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Doctoral thesis

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Abstract

Aims

Norway's goal is to be climate neutral within the year 2030 according to Stortingsmelding nr. 39 (Det Kongelige Landbruks- og Matdepartementet 2008-2009) and agriculture needs to be a part of the solution. This includes livestock manure that amounts to ~40 % of the total energy potential for biogas production in Norway of which only an insignificant fraction of this is exploited today.

The aim of this thesis was to contribute to sustainable energy production from manure by evaluating the microbial communities in compact anaerobic digestion sludge bed reactors intended for implementation into existing farm infrastructure for manure slurry treatment.

Materials and methods

Two long term experiments were run; the high rate experiment and the high ammonia experiment.

In the high rate experiment, the filtrate of sieved dairy cow manure was used as influent for a period of 96 days in four reactors. Another four reactors were fed pig manure slurry supernatant for 106 days with unadapted and adapted microbial communities. HRT decreased by 5% per day. A PCR/denaturing gradient gel electrophoresis (DGGE) strategy was employed to characterize the microbial communities, and to evaluate the time needed for adaptation of the granular inoculum to the conditions in the manure-fed anaerobic digestion (AD) reactors.

In the high ammonia experiment, four laboratory scale upflow anaerobic sludge bed (UASB) reactors treating pig manure slurry supernatant were operated at different ammonia concentrations and at variable temperatures over a time period of 358 days. High-throughput sequencing of 16S rRNA gene amplicons was applied for both bacterial and archaeal communities to investigate the microbial community dynamics in response to operational variables.

Results and discussion

Multiple operational parameters were tested, like different influents (dairy cow or pig manure supernatant), a wide range of loading rates (up to 400 g COD L^{-1} reactor d^{-1}), extremely low hydraulic retention times (HRTs; 1.7h), high levels of total ammonia nitrogen (TAN; 3.7 ± 0.2 g NH_4-N L^{-1}) and extreme levels of free ammonia nitrogen (FAN; 1.2 ± 0.3 g NH_3-N L^{-1}). Overall, the reactors performed very well under extreme conditions. No foaming and no significant drop or increase in pH were observed even without pH control. The reactors showed remarkable stability and adaptation to changes in loading rate. The process did not fail even at the highest organic loading rate (OLR) tested, implying that supernatant AD is a very robust process. The methane yields obtained at high pig manure OLRs compared to the more particle rich dairy manure indicated that pig manure supernatant was more suitable than dairy manure filtrate as UASB influent. The reactors run on dairy manure needed longer time to stabilize the methane yield, acetate removal and propionate removal, which may be a consequence of the higher fraction of slowly degradable particles in dairy manure.

The results illustrated that microbial communities in the reactor sludge seemed to be more similar to the communities in the influent than those associated with the granular sludge inoculum. This suggests that the influent had a higher impact on microbial community composition in the reactors

than the granular sludge inoculum. Also, non-granular particles accumulated in the reactors and eventually made up a considerable portion of the solid fraction in the reactors. The non-granular particles most likely served as both slowly degradable substrate and as carriers for biofilm growth, ensuring stable operation of the reactors. The observation that methane production significantly exceeded the biogas potential of dissolved organics implies that the feed particles were efficiently retained and degraded. Unique bacterial communities evolved in the reactor liquids despite low HRT, shaped by the selection pressure and conditions in the reactors.

Ammonia inhibition was much reduced after around 200 days of adaptation, allowing methane production at a rather extreme FAN level. The methane production, COD, propionate and acetate removal increased substantially in the high ammonia (HA) reactors from around day 300. The bacterial and archaeal communities adapted into new unique microbiota as a response to the feed composition and elevated concentrations of ammonia. The archaeal operational taxonomic unit (OTU) no. 2, classified as *Methanosaeta*, was the second most dominating OTU across all solid fraction reactor samples and its abundance increased towards the end of the high ammonia experiment, contradicting previous studies. A possible reason for the apparent high ammonia tolerance we observed for *Methanosaeta* in this study could be protection obtained by growing in aggregates with other microbes in the granules and colonized non-granular particles.

We found that a defined group of OTUs representing archaeal taxa outside the Euryarchaeota was highly abundant in the UASB reactors at high ammonia concentrations. We speculate that they have a functional role, either directly as undiscovered methanogenic archaea or indirectly through syntrophic associations. No indications were found of previously described syntrophic acetate oxidizing bacteria (SAOB) associated with methane production in the HA reactors, though we cannot rule out that putative SAOB could be hiding among the numerous unclassified OTUs.

Populærvitenskapelig sammendrag

Norge har som mål å være klimanøytral innen år 2030. Landbruket skal være en del av løsningen ved å produsere biogass av husdyrgjødsel. I dag blir en ubetydelig andel husdyrgjødsel utnyttet. I Europa kjøres gjødselen til store sentraliserte anlegg hvor den behandles. Det fungerer ikke i Norge siden gårdene er små og ligger spredt mellom fjorder og fjell. Her er det mer hensiktsmessig å benytte små biogassanlegg som kan integreres på bondegården.

Vi har designet små anlegg som kan produsere biogass i høyt tempo ved å bare behandle væskefraksjonen av gjødselen. Dette konseptet er stabilt selv ved rask innmating på anlegget og ved høyt innhold av ammoniakk i gjødselen, noe som er vanlig i gjødsel fra bl.a. griser.

Biogass produseres av små mikrober som bakterier og arker. Arker er like små som bakterier, men DNAet deres er mer likt vårt enn bakterienes DNA. Vi har undersøkt det mikrobielle samfunnet i de små anleggene og undersøkt hvordan de endrer seg ved ulike prosessbetingelser. Smårusk samlet seg opp inne i reaktorene og gav mikrobene noe å både vokse og spise på. Dermed bidro smårusket positivt ved at mikrobene fikk stabile vekstforhold. Dette bidro også til at ulike mikrobielle samfunn etablerte seg i forskjellige fraksjoner i reaktoren.

Ammoniakk påvirker det mikrobielle samfunnet negativt. Vi oppdaget at etter 200 dager hadde samfunnet tilpasset seg slik at det kunne produsere gradvis mer biogass selv om det var mye ammoniakk i reaktorene. Det mest interessante var at vi fant en metanproduserende arke (*Methanosaeta*) som vanligvis blir hemmet av mye ammoniakk, men i våre reaktorer trivdes den godt. Det kan kanskje forklares med at denne arken vokste sammen med andre mikrober i midten av store mikrobeklumper (granuler) som skjermet den fra å komme i kontakt med ammoniakk.

Preface

This dissertation is submitted for the degree of Philosophiae Doctor (PhD). The work is divided into two parts. Part I consists of background theory, an overview of the research project, overall discussion and future aspects. Part II contains the four articles that the thesis is based on.

All research was carried out in close collaboration with the University College of Southeast Norway (USN). The reactors were operated at USN) Porsgrunn, Norway, supervised by PhD Wenche Hennie Bergland (main author of article I). All the microbial analyses were executed at Norwegian University of Science and Technology, Trondheim, Norway.

During the thesis, I have participated at three conferences, one local and two international on anaerobic digestion. I presented posters at the international conferences. In addition, I have written several popular science items like blogposts, student paper articles, etc.

List of papers

Bergland, W.H., Dinamarca, C., Toradzadegan, M., Nordgård, A.S.R., Bakke, I. and Bakke, R. (2015) High rate manure supernatant digestion. *Water Research* **76:** 1-9.

Nordgård, A.S., Bergland, W.H., Bakke, R., Vadstein, O., Østgaard, K. and Bakke, I. (2015) Microbial community dynamics and biogas production from manure fractions in sludge bed anaerobic digestion. *Journal of Applied Microbiology* **119**: 1573-1583.

Nordgård, A.S., Bergland, W.H., Vadstein, O., Mironov, V., Bakke, R., Østgaard, K. and Bakke, I. (submitted) Anaerobic digestion of pig manure supernatant in UASB reactors at high ammonia concentrations characterized by high abundances of *Methanosaeta* and non-euryarchaeotal archaea. *Scientific Reports.*

Nordgård, A.S., Bergland, W.H., Mironov, V., Bakke, R., Østgaard, K. and Bakke, I. Mapping anaerobic sludge bed community adaptations to manure supernatant in biogas reactors (working title). *Manuscript in preparation for Applied Microbiology and Biotechnology.*

List of abbreviations

Anaerobic digestion	AD
Canonical correspondence analysis	CCA
Chemical oxygen demand	COD
Coenzyme M methyltransferase	MTR
Continuously stirred tank reactor	CSTR
Denaturing gradient gel electrophoresis	DGGE
Digital droplet polymerase chain reaction	ddPCR
Direct interspecies electron transfer	DIET
Fluorescence *in situ* hybridization	FISH
Free ammonia nitrogen	FAN
High ammonia	HA
High-throughput sequencing	HTS
Hydraulic retention time	HRT
Interspecies hydrogen transfer	IHT
Low ammonia	LA
Methyl-coenzyme M reductase complex	MCR
Miscellaneous Crenarchaeotal Group	MCG
No template control	NTC
Non-methanogenic archaea	NMA
Operational taxonomic unit	OTU
Organic loading rate	OLR
Polymerase chain reaction	PCR
Ribosomal Database Project	RDP
Similarity Percentage	SIMPER
Stable isotope probing	SIP
Sulfate reducing bacteria	SRB
Syntrophic acetate oxidation	SAO
Syntrophic propionate oxidation	SPO
Syntrophic acetate oxidizing bacteria	SAOB
Syntrophic propionate oxidizing bacteria	SPOB
Total ammonia nitrogen	TAN
Total solids	TS
Upflow anaerobic sludge blanket	UASB
Volatile fatty acids	VFA
Volatile suspended solids	VSS

Contents

1 Introduction

Norway's goal is to be climate neutral within the year 2030 according to Stortingsmelding nr. 39 (Det Kongelige Landbruks- og Matdepartementet 2008-2009). The Government wants to prioritize actions that have a positive effect on the environment. All sectors must make cuts in their CO_2 release and the agriculture must also do its part. Cultured land makes out only three percent of the Norwegian land area and it is mostly used for food production. Still there is a good potential for exploiting resources from agriculture for production of bioenergy such as biogas. This includes livestock manure. Biogas can be used for production of heat, electricity and fuel. Livestock manure amounts to 42 % of the total energy potential for biogas production in Norway (Det Kongelige Landbruks- og Matdepartementet 2008-2009). Still only an insignificant fraction of this is exploited today, though it is a goal that 30 % should be treated in biogas facilities within the year 2020. Establishment of biogas facilities based on livestock manure will give a double gain since it also contributes to the reduction of methane emissions from the agriculture. Anaerobic digestion (AD) is the default process for biological conversion of residue organic matter like manure to methane. Biogas technology offers unique possibilities to manage environmental issues as it encompasses renewable energy production, waste treatment, and nutrient recycling.

1.1 Anaerobic digestion

The conversion of complex organic matter to methane is done through the cooperation of a wide range of bacteria and archaea. Bacteria convert organic matter into volatile fatty acids (VFAs), one carbon substrates, CO_2 and H_2 via hydrolysis, acidogenesis and acetogenesis, while archaea use these substrates to produce methane as illustrated in Figure 1 (Stams 1997). Each step will be explained in detail in the following chapters.

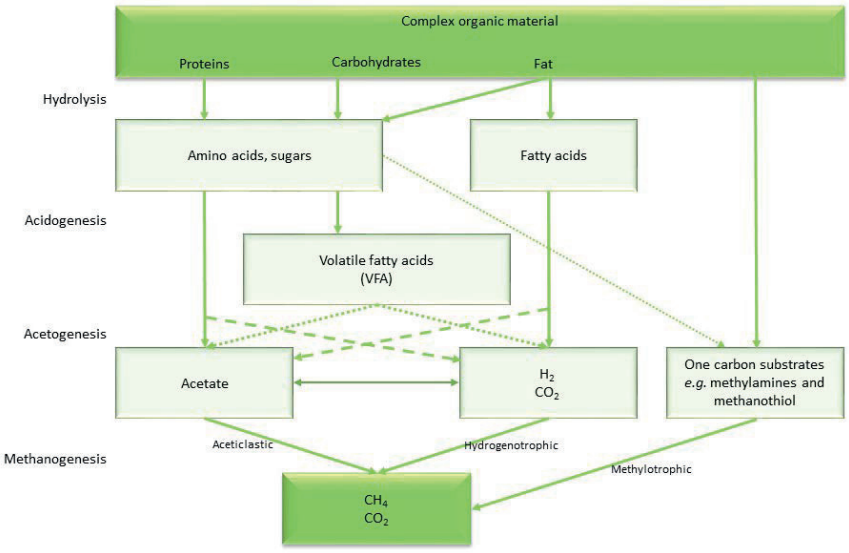

Figure 1: Biological conversion of complex organic matter to methane (CH_4) and CO_2. The figure is adapted from (Stams 1997).

1.1.1 Hydrolysis and acidogenesis by bacteria

Hydrolysis is the conversion of complex organic matter like proteins, carbohydrates and fat into smaller molecules, *i.e.* amino acids, sugars and fatty acids. This is usually done by extracellular enzymes to facilitate transport into the cell where these molecules are converted into volatile fatty acids (VFA). VFA are short chain fatty acids of 2-6 carbon atoms in length (Larreta *et al.* 2006). They are intermediates in the conversion of organic matter and thus important to monitor (Ahring *et al.* 1995). If the rate of VFA production is higher than their consumption, this will be seen as accumulation of butyrate, propionate and acetate. There are multiple possible causes for VFA accumulation, *e.g.* elevated concentrations of ammonia, hydraulic or organic overload (more on this in chapter 1.1.4.1.).

Hydrolysis and acidogenesis are commonly performed by the same bacterial taxa. Riviere *et al.* (2009) have suggested a core group of bacteria as they found these to be common in seven anaerobic sludge digesters with different technology, type of sludge, process and water quality. This core was represented by Chloroflexi, Betaproteobacteria, Bacteroidetes and Synergistetes. Bacteroidetes ferment a range of carbohydrates, while Chloroflexi includes bacteria with various metabolic features. Synergistetes are mainly obligate anaerobic aminolytic bacteria usually found at low abundances in all habitats as well as in AD (Rosenberg *et al.* 2014). Several studies with elevated levels of ammonia report Firmicutes as the dominant bacterial phylum (Niu *et al.* 2013, Chen *et al.* 2016, de Jonge *et al.* 2017, De Vrieze *et al.* 2017, Tsapekos *et al.* 2017). Firmicutes are capable of hydrolyzing carbohydrates and proteins and play a significant role in VFA degradation (Rosenberg *et al.* 2014). Many of these also report Actinobacteria as an important phylum in addition to the phyla already mentioned by Riviere *et al.* (2009).

1.1.2 Acetogenesis

Acetogenesis is the bacterial generation of acetate either via the conversion of VFA by β-oxidation (Schink 1997) or from CO_2 and H_2 via the Wood-Ljungdahl (WL) pathway (Muller *et al.* 2013). In β-oxidation, one moiety of acetate is cleaved off the fatty acid per round until only acetate or propionate remains. Two moieties of propionate are then coupled to a C6 molecule and three acetate molecules are subsequently produced (de Bok *et al.* 2001). The WL pathway, on the other hand, enables acetogenic bacteria to use hydrogen as an electron donor and CO_2 as an electron acceptor and as building blocks for biosynthesis. The pathway may be viewed as a series of reactions where two molecules of CO_2 are reduced and four molecules of H_2 are oxidized to produce acetyl-CoA and eventually acetate. WL is also referred to as the reductive acetyl-coenzyme A pathway and it is illustrated in Figure 2.

The WL pathway is utilized in its reductive direction by acetogenic bacteria, in its oxidative direction by syntrophic acetate oxidizing bacteria (SAOB, see chapter 1.1.2.2) and certain sulfate reducers, and even in part by aceticlastic methanogenic archaea (Muller 2003, Muller *et al.* 2013). Acetogenic bacteria may be divided in two groups based on energy conservation (Muller 2003); Na^+- dependent with *Acetobacterium woodii, Thermoanaerobacter kivui* and *Ruminococcus productus* as examples, and H^+-dependent with *Moorella* spp. as examples.

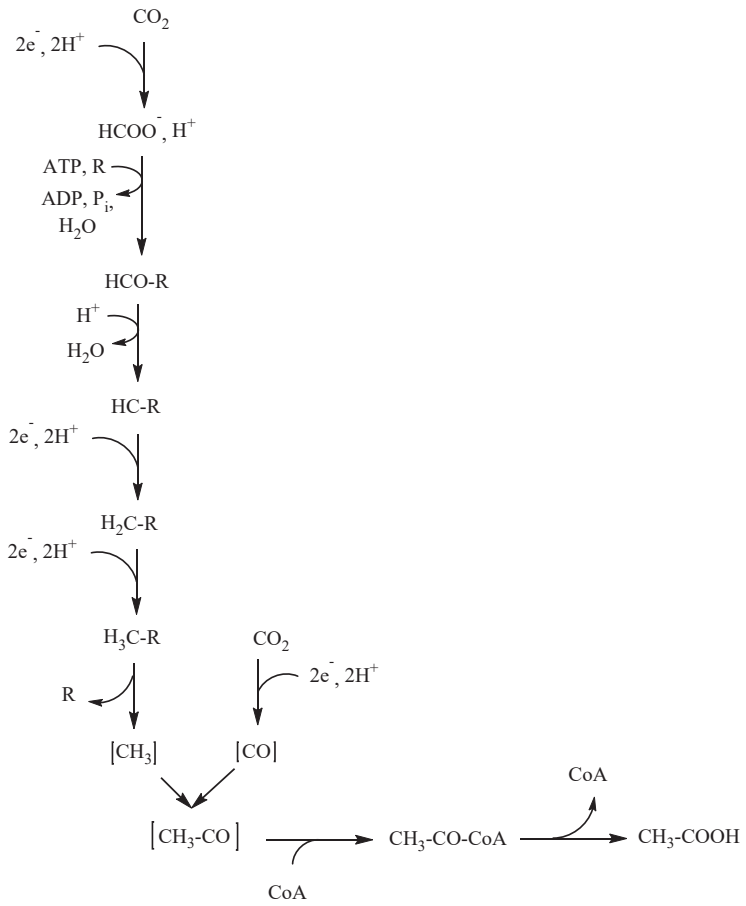

Figure 2: The Wood-Ljungdahl pathway where acetic acid is produced from CO_2 and hydrogen. $2e^-$: reducing equivalents. R: tetrahydrofolate. CoA: coenzyme A. The figure is adapted from Muller *et al.* (2013).

Acetogenic metabolisms under methanogenic conditions face two types of obstacles: thermodynamically unfavorable electron disposal and energy acquisition from limited energy margins. Coupling reoxidation of the general physiological electron carrier, NADH, with respective reduction of H^+ and CO_2 to H_2 and formate is thermodynamically unfavorable. The hydrogen gas level must be kept sufficiently low to give a net release of free energy. The free-energy values given in Table 1 (Vaccari *et al.* 2005) illustrate this dependence. In the absence of methanogenic consumption of hydrogen gas during AD, the free-energy release for the fermentation of either propionate or butyrate would be positive in value and these reactions would simply not occur. By maintaining the intermediate hydrogen gas levels at an acceptably low level, the conversions will be energetically favorable (*i.e*. negative free-energy release). This interplay between H_2-producing and H_2-consuming anaerobes is known as interspecies hydrogen transfer (IHT). Without IHT, the overall process of anaerobic digestion would not be possible.

Table 1: Fermentation free-energy changes for standard *vs* typical reactor values (kJ/reaction)(Vaccari *et al.* 2005)

Fermentation type	Reaction	ΔG^a	ΔG^b
Propionate to acetate, CO_2 and H_2	Propionate + $3H_2O$ → acetate + H^+ + $3H_2$ + HCO_3^-	+76.2	-5.5
Butyrate to acetate, CO_2 and H_2	Butyrate + $2H_2O$ → 2 acetate + H^+ + H_2	+48.2	-17.6

a ΔG^0, Standard free-energy release with 1M fatty acid and H_2 at 1 atm.

b ΔG, free-energy release with typical reactor conditions (1mM fatty acid and H_2 at 10^{-3} atm).

Schink (1997) stated that syntrophy is "a special case of symbiotic cooperation between two metabolically different types of bacteria which depend on each other for degradation of a certain substrate, typically for energetic reasons". The acetogenic bacteria responsible for fatty acid fermentations and H_2 production are referred to as syntrophs (Vaccari *et al.* 2005) and they are dependent on IHT with hydrogenotrophic methanogenic archaea. Hence, they need to stay very close together. Small distances may be obtained by growing in flocs, aggregates or granules (see chapter 1.2.1). Syntrophic associations between bacteria and archaea are crucial for VFA conversion, however, there is quite little information about them. Although Nobu *et al.* (2015) has made a substantial effort to shed some light on the complex syntrophic networks in methanogenic bioreactors and found that the holistic carbon flux from terephthalate to CH_4 and CO_2 may require primary terephthalate degraders, secondary degraders, detritus scavengers and methanogens to form syntrophic networks. Nobu *et al.* (2015) employed state-of-the-art omics tools to generate draft genomes and transcriptomes for uncultivated organisms spanning 15 phyla and was able to suggest functions for several poorly understood phyla. The *Mesotoga* and *Pseudothermotoga* members may be able to syntrophically oxidize acetate through a previously uncharacterized acetate-oxidizing pathway, while a *Syntrophus*-related clade likely performs syntrophic degradation of butyrate and branched-chain fatty acids. Chloroflexi may be capable of homoacetogenesis, and they infer that Hydrogenedentes syntrophically degrades glycerol and lipids derived from detrital biomass. They proposed that Marinimicrobia syntrophically and fermentatively degrades amino acids through proteolysis of protein bound in the community detritus. Atribacteria and Cloacimonetes may perform syntrophic propionate metabolism, and they speculated that chaining syntrophic interactions (secondary syntrophy) and substrate exchange may facilitate proteinaceous detritus metabolism. Anaerobic digestion is still mainly treated as a black box by engineers, but it is essential to understand how this black box works in order to maintain a stable ecosystem in the biogas reactors and to be able to optimize the process.

1.1.2.1 Syntrophic propionate oxidizing bacteria

Propionic acid is one of the major intermediates in AD. According to Lawrence & McCarty (1969), it is a precursor of ca 35 mol% methane (ca 85 mol% together with acetic acid). Propionate oxidizing bacteria oxidize propionate in co-culture with a hydrogen/formate utilizing methanogen or with a suitable chemical compound as electron acceptor. Two propionate-oxidizing pathways have been proposed for mesophilic syntrophic bacteria, *i.e.* the methylmalonyl- coenzyme A pathway (Kosaka *et al.* 2006) and a pathway via a six-carbon intermediate metabolite (de Bok *et al.* 2001). The methylmalonyl- coenzyme A pathway transform propionate into acetate, H_2 and CO_2 via several enzymatic steps including methylmalonyl-CoA, succinate, fumarate, malate, oxaloacetate and pyruvate.

Mostly, syntrophic propionate oxidizing bacteria (SPOB) belong to the Syntrophobacterales order (Deltaproteobacteria) or the *Peptococcaceae* family (Firmicutes) (Felchner-Zwirello 2013). Bacteria with known propionate oxidizing abilities are listed in Table 2. Most syntrophic propionate oxidizing bacteria (SPOB) are also able to reduce sulfate which makes them a special case of sulfate reducing bacteria (SRB). Propionate degradation coupled with sulfate (SO_4^{2-}) reduction to sulfide (H_2S) is energetically more favorable than syntrophic propionate oxidation (SPO). SRBs compete with methanogens for hydrogen and, in some cases, acetate. Thus, the activity of SRBs may be the cause of reactor imbalance in cases where the influent has a high S content (Muyzer & Stams 2008).

Table 2: Syntrophic propionate oxidizing bacteria and their sulfate reduction abilities. Delta; Deltaproteobacteria. Firm.; Firmicutes.

Name	Phylum	Sulfate red.	Reference
Syntrophobacter wolinii	Delta	+	(Boone & Bryant 1980, Wallrabenstein *et al.* 1994)
Syntrophobacter pfennigii		+	(Wallrabenstein *et al.* 1995)
Syntrophobacter fumaroxidans		+	(Harmsen *et al.* 1998)
Syntrophobacter sulfatireducens		+	(Chen *et al.* 2005)
Smithella propionica		-	(Liu *et al.* 1999, de Bok *et al.* 2001)
Pelotomaculum schinkii	Firm.	-	(de Bok *et al.* 2005)
Pelotomaculum thermopropionicum		-	(Imachi *et al.* 2002)
Pelotomaculum propionicum		-	(Imachi *et al.* 2007)
Desulfotomaculum thermocisternum		+	(Nilsen *et al.* 1996)
Desulfotomaculum thermobenzoicum subsp. *thermosyntrophicum*		+	(Plugge *et al.* 2002)
Atribacteria spp.			(Nobu *et al.* 2015)
Cloacimonetes spp.			

Syntrophobacter wolinii was the very first SPOB to be described. It was isolated from an anaerobic digester running on municipal sewage and grown in a co-culture with *Desulfovibrio* sp. (Boone & Bryant 1980). All the *Syntrophobacter* species listed in Table 2 and also *Smithella propionica*, *Pelotomaculum schinkii* and *P. propionicum* have been described in co-cultures with *Methanospirillum hungatei*. *Pelotomaculum thermopropionicum* grew in co-culture with *Methanothermobacter thermautotrophicum* while the two *Desulfotomaculum* species in Table 2 were described in co-culture with *Methanobacterium thermoautotrophicum.* Members of the genus *Syntrophobacter* are phylogenetically and physiologically related to SRBs and can couple propionate oxidation with sulfate reduction (Stams & Plugge 2009). The only member of the *Syntrophobacterales* which cannot reduce sulfate, is *Smithella propionica* (McInerney *et al.* 2008). The genera *Syntrophobacter* and *Smithella* are mesophilic while *Desulfotomaculum* and *Pelotomaculum* are thermophilic.

1.1.2.2 Syntrophic acetate oxidizing bacteria

Most syntrophic acetate oxidizing bacteria (SAOB) characterized to date are affiliated to the physiological group of acetogens. Only a few SAOBs have been isolated and characterized so far, but several taxonomic groups have recently been suggested to harbor novel SAOBs. These are listed in Table 3. SAOB normally grow as lithotrophs or heterotrophs while producing acetate through the Wood‑Ljungdahl pathway (Figure 2). However, under certain conditions like high levels of ammonia and/or thermophilic temperatures, they grow in syntrophy with hydrogenotrophic methanogenic archaea using the WL pathway in its reverse direction to oxidize acetate to hydrogen and CO_2 (Muller *et al.* 2013). The details in the biochemical and regulatory mechanisms behind the shift and the way in which the bacteria regain energy remain largely unknown and hence they provide interesting opportunities for future research. It seems like syntrophic acetate oxidation (SAO) is not a common physiological feature of acetogens as other acetogens such as *Moorella thermoacetica*, *Thermoanaerobacter kivui* and *Acetobacterium woodii* are unable to use the WL pathway in its reverse direction and to grow syntrophically with hydrogenotrophic methanogens (Muller *et al.* 2013). Hagen *et al.* (2017) suggest a metabolic scenario where multiple uncultured SAOBs are capable of syntrophically oxidizing acetate as well as long-chain fatty acids (LCFA) via β-oxidation and the WL pathways to H_2 and CO_2. Such an arrangement differs from known syntrophic oxidizing bacteria.

Two pathways have been suggested for syntrophic acetate oxidation (Muller *et al.* 2016). The first employs a reversed WL pathway which has been established in *Thermacetogenium phaeum* and *Clostridium ultunense*. *Tepidanaerobacter acetatoxydans* harbors a truncated WL pathway. The second pathway circumvents the carbonyl branch of the WL pathways by combining the glycine cleavage system with the methyl branch of the WL pathway and has been suggested for a *Mesotoga* community and *P. lettingae* (Nobu *et al.* 2015).

In the case of the mesophilic *S. schinkii*, a study using genome walk approach proved the presence and expression of the formyltetrahydrofolate synthetase gene (FTHFS) under heterotrophic and syntrophic growth conditions (Muller *et al.* 2013). Formyltetrahydrofolate synthetase is a key enzyme of both acetogenic and SAO metabolism. Both SAO pathways require FTHFS activity, catalyzing the reversible ATP-dependent activation of formate, and thus the *fhs* gene is a suitable functional marker for both acetogenic and SAOB communities (Westerholm *et al.* 2011b, Muller *et al.* 2016).

Most of the isolated and characterized SAOBs have been isolated from biogas reactors except *Candidatus Contubernalis alkalaceticum* which was retrieved from the silty sediments of the Khatyn soda lake in Russia. Ito *et al.* (2011) discovered that the Synergistes group 4/PD-UASB-13 must harbor SAOB by employing RNA-SIP (stable isotope probing) with 13C6-glucose and 13C3-propionate as sole carbon source to identify the acetate-utilizing unidentified bacteria. To confirm that this bacterial group could utilize acetate, specific fluorescence *in situ* hybridization (FISH) probes targeting Synergistes group 4 was applied to the sludge incubated with 14C-acetate for MAR-FISH. Interestingly, they found that Synergistes group 4/PD-UASB-13 had high Km for acetate and maximum utilization rate. Therefore, they were more competitive for acetate than *Methanosaeta* at high acetate concentrations.

Table 3: Syntrophic acetate oxidizing bacteria (SAOB)

Status	Name	Reference
Isolated and characterized	Strain AOR	(Lee & Zinder 1988)
	Clostridium ultunense	(Schnürer *et al.* 1996)
	Thermacetogenium phaeum	(Hattori *et al.* 2000)
	Pseudothermotoga lettingae	(Balk *et al.* 2002)
	Candidatus Contubernalis alkalaceticum	(Zhilina *et al.* 2005)
	Syntrophaceticus schinkii	(Westerholm *et al.* 2010)
	Tepidanaerobacter acetatoxydans	(Westerholm *et al.* 2011c)
Suggested	Synergistes group 4/PD-UASB-13	(Ito *et al.* 2011)
	Coprothermobacter spp.	(Ho *et al.* 2014)
	Mesotoga spp.	(Nobu *et al.* 2015)
	Pseudothermotoga spp.	(Nobu *et al.* 2015)
	unFirm_1	(Frank *et al.* 2016)
	Five subspecies of Clostridia	(Mosbæk *et al.* 2016)
	Putative SAOB cluster I, II and III	(Muller *et al.* 2016)
	unFi_c1 and unFi_c2	(Hagen *et al.* 2017)

Muller *et al.* (2016) revealed three clusters of putative SAOB affiliating with Thermoanaerobacterales (pSAOB I), *Thermoanaerobacteraceae* (pSAOB II) and *Clostridiaceae* (pSAOB III). In addition, they found OTUs affiliating to *Tepidimicrobium* (*Tessierellaceae*), *Thermacetogenium* (*Thermoanaerobacteraceae*), *Alkaliphilus* (*Clostridiaceae*), *Acholeplasmataceae*, MBA08 (Clostridia), SHA-98 (Clostridia), OPB54 (Firmicutes) and Thermanaerobacterales became more abundant at the same time point as SAO became the dominant methane producing pathway. Hence, both the entire bacterial community and the *fhs*-harbouring sub-community responded by a complete community change to elevated levels of ammonia. Werner *et al.* (2014) set forth a hypothesis where they present the idea of a microbial community with SAO function: "…individual syntrophic strains have been enriched, it remains elusive whether or not enriched strains represent keystone populations in reactor microbiomes or if syntrophic acetate oxidation is a function of a heterogeneous and dynamic community." The results of the study conducted by Muller *et al.* (2016), however, point to a keystone community specialized in SAO rather than a versatile, heterogeneous community performing SAO as a metabolic option.

Frank *et al.* (2016) showed that the microbiome of a commercial, ammonia-tolerant biogas reactor harbored a deeply branched, uncultured phylotype unFirm_1 with high SAO activity. Culturable SAOBs were identified in genomic analyses of the reactor but their limited proteomic representation suggested that unFirm_1 played an important role in channeling acetate toward methane. Notably, they found unFirm_1-like populations in other high-ammonia biogas installations, conjecturing a broader importance for this novel clade of SAOB in anaerobic fermentations.

Mosbæk *et al.* (2016) used a combination of metagenomics, amplicon sequencing and protein-SIP. The proteins from actively incorporating organisms were mapped onto a binned metagenome for identification of the bacteria involved. The results revealed that five subspecies of Clostridia were actively involved in acetate turnover. The acetate consuming organisms all contained the FTFHS gene, indicating that these organisms are possible SAOB.

Hagen *et al.* (2017) used a combination of high-throughput 16S rRNA gene sequencing and total metagenome analyses, which allowed generation of population genome bins of both classified and novel phylotypes. They identified two uncultured phylotypes affiliated with the Firmicutes phylum (unFi_c1 and unFi_c2). The detection of nearly all enzymes required for WL pathway gave strong indications towards their role as an SAOB.

1.1.3 Methanogenesis

1.1.3.1 Methanogenic pathways

Methane is produced by methanogenic archaea via three pathways (Garcia *et al.* 2000, Borrel *et al.* 2013b); aceticlastic, hydrogenotrophic and methylotrophic methanogenesis. All are illustrated in Figure 3. The last step of all three pathways consists of the conversion of methyl-coenzyme M (methyl-S-CoM) into methane and is performed by the same enzymatic complex in all methanogens, the methyl-coenzyme M reductase (MCR).

In the hydrogenotrophic pathway, CO_2 is reduced to methyl-S-CoM via six steps and the electrons required to reduce methyl-S-CoM to methane derive from an external H_2 source, *e.g.* acetogenic bacteria. In the aceticlastic pathway, the methyl group of acetate is converted into methane and the carboxyl group into CO_2. The electrons required to reduce methyl-S-CoM to methane are derived from the oxidation of the carboxyl group of the acetate (Borrel *et al.* 2013b).

Lastly, in methylotrophic methanogenesis, methyl-CoM is produced via transfer of a methyl group from *e.g.* methanol, methylamines (mono-, di-, and trimethylamine), or dimethyl sulfide to HS-CoM by enzymes specific for each substrate. Methyl-CoM is subsequently disproportionated in a 3:1 ratio; one mole of methyl-CoM is oxidized to CO_2 for every three moles of methyl-CoM reduced to methane. Methyl-CoM oxidation occurs through a reverse of the CO_2 reduction pathway to generate reducing equivalents for methyl reduction. Regeneration of HS-CoM occurs via a heterodisulfide of coenzyme M and B (CoM-S-S-CoB) (Borrel *et al.* 2013b). This last step is illustrated in Figure 3 and it is present in all the three methanogenic pathways. Methylotrophic methanogens are found in the orders Methanosarcinales, Methanobacteriales and Methanomassiliicoccales, and can be divided into two groups: Methylotrophs without cytochromes which are obligately H_2-dependent, and those that possess cytochromes (that is, members of the Methanosarcinales), which also can oxidize methyl groups to CO_2 via a membrane-bound electron transport chain (Vanwonterghem *et al.* 2016). All known methanogens use cofactor F_{420} (functionally analogous to NAD^+), which results in a bright blue-green autofluorescence of the cells when illuminated at 420 nm.

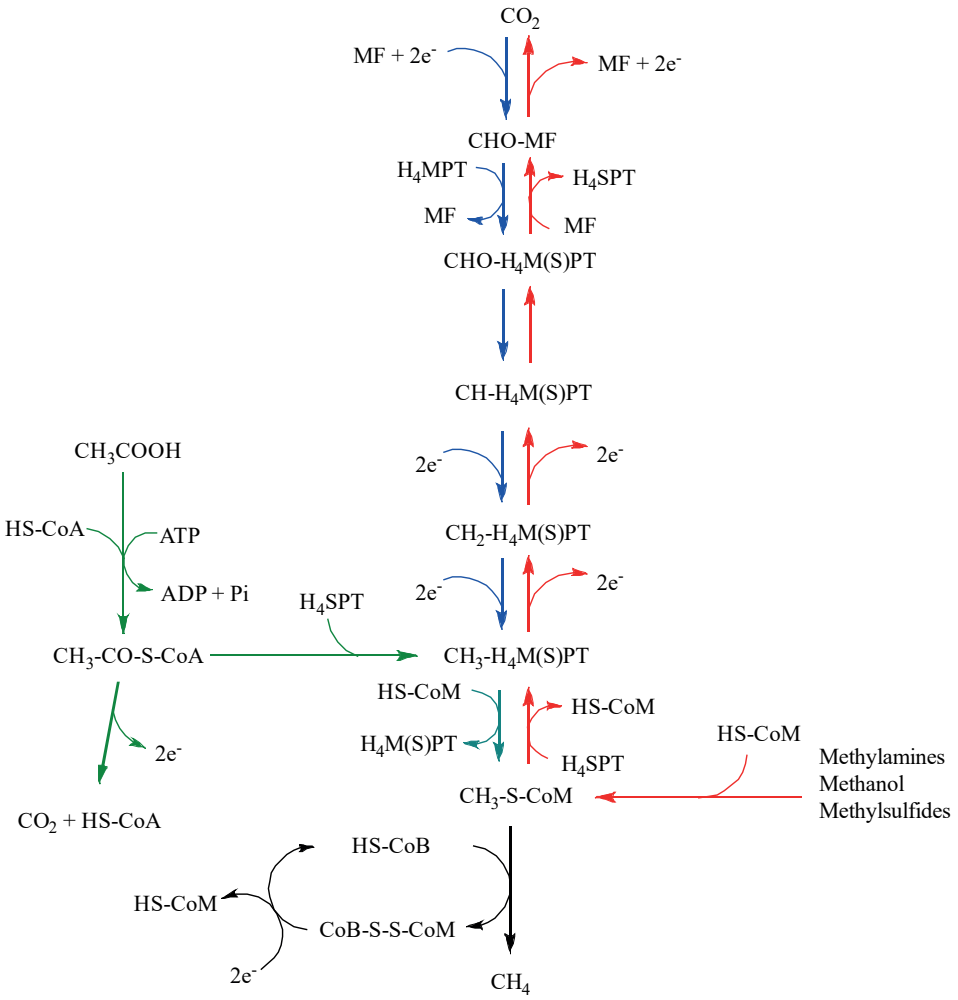

Figure 3: The aceticlastic (green), hydrogenotrophic (blue) and methylotrophic (red) methanogenesis pathways. Black arrows indicates reactions that are common to all pathways while the turquoise arrows are common to the aceticlastic and hydrogenotrophic pathways. $2e^-$: reducing equivalents. MF: methanofuran. H_4MPT: tetrahydromethanopterin. H_4SPT: tetrahydrosarcinapterin, a functional analogue of H_4MPT found in Methanosarcinales. CoM/B/A: coenzyme M/B/A. CoB-S-S-CoM: heterodisulfide of coenzyme B and M. The figure is adapted from (Browne & Cadillo-Quiroz 2013).

It has generally been believed that aceticlastic methanogenesis has been the dominant pathway in most biogas reactors producing approximately 70 % of the collected methane while 30 % was produced via the hydrogenotrophic pathway (Stams 1997). A recent study conducted in Sweden using samples from nine randomly selected large-scale digesters revealed that methane was generated through SAO rather than aceticlastic methanogenesis (unpublished). Thus, SAO appears to be of much greater importance for the biogas process than assumed previously and therefore maybe aceticlastic methanogenesis should no longer be considered to be the main pathway (Muller *et al.* 2013).

1.1.3.2 Overview of methanogenic archaea

Methanogenesis coupled to the WL pathway is one of the most ancient metabolisms for energy generation and carbon fixation in the Archaea. Acetogenic bacteria use the WL pathway to generate acetate while most archaea produce methane. The WL pathway has therefore been traditionally linked to methanogenesis in the Archaea. All known methanogenic archaea (Table 4) have until recently been classified in the archaeal phylum Euryarchaeota (Borrel *et al.* 2016). They have traditionally been divided into two phylogenetically distinct classes (Bapteste *et al.* 2005). Class I harbored Methanobacteriales, Methanococcales and Methanopyrales while Class II was comprised of Methanomicrobiales, Methanocellales and Methanosarcinales (*i.e.* the taxonomic class of Methanomicrobia). Independent of the type of methanogenesis performed, the representatives of these two classes have been consistently found to share a common set of enzymes for methanogenesis: the methyl-branch of the archaeal type WL pathway, the coenzyme M methyltransferase complex (MTR) and the methyl-coenzyme M reductase complex (MCR) (Borrel *et al.* 2016).

Our knowledge of methanogenic substrate utilization and energy conservation is still incomplete but rapidly expanding through the development of new molecular techniques such as metagenomics. This includes the surprising discovery of a complete set of genes for the enzymes necessary for the reduction of carbon to methane encoded in *Methanosaeta*, a genus originally thought to be strictly aceticlastic (Zhu *et al.* 2012, Rotaru *et al.* 2014). Rotaru *et al.* (2014) have demonstrated that *Methanosaeta harundinacea* accept electrons via direct interspecies electron transfer (DIET) for reduction of CO_2 to methane. It has generally been assumed that only H_2 or formate could serve as interspecies electron carriers. However, the discovery that *Methanosaeta* species are able to make direct electrical connections with *Geobacter* species and accept electrons for the reduction of CO_2 to methane, demonstrates that DIET is an alternative to interspecies H_2/formate transfer. In other words, *Methanosaeta* is still not a hydrogenotrophic methanogen, but rather a CO_2-reducing and aceticlastic methanogen.

Metagenomic sequencing lead to the discovery of several new methanogens, *e.g. Candidatus Methanoflorens stordalenmirensis* by Mondav *et al.* (2014). This novel methanogen belonged to the uncultivated lineage “Rice cluster II”, now called *Candidatus Methanoflorentaceae*. *Candidatus Methanoflorentaceae* are widespread in high methane-flux habitats suggesting that this lineage is both prevalent and a major contributor to global methane production. *Candidatus M. stordalenmirensis* was placed in the order Methanocellales and its genome revealed all of the genes required for hydrogenotrophic methanogenesis.

Table 4: Methanogenic archaea. The number of genera is listed if it exceeds four genera, except *Methanoculleus* and *Methanosarcina* as they are of special interest in digesters with high ammonia levels. Only discoveries made after 2012 are included with references.

Phylum	Class	Order	Family	Genus	References
Euryarchaeota	Methanobacteria	Methanobacteriales	*Methanobacteriaceae*	*Methanobacterium* *Methanobrevibacter* *Methanosphaera* *Methanothermobacter*	
			Methanothermaceae	*Methanothermus*	
	Methanococci	Methanococcales	*Methanocaldococcaceae*	*Methanocaldococcus* *Methanotorris*	
			Methanococcaceae	*Methanococcus* *Methanothermococcus*	
	Methanomicrobia	Methanosarcinales	*Methanosarcinaceae*	*Methanosarcina** + 7	
			Methanosaetaceae	*Methanosaeta**	
			Methermicoccaceae	*Methermicoccus*	
		Methanomicrobiales	*Methanocorpusculaceae*	*Methanocorpusculum*	
			Methanomicrobiaceae	*Methanoculleus* + 5	
			Methanospirillaceae	*Methanospirillum*	
			Methanoregulaceae	*Methanosphaerula* *Methanolinea* *Methanoregula*	
			Incertae-sedis	*Methanocalculus*	
		Methanocellales	*Methanocellaceae*	*Methanocella* *Methanoflorens*	(Mondav *et al.* 2014)
	Methanopyri	Methanopyrales	*Methanopyraceae*	*Methanopyrus*	
	Ca. Methanofastidiosa			*Ca. Methanofastidiosum*	(Nobu *et al.* 2016)
	Thermoplasmata	Methanomassiliicoccales	*Methanomassiliicoccaceae*	*Methanomassiliicoccus* *Ca. Methanogranum* *Ca. Methanomethylophilus* *Ca. Methanoplasma*	(Borrel *et al.* 2012, Dridi *et al.* 2012, Borrel *et al.* 2013b, Iino *et al.* 2013, Lang *et al.* 2015)
Bathyarchaeota				BA1 and BA2 **	(Evans *et al.* 2015)
Ca. Verstraetearchaeota	*Ca.* Methanomethylia	*Ca.* Methanomethyliales	*Ca. Methanomethyliaceae*	*Ca. Methanomethylicus* *Ca. Methanosuratus*	(Vanwonterghem *et al.* 2016)

* acetate utilizing

** near-complete reconstructed genomes

Borrel *et al.* (2013b) established the existence of a seventh order of methanogenic archaea, namely the Methanomassiliicoccales, which did not cluster with neither Class I nor Class II. Methanomassiliicoccales rather belonged to the recently proposed superclass Diaforarchaea and has so far one family, *Methanomassiliicoccaceae*, with one characterized member *Methanomassiliicoccus luminyensis* (Dridi *et al.* 2012) and four *candidatus* members; *Methanomassiliicoccus intestinalis* (Borrel *et al.* 2013a), *Methanomethylophilus alvus* (Borrel *et al.* 2012), *Methanogranum caenicola* (Iino *et al.* 2013) and *Methanoplasma termitum* (Lang *et al.* 2015). The Methanomassiliicoccales are obligate H_2-utilizing methylotrophic methanogens. Amazingly, they lack the methyl-branch of the archaeal type WL pathway and the MTR complex. For methanogenesis, they use methyltransferases and corrinoid proteins allowing the transfer of methyl-groups from methanol, methylated-amines, and dimethyl sulfide to H-S-CoM.

The larger phylogenetic distribution of methanogens without WL or MTR underlines the increasing importance of methanogenesis based on the reduction of methyl compounds by H_2. This is supported by the recent report of this metabolic conformation in a proposed sixth Euryarchaeota class of methanogens named *Candidatus Methanofastidiosa* (formerly known as WSA2 or Arc I) by Nobu *et al.* (2016) with the species *Candidatus Methanofastidiosum methylthiophilus*. They recovered eight draft genomes for four WSA2 populations. Taxonomic analyses indicated that WSA2 is a distinct class from other Euryarchaeota. All the genomes consistently encoded a methylated thiol coenzyme M methyltransferase that suggests that WSA2 is the first discovered methanogens restricted to methanogenesis by methylated thiol reduction.

1.1.3.3 Methanogenic archaea outside Euryarchaeota

An astonishing discovery was made by Evans *et al.* (2015) as they were the first to report the existence of methanogenic archaea outside the Euryarchaeota phylum. This discovery challenged the current idea that methanogenesis originated early in the evolution of Euryarchaeota. Vanwonterghem *et al.* (2016) made an important contribution to this challenge when they described a whole new phylum harboring two new genera.

Evans *et al.* (2015) performed metagenomic sequencing of a deep aquifer in Australia. From their samples, they recovered two near-complete genomes, BA1 and BA2, belonging to the archaeal phylum Bathyarchaeota (formerly known as the Miscellaneous Crenarchaeotal Group). These genomes contained divergent homologs of the genes necessary for methane metabolism, including those that encode the MCR complex. BA1 and BA2 appear to have the ability to carry out complex fermentation and β-oxidation. This is unique among archaeal methanogens. Evans *et al.* (2015) hypothesize that BA1 is a methylotrophic methanogen that use reduced ferredoxin, generated during fermentation of amino acids and maltose, to reduce methyl groups from diverse organic sources to methane. In contrast, BA2 most likely derives its energy from fatty acid metabolism, and the presence of a gene for acetyl-CoA synthase suggests that carbon can be incorporated into biomass from acetate. Unlike euryarchaeotal methanogens, BA1 may use lactate, and BA2 may use peptides, monosaccharides, and pyruvate for energy production. It thus appears that BA1 and BA2 have broader substrate spectra than euryarchaeotal methanogens and do not catabolize the classical fermentation end products H_2/CO_2 and acetate used by many euryarchaeotal methanogens. However, both appears to have the potential for methylotrophic methanogenesis using a wide range of methylated compounds. Moreover, in

unrooted phylogenies of MCR subunits, Bathyarchaeota sequences are very divergent with respect to Euryarchaeota sequences, suggesting vertical inheritance of these genes in BA1 and BA2. It was proposed that methanogenesis in these Bathyarchaeota could occur via reduction of methyl compounds by H_2, similar to Methanomassiliicoccales (Evans *et al.* 2015).

Vanwonterghem *et al.* (2016) further strengthened the growing importance of methylotrophic methanogenesis. They analyzed divergent MCR genes from several distinct habitats ranging from anaerobic digesters to waters of coalbed methane wells. They were able to establish a whole new phylum (see Table 4), *Candidatus* Verstraetearchaeota, a new order, a new family, and two new genera; *Candidatus Methanomethylicus* with two species and *Candidatus Methanosuratus* with one species. In addition to methane metabolism, the Verstraetearchaeota appear to be capable of utilizing sugars as a carbon source and generating acetyl-CoA.

Future research will reveal how widely distributed novel groups of methane cycling archaea are (both phylogenetically and environmentally), what their contribution is to global methane production, which energy substrates they use and why they have so far evaded isolation.

1.1.3.4 Non-methanogenic archaea

The domain Archaea has historically been divided into two phyla, the Crenarchaeota and Euryarchaeota. In anaerobic digestion processes, methanogens belonging to Euryarchaeota were the predominant archaeal populations. Studies based on genomes, 16S rRNA gene sequences, and the "omics era" have dramatically expanded and reshaped the archaeal tree, and several new phylum-level lineages and two superphyla have been proposed. The first superphylum is referred to as TACK and is comprised of Thaumarchaeota, Aigarchaeota, Crenarchaeota and Korarchaeota. The second one is named DPANN and was originally made up of Diapherotrites, Parvarchaeota, Aenigm-archaeota, Nanoarchaeota and Nanohaloarchaea. Now Pacearchaeota and Woesearchaeota have also been added to DPANN (Castelle *et al.* 2015).

Non-methanogenic archaea (NMA) in anaerobic digestion have previously been identified as Crenarchaeota (Riviere *et al.* 2009, Chouari *et al.* 2015). Some of these organisms are characterized by an ammonia oxidation metabolism, which are now classified as Thaumarchaeota (Brochier-Armanet *et al.* 2008, Pester *et al.* 2011, Kerou *et al.* 2016). Chouari *et al.* (2015) made an effort to define a core of NMA OTUs present in anaerobic digestion when they sampled 22 digesters from all over the world. They uncovered two crenarchaeotal OTUs representing up to 33.6 % of all archaeal sequences in their samples in almost half of the digesters sampled. Interestingly, Thermoplasmata was less abundant but were always detected when Crenarchaeota were present. Three OTUs were identified for Thermoplasmata. Moreover, Crenarchaeotes coexisted with methanogens and were particularly abundant when *Candidatus Methanofastidiosa* was dominant in the digesters.

Chen & He (2016) tried to gain insight into the ecophysiology of these uncharacterized archaeal populations, and presented a phylogenetic analysis performed on a collection of presumably non-methanogenic archaeal 16S rRNA gene sequences from anaerobic digesters of broad geographic distribution. They revealed a distinct clade in subgroup 6 of the Miscellaneous Crenarchaeotal Group (MCG) in the newly proposed archaeal phylum Bathyarchaeota. These archaeal populations were minor constituents of the archaeal communities, and their abundance remained relatively constant irrespective of process perturbations. Furthermore, these NMAs appeared to favor co-occurrence with aceticlastic methanogens. Interestingly, NMA have been reported as the dominant archaeal

population in several anaerobic processes (Collins *et al.* 2005, Enright *et al.* 2009, Schauer-Gimenez *et al.* 2010, Acharya *et al.* 2011, Siggins *et al.* 2011). Hence, they could be exploiting a metabolic pathway that has yet to be identified in AD. In order to support NMA populations at high abundance in AD, Chen & He (2016) hypothesized that these NMA populations could be involved in carbon metabolism, which is central in the methanogenic anaerobic food web. Their results indicated that NMA appeared to have low abundance in AD processes run on animal manure and suggested that this discrepancy might be attributed to the differences in feedstock. None of the studies with high abundance of NMA utilized animal waste as influent.

1.1.4 Common challenges in anaerobic digestion

1.1.4.1 Accumulation of volatile fatty acids

Accumulation of VFA is usually a sign of process imbalance and may cause a drop in pH. Volatile fatty acid accumulation reflects a kinetic uncoupling between acid producers and consumers and is typical for stress situations (Ahring *et al.* 1995, Vaccari *et al.* 2005). Acidic pH levels have negative impact on these reactions. Methanogens are sensitive to pH outside the range 6 to 8, hence, a decrease in pH below 6 reduces the activity of the methanogens more than that of the acidogens/acetogens. This causes a buildup of organic acids, further reducing pH. Thus, anaerobic digestion is unstable when confronted with pH disturbances. A decrease in pH could be caused by too high organic loading rate with an easily fermented influent. In such cases, VFAs are being produced faster than the methanogens are able to consume them. VFA accumulation could also be caused by too high H_2 partial pressure disrupting the delicate balance between H_2 production and consumption.

1.1.4.2 High ammonia concentration

Ammonia is produced by the biological degradation of the nitrogenous matter, mostly in the form of proteins and urea. It is generally believed that ammonia concentrations below 200 mg/L are beneficial to anaerobic process since nitrogen is an essential nutrient for anaerobic microorganisms (Chen *et al.* 2008).

High total ammonia nitrogen levels (TAN, *i.e.* $NH_4^+ + NH_3$; > 3000 mg/L) have been shown to be an important factor regulating the shift from aceticlastic methanogenesis to syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis in mesophilic biogas processes (Schnürer & Nordberg 2008).The shift is probably a consequence of inhibition of the activity of the aceticlastic methanogens. Free ammonia nitrogen (FAN, *i.e.* NH_3) has been considered as the main cause of ammonia inhibition as it may freely pass through the cell membrane. Several mechanisms for ammonia inhibition have been proposed, such as a change in the intracellular pH, increase of maintenance energy requirement, and inhibition of a specific enzyme reaction (Chen *et al.* 2008). It is believed that aceticlastic methanogens are more sensitive to high ammonia levels than hydrogenotrophic methanogens and that methanogens in general are more sensitive than SAOBs (Fotidis *et al.* 2013). However, there are indications that some hydrogenotrophic methanogens are equally or even more tolerant to high TAN than SAOBs (Wang *et al.* 2015). This makes it hard to postulate generalizations on ammonia inhibition in AD. Inhibition of the AD process is usually indicated by a decrease in the steady state methane production rates and increase in the intermediate digestion products like VFA concentrations, particularly acetate and propionate.

A good choice of temperature, control of pH and C/N ratio, and utilization of acclimatized microflora to higher ammonia concentrations may ensure a stable and undisturbed digestion (Rajagopal *et al.*

2013). Since FAN has been suggested to be the actual toxic agent, an increase in pH would result in increased toxicity as described in equation (1).

$$FAN = \frac{TAN}{1+\frac{10^{-pH}}{K_a}} \quad (1)$$

Process instability due to ammonia often results in VFA accumulation, which again leads to a decrease in pH and thereby declining concentration of FAN. Both microbial growth rates and FAN concentration are affected by temperature change since K_a is dependent on temperature. An increased process temperature in general has a positive effect on the metabolic rate of the microorganisms but also results in a higher concentration of FAN. In anaerobic digesters operated at pH 7 and 35°C, FAN represents less than 1% from the total ammonia, while, at the same temperature, but pH 8 the FAN increases to 10% (Rajagopal *et al.* 2013). In other words, an increase in pH from 7 to 8 will lead to an eightfold increase of the FAN levels in mesophilic conditions and even more at thermophilic temperatures.

Several methods have been suggested to counteract ammonia inhibition (Chen *et al.* 2008, Rajagopal *et al.* 2013). Certain ions such as Na^+, Ca^{2+}, and Mg^{2+} have antagonistic effects on ammonia inhibition, a phenomenon in which the toxicity of one ion is decreased by the presence of other ion(s). Air stripping and chemical precipitation may remove ammonia from the substrate. Dilution reduce the concentration of ammonia, but it also increases the waste volume. The use of struvite precipitation, anammox, zeolite and carbon fiber textiles has also been proposed (Rajagopal *et al.* 2013). Another common method is to increase the retention time of the biomass either by enhanced sedimentation or by immobilization. Acclimation of methanogenic communities is a proven useful and economical method but it is not yet clear whether the adaptation is a consequence of metabolic transition of already existing microbial populations or from changes in the community composition due to different ammonia concentrations. TAN concentrations of around 1700–1800 mg/L may be completely inhibitory with unacclimated inoculum, although with acclimation, inhibitory TAN levels could surpass 5000 mg/L (Yenigun & Demirel 2013).

1.1.4.3 Reduction of sulfate

There are three possible sulfur sources in anaerobic digestion (Vaccari *et al.* 2005). Sulfides may be introduced to anaerobic digesters via the sludge streams as

i) constitutive cellular elements (*e.g.* sulfur-bearing amino acids) released directly from lysed microbial cells found in the secondary sludge
ii) reduced metallic-sulfide (*e.g.* FeS) precipitates found within reduced sludges
iii) incoming soluble sulfates being converted directly to sulfides due to the highly reducing conditions within the digester

Whatever their source, sulfides within anaerobic digesters may then undergo a variety of important transformations.

Sulfate reducing bacteria (SRB) may reduce sulfate to sulfide. SRB are very diverse in terms of their metabolic pathways and may completely or partially degrade branched-chain and long chain fatty acids, ethanol and other alcohols, organic acids, and aromatic compounds (Chen *et al.* 2008). SRB may compete with methanogens, acetogens, or fermentative microorganisms for available acetate, H2, propionate, and butyrate in anaerobic systems. The outcome of the competition determines the concentration of sulfide in the reactor system. Sulfide is toxic to methanogens as well as to the SRB themselves.

Sulfide-based precipitation may occur to an extent that could reduce the net solubility of many metal species (*e.g.* iron, copper, nickel and zinc), possibly even to a degree that would reduce their availability as metabolically necessary trace elements. Hydrolysis of the sulfide may also take place given the slightly acidic conditions found in many anaerobic digesters (*i.e*. due to the low acid ionization constant for sulfide hydrolysis, $H_2S \rightarrow HS^- + H^+$). This would lead to gas exchange via stripping into the headspace and contamination of the methane product to an extent that could necessitate removal prior to using the methane as an energy-rich feed gas (Vaccari *et al.* 2005).

1.2 High rate UASB reactors integrated with existing farm infrastructure

Manure storage tanks with 8 months minimum HRT capacity are already installed in cold climate countries like Norway to comply with government regulations to avoid use as fertilizer outside the short growth season. This storage may serve as a first step in an AD treatment line and/or be used for effluent storage. It has been observed that manure particles disintegrate and hydrolyze during such storage, thereby improving its quality as AD feed (Bergland *et al.* 2014). The manure in the storage separates spontaneously into a floating layer (straw, wood chips, *etc.*), a bottom sediment layer and a middle layer with much less suspended solids than the floating and bottom layers (Figure 4). High rate AD feed can be siphoned from the middle layer at no extra cost.

The original and most extensively used high rate reactor is the upflow anaerobic sludge blanket (UASB). High rate UASB AD could be used in combination with a particle-liquid separation process and be implemented into existing farm infrastructure. This scenario is illustrated in Figure 5. A set up like this could be economically feasible and manageable for a single farm. UASB reactors can be more than fifty times more efficient than traditional completely mixed biogas reactors consistently used in agriculture and wastewater sludge treatment at present (Lettinga *et al.* 1997, Barber & Stuckey 1999, Tchobanoglous *et al.* 2003, von Sperling & Oliveira 2009). Such sludge blanket reactors are used to treat the liquid fraction of organic waste containing small amounts of suspended solids (Tchobanoglous *et al.* 2003). The particle content of settled manure is higher (> 6 g TSS L^{-1}) than recommended for UASB treatment (Tchobanoglous *et al.* 2003). Alternative high rate AD designs, such as fixed biofilm reactors, have been tested on such wastes but solids build up blocking the void spaces in the filter medium making such alternatives less promising. The UASB is, however, the standard of high rate AD, so a small UASB like sludge bed reactor design was chosen for the present project to test the possibilities of high rate AD slurry treatment.

Figure 4: Pig manure sample from the bottom of a storage tank. The particles sediment into a bottom layer leaving a middle layer (upper part of the picture) suitable as high rate UASB AD feed.

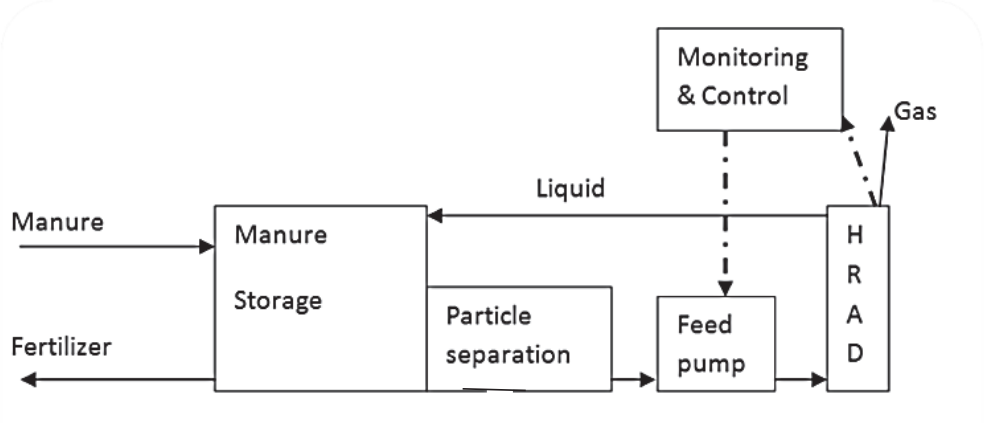

Figure 5: Manure storage with particle-liquid separation when required and high rate anaerobic digestion (HRAD; *e.g.* UASB) in combination with automation for process control.

1.2.1 Anaerobic granules

The separation of metabolic functions and their distribution among metabolically different microorganisms, *i.e.* substrate oxidation and hydrogen formation in the one and hydrogen oxidation and CO_2 reduction in the other, is a rather unusual strategy that is not observed in the same extent in oxic environments (Schink & Stams 2006). However, it may have its advantages as it allows for a higher degree of metabolic specialization within each species. The metabolic efficiency of such cooperating organisms depends on efficient metabolite transfer between the involved partners. The flux of H_2 between acetogenic bacteria and hydrogenotrophic methanogens is inversely proportional to the distance between them. Optimal metabolite transfer can be achieved best when the partners are in close contact with each other like in aggregates, flocs and granules (Schink & Stams 2006).

Granules built and maintained by microorganisms are essential in high rate UASB anaerobic digestion. Generally, microorganisms including acidogens, acetogens and methanogens, aggregate into granules of 0.5–3 mm size, giving a sedimentation velocity high enough to avoid washout even under high hydraulic load (Lu *et al.* 2013). Syntrophic acetogenic bacteria and their methanogenic partners are dependent on interspecies hydrogen transfer (IHT) which forces them to live close together. Granules in UASB reactors accommodate this requirement and are considered as units that enhance syntrophic degradation of metabolites.

The structure of granules and biofilms has been a research topic for many years and has been analyzed by many means; cross sections analyzed by fluorescence *in situ* hybridization (Sekiguchi *et al.* 1999), mathematical modeling (van Loosdrecht *et al.* 2002) and shearing of the outer granule layers to enable selective microbial sampling and analysis (Lu *et al.* 2013). It has been proposed that granules have a layered structure with aceticlastic methanogens, *i.e. Methanosaeta* spp., in their core surrounded by acetogenic bacteria and hydrogenotrophic methanogens (Liu *et al.* 2003, McHugh *et al.* 2003, Zheng *et al.* 2006), illustrated in Figure 6.

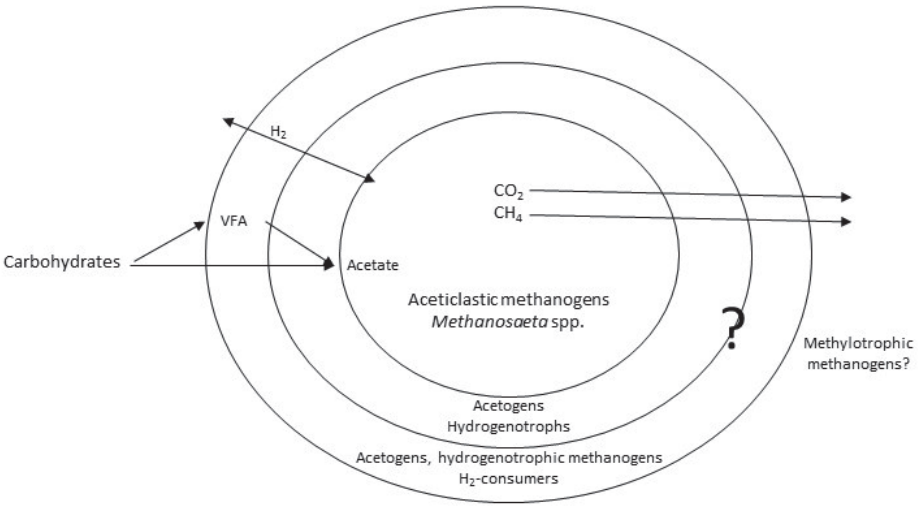

Figure 6: Schematic of a granule's layered structure and the microbes that reside in the different layers, adapted from (Liu *et al.* 2003, Felchner-Zwirello 2013).

2 The aim of the thesis

The aim of this thesis was to contribute to sustainable energy production from manure by evaluating the microbial communities in compact anaerobic digestion sludge bed reactors intended for implementation into existing farm infrastructure for manure slurry treatment.

The project was designed to get a better understanding of the underlying mechanisms of ammonia tolerant AD by observing microbial community adaptations to feed composition and process configuration. Such knowledge can be used to optimize full scale AD design and operation. The project can therefore make a significant contribution to the knowledge of high efficiency agricultural AD.

Specific research objectives

- Examine the efficiency, flexibility and stability of manure supernatant AD treatment in sludge bed reactors. Evaluate the process capacity and robustness by measuring manure degradation and product formation for a wide range of loading rates, including loads that are much higher than what is expected to be required or optimal. Employ a PCR/DGGE strategy to characterize the microbial communities, and to evaluate the time needed for adaptation of the granular inoculum to the conditions in the manure-fed AD reactors.
- Elucidate how granular sludge inoculum, different influents (supernatant of dairy cow and pig manure) and OLR influence the structure of the bacterial and archaeal communities in the biogas reactors.
- Examine how temperature and ammonia concentrations influence the performance of high rate UASB reactors treating pig manure slurry supernatant. Characterize the bacterial and archaeal communities using Illumina sequencing of 16S rRNA gene amplicons to identify key players in methanogenesis at high ammonia concentrations in four reactors at two ammonium concentrations and at variable temperatures over a period of 358 days.
- Characterize microbial communities associated with granules, solid and liquid fractions in the UASB reactors after adaptation to treatment of pig manure supernatant at high and medium ammonium concentrations.

3 Materials and methods

An overview of the experiments, materials and methods is given here. Details are provided in the articles.

3.1 Organization

The work was coordinated from University College of Southeast Norway (USN), Porsgrunn, Norway, and the reactors were operated there. USN performed measurements of COD, pH, VFA, ammonia content, monitored the gas production and provided samples for microbial analysis.

The microbial analyses including DNA isolation, PCR, DGGE, Illumina library preparation and analysis, and ddPCR were executed at the Norwegian University of Science and Technology, Trondheim, Norway.

3.2 Manure influent

3.2.1 Pig manure

Manure slurry was collected from a pig production farm in Porsgrunn, Norway. The manure came from barns that contain 105 sows, 315 "farrow to finish" and 545 weaners that were fed protein concentrate (14.6 % crude protein) added some grass/straw. Wood shavings and straw were used as bedding material. The manure was transported into a storage pit where it was diluted about 30 % by wash water from regular barn washing routines. This mixture was defined as manure slurry. The HRT of the storage pit varied from 70 to 90 days. The liquid manure was stored at 4°C until use.

Pig manure supernatant was used as influent in all articles.

3.2.2 Cow manure

Dairy cow manure was collected from Foss Farm, Skien, Norway. The cows were fed 25 % dairy concentrate (19 % protein) while the rest was grass/clover ensilage. The manure handling included flushing the manure into an indoor temporarily tank using small quantities of water (diluting the manure by approx. 14 % on average). Solids were removed from the manure by a drum filter and the filtrate was stored at 4°C until use as influent.

The results from reactors run on the filtrate of sieved dairy cow manure is reported in article II only.

3.3 Reactor model systems

The reactors were a simplified UASB made of a 370 mL glass vessel as illustrated in Figure 7a. The lab-scale process line is presented in Figure 7b. Suspended solids were separated inside the reactors to retain biomass while the gas and liquid were separated outside the reactors to ease operation in such small scale reactors. The inoculum was based on granules (70 g L^{-1} VSS) from a UASB reactor treating pulp and paper process wastewater at Norske Skog Saugbrugs in Halden, Norway. See Figure 8. Half of the reactor volumes were filled with granules.

Details are given in article I.

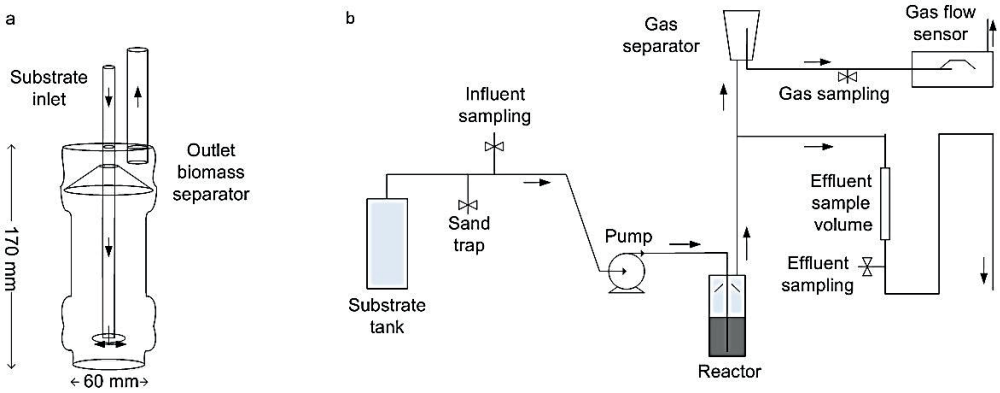

Figure 7: Reactor set up. a) Sketch of lab-scale AD reactor with central inlet and separator. b) Diagram of lab-scale process line.

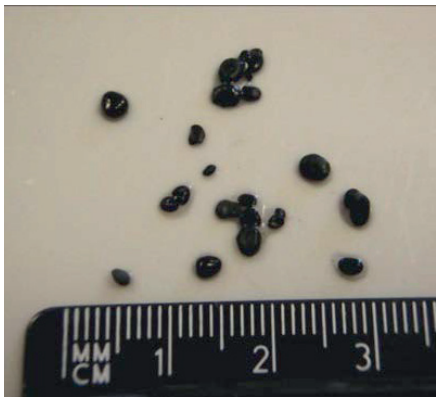

Figure 8: Granules from a UASB reactor treating pulp and paper process wastewater at Norske Skog Saugbrugs, Halden, Norway, were used as inoculum.

3.4 Experiments

3.4.1 The high rate experiment – article I and II

The experiment was designed to examine the efficiency, flexibility and stability of manure supernatant AD treatment in sludge bed reactors. The process capacity and robustness was evaluated by measuring manure degradation and product formation for a wide range of loading rates, including loads that are much higher than what is expected to be required or optimal (article I). A PCR/DGGE strategy was employed to evaluate the time needed for adaptation of the granular inoculum to the conditions in the manure-fed AD reactors (article I) and to elucidate how the influent composition, granular sludge inoculum and organic loading rate affected the structure of the bacterial and archaeal communities in the biogas reactors (article II).

A total of eight laboratory scale reactors were studied. The filtrate of sieved dairy cow manure was used as influent for a period of 96 days in four reactors (CA1, CA2, CB1 and CB2) The reactors were abbreviated as follows; the C reactors – all four reactors, CA – the A1 and A2 parallels, CB – the B1 and B2 parallels. The other four reactors (PA1, PA2, PB1 and PB2) were fed pig manure slurry supernatant for 106 days. They were abbreviated by the same pattern as above (P reactors, PA and PB). For six of the reactors (C reactors and PB), the granular inoculum had been stored for 6 months at 11 °C prior to the experiment. The remaining two reactors (PA) were run on pig manure influent six months prior to the experiment to adapt the granular sludge inoculum. The C reactors were all started at HRT 1.77 days together with PB, while the PA reactors previously adapted to pig manure were started at 0.35 days HRT and decreased by 5% per day. Details on HRT and OLR for all reactors are given in Table 2, article II.

3.4.2 The high ammonia experiment – article III, IV and unpublished results

Four laboratory scale UASB reactors treating pig manure slurry supernatant were operated at different ammonia concentrations and at variable temperatures over a time period of 358 days. High-throughput sequencing of 16S rRNA gene amplicons was applied for both bacterial and archaeal communities to investigate the microbial community dynamics in response to operational variables (article III). In our UASB reactors treating manure supernatant, suspended solids accumulated in the reactors and formed a solid fraction together with the granules. The role of this fraction is unclear. Insight into the microbial community dynamics of the granules, as well as those of the liquid and solid fractions of the sludge, is therefore essential to understand the process to the level needed for optimal design and operation (article IV).

The four reactors (High Ammonia (HA)1, HA2, Low Ammonia (LA)1 and LA2) were fed pig manure slurry supernatant. The influent for the two HA reactors was added urea to increase the ammonium concentration in the reactors. Increasing concentration of urea added during days 69-107 resulted in increasing ammonium concentration in the substrate. Details are given in article III. The LA reactors were fed pig manure supernatant as collected. The reactors were all operated at HRT 1.0 day.

4 Summary of results

4.1 Article I: High rate manure supernatant digestion

In this article, we evaluated the process capacity and robustness of pig manure supernatant with particulate contents initially deemed too high for treatment in UASB reactors. All four reactors produced biogas from day one and stabilized after 35 days of constant hydraulic load. This article focused on the results from the subsequent 33 days with 5 % daily feed flow increase giving the reactors HRTs from 42 to 8.5 h for the two medium rate reactors and from 8.5 to 1.7 h for the two high rate reactors. The high rate reactors reached a biogas production rate of 97 g COD L^{-1} reactor d^{-1} at the highest load at which process stress signs were apparent. The first stress symptom occurred as reduced methane yield when HRT dropped below 17 h. When HRT dropped below 4 h the propionate removal stopped. Propionate removal was reduced with the load increase, but this did not cause other instability symptoms than lowered methane yield. The reduced propionate removal can be explained by low growth rate and inhibition due to high levels of acetate and/or hydrogen. The increasing feed flow rate used to induce the load increase could also have caused a washout of some dispersed biomass especially at the higher flows, worsening the situation for the slow growing propionate removal organisms.

A PERMANOVA test confirmed that there were significant differences in microbial communities between the reactors inoculated with pre-adapted granules and the reactors inoculated with non-adapted granules both for bacteria and archaea, hence the six months pre-adaptation period of the high rate reactors had a significant impact on the reactor microbial community. The average Bray-Curtis similarities show that the microbial communities in the high rate reactors and the medium rate reactors became rapidly more similar with time. This implies that a long-lasting adaptation of the granular inoculum from pulp and paper mill UASB wastewater treatment is not needed to make it capable of treating manure.

4.2 Article II: Microbial community dynamics and biogas production from manure fractions in sludge bed anaerobic digestion

This article focused on elucidating how granular sludge inoculum and particle rich organic loading affected the structure of the microbial communities and process performance in UASB reactors. We investigated four reactors run on sieved dairy cow manure filtrate and four on pig manure supernatant for three months achieving similar methane yields. The end of the 5 % increase period resulted in a maximum OLR of 397 g COD_T L^{-1} d^{-1} for PA reactors with HRT 0.07 days (4 h). The PB reactors which started at a lower load had a maximum OLR of 163 g COD_T L^{-1} d^{-1} at day 106 with HRT 0.17 days. Reduced methane production and propionate removal at the highest loads of the PA reactors were signs of stress. The PB reactors (without preadapted inoculum and not exposed to such extreme loads) achieved higher methane yield than PA except for the first two weeks. The C reactors, all inoculated with non-adapted granules, never reached stable biogas production. The CB reactors were therefore kept at constant load of 29 g COD_T L^{-1} d^{-1}. From day 71 the CA reactors got 5 % daily influent flow increase from HRT 1.77 to 0.45 days and OLR 29 to 107 g COD_T L^{-1} d^{-1}. Acetate concentrations for the C reactors showed increasing removal with stabilized 83 % acetate removal after day 57. Insignificant

propionate removal was observed until day 80 after which 73-92 % propionate removal were observed at day 96 for the CB reactors. Propionate removal did not occur in the two CA reactors exposed to load increase after day 71.

The microbial communities differed significantly between the reactor sludge bacterial and archaeal communities run on dairy and pig manure. This was confirmed by a PERMANOVA test and ordination by principal coordinate analysis (PcoA) based on Bray-Curtis similarities for the microbial communities associated with the influent, the granular inoculum and the reactor slurries. The microbial communities in the reactor sludge seemed to be more similar to the communities in the influent than those associated with the granular sludge inoculum. This suggested that the influent had a higher impact on microbial community composition in the reactors than the granular sludge inoculum.

The PA and PB reactors were run with the same influent under stable and similar conditions except for different granular sludge inoculum (preadapted for PA and non-adapted for PB), and varying OLRs and HRTs. The PcoA ordination based on Bray-Curtis similarities indicated a difference in the microbial community profiles between the PA and PB reactors for both bacteria and archaea. This was confirmed by a PERMANOVA test. Variance partitioning was used to evaluate the influence of running time, OLR and type of granular sludge inoculum on the variation in microbial community composition in the PA and PB reactors. Only 26 and 31 % of the variation in microbial community structure for bacteria and archaea, respectively, was explained by these three variables. Most of this variance was explained by interaction effects.

4.3 Article III: Anaerobic digestion of pig manure supernatant at high ammonia concentrations is characterized by high abundances of *Methanosaeta* and non-euryarchaeotal archaea

This study aimed to get a better understanding of the underlying microbial processes of ammonia tolerant AD in UASB reactors run on particle rich influent. We operated four UASB reactors treating pig manure supernatant rich in suspended solids. The TAN concentration was increased from 1.9 to 3.7 g L^{-1} at day 69 of a 358 days experiment for two of the reactors (High ammonia; HA) while the other two (Low ammonia; LA) served as control. Average FAN concentrations were 0.8±0.2 and 0.14±0.10 g L^{-1} for the HA and LA reactors, respectively. The reactors were operated at 35 °C from startup, 30 °C during days 119-146, 25 °C at days 146-296, 30 °C at days 296-322 and 35 °C again during days 322-358. After urea addition, acetate accumulated in the HA reactors while in LA the acetate concentration continued to decrease to approx. 0.5 g L^{-1}. The biogas yield from the HA reactors increased slightly during the first 77 days of constant urea addition and stabilized at 0.53 ± 0.1 NL* methane L^{-1} substrate during the next 150 days (i.e. days 146-296) when the reactors were operated at 25 °C. The biogas yield from the LA reactors was seven times higher during the same 150 days period (3.7 ± 0.8 NL methane L^{-1} substrate).

Firmicutes was the most abundant bacterial phylum in all reactor samples, and accounted for almost half of the sequence reads (44.8 ± 9.1%). A major fraction of these reads was classified as Clostridia at class level. In addition, the phyla Bacteroidetes, Cloroflexi, Synergistetes, Aminicenantes, Cloacimonetes, Actinobacteria, and Proteobacteria were observed in all reactor samples. A large number of OTUs was only classified as Bacteria or not even at the domain level. Some of them were abundant, and they accounted for on average as much as 19.1 ± 8.2% of the reads in the samples.

The archaeal communities were generally dominated by Euryarchaeotal taxa such as *Methanosarcinaceae*, *Methanosaeta* and *Methanobacterium*, but the abundance clearly varied according to the ammonium concentration in the reactors. OTUs that could not be taxonomically assigned were highly abundant, particularly in the HA reactor samples. Most of these reads were accounted for by 13 OTUs, which were found to be monophyletic and related to Thaumarchaeota in phylogenetic analysis.

Canonical correspondence analyses (CCA) suggested that TAN and acetate concentration played fairly equal roles in structuring the bacterial and archaeal communities, while temperature had less impact. The bacterial communities in LA and HA reactors appeared to diverge from each other already at day 114 while the archaeal HA and LA communities seemed to diverge from day 230. SIMPER analysis for archaeal communities from day 230 and onwards showed that high abundance of the *Methanosarcinaceae* OTU 1 in LA and high abundance of the *Methanosaeta* OTU 2 in HA reactors. This indicated that *Methanosaeta*, but not *Methanosarcina*, was able to adapt to very high ammonia concentrations. The methane yield in the HA reactors were low until it started increasing from around day 300. Spearman correlation analysis, including only HA reactor samples from day 230 to 347, identified the *Methanosaeta* OTU 2 as one of four archaeal OTUs that were positively correlated to methane yield. The other three OTUs were classified as *Methanoculleus*, *Methanobacteriaceae* and *Methanobrevibacter*.

* In this thesis, "NL" should be read as "normal liter". Normal refers to the conditions 0 °C and 1 atm.

The archaeal communities developed differently in the LA1 and LA2 reactors, even though they were operated under the same conditions and had similar methane yields. The most striking difference was the presence of an OTU representing *Methanospirillum* in LA1 in abundances up to 36%. In LA2, *Methanospirillum* never exceeded 1.6%, while it was barely detected in the HA reactors.

We examined the bacterial OTU table for the presence of genera including previously described syntrophic acetate oxidizing bacteria (SAOBs) and found four OTUs classified as *Syntrophaceticus* or *Tepidanaerobacter* but all had low abundances. Hence, we found no indications that previously described SAOBs were associated with the methane production in the HA reactors.

4.4 Article IV: Mapping anaerobic sludge bed community adaptations to manure supernatant in biogas reactors

The objective was to characterize microbial communities associated with different fractions in UASB reactors adapting to treatment of pig manure supernatant at high and medium ammonium concentrations. The granules used as inoculum originated from a pulp and paper industrial process characterized by a high C/N ratio. These were subjected to fundamental different selection regimes in this process, with low C/N ratio, higher HRT and high content of solids. All reactors produced methane at the end of the 358 days experiment, although better methane yields were obtained for the LA reactors. High organic loading rate and high methane production was achieved at high ammonia levels.

Richness (Chao1), evenness and Shannon's diversity suggested a considerable increase of diversity for granular bacterial communities after nearly one year in the reactors. A similar trend, although less pronounced, was found for the archaeal communities. Clostridia (Firmicutes), Bacteriodetes OTUs classified at phylum level only and OTUs classified only as Bacteria were the three most abundant bacterial taxa. *Methanosaeta* was the most abundant archaeal genus across all samples, then followed OTUs classified only on domain level as Archaea and *Methanocorpusculum* as the second and third most abundant taxa.

The principal coordinate analysis (PcoA) plot based on Bray-Curtis similarities illustrated that the bacterial community profiles in the PP granules were clearly separated from those in the reactor granules after one year of operation. This was corroborated by Bray-Curtis similarities as low as 0.10±0.05 and 0.07±0.02 for comparisons of the PP granules and the HA1 and LA1 granules, respectively. It was also interesting to note that only a small fraction of the bacterial OTUs were shared by the PP and reactor granules and as much as 60% of the bacterial PP granule OTUs were unique to the PP granules. SIMPER analysis was performed to identify the OTUs contributing most to the difference between the bacterial communities in the PP granules *versus* HA1 and the LA1 reactor granules. We found that eight and 12 OTUs, respectively, were responsible for 50% of the Bray-Curtis dissimilarity.

Nearly 70 % of the archaeal OTUs were common to all the granules. Bray-Curtis similarities indicated higher similarities for comparisons between the archaeal communities of the PP and HA1 reactor granules (0.70±0.01) compared to those between the PP and LA1 reactor granules (0.39±0.02). This was corroborated by the low Bray-Curtis similarities when comparing the HA1 and LA1 reactor granules (0.46) and illustrated by the PcoA plot. Hence, the differences in ammonia concentration in

the HA and LA reactors caused the inoculum to adapt into two new unique microbiota. SIMPER analysis showed that only four and three OTUs explained as much as 50% of the Bray-Curtis dissimilarity between the archaeal communities in the PP granules and HA1 granules, and between PP granules and LA1 granules, respectively.

The accumulating solids in the reactor did not merely represent the microbes associated with particle-rich manure supernatant influent. PcoA plots and comparisons of Bray-Curtis similarities indicated that the non-granular solid fraction in the reactors represented a distinct methanogenic community.

The archaeal communities present in the liquid fractions of the reactor samples were strikingly similar to those of found in the liquid fraction of influent samples, as illustrated by PcoA plot and high Bray-Curtis similarities (around 0.7). In contrast, the bacterial liquid fraction of the reactor was not just a mirror image of the influent passing through the digester.

4.5 Unpublished results – Quantification of AD key taxa by droplet digital PCR (ddPCR)

During the last decade, deep sequencing of 16S rRNA gene amplicons has become the golden standard for characterizing the composition of microbial communities. The most abundant approach is to apply broad-coverage bacterial primers to target conserved regions of the 16S rRNA gene, resulting in PCR products representing the majority of bacteria present in DNA templates extracted from an environmental sample. The resulting community profile is considered to be semi-quantitative, and the abundance of the OTUs is assumed to reflect the presence of the actual bacteria in the original sample. Still, this is an end-point PCR approach, and it is therefore not expected to provide a quantitative presentation of the original community.

In quantitative PCR approaches the objective is not to generate an amplified DNA product, but rather to determine accurate quantities of target DNA in a template sample. The traditional real-time PCR method is based on the fact that the onset of exponential amplification, rather than the amount of endpoint PCR product, reflects the amount of target DNA in the sample. The amplification of DNA is monitored in "real-time" by fluorescent labelling of the amplified DNA (most commonly non-specific fluorescent dyes that intercalate with double-stranded DNA), and a threshold cycle (C_t) is determined at which the amplification curve crosses a given threshold of the fluorescent signal (Peirson & Butler 2007). This monitored C_t will depend on the amplification efficiency and the length of the PCR product, because longer PCR products will be more stained than shorter ones. Therefore, in relative quantification, for example when quantifying the relative abundance of two gene products (*e.g*. two microbial taxa) by applying two different primer pairs, a third PCR product is needed for normalization (often denoted "the endogenous control") (Pfaffl 2001).

The newly developed quantitative PCR approach, droplet digital PCR (ddPCR), is based on a fundamentally distinct principle. A water-oil emulsion droplet system ensures clonal amplification of template DNA molecules. The fraction of positive droplets with amplification products are determined and used for calculation of the template DNA concentration in the original sample. Quantification is therefore independent of amplification efficiency, and neither standard curves nor an endogenous control is needed (Hindson *et al.* 2011). This makes ddPCR particularly suitable for quantification of microbial taxa in samples representing microbial communities. By applying taxa-specific primer pairs for a given template, for example targeting the 16S rRNA gene, relative amounts of different taxa may be determined. ddPCR has already been applied for quantifying microbial groups. Kim *et al.* (2015) designed a ddPCR-based assay based on Taqman probes for quantifying 23 methanogenic taxa.

Here, we investigate the potential of using ddPCR for quantifying microbial taxa by applying taxon-specific PCR primers and a dsDNA-binding dye (EvaGreen). We designed specific PCR primers targeting the 16S rRNA gene for three archaeal methanogenic genera (*Methanosaeta*, *Methanoculleus* and *Methanosarcina*) and three bacterial genera previously demonstrated to include syntrophic acetate oxidizing bacteria (*Thermacetogenium*, *Syntrophaceticus* and *Tepidanaerobacter*). We further designed broad-coverage PCR primers for both Archaea and Bacteria, and finally "universal" prokaryotic primers targeting both Archaea and Bacteria 16S rRNA gene sequences.

4.5.1 Materials and methods

4.5.1.1 Selected microbial strains and microbial community samples

Selected bacterial and archaeal strains were grown and used as DNA templates in ddPCR assay. The strains are listed in Table 5 along with a short description of how they were grown. DNA was extracted using the Power Soil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA) as described by the manufacturer. DNA concentration was measured by NanoDrop Spectrometer ND-1000 (NanoDrop Technologies, USA).

DNA extracted from two reactor samples were chosen for testing of the ddPCR protocol; HA1 (high ammonia) and LA1 (low ammonia) of day 347 from the high ammonia experiment. These are described in article III and IV.

Table 5: Selected bacteria and archaea for testing of ddPCR assay.

Name	Strain	Growth procedure
Escherichia coli	DH5α	Grown on LA agar plates, single colony transferred to 8 ml medium LB tubes (20 °C, 48 h). DNA extracted from cell pellet.
Vibrio sp	RD5-30	
Microbacterium sp	ND2-7	
Roseobacter sp	RA4-1	
Clostridium ultunense	Esp	Culture donated by Professor Anna Schnürer (Swedish University of Agricultural Sciences). Cells pelleted by centrifugation and DNA extracted.
Syntrophaceticus schinkii	Sp	
Tepidanaerobacter acetatoxydans	Re1	
Methanoculleus bourgensis	MAB1	
Methanosarcina barkeri		
Methanosaeta concilii		

4.5.1.2 Primer design

Primers designed during this study yielded both broad coverage primers covering both the bacterial and archaeal domain, general archaeal primers that covered the archaeal domain and six primers covering specific bacterial and archaeal genera.

Conserved regions of the bacterial and archaeal 16S rRNA gene were identified by aligning diverse sequences using the program Clone Manager 9 (Scientific & Educational software). For further optimization of primer sequences to improve coverage, we used the Probematch tool (Cole *et al.* 2014) of the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) together with sequence alignments including numerous 16S rRNA gene sequences for relevant bacterial and archaeal taxa. For the genus specific primers, 16S rRNA gene sequences representing strains within the relevant genus and strains representing related genera (identified using the SeqMatch tool at the RDP) were used to generate sequence alignments. Genus specific regions of the 16S rRNA gene were identified and the primers were designed to target these regions.
The resulting primers are listed in Table 6. The primers targeting *Clostridium ultunense* were designed by Westerholm *et al.* (2011a).

Table 6: Primers for ddPCR assays

Primer Name	Sequence 5' to 3'	Target	Taxon
CultF CultR	ccttcgggtggaatgataaa tcatgcgattgctaagtttca	*Clostridium ultunense*	Species
SAOBtherm-570F SAOBtherm-736R	ttcgagtcatctgtgaaagccg caggtagaggccagaaggc	*Thermacetogenium*	Genus
SAOBsynth-615F SAOBsynth-817R	cttgaggcaaggagaggaag acagatggkkttgawaccacct	*Syntrophaceticus*	Genus
SAOBtepid-430F SAOBtepid-625R	tygtaaagctcagtcatyggg cagttttatcggcaagccc	*Tepidanaerobacter*	Genus
Mcul-495-F Mcul-948-R	gctgggtyrttaagtcycttgg gtcatgcagagtcttcagccc	*Methanoculleus*	Genus
Msarc-78-F Msarc-433-R	yatntgctggaatgctttmtgskt acccttagacccaataatmacgatc	*Methanosarcina*	Genus
Msaeta-1159-F Msaeta-1302-R	caatggtyggtacaatgggyat ggacgtattcaccgcgttctg	*Methanosaeta*	Genus
B+A340F B+A800R	shcctaygggdbgcascagk ggactacnvgggtatctaakcc	Bacteria + Archaea	Domain
Ark-340F Ark-880R	ggygcascagncgvgaaa cccgycaattcctttaagtttc	Archaea	Domain

4.5.1.3 Droplet digital PCR workflow and data analysis

Triplicate reaction mixtures of 24 µL were prepared as follows: 12 µL QX200 EvaGreen supermix, 3 µL of each primer (resulting in 0.3 µM primers per reaction) and 6 µL template (0.1 ng/µL unless otherwise stated). A 20 µL aliquot was taken from each of the assembled reaction mixtures and pipetted into each sample well of an eight-channel disposable droplet generator cartridge (Bio- Rad, Hercules, CA, USA). A 70 µL volume of Droplet Generation Oil (Bio-Rad) was then loaded into each of the eight oil wells. The cartridge was placed into the droplet generator (Bio-Rad). The resulting droplets were transferred with a multichannel pipet to a 96-well plate. The plate was heat-sealed with foil using a PX1 PCR Plate Sealer (Bio-Rad) and placed in a conventional thermal cycler. Thermal cycling conditions for all EvaGreen assays consisted of an activation period (5 min at 95 °C) followed by 40 cycles of a denaturation step (30 s at 95 °C) and a combined annealing-extension step (60 s at 60 °C unless otherwise stated). A dye-stabilization step was also included at the end (4 °C for 5 min then, 95 °C for 5 min, and finally a 4 °C indefinite hold). A 2 °C per second ramp rate was applied for all thermal cycling steps. After PCR, the 96-well plate was loaded into the QX200 Droplet Reader (Bio-Rad). Droplets were automatically aspirated from each well and streamed past a fluorescence detector. Analysis of the ddPCR data was performed with the QuantaSoft analysis software (Bio-Rad) that accompanied the QX200 Droplet Reader. Thresholds separating positive from negative droplets were set manually for all assays based on the signals observed in the no template controls (NTC).

4.5.2 Results

4.5.2.1 Primer specificity and identification of optimal annealing temperature

Primer specificity testing was done using conventional PCR. Optimal annealing temperature was screened for using gradient PCR (52-54-56-58-60 °C) for the genus and species specific primers. Similar product amounts were obtained for all annealing temperatures, hence 60 °C was chosen for all the genera specific primers to maximize stringency. The primers were also tested for specificity using specific and non-specific DNA templates (Table 7). All primer sets amplified the specific DNA template while the results were negative for the non-specific DNA templates.

The universal primers (B+A) targeting all bacteria and archaea, and the general primers targeting all archaea (Ark) were also tested for optimal annealing temperature and specificity using conventional PCR and temperature gradients (56-58-60-62 °C). The optimal annealing temperature for the Ark primers was 60 °C, for which the primers amplified the specific DNA template, but not the non-specific template. It proved difficult to find an annealing temperature where the B+A primers amplified both bacteria and archaea satisfactory. We therefore decided to run a gradient temperature ddPCR assay (56-58-60 °C) with the B+A primers to find the optimal annealing temperature. Unfortunately, the results were so heavy with rain (*i.e.* droplets with intermediate signal) that is was impossible to distinguish a negative droplet bar from a positive droplet bar as illustrated in Figure 9 for 56 °C. All the droplets were displayed as one continuous smear. The no template control (NTC) of each temperature was used to force a manual global threshold (*i.e.* cut-off values for positive droplets).

Table 7: Specific and non-specific DNA template for the different primers

Primer Name	Specific DNA template	Non-specific DNA template
Cult	*Clostridium ultunense*	All bacteria listed in Table 5 except *C. ultunense*
SAOBtherm	No available specific DNA	All bacteria listed in Table 5
SAOBsynth	*Syntrophaceticus schinkii*	All bacteria listed in Table 5 except *S. schinkii*
SAOBtepid	*Tepidanaerobacter acetatoxydans*	All bacteria listed in Table 5 except *T. acetatoxydans*
Mcul	*Methanoculleus bourgensis*	All archaea listed in Table 5 except *M. bourgensis*
Msarc	*Methanosarcina barkeri*	All archaea listed in Table 5 except *M. barkeri*
Msaeta	*Methanosaeta concilii*	All archaea listed in Table 5 except *M. concilii*
B+A	All listed in Table 5	-
Ark	All archaea listed in Table 5	All bacteria listed in Table 5

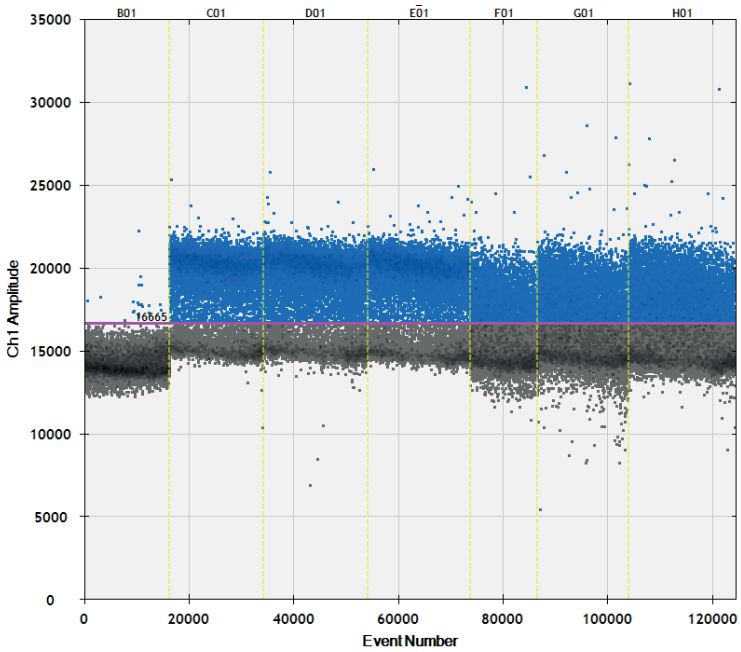

Figure 9: Results of B+A primers at 56 °C with NTC in the first well (B01), then follows triplicates of bacterial and archaeal DNA templates respectively. Rain (*i.e.* droplets with intermediate signal) made it impossible to distinguish a negative droplet bar from a positive droplet bar. The NTC was used to force a manual global threshold (pink line).

4.5.2.2 Genera specific ddPCR assays

Dilution series assays

To assess the ability of the primers to efficiently quantify the specific DNA templates, dilution series were run using ddPCR for the genus and species specific primer sets. The SAOBtherm primers were not included because no specific DNA template was available. Template concentrations of specific DNA ranging from 1.0 to $1 \cdot 10^{-4}$ ng/µl were used but the results from 1.0 ng/µl specific template was excluded due to oversaturation (*i.e.* all droplets were positive), which made it impossible to calculate concentration. Non-specific DNA templates of 1.0 ng/µL was also included to check for non-specific amplification. The thresholds were set manually based on signal levels of the NTCs. The results are listed in Table 8. Linear regression was performed for all dilution series, and yielded $R^2 \geq 0.99$. Very small amounts of non-specific DNA was amplified, implying that the primers and method functioned as intended.

Table 8: Dilution series results [copies/µl]

DNA template	0.1 ng/µl	0.01 ng/µl	0.001 ng/µl	0.0001 ng/µl	Non-specific 1.0 ng/µl	R^2
Cult	314	39.4	4.3	0.43	0.24	0.9994
SAOBsynth	12 800	1 708	125.8	17.6	0.09	0.9989
SAOBtepid	6 430	622	61.6	6.5	1.62	1.0
Mcul	968	122.7	3.24	0.98	0.12	0.9991
Msarc	2 044	336	24.5	1.38	0.35	0.9958
Msaeta	6 090	358	21.8	1.71	0.12	0.9986

Mock community assays

DNA isolated from *Methanoculleus bourgensis, Methanosarcina barkeri* and *Methanosaeta concilii* were used to design two mock communities for testing the accuracy of the quantