao et al., 2010), the synergetic stress of acids and NH_4^+ (Lü et al., 2013), dilution rate (Shigematsu et al., 2004) and temperature (Schink, 1997; Schink and Stams, 2013).

1.2.5 Syntrophic acetate oxidizing bacteria

A few SAOB are currently isolated and characterized from differently operated biogas reactors. *Thermacetogenium phaeum* (Hattori et al., 2000) and *Thermotoga lettinga* (Balk et al., 2002) were initially isolated from thermophilic reactors. *Tepidanaerobacter acetatoxydans* is identified as thermotolerant, and *Clostridium ultunense* and *Syntrophaceticus schinkii* are isolated from reactors operated in the mesophilic range (Schnürer et al., 1996; Westerholm et al., 2010, 2011c). However, observations of SAO in anaerobic digesters, and recent detections of novel syntrophic populations suggests that methane formation via this pathway appears to be quite common (Frank et al., 2016; Lee et al., 2015).

The growth of SAOB are normally lithotrophic or heterotrophic, and they produce acetate through the Wood–Ljungdahl pathway involving the key enzyme Acetyl Co enzyme A (Müller et al., 2013). However, when they grow in a syntrophic relation with a methanogen, they reverse this pathway and oxidize acetate to H_2 and CO_2. Growth of SAOB in co-culture with hydrogenotrophic methanogens have been investigated, and a doubling time of 28 days for *C. ultunese* is reported (Schnürer et al., 1994), while the generation time of *S. schinkii* in co-culture

with *Methanoculleus* sp. MAB1 is calculated to be as long as 69-78 days (Westerholm, 2012). The doubling time of *T. acetatoxydans* during acetate oxidation is currently not known (Westerholm et al., 2011c). The slow doubling times for SAOB suggest that a long retention time is essential to avoid washout of these bacteria from continuous biogas reactors. However, SAOB have been detected in a number of different biogas reactors operated under a wide range of hydraulic retention times (HRTs), suggesting that other parameters might also influence the presence of these bacteria (Karlsson et al., 2012; Moestedt et al., 2014; Sun et al., 2014; Westerholm et al., 2012, 2011b). Moreover, a possible reason that slow growing microorganisms are able to retain in continuous processes with low HRTs might be the formation of biofilms. It is known that microbes in biogas reactors tend to grow in biofilm structures that attach to surfaces (Davey and O'toole, 2000) and such attachments in biogas reactors would make the microorganisms more resistant to washout.

1.2.6 Ammonium tolerance and inhibition

During hydrolysis and fermentation, proteins are degraded to amino acids and peptides by bacterial proteases and peptidases (Kovács et al., 2013). During fermentation, the amino acids are further broken down, and amine groups (NH_2) are released as NH_4^+; ammonium (NH_4^+) and ammonia (NH_3). NH_4^+ and NH_3 are in equilibrium, and the dominating form is highly dependent on pH and temperature (Gerardi, 2003; Speece, 1996). Some NH_4^+ is essential for bacterial growth, but elevated concentrations, especially in the form of NH_3, inhibit methanogenic activity in biogas reactors (Rajagopal et al., 2013; Yenigün and Demirel, 2013) and leads to an unstable biogas process. The mechanism of inhibition is not fully known, but some suggestions have been

presented. Investigations of pure cultures have shown that NH_4^+ can affect methanogenic microbes by direct inhibition of NH_4^+ on methanogenic enzymes, and by diffusion of NH_3 into bacterial cells, causing pH and proton imbalance and potassium loss (Gallert et al., 1998; Sprott and Patel, 1986; Whitman et al., 2006). NH_3, which is uncharged, can enter the microbial cell (Figure 3) and subsequently be converted to NH_4^+. Hydrogen ions (H^+) are consumed in this reaction with concomitant increase in pH. To compensate for the change in pH, the cell pumps in H^+ from the environment. At the same time, potassium (K^+) is pumped out, and the cell will eventually suffer from potassium loss (Sprott and Patel, 1986). The initial potassium content within a cell will have an impact on the degree of inhibition from NH_3, and methanogens that use acetate for CH_4 production generally contains lower levels of potassium than hydrogenotrophic methanogens. The former methanogenic group is thus more sensitive to NH_3.

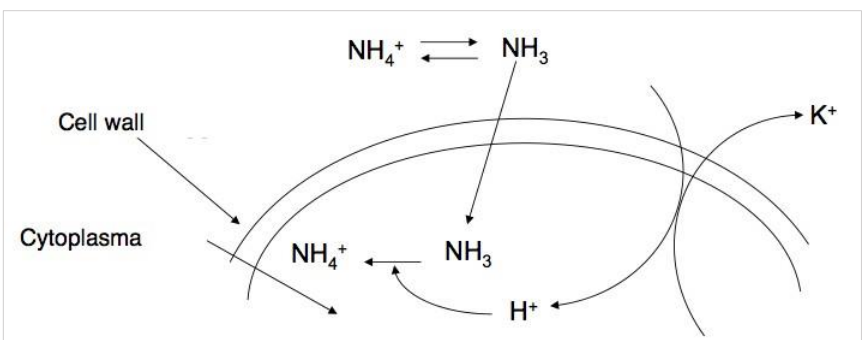

Figure 3. The effect of ammonia in methanogenic microbial cell. Hypothesis proposed by (Sprott and Patel, 1986). Figure adopted from (Schnurer and Jarvis, 2010).

Optimal NH_4^+ levels provides a valuable nitrogen source for the microbes involved, and also a sufficient buffer capacity for low pH levels. However, elevated NH_4^+ concentrations is a

commonly reported reason for biogas process failure (Chen et al., 2008; Hejnfelt and Angelidaki, 2009). NH_4^+ inhibition is usually indicated by a decrease in steady state CH_4 yields together with accumulation of other intermediate products such as VFA (Angelidaki and Ahring, 1994; Calli et al., 2005). Inhibitory levels of NH_4^+ in biogas reactors have been investigated, and the reported results are non-consistent. Concentrations from around 1.5 and up to 7 grams NH_4^+ / L has been reported as inhibiting (Rajagopal et al., 2013). These variable reported levels of inhibition by NH_4^+ are most likely due to the impact of other factors such as pH-levels, temperature, type of inoculums and acclimation time (Chen et al., 2008). The share of NH_3 increases with elevated temperature and pH levels, and a biogas reactor operated in the mesophilic range (~ 35 – 40 °C) will maintain stable at higher NH_4^+ concentrations than a reactor operated in the thermophilic range (~ 55 – 60 °C). If the pH level is increased from 7 to 8 in a biogas reactor operated in the mesophilic range, the share of NH_3 will increase from approx. 1 to 10 % of NH_4^+ (Fernandes et al., 2012). Adaptation of the microbial consortia is of great importance to avoid process failure from NH_4^+ inhibition. Several studies have reported successful adaptation of microbial communities to elevated NH_4^+ levels (Angelidaki and Ahring, 1993; Angenent et al., 2002; Calli et al., 2005), and the required adaptation time is reported to be as long as 2 months or even longer (Hansen et al., 1998). However, even after adaptation, low CH_4 production is obtained at elevated NH_4^+ levels compared to an undisturbed system (Van Velsen, 1979).

It has not been clear whether NH_4^+ tolerance can develop in an already existing population, or if adaptation occurs due to establishment of new microbes. Studies investigating the effect of accumulated NH_4^+ concentrations in methanogenic environments have demonstrated that the main pathway for CH_4 production is through the syntrophic relations between acetate oxidizing bacteria, SAOB and H_2 utilizing methanogens (Angelidaki and Ahring, 1994; Angenent et al.,

2002; Schnürer et al., 1999, 1994; Werner et al., 2014; Westerholm et al., 2011a). The presence of SAO and SAOB has been investigated in biogas reactors operated at high NH_4^+ levels, and a shift in CH_4 production pathway and methanogenic community structure is typically detected when the NH_4^+ concentrations exceed 3 – 4 grams / L (Schnürer and Nordberg, 2008; Westerholm et al., 2011a). This shift in methanogenic community to slow growing SAOB may explain the long adaptation time required for NH_4^+ tolerance to develop.

1.3 Anaerobic digestion process operation and substrate loading

The choice of biogas reactor design and operation is usually based on a compromise between maximizing CH_4 yield, sufficient organic material degradation, and process economy. The hydrolytic and fermentative bacteria differ widely from the methanogenic Archaea in their preferred environment, such as optimal pH range and nutrient requirements. The main energy components in substrates for AD are carbohydrates, fat and proteins, and they represents theoretical biogas potentials of approx. 0.4, 1.0 and 0.5 m^3 biogas / kg VS, respectively (Berglund and Bôrjesson, 2003). For a successful optimization of a biogas process, it is necessary to adjust the parameters so that important microbes and pathways are maintained. The number of different factors that affects AD stability is extensive, and mostly in close relation to each other. Factors such as process and reactor design, substrate type and mixes, loading of potentially inhibiting compounds together with other operational parameters are of crucial importance for a successful AD process. A large variety and combinations of biogas reactors are available, and the most common reactor type for slow rate processes (i.e. long HRT and high solid loading) has traditionally been the continuously stirred tank reactor (CSTR)

(Weiland, 2010). Such reactors typically consist of a cylindrical tank with in- and outlet valves, and a steering mechanism (Figure 4). The principles of a CSTR is that AD takes place at constant rate and temperature in a completely mixed reactor (Froment et al., 2009). The main advantage of the CSTR is that it is typically robust and easy to operate, due to a relatively simple design.

Figure 4. Continuously stirred tank reactor, CSTR.

1.3.1 Operational parameters

A wide number of operational parameters such as biogas production, substrate loading, operational pressure, and reactor capacity are commonly adjusted and monitored in a biogas reactor (Al Seadi et al., 2008). Monitoring and adjustment of operational parameters is crucial for process maintenance, i.e. facilitate sufficient microbial activity and biogas yields.

CH_4 production takes place at different temperature ranges, but most biogas reactors are operated either in the mesophilic or thermophilic range, at temperatures of 35-37 and 55-60 ℃, respectively (Bouallagui et al., 2004). Digestion under thermophilic condition has several benefits, such as higher metabolic rates and CH_4 yields, and a high destruction of pathogens and weed seeds (Zabranska et al., 2002). However, thermophilic conditions in biogas reactors have some drawbacks compared to mesophilic conditions. The rate of metabolism in hydrolytic and fermentative bacteria is increased with elevated temperatures (Ahring et al., 2001), and elevated concentrations of intermediate products (e.g. NH_4^+, LCFA and VFA) from these bacterial groups are reported to inhibit AD at high temperatures (Ahring et al., 2001; Angelidaki and Ahring, 1993, 1992; Sung and Liu, 2003). The NH_3 share of NH_4^+ is increased at elevated temperature. It has been reported that AD processes operated at high NH_4^+ concentrations are more easily inhibited and less stable at thermophilic temperatures than at mesophilic temperatures (Braun et al., 1981). Moreover, decreasing the temperature in thermophilic biogas reactor suffering from NH_4^+ inhibition can restore process stability (Angelidaki and Ahring, 1994; Hansen et al., 1999). Hence, AD is considered more robust in the mesophilic range than at high temperatures, although lower CH_4 yields are obtained.

Hydraulic retention time (HRT) is an important parameter for dimensioning of a biogas reactor. The HRT is the residence time of a substrate in the reactor, and it is correlated to the reactor volume and the volume of substrate fed per unit of time (Al Seadi et al., 2008). The HRT in CSTRs is typically 15 to 30 days in mesophilic reactors, and 10 to 20 days in thermophilic reactors (Angelidaki et al., 2011). In addition to dimensioning and scaling, the HRT is regarded to be an important factor for the microbial community in a biogas reactor. HRT is regulated based on several different factors, such as process temperature and substrate

properties, and the microbial population is highly dependent on these parameters. The microbes involved in AD need a uniform flow of substrates and effluents to maintain stable activity, and they require a certain amount of time for sufficient degradation of the substrates. Slowly degradable materials (e.g. poorly bioavailable carbohydrates and fat) will need a longer HRT than more easily degradable material. Due to an increased metabolic rate at elevated temperatures, a thermophilic process is typically operated at a shorter HRT than a corresponding mesophilic process (Kim et al., 2006). However, reactor operation with short HRT and elevated temperature will increase the risk of inhibition from accumulation of hydrolysis and fermentation intermediates. Moreover, a short HRT is likely to increase washout of the microbial population (Sreekrishnan et al., 2004), which can result in removal of key functioning microbial groups in a biogas reactor.

The organic loading rate (OLR) is a measure for amount of organic material added to a process, commonly designated as volatile solids (VS) or chemical oxygen demand (COD) / volume / time (Al Seadi et al., 2008). For most CSTRs, the OLR is completely correlated with the volumetric loading, and hence also the HRT. Operation at short HRT corresponds to a high volumetric load and thus an elevated OLR. Different reactors are operated at various OLR, and typically, mesophilic wet fermenting CSTRs are stable at OLRs between 2 and 4 kg VS / m^3 (Weiland, 2010). Increased OLR will increase biogas yields. However, biogas reactors loaded with extended high input of organic material have an increased risk of failure. Organic overload occurs when the amount of organic material added to the reactor exceeds the degradation capacity of the microbes to produce biogas. Elevated OLR is causing instability due to increased concentrations of fatty acids (Blume et al., 2010; Marchaim and Krause, 1993), NH_4^+ (Kovács et al., 2013), or a combination of these (Lü et al., 2013), depending on the substrate

composition. Process instability due to accumulation of NH_4^+ often results in VFA accumulation, which again may lead to a decrease in pH (Chen et al., 2008) and process failure.

1.3.2 Proteinaceous organic material

Nitrogen and carbon are essential elements in biogas substrates, and the yield of biogas from any substrate is highly dependent on the ratio of carbon/nitrogen (C/N). Proteinaceous substrates, such as slaughterhouse wastes and animal offals, fish waste and some types of food wastes are energy rich, and usually consist of relatively low levels of carbon in relation to nitrogen (Cuetos et al., 2010; Divya et al., 2015). The effect of C/N ratios on CH_4 production has been extensively studied. Optimal C/N ratios are suggested to be in the range between 15 and 30 (Weiland, 2010), however, stable AD of proteinaceous substrates have been achieved at lower C/N values (Mshandete et al., 2004). During AD of substrates with a low C/N ratio, accumulation of inhibiting NH_4^+ can occur, and the risk of process instability increases. However, although high NH_4^+ concentrations can lead to inhibition, it can also provide increased buffering capacity. In processes operated with high organic load and elevated concentrations of fatty acids, NH_4^+ can provide maintenance of neutral pH level and thus potentially minimize the inhibitory effect of VFA and LCFA (Marchaim, 1992).

In addition to balancing the macronutrients C and N, certain micronutrients and trace elements also affects the AD process and biogas production (Feng et al., 2010; Wintsche et al., 2016), especially in processes operated with high concentrations of fatty acids and nitrogen (Banks et al., 2012). Trace elements required for enzyme activity in methanogenic systems are, for example, Co, Ni, Fe, Zn, Se, B, Mo and W (Feng et al., 2010; Schattauer et al., 2011). Studies

have shown that trace elements can be depleted in biogas reactors (Schattauer et al., 2011), and that organic loading and biogas yields can be increased and inhibition can be minimized by addition and balancing of micronutrients (Banks et al., 2012; Feng et al., 2010).

1.3.3 Fish waste

Organic waste from aquaculture is produced in large quantities, and the focus on sustainable management of these fractions is increasing. A significant portion of such waste is represented by slaughter waste and offal from fish processing, and dead fish. These fish wastes are energy rich, i.e. they contain large amounts of protein and fat (Callaghan et al., 1998), and thus have a high biogas potential. A portion of the fish waste is utilized as animal feed, and processing for such purposes is typically carried out by separating the fish oils during fishmeal production. The majority of fish oils are used in aquaculture feeds, and the fishmeal is used in livestock animal feed manufacturing (Arvanitoyannis and Kassaveti, 2008). In Europe and Norway, fish waste (i.e. offals and dead fish) is divided into different quality categories, and category 1 and 2 consist mainly of disease infected self-dead fish, and can thus not be utilized for animal feed (Grøntvedt et al., 2010). The fish waste in Norway that is used for biogas production is pretreated by ensilation for stabilization (i.e. to minimize microbial activity). Ensiled fish waste is a liquid product produced from the fish or fish parts, and the liquefaction of the biomass is initiated by the interaction of added acids and enzymes from the fish (Arruda et al., 2007). The process involves grinding and mixing of the fish waste with formic acid to a pH below 4.0, followed by thermal treatment, according to regulations. The fish silage processing method

(FSPM) is a Norwegian sanitation method approved by the Norwegian Animal Health Authority, originally adapted from the EU legislation (Møretrø et al., 2010).

CH_4 potentials from fish waste (e.g. various types of fish offal's) have been investigated, and reported to be in the range between 400 and 800 L / kg VS (Callaghan et al., 1999; Gunnarsdóttir et al., 2014; Kafle et al., 2013; Mshandete et al., 2004), which is high, compared to e.g. animal manure. However, loading of a continuous biogas reactor with fat- and protein rich substrates can be challenging due to accumulation of and inhibition from fatty acids and NH_4^+ (Weiland, 2010; Yenigün and Demirel, 2013). In addition, during AD of ensiled (i.e. acidified) substrates, the pH levels might drop significantly. Such conditions will inhibit methanogenic activity and increase the risk of biogas process failure. A possible way to enable sufficient AD of fat- and proteinaceous substrates is by co-digesting them with a carbon rich substrate with high buffer capacity, such as manure.

1.3.4 Manure from cows and other livestock

The largest fraction of organic material for biogas production in Europe is animal manure from cattle, pig and poultry (Faostat, 2014). As the focus on energy production from biomass has increased, the use of the organic fractions from agriculture, industry and households for CH_4 production has gained interest. Untreated and poorly managed animal manure is a source of air and water pollution in the form of nutrient leachate rich in nitrogen and phosphorous. A large portion of animal manure in Europe is handled as slurries; liquid mixtures of urine, feces, water and bedding material (Holm-Nielsen et al., 2009), consisting of mainly carbohydrates, proteins and lipids (Møller et al., 2004). When handled properly, this is an organic fraction suitable for

energy production and a source of nutrients for agriculture. Animal manure as a biogas substrate is widely investigated (Alvarez et al., 2006; Artanti et al., 2012; Lehtomäki et al., 2007; Møller et al., 2004; Wu et al., 2010), and the CH_4 yields depends on several factors, e.g. livestock feeding regimes, animal metabolism and energy uptake. Among the different animal waste fractions, manure from cows represents the largest volume, and CH_4 yields between 150 and 300 L CH_4 / kg VS are frequently reported from this material (Amon et al., 2007; Lehtomäki et al., 2007; Møller et al., 2004). Manures from pigs and poultry are reported to contain higher concentrations of NH_4^+ than cattle manure (Hansen et al., 1998; Kelleher et al., 2002), and moreover, pig manure contains higher levels of lipids than manure from cows (Møller et al., 2004). Since a large portion of cattle are fed mainly with roughage, the levels of organics with low bioavailability (e.g. lignin) is frequently high in cow manure (Lehtomäki et al., 2007; Møller et al., 2004). In general, cow manure is considered to be a low energy biogas substrate. When this fraction is added to a biogas reactor, it has already passed through the digestive system of the animal, and most of the energy-rich substances (i.e. proteins and easy carbohydrates) contained in the feed (crops) have already been digested. However, although cow manure does not contribute to very high CH_4 yields, it is a highly suitable co-substrate, providing increased buffer capacity and nutrient balance in a reactor feedstock (Alvarez and Lidén, 2008; Lehtomäki et al., 2007).

1.3.5 Co – digestion

Co-digestion of substrates with different properties provide several benefits. AD of energy poor material, such as manure from ruminant livestock, is usually stable, but results in relatively low CH_4 yields (Alvarez and Lidén, 2008; Amon et al., 2007; Artanti et al., 2012). Thus, many

agricultural biogas plants add co-substrates to the process to increase the content of energy and hence obtain higher CH_4 yields (Weiland, 2010, 2003). Due to improved balance of nutrients, minerals and trace metals (Braun and Wellinger, 2003), and thus a more complete medium for microbial metabolism and growth, CH_4 yields from mixed substrates are frequently reported to be higher than the sum of CH_4 yields from digestion of individual substrates (Carucci et al., 2005; Neves et al., 2009; Parawira et al., 2004). In addition, co-digestion of high-energy content substrates with energy - poor substrates will dilute the concentration of possibly inhibiting compounds such as NH_4^+ and fatty acids.

Cow manure as a co-substrate provide buffering capacity due to its alkalinity, and a wide range of nutrients (Lehtomäki et al., 2007). The buffering capacity of cow manure can play an important role during co-digestion by decreasing the risk of pH drop, and hence inhibition from fatty acids. The extracellular enzymes produced by microorganisms during AD are affected by pH, with minimum, maximum, and optimal pH for activity (Lay et al., 1997). The pH interval for methanogenic activity is quite narrow ranging from 5.5 to 8.5 with an optimal range of 6.5-8.0 (Nielsen, 2006). For hydrolyzing and fermentative bacteria a much wider pH range between 4 and 8 is observed (Hwang et al., 2004), and it is reported that optimal pH levels in a mixed-culture AD is between 6.5 and 7.5 (Moosbrugger et al., 1993). In addition to buffer capacity, cow manure usually have a balanced C/N ratio with values between 16 and 25 (Divya et al., 2015). The use of co-substrates with low protein content and a sufficient portion of carbohydrates (e.g. manure) to blend with proteinaceous material will increase the C/N ratio and thus reduce the risk of potential problems associated with accumulation of NH_4^+.

2. Project outline and objectives

2.1 The research project

This PhD project was part of a Norwegian research project (Biogas reactor technology for Norwegian agriculture, BIONA) aiming to make biogas reactor technology in Norwegian agriculture more economically viable by testing and evaluating different methods for increasing biogas yields.

In order to reduce the greenhouse gas emissions from agriculture, the Norwegian Government has set ambitious goals for AD treatment of animal manure in the future (Klima- og Miljødepartementet, 2014; Landbruks- og matdepartementet, 2009). Due to low energy prices, and because Norwegian agriculture is dominated by small and scattered farms, only a few farms have installed biogas plants. To establish more farm-based biogas plants the AD economy must be improved. Thus, higher biogas- and energy yields are needed.

2.2 The PhD project; outline and objectives

One possible way to increase methane and energy yields in the agricultural sector is co-digesting animal manure with energy rich substrates like fish offals. Such co-digestion of organic waste fractions from agriculture and aquaculture could add value to both sectors. However, co-digestion with such protein rich fractions stands the risk of elevated NH_4^+ concentrations, which might cause process inhibition. Although biogas production can occur in

environments with elevated NH_4^+ levels, sufficient stabilization and adaptation periods are required, and even after adaptation, relatively low CH_4 productions may be achieved (Van Velsen, 1979). The microbial adaptation to elevated NH_4^+ concentrations is probably due to the development of an alternative mechanism for CH_4 production called syntrophic acetate oxidation (Schnürer et al., 1999; Schnürer and Nordberg, 2008). In this pathway, acetate is not directly converted to biogas, but initially converted to H_2 and CO_2 by syntrophic acetate oxidizing bacteria (SAOB), followed by the subsequent reduction of CO_2 to CH_4 by H_2-utilizing methanogenic partners. Thus, establishment of such syntrophic microbial consortia offers a way to run high NH_4^+ AD processes. However, due to their slow growth, wash-out of continuous flow reactors can be a challenge. Factors like sufficient adaptation, long HRTs and biofilm formation may facilitate and retain the syntrophic microbial community in the reactor, allowing stable operation even at high levels of NH_4^+.

The main objective of this PhD project was to make biogas production from protein rich fish waste efficient and robust by blending it with cow manure to develop a stable co-digestion processes. Secondary objectives were to optimize fish waste and manure ratios, investigate microbial adaptation during startup period and characterize microbial communities and key syntrophic microorganism in co-digestion processes. The first part of this work focused on finding optimal proportions of fish waste silage and cow manure for efficient biogas production (**Paper I**). In the next part, the microbial communities in biogas reactors added fish waste silage and manure during a startup phase were characterized and compared to the original inoculum (**Paper II**). In the last part of this work, the dynamics of important NH_4^+ tolerant microorganisms in reactors with different hydraulic retention times and in reactors added increasing ratios of fish waste silage were investigated (**Paper III**).

3. Main results and discussion

This PhD study is based on three research papers, and the main findings are summarized in this section.

3.1 Effects of increased loads of fish waste silage in co digestion with manure on methane production (Paper I):

In this paper the effects of increased loads of nitrogen-rich fish waste on AD and CH_4 production was examined. Co-digestion of fish waste silage (FWS) and cow manure was studied in two parallel laboratory-scale continuously stirred, semi-continuously fed tank reactors, and compared to AD of cow manure alone. The reactors were operated in the mesophilic range (37 °C) with a hydraulic retention time of 30 days, and the entire experiment lasted for 450 days. The rate of organic loading was raised by increasing the content of FWS in the substrate blend. During the experiment, the amount (volume %) of FWS was increased stepwise in the following order: 3% – 6% – 13% – 16%, and 19%. CH_4 production and pH were continuously monitored while the content of VFA and NH_4^+ were analyzed. The highest CH_4 production from co-digestion of FWS and manure was approx. 0.400 L CH_4 / gram VS, obtained during a period with loading of 16% FWS. Compared to mono-digestion of manure, the CH_4 production was increased up to 100% when FWS was added to the reactors. At very high FWS loadings between 16% and 19%, the biogas processes failed due to accumulation of NH_4^+ and VFA. During a period of loading the

reactors with a substrate mix of 13 % FWS and 87 % manure, a stable AD operation and relatively high CH_4 yields were observed (**Figure 3.1 A**). The effluent quality was within a range typically tolerated by methanogenic bacteria, i.e. the concentrations of VFAs and NH_4^+ did not exceed inhibitory levels, and the pH levels were stable in the range of between 7 and 8. Overall, this study clearly shows that FWS in mix with manure increases the biogas yields, and that the AD process is stable at a substrate mixing ratios of up to 13 % FWS, and can be recommended for scaled up production. However, higher levels FWS lead to accumulation of NH_4^+ and VFAs, which caused CH_4 production failure. At loadings of between 16% and 19% FWS, the NH_4^+ concentrations reached 7-8 g/L (**Figure 3.1 B**). The VFA levels, mainly acetic- and propionic acid, increased to concentrations of 15-20 g/L, causing a marked drop in pH.

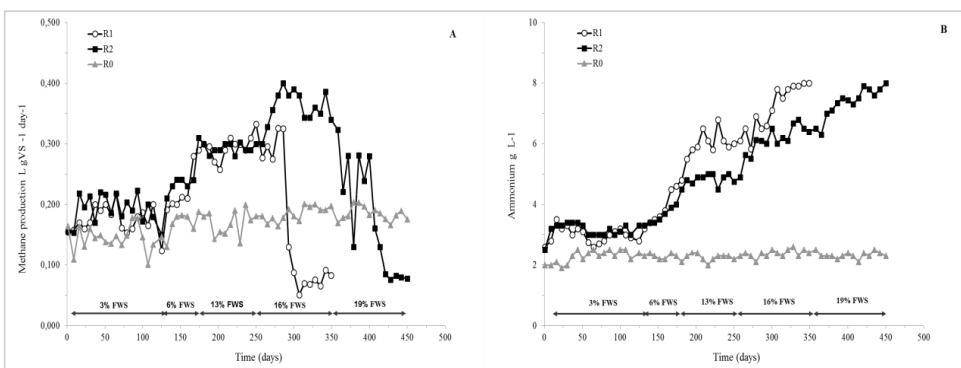

Figure 3.1. Process performance of two parallel experimental CSTR biogas reactors fed with increased ratios of fish waste silage, and one CSTR biogas reactor fed with manure only (control). **A:** Specific methane production, L CH_4 / gram VS / day. **B:** Ammonium concentrations, gram NH_4^+ / L.

3.2 A metagenomic study of the microbial communities in four parallel biogas reactors (Paper II):

The biogas process performance during the startup phase of four parallel continuously stirred tank reactors co-digesting FWS and manure was studied in Paper II. The reactors were operated in the mesophilic range, with a substrate blend (volume) of 13 % FWS and 87 % manure, based on the stable process achieved by operating reactors with this feedstock blend described in Paper I. The microbial communities found in the inoculum (day 0) and the reactors at day 59 were characterized and compared using 454 FLX Titanium pyrosequencing. In the inoculum and the reactor samples, bacteria belonging to the genera *Clostridium* and *Syntrophomonas* were highly abundant (**Figure 3.2**), while the dominating methanogen was the hydrogenotrophic *Methanoculleus*. Syntrophic prokaryotes frequently found in biogas reactors with high concentrations of NH_4^+ and VFA were detected in all samples. Compared to the initial inoculum, the experimental reactors showed somewhat different microbial composition. In particular, the *Candidatus Cloacamonas* belonging to the candidate phylum *Cloacimonetes* (WWE1) increased in all reactors and was the dominating bacterium at day 59. Interestingly, this bacterium showed a significantly higher abundance in one reactor. *Candidatus Cloacimonas acidaminovorans* is described to be a syntrophic bacterium, obtaining most of its energy from the fermentation of amino acids, and can ferment propionate to acetate, H_2, and CO_2 in syntrophy with H_2-consumers (Bengelsdorf et al., 2015). CH_4 production and the reactor effluent characteristics were monitored over the experimental period, showing stable operation and similar characteristics for all four reactors. The average CH_4 production in the reactors varied between 0.278 and 0.296 L/g VS. Thus, this study showed that four parallel reactors co-

digesting manure and FWS operated stably and similar during a startup phase. Several important Archaea and Bacteria degrading the protein-rich substrate were identified, and analysis of metabolic systems showed that highest percentage of reads in the samples were related to amino acid metabolism.

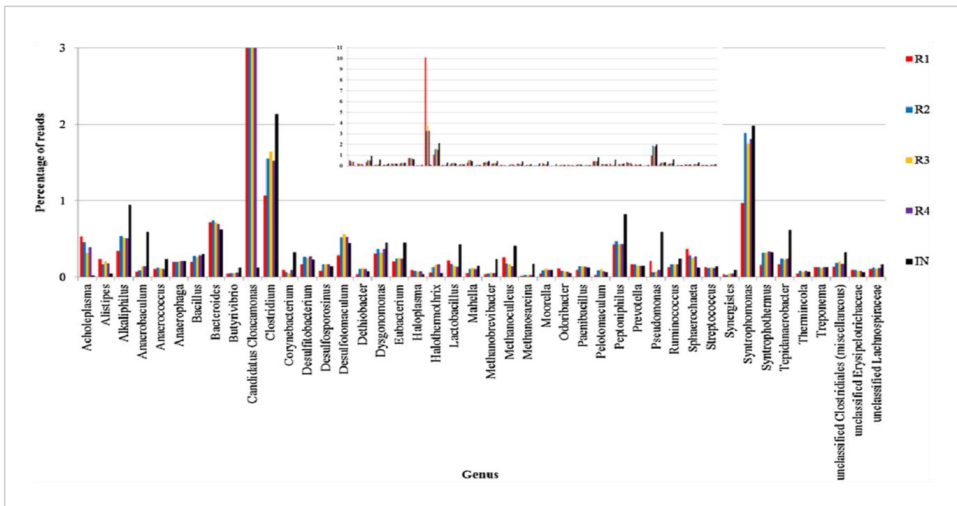

Figure 3.2. Microbial composition at genus level of four parallel biogas reactors after 59 days of stabilization, and in the initial inoculum sample. Percentage of reads assigned to the 44 genera with more than 0.1% reads assigned. Insert shows full scale of the Y-axes.

3.3 Process stability and dynamics of ammonium-tolerant microorganisms during co-digestion of fish waste and manure (Paper III):

Co-digestion of FWS and manure with a mixing ratio of 13 and 87 %, respectively (volume based), was carried out at different hydraulic retention times (HRT) and organic loading rates (OLR). The effects on CH_4 production, NH_4^+ accumulation and composition of NH_4^+-tolerant microorganisms (syntrophic acetate oxidizing bacteria, SAOB, and hydrogenotrophic methanogens) were investigated. The microbial composition in reactors operated at different HRTs and equal ratios of FWS in mix with manure, were compared to the microbial composition in reactors operated at equal HRTs with a feedstock of increasing ratios of FWS in mix with manure. In the latter mentioned reactors, the ratio of FWS were increased gradually from 3 to 19 % (volume based), and this experiment is described in detail in Paper I. The microbial composition and density of SAOB and methanogens (**Figure 3.3 A & B**), NH_4^+ concentrations and process performance were stable throughout the experiment in reactors operated with equal ratios of FWS and different HRTs. In comparison, in the reactors loaded with increasing ratios of FWS, it was observed a gradual increase of NH_4^+, and in the abundance of NH_4^+-tolerant microorganisms (**Figure 3.3 C & D**). The methanogenic groups of *Methanosarcinaceae* and *Methanosaetaceae* dominated at low levels of NH_4^+ and low loadings of FWS, while the hydrogenotrophic methanogenic group of Methanomicrobiales increased in abundance in response to higher FWS loading rates and NH_4^+ concentrations. In the reactors operated with increased ratios of FWS, the CH_4 yield increased until a load of around 16 % volume FWS was added. A further increase of FWS in the feedstock resulted in elevated NH_4^+ levels with maximum concentrations of 7-8 g/L, with a subsequent instability in microbial composition and biogas performance, and ultimately AD process failure. The

microbial composition changed when the reactor process got unstable, and the predominant change was the decrease in density of the SAOB *Tepidanaerobacter acetatoxydans*. High NH_4^+ concentrations as a result of an increased load of FWS caused an increase in the abundance of different SAOB and the hydrogenotrophic methanogenic order Methanomicrobiales. Overall, different HRTs had little effect on methane yield and microbial community composition, but elevated ratios of FWS inhibited activity of syntrophic microorganisms and caused instability and failure of the anaerobic process. The results showed that increasing the total volume of a fixed FWS/manure-blend is less inhibiting for the syntrophic microbial community and the biogas process performance than raising the proportion of FWS in the feedstock. This experiment showed that it is possible to utilize fat- and protein-rich FWS as biogas substrates at relatively low HRT and high OLR. By decreasing the HRT from 30 to 20 days and keeping the substrate proportions equal, the loading volume of FWS can be increased by approx. 50% without process failure, which can allow management of increased waste volumes.

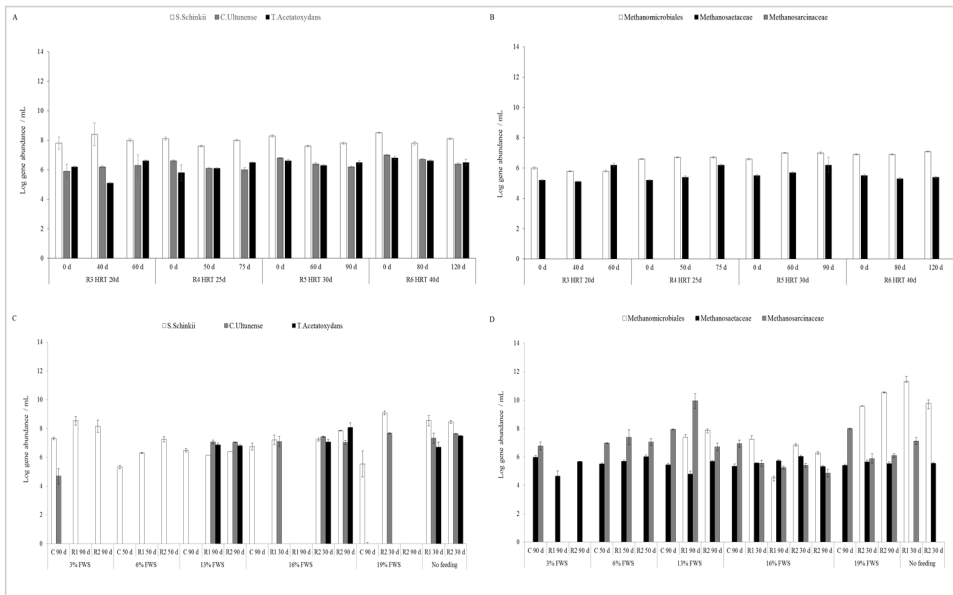

Figure 3.3. Concentrations (average log gene abundance / mL) of targeted NH_4^+ tolerant key functioning microorganisms in continuous biogas reactors operated with feedstock mixes of fish waste silage and manure. **A and B:** abundance of SAOB and methanogens, respectively, in four experimental reactors operated at different HRTs and fed with equal ratios of fish waste silage in mix with manure. **C and D:** abundance of SAOB and methanogens, respectively, in two experimental parallel reactors fed with increased ratios of fish waste silge in mix with manure, and one control reactor fed with manure only.

4. Concluding remarks and future perspectives

The Norwegian Government's goal of reducing GHG and nutrient emissions requires increased focus on our environmental challenges, such as management of the organic waste from the agri- and aquaculture sectors. AD is an applicable strategy for waste treatment, and increased energy yields are necessary for profitability. Aquaculture is one of the largest industries in Norway, and Norway is the world's foremost producer of Atlantic salmon. With the planned increase of aquaculture production in Norway, the need for waste treatment will increase. This PhD project has addressed some of the challenges regarding AD of proteinaceous organic waste. It has been shown that waste fractions produced in aquaculture can be treated with manure in anaerobic co-digestion processes and increase biogas yields, and that the microbial communities in such biogas reactors are able to adapt to this feedstock. However, only relatively low loadings of such proteinaceous wastes can be added to a biogas reactor before instability occurs due to accumulation of NH_4^+ and fatty acids.

The objective of establishing a stable mesophilic co-digestion process was achieved by gradually increasing the amount of fish waste in mix with manure, and a fish waste content between 13% and 16% volume was determined as the maximum in continuous biogas reactors operated at a HRT of 30 days and total OLR of approx. 3 (**Paper I**). Moreover, when adding a feedstock with a ratio of 13% fish waste, it was observed that by decreasing the HRT from 30 to 20 days, the feedstock loading could be further increased to a total OLR of approx. 4 without markedly inhibiting the AD process performance (**Paper III**). Compared to AD of manure alone, the methane yields could be increased by approx. 100% when fish waste was added to biogas reactors (**Paper I**). Nevertheless, high loads of fish waste to a biogas reactor will inhibit the methane production process stability due to accumulation of NH_4^+ and fatty acids (**Paper I**).

The studies of the microbial population dynamics in reactors operated at high protein loading and NH_4^+ concentrations showed that metabolic relations between syntrophic bacteria and hydrogenotrophic methanogens plays an important role under such conditions (**Paper II and III**). Investigation of the microbial structure in biogas reactors operated at high protein loading during a start-up phase showed that the total methanogenic community changed over time (**Paper II**), e.g there was detected a significant transition in dominance of the genus *Candidatus Cloacamonas* in continuously operated reactors compared to the initial inoculum. This study also revealed that even in parallel reactors initiated from the same inoculum and showing similar and stable process performance, the microbial communities can be different (**Paper II**). However, the study of key functioning microbes involved in syntrophic reactions during AD at high NH_4^+ concentrations showed that the process performance can be related to the development, establishment and maintenance of certain specialized bacteria and their methanogenic partners. In particular, the syntrophic relation between *Tepidanaerobacter Acetatoxydans* and the hydrogenotrophic Methanomicrobiales seemed important in these reactors. (**Paper III**).

Overall, this work has emphasized that to enable development of microbial communities adapted to stressful environments, such as biogas reactors operated at high NH_4^+ concentrations, process parameters must be carefully controlled. Additionally, the microbial community structure, especially the development of certain key functioning microorganisms, are important elements for successful AD operations. A main conclusion is that stable AD operation and relatively high methane yields can be achieved from co-digesting fish waste and manure due to the adaptation of NH_4^+-tolerant microbial consortia. Collectively, these findings

provides useful information and guidelines for operation of biogas plants that use aquaculture wastes and other protein-rich substrates as feedstocks.

However, further research focusing on AD of proteinaceous materials such as aquaculture waste is needed to improve waste management and exploit the energy potential from such fractions.

The aquacultural sector is producing large amounts of proteinaceous organic waste in the form of slaughtery waste and sludge, and management of such waste will require development and use of new methods, and implementation of established methods in new sectors. Successful treatment and management of such waste fractions by AD require an interdisciplinary approach where microbial, chemical and mechanical engineering disciplines are important.

One possible way of establishing key microbes in high NH_4^+ processes is direct addition of specific pre-cultured bacteria and methanogenic partners. This approach, called bioaugmentation, have been investigated and shown as a promising solution for AD process improvement, or even a method for process stability recovery. From an industrial point of view, the addition of NH_4^+ tolerant microbial populations could be a way to increase energy yields or prevent process failures. Known key functioning microorganisms in high NH_4^+ AD processes, SAOB and hydrogenotrophic methanogens, are slow growing. One possible approach to facilitate and retain these bacteria and methanogenic partners in such systems would be application of adapted biofilms in biogas reactors. Such biofilms have shown to enhance the performance in AD by providing an increased surface area for attached growth of microorganisms which can lead to increased population densities in the reactor. Most research on AD and biogas-production is performed using traditional lab scale semi-continuously fed

CSTRs, and there is a lack of knowledge about whether process stability can be improved by using other types of reactors and feeding regimes during AD of proteinaceous materials.

For AD of aquacultural wastes, not only biological aspects, but also economical and logistical challenges need to be addressed. In areas with close proximity of operations, joint biogas plants between aquaculture and agriculture could positively influence several problematic aspects such as waste management, nutrient recycling and nutrient- and GHG emissions. In addition, renewable energy in the form of methane would be produced. Many aquaculture farms are located in remote areas where larger-scale biogas plant solutions is not possible due to the limited amount of waste produced. However, many such farms are close to agricultural activity, meaning that livestock- and fish farmers could combine their waste and make the establishment of a biogas plant possible. This is an important perspective for the future, where industrial symbioses will have the possibility to improve the sustainability and economy of both aqua – and agriculture.

References

Ahring, B., Schmidt, J., Winther-Nielsen, M., Macario, A., de Macario, E.C., 1993. Effect of medium composition and sludge removal on the production, composition, and architecture of thermophilic (55 degrees C) acetate-utilizing granules from an upflow anaerobic sludge blanket reactor. Appl. Environ. Microbiol. 59, 2538–2545.

Ahring, B.K., 1995. Methanogenesis in thermophilic biogas reactors. Antonie Van Leeuwenhoek 67, 91–102.

Ahring, B.K., Ibrahim, A.A., Mladenovska, Z., 2001. Effect of temperature increase from 55 to 65 C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. Water Res. 35, 2446–2452.

Al Seadi, T., Rutz, D., Prassl, H., Kôttner, M., Finsterwalder, T., Folk, S., Rainer, J., 2008. Biogas handbook. University of Southern Denmark, Esbjerg.

Ali Shah, F., Mahmood, Q., Maroof Shah, M., Pervez, A., Ahmad Asad, S., 2014. Microbial ecology of anaerobic digesters: the key players of anaerobiosis. Sci. World J. 2014.

Alvarez, R., Lidén, G., 2008. Semi-continuous co-digestion of solid slaughterhouse waste, manure, and fruit and vegetable waste. Renew. Energy 33, 726–734.

Alvarez, R., Villca, S., Lidén, G., 2006. Biogas production from llama and cow manure at high altitude. Biomass Bioenergy 30, 66–75.

Amon, T., Amon, B., Kryvoruchko, V., Zollitsch, W., Mayer, K., Gruber, L., 2007. Biogas production from maize and dairy cattle manure—influence of biomass composition on the methane yield. Agric. Ecosyst. Environ. 118, 173–182.

Angelidaki, I., Ahring, B., 1994. Anaerobic thermophilic digestion of manure at different ammonia loads: effect of temperature. Water Res. 28, 727–731.

Angelidaki, I., Ahring, B., 1993. Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. Appl. Microbiol. Biotechnol. 38, 560–564.

Angelidaki, I., Ahring, B., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Appl. Microbiol. Biotechnol. 37, 808–812.

Angelidaki, I., Ellegaard, L., 2003. Codigestion of manure and organic wastes in centralized biogas plants. Appl. Biochem. Biotechnol. 109, 95.

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., Stams, A.J., 2011. Biomethanation and its potential. Methods Enzym. 494, 327–351.

Angenent, L.T., Sung, S., Raskin, L., 2002. Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Water Res. 36, 4648–4654.

Arruda, L.F. de, Borghesi, R., Oetterer, M., 2007. Use of fish waste as silage: a review. Braz. Arch. Biol. Technol. 50, 879–886.

Artanti, D., Saputro, R., Budiyono, B., 2012. Biogas production from cow manure. Int. J. Renew. Energy Dev. 1, 61.

Arvanitoyannis, I.S., Kassaveti, A., 2008. Fish industry waste: treatments, environmental impacts, current and potential uses. Int. J. Food Sci. Technol. 43, 726–745. doi:10.1111/j.1365-2621.2006.01513.x

Bagi, Z., Ács, N., Bálint, B., Horváth, L., Dobó, K., Perei, K.R., Rákhely, G., Kovács, K.L., 2007. Biotechnological intensification of biogas production. Appl. Microbiol. Biotechnol. 76, 473–482.

Balk, M., Weijma, J., Stams, A.J., 2002. Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int. J. Syst. Evol. Microbiol. 52, 1361–1368.

Banks, C.J., Zhang, Y., Jiang, Y., Heaven, S., 2012. Trace element requirements for stable food waste digestion at elevated ammonia concentrations. Bioresour. Technol. 104, 127–135.

Batstone, D.J., Hülsen, T., Mehta, C.M., Keller, J., 2015. Platforms for energy and nutrient recovery from domestic wastewater: A review. Chemosphere 140, 2–11. doi:http://dx.doi.org/10.1016/j.chemosphere.2014.10.021

Bengelsdorf, F.R., Gabris, C., Michel, L., Zak, M., Kazda, M., 2015. Syntrophic microbial communities on straw as biofilm carrier increase the methane yield of a biowaste-digesting biogas reactor. AIMS Bioeng. 2, 264–276.

Berglund, M., Bôrjesson, P., 2003. "Energianalys av biogassystem" , 2003. (No. 44). Dept. teknik och samhälle, Lund Univeristy. Sweden.

Beschkov, V., 2017. Biogas, Biodiesel and Bioethanol as Multifunctional Renewable Fuels and Raw Materials, in: Frontiers in Bioenergy and Biofuels. InTech.

Blume, F., Bergmann, I., Nettmann, E., Schelle, H., Rehde, G., Mundt, K., Klocke, M., 2010. Methanogenic population dynamics during semi-continuous biogas fermentation and acidification by overloading. J. Appl. Microbiol. 109, 441–450.

Bond, T., Templeton, M.R., 2011. History and future of domestic biogas plants in the developing world. Energy Sustain. Dev. 15, 347–354.

Bouallagui, H., Haouari, O., Touhami, Y., Cheikh, R.B., Marouani, L., Hamdi, M., 2004. Effect of temperature on the performance of an anaerobic tubular reactor treating fruit and vegetable waste. Process Biochem. 39, 2143–2148.

Braun, R., Huber, P., Meyrath, J., 1981. Ammonia toxicity in liquid piggery manure digestion. Biotechnol. Lett. 3, 159–164.

Braun, R., Wellinger, A., 2003. Potential of co-digestion. Presented at the IEA Bioenergy, Task.

Callaghan, F., Wase, D., Thayanithy, K., Forster, C., 1999. Co-digestion of waste organic solids: batch studies. Bioresour. Technol. 67, 117–122.

Callaghan, F., Wase, D., Thayanithy, K., Forster, C., 1998. An examination of the continuous anaerobic co-digestion of cattle slurry and fish offal. Process Saf. Environ. Prot. 76, 224–228.

Calli, B., Mertoglu, B., Inanc, B., Yenigun, O., 2005. Effects of high free ammonia concentrations on the performances of anaerobic bioreactors. Process Biochem. 40, 1285–1292.

Carucci, G., Carrasco, F., Trifoni, K., Majone, M., Beccari, M., 2005. Anaerobic digestion of food industry wastes: effect of codigestion on methane yield. J. Environ. Eng. 131, 1037–1045.

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99, 4044–4064.

Cordell, D., Drangert, J.-O., White, S., 2009. The story of phosphorus: Global food security and food for thought. Glob. Environ. Change 19, 292–305. doi:http://dx.doi.org/10.1016/j.gloenvcha.2008.10.009

Cuetos, M., Gómez, X., Otero, M., Morán, A., 2010. Anaerobic digestion and co-digestion of slaughterhouse waste (SHW): influence of heat and pressure pre-treatment in biogas yield. Waste Manag. 30, 1780–1789.

Davey, M.E., O'toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64, 847–867.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev. Environ. Sci. Biotechnol. 7, 173–190.

Divya, D., Gopinath, L., Christy, P.M., 2015. A review on current aspects and diverse prospects for enhancing biogas production in sustainable means. Renew. Sustain. Energy Rev. 42, 690–699.

Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., 2006. The Prokaryotes: Vol. 3: Archaea. Bacteria: Firmicutes, Actinomycetes. Springer Science & Business Media.

ENOVA, 2014. ENOVA.

European Biogas Association, 2016. EBA Annual Report 2015, EBA Annual Report for 2015 Biomethane & Biogas Report. European Biogas Association.

Faostat, 2014. Faostat – Food and Agriculture Organization of the United Nations, FAO Statistical Databases, numbers for 2014. Available from: http://www.fao.org/faostat/en/#data/QA.

Feng, X.M., Karlsson, A., Svensson, B.H., Bertilsson, S., 2010. Impact of trace element addition on biogas production from food industrial waste–linking process to microbial communities. FEMS Microbiol. Ecol. 74, 226–240.
Fernandes, T.V., Keesman, K.J., Zeeman, G., van Lier, J.B., 2012. Effect of ammonia on the anaerobic hydrolysis of cellulose and tributyrin. Biomass Bioenergy 47, 316–323.
Frank, J., Arntzen, M., Sun, L., Hagen, L., McHardy, A., Horn, S., Eijsink, V.G., Schnürer, A., Pope, P.B., 2016. Novel Syntrophic Populations Dominate an Ammonia-Tolerant Methanogenic Microbiome. mSystems 1, e00092-16.
Froment, G., Bischoff, K.B., De Wilde, J., 2009. Chemical reactor analysis and design. John Wiley & Sons, Inc.
Gallert, C., Bauer, S., Winter, J., 1998. Effect of ammonia on the anaerobic degradation of protein by a mesophilic and thermophilic biowaste population. Appl. Microbiol. Biotechnol. 50, 495–501.
Gebauer, R., 2004. Mesophilic anaerobic treatment of sludge from saline fish farm effluents with biogas production. Bioresour. Technol. 93, 155–167.
Gebauer, R., Eikebrokk, B., 2006. Mesophilic anaerobic treatment of sludge from salmon smolt hatching. Bioresour. Technol. 97, 2389–2401.
Gerardi, M.H., 2003. The microbiology of anaerobic digesters. John Wiley & Sons.
Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L., 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol. Bioeng. 57, 342–355.
Grøntvedt, H., Haugen, R., Langvik, M., Nestaas, E., Reppe, S., Wollan, H., 2010. Bioenergi fra biprodukt av laks (No. 194). RUBIN.
Gunnarsdóttir, R., Heiske, S., Jensen, P.E., Schmidt, J.E., Villumsen, A., Jenssen, P.D., 2014. Effect of anaerobiosis on indigenous microorganisms in blackwater with fish offal as co-substrate. Water Res. 63, 1–9.
Hanaki, K., Matsuo, T., Nagase, M., 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnol. Bioeng. 23, 1591–1610.
Hansen, K.H., Angelidaki, I., Ahring, B.K., 1999. Improving thermophilic anaerobic digestion of swine manure. Water Res. 33, 1805–1810.
Hansen, K.H., Angelidaki, I., Ahring, B.K., 1998. Anaerobic digestion of swine manure: inhibition by ammonia. Water Res. 32, 5–12.
Hao, L.-P., Lü, F., He, P.-J., Li, L., Shao, L.-M., 2010. Predominant contribution of syntrophic acetate oxidation to thermophilic methane formation at high acetate concentrations. Environ. Sci. Technol. 45, 508–513.
Hatti-Kaul, R., Mamo, G., Mattisson, B., 2016. Anaerobes in biotechnology.
Hattori, S., Kamagata, Y., Hanada, S., Shoun, H., 2000. Thermacetogenium phaeum gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. Int. J. Syst. Evol. Microbiol. 50, 1601–1609.
Hattori, S., Luo, H., Shoun, H., Kamagata, Y., 2001. Involvement of formate as an interspecies electron carrier in a syntrophic acetate-oxidizing anaerobic microorganism in coculture with methanogens. J. Biosci. Bioeng. 91, 294–298.
Hejnfelt, A., Angelidaki, I., 2009. Anaerobic digestion of slaughterhouse by-products. Biomass Bioenergy 33, 1046–1054.
Holm-Nielsen, J.B., Al Seadi, T., Oleskowicz-Popiel, P., 2009. The future of anaerobic digestion and biogas utilization. Bioresour. Technol. 100, 5478–5484.
Hwang, M.H., Jang, N.J., Hyun, S.H., Kim, I.S., 2004. Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. J. Biotechnol. 111, 297–309.
Jensen, P.D., Sullivan, T., Carney, C., Batstone, D.J., 2014. Analysis of the potential to recover energy and nutrient resources from cattle slaughterhouses in Australia by employing anaerobic digestion. Appl. Energy 136, 23–31. doi:http://dx.doi.org/10.1016/j.apenergy.2014.09.009

Kafle, G.K., Kim, S.H., Sung, K.I., 2013. Ensiling of fish industry waste for biogas production: a lab scale evaluation of biochemical methane potential (BMP) and kinetics. Bioresour. Technol. 127, 326–336.
Kampmann, K., Ratering, S., Baumann, R., Schmidt, M., Zerr, W., Schnell, S., 2012. Hydrogenotrophic methanogens dominate in biogas reactors fed with defined substrates. Syst. Appl. Microbiol. 35, 404–413. doi:http://dx.doi.org/10.1016/j.syapm.2012.07.002
Karakashev, D., Batstone, D.J., Angelidaki, I., 2005. Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl. Environ. Microbiol. 71, 331–338.
Karlsson, A., Einarsson, P., Schnürer, A., Sundberg, C., Ejlertsson, J., Svensson, B.H., 2012. Impact of trace element addition on degradation efficiency of volatile fatty acids, oleic acid and phenyl acetate and on microbial populations in a biogas digester. J. Biosci. Bioeng. 114, 446–452.
Kelleher, B., Leahy, J., Henihan, A., O'dwyer, T., Sutton, D., Leahy, M., 2002. Advances in poultry litter disposal technology–a review. Bioresour. Technol. 83, 27–36.
Kim, J.K., Oh, B.R., Chun, Y.N., Kim, S.W., 2006. Effects of temperature and hydraulic retention time on anaerobic digestion of food waste. J. Biosci. Bioeng. 102, 328–332.
Klima- og Miljødepartementet, 2014. Nasjonal tverrsektoriell biogasstrategi (No. ISBN: 978-82-457-0485-3 (PDF)). Klima- og miljødepartementet, Oslo.
Klocke, M., Mähnert, P., Mundt, K., Souidi, K., Linke, B., 2007. Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate. Syst. Appl. Microbiol. 30, 139–151.
Klocke, M., Nettmann, E., Bergmann, I., Mundt, K., Souidi, K., Mumme, J., Linke, B., 2008. Characterization of the methanogenic Archaea within two-phase biogas reactor systems operated with plant biomass. Syst. Appl. Microbiol. 31, 190–205. doi:http://dx.doi.org/10.1016/j.syapm.2008.02.003
Kovács, E., Wirth, R., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2013. Biogas production from protein-rich biomass: fed-batch anaerobic fermentation of casein and of pig blood and associated changes in microbial community composition. PLoS One 8, e77265.
Krause, L., Diaz, N.N., Edwards, R.A., Gartemann, K.-H., Krömeke, H., Neuweger, H., Pühler, A., Runte, K.J., Schlüter, A., Stoye, J., 2008. Taxonomic composition and gene content of a methane-producing microbial community isolated from a biogas reactor. J. Biotechnol. 136, 91–101.
Kröber, M., Bekel, T., Diaz, N.N., Goesmann, A., Jaenicke, S., Krause, L., Miller, D., Runte, K.J., Viehöver, P., Pühler, A., 2009. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J. Biotechnol. 142, 38–49.
Lanari, D., Franci, C., 1998. Biogas production from solid wastes removed from fish farm effluents. Aquat. Living Resour. 11, 289–295.
Landbruks- og matdepartementet, 2009. St.meld.nr.39 Klimautfordringene - landbruket en del av løsningen.
Lay, J., Li, Y., Noike, T., Endo, J., Ishimoto, S., 1997. Analysis of environmental factors affecting methane production from high-solids organic waste. Water Sci. Technol. 36, 493–500.
Lee, S.-H., Park, J.-H., Kim, S.-H., Yu, B.J., Yoon, J.-J., Park, H.-D., 2015. Evidence of syntrophic acetate oxidation by Spirochaetes during anaerobic methane production. Bioresour. Technol. 190, 543–549.
Lehtomäki, A., Huttunen, S., Rintala, J., 2007. Laboratory investigations on co-digestion of energy crops and crop residues with cow manure for methane production: effect of crop to manure ratio. Resour. Conserv. Recycl. 51, 591–609.
Li, J., Ban, Q., Zhang, L., Jha, A.K., 2012. Syntrophic Propionate Degradation in Anaerobic Digestion: A Review. Int. J. Agric. Biol. 14.

Liaset, B., Espe, M., 2008. Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials. Process Biochem. 43, 42–48.
Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189.
Lü, F., Hao, L., Guan, D., Qi, Y., Shao, L., He, P., 2013. Synergetic stress of acids and ammonium on the shift in the methanogenic pathways during thermophilic anaerobic digestion of organics. Water Res. 47, 2297–2306.
Marchaim, U., 1992. Biogas processes for sustainable development. Food & Agriculture Org.
Marchaim, U., Krause, C., 1993. Propionic to acetic acid ratios in overloaded anaerobic digestion. Bioresour. Technol. 43, 195–203.
Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., Suzuki, A., 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann. Bot. mcq028.
McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J., Schink, B., Rohlin, L., Gunsalus, R.P., 2008. Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. Ann. N. Y. Acad. Sci. 1125, 58–72.
McMahon, K.D., Stroot, P.G., Mackie, R.I., Raskin, L., 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—II: microbial population dynamics. Water Res. 35, 1817–1827.
Miljødirektoratet, 2016. Utslipp av næringssalter fra fiskeoppdrett.
Moestedt, J., Nordell, E., Schnürer, A., 2014. Comparison of operating strategies for increased biogas production from thin stillage. J. Biotechnol. 175, 22–30.
Møller, H.B., Sommer, S.G., Ahring, B.K., 2004. Methane productivity of manure, straw and solid fractions of manure. Biomass Bioenergy 26, 485–495.
Möller, K., Müller, T., 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Eng. Life Sci. 12, 242–257. doi:10.1002/elsc.201100085
Moosbrugger, R., Wentzel, M., Ekama, G., Marais, G. v R., 1993. A 5 pH point titration method for determining the carbonate and SCFA weak acid/bases in anaerobic systems. Water Sci. Technol. 28, 237–245.
Møretrø, T., Hjeltnes, B., Lunestad, B.T., Nesse, L.L., 2010. Assessment of the Fish Silage Processing Method (FSPM) for treatment of category 2 and 3 material of fish origin (ISBN: 978-82-8082-368-7 No. 08/608). Norwegian Scientific Committee for Food Safety.
Mshandete, A., Kivaisi, A., Rubindamayugi, M., Mattiasson, B., 2004. Anaerobic batch co-digestion of sisal pulp and fish wastes. Bioresour. Technol. 95, 19–24.
Müller, B., Sun, L., Schnürer, A., 2013. First insights into the syntrophic acetate-oxidizing bacteria–a genetic study. Microbiologyopen 2, 35–53.
Müller, N., Worm, P., Schink, B., Stams, A.J., Plugge, C.M., 2010. Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. Environ. Microbiol. Rep. 2, 489–499.
Neves, L., Oliveira, R., Alves, M., 2009. Co-digestion of cow manure, food waste and intermittent input of fat. Bioresour. Technol. 100, 1957–1962.
Nielsen, H.B., 2006. CONTROL PARAMETERS FOR UNDERSTANDING AND PREVENTING PROCESS IMBALANCES IN BIOGAS PLANTS. EMPHAS IS ON VFA DYNAMICS.
Parawira, W., Murto, M., Zvauya, R., Mattiasson, B., 2004. Anaerobic batch digestion of solid potato waste alone and in combination with sugar beet leaves. Renew. Energy 29, 1811–1823.
Pettersen, I., Grønlund, A., Stensgård E, A., Walland, F., 2017. Klimatiltak i jordbruk og matsektoren. Kostnadsanalyse av fem tiltak (No. 3/2/2017), 978-82-17-01771-4. NIBIO.
Raadal, H.L., Schakenda, V., Morken, J., 2008. Potensialstudie for biogass i Norge (No. OR 21.08). Østfoldforskning.
Rajagopal, R., Massé, D.I., Singh, G., 2013. A critical review on inhibition of anaerobic digestion process by excess ammonia. Bioresour. Technol. 143, 632–641.

Rosten, T.W., Henriksen, K., Skontorp Hognes, E., 2013. Land Based RAS and Open Pen Salmon Aquaculture: Comparative Economic and Environmental Assessment.
RUBIN, 2012. Varestrømsanalyse for 2011.
Schattauer, A., Abdoun, E., Weiland, P., Plöchl, M., Heiermann, M., 2011. Abundance of trace elements in demonstration biogas plants. Biosyst. Eng. 108, 57–65.
Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiol. Mol. Biol. Rev. 61, 262–280.
Schink, B., Stams, A.J., 2013. Syntrophism among prokaryotes. Springer.
Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.-H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., 2008. The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. J. Biotechnol. 136, 77–90.
Schmidt, A., Müller, N., Schink, B., Schleheck, D., 2013. A proteomic view at the biochemistry of syntrophic butyrate oxidation in Syntrophomonas wolfei. PloS One 8, e56905.
Schnürer, A., Houwen, F.P., Svensson, B.H., 1994. Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. Arch. Microbiol. 162, 70–74.
Schnurer, A., Jarvis, A., 2010. Microbiological handbook for biogas plants. Swed. Waste Manag. U 2009, 1–74.
Schnürer, A., Nordberg, Å., 2008. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Sci. Technol. 57, 735–740.
Schnürer, A., Schink, B., Svensson, B.H., 1996. Clostridium ultunense sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. Int. J. Syst. Evol. Microbiol. 46, 1145–1152.
Schnürer, A., Svensson, B.H., Schink, B., 1997. Enzyme activities in and energetics of acetate metabolism by the mesophilic syntrophically acetate-oxidizing anaerobe Clostridium ultunense. FEMS Microbiol. Lett. 154, 331–336.
Schnürer, A., Zellner, G., Svensson, B.H., 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol. Ecol. 29, 249–261.
Shigematsu, T., Tang, Y., Kobayashi, T., Kawaguchi, H., Morimura, S., Kida, K., 2004. Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation. Appl. Environ. Microbiol. 70, 4048–4052.
Sletten, T.M., Maas, C., 2013. Underlagsmateriale til tverrsektoriell biogass-strategi (No. 3020/2013). Klima- og forurensningsdirektoratet.
Smil, V., 2000. Phosphorus in the environment: natural flows and human interferences. Annu. Rev. Energy Environ. 25, 53–88.
Speece, R.E., 1996. Anaerobic biotechnology for industrial wastewaters. Archae Press, Nashville, Tenn.
Sprott, G.D., Patel, G.B., 1986. Ammonia toxicity in pure cultures of methanogenic bacteria. Syst. Appl. Microbiol. 7, 358–363.
Sreekrishnan, T., Kohli, S., Rana, V., 2004. Enhancement of biogas production from solid substrates using different techniques—a review. Bioresour. Technol. 95, 1–10.
Stams, A.J., 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek 66, 271–294.
Statistics Norway, 2016. Akvakultur, 2015. Endelige tall. Statistics Norway, SSB. https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar/2016-10-28?fane=tabell#content.
Stroot, P.G., McMahon, K.D., Mackie, R.I., Raskin, L., 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—I. Digester performance. Water Res. 35, 1804–1816.
Sun, L., Müller, B., Westerholm, M., Schnürer, A., 2014. Syntrophic acetate oxidation in industrial CSTR biogas digesters. J. Biotechnol. 171, 39–44.

Sung, S., Liu, T., 2003. Ammonia inhibition on thermophilic anaerobic digestion. Chemosphere 53, 43–52.
Surendra, K., Takara, D., Hashimoto, A.G., Khanal, S.K., 2014. Biogas as a sustainable energy source for developing countries: Opportunities and challenges. Renew. Sustain. Energy Rev. 31, 846–859.
Thauer, R.K., 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson: 1998 Marjory Stephenson Prize Lecture. Microbiology 144, 2377–2406.
Van Velsen, A., 1979. Adaptation of methanogenic sludge to high ammonia-nitrogen concentrations. Water Res. 13, 995–999.
Weiland, P., 2010. Biogas production: current state and perspectives. Appl. Microbiol. Biotechnol. 85, 849–860.
Weiland, P., 2003. Production and energetic use of biogas from energy crops and wastes in Germany. Appl. Biochem. Biotechnol. 109, 263–274.
Werner, J.J., Garcia, M.L., Perkins, S.D., Yarasheski, K.E., Smith, S.R., Muegge, B.D., Stadermann, F.J., DeRito, C.M., Floss, C., Madsen, E.L., 2014. Microbial community dynamics and stability during an ammonia-induced shift to syntrophic acetate oxidation. Appl. Environ. Microbiol. 80, 3375–3383.
Westerholm, M., 2012. Biogas production through the syntrophic acetate-oxidising pathway.
Westerholm, M., Dolfing, J., Sherry, A., Gray, N.D., Head, I.M., Schnürer, A., 2011a. Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes. Environ. Microbiol. Rep. 3, 500–505.
Westerholm, M., Levén, L., Schnürer, A., 2012. Bioaugmentation of syntrophic acetate-oxidizing culture in biogas reactors exposed to increasing levels of ammonia. Appl. Environ. Microbiol. 78, 7619–7625.
Westerholm, M., Moestedt, J., Schnürer, A., 2016. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl. Energy 179, 124–135.
Westerholm, M., Muller, B., Schnurer, A., 2011b. Changes in the acetogenic population in a mesophilic anaerobic digester in response to increasing ammonia concentration. Microbes Environ 26, 347–353.
Westerholm, M., Roos, S., Schnürer, A., 2011c. Tepidanaerobacter acetatoxydans sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from two ammonium-enriched mesophilic methanogenic processes. Syst. Appl. Microbiol. 34, 260–266.
Westerholm, M., Roos, S., Schnürer, A., 2010. Syntrophaceticus schinkii gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. FEMS Microbiol. Lett. 309, 100–104.
Whalen, J.K., Chang, C., Clayton, G.W., Carefoot, J.P., 2000. Cattle manure amendments can increase the pH of acid soils. Soil Sci. Soc. Am. J. 64, 962–966.
Whitman, W.B., Bowen, T.L., Boone, D.R., 2006. The methanogenic bacteria, in: The Prokaryotes. Springer, pp. 165–207.
Wintsche, B., Glaser, K., Sträuber, H., Centler, F., Liebetrau, J., Harms, H., Kleinsteuber, S., 2016. Trace elements induce predominance among methanogenic activity in anaerobic digestion. Front. Microbiol. 7.
Wirth, R., Kovács, E., Maróti, G., Bagi, Z., Rákhely, G., Kovács, K.L., 2012. Characterization of a biogas-producing microbial community by short-read next generation DNA sequencing. Biotechnol. Biofuels 5, 41.
Wu, X., Yao, W., Zhu, J., Miller, C., 2010. Biogas and CH 4 productivity by co-digesting swine manure with three crop residues as an external carbon source. Bioresour. Technol. 101, 4042–4047.
Yenigün, O., Demirel, B., 2013. Ammonia inhibition in anaerobic digestion: a review. Process Biochem. 48, 901–911.

Zabranska, J., Dohanyos, M., Jeníček, P., Zaplatilkova, P., Kutil, J., 2002. The contribution of thermophilic anaerobic digestion to the stable operation of wastewater sludge treatment. Water Sci. Technol. 46, 447–453.
Zheng, D., Raskin, L., 2000. Quantification of Methanosaeta species in anaerobic bioreactors using genus-and species-specific hybridization probes. Microb. Ecol. 39, 246–262.
Zhu, C., Zhang, J., Tang, Y., Zhengkai, X., Song, R., 2011. Diversity of methanogenic archaea in a biogas reactor fed with swine feces as the mono-substrate by mcrA analysis. Microbiol. Res. 166, 27–35. doi:http://dx.doi.org/10.1016/j.micres.2010.01.004
Ziganshin, A.M., Ziganshina, E.E., Kleinsteuber, S., Nikolausz, M., 2016. Comparative Analysis of Methanogenic Communities in Different Laboratory-Scale Anaerobic Digesters 2016.
Zinder, S.H., 1993. Physiological ecology of methanogens, in: Methanogenesis. Springer, pp. 128–206.
Zinder, S.H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch. Microbiol. 138, 263–272.

PAPER I

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Effects of a gradually increased load of fish waste silage in co-digestion with cow manure on methane production

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ABSTRACT

This study examined the effects of an increased load of nitrogen-rich organic material on anaerobic digestion and methane production. Co-digestion of fish waste silage (FWS) and cow manure (CM) was studied in two parallel laboratory-scale (8 L effective volume) semi-continuous stirred tank reactors (designated R1 and R2). A reactor fed with CM only (R0) was used as control. The reactors were operated in the mesophilic range (37 °C) with a hydraulic retention time of 30 days, and the entire experiment lasted for 450 days. The rate of organic loading was raised by increasing the content of FWS in the feed stock. During the experiment, the amount (volume%) of FWS was increased stepwise in the following order: 3% – 6% – 13% – 16%, and 19%. Measurements of methane production, and analysis of volatile fatty acids, ammonium and pH in the effluents were carried out. The highest methane production from co-digestion of FWS and CM was 0.400 L CH4 gVS^{-1}, obtained during the period with loading of 16% FWS in R2. Compared to anaerobic digestion of CM only, the methane production was increased by 100% at most, when FWS was added to the feed stock. The biogas processes failed in R1 and R2 during the periods, with loadings of 16% and 19% FWS, respectively. In both reactors, the biogas processes failed due to overloading and accumulation of ammonia and volatile fatty acids.

1. Introduction

Salmon farms in Norway are experiencing reduced production due to the large number of fish which die at these facilities. In 2012 a loss of 27.412 million salmon was reported. Until recently such losses were disposed of as waste, despite it representing a lot of organic material (Statistics Norway, 2013). This waste contains large amounts of fat and protein, and can therefore be used as an energy-rich substrate for biogas production. In turn, the digestate from the anaerobic biogas process contains high levels of nitrogen, making it useful as a fertilizer. In Rogaland in western Norway, fish waste is to some extent utilized in a biogas pilot plant (320 m^3) located at Åna Kretsfengsel (a district prison). The fish waste is ensiled (acidified) to avoid microbial growth, and this pretreatment lowers the pH to approximately 3 (Alwan et al., 1993). The combination of acidity and high levels of fat and protein make the FWS difficult to digest as a sole substrate (Nges et al., 2012). Methane production takes place at pH levels from 6.5 to 8.5, and the optimal levels for methane production is between 7 and 8. (Weiland, 2010). The steps in anaerobic degradation of organic material roughly consist of hydrolysis, fermentation, and methanogenesis, which involve several groups of microorganisms (Gujer and Zehnder, 1983), and, accordingly, the performance of an anaerobic digestion process depends largely on the activity of these microorganisms. In general, the microorganisms involved in anaerobic digestion differ widely with respect to their physiology, nutritional needs, growth kinetics, and sensitivity to environmental conditions (Chen et al., 2008), and failure to maintain the balance between different groups of microorganisms is the primary cause of reactor instability (Demirel and Yenigun, 2002). In addition, parameters such as temperature and stirring, hydraulic retention time, and organic loading rate is also of importance for the performance of the process (Appels et al., 2008). Process breakdown induced by accumulation of toxic compounds such as NH_3 and fatty acids, are often the result of overloading with energy-rich substrates (Ortega et al., 2008).

It has been demonstrated that the optimum C/N is between 20 and 30 (Parkin and Owen, 1986), and if the C/N is too low, the process may be inhibited by accumulation of NH_3 produced from protein degradation (Angelidaki and Ahring, 1993; Angelidaki

Abbreviations: FWS, fish waste silage; CM, cow manure; sCSTR, semi-continuously stirred tank reactor; OLR, organic loading rate ($g\ L^{-1}\ d^{-1}$); HRT, hydraulic retention time (d); VFA, volatile fatty acids ($g\ L^{-1}$).

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et al., 2004; Yen and Burne, 2007). Another important parameter is the content of lipids. During anaerobic degradation, lipids are hydrolyzed to long chained fatty acids (LCFA) (Angelidaki and Ahring, 1992) and VFAs (Biebl, 2001). Both LCFAs and VFAs are detected as accumulating intermediates in unstable biogas reactors, and can give rise to unstable processes and biogas production failure (Karlsson et al., 2012).

One way to overcome the problems with anaerobic digestion of protein and lipid rich waste materials (energy- rich materials) is to use a mixture of substrates with different properties. Co-digestion may improve the anaerobic digestion process by creating a better nutrient balance, diluting toxic compounds, and stimulating synergistic effects of microorganisms (Chen et al., 2010; El-Mashad and Zhang, 2010; Lehtomaki et al., 2007), and possibly also increase the stability of the system and the methane production.

In addition, to enable the adjustment of the process parameters, inocula adapted to high concentrations of certain compounds can enhance production of biogas from energy-rich substrates (Goncalves et al., 2009; Toreci et al., 2011). Continuous anaerobic co-digestion of different substrates has been studied extensively (Ashekuzzman and Poulsen, 2011; Lehtomaki et al., 2007; Møller et al., 2004), and a few investigations dealing with co-digestion of fish waste or ensiled fish waste and manure have been carried out, mostly through batch experiments. The results of these investigations show that fish waste in general contains high concentrations of fat and protein, and that there is a large risk for accumulation of fatty acids and NH_3 when these types of substrates are anaerobically digested (Gebauer, 2004; Gebauer and Eikebrokk, 2006; Kafle et al., 2013; Nges et al., 2012). Consequently, the exploitation of the promising waste management and biogas potentials from FWS is limited. The present study has three main objectives: (i) to determine the methane production from co-digestion of FWS and CM; (ii) to ascertain optimal mixing ratios of FWS and CM as a reactor feedstock by evaluating methane productions and effluent composition; (iii) to identify a threshold level for the amount of FWS that can be added to an anaerobic reactor.

2. Materials and methods

In general, the present experiment was designed to study the methane production from co-digestion of CM and FWS in laboratory-scale (8-L) s-CSTR reactors. The amount of FWS in the feed stocks was gradually increased over the 450 days of operation.

2.1. Description and chemical analysis of raw materials

The starter culture for the experimental reactors (R1 and R2), and raw materials for feedstocks, were collected in June 2009 from a biogas pilot plant (320 m^2) located in Åna, Rogaland, Norway. The pilot plant is operated with co-digestion of FWS and CM under mesophilic conditions (37 °C). The raw materials used in the pilot plant is manure from dairy cows and fish waste (category 2), the latter consisting mainly of dead salmon from fish farms located on the western and northwestern coast of Norway. The fish waste (not defatted) is pretreated by ensiling with formic acid under pressure (3 bars) and high temperature (133 °C) for 20 min.

Before the start of the experiment, the raw materials were analyzed for content of DM, VS, fat, protein, NH_4^+, and pH levels (Table 1). Content of DM, VS, and pH levels were determined according to methods specified by the International and European Organization for Standardization (ISO 11465: 1993; NS-EN 15935: 2012; ISO 10390: 2005). NH_4^+ concentrations were analyzed by using an NH_4^+ selective electrode (Thermo Scientific Orion ISE/NH4) in diluted (1:10) samples held at 20 °C, and supplemented with an ionic strength adjustor (ISA; 10 mL of ISA per 100 mL of sample); ISA stabilizes NH_4^+. The analyses were performed on triplicate samples. The amount of fat and protein in the raw materials was measured by Eurofins AS Norway, on single samples. Determination of raw fat was carried out by using the SBR method (NMKL, 1989), and the crude protein was determined by using the Kjeldahl N method. The methods for determination of fat and protein have measurement uncertainties of 9–30% and 10–20%, respectively. The characteristics of the raw materials are shown in Table 1.

2.2. Anaerobic reactor setup and feeding strategy

The reactor tanks used in the experiment are previously described in detail by Bergersen et al. (2012). Three reactors were used, two parallel added FWS in co-digestion with CM (R1 and R2), and one additional control reactor added CM only (R0). Each reactor (total volume 10 L, effective volume 8 L, headspace 2 L) was stirred continuously throughout the experiment (30–40 rpm), operated at 37 °C, and fed manually with 267 mL of substrate daily, with an HRT of 30 days. The amount (volume%) of FWS in the feedstock mixture was increased five times during the experiment, in the following manner: 3% FWS for 125 days, 6% FWS for 48 days, 13% FWS for 93 days, 16% FWS for 94 days, and 19% FWS for 90 days. The volume percentages of FWS in the feed stocks corresponds to total gVS L^{-1} day^{-1}(OLR) of respectively 2, 2.3, 3, 3.5 and 4.3. Semi-continuous feeding of the reactors was initiated four days after adding 8 L starter culture, and the reactors were loaded with 3% FWS from startup. Each feedstock mixture was, ahead of every new period with an increase of FWS, prepared in large (50 L) batches and divided into smaller portions (0.5 L plastic containers). The 0.5 L containers were stored at 4 °C to avoid hydrolysis of the solids. The feed stock materials were analyzed for content of DM, VS and pH levels twice during each period with different amounts of FWS, and no particular degradation was detected.

2.3. Chemical analysis of feedstocks and effluents

The feedstock mixtures were analyzed for content of DM (data not shown), VS (data not shown), carbon and nitrogen, and pH levels at the start of each new period with a change of feedstock composition. Determination of total nitrogen and carbon was performed by Eurofins AS Norway, based on the total Kjeldahl nitrogen content (spectrophotometric analysis) and the content of combustible material (gravimetric analysis).

Reactor effluent samples were analyzed for content of DM (data not shown), VS (data not shown), NH_4^+, and pH levels every week during the experiment (The analysis methods are described in section 2.2). The NH_3 concentrations in the effluents were calculated from NH_4^+ concentrations, pH and temperatures. The VS removal was calculated with respect to DM and VS content in the reactor feed stocks and effluents.

Effluent samples for VFA measurements were collected twice during the last week of each period with different feedstock composition. In the period with loading of 16% FWS in R1, and that with 19% FWS in R2, the VFA levels were measured on day 30 of the first HRT.

Concentrations of VFAs (acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, iso-caproic acid, n-caproic acid, and heptanoic acid) were analyzed at Telemark University College (HIT), by gas chromatography (Hewlett Packard 6890) with a flame ionization detector and a capillary column (FFAP 30 m, inner diameter 0.250 mm, film 0.25 μm). The oven was programmed from 80 °C for 1 min, to 180 °C at a rate of 30 °C min^{-1}, and then 230 °C at a rate of 100 °C min^{-1}. The carrier gas was helium, at a flow rate of 24 mL min^{-1}. The injector and detector temperatures were set at 200 and 250 °C, respectively. The samples (50 mL) were prepared by centrifugation (1300 rpm,

Table 1
Characteristics of the raw materials, cow manure (CM) and fish waste silage (FWS) used in the experiment.

	DM (% WW)	VS (% DM)	PH	NH_4^+ (g L^{-1})	Fat* (% WW)	Protein* (% WW)
CM	6.2 (0.5)	78.4 (0.7)	7.8 (0.5)	2.0 (0.7)	0.6	2.3
FWS	35.1 (0.2)	93.3 (0.4)	4.3 (0.3)	0.4 (0.1)	19.4	14.2

*Euro fins AS.

10 min) followed by filtration (pore size 0.45 μm) before dilution. To stabilize the VFAs, 1 mL of 0.65 M formic acid was added to the samples. All analysis of feed stocks and effluents were performed on triplicate samples, and the average standard deviations (SD) were calculated. Table 2 presents data on the duration and HRT (days) of the experiment, OLR, (g $L^{-1}d^{-1}$), content of total nitrogen (g L^{-1}), C/N ratio, and pH levels in the feedstock mixtures.

2.4. Gas measurements

The biogas was collected in 25-L Tedlar bags. CH_4 and CO_2 (volume%) was measured once a day by use of a GA2000 Landfill Gas Analyser (Geotechnical Instruments Ltd., UK). Total gas volume L d^{-1} was calculated from flow measurements (rate 300 cm^3/min) as follows: ((pump-number/60 s $*$ 300 $cm^{3\text{-min}}$)/(1000 mL).

3. Results

3.1. Methane productions

The methane production (L CH_4 gVS^{-1}) in R1, R2 and R0 is shown in Fig. 1A. During the periods with loading of 3%, 6% and 13% FWS, the methane productions in R1 increased to respectively 0.217, 0.212 and 0.333 L CH_4 gVS^{-1} day^{-1}. In R2, the methane productions during the corresponding periods increased to 0.223, 0.241, and 0.310 CH_4 gVS^{-1} day^{-1}. In R0, the methane production was in average 0.200 L CH_4 gVS^{-1} day^{-1} throughout the experiment. In R2, during the period with loading of 16% FWS, the methane productions were 0.400 L CH_4 $gVS^{-1}day^{-1}$, which was the highest methane production obtained during the experiment. Compared to anaerobic digestion of CM only, co-digestion of 3%, 6% and 13% FWS with CM in R1 and R2, increased the methane production by respectively 10%, 13%, and 60% (average for R1 and R2). In addition, co digestion of 16% FWS with CM in R2, gave an increase in methane production of 100%. The percentage of methane content in the biogas increased from around 65% to 70% in R1 and R2 when the content of FWS was increased from 3% to 13%, and in R0, the percentage of methane content in the biogas was ranging between 60% and 65% throughout the experiment (data not shown). During the period with loading of 16% and 19% FWS, the biogas process failed in R1 and R2 respectively.

3.2. Effluent composition

The VS removal (volume%), pH, and NH_4^+ (g L^{-1}) levels measured during the experiment is shown in Fig. 1B–D. The effluents concentrations of VFAs is shown in Table 3. In the period with loading of 3% FWS, the average NH_4^+ concentrations in R1 and R2 were respectively 2.99 and 3.14 g L^{-1} (Fig. 1D), (corresponding to 0.36 and 0.45 g NH_3 L^{-1}). At the end of the period with loading of 13% FWS, the NH_4^+ concentrations in R1 and R2 were respectively 5.7 and 4.7 g L^{-1} (corresponding to 0.75 and 0.55 g NH_3 L^{-1}), and during the period with loading of 16% FWS, the NH_4^+ concentrations in R1 increased to a maximum of 8 g L^{-1} (corresponding to 1.35 g NH_3 L^{-1}). The NH_4^+ concentration in R2 increased to a maximum of 7.0 g L^{-1} (corresponding to 1.27 g NH_3 L^{-1}), during the period with loading of 16% FWS, and at the end of the period with loading of 19% FWS, the NH_4^+ concentration was around 8 g L^{-1} (0.60 g NH_3 L^{-1}). The concentrations of VFAs also increased in both experimental reactors when the amount of FWS in the feed stock was increased. The content of acetic acid and propionic acid showed the most significant increase, compared to the other VFAs. From startup and throughout the period with loading of 13% FWS, the total VFA concentration increased from 1.18 to 6.03 g L^{-1} in R1, and 1.25 to 4.77 g L^{-1} in R2 (Table 3). During the period with loading of 16% FWS, the total VFA concentration in R1 increased to 19.81 g L^{-1}. In R2, from the period with loading of 16–19% FWS, the total VFA concentration increased from 8.16 to 16.42 g L^{-1} (Table 3).

The pH levels in the reactors were stable around 8 from startup and throughout the period with loading of 13% FWS (Fig. 1C). The pH level decreased in R1and R2 during the periods with loadings of respectively 16% and 19% FWS, when the NH_4^+ and VFA concentrations increased. From the experiment startup and throughout the period with loading of 3% FWS, the VS removal% was between 30% and 40% in R1 and R2 (Fig. 1B). At the end of the periods with loading of 13% FWS, the VS removal% was around 60 in R1 and R2. In R2, during the period with loading of 16% FWS, the VS removal% was between 63% and 70%. During the period with loading of 16% and 19% FWS, the VS removal in R1 and R2 respectively decreased to around 16%. The control reactor (R0) effluent composition was stable throughout the experiment, with NH_4^+ concentrations around 2.6 g L^{-1}, and the pH levels ranged between 7.9 and 8.2. The total VFA concentration in R0 was 0.60 g L^{-1}, and the VS removal ranged between 30% and 35% (Fig. 1, Table 3).

4. Discussion

FWS is an energy- and N-rich substrate that will come to serve as one of the organic fractions used together with CM for production of biogas in Norway. The results of the present study demonstrate that FWS has a potential as feedstock for methane production when it is co-digested with CM. The study also

Table 2
Feedstock composition presented as the input parameters HRT (days), OLR (gVS L^{-1} day^{-1}), Tot N (g L^{-1}), C/N ratio, and pH levels.

Feedstock period	Input parameters				
	Days (HRT = 30)	OLR (gVS L^{-1} day^{-1})	Tot N[a] (g L^{-1})	C/N[a]	pH
3% FWS	125	2.00	2.4 (0.5)	17.00	6.64
6% FWS	48	2.30	2.6 (0.5)	15.90	6.15
13% FWS	93	3.00	4.0 (0.9)	8.50	6.30
16% FWS	94	3.50	4.6 (0.9)	8.00	6.10
19% FWS	90	4.30	5.1 (1.0)	6.30	5.90
Control CM	450	1.50	2.3 (0.5)	17.50	7.80

[a] Euro fins AS.

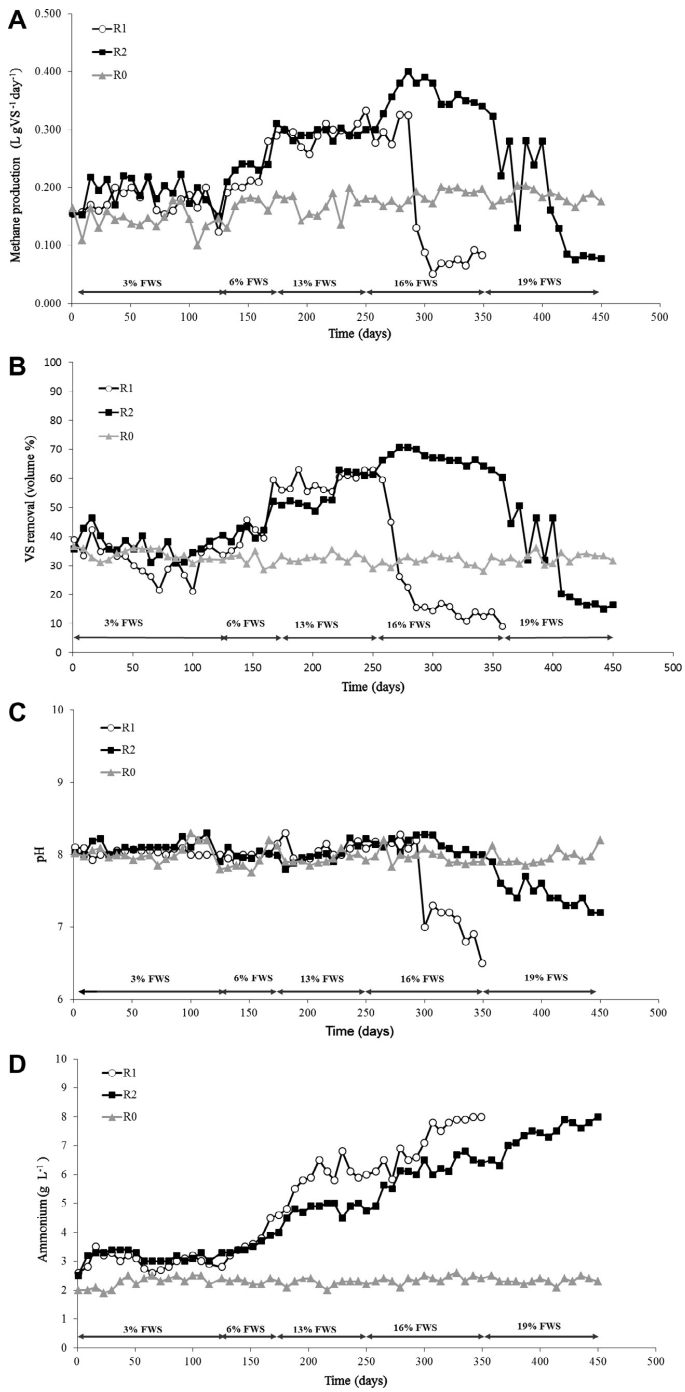

Fig. 1. Methane production, L gVS^{-1} day^{-1} (A), VS removal, volume% (B), pH levels, (C), ammonium concentration, g L^{-1}(D) from reactors R1, R2 and R0, during the 450 days of adding feedstock with increasing amounts (volume%) of fish waste silage (FWS), in co digestion with cow manure (CM); 3% FWS (125), 6% FWS (48), 13% FWS (93), 16% FWS (94), and 19% FWS (90). The values represent weekly average measurements.

Table 3
Concentration of VFA (g L^{-1}) in effluent samples from R1 and R2 at the end of each period with different loading of fish waste silage (FWS). STDEV are given in brackets, (n = 3).

Parameters (g L^{-1})	3%		6%		FWS (% volume) 13%		16%		19%	Control CM
	Rl	R2	Rl	R2	Rl	R2	Rl	R2	R2	R0
Acetic acid	0.93 (0.06)	0.99 (0.06)	0.50 (0.03)	0.90 (0.06)	3.74 (0.10)	2.40 (0.75)	8.74 (0.70)	4.17 (0.05)	9.34 (0.35)	0.50 (0.03)
Propionic acid	0.11 (0.03)	0.12 (0.05)	0.34 (0.03)	0.57 (0.05)	1.09 (0.02)	0.78 (0.01)	3.40 (0.03)	1.86 (0.02)	3.80 (0.10)	0.10 (0.03)
Iso-Butyric acid	0.04 (0.01)	0.04 (0.01)	nd	0.03 (0.01)	0.30 (0.01)	0.80 (0.01)	3.50 (0.01)	1.00 (0.01)	nd	nd
n-Butyric acid	0.03 (0.01)	0.03 (0.01)	nd	0.04 (0.02)	0.03 (0.01)	0.07 (0.01)	1.72 (0.02)	0.15 (0.01)	nd	nd
Iso-Valeric acid	0.08 (0.02)	0.08 (0.01)	0.06 (0.01)	0.04 (0.01)	0.68 (0.02)	0.61 (0.05)	2.10 (0.03)	0.74 (0.01)	3.28 (0.13)	nd
n-Valeric acid	nd	nd	nd	nd	0.05 (0.01)	0.04 (0.01)	0.12 (0.01)	0.10 (0.01)	nd	nd
Iso-Caprionic acid	nd	nd	nd	nd	0.09 (0.01)	0.08 (0.02)	0.07 (0.01)	0.14 (0.07)	nd	nd
n-Caprionic acid	nd	nd	nd	nd	nd	nd	0.18 (0.06)	nd	nd	nd
Tot. id VFA	1.18	1.25	0.90	1.58	6.03	4.77	19.81	8.16	16.42	0.60

nd = not detected.
Tot. id VFA = total identified volatile fatty acids.

indicates the amount of FWS that can be mixed with CM to create a feedstock for use in an anaerobic digester.

As described, starter culture from the biogas pilot plant at the Åna facilities was added to the two reactors, and, after stabilization, co-digestion of FWS and CM was initiated. Fig. 1A shows that the CH_4 production increased significantly when FWS was added to the feedstocks. In short, it was detected that increasing the proportion of FWS in the feedstock stepwise from 3% to 6% and then 13%, with a consequent increase of NH_4^+, NH_3 and VFA concentrations in the reactors, gave corresponding rises in the CH_4 productions from approximately 0.200 to 0.300 L gVS^{-1} day^{-1} (Fig. 1A) in R1 and R2. This shows that the biogas process gradually adapted to high concentrations of NH_4^+, NH_3 and VFA without markedly inhibiting the methanogenic microbial community. The CH_4 content (volume%) increased from around 65% to 70% in R1 and R2 from the period with loading of 3% to 13% FWS. In R0 (without FWS) the CH_4 content was ranging between 60% and 65%.

Some biogas experiments with different kinds of fish waste have previously been conducted. Kafle et al. (2013) performed a batch experiment with fish waste silage as a substrate, and the methane yields obtained from this substrate was 0.441–0.482 L CH_4 gVS^{-1}. It was found that inhibition occurred from high concentrations of LCFAs in one of the experiments were fish waste (80%) was mixed with bread (20%). Nges et al. (2012) conducted a batch experiment with fish sludge and fish waste as substrates, and the methane yields obtained were respectively 0.742 and 0.828 L CH_4 gVS^{-1}, and no markedly inhibition of the methane production was observed. Salam et al. (2009) presented an experiment where cow dung and fish waste were used as a mixed substrate for biogas production. The highest gas production obtained was 2 L biogas kg^{-1} waste, with a ratio of 1:1.2 fish waste: cow dung. In this experiment it was observed that when only fish waste was used, the gas yield obtained was as low as 150 mL biogas kg^{-1} waste. The results from these previous studies reflect the results from our experiments, showing that fish waste has a large biogas potential when it is co-digested with manure.

In our experiment, the biogas process failed in R1 and R2 during the periods of loading 16% and 19% FWS, respectively. Failure was probably due to overloading of nutrients, and the accumulation of NH_4^+, NH_3 and VFAs. By comparison, all the results for the control reactor were as expected in regard to both methane production and effluent composition, the former stable at values of between 0.150 and 0.200 L CH_4 gVS^{-1} day^{-1} (Fig. 1A), which coincides with results on methane production from cow manure reported by other researchers (Lehtomaki et al., 2007; Monteiro et al., 2011; Nasir et al., 2012). Previous experiments with a focus on biogas reactor performance and process failure, have shown patterns in overloaded reactors similar to those observed in our study (Chua et al., 1997; Banks et al., 2011; Neves et al., 2009). In our reactors, the biogas process failed when the load of FWS was between 16% and 19%, with OLRs between 3.5 and 4.3 g L^{-1} day^{-1} (Table 2). Braun et al. (2003) carried out a batch experiment with co-digestion of proteinaceous industrial waste with cow manure and sewage sludge, and it was found that about 5–15% co-substrate addition proved to be best suited, without causing any detrimental effects on the digestion process or on further use of the digestate.

Alvarez et al. (2010) investigated biodegradability and methane production from co-digestion of manure, fish waste and biodiesel, and found that the highest biodegradation potential was reached with a mixture containing 84% pig manure, 5% fish waste and 11% biodiesel waste, while the highest methane production rate was obtained with a mixture containing 88% pig manure, 4% fish waste and 8% biodiesel waste.

In another experiment, where fish and biodiesel wastes were used as co-substrates in pig manure anaerobic digestion, it was found that co-substrates improved the methane production when the mixing ratio was adjusted to 90% pig manure and 10% fish (w/w). The experiment also showed that higher loading of fish and biodiesel caused VFA and NH_3 accumulation (Regueiro et al., 2012). These findings reflect the results from our experiment.

Our results show that the VS removal values increased from around 40% to 60% in R1 and R2 (Fig. 1B) during the periods with loading of 3%, 6% and 13% FWS, with a maximum of around 70% VS removal in R1 during the period with loading of 16% FWS. When the biogas production failed, the VS removal decreased to around 16% in both R1 and R2. The maximum VS removal values in R0 (Fig. 1B) were low (30–35%) compared to the VS removal values in R1 and R2, which is in accordance to previous experiment evaluating VS removal in cow manure (Abubakar and Ismail, 2012; Sánchez-Hernández et al., 2013). The results on VS removal in R1 and R2 compared to R0 show that adding FWS largely increased the feed stock nutrient content, and also the anaerobic degradation rates. The results obtained in our experiment coincide with results from previous experiments where co-digestion of fat and protein rich substrates and cow manure have been investigated in regard to VS removal. (Liua et al., 2012; Razaviarani et al., 2013).

The C/N ratio decreased with increasing OLR in R1 and R2, and other investigations have indicated that a C/N value between 15 and 30 is optimal for biogas production, and that low C/N ratio feed stock can result in high NH_3 release and accumulation of VFAs (Yen and Burne, 2007). Our reactors performed satisfactorily when feedstocks with C/N ratio from 17 to 8.5 were added (Table 2). The tolerance for the low C/N ratios can probably be explained by microbial adaptation to the nitrogen rich feedstocks.

Many investigations have examined the inhibitory impact of NH_4^+ and NH_3 on methane production, and the reported results are somewhat variable. Free NH_3 is the active component that interferes with the methane production process (Angenent et al.,

2002), and it is known that the fraction of free NH_3 rises with increasing pH. Inhibition of methane production is reported to start at NH_3 concentrations of 0.15–2.5 g L^{-1} (Braun et al., 1981; Hashimoto, 1986; Van Velsen, 1979). However, NH_3 concentrations of up to 4 g L^{-1} have been detected in stable biogas reactors (Angelidaki and Ahring, 1993). Koster and Lettinga (1984) found that inhibition of the biogas process occurred at an NH_3 concentration of 1.7 g L^{-1} and a pH of 7.5.

Schnurer and Nordberg (2008) found that the activation of syntrophic acetate oxidizing bacteria (SAOB) occurs when NH_4^+ levels exceed 3 g L^{-1}, and that the methane production decreases when the anaerobic pathway changes from acetoclastic to syntrophic acetate oxidation (SAO). Borja et al. (1996) studied a thermophilic reactor and found that the VFA levels increased with rising NH_4^+ concentrations, and also noted that the activity of acetoclastic methanogens, microorganisms that are normally responsible for two-thirds of the methane production, had decreased by 72% at an NH_4^+ level of 7 g L^{-1}. By comparing the previous results with the results obtained in this study, it is reasonable to assume that a change in methane production pathway from acetoclastic to SAO occurred in our reactors as the NH_4^+ levels gradually increased to maximum values of 8 g L^{-1} (Fig. 1D).

In both R1 and R2, the acidity was stable around pH 8 throughout the periods with loading of 3%, 6% and 13% (Fig. 1C), although both NH_4^+, NH_3 and VFA concentrations increased. Methane production in R1 failed during the period with loading of 16% FWS, when the NH_4^+ concentrations reached 8 g L^{-1} (1.35 g NH_3 L^{-1}), with subsequent decrease in pH to a minimum of 6.5 (Fig. 1C and D). In R2, the concentration of NH_4^+ reached 7 g L^{-1} (1.27 g NH_3 L^{-1}) during the period with loading of 16% FWS (Fig. 1D). This was the highest NH_3 concentration detected in R2, and methane production slowly decreased from this point. During the period with loading of 19% FWS in R2, the pH levels decreased to a minimum of 7.2, and the NH_4^+ concentration increased to 7.9 g L^{-1} (0.60 g NH_3 L^{-1}). The elevated NH_4^+ levels at high pH values probably induced the inhibitory effect of free NH_3 on acetoclastic methanogenic activity in our reactors.

Considering the effects of VFAs, the inhibition of biogas processes from the accumulation of propionic acid has been investigated extensively (Chen et al., 2008; Retfalvi et al., 2011; Wang et al., 2009), and is considered to be the most toxic of all VFAs found in anaerobic digesters (Kaushalya et al., 2011). Inhibitory levels of propionic acid have varied between studies, from 0.8 g L^{-1} (Ma et al., 2009) to 6 g L^{-1} (Gallert and Winter, 2008). Ma et al. (2009) reported that inhibition from propionic acid on methane production usually occurs at concentrations of around 1–2 g L^{-1}. In our experiments, process failure occurred when the propionic acid concentration was 3.4 and 3.8 g L^{-1} in R1 and R2 respectively (Table 3).

The ratio between propionic and acetic acid increased from about 0.1 at startup to 0.4 at process failure in our reactors. In stable biogas reactors, there are often variable levels of acetic acid, but very low concentrations of propionic acid, and there is evidence that the ratio between propionic and acetic acid is critical for reactor stability (Marchaim and Krause, 1993). In addition to elevated levels of propionic acid, there was also an accumulation of iso-valeric and iso-butyric acids during process failure in our reactors. In R1, iso-valeric acid concentration increased from 0.08 to 2.10, and iso-butyric acid concentration increased from 0.04 to 3.50 g L^{-1} from the period of loading 3% to 16% FWS (Table 3). In R2, the iso-valeric acid concentration increased from 0.08 to 3.28 g L^{-1} from the period of loading 3% to 19% FWS, and the iso-butyric acid concentration increased from 0.04 to 1.00 g L^{-1} from the period of loading 3% to 16% FWS (Table 3). Iso-butyric acid was not detected in R2 during the period with loading of 19% FWS. These results are in accordance with previous studies, which show that the iso-VFAs are slowly degradable (Retfalvi et al., 2011) and that accumulation of iso-butyric acid impedes methane production (Aguilar et al., 1995; Chen et al., 2008). It has also been found that when NH_3 is added to biogas reactors, a significant increase in levels of propionic, butyric, and valeric acids can be observed (Poggi-Varaldo et al., 1996), and this coincides with our results which show increasing concentrations of NH_4^+, NH_3 and VFA in the reactors fed with FWS. According to Inac et al. (1999), acetic acid concentrations of up to 10 g L^{-1} can be tolerated in anaerobic digesters, which is in accordance with the acetic acid levels detected in the effluents from our reactors. Considering both the present results on methane production and effluent quality, and the results from other investigations on the same topic, it can be assumed that the accumulation of free NH_3 was the initial factor inhibiting the methanogenic microbial process, followed by the negative effects of high VFA concentrations and low pH levels in R1 and R2. The methane production process failed somewhat earlier in R1 than in R2, which can be explained by the fact that the concentrations of NH_4^+, NH_3 and VFA accumulated to markedly high concentrations at an earlier stage in the aforementioned reactor. It is not clear as to why the methane production performance and effluent quality was maintained longer in R2 than in R1, but a likely explanation is that the microbial communities, and especially key controlling microorganisms, developed differently in the two reactors. To address this question, further research is needed.

5. Conclusions

The present study demonstrates that co-digestion of FWS and CM has a high methane potential. The highest methane production (0.400 L CH_4 gVS^{-1}) was obtained in one reactor when the feed stock mix was 16% FWS and 84% CM. Addition of this feed stock composition increased the methane production by 100%, in comparison to the methane production obtained from CM only. Our results show that an excessive increase of FWS in the feed stock leads to an accumulation of NH_4^+, NH_3 and VFA, which will cause methane production failure.

In order to optimize methane production and avoid evident inhibition, FWS and CM should be mixed at ratios of between 13% and 16% FWS with 87% and 84% CM (vol.). Performance data from reactors using different loadings of FWS can provide guidelines for operation of biogas plants that use such material and other protein-rich substrates as feedstocks.

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References

Abubakar, B.S.U.I., Ismail, N., 2012. Anaerobic digestion of cow dung for biogas production. J. Eng. Appl. Sci. 7, 1819–6608.

Aguilar, A., Casas, C., Lema, J.M., 1995. Degradation of volatile fatty acids by differently enriched methanogenic cultures: kinetics and inhibition. Water Res. 29, 505–509.

Alvarez, J.A., Otero, L., Lema, J.M., 2010. A methodology for optimising feed composition for anaerobic co-digestion of agro-industrial wastes. Bioresour. Technol. 10, 1153–1158.

Alwan, S.R., Buckley, D.J., O'Connor, T.P., 1993. Silage from fish waste: chemical and microbiological aspects. Irish J. Agric. Food Res. 32, 75–81.

Angelidaki, I., Ahring, B.K., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Appl. Microbiol. Biotechnol. 37, 808–812.

Angelidaki, I., Ahring, B.K., 1993. Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. Appl. Microbiol. Biotechnol. 38, 560–564.
Angelidaki, I., Ellegaard, L., Ahring, B.K., 2004. A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnol. Bioeng. 42, 159–166.
Angenent, L.T., Sung, S., Raskin, L., 2002. Methanogenic population dynamics during startup of a full scale anaerobic sequencing batch reactor treating swine manure. Water Res. 36, 4648–4654.
Appels, L., Baeyens, J.C., Degreve, J., Dewil, R., 2008. Principles and potential of the anaerobic digestion of waste-activated sludge. Prog. Energy Combust. Sci. 34, 755–781.
Ashekuzzman, S.M., Poulsen, Tjalfe G., 2011. Optimizing feed composition for improved methane yield during anaerobic digestion of cow manure based waste mixtures. Bioresour. Technol. 102, 2213–2218.
Banks, C.J., Chessire, M., Heaven, S., Arnold, R., 2011. Anaerobic digestion of source segrated domestic food waste: performance assessment by mass and energy balance. Bioresour. Technol. 102, 612–620.
Bergersen, O., Hanssen, K.Ø., Vasskog, T., 2012. Anaerobic treatment of sewage sludge containing selective serotonin reuptake inhibitors. Bioresour. Technol. 117, 325–332.
Biebl, H., 2001. Fermentation of glycerol by Clostridium pasteurianum — batch and continuous culture studies. J. Ind. Microbiol. Biotechnol. 27, 18–26.
Borja, R., Sanchez, E., Weiland, P., 1996. Influence of ammonia concentration on thermophilic anaerobic digestion of cattle manure in upflow anaerobic sludge blanket (UASB) reactors. Process Biochem. 31, 477–483.
Braun, R., Huber, P., Meyrath, J., 1981. Ammonia toxicity in liquid piggery manure digestion. Biotechnol. Lett. 3, 159–164.
Braun, R., Brachtl, E., Grasmug, M., 2003. Codigestion of proteinaceous industrial waste. Appl. Biochem. Biotechnol. 109, 139–153.
Chen, Y., Cheng, J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99, 4044–4064.
Chen, G., Zheng, Z., Yang, S., Fang, C., Zou, X., Zhang, J., 2010. Improving conversion of Spartina alterniflora into biogas by co-digestion with cow feces. Fuel Process Technol. 91, 1416–1421.
Chua, H., Hu, W.F., Yu, P.H.F., Cheung, M.W.L., 1997. Responses of an anaerobic fixed film reactor to hydraulic shock loadings. Bioresour. Technol. 61, 79–83.
Demirel, B., Yenigun, O., 2002. Two-phase anaerobic digestion process: a review. J. Chem. Technol. Biotechnol. 77, 743–755.
El-Mashad, H.M., Zhang, R., 2010. Biogas production from co-digestion of dairy manure and food waste. Bioresour. Technol. 101, 4021–4028.
Gallert, C., Winter, J., 2008. Propionic acid accumulation and degradation during restart of a full scale anaerobic biowaste digester. Bioresour. Technol. 99, 170–178.
Gebauer, R., Eikebrokk, B., 2006. Mesophilic anaerobic treatment of sludge from salmon smolt hatching. Bioresour. Technol. 97, 2389–2401.
Gebauer, R., 2004. Mesophilic anaerobic treatment of sludge from saline fish farm effluents with biogas production. Bioresour. Technol. 93, 155–167.
Goncalves, M.R., Costa, J.C., Marques, I.P., Alves, M.M., 2009. Innoculum acclimation to oleate promotes the conversion of olive mill wastewater to methane. Bioresour. Technol. 61, 79–83.
Gujer, W., Zehnder, A.J.B., 1983. Conversion processes in anaerobic digestion. Water Sci. Technol. 8–9, 127–167.
Hashimoto, A.G., 1986. Ammonia inhibition of methanogenesis from cattle wastes. Agric. Wastes 17, 241–261.
Inac, B., Matsui, S., Ide, S., 1999. Propionic acid accumulation in anaerobic digestion of carbohydrates: an investigation on the role of hydrogen gas. Water Sci. Technol. 40, 93–100.
ISO 10390: 2005. Soil quality. Determination of pH.
ISO 11465: 1993. Soil quality – determination of dry matter and water content on a mass basis. Gravimetric method.
Kafle, G.K., Kim, S.H., Sung, K.I., 2013. Ensiling of fish industry waste for biogas production: a lab scale evaluation of biochemical methane potential (BMP) and kinetics. Bioresour. Technol. 127, 326–336.
Karlsson, A., Einarsson, P., Schnurer, A., Sundberg, C., Ejlertsson, J., Svensson, B.H., 2012. Impact of trace element addition on degradation efficiency of volatile fatty acids, oleic acid and phenyl acetateand on microbial population in a biogas digester. J. Biosci. Bioeng. 114, 446–452.
Kaushalya, W., Visvanathan, C, Abeynayaka, A., 2011. Effect of organic loading rate on VFA production, organic matter removal and microbial activity of a two stage thermophilic anaerobic membrane bioreactor. Bioresour. Technol. 102, 5353–5360.
Koster, I.W., Lettinga, G., 1984. The influence of ammonium–nitrogen on the specific activity of pelletized methanogenic sludge. Agric. Wastes. 9, 205–216.
Lehtomaki, A., Huttunen, S., Rintala, J.A., 2007. Laboratory investigations of energy crops and crop residues with cow manure for methane production: Effect of crop to manure ratio. Resour. Conserv. Recycl. 51, 591–609.
Liua, X., Wanga, W., Shi, Y., Zhenga, L., Gao, X., Qiaoc, W., Zhoud, Y., 2012. Pilot-scale anaerobic co-digestion of municipal biomass waste and waste activated sludge in China: effect of organic loading rate. Waste Manage. 32, 2056–2060.
Ma, J., Carballa, M., De Caveye, P.V., Verstraete, W., 2009. Enhanced propionic acid degradation (EPAD) system: proof of principle and feasibility. Water Res. 43, 3239–3248.
Marchaim, U., Krause, K., 1993. Propionic to acetic acid ratios in overloaded anaerobic digestion. Bioresour. Technol. 43, 195–203.
Møller, H.B., Sommer, S.G., Ahring, B.K., 2004. Methane productivity of manure, straw and solid fractions of manure. Biomass Bioenergy. 26, 485–495.
Monteiro, E., Mantha, V., Rouboa, A., 2011. Prospective application of farm cattle manure for bioenergy production in Portugal. Renew. Energy 36, 627–631.
Nasir, I.M., Mohd Gahzi, T.I., Omar, R., Ildris, A., 2012. Palm oil mill effluent as an additive with cattle manure in biogas production. P Eng. 50, 904–912.
Neves, L., Oliveira, R., Alves, M.M., 2009. Co-digestion of cow manure, food waste and intermittent input of fat. Bioresour Technol. 100, 1957–1962.
Nges, I., Mbatia, B., Bjørnsson, L., 2012. Improved utilization of fish waste by anaerobic digestion following omega-3 fatty acids extraction. J. Environ. Manage. 110, 159–165.
NMKL methods, 1989. Fett. Bestämning enligt SBR (Shmid–Bondzynski–Ratslaff) I kött och köttvaror. Fat. Determination according to SBR in meat and meat products. No. 131.
NS-EN 15935: 2012. European Standard method for the determination of the loss on ignition (LOI) of dry matter at 550 & #xB0;C.
Ortega, L., Husser, C., Barrington, S., Guiot, S.R., 2008. Evaluating limiting steps of anaerobic degradation of food waste based on methane production tests. Water Sci. Technol. 57, 419–422.
Parkin, G.F., Owen, V.F., 1986. Fundamentals of anaerobic digestion of waste – water sludges. J. Environ. Eng. 12, 867–920.
Poggi-Varaldo, H.M., Rodríguez-Vázquez, R., Fernández-Villagómez, G., Esparza-García, F., 1996. Inhibition of mesophilic solid-substrate anaerobic digestion by ammonia nitrogen. Appl. Microbiol. Biotechnol. 47, 284–291.
Razaviarani, Vahid, Buchanana, I.D., Malik, Shahid, Katalambula, Hassan, 2013. Pilot-scale anaerobic co-digestion of municipal wastewater sludge with restaurant grease trap waste. J. Environ. Manage. 123, 26–33.
Regueiro, L., Carballa, M., Alvarez, J.A., Lema, J.M., 2012. Enhanced methane production from pig manure anaerobic digestion using fish and biodiesel wastes as co-substrates. Bioresour. Technol. 123, 507–513.
Retfalvi, T., Tukacs-Hajos, A., Levente, A., Marosvolgyi, B., 2011. Laboratory scale examination of the effects of overloading on the anaerobic digestion by glycerol. Bioresour. Technol. 102, 5270–5275.
Salam, B., Islam, M., Rahman, T. Biogas from Anaerobic Digestion of Fish Waste. In: Proceedings of the International Conference on Mechanical Engineering (ICME2009) 26–28 December 2009, Dhaka, Bangladesh.
Sánchez-Hernández, E.P., Weiland, P., Borja, R., 2013. The effect of biogas sparging on cow manure characteristics and its subsequent anaerobic biodegradation. Int. Biodet. Biodeg. 83, 10–16.
Schnurer, A., Nordberg, Å., 2008. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at at mesophilic temperature. Water Sci. Technol. 57, 735–740.
Statistics Norway. SSB.2013. 07516: Akvakultur. Tap/svinn i matfiskproduksjon, etter fiskeslag (F). http://www.ssb.no/sok?sok=d%C3%B8d+laks+.
Toreci, I., Droste, R.L., Kennedy, K.J., 2011. Mesophilic anaerobic digestion with high temperature microwave pretreatment and importance of inoculum acclimation. Water Environ. Res. 83, 559–594.
Van Velsen, A.F.M., 1979. Adaptation of methanogenic sludge to high ammonia–nitrogen concentrations. Water Res. 13, 995–999.
Wang, Y., Zhang, Y., Wang, J., Meng, L., 2009. Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria. Biomass Bioenergy 33, 848–853.
Weiland, P., 2010. Biogas production: current state and perspectives. Appl. Microbiol. Biotechnol. 85, 849–860.
Yen, H.W., Burne, D.E., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour. Technol. 98, 130–134.

PAPER II

II

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RESEARCH ARTICLE Open Access

A metagenomic study of the microbial communities in four parallel biogas reactors

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Abstract

Background: Biogas is a renewable energy carrier which is used for heat and power production or, in the form of purified methane, as a vehicle fuel. The formation of methane from organic materials is carried out by a mixed microbial community under anaerobic conditions. However, details about the microbes involved and their function are limited. In this study we compare the metagenomes of four parallel biogas reactors digesting a protein-rich substrate, relate microbiology to biogas performance, and observe differences in these reactors' microbial communities compared to the original inoculum culture.

Results: The biogas process performance during the startup phase of four parallel continuous stirred tank reactors (designated R1, R2, R3, and R4) co-digesting fish waste and cow manure was studied. The microbial composition of the inoculum (day 0) and the four reactors at day 59 was studied and compared using 454 FLX Titanium pyrosequencing. In the inoculum and the reactor samples, the Bacteria *Clostridium* and *Syntrophomonas* were highly abundant, and the dominating methanogen was the hydrogenotrophic *Methanoculleus*. Syntrophic prokaryotes frequently found in biogas reactors with high concentrations of ammonium and volatile fatty acids were detected in all samples. The species *Candidatus Cloacimonas acidaminovorans* of the candidate phylum Cloacimonetes (WWE1) increased in all reactors and was the dominating bacterium at day 59. In particular, this bacterium showed a very high abundance in R1, which distinguished this reactor significantly from the other reactors in terms of microbial composition. Methane production and the reactor slurry characteristics were monitored in the digestion period. Generally all four reactors operated stably and showed rather similar characteristics. The average methane production in the reactors varied between 0.278 and 0.296 L gVS^{-1}, with the lowest production in R1.

Conclusions: This study showed that four parallel reactors co-digesting manure and fish waste silage operated stably during a startup phase. Several important Archaea and Bacteria degrading the protein-rich substrate were identified. In particular, microorganisms involved in syntrophic methane production seemed to be important. The detailed characterization of the microbial communities presented in this work may be useful for the operation of biogas plants degrading substrates with high concentrations of proteins.

Keywords: Anaerobic digestion, Syntrophic oxidation, Metagenomic, Biogas, Taxonomic structure, Biofuel, Biorefinery, Methane

Background

Anaerobic digestion of organic materials from agriculture and industry may reduce local pollution and provide energy in the form of methane. Large amounts of organic materials are produced and disposed as waste every year. In Norway organic materials such as cattle manure and dead fish from fish farms are in large supply. In 2012 a loss of 27.4 million dead salmon was reported from Norwegian fish farms [1], and the total annual amount of organic waste in Norway is 1.45 million tons [2].

During anaerobic digestion organic materials are converted to methane and carbon dioxide plus small amounts of other gases by a microbial community through four main reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The anaerobic degradation process is initiated by hydrolysis, where complex molecules like carbohydrates, lipids, and proteins are depolymerized into soluble compounds by a range of enzymes produced by

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the Bacteria. The hydrolyzed compounds are further fermented into acetate, propionate, butyrate, lactate, ethanol, methanol, ammonia, hydrogen, and carbon dioxide. Acetogenesis is the reaction in which acetate is produced from hydrogen and carbon sources by acetogenic Bacteria [3].

Methanogens belong to the Archaeal phylum Euryarchaeota [4], and methane is produced in the last step of the anaerobic process. The methane-producing microorganisms that usually dominate in biogas reactors are the acetoclastic methanogens [5]. The acetoclastic pathway is carried out by the order Methanosarcinales [6,7]. The primary substrate for methane production by the hydrogenotrophic methanogens is CO_2 and H_2, and this group consists of several methanogenic orders: Methanobacteriales, Methanococcales, and Methanomicrobiales [6,7]. An alternative methane production pathway, called syntrophic acetate oxidation, is known to take place in reactors with a high content of ammonia and fatty acids. The reaction includes conversion of acetate to H_2 and CO_2 by syntrophic acetate-oxidizing Bacteria, such as *Clostridium ultunense*, *Tepidanaerobacter acetatoxydans*, and *Syntrophaceticus schinkii*, followed by methane production by a hydrogenotrophic methanogen (for example, members of the orders Methanomicrobiales and Methanobacteriales) [8-11].

The acetogenic Bacteria and the methanogenic Archaea differ largely in terms of nutritional needs and sensitivity to environmental conditions [12]. Additionally, the methanogens have a slower growth rate than the acidogenic Bacteria [13], which in turn may result in accumulation of intermediate degradation products. A common reason for biogas reactor instability is failure to maintain the balance between these two groups of microorganisms [14].

The various complex anaerobic reactions that lead to methane formation are to a large extent performed through syntrophy between Bacteria and methanogenic Archaea. These syntrophic relationships provide the methanogens with their substrates and remove metabolic products from the acid-forming Bacteria [15]. Analyses of microbial communities have shown that elevated concentrations of ammonia in biogas reactors trigger the syntrophic acetate oxidation pathway, where acetate is transformed to CO_2 and H_2 before methane is produced by hydrogenotrophic methanogens [8,9]. The syntrophic degradation of other short chain fatty acids during anaerobic digestion has also been described [16,17], and several Bacterial strains and groups of methanogens are identified as having key roles in various syntrophic reactions.

Ensilaged fish waste contains large amounts of fat and protein [1], making it an energy-rich substrate that is suitable for biogas production. However, high inputs of fat and protein to a biogas reactor may cause accumulation of ammonia and fatty acids, potentially yielding unstable methane production and biogas reactor failure [15,18]. Generally, methanogens, and thus methane production, are inhibited by ammonia (NH_3) formed in the process of protein degradation [19-21]. Long chain fatty acids (LCFAs) [22] and volatile fatty acids (VFAs) [23] formed from lipid degradation may also lead to inhibition.

The low pH of the ensilaged fish waste and the high concentrations of fat and protein make the substrate suitable for co-digestion with an alkaline organic material like cow manure. Co-digestion may improve the anaerobic digestion process by creating a better nutrient balance, diluting toxic compounds, and stimulating synergistic effects of microorganisms [24-26], and may possibly also increase the stability of the system and the methane production.

The startup is a critical phase in biogas reactors [13,27,28], and inoculum stability is highly important. Anaerobic microbial communities can adapt to high concentrations of ammonia and fatty acids [29], if a strategy of gradual acclimatization and proper adjustment of operational parameters such as substrate composition, organic loading rate (OLR), and hydraulic retention time (HRT) is applied [30]. During startup of a biogas reactor, many different groups of microorganisms with varying requirements for biochemical and physical conditions are introduced, and the initial one to three weeks are considered to be a reactor's startup period [13]. Several experiments have dealt with startup dynamics in anaerobic digestion [13,31,32], but to our best knowledge, no metagenome analyses of microbial community structure in parallel continuously stirred tank reactors (CSTR) have been carried out.

The objectives of this study were to use metagenomic sequencing analysis to examine the microbial composition of a methane-producing inoculum, and to investigate the development of the inoculum through a stabilization period of 59 days in four parallel biogas reactors added protein-rich substrate under mesophilic conditions. The goal was also to compare the four reactors to investigate if the development of the microbial communities was similar in reactors running under the same conditions.

Results and discussion

Methane production and reactor slurry characteristics

The performance of four parallel biogas reactors during semicontinuous addition of fat and protein-rich materials (Table 1) was studied (Figure 1). The biogas volume and the CH_4 and CO_2 concentrations were measured once a day. In Figure 1A the average methane production is shown every fourth day. Although the CH_4 production was quite similar in the four reactors, a somewhat lower CH_4 production was observed from day 47 for R1. The average values of methane production in R1 and R2 were 0.282 (±0.039) and 0.297 (±0.042) L gVS^{-1}, respectively (Figure 1A). These CH_4 yields are in accordance with

Table 1 Chemical characterization of reactors' substrate

Dry matter (%)	Volatile solids* (%)	Total nitrogen (g L^{-1})	Total carbon (g L^{-1})	Carbon/nitrogen	Acetic acid (g L^{-1})	Propionic acid (g L^{-1})	Ammonium (g L^{-1})	pH
9.2	84.1	4.06	47.09	11.80	2.88	1.69	2.90	6.20

*Percentage of dry matter.

previous experiments on anaerobic co-digestion of the same substrates, where the yield was between 0.250 and 0.300 L CH_4 gVS^{-1} [1]. The production of CO_2 in the four reactors was on average between 0.142 and 0.161 L CO_2 gVS^{-1} during the experiment (data not shown).

The NH_4^+, VFA, pH, and volatile solids (VS) reductions were measured every fourth day during the experimental period. The pH was around 8 in all the reactors during the entire period (Figure 1C). The average NH_4^+ concentrations were highest in R1, with a concentration

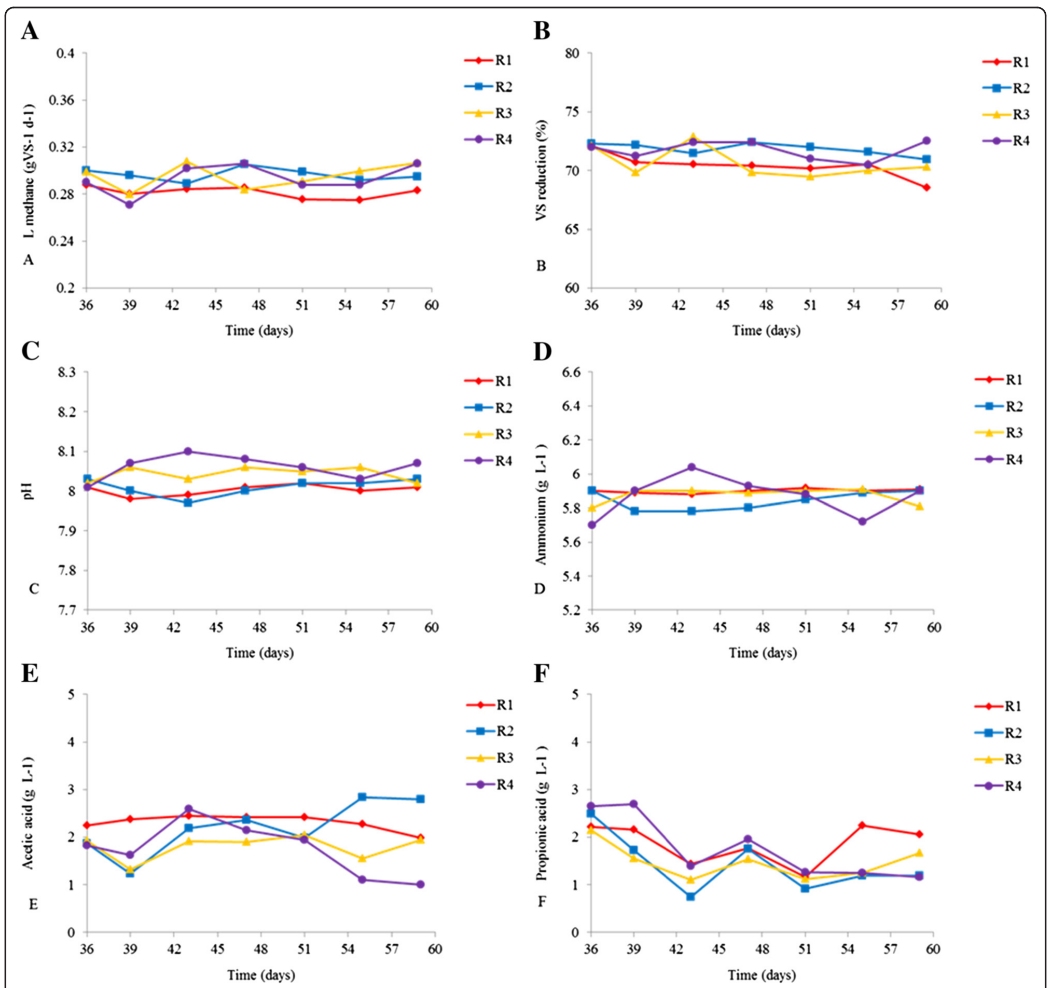

Figure 1 Anaerobic process performance in R1, R2, R3, and R4 during 28 days of continuous operation (day 36 to day 59). A) Methane productions, **B)** % volatile solid (VS) removal, **C)** pH values, **D)** NH_4^+ concentrations, **E)** acetic acid concentration, and **F)** propionic acid concentration.

of 5.90 (±0.08) g L^{-1}, and lowest in R2, with a value of 5.84 (±0.042) g L^{-1} (Figure 1D), with corresponding NH_3 concentrations in the range 0.67 to 0.75 g L^{-1} (data not shown). Previous experiments show that inhibition of methane production has been reported to take place at NH_3 concentrations of 0.7 to 2.0 g L^{-1} [19,29,33,34]. On average, the concentrations of acetic acid in the reactors were lowest in R4 and highest in R1, ranging between 1.75 (±0.430) and 2.31 (±0.120) g L^{-1} (Figure 1E). The average concentrations of propionic acid varied between 1.43 (±0.482) and 1.86 (±0.351) g L^{-1} (Figure 1F), with the highest levels in R1 and the lowest in R2.

High levels of acetate are common in stable biogas reactors, while propionic acid has been reported to inhibit methanogenic activity in the range 0.8 g L^{-1} [35] to 6 g L^{-1} [36]. Previous studies investigating methanogenic populations' adaptation capabilities to NH_4^+, NH_3, and VFAs have shown that methane production can be maintained in environments with high concentrations of these compounds [29]. The concentrations of NH_4^+ and VFAs observed in this study (Figure 1) were not alarmingly high, and the stable performance of the reactors suggest that the microbial communities in the reactors adapted to these conditions.

The amount of VS reduction (Figure 1B) supports the results of the other parameters measured, showing that the anaerobic degradation was somewhat lower in R1 than in the other reactors. The VS reduction in R1 decreased from 72.1 to 68.5 % from the startup of the continuous process to day 59, and the average VS reduction value in this reactor was 70.4 (±0.7)% (Figure 1B). In R2, R3, and R4, the VS removal values were quite similar and stable, with average values of 71.8 (±0.4), 70.6 (±1.0), and 71.7 (±0.7)%, respectively.

Sequencing, coverage, and taxonomic richness

The results from pyrosequencing of the inoculum and the four reactors (day 59) before and after quality filtering are shown in Table 2. Unless otherwise specified, all percentages in the following text refer to the total number of reads in each of the filtered datasets.

Rarefaction analysis in the program MEGAN was used to characterize the richness of taxa in the five metagenomes at the genus level and at the fully resolved level, where all species and strains were included (Figure 2). At the genus level, the curves were leveling off, indicating acceptable sampling and coverage of the richness in the samples. We detected from 324 (R3) to 496 (R2) genera (given as number of leaves in Figure 2). At the fully resolved level the number of taxa was in the range of 519 (R3) to 906 (R2). The richness in the samples was approximately proportional to the number of reads in the datasets (Table 2), and this may explain some of the variation in the number of taxa detected in the different samples. The high taxonomic richness shows that the samples harbor complex prokaryotic communities. The taxonomic richness in the inoculum (IN) was in the range of the reactor samples (R1 to R4).

Effective genome size (EGS) is a computational method to predict the average genome size, including multiple plasmid copies, inserted sequences, and associated phages and viruses, from short sequencing reads of metagenomes. EGS has been suggested as a link between the genome size and the functional repertoire of the metagenome; the greater the functional complexity, the greater the EGS [37]. In addition to the EGS values (Table 3), we calculated the probability (P) for detection of hits to a theoretical gene (X) of 1,000 bp. The expected number of hits to this gene X was calculated, assuming one copy number of this gene was present in all organisms in our communities. In the reactor samples (R1 to R4) the average EGS was 2.5. The slightly greater EGS in the inoculum (IN) than in the reactor samples may therefore indicate greater functional complexity in the inoculum compared to the reactor samples, which have experienced selective pressure in the 59-day stabilization period.

Taxonomic structure

The taxonomy at the domain level in the reactor samples and in the inoculum is shown in Figure 3. 75.64 to 78.48% of the total reads were assigned to taxa in MEGAN, while 21.46 to 24.31% were assigned to no hits. From 69.33% to 71.84% of the total reads were Bacterial, while 0.71% to 1.25% were assigned to Archaea. Although Archaea is usually less abundant than Bacteria in biogas reactors [38], the reads assigned to Archaea in our reactors are in the lower range of earlier reports. Typically, Archaea in biogas

Table 2 Characteristics of metagenomic reads before and after quality filtering derived from DNA extracted from the four biogas reactors and their inoculum

Metagenome	Raw dataset	Filtered dataset			
	Number of reads	Number of reads	Reads (%)	Mean sequence length (bp)	Mean GC ratio (%)
R1	245499	177017	72.10	413	43.08
R2	548434	390641	71.23	417	42.86
R3	182122	130610	71.72	410	43.75
R4	286008	205035	71.72	413	43.65
IN	241804	172150	71.19	409	43.10

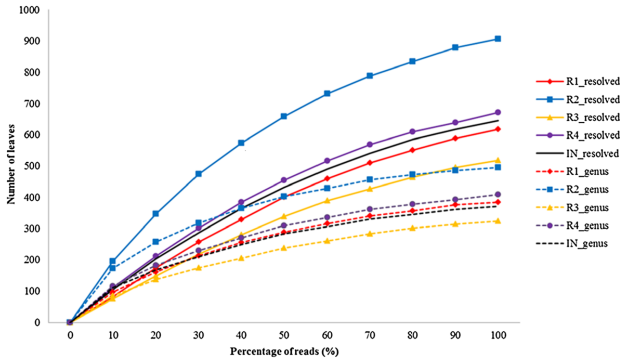

Figure 2 Rarefaction curves of taxonomic richness in the samples at the genus and the fully resolved level in MEGAN.

reactors is reported to be around 10% of the total reads [38-40]. However, other studies have reported Archaeal reads as low as 0.5% [41].

Eukaryota and viruses were also present in the metagenomes, representing from 0.44% to 0.58% and from 0.11% to 0.14%, respectively. Sample IN differed from the reactor samples by slightly greater percentages of reads assigned to Bacteria, Archaea, Eukaryota, and viruses, resulting in a corresponding reduction in reads with no hits.

A comparison of the taxonomic structures in the samples of phyla with more than 0.1% of the total number of reads assigned, in at least one metagenome, are given in Figure 4. The most abundant phyla in all the reactor samples were Firmicutes followed by Bacteroidetes and Cloacimonetes (WWE1). Together these phyla represented about 40 to 50% of all reads. This is in agreement with other investigations, which report that in nearly all microbial populations in methane-producing reactors, species from Firmicutes and Bacteroidetes are dominant [40,42]. It is therefore likely that these phyla are ubiquitous in all biogas reactors.

The structure in the inoculum (IN) differed from that of the reactor samples (R1 to R4) in several ways. IN harbored more of Firmicutes, Proteobacteria, Euryarchaeota, Actinobacteria, and Synergistetes, compared to the reactor samples. Comparison of the reactor samples only showed that R1 differed from the other samples. In particular, the abundance of Firmicutes is lower and the level of the *in silico* phylum, Cloacimonetes (WWE1), is greater in R1 than in the other reactor metagenomes. Proteobacteria, Euryarchaeota, Spirochaetes, and Tenericutes were also more abundant in R1 compared to the other reactors.

Table 3 Effective genome size of the metagenomes

Metagenome	Effective genome size (Mbp)	Probability (P) of hitting gene X	Expected hits to gene X
R1	2.2	0.000452462	80.0934268
R2	2.5	0.000404997	71.69129853
R3	2.6	0.000377457	66.81634238
R4	2.5	0.000394418	69.8187181
IN	3.2	0.000314795	55.72407323

Due to the complexity of the metagenomes, a principal component analysis (PCA) plot, at the phylum level, was constructed to view the clustering of the five samples (Figure 5). The reactor samples R2, R3, and R4 were highly similar and clustered closely in the lower right quadrant, while sample R1, located in the upper left quadrant, differed in several ways from the other reactor metagenomes. As expected, the inoculum sample separated from all the reactor samples in the PCA plot and was positioned in the upper right quadrant. The abundances of Firmicutes and Cloacimonetes (WWE1) were the most important parameters for positioning of the samples along the first principal component (PC1). Firmicutes, Actinobacteria, and Synergistetes all had positive scores along PC1, indicating that the samples placed on the right section of the PCA plot (IN, R2,R3,R4) had relatively high abundances of these taxa compared to sample R1. Proteobacteria and Euryarchaeota have positive scores along PC1 but also strong positive scores at PC2, indicating a greater abundance of these phyla in R1 and IN compared to the other samples. The separation of R1 from the other reactor samples (R2, R3, and R4) is mainly due to its high content of Cloacimonetes (WWE1) but also of Bacteroidetes.

Of the 324 to 496 genera detected in the rarefaction analysis (Figure 2), 44 genera were characterized as highly abundant as each of them harbored ≥0.1% of the reads in one or more of the metagenomes (Figure 6). *Candidatus Cloacimonas* (of the phylum Cloacimonetes (WWE1)) [43] is the most abundant genus in the reactor samples, where it represented from 3.26% (R2) to 10.10 % (R1) of the reads.

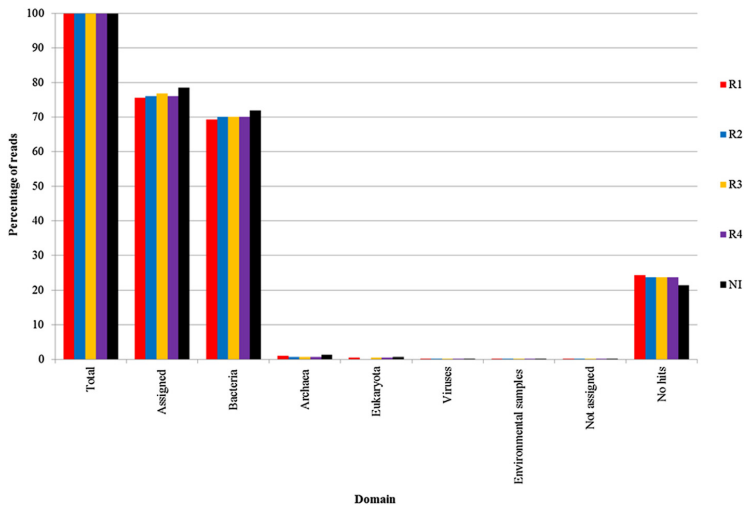

Figure 3 Taxonomic distribution at the domain level in MEGAN. Reads assigned at the domain level given as percentage of total reads in each metagenome. "No hits" are reads without hits in the BLAST search. "Not assigned" are reads with a hit in BLAST, but with no assignment to a taxon due to the settings in MEGAN. "Environmental samples" are reads with hits in other metagenome sequences with unknown biological classification.

The abundance of this taxon in the inoculum (IN) is considerably lower (0.12%). An increasing abundance of phylum Cloacimonetes (WWE1) over longer anaerobic digestion periods has been observed previously [44]. The species *Candidatus Cloacimonas acidaminovorans* has not been cultivated, and the complete genome was reconstructed from a metagenomic analysis of a biogas reactor digesting municipal wastewater [45]. *In silico* proteome analysis indicated that this bacterium derived most of its carbon and energy from the fermentation of amino acids. The gene content suggests *Candidatus Cloacimonas acidaminovorans* to be a syntroph producing H_2 and CO_2 from formate, and this strain is probably present in many anaerobic digesters [45].

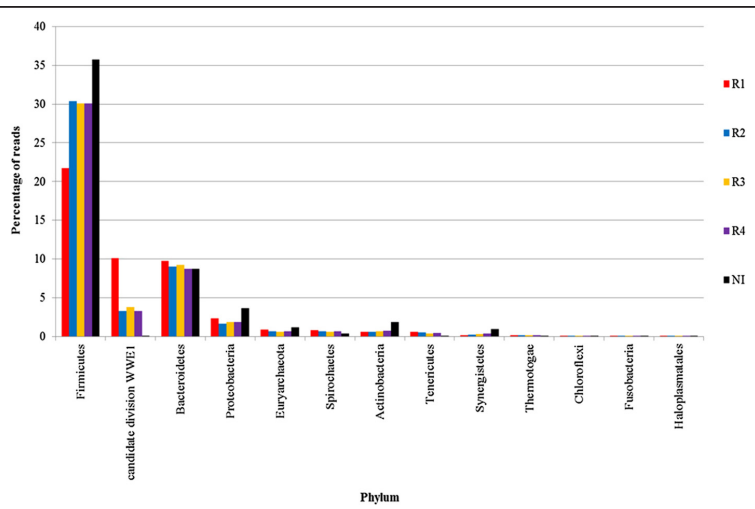

Figure 4 Percentage of reads assigned to prokaryotic phyla with more than 0.1% of total reads assigned.

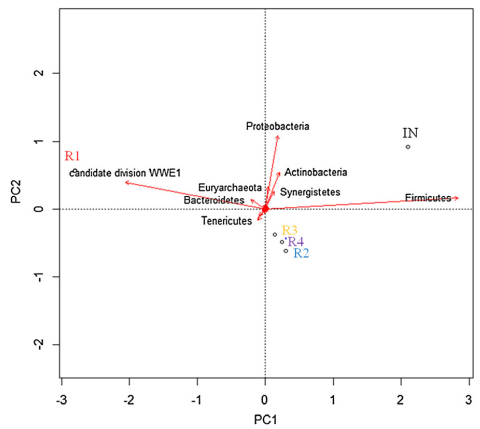

Figure 5 PCA of phyla with Euclidean distance greater than 0.1 from origo. Reads with no hits in the blast search and reads not assigned by MEGAN are excluded. The metagenomic parameters are represented by red arrows. Labels are shown for parameters with Euclidean distance over 0.1 from origo. All metagenome data were given as percentage of total reads.

Altogether 25 genera of the phylum Firmicutes were among the highly abundant genera (Figure 6). The two genera, *Clostridium* and *Syntrophomonas*, each accounted for about 1 to 2% of the reads in all the five metagenomes. The 23 remaining highly abundant Firmicutes genera were: *Alkaliphilus*, *Anaerococcus*, *Bacillus*, *Dethiobacter*, *Butyrivibrio*, *Desulfitobacterium*, *Desulfosporosinus*, *Desulfotomaculum*, *Eubacterium*, *Halothermothrix*, *Lactobacillus*, *Mahella*, *Moorella*, *Paenibacillus*, *Pelotomaculum*, *Peptoniphilus*, *Ruminococcus*, *Streptococcus*, *Syntrophothermus*, *Tepidanaerobacter*, unclassified Clostridiales (miscellaneous), unclassified Erysipelotrichaceae, and unclassified Lachnospiraceae. The following six genera of the phylum Bacteroidetes were also among the highly abundant taxa (Figure 6): *Alistipes*, *Bacteroides*, *Dysgonomonas*, *Odoribacter*, *Porphyromonas*, and *Prevotella*. Three genera of the phylum Synergistetes (*Anaerobaculum*, *Anaerophaga*, *Synergistes*), three genera of the Archaeal phylum Euryarchaeota (*Methanobrevibacter*, *Methanoculleus*, *Methanosarcina;* all methane producers) and two genera of the phylum Spirochaetes (*Treponema* and *Sphaerochaeta*) were also highly abundant in the metagenomes. In addition, we detected the following genera as highly abundant (phylum indicated in brackets): *Acholeplasma* (Tenericutes), *Corynebacterium* (Actinobacteria), *Haloplasma* (unclassified Bacteria), and *Pseudomonas* (Proteobacteria).

The most abundant genus of Firmicutes in the biogas reactors was *Clostridium*. In general, Clostridia are known to be involved in the hydrolytic digestion of macromolecular compounds in the first step of a fermentation process, and therefore play a crucial role in biogas production [38,46,47].

The taxonomic analysis revealed great diversity of highly abundant genera in all samples. Still the high

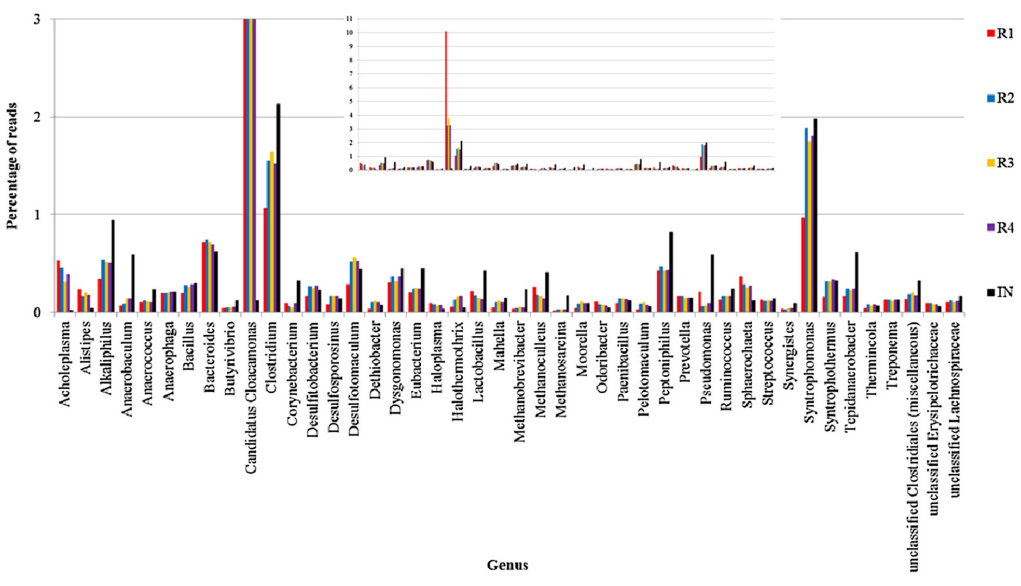

Figure 6 Percentage of reads assigned to the 44 genera with more than 0.1% reads assigned. Insert shows full scale of the Y-axes.

abundance of *Candidatus Cloacimonas*, *Clostridium*, and *Syntrophomonas* indicated a major role of these genera in the biogas reactors and in the inoculum. Abundance shifts in the reactor samples compared to the inoculum at the genus level are illustrated in Figure 7. The predominant change is the large increase of *Candidatus Cloacimonas* in the reactor samples, especially R1, indicating an important role of this genus in the reactors. There is also a relatively large increase in the abundance of *Acholeplasma*, while *Pseudomonas*, *Anaerobaculum*, *Corynebacterium*, *Methanobrevibacter*, and *Methanosarcina* are among the genera most reduced in their abundance compared to the inoculum.

To further study the clustering of the metagenomes, a PCA plot at the genus level was constructed. All genera were included, but reads with no hits were excluded (Figure 8). When the genus level was used, the metagenomes of R2, R3, and R4 clustered more closely than in the PCA plot at the phylum level (Figure 6). The overall clustering pattern of the samples at the genus level is however similar to the clustering detected at the phylum level. This supports consistency in the clustering analysis using PCA and shows that the same clustering is expressed at two quite different taxonomic levels of these complex metagenomes. Figure 8 shows that it is the significantly higher abundance of the genus *Candidatus Cloacimonas* in R1 that gives the major contribution to R1's separation from samples R2, R3, and R4. Inspection of the MEGAN charts showed that all reads of this genus were further assigned to the strain *Candidatus Cloacimonas acidaminovorans* str. Evry. As suggested from a reconstruction of the complete genome [45], this uncultivated strain is probably a syntrophic bacterium that is present in many anaerobic digesters.

In a previous study carried out by Kovács *et al.* [48], changes in the composition of the microbial community were detected through the use of a highly parallel SOLiD® (Sequencing by Oligo Ligation and Detection) next generation DNA sequencing on samples from fed-batch reactors fed with a low C/N ratio substrate. It was found that the bacterium *Candidatus Cloacimonas acidaminovorans* disappeared when the reactors were added a protein monosubstrate with a C/N ratio of 3. This bacterium is not capable of producing polyamines and a number of other cofactors. In our experiments we observed an increase in the abundance of *Candidatus Cloacimonas acidaminovorans* in all reactors (Figure 6).

The Firmicutes genus *Syntrophomonas* strongly influenced the clustering of R2, R3, and R4 in the lower right quadrant. It should be noted that the abundance of this genus is much less in R1 compared to the other reactors and the inoculum. Inspection of the MEGAN charts revealed the strain *Syntrophomonas wolfei* as the predominant *Syntrophomonas* in all the five metagenomes.

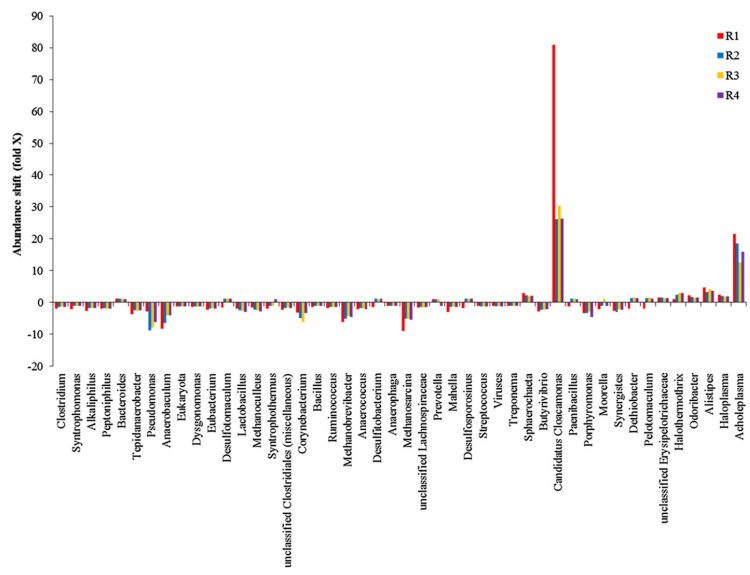

Figure 7 Abundance shift compared to inoculum at the genus level. This figure shows the fold change in abundant genera (>0.1% in one or more metagenomes) in the reactor samples compared to the inoculum. Fold change values less than 1 were replaced by the negative of their inverse.

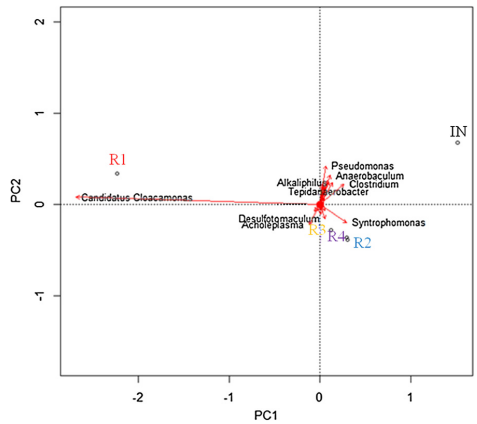

Figure 8 PCA of genera with Euclidean distance from origo greater than 0.1. Reads with no hits in the BLAST search and reads not assigned by MEGAN are excluded. The metagenomic parameters are represented by red arrows. Labels are shown for parameters with Euclidean distance over 0.1 from origo. All metagenome data were given as percentage of total reads.

S. wolfei is a Gram-negative bacterium isolated from anaerobic environments like aquatic sediments or sewage sludge [49]. This organism is able to beta-oxidize saturated fatty acids (C4 to C8 fatty acids) anaerobically to acetate, or to acetate and propionate, in the presence of a syntrophic partner [50]. Fatty acid degradation also leads to production of H_2, which is consumed by a syntrophic methanogenic partner (the Methanomicrobiales strain *Methanospirillum hungatei* has been reported) [51]. The syntrophic H_2 transfer mechanism from *Syntrophomonas* to the methanogen is probably mediated by format because H_2 cannot diffuse rapidly enough to account for the level of methane synthesis in methanogenic cultures [52]. Another synergist known to be involved in syntrophic acetate oxidation under high NH_4^+ concentrations, *Tepidanaerobacter acetatoxydans* [10,11], was detected in our biogas reactors, with higher abundance in R2, R3, and R4 than in R1. Potential methanogenic syntrophic partners to *Syntrophomonas* were also present in the metagenomes. The methanogenic genus *Methanospirillum* was present with low density in all the reactor samples in this study (data not shown), but the genera *Methanoculleus* and *Methanobrevibacter* (Figure 6) were abundant. Overall, the high abundance of syntrophic Bacteria indicates that syntrophic methane production is important in these reactors.

Methanogenesis and subsystems of metabolism

The methanogenic Archaea play a major role in the global carbon cycle by carrying out the final methane-producing step in the anaerobic degradation of organic materials. Methanogens typically thrive in environments where all electron acceptors other than CO_2 are depleted.

Inspection of the MEGAN charts of *Euryarchaeota* at the genus level revealed great diversity in all metagenomes. The genus *Methanoculleus* of the order Methanomicrobiales, followed by the genus *Methanobrevibacter* of the order Methanobacteriales (both orders are known to produce methane from H_2 and CO_2) were the most abundant in all the samples (Table 4). *Methanosarcina* and *Methanosaeta* of the order Methanosarcinales were present, but the abundance was significantly lower. Members of the genus *Methanoculleus* are among the most prevalent methanogens found in wastewater, sewage bioreactors, and landfills [53]. All reads of the genus *Methanoculleus* in the MEGAN analyses were further assigned to the species *Methanoculleus marisnigri* JR1. This organism has all genes required for methanogenesis from hydrogen and CO_2 [54]. In addition this organism can use formate and secondary alcohols such as propanol and butanol as electron donors in methanogenesis. The high abundance of Methanomicrobiales in the reactor samples is in consistence with the relative high VFA levels in the reactors, which indicate high hydrogen production. The high levels of acetate in the reactors are in accordance with the abundance of the methanogenic genus *Methanosarcina* (*M. acetivorans, M. barkeri,* and *M. mazei*). These methanogens are known to be capable of using all the three degradation pathways for methane formation (acetate, methyl, and hydrogen). Acetate cleavage has been regarded to be dominated by Methanosarcinaceae at high acetate concentrations and by Methanosaetaceae at low acetate concentrations [55]. Absence of Methanosaetaceae is also correlated with acetate oxidation [55].

The abundance of *Methanoculleus*, *Methanobrevibactor, Methanosarcina*, and *Methanosaeta* in the reactor samples indicates that the methane production was carried out by both hydrogenotrophic and acetoclastic methanogenesis. Figure 9 shows the results from a KEGG analysis of functional enzymes involved in methane production. Enzymes for methane formation from both CO_2 and hydrogen, and acetate were present in the reactors. These results support the assumption that methane was formed from both hydrogenotrophic and acetoclastic reaction pathways in the reactors.

Figure 10 shows the results from the KEGG analysis of metabolic systems that are related to methane production, including metabolism of amino acids, energy, carbohydrates, nucleotides, lipids, cofactors, vitamins, polyketides, terpenoids, glycan, and xenobiotics. These metabolic activities are associated with the conversion of biomass into methane during anaerobic fermentation. The results show that a large amount of reads are distributed among amino acid metabolism and carbohydrate metabolism.

Table 4 Percentage of reads assigned to the most abundant methanogenic genera

Metagenome	*Methanoculleus*	*Methanobrevibacter*	*Methanosarcina*	*Methanosaeta*
R1	0.264	0.038	0.019	0.021
R2	0.183	0.046	0.034	0.017
R3	0.164	0.057	0.034	0.011
R4	0.145	0.052	0.031	0.018
IN	0.417	0.236	0.171	0.017

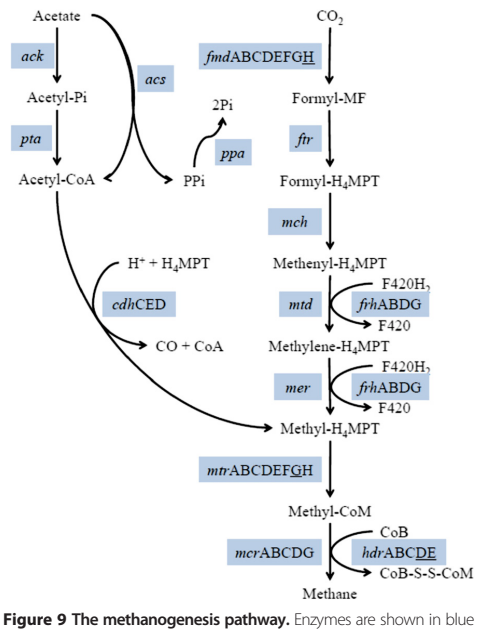

Figure 9 The methanogenesis pathway. Enzymes are shown in blue boxes. Subunits missing in all our datasets (R1, R2, R3, R4, and IN) after search against the KO database at MG-RASTare underlined. Abbreviations used in the figure are Acetyl-Pi: acetyl phosphate; ack: acetate kinase; acs: acetyl-CoA synthetase; cdh: acetyl-CoA decarbonylase/synthase; CO: carbon monoxide; CoA: coenzyme A; CoB: coenzyme B; CoB-S-S-CoM: coenzyme M 7-mercaptoheptanoylthreonine-phosphate heterodisulfide; F420: coenzyme F420; fmd: formylmethanofuran dehydrogenase; Formyl-H4MPT: 5-formyl-5,6,7,8-tetrahydromethanopterin; Formyl-MF: formylmethanofuran; frh: coenzyme F420 hydrogenase; ftr: formylmethanofuran-tetrahydromethanopterin N-formyltransferase; H4MPT: 5,6,7,8-tetrahydromethanopterin; hdr: heterodisulfide reductase; mch: methenyltetrahydromethanopterin cyclohydrolase; mcr: methyl-coenzyme M reductase; mer: 5,10-methylenetetrahydromethanopterin reductase; Methenyl-H4MPT: 5,10-methenyl-5,6,7,8-tetrahydromethanopterin; Methyl-CoM: methylcoenzyme M; Methylene-H4MPT: 5,10-methylenetetrahydromethanopterin; Methyl-H4MPT: 5-methyl-5,6,7,8-tetrahydromethanopterin; mtd: methylenetetrahydromethanopterin dehydrogenase; mtr: tetrahydromethanopterin S-methyltransferase; ppa: inorganic diphosphatase; pta: phosphate acetyltransferase.

This observation is consistent with the finding that many species found in the samples are involved in amino acid and carbohydrate digestion. The amount of protein in the fish waste silage that was added to our reactors during the experiment is 15% (ww), and the high content of protein in the substrate is consistent with the abundant reads for enzymes involved in the amino acid metabolism. In a previous study, Li *et al.* [41] used fat- and protein-rich food waste as a biogas substrate, and they found that a significant amount of reads were obtained for the processes involved in the protein degradation pathway. Among the genes involved in the carbohydrate metabolism, those that degrade cellulose are particularly important for the efficient breakdown of substrates such as co-manure. The high percentage of reads assigned to carbohydrate metabolism and the abundance of the Firmicutes phylum and *Clostridium* genus in our reactors demonstrate the importance of carbohydrate and cellulose degradation by the anaerobic microbial community.

The result on carbohydrate metabolism is in agreement with previous investigations [47,56,57], and the evaluation of metabolic pathways shows that the capabilities of metabolism varied somewhat in the different reactors. Enzymes related to metabolism of amino acids and cofactors and vitamins were highest in the IN sample. Among the reactor samples, R1 in general had the fewest reads assigned to metabolic subsystems, while the highest number of reads assigned to subsystems was detected in R2 and R4.

Methane production in R1 was somewhat lower than in the other reactors. The propionic acid concentration in this reactor was somewhat higher than in the other reactors. This, together with the high abundance of *Candidatus Cloacimonas acidaminovorans* in R1 may have had an impact on this reactor's somewhat lower metabolic capabilities. The high prevalence of the bacterium might have been an inhibiting factor in R1.

Conclusions

Taxonomic and functional studies of inoculum and reactor samples showed that the microbial consortium changed over time in the four reactors during the digestion phase. The results show that the microbial community in the four biogas reactors after 59 days of operation was different

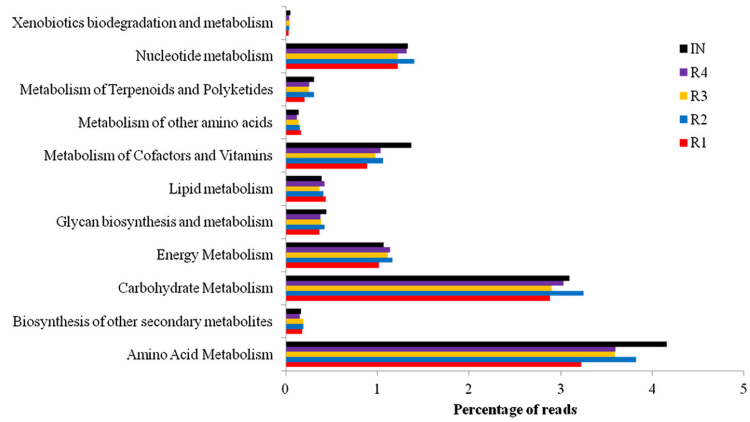

Figure 10 Reads assigned to level 2 metabolism subsystems at MG-RAST (KO database).

from the microbial composition in the inoculum. A greater functional complexity was detected in the inoculum compared to the reactor samples. Microbial communities tend to become more specific and less complex over time when degrading the same substrate. The results showed that the microbial composition developed quite similarly in three of the four parallel reactors during the experiment.

Bacteria from the phylum Firmicutes were most abundant in the reactors, followed by the phyla Bacteroidetes and Proteobacteria. In particular, the species from genera *Clostridium* and *Syntrophomonas* play key roles in the initial degradation of protein, cellulose, and other polysaccharides. These results were further supported by gene functional annotation, where we detected many enzymes involved in protein degradation and carbohydrate metabolism. The dominant methanogens present in the reactors were from Methanomicrobiales, and the most prevalent genus appeared to be *Methanoculleus. Methanobrevibacter, Methanosarcina*, and *Methanosaeta* were also detected in the samples. These methanogens use versatile substrates and contain both acetotrophic and hydrogenotrophic pathways for methane production.

The microbial composition in one reactor (R1) differed from those of the others, especially in relation to the high prevalence of the Bacterium *Candidatus Cloacimonas acidaminovorans*. This reactor also showed lower average methane production and VS removal than the other three reactors, and this might be linked to the difference in microbiology. One possible theory for the dissimilarity is that the high density of the *Candidatus Cloacimonas acidaminovorans* in R1 may have had a negative impact on the syntrophic relationships between Bacteria and methanogens in this reactor. R1 had, in addition to very high values of Cloacimonetes (WWE1), also a low density of Firmicutes, a phylum consisting of many important syntrophic members of the class Clostridia (e.g. *Syntrophomonas*). The reactor also had a low density of the syntrophic acetate-oxidizing bacteria *Tepidanaerobacter acetatoxydans*. It should be noted that the 454 sequencing in this study was not done in replicate, mainly due to the cost of the analysis. While the method is known to be very reproducible [58], future studies of parallel reactors should ideally also include replicate sequencing.

This study showed that four parallel reactors co-digesting manure and fish waste silage operated stably during a startup phase. Clear changes in the microbial population were seen in all four reactors, the most pronounced being the increased abundance of *Candidatus Cloacimonas acidaminovorans*. Additionally, several important Archaea and Bacteria degrading the protein-rich substrate were identified. In particular, microorganisms involved in syntrophic methane production seemed to be important. These results give leads for the design of well-functioning microbial communities for biogas plants digesting similar substrates.

Materials and methods

Inoculum, substrate, and reactors operation

Slurry from a biogas reactor co-digesting a mix of 19% (v/v) fish waste silage and 81% cow manure [1] was used as the inoculum (IN). In a previous study the high amount of amount of fish waste silage led to process inhibition due to overloading of protein and fat. The slurry from this reactor was kept without any addition of substrate for 50 days, until startup of the present experiment. The chemical composition of the inoculum was measured in triplicate samples, and had the following characteristics: pH =8.1 (±0.09), NH_4^+ (g L^{-1}) =5.5 (±0.08), DM (%) =6.3 (±0.06), VS of DM (%) =73.6 (±0.51). The substrate used was a mix of 87% cow manure and 13 % fish waste silage (v/v). The substrate

chemical composition was measured in triplicate samples, and its characteristics are shown in Table 1.

To four 10-L continuously stirred tank reactors, designated R1, R2, R3, and R4, were added 2.55 L inoculum (day 0). From day one substrate was added to the reactors every day until the effective reactor volume was 8 L. The amount of substrate added each day was calculated from the reactors' increasing effective volume and 30 days HRT (for example, based on a reactor with 2.55 L effective volume and an HRT of 30 days: 2.55 L/30 d =85 mL substrate/reactor/day). At day 36 the reactors were fed with 266.7 mL substrate, yielding a final effective volume of 8 L. From day 36 to day 59 (28 days), the reactors were fed at a fixed rate of 266.7 mL substrate/reactor/day (this was based on reactors with 8 L effective volume, and an HRT of 30 days: 8 L/30 d =266.7 mL substrate/reactor/day). The same amount of reactor slurry was removed (prior to substrate addition) each day to maintain the volume at 8 L. The reactors were operated anaerobically at 37°C with a stirring speed of 150 rpm. The total carbon and nitrogen in the substrate was determined in single samples, and the carbon:nitrogen (C/N) ratio was calculated. Approximately 20 g inoculum was collected at day 0, and 50 g slurry from each of the four reactors (R1, R2, R3, and R4) were collected at day 59, and stored frozen (-20°C) in 50 mL Nunc centrifuge tubes prior to DNA extraction.

Chemical analysis procedures

The content of DM and VS, and the pH in the inoculum, the reactor slurries, and in the substrate were determined according to standardized methods [59-61] every fourth day, in triplicate samples.

The NH_4^+ and VFA concentrations in the inoculum and in the reactor slurries were determined every fourth day. The concentration of NH_4^+ was determined in triplicate samples by use of an ammonium selective electrode (Thermo Scientific Orion ISE/NH4). Samples for NH_4^+ analysis were diluted (1:10) in distilled water and measured at 20°C and supplemented with an ionic strength adjustor (28.7 g glacial acetic acid L^{-1} and 53.6 g magnesium acetate L^{-1}), using 10 mL of ionic strength adjustor per 100 mL of sample, for stabilization of NH_4^+. The NH_3 concentrations were calculated based on the NH_4^+ concentrations. The average deviations between the triplicate samples (not shown) were <0.5. Samples for VFA (acetic acid and propionic acid) analysis were centrifuged (13,000 rpm) and filtrated (0.45 μm) prior to analysis. The concentrations of VFAs were determined in single samples, by use of a Rezex RFQ Fast Acid H + (8%) 100 × 7.8 mm HPLC (Phenomenex, Torrance, CA, USA), operated at a temperature of 85°C, with an Ultimate 3000RS column and UV detection at 210 nm (Dionex, Sunnyvale, CA, USA). The samples were diluted with sulfuric acid (8 μL total) before analysis.

The elemental composition of carbon, hydrogen, and nitrogen was determined in the reactors' substrate by combustion using a LECO CHN-1000 instrument (St. Joseph, MI, USA).

The biogas was collected in 25-L Tedlar bags (Tedlar® Gas Sampling Bag, Sigma-Aldrich, St. Louis, MO, USA). CH_4 and CO_2, as a percentage of the gas volume of samples, were measured once a day with a GA2000 Landfill Gas Analyzer (Geotechnical Instruments Ltd., UK). The total gas production rate volume (L/d) was calculated from flow measurements (rate 300 cm^3/min) as follows: (pump-number/60 seconds * 300 cm^3-min) / (1000 mL).

DNA extraction from reactor samples

All samples for DNA extraction were collected at the same time and treated in exactly the same way. In order to achieve homogeneous and representative samples, the inoculum and the reactor slurries were thoroughly stirred before and during sampling. The samples were collected in 100-mL plastic bottles and frozen. The frozen samples of the inoculum (IN) and reactors (R1 to R4) were slowly thawed before the total genomic DNA was extracted from duplicate subsamples using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA), according to the producer's instruction. Lysis and homogenization of the samples were performed in a Bertin Technologies (Rockville, MD) Precellys 24 system, for 2 × 20 seconds at speed 5400. Each subsample was eluted from the columns with 100 μL DNase/pyrogen-free water (DES). The combined eluates were purified using a Wizard® DNA Clean-Up System (Promega, Madison, WI) and finally eluted from the Wizard column with 50 μl DES. The DNA purity and concentrations were measured in a NanoVue spectrophotometer and Qubit assay using the Qubit® 2.0 Fluorometer. The DNA quality and chain length were inspected in 1.2% agarose (Biozyme RESult, LE General Purpose Agarose) gel in 1× Tris-acetate-EDTA with added 20 μL SYBR Safe DNA gel stain, 10,000 concentration in DMSO (Life Technologies, Grand Island, NY) to a final gel volume of 200 mL. DNA extracts were added using TrackIt™ Cyan/Yellow Loading buffer (Invitrogen) to a final volume of 10 μL. Three microliters of Trackit™ 100 bp DNA Ladder (Invitrogen) were used. The agarose gel was run at 100 V for 90 minutes. Images of the gel were made using a KODAK Gel Logic 212 Imaging System for inspection of the chain length prior to 454 pyrosequencing.

454 pyrosequencing

Sample preparation and sequencing of extracted DNA were performed at the High Throughput Sequencing Centre at CEES, University of Oslo [62], according to standard 454 GS FLX Titanium protocol. The five samples were tagged, mixed, and sequenced on a 70 × 75 format PicoTiterPlate™ on a GS FLX Titanium instrument. The

sequence data have been submitted to the NCBI database (http://www.ncbi.nlm.nih.gov) under BioProject accession number PRJNA261310.

Quality filtering

The complete datasets were analyzed with PRINSEQ [63] to determine the sequence quality scores. For each sample we performed quality filtering to remove low quality reads (reads containing ≥10 ambiguous bp, homopolymers of ≥10 bp, and sequence length <100 bp) in mothur v.1.25.1 [64]. The trimmed files were checked for artificial replicates using cdhit-454 with standard settings [65]. The cleaned files were analyzed with PRINSEQ before the files were uploaded at the Bioportal computer service [66] for Blast X against the NCBI non-redundant Protein database (ncbiP-nr). The maximum expectation value was set to 10^{-3}, and a maximum of 25 alignments were reported per hit.

Effective genome size

The effective genome size (EGS) for each metagenome was estimated according to the method developed by Raes *et al.* [37], using the constants a =18.26, b =3650, and c =0.733. A protein reference database containing the 35 single copy COGs in question was downloaded from STRING (v. 9.0) [67]. BlastX was conducted at the freely available Bioportal computer service [68]. The sampling probability of a random universal single copy gene (1000 bases) and expected number of reads detected were calculated according to Beszteri *et al.* [69].

Taxonomic classification

The BlastX output files were analyzed according to NCBI taxonomy in the program MEGAN, version 4 [70,71] with default LCA parameters (Min Score: 35, Top Percent: 10.0, and Min Support: 5). All taxa were enabled.

Principal component analysis

The PCA plots were created using the vegan library in R [72] as previously described [73]. The ordination was based on reads assigned to the phylum and to the genus level in MEGAN. All metagenome data were given as a percentage of total reads.

Metabolic annotation

The metagenomic reads were assigned to subsystems on the MG-RAST server [74] (version 3.3.9) [75]. The KEGG Orthology (KO) reference database was used. The maximum expectation value was set to 10^{-5}, the minimum alignment length was set to 50 bases, and the minimum percentage identity was set to 50%. We used the same settings to search the metagenomes for key genes involved in methanogenesis.

Abbreviations

Acetyl-Pi: acetyl phosphate; ack: acetate kinase; acs: acetyl-CoA synthetase; cdh: acetyl-CoA decarbonylase/synthase; CO: carbon monoxide; CoA: coenzyme A; CoB: coenzyme B; CoB-S-S-CoM: coenzyme M 7-mercaptoheptanoylthreonine-phosphate heterodisulfide; DES: DNase/pyrogen-free water; DM: dry matter; DMSO: dimethyl sulfoxide; F420: coenzyme F420; fmd: formylmethanofuran dehydrogenase; Formyl-H4MPT: 5-formyl-5,6,7,8-tetrahydromethanopterin; Formyl-MF: formylmethanofuran; frh: coenzyme F420 hydrogenase; ftr: formylmethanofuran-tetrahydromethanopterin N-formyltransferase; H4MPT: 5,6,7,8-tetrahydromethanopterin; hdr: heterodisulfide reductase; mch: methenyltetrahydromethanopterin cyclohydrolase; mcr: methyl-coenzyme M reductase; mer: 5,10-methylenetetrahydromethanopterin reductase; Methenyl-H4MPT: 5,10-methenyl-5,6,7,8-tetrahydromethanopterin; Methyl-CoM: methylcoenzyme M; Methylene-H4MPT: 5,10-methylenetetrahydromethanopterin; Methyl-H4MPT: 5-methyl-5,6,7,8-tetrahydromethanopterin; mtd: methylenetetrahydromethanopterin dehydrogenase; mtr: tetrahydromethanopterin S-methyltransferase; ppa: inorganic diphosphatase; pta: phosphate acetyltransferase; VFA: volatile fatty acid; VS: volatile solids.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SJH and AGR conceptualized the work and wrote parts of the manuscript. LS did all the experimental work with the biogas reactors and wrote most of the manuscript. AGR planned and carried out the DNA extraction and metagenome analysis. OEH contributed to the metagenome analysis and made several of the figures. All authors read and approved the final manuscript.

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References

1. Solli L, Bergersen O, Sørheim R, Briseid T: **Effects of a gradually increased load of fish waste silage in co-digestion with cow manure on methane production.** *Waste Manage* 2014, **34:**1553–1559.
2. **Statistics Norway. SSB. 2013.Avfallsregnskapet 2011.** http://www.ssb.no/natur-og-miljo/statistikker/avfregno.
3. Daniel SL, Drake HL: **Oxalate- and glyoxylate-dependent growth and acetogenesis by *Clostridium thermoaceticum*.** *Appl Environ Microbiol* 1993, **59:**3062–3069.
4. Anderson I, Ulrich LE, Lupa B, Susanti D, Porat I, Hooper SD, Lykidis A, Sieprawska-Lupa M, Dharmarajan L, Goltsman E, Lapidus A, Saunders E, Han C, Land M, Lucas S, Mukhopadhyay B, Whitman WB, Woese C, Bristow J, Kyrpides N: **Genomic characterization of methanomicrobiales reveals three classes of methanogens.** *PLoS One* 2009, **4:**6.
5. Zinder SH: **Physiological ecology of methanogenesis.** In *Methanogenesis. Ecology, Physiology, Biochemistry and Genetics. Volume 1.* 1st edition. Edited by Ferry JG. New York: Chapman and Hall; 1993:128–206.
6. Garcia JL, Patel BKC, Ollivier B: **Taxonomic, phylogenetic and ecological diversity of methanogenic Archaea.** *Anaerobe* 2000, **6:**105–226.

7. Liu Y, Whitman WB: **Metabolic, phylogenetic, and ecological diversity of the methanogenic Archaea.** *Annu N Y Acad Sci* 2008, **1125:**171–189.
8. Schnurer A, Zellner G, Svensson BH: **Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors.** *FEMS Microbiol Ecol* 1999, **29:**249–261.
9. Schnurer A, Nordberg Å: **Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature.** *Water Sci Technol* 2008, **57:**735–740.
10. Westerholm M, Dolfing J, Sherry A, Grey ND, Head IM, Schnurer A: **Quantification of syntrophic acetate oxidizing microbial communities in biogas process.** *Environ Microbiol Reports* 2011, **3:**500–505.
11. Westerholm M, Muller B, Arthurson V, Schnurer A: **Changes in the acetogenic population in a mesophilic anaerobic digester in response to increasing ammonia concentration.J.** *Microbes Environ* 2011, **26:**347–353.
12. Chen Y, Cheng JJ, Creamer KS: **Inhibition of anaerobic digestion process: a review.** *Bioresource Technol* 2008, **99:**4044–4064.
13. Pandey PK, Ndegwa PM, Soupir ML, Alldrege RJ, Pitts MJ: **Efficacies of inocula on the startup of anaerobic reactors treating dairy manure under stirred and unstirred conditions.** *Biomass Bioenerg* 2011, **35:**2705–2720.
14. Demirel B, Yenigun O: **Two-phase anaerobic digestion process: a review.** *J Chem Technol Biotechnol* 2002, **77:**743–755.
15. Weiland P: **Biogas production: current state and perspectives.** *Appl Microbiol Biot* 2010, **85:**849–860.
16. Harmsen HJN, Kengen HMP, Akkermans ADL: **Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16 s rRNA-based oligonucleotide probes.** *Appl Environ Microbiol* 1996, **62:**1656–1663.
17. Amani T, Nosrati M, Mousavi SM, Kermanshahi RK: **Analysis of the syntrophic anaerobic digestion of volatile fatty acids using enriched cultures in a fixed-bed reactor.** *Water Environ Res* 2012, **84:**460–472.
18. Nielsen HB, Angelidaki I: **Strategies for optimizing recovery of the biogas process following ammonia inhibition.** *Bioresource Technol* 2007, **99:**7995–8001.
19. Angelidaki I, Ahring BK: **Thermophilic anaerobic digestion of livestock waste: the effect of ammonia.** *Appl Microbiol Biot* 1993, **38:**560–564.
20. Angelidaki I, Ellegaard L, Ahring BK: **A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition.** *Biotechnol Bioeng* 2004, **42:**159–166.
21. Yen HW, Brune DE: **Anaerobic co-digestion of algal sludge and waste paper to produce methane.** *Bioresource Technol* 2007, **98:**130–134.
22. Angelidaki I, Ahring BK: **Effects of free long-chain fatty acids on thermophilic anaerobic digestion.** *Appl Microbiol Biot* 1992, **37:**808–812.
23. Biebl H: **Fermentation of glycerol by*Clostridium pasteurianum*- batch and continuous culture studies.** *J Ind Microbiol Microbiot* 2001, **27:**18–26.
24. El-Mashad HM, Zhang R: **Biogas production from co-digestion of dairy manure and food waste.** *Bioresourse Technol* 2010, **101:**4021–4028.
25. Chen G, Zheng Z, Yang S, Fang C, Zou X, Zhang J: **Improving conversion of*Spartina alterniflora*into biogas by co-digestion with cow feces.** *Fuel Process Technol* 2010, **91:**1416–1421.
26. Lehtomaki A, Huttunen S, Rintala JA: **Laboratory investigations of energy crops and crop residues with cow manure for methane production: effect of crop to manure ratio.** *Resour Conserv Recy* 2007, **51:**591–609.
27. Regueiro L, Veiga P, Figueroa M, Alonso-Gutierrez J, Stams AJM, Lema JM, Carballa M: **Relationship between microbial activity and microbial community structure in six full-scale anaerobic digesters.** *Microbiol Res* 2012, **167:**581–589.
28. Ike M, Inoue D, Miyano T, Liu TT, Sei K, Soda S, Kadoshin S: **Microbial population dynamics during startup of a full-scale anaerobic digester treating industrial food waste in Koyoto eco-energy project.** *Bioresource Technol* 2010, **101:**3952–3957.
29. Van Velsen AFM: **Adaptation of methanogenic sludge to high ammonia-nitrogen concentrations.** *Water Res* 1979, **13:**995–999.
30. Griffin ME, McMahon KD, Mackie RI, Raskin L: **Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids.** *Biotechnol Bioeng* 1998, **57:**342–355.
31. Raposo F, Borja R, Martín MA, Martín A, de la Rubia MA, Rincón B: **Influence of inoculum to substrate ratio on the anaerobic digestion of sunflower oil cake in batch mode: process stability and kinetic evaluation.** *Chem Eng J* 2009, **149:**70–77.
32. Lins P, Reitschuler C, Illmer P: **Development and evaluation of inocula combating high acetate concentrations during the start-up of an anaerobic digestion.** *Bioresource Technol* 2012, **110:**167–173.
33. Hansen KH, Angelidaki I, Ahring BK: **Anaerobic digestion of swine manure: inhibition by ammonia.** *Water Res* 1998, **32:**5–12.
34. Hashimoto AG: **Ammonia inhibition of methanogenesis from cattle wastes.** *Agric Wastes* 1986, **17:**241–261.
35. Ma J, Carballa M, De Caveye PV, Verstraete W: **Enhanced propionic acid degradation (EPAD) system: Proof of principle and feasibility.** *Water Res* 2009, **43:**3239–3248.
36. Gallert C, Winter J: **Propionic acid accumulation and degradation during restart of a full scale anaerobic biowaste digester.** *Bioresource Technol* 2008, **99:**170–178.
37. Raes J, Korbel JO, Lercher MJ, vor Mering C, Bork P: **Prediction of effective genome size in metagenomic samples.** *Genome Biol* 2007, **8:**19.
38. Wirth R, Kovacs E, Maróti G, Bagi Z, Rakhely G, Kovacs KL: **Characterization of a biogas producing microbial community by short read next generation DNA sequencing.** *Biotechnol Biofuels* 2012, **5:**1–16.
39. Krause L, Diaz NN, Edwards RA, Gartemann K-H, Krömeke H, Neuwger H, Pühler A, Runte KJ, Schlüter A, Stoye J, Szczepanowski R, Tauch A, Goesmann A: **Taxonomic composition and gene content of a methaneproducing microbial community isolated from a biogas reactor.** *J Biotech* 2008, **136:**91–101.
40. Sundberg C, Al-Soud WA, Larsson M, Alm E, Yekta SS, Svensson BH, Sørensen SJ, Karlsson A: **454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters.** *FEMS Microbiol Ecol* 2013, **85:**612–626.
41. Li A, Chu YN, Wang X, Ren L, Yu J, Liu X, Yan J, Zhang L: **A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor.** *Biotechnol Biofuels* 2013, **6:**3.
42. Klocke M, Mähnerta P, Mundt K, Souidia K, Linke B: **Microbial community analysis of a biogas-producing completely stirred tank reactor fed continuously with fodder beet silage as mono-substrate.** *Syst Appl Microbiol* 2007, **30:**139–151.
43. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu W-T, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T: **Insights into the phylogeny and coding potential of microbial dark matter.** *Nature* 2013, **499:**431–437.
44. Li YF, Chen PH, Yu Z: **Spatial and temporal variations of microbial community in a mixed plug-flow loop reactor fed with dairy manure.** *Microbial Biotechnol* 2014. doi:10.1111/1751-7915.12125.
45. Pelletier E, Kreymeyer A, Bocs S, Rouy Z, Gyapay G, Chouari R, Riviere D, Ganesan A, Daegelen P, Sghir A, Cohen GN, Medigue C, Weissenbach J, Paslier DL: **"Candidatus Cloacamonas acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division.** *J Bacteriol* 2008, **190:**2572–2579.
46. Zakrzewski M, Goesmann A, Jaenicke S, Junemann S, Eikmeyer F, Szczepanowski R, Al-Soud WA, Sørensen S, Pühler A, Schluter A: **Profiling the metabolically active community from a production scale biogas plant by means of high throughput metatranscriptome sequencing.** *J Biotechnol* 2012, **158:**248–258.
47. Schluter A, Bekel T, Diaz NN, Dondru M, Eichenlaub R, Gartemann KH, Krahn I, Krause L, Kromeke H, Kruse O, Mussgnug JH, Neuweger H, Niehaus K, Pühler A, Runte KJ, Szczepanowski R, Tauch A, Tilker A, Viehover P, Goesmann A: **The metagenome of a biogas producing microbial community of a production scale biogas plant fermenter analyzed by the 454-pyrosequencing technology.** *J Biotechnol* 2008, **136:**77–90.
48. Kovács E, Wirth R, Maróti G, Bagi Z, Rákhely G, Kovács KL: **Biogas production from protein-rich biomass: fed-batch anaerobic fermentation of casein and of pig blood and associated changes in microbial community composition.** *PLoS One* 2013, **8:**10.
49. McInerney MJ, Bryant MP, Hespell RB, Costerton JW: ***Syntrophomonas wolfei*gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium.** *Appl Environ Microbiol* 1981, **41:**1029–1039.
50. Sousa DZ, Smidt H, Alves M, Stams AJM: **Ecophysiology of syntrophic communities that degrade saturated and unsaturated long-chain fatty acids.** *FEMS Microbiol Ecol* 2009, **68:**257–272.
51. Scholten JCM, Conrad R: **Energetics of syntrophic propionate oxidation in defined batch and chemostat cocultures.** *Appl Environ Microbiol* 2000, **66:**2934–2942.
52. Boone DR, Johnson RL, Liu Y: **Diffusion of the interspecies electron carriers H(2) and formate in methanogenic ecosystems and its**

implications in the measurement of K(m) for H(2) or formate uptake. *Appl Environ Microbiol* 1989, **55:**1735–1741.
53. Tabatabaeia M, Rahimc RA, Abdullahd N, Wrighte ADG, Shiraif Y, Sakaig K, Sulaimanh A, Hassan MA: **Importance of the methanogenic Archaea populations in anaerobic wastewater treatments.** *Process Biochem* 2010, **45:**1214–1225.
54. Sun L, Müller B, Westerholm M, Schnürer A: **Syntrophic acetate oxidation in industrial CSTR biogas digesters.** *J Biotechnol* 2013, **171:**39–44.
55. Karakashey D, Batstone DJ, Trably E, Angelidaki I: **Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae.** *Appl Environ Microbiol* 2006, **72:**5138–5141.
56. Kröber M, Bekel T, Diaz NN, Goesmann A, Sebastian J: **Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing.** *J Biotech* 2009, **142:**38–49.
57. Jaenicke S, Ander C, Bekel T, Bisdorf R, Dröge M, Gartemann K-H, Jünemann S, Kaiser O, Krause L, Tille F, Zakrzewski F, Pühler A, Schlüter A, Goesmann A: **Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing.** *PLoS One* 2011, **6:**1.
58. Pilloni G, Granitsiotis MS, Engel M, Lueders T: **Testing the limits of 454 pyrotag sequencing: reproducibility. Quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes.** *PLoS One* 2012, **7:**7.
59. ISO 11465: **Soil quality. Determination of dry matter and water content on a mass basis. Gravimetric method.** 1993.
60. NS-EN 15935: **European Standard method for the determination of the loss on ignition (LOI) of dry matter at 550°C.** 2012.
61. ISO 10390: **Soil quality. Determination of pH.** 2005.
62. **Norwegian Sequencing Centre.** http://www.sequencing.uio.no.
63. Schmieder R, Edwards R: **Quality control and preprocessing of metagenomic datasets.** *Biogeosciences* 2011, **27:**863–864.
64. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF: **Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities.** *Appl Environ Microbiol* 2009, **75:**7537–7541.
65. Niu BF, Fu LM, Sun SL, Li WZ: **Artificial and natural duplicates in pyrosequencing reads of metagenomic data.** *BMC Bioinformat* 2010, **11:**187–198.
66. **BIOPORTAL.UIO.NO.** http://www.mn.uio.no/ibv/bioportal/index.html.
67. **STRING - Known and Predicted Protein-Protein Interactions.** http://string-db.org/.
68. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool.** *J Mol Biol* 1990, **215:**403–410.
69. Beszteri B, Temperton B, Frickenhaus S, Giovannoni SJ: **Average genome size: a potential source of bias in comparative metagenomics.** *ISME J* 2010, **4:**1075–1077.
70. Huson DH, Auch AF, Qi J, Schuster SC: **MEGAN analysis of metagenomic data.** *Genome Res* 2007, **17:**377–386.
71. Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC: **Integrative analysis of environmental sequences using MEGAN4.** *Genome Res* 2011, **21:**1552–1560.
72. **The R Project for Statistical Computing.** http://www.r-project.org.
73. Havelsrud OE, Haverkamp THA, Kristensen T, Jakobsen K, Rike AG: **Metagenomic and geochemical characterization of pockmarked sediments overlaying the troll petroleum reservoir in the North Sea.** *BMC Microbiol* 2012, **12:**203.
74. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA: **The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes.** *BMC Bioinformat* 2008, **9:**386.
75. **MG-RAST. Metagenomic Analysis Server.** http://metagenomics.anl.gov/.

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PAPER III

III

Process performance and population dynamics of ammonium tolerant microorganisms during co-digestion of fish waste and manure

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Abstract

Four lab scale biogas reactors (CSTR) were operated with hydraulic retention times of 20, 25, 30 and 40 days, and fed with a substrate composition of equal ratios of fish waste silage and manure fixed at 13 and 87 volume %, respectively. Co digestion of fish waste silage and manure at different HRTs was evaluated with regard to biogas process performance and stability in terms of methane yields, NH_4^+ accumulation and abundance of NH_4^+-tolerant microorganisms. The process performance in reactors operated with different HRTs were compared to the process performance and abundance of NH_4^+-tolerant microorganisms in reactors operated with equal HRT, fed with increased ratios of fish waste silage in co digestion with manure. The process performance and abundance of NH_4^+-tolerant microorganisms were stable in reactors operated with different HRTs and equal ratios of fish waste and manure in the feedstock. In the reactors added increased ratios of fish waste, it was observed an elevated concentration of NH_4^+ and abundance of NH_4^+-tolerant acetate oxidizing bacteria. The biogas process failed in these reactors simultaneously with an observed shift in microbial composition when the ratio of fish waste silage was increased. In particular, the bacterium *Tepidanaerobacter Acetatoxydans* seemed to affect the biogas process stability at high NH_4^+ concentrations. The methanogens *Methanosarcinaceae* and *Methanosaetaceae* dominated at low levels of NH_4^+ and loadings of fish waste, while the hydrogenotrophic Methanomicrobiales increased in abundance in response to higher fish waste loading rates and NH_4^+ concentrations. This study showed that it is possible to utilize fat- and protein-rich fish waste as a biogas substrate, even at low HRT. By decreasing the HRT from 30 to 20 days in reactors added 13% fish waste in

mix with manure, the loading volume of fish waste can be increased without process failure.

Keywords: fish waste silage, anaerobic digestion, biogas, SAOB, ammonium

1. Introduction

Fishery and aquaculture are growing industries with high nutritional and commercial value [1]. However, it is produced huge amounts of organic waste, both from fishery and fish farming, and the two main waste streams from these industries are offals and sludge. Processing the organic waste from fish industries is challenging, and focus on waste management, energy production and nutrient recycling to achieve sustainable operation has increased during recent years. Norway is the world's largest producer of Atlantic salmon [2] and the Norwegian government aims to further increase the production from fish industry. During processing around 40% of each fish is disposed of as waste [3] and moreover, a significant proportion of farmed fish die mainly due to parasites and other diseases [2], [4] For example, in 2015 a total of more than 50 million dead salmon were reported by Norwegian fish farms [5]. This organic waste represents a potential valuable resource and alternative usage would also reduce the disposal costs. During recent years, use of fish waste as a source of renewable energy production has been exploited, in particular for production of biodiesel and biogas [6]. Some of the dead fish waste from Norwegian salmon production is ensiled with formic acid to stabilise it during storage. Such ensiled fish waste contains large amounts of energy-rich proteins and fats and has been shown to have large biogas potential [7]. However,

proteins and fats are also components that can create problems during anaerobic digestion, due to release of total ammonium, NH_4^+ (NH_3 and NH_4^+) and fatty acids (LCFA and VFA), respectively. Formation of NH_4^+ may initially have a positive impact on the anaerobic digestion process by buffering the low pH of ensiled fish waste and also results in a nitrogen-rich digestate with high value as a fertiliser [8]. However, NH_4^+ can also cause severe problems due to inhibition of the microbial community, specifically methanogens [9]. Inhibition of methanogens by NH_4^+ accumulation is a common cause of biogas process failure in reactors fed protein-rich substrate [9]–[11]. LCFA can be degraded anaerobically, but are also known to cause inhibition of the biogas process, in particular inhibition of gram-positive bacteria and methanogens [12]. However, anaerobic digestion of fish waste has been shown to be stable and efficient in co-digestion with manure, a substrate with low protein and fat concentrations and high buffering capacity [7], [13]–[15]. Anaerobic digestion proceeds through a series of degradation steps engaging four main groups of microorganisms: hydrolytic bacteria, fermenting bacteria, organic acid-oxidising bacteria and methanogenic Archaea [9], [16]. In the last step, methane is produced from acetate and hydrogen by acetoclastic and hydrogenotrophic methanogens, respectively. In addition, acetate can be converted to methane via syntrophic acetate oxidation (SAO), a two-step process which involves syntrophic acetate-oxidising bacteria (SAOB) and a hydrogenotrophic partner methanogen [17]. NH_4^+ is a strong regulating factor for development of SAO, possibly as a consequence of inhibition of acetoclastic methanogens, which are known to be more sensitive to NH_4^+ than hydrogenotrophs [18], [19]. This shift in pathway from acetoclastic methanogenesis to SAO, typically occurring at NH_4^+ concentrations around 3-4 g/l [20], [21], probably explains the long adaptation period needed for biogas

production at high NH_4^+ concentrations [22], [23]. To date, only a few SAOB have been isolated and characterised. These are the thermophilic *Thermacetogenium phaeum* [24] and *Thermotoga lettinga*, [25] the thermotolerant *Tepidanaerobacter acetatoxydans* and the mesophilic *Clostridium ultunense* and *Syntrophaceticus schinkii* [26]. These bacteria are typically slow growing and thus long retention times have been suggested to be essential in preventing wash-out in continuous biogas processes [27]. However, SAOB have been detected in a number of different biogas reactors operated under a wide range of hydraulic retention times (HRTs), suggesting that other parameters might also influence the presence of these bacteria [19], [28]–[31]. Moreover, observations of SAO in anaerobic digesters, and recent detections of novel syntrophic populations suggests that methane formation via this pathway appears to be quite common [32], [33].

The aim of this study was to examine the NH_4^+ tolerant microbial community in biogas reactors operated at different HRTs and loading ratios of protein rich fish waste silage. Moreover, the aim was to assess the reactor performance, particularly focusing on how the process can be managed and maintained in order to enable an increased load of fish waste silage. Process limitations due to high nitrogen levels, and the abundance of microbial groups previously shown to be influenced by the NH_4^+ level in mesophilic conditions was investigated and evaluated.

2. Material and Methods

This study describes the performance in terms of biogas process stability and effluent quality in four biogas reactors fed with a mix of fish waste silage (FWS) and cow manure (CM). The reactors were operated in the mesophilic range with fixed HRTs of

20, 25, 30 and 40 days, and the feedstock was composed of 13 % FWS and 87 % CM (volume based). Quantification of NH_4^+-tolerant microorganisms in effluents from the reactors operated at different HRTs and equal ratios of FWS and CM were carried out. Moreover, NH_4^+-tolerant microorganisms in two additional experimental reactors were quantified. These reactors were operated with HRTs of 30 days, and loaded with increased ratios of FWS in mix with CM.

2.1 Biogas reactors operated with equal HRTs

The reactor experiments were performed in 8-L laboratory-scale semi continuously stirred tank reactors (CSTR) previously described by Bergersen et al [34].

Two experimental reactors operated with HRTs of 30 days were used to investigate anaerobic digestion of increased ratios of FWS in mix with CM. The inoculum and substrates used, and the performance of the reactors are described in detail in a previously published paper [7]. In short, two parallel experimental reactors (R1 and R2) were fed with a substrate blend of FWS and CM, with gradually increased amounts of FWS (in addition one reactor, R0, was fed with CM only, as a control). The amount of FWS in the substrate blend was increased in the experimental reactors from: 3% by volume to 6%, 13%, 16% and 19%, with total OLR corresponding to approx. 2.0, 2.3, 3.0, 3.5 and 4.3 g volatile solids (VS) /l/d, respectively. The reactors were operated in the mesophilic range, at a HRT of 30 days throughout the experiment, with a total duration of 450 days. Stable biogas process was obtained in the reactors with up to between 13 and 16% FWS in the feed stock, and the highest loads of FWS (16-19%)

resulted in process failure. Effluent samples from the reactors were collected during each phase with increased ratios of FWS in the feedstocks.

2.2 Biogas reactors operated with different HRTs

Following the initial reactor experiment investigating the effect of an increased ratio of FWS in the feedstock on anaerobic digestion, four reactors (R3, R4, R5 and R6) were used to evaluate the effect of different HRTs on process stability. Moreover, samples collected from the reactors R0, R1, R2 and R3, R4, R5 and R6 were analysed to quantify and compare the abundance of NH_4^+ tolerant microorganisms in the reactors.

For start-up, the reactors R3-R6 were inoculated with a mixed culture from the two experimental reactors R1 and R2, and fed a mixture of 13% FWS and 87% CM (by volume), and operated at 37 °C. During the initial start-up phase of 59 days, the reactors were operated at an HRT of 30 days and OLR of approx. 2.8 g VS /l/d, after which the HRT immediately was changed to 20, 25 and 40 days, and OLRs to approx. 4, 3.2 and 2 g VS /l/d in R3, R4 and R6, respectively. The HRT and OLR was modified simultaneously to imitate a large scale CSTR biogas process. Reactor R5 was kept at the same HRT and OLR as in the start-up phase. The total duration of the experiment with fixed HRT in R3-R6 was 150 days.

2.3 Analyses and sampling

Gas production (volume and content of methane (CH_4) and carbon dioxide (CO_2) from the reactors was measured daily. The concentrations of dry matter (DM), VS and NH_4^+

and the pH level in the reactors were measured once every week, and the concentration of VFAs in the reactors was measured at the end of every HRT. Samples of reactor contents for DNA analyses were collected from the experimental reactors (R1-R6) and the control reactor (R0) and stored at -20 °C. DNA isolation and qPCR analysis were performed on samples collected from the reactors at the end of every HRT. The biogas was collected in 25-L Tedlar bags (Tedlar® Gas Sampling Bag, Sigma-Aldrich, St. Louis, MO, USA). Concentrations (vol.-%) of CH_4 and CO_2 were measured once a day using a GA2000 Landfill Gas Analyzer (Geotechnical Instruments Ltd., UK). Total gas production rate volume (l/d) was calculated from flow measurements (rate 300 mL min^{-1}) as: Time (min) x 300 (mL/min)/1000 mL. Content of DM, VS concentration and pH level were determined according to methods specified by the International and European Organization for Standardization [35]. NH_4^+ concentration was analysed using an NH_4^+ selective electrode (Thermo Scientific Orion ISE/NH4) in diluted (1:10) reactor digestate samples held at 20 °C and supplemented with an ionic strength adjustor (ISA; 10 mL of ISA per 100 mL of sample) for stabilisation of NH_4^+. Serial dilutions of 0.1 M NH_4^+ standards were used for calibration of the electrode. Samples for VFA analysis were centrifuged (13,000 rpm) and filtered (0.45 μm) prior to analysis. The concentrations of different VFAs were determined using a Rezex RFQ Fast Acid H+ (8%) 100 × 7.8 mm HPLC (Phenomenex, Torrance, CA, USA), operated at a temperature of 85 °C, with an Ultimate 3000RS column and UV detection at 210 nm (Dionex, Sunnyvale, CA, USA). The samples were diluted with sulphuric acid (8 μL total) before analysis and quantification using VFA standards.

2.4 DNA isolation and q-PCR analysis

The abundance of microorganisms was determined by quantitative PCR (qPCR). Frozen digestate samples from different sampling times were thawed and total genomic DNA samples were extracted from triplicate aliquots of 0.3 mL using the FastDNA Spin kit for soil according to the manufacturer's protocol (Qbiogene, Illkrich, France). The SAOB *C. ultunense*, *S. schinkii* and *T. acetatoxydans* and the methanogenic groups *Methanosarcinaceae*, Methanomicrobiales and *Methanosaetaceae* were quantified using specific primers targeting 16s ribosomal RNA (rRNA) genes and q-PCR analysis. Different DNA dilutions were evaluated for the PCR reaction to test possible inhibition. Standard curves were prepared from plasmid DNA as described previously [23]. The standard curves had a linear correlation coefficient (r^2) ranging between 0.9 and 1. The calculated qPCR efficiency of the reactions varied between 85 and 105 %. At the end of each qPCR analysis, a temperature melt curve was performed to verify reaction quality. Primers and PCR conditions were as described by Westerholm et al [23].

3. Results and discussion

3.1 Biogas process performance

In the reactors operated at HRTs from 20 to 40 days fed with a substrate mix of 13% FWS (R3-R6), the average total CH_4 production was approx. 8, 7.5, 6.5 and 5 l CH_4 / day in R3-R6, respectively (data not shown). These production results show that the total methane yields were increased by more than 60 % when the volumetric loading was doubled as a result of decreasing the HRT from 40 to 20 days. The specific methane production was 0.25, 0.29 0.30 and 0.31 l CH_4 /g VS, respectively (Table 2).

The NH_4^+ concentrations were stable in R3-R6 during the entire experiment, with values of 5.92, 5.68, 5.52 and 5.48 g/l in R3, R4, R5 and R6 days, respectively (Table 2). In the previously published experiment, were the load of FWS was increased to a maximum of 19 volume %, the NH_4^+-concentrations were elevated to over 7 g/l and the process failed. The volumetric loading was constant at 267 mL / day (HRT 30 days). These results show that the NH_4^+ concentrations in R3 and R4 (20 and 25 days HRT) stabilized at lower concentrations although they were operated with higher volumetric loading and volume of FWS and proteins. This could be explained either by an inhibited or reduced mineralization of nitrogen in the reactors due to the high loading, and also possibly by the direct effect of a shorter HRT, i.e. a shorter time for the microbes to degrade a higher load of proteins.

Some VFA accumulated during operation of R3-R6 and the levels were stable over time. Increased concentrations of VFAs in the reactors were correlated with somewhat lower methane yield (Table 2). Acetic acid concentration varied between 2.2 and 4.6 g/l in the reactors, while propionic acid concentration varied between 1.0 and 2.6 g/l and was highest in R3, the reactor with the lowest specific methane production. It has been suggested that elevated propionic acid levels is an indicator of an unstable methane production process [36], which suggests that the OLR in reactor R3 was too high and/or the HRT was too short. Inhibition of biogas processes by accumulation of propionic acid has been extensively investigated in previous studies [9], [37]–[40], and propionic acid is considered to be the most toxic of all VFAs found in anaerobic reactors [41]. The reported inhibitory level of propionic acid varies between studies, from 0.8 g/l [42] to 6 g/l [43].

Comparing the results from the reactors used in the two continuous experiments show that increasing the total volume of the FWS/CM-blend by modifying HRT (R3-R6) is less important for the biogas process performance than increasing the ratio of FWS in the feedstock (R1-R2). This is probably due to a higher buffer capacity in the feedstock when the ratio of CM and FWS is kept the same, as compared to the experiments with increased ratio of FWS. Manure is commonly reported to help stabilize biogas processes due to its high buffering capacity [44]–[46].

During the previously published reactor experiment were different substrate mixtures were evaluated, stable biogas production was seen in the parallel reactors R1 and R2, when FWS comprised up to 13% of the substrate mix with CM [7]. These reactors were operated at 37 °C with an HRT of 30 days and a total OLR corresponding to approx. 3 g VS l/d (Table 1). Specific methane production in R1 and R2 ranged from approx. 0.2 L CH_4 / g VS (3% FWS) to a maximum of approx. 0.4 L CH_4 / g VS in R2 (16% FWS). The NH_4^+ concentration in the reactors increased during the operating period from approx. 3 g/l in R1 and R2 with a load of 3% FWS to 5.7 (R1) and 4.6 (R2) g/l with a load of 13% FWS (Table 2). Operation with 16% and 19% FWS was also evaluated but eventually resulted in process failure in R1 and R2, respectively. The VFA accumulated, yielding total concentrations between 15 and 20 g/l (mainly acetic and propionic acids), and reduced methane yields were followed by process failure after 1 HRT in the reactors. High NH_4^+ levels of between 7 and 8 g/l (Table 2) most likely caused this instability. For R0, operating solely with CM, the CH_4 yield was approx. 0.2 l/g VS and the NH_4^+ concentration was around 2 g/l (Table 2). These are in line with values reported in previous studies [47]–[49], and illustrate the low gas yield obtained when using only CM as a biogas substrate.

Replacing part of the CM with FWS in R1 and R2 increased the methane yield by almost 100% at the highest FWS load (Table 2). Increasing the OLR to the same extent with CM alone would have resulted in a significant decrease in HRT and, as a consequence, most likely decreased degradation efficiency. Moreover, in addition to increased methane yield, including FWS in the substrate resulted in residues with high NH_4^+ content, representing a product with higher value as a fertiliser [8], [50], [51].

3.2 Microbial community analysis - SAOB

q-PCR analysis of three SAOB (Fig. 1A) showed a change in composition of the microbial community as a function of increasing amounts of FWS during the initial reactor experiment. *S. schinkii* was detected in both R1 and R2 during the periods with loading of 3, 6 and 13 % FWS, with NH_4^+ concentrations ranging from around 2 to 6 g/l. However, *C. ultunense* and *T. acetatoxydans* (Fig. 1A) were not detected in any of the experimental reactors until the period of loading with 13% FWS, when the NH_4^+ concentration was 5.72 and 4.61 g/l in R1 and R2, respectively. *T. acetatoxydans* was present in both experimental reactors after 90 days of loading with 13% FWS, with levels of 6.86 and 6.81 average log gene abundance / mL in R1 and R2, respectively. For *C. ultunense*, levels of 7.07 and 7.05 average log gene abundance / mL were observed in R1 and R2, respectively. Both *S. schinkii* and *C. ultunense* were detected in the control reactor (R0) during the first 90 days of operation, at levels of 7.7 and 4.3 average log gene abundance / mL, respectively, but for the rest of the experimental period *S. schinkii* was the only SAOB detected in R0.

Previous studies have shown that different SAOB are found in reactors with different NH_4^+ concentrations and it has been observed that *S. schinkii*, *C. ultunense* and *T. acetatoxydans* in co-culture can tolerate total NH_4^+ concentrations equivalent to between 0.6 and 1.0 M NH_4Cl [21], [52]. *S. schinkii* has also been shown to be present in reactors operating at lower NH_4^+ levels and with acetoclastic methanogenesis as the dominant pathway for methane production, but typically at lower abundance than in operation at higher NH_4^+ levels and with SAO as the dominant pathway for methanogenesis [19], [21], [30]. *S. schinkii* has been found in reactors with total ammonia concentrations ranging between 0.65 and 7 g NH_4^+ / l (at neutral pH) [23], [52]. *C. ultunense* and *T. acetatoxydans* are typically found in reactors with high NH_4^+ concentrations and with SAO as the dominant methane production pathway [19], [21], [30]. Similarly, the present study showed presence of *S. schinkii* at relatively low NH_4^+ concentrations (2-3 g/l) and presence of *T. acetatoxydans* and *C. ultunense* when the NH_4^+ concentration was elevated to 4-5 g/l (Fig. 1A).

Although a few cultured SAOB are somewhat firmly characterized there are probably several undetected microorganisms involved in SAO at high NH_4^+ concentrations. A recent proteomic study investigating the syntrophic microbial community detected that the microbiome of an NH_4^+ tolerant biogas reactor harboured an uncultured phylotype capable of SAO [32]. Previously characterized SAOB were also identified in the reactor in limited representation compared to the uncultured phylotype, suggesting that the novel phylotype played an important role. This finding emphasize that although a small number of SAOB are currently characterized, there are probably many undetected microorganisms involved in SAO.

During the periods of loading with 16 and 19% FWS, when methane formation failed in R1 and R2, respectively [7], quantification of SAOB was performed after 30 and 90 days of operation (1 and 3 HRTs). In R1, after 30 days of loading with 16% FWS and 296 days of total operation *S. schinkii* and *C. ultunense* were still abundant, with levels of 7.21 and 7.10 average log gene abundance / mL, respectively, but the level of *T. acetatoxydans* at that time was below the detection limit (Fig. 1A). After 90 days and 356 days of total operation, none of the SAOB analysed were found in this reactor. However, when R1 failed and collapsed the feeding was stopped, and after a period of 30 days without any feeding and 386 days of total operation, *S. schinkii*, *C. ultunense* and *T. acetatoxydans* were again detected in the reactor, with values of 8.55, 7.34 and 6.71 average log gene abundance / mL, respectively.

The abundance of SAOB in R2 did not change significantly when the amount of FWS was increased from 13 to 16%. Moreover, compared to R1, the increased load of FWS from 13 to 16% in R2 resulted in lower levels of accumulated NH_4^+ and VFAs (table 2). However, after 30 days of loading with 19% FWS and 390 days of total operation, *T. acetatoxydans* was not detected in R2, while *S. schinkii* and *C. ultunense* were present at levels of 9.09 and 7.68 average log gene abundance / mL, respectively. After 90 days of loading with 19% FWS and 450 days of total operation, none of the SAOB was detected in R2 but, as seen for R1, after 30 days without any feeding *S. schinkii*, *C. ultunense* and *T. acetatoxydans* were again found in high abundance (Fig. 1A). The abundance of the SAOB decreased at different times in R1 and R2, but in both reactors when the NH_4^+ concentration reached approximately 7 g/l. Thus elevated NH_4^+ concentration seemed to be the main cause of the observed decrease in abundance of these syntrophic bacteria.

In reactors R3-R6, operating at different HRT and OLR but with the same substrate mix (13% FWS), the different targeted SAOB were detected in all reactors and at all sampling points and remained at similar levels throughout the experiment (Fig. 2A). These results indicate that the target SAOB all had the ability to persist in the reactors, even at rather short HRT and also despite high NH_4^+ levels. As mentioned earlier, the growth rate of the SAOB is quite low and, as a consequence, HRT has been suggested to be a critical parameter influencing the degree of SAO [16], [27], [29], [53]. Studies evaluating growth of SAOB in co-culture with hydrogenotrophic methanogens have reported a doubling time of 28 days for *C. ultunense* [54] while the generation time of *S. schinkii* in co-culture with *Methanoculleus* sp. MAB1 is calculated to be as long as 69-78 days [27]. The doubling time of *T. acetatoxydans* during acetate oxidation is currently not known [21]. These findings suggest that an HRT of 20-30 days might lead to wash-out of SAOB in continuous reactors. However, Sun et al. [19] investigated different large-scale reactors and detected quite high abundance of the known SAOB at various HRT (20-110 days). Moreover, Moestedt et al. [29] studied the effect of HRT on process performance at high NH_4^+ levels and presence of SAOB and found that the HRT could be reduced from 45 to 25 days, reaching an OLR of 4.6 kg VS /m^3/ d, without any disturbance of the anaerobic process or decrease in levels of the SAOB. The undiminished levels of SAOB even at rather short HRT might be caused by an interactive effect between these bacteria, as described by Westerholm et al. [27] for a syntrophic acetate-degrading culture containing *C. ultunense*, *T. acetatoxydans*, *S. schinkii* and *Methanoculleus* sp. MAB1. The doubling time in that co-culture, represented by the methane formation rate in the exponential phase, was found to be only 9 days. Another explanation for the persistence of these slow growing bacterias in

reactors operated at short HRTs could be the aspect of biofilm formation. Studies of microbial communities in different environments has shown that microorganisms often tend to grow in biofilms on different surfaces [55]–[57], and it can be assumed that the microbial communities in CSTR biogas process forms biofilm and are thus enabled to retain in the system.

3.4 Microbial community analysis - methanogens

q-PCR analysis of the methanogens in R1 and R2 fed with increasing ratios of FWS (Fig. 1B) revealed that the acetoclastic group of *Methanosaetaceae* was the most abundant methanogen during the period of loading with 3% FWS. This methanogen was present in both R1 and R2, at levels of around 4-6 average log gene abundance / mL throughout the experiment (Fig. 1B). When the load of FWS was increased from 3 to 6 %, the level of *Methanosarcinaceae* increased to 7.38 and 7.05 average log gene abundance / mL in R1 and R2, respectively (Fig. 1B). After 90 days of loading with 13% FWS, Methanomicrobiales was also detected, at levels of 7.39 and 7.88 average log gene abundance / mL in R1 and R2, respectively. This group of methanogens was not detected at the lower loading ratio of FWS. The generally higher levels of *Methanosarcinaceae* compared with *Methanosaetaceae* in R1 and R2 are in accordance with the higher level of acetate, as this methanogen has a comparatively higher threshold for acetate consumption [38], [58]. In addition, the *Methanosarcinaceae* also have a comparatively higher tolerance to NH_4^+ [59], which is in consistence with the elevated NH_4^+ concentrations observed in R1 and R2 when the ratio of FWS was increased (table 2). Karakashev et al. [58] investigated methanogen composition in

differently treated reactors and reported that those with high levels of NH_4^+ and VFA were dominated by members of the *Methanosarcinaceae*, while those with low levels of NH_4^+ and VFA were dominated by members of the *Methanosaetaceae*. The *Methanosaetaceae* are known to be sensitive to increasing NH_4^+ concentrations [60], [61] and it is therefore somewhat surprising that this methanogen was also detected in the reactors with high NH_4^+ concentrations (Figs. 1 and 2). However, the *Methanosaetaceae* were not the dominant methanogen in the reactors when the NH_4^+ concentrations increased and their presence has also previously been observed in high-NH_4^+ processes [19], [44], indicating that there are some members of this group that can tolerate higher NH_4^+ concentrations.

The hydrogenotrophic group of Methanomicrobiales appeared simultaneously with the SAOB *C. ultunense* and *T. acetatoxydans* and at a time when the NH_4^+ concentration had increased above 5 and 4 g/l in R1 and R2 (table 2). These results are in agreement with other experiments investigating the development and abundance of SAOB and methanogens, indicating that representatives of Methanomicrobiales are most likely a partner in SAO in mesophilic biogas reactors [23], [62]–[65]. More specifically, in several studies *Methanoculleus* sp. have been shown to increase in abundance in line with increasing NH_4^+ levels and the establishment of SAO. Moreover, *Methanoculleus* sp. has been detected at high abundance in different biogas reactors operating at high NH_4^+ concentrations, showing the high NH_4^+ tolerance of this methanogen [17], [30], [62]. Interestingly, the abundance of all target methanogens was stable in both R1 and R2 when process instability occurred, i.e. the methanogens did not seem to be affected to the same extent as the SAOB when the concentrations of NH_4^+ and VFA increased. The decrease in the SAOB was possibly the cause of accumulation of acetate in the

reactors at this time, and when the feeding of R1 and R2 was stopped, the SAOB were again detected in the reactors. Other experiments investigating changes in the methanogenic community during biogas reactor instability have reported somewhat similar results. For example, Munk and Lebuhn [66] investigated the quantity of methanogenic Archaea in differently treated biogas reactors and found no major differences in concentration, although when the OLR was raised the Archaeal population moderately increased and when the OLR was further increased the population decreased, with methane productivity becoming unstable at OLR between 3 and 4 kg VS / m^3 / d.

In R3-R6, Methanomicrobiales and *Methanosaetaceae* were detected at similar levels at the different HRT and OLR, while the abundance of *Methanosarcinaceae* was below the detection level (Fig. 2B). The absence of *Methanosarcinaceae* was somewhat surprising, but a previous metagenomic sequence analysis of these reactors at start-up (inoculum, day 0) and after 59 days showed that *Methanosarcinaceae* were present at day 0, but that their abundance decreased throughout the 59-day start-up phase [15]. Thus it seems that *Methanosarcinaceae* gradually disappeared from the reactors, in pace with the decrease in HRT and increase in OLR. *Methanosarcinaceae* is a group of methanogens described as a robust and often persistent microbe in different biogas reactors [59], and the observed decrease of this methanogen in R3 – R6 is contradictory to previous findings [67] for a high- NH_4^+ (5-6 g/kg) biogas process operating with stillage, where the *Methanosarcinaceae* increased in abundance with a decrease of the HRT from 45 to 24 and an increase in OLR from 3.2 to 6 g/l/d. It is not clear why the density of *Methanosarcinaceae* decreased in R3-R6. However, during long adaptations and adjustment to specific conditions such as nutrient composition it has been observed

exchanges of functioning microbial groups [15], [68], and it can be assumed that the majority of *Methanosarcinaceae* members were not key acting and thus defeated by other more specialized microbes in the reactors operated in the present experiment.

4. Conclusions

This study showed that it is possible to utilise fat- and protein-rich fish waste silage as a biogas substrate, even at low HRT and high total OLR. High NH_4^+ concentrations as a result of elevated loading of FWS caused an increase in the abundance of different SAOB and the hydrogenotrophic methanogenic order Methanomicrobiales. Stable process performance seemed to be related to the presence of *T. acetatoxydans* together with the hydrogenotrophic methanogen. Different HRTs had little effect on methane yield and microbial community composition, but increased ratio loading of FWS at a fixed HRT inhibited activity of syntrophic microorganisms and caused instability and failure of the biogas process. The results thus showed that increasing the total volume of a fixed FWS/CM-blend is of less importance for the syntrophic microbial community and the biogas process performance than raising the proportion of FWS in the feed stock. By decreasing the HRT from 30 to 20 days in reactors added a feedstock composed of 13 % FWS, it is possible to increase the loading volume of FWS by approx. 50% without markedly inhibiting the microbial community and biogas process. Hence, increased waste volumes of FWS can be managed by decreasing the reactor HRT.

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Figure captures

Figure 1. Concentrations (average log gene abundance / mL) of the target micoorganisms in the control reactor (R0) and experimental parallel reactors (R1 and R2) as a function of incubation time (days) at the different feeding blends and the sampling point for the microbial analyses. The reactors received manure mixed with (volume basis): 3% fish waste silage (FWS), 6% FWS, 13% FWS, 16% FWS and 19% FWS. **A)** The SAOBs *S. schinkii*, *C. ultunense* and *T. acetatoxydans* and **B)** the methanogens Methanomicrobiales, *Methanosaetaceae* and *Methanosarcinaceae*. The experimental reactors R1 and R2 collapsed during the period with addition of 16% and 19% FWS, respectively. When the reactors collapsed, feedstock addition was stopped.

Figure 2. Concentrations (average log gene abundance / mL) of the target microorganisms in experimental reactors R3, R4, R5 and R6 as a function of incubation time (days) at the different HRTs and sampling point for the microbial analyses. The reactors were operated at HRT 20, 25, 30 and 40 days, respectively. **A)** The SAOBs *S. schinkii*, *C. ultunense* and *T. acetatoxydans* and **B)** the methanogens Methanomicrobiales, *Methanosaetaceae* and *Methanosarcinaceae.*

References

[1] Faostat, “Faostat – Food and Agriculture Organization of the United Nations, FAO Statistical Databases, numbers for 2014. Available from: http://www.fao.org/faostat/en/#data/QA.” 2014.

[2] S. Soares, D. M. Green, J. F. Turnbull, M. Crumlish, and A. G. Murray, “A baseline method for benchmarking mortality losses in Atlantic salmon (Salmo salar) production,” *Aquaculture*, vol. 314, no. 1, pp. 7–12, 2011.

[3] B. Liaset and M. Espe, “Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials,” *Process Biochem.*, vol. 43, no. 1, pp. 42–48, 2008.

[4] R. Kilburn, A. Murray, M. Hall, D. Bruno, D. Cockerill, and R. Raynard, “Analysis of a company’s production data to describe the epidemiology and persistence of pancreas disease in Atlantic salmon (Salmo salar L.) farms off Western Scotland,” *Aquaculture*, vol. 368, pp. 89–94, 2012.

[5] SSB, “Statistics Norway, SSB. https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar.” 2017.

[6] K. Jayathilakan, K. Sultana, K. Radhakrishna, and A. Bawa, “Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review,” *J. Food Sci. Technol.*, vol. 49, no. 3, pp. 278–293, 2012.

[7] L. Solli, O. Bergersen, R. Sørheim, and T. Briseid, “Effects of a gradually increased load of fish waste silage in co-digestion with cow manure on methane production,” *Waste Manag.*, vol. 34, no. 8, pp. 1553–1559, 2014.

[8] V. Arthurson, “Closing the global energy and nutrient cycles through application of biogas residue to agricultural land–potential benefits and drawback,” *Energies*, vol. 2, no. 2, pp. 226–242, 2009.

[9] J. L. Chen, R. Ortiz, T. W. Steele, and D. C. Stuckey, “Toxicants inhibiting anaerobic digestion: a review,” *Biotechnol. Adv.*, vol. 32, no. 8, pp. 1523–1534, 2014.

[10] A. Hejnfelt and I. Angelidaki, “Anaerobic digestion of slaughterhouse by-products,” *Biomass Bioenergy*, vol. 33, no. 8, pp. 1046–1054, 2009.

[11] R. Rajagopal, D. I. Massé, and G. Singh, “A critical review on inhibition of anaerobic digestion process by excess ammonia,” *Bioresour. Technol.*, vol. 143, pp. 632–641, 2013.

[12] D. Z. Sousa *et al.*, “Activity and viability of methanogens in anaerobic digestion of unsaturated and saturated long-chain fatty acids,” *Appl. Environ. Microbiol.*, vol. 79, no. 14, pp. 4239–4245, 2013.

[13] G. K. Kafle, S. H. Kim, and K. I. Sung, “Ensiling of fish industry waste for biogas production: a lab scale evaluation of biochemical methane potential (BMP) and kinetics,” *Bioresour. Technol.*, vol. 127, pp. 326–336, 2013.

[14] B. Salam, M. Islam, and M. Rahman, “Biogas from anaerobic digestion of fish waste,” presented at the Proceedings of the International Conference on Mechanical Engineering, 2009, vol. 26, pp. 1–3.

[15] L. Solli, O. E. Håvelsrud, S. J. Horn, and A. G. Rike, “A metagenomic study of the microbial communities in four parallel biogas reactors,” *Biotechnol. Biofuels*, vol. 7, no. 1, p. 146, 2014.

[16] I. Angelidaki, D. Karakashev, D. J. Batstone, C. M. Plugge, and A. J. Stams, “Biomethanation and its potential,” *Methods Enzym.*, vol. 494, no. 16, pp. 327–351, 2011.

[17] A. Schnürer, G. Zellner, and B. H. Svensson, “Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors,” *FEMS Microbiol. Ecol.*, vol. 29, no. 3, pp. 249–261, 1999.

[18] I. Angelidaki and B. Ahring, “Thermophilic anaerobic digestion of livestock waste: the effect of ammonia,” *Appl. Microbiol. Biotechnol.*, vol. 38, no. 4, pp. 560–564, 1993.

[19] L. Sun, B. Müller, M. Westerholm, and A. Schnürer, “Syntrophic acetate oxidation in industrial CSTR biogas digesters,” *J. Biotechnol.*, vol. 171, pp. 39–44, 2014.
[20] A. Schnürer and Å. Nordberg, “Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature,” *Water Sci. Technol.*, vol. 57, no. 5, pp. 735–740, 2008.
[21] M. Westerholm, S. Roos, and A. Schnürer, “Tepidanaerobacter acetatoxydans sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from two ammonium-enriched mesophilic methanogenic processes,” *Syst. Appl. Microbiol.*, vol. 34, no. 4, pp. 260–266, 2011.
[22] A. Van Velsen, “Adaptation of methanogenic sludge to high ammonia-nitrogen concentrations,” *Water Res.*, vol. 13, no. 10, pp. 995–999, 1979.
[23] M. Westerholm, J. Dolfing, A. Sherry, N. D. Gray, I. M. Head, and A. Schnürer, “Quantification of syntrophic acetate-oxidizing microbial communities in biogas processes,” *Environ. Microbiol. Rep.*, vol. 3, no. 4, pp. 500–505, 2011.
[24] S. Hattori, Y. Kamagata, S. Hanada, and H. Shoun, “Thermacetogenium phaeum gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium.,” *Int. J. Syst. Evol. Microbiol.*, vol. 50, no. 4, pp. 1601–1609, 2000.
[25] M. Balk, J. Weijma, and A. J. Stams, “Thermotoga lettingae sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor.,” *Int. J. Syst. Evol. Microbiol.*, vol. 52, no. 4, pp. 1361–1368, 2002.
[26] M. Westerholm, S. Roos, and A. Schnürer, “Syntrophaceticus schinkii gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter,” *FEMS Microbiol. Lett.*, vol. 309, no. 1, pp. 100–104, 2010.
[27] M. Westerholm, *Biogas production through the syntrophic acetate-oxidising pathway*, vol. 2012. 2012.
[28] A. Karlsson, P. Einarsson, A. Schnürer, C. Sundberg, J. Ejlertsson, and B. H. Svensson, “Impact of trace element addition on degradation efficiency of volatile fatty acids, oleic acid and phenyl acetate and on microbial populations in a biogas digester,” *J. Biosci. Bioeng.*, vol. 114, no. 4, pp. 446–452, 2012.
[29] J. Moestedt, S. N. Påledal, A. Schnürer, and E. Nordell, “Biogas production from thin stillage on an industrial scale—experience and optimisation,” *Energies*, vol. 6, no. 11, pp. 5642–5655, 2013.
[30] M. Westerholm, L. Levén, and A. Schnürer, “Bioaugmentation of syntrophic acetate-oxidizing culture in biogas reactors exposed to increasing levels of ammonia,” *Appl. Environ. Microbiol.*, vol. 78, no. 21, pp. 7619–7625, 2012.
[31] M. Westerholm, B. Muller, and A. Schnurer, “Changes in the acetogenic population in a mesophilic anaerobic digester in response to increasing ammonia concentration,” *Microbes Environ*, vol. 26, pp. 347–353, 2011.
[32] J. Frank *et al.*, “Novel Syntrophic Populations Dominate an Ammonia-Tolerant Methanogenic Microbiome,” *mSystems*, vol. 1, no. 5, pp. e00092-16, 2016.
[33] S.-H. Lee, J.-H. Park, S.-H. Kim, B. J. Yu, J.-J. Yoon, and H.-D. Park, “Evidence of syntrophic acetate oxidation by Spirochaetes during anaerobic methane production,” *Bioresour. Technol.*, vol. 190, pp. 543–549, 2015.
[34] O. Bergersen, K. Ø. Hanssen, and T. Vasskog, “Anaerobic treatment of sewage sludge containing selective serotonin reuptake inhibitors,” *Bioresour. Technol.*, vol. 117, pp. 325–332, 2012.
[35] “ISO 10390: 2005; ISO 11465: 1993; NS-EN 15935: 2012.” .
[36] U. Marchaim and C. Krause, “Propionic to acetic acid ratios in overloaded anaerobic digestion,” *Bioresour. Technol.*, vol. 43, no. 3, pp. 195–203, 1993.
[37] Y. Chen, J. J. Cheng, and K. S. Creamer, “Inhibition of anaerobic digestion process: a review,” *Bioresour. Technol.*, vol. 99, no. 10, pp. 4044–4064, 2008.
[38] J. Ma *et al.*, “Methanosarcina domination in anaerobic sequencing batch reactor at short hydraulic retention time,” *Bioresour. Technol.*, vol. 137, pp. 41–50, 2013.

[39] T. Rétfalvi, A. Tukacs-Hájos, L. Albert, and B. Marosvölgyi, “Laboratory scale examination of the effects of overloading on the anaerobic digestion by glycerol,” *Bioresour. Technol.*, vol. 102, no. 8, pp. 5270–5275, 2011.
[40] Y. Wang, Y. Zhang, J. Wang, and L. Meng, “Effects of volatile fatty acid concentrations on methane yield and methanogenic bacteria,” *Biomass Bioenergy*, vol. 33, no. 5, pp. 848–853, 2009.
[41] K. C. Wijekoon, C. Visvanathan, and A. Abeynayaka, “Effect of organic loading rate on VFA production, organic matter removal and microbial activity of a two-stage thermophilic anaerobic membrane bioreactor,” *Bioresour. Technol.*, vol. 102, no. 9, pp. 5353–5360, 2011.
[42] J. Ma, M. Carballa, P. Van De Caveye, and W. Verstraete, “Enhanced propionic acid degradation (EPAD) system: Proof of principle and feasibility,” *Water Res.*, vol. 43, no. 13, pp. 3239–3248, 2009.
[43] C. Gallert and J. Winter, “Propionic acid accumulation and degradation during restart of a full-scale anaerobic biowaste digester,” *Bioresour. Technol.*, vol. 99, no. 1, pp. 170–178, 2008.
[44] M. Westerholm, M. Hansson, and A. Schnürer, “Improved biogas production from whole stillage by co-digestion with cattle manure,” *Bioresour. Technol.*, vol. 114, pp. 314–319, 2012.
[45] G. K. Kafle and S. H. Kim, “Anaerobic treatment of apple waste with swine manure for biogas production: batch and continuous operation,” *Appl. Energy*, vol. 103, pp. 61–72, 2013.
[46] I. Angelidaki and L. Ellegaard, “Codigestion of manure and organic wastes in centralized biogas plants,” *Appl. Biochem. Biotechnol.*, vol. 109, no. 1–3, p. 95, 2003.
[47] R. Alvarez, S. Villca, and G. Lidén, “Biogas production from llama and cow manure at high altitude,” *Biomass Bioenergy*, vol. 30, no. 1, pp. 66–75, 2006.
[48] T. Amon, B. Amon, V. Kryvoruchko, W. Zollitsch, K. Mayer, and L. Gruber, “Biogas production from maize and dairy cattle manure—influence of biomass composition on the methane yield,” *Agric. Ecosyst. Environ.*, vol. 118, no. 1, pp. 173–182, 2007.
[49] A. Lehtomäki, S. Huttunen, and J. Rintala, “Laboratory investigations on co-digestion of energy crops and crop residues with cow manure for methane production: effect of crop to manure ratio,” *Resour. Conserv. Recycl.*, vol. 51, no. 3, pp. 591–609, 2007.
[50] K. Svensson, M. Odlare, and M. Pell, “The fertilizing effect of compost and biogas residues from source separated household waste,” *J. Agric. Sci.*, vol. 142, no. 4, pp. 461–467, 2004.
[51] K. Yetilmezsoy and Z. Sapci-Zengin, “Recovery of ammonium nitrogen from the effluent of UASB treating poultry manure wastewater by MAP precipitation as a slow release fertilizer,” *J. Hazard. Mater.*, vol. 166, no. 1, pp. 260–269, 2009.
[52] A. Schnürer, B. Schink, and B. H. Svensson, “Clostridium ultunense sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium,” *Int. J. Syst. Evol. Microbiol.*, vol. 46, no. 4, pp. 1145–1152, 1996.
[53] T. Shigematsu, Y. Tang, T. Kobayashi, H. Kawaguchi, S. Morimura, and K. Kida, “Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation,” *Appl. Environ. Microbiol.*, vol. 70, no. 7, pp. 4048–4052, 2004.
[54] A. Schnürer, F. P. Houwen, and B. H. Svensson, “Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration,” *Arch. Microbiol.*, vol. 162, no. 1, pp. 70–74, 1994.
[55] G. O’Toole, H. B. Kaplan, and R. Kolter, “Biofilm formation as microbial development,” *Annu. Rev. Microbiol.*, vol. 54, no. 1, pp. 49–79, 2000.
[56] M. E. Davey and G. A. O’toole, “Microbial biofilms: from ecology to molecular genetics,” *Microbiol. Mol. Biol. Rev.*, vol. 64, no. 4, pp. 847–867, 2000.

[57] N. Qureshi, B. A. Annous, T. C. Ezeji, P. Karcher, and I. S. Maddox, “Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates,” *Microb. Cell Factories*, vol. 4, no. 1, p. 24, 2005.
[58] D. Karakashev, D. J. Batstone, E. Trably, and I. Angelidaki, “Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae,” *Appl. Environ. Microbiol.*, vol. 72, no. 7, pp. 5138–5141, 2006.
[59] J. De Vrieze, T. Hennebel, N. Boon, and W. Verstraete, “Methanosarcina: the rediscovered methanogen for heavy duty biomethanation,” *Bioresour. Technol.*, vol. 112, pp. 1–9, 2012.
[60] B. Calli, L.-C. Chung, D. Arslan, and K. Vanbroekhoven, “H2 production potential in thermophilic mixed fermentation,” *J. Environ. Sci. Health Part A*, vol. 44, no. 1, pp. 78–86, 2009.
[61] G. D. Sprott and G. B. Patel, “Ammonia toxicity in pure cultures of methanogenic bacteria,” *Syst. Appl. Microbiol.*, vol. 7, no. 2–3, pp. 358–363, 1986.
[62] L. T. Angenent, S. Sung, and L. Raskin, “Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste,” *Water Res.*, vol. 36, no. 18, pp. 4648–4654, 2002.
[63] J. Moestedt, B. Müller, M. Westerholm, and A. Schnürer, “Ammonia threshold for inhibition of anaerobic digestion of thin stillage and the importance of organic loading rate,” *Microb. Biotechnol.*, vol. 9, no. 2, pp. 180–194, 2016.
[64] T. Shimada *et al.*, “Syntrophic acetate oxidation in two-phase (acid–methane) anaerobic digesters,” *Water Sci. Technol.*, vol. 64, no. 9, pp. 1812–1820, 2011.
[65] M. Westerholm, B. Müller, S. Isaksson, and A. Schnürer, “Trace element and temperature effects on microbial communities and links to biogas digester performance at high ammonia levels,” *Biotechnol. Biofuels*, vol. 8, no. 1, p. 154, 2015.
[66] B. Munk and M. Lebuhn, “Process diagnosis using methanogenic Archaea in maize-fed, trace element depleted fermenters,” *Anaerobe*, vol. 29, pp. 22–28, 2014.
[67] J. Moestedt, E. Nordell, and A. Schnürer, “Comparison of operating strategies for increased biogas production from thin stillage,” *J. Biotechnol.*, vol. 175, pp. 22–30, 2014.
[68] D. Karakashev, D. J. Batstone, and I. Angelidaki, “Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors,” *Appl. Environ. Microbiol.*, vol. 71, no. 1, pp. 331–338, 2005.

Figure 1.

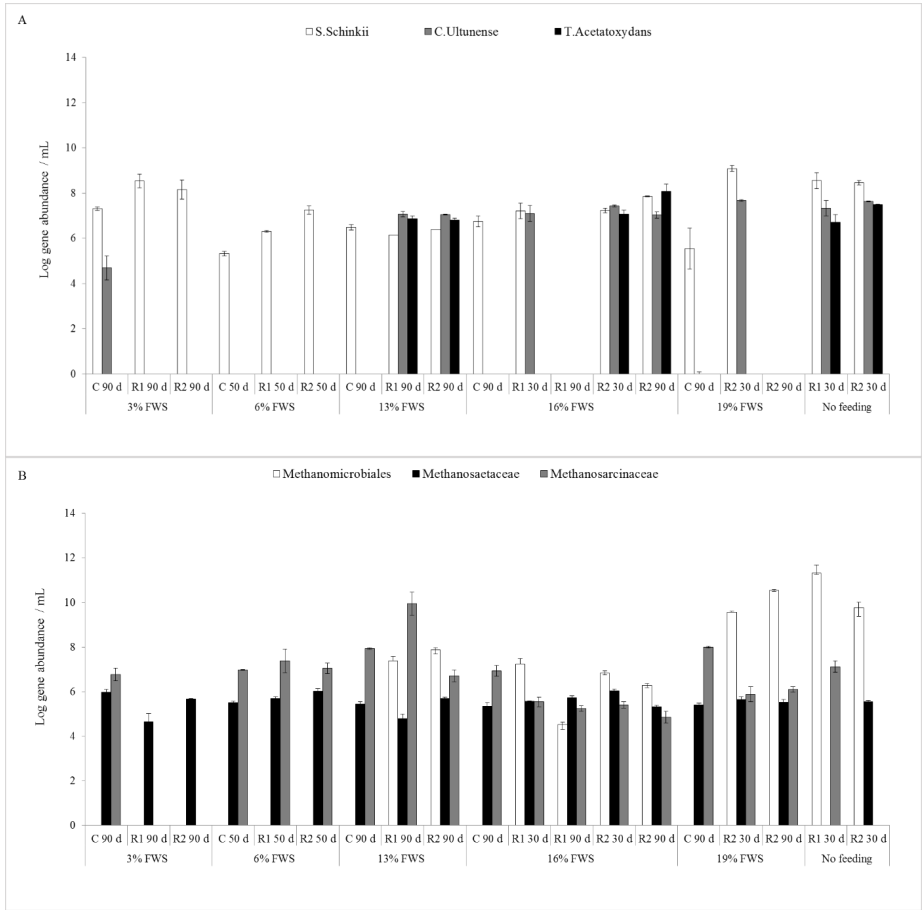
A
S.Schinkii
C.Ultunense
T.Acetatoxydans
Log gene abundance / mL
0
2
4
6
8
10
12
14
C 90 d
R1 90 d
R2 90 d
C 50 d
R1 50 d
R2 50 d
C 90 d
R1 90 d
R2 90 d
C 90 d
R1 30 d
R1 90 d
R2 30 d
R2 90 d
C 90 d
R2 30 d
R2 90 d
R1 30 d
R2 30 d
3% FWS
6% FWS
13% FWS
16% FWS
19% FWS
No feeding
B
Methanomicrobiales
Methanosaetaceae
Methanosarcinaceae
Log gene abundance / mL
0
2
4
6
8
10
12
14
C 90 d
R1 90 d
R2 90 d
C 50 d
R1 50 d
R2 50 d
C 90 d
R1 90 d
R2 90 d
C 90 d
R1 30 d
R1 90 d
R2 30 d
R2 90 d
C 90 d
R2 30 d
R2 90 d
R1 30 d
R2 30 d
3% FWS
6% FWS
13% FWS
16% FWS
19% FWS
No feeding

Figure 2.

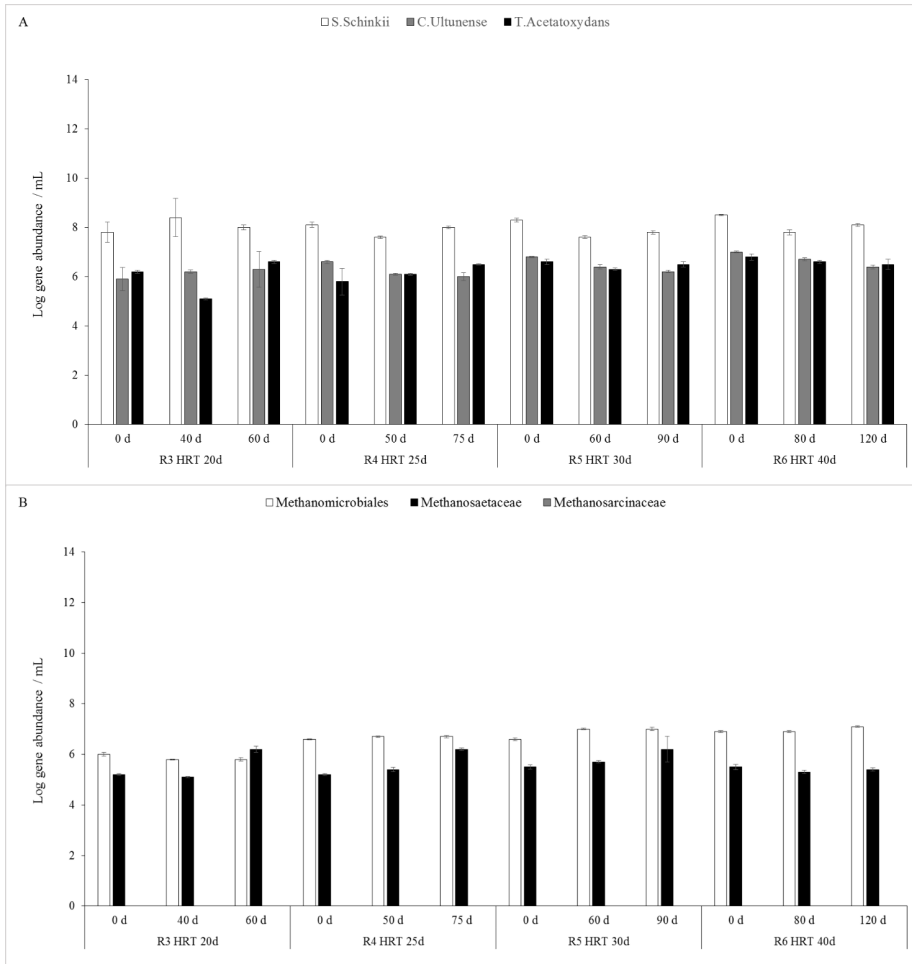
A
S.Schinkii
C.Ultunense
T.Acetatoxydans
Log gene abundance / mL
0 d
40 d
60 d
0 d
50 d
75 d
0 d
60 d
90 d
0 d
80 d
120 d
R3 HRT 20d
R4 HRT 25d
R5 HRT 30d
R6 HRT 40d
B
Methanomicrobiales
Methanosaetaceae
Methanosarcinaceae
Log gene abundance / mL
0 d
40 d
60 d
0 d
50 d
75 d
0 d
60 d
90 d
0 d
80 d
120 d
R3 HRT 20d
R4 HRT 25d
R5 HRT 30d
R6 HRT 40d

Table 1. Substrate composition and operational parameters of the biogas reactors. Substrate characteristics and operational parameters for R0, R1 and R2 are previously published [7].

REACTOR	FWS [A] (V%)	HRT [B]	PH	C/N RATIO	WW [C] (G D^{-1})	DURATION (DAYS)	OLR [D] G L^{-1} D^{-1}
R0	0	30	7.80	17.5	267	450	1.5
R1 R2	3	30	6.64	17.0	267	125	2.0
R1 R2	6	30	6.15	15.9	267	48	2.3
R1 R2	13	30	6.30	8.5	267	93	3.0
R1 R2	16	30	6.10	8.0	267	94	3.5
R1 R2	19	30	5.90	6.3	267	90	4.3
R3	13	20	6.19	11.8	400	150	4.0
R4	13	25	6.19	11.8	320	150	3.2
R5	13	30	6.19	11.8	267	150	2.8
R6	13	40	6.19	11.8	200	150	2.0

[a] Fish Waste Silage, [b] Hydraulic Retention Time, [c] Wet Weight, [d] Organic Loading Rate

Table 2. Methane production performance and reactor composition (average values). Process performance results for R0, R1 and R2 are previously published [7].

FWS [A] V%	HRT [B]	REACTOR	CH_4 (L G VS^{-1})	NH_4^+-N (G L^{-1})	ACETIC ACID (G L^{-1})	PROPIONIC ACID (G L^{-1})	PH
0	30	R0	0.19 (+/- 0.02)	2.3 (+/- 0.12)	0.50 (+/- 0.03)	0.10 (+/- 0.03)	7,97 (+/- 0.01)
3	30	R1	0.18 (+/- 0.02)	2.99 (+/- 0.21)	0.93 (+/- 0.06)	0.11 (+/- 0.03)	8.0 (+/- 0.06)
		R2	0.19 (+/- 0.02)	3.14 (+/- 0.22)	0.99 (+/- 0.06)	0.12 (+/- 0.05)	8.1 (+/- 0.07)
6	30	R1	0.19 (+/- 0.02)	3.38 (+/- 0.26)	0.50 (+/- 0.03)	0.34 (+/- 0.03)	7.9 (+/- 0.03)
		R2	0.22 (+/- 0.02)	3.43 (+/- 0.12)	0.90 (+/- 0.06)	0.57 (+/- 0.05)	8.0 (+/- 0.06)
13	30	R1	0.294 (+/- 0.01)	5.72 (+/- 0.53)	3.74 (+/- 0.10)	1.09 (+/- 0.02)	8.0 (+/- 0.09)
		R2	0.290 (+/- 0.01)	4.61 (+/- 0.37)	2.40 (+/- 0.75)	0.78 (+/- 0.01)	7.9 (+/- 0.09)
16	30	R1	0.15 (+/- 0.08)	7.26 (+/- 0.67)	8.74 (+/- 0.70)	3.40 (+/- 0.02)	7.5 (+/- 0.62)
		R2	0.36 (+/- 0.02)	6.20 (+/- 0.45)	4.17 (+/- 0.05)	1.86 (+/- 0.02)	8.1 (+/- 0.08)
19	30	R1	-	-	-	-	-
		R2	0.10 (+/- 0.06)	7.36 (+/- 0.42)	9.34 (+/- 0.35)	3.80 (+/- 0.10)	7.51 (+/- 0.20)
13	20	R3	0.253 (+/- 0.03)	5.92 (+/- 0.13)	4.17 (+/- 1.02)	2.61 (+/- 0.13)	7.77 (+/- 0.46)
	25	R4	0.295 (+/- 0.03)	5.68 (+/- 0.10)	4.63 (+/- 0.95)	2.13 (+/- 0.20)	7.80 (+/- 0.41)
	30	R5	0.300 (+/- 0.02)	5.52 (+/- 0.10)	3.55 (+/- 0.66)	1.80 (+/- 0.27)	7.82 (+/- 0.31)
	40	R6	0.312 (+/- 0.03)	5.48 (+/- 0.07)	2.24 (+/- 0.55)	1.01 (+/- 0.32)	7.98 (+/- 0.38)

[a] Fish Waste Silage [b] Hydraulic Retention Time

Errata list

P. nr	Paragraph	Changed from	Changed to
-	Table of con tents	PAPERS….I-II	PAPERS….I-III
ix	List of papers	1.List of papers	List of papers
2	1.1.1	Anaerobic digestion in Norway and Europe	Anaerobic digestion in Norway and Europe – exploitations and potentials
6	1.1.2	a potentially energy rich biogas substrate	potentially energy rich biogas substrates
6	1.1.2	as a co-substrate	as co-substrates
8	1.2.1	first reaction step of AD	first reaction steps of AD
8	1.2.1	fermentative bacteria convert	fermentative bacteria converts
9	1.2.2	degradations steps	degradation steps
11	1.2.3	these reactions is	these reactions are
12	1.2.3	syntrophic operations in biogas reactor	syntrophic operations in biogas reactors
15	1.2.5	in a biogas reactor	in biogas reactors
15	1.2.6	and lead to an unstable	and leads to an unstable
16	1.2.6	elevated NH_4^+ concentrations are a commonly reported reason	elevated NH_4^+ concentrations is a commonly reported reason
18	1.2.6	3 – 4 grams / liter	3 – 4 grams / L
18	1.3	principals	principles
19	1.3.1	operational parameters are crucial	operational parameters is crucial
20	1.3.1	high NH_4^+ concentrations is more easily inhibited	high NH_4^+ concentrations are more easily inhibited
20	1.3.1	the HRT in CSTRs are typically	the HRT in CSTRs is typically
22	1.3.2	also affect the AD process	also affects the AD process
23	1.3.3	in Norway that are used for biogas production	in Norway that is used for biogas production
24	1.3.4	household for CH_4	household for CH_4 production
26	1.3.5	by decrease the risk	by decreasing the risk
27	2.2	The PhD project: outline and objectives	The PhD project; outline and objectives
31	3.2	bacteria belonging to the genuses	bacteria belonging to the genera
40	References	5. References	References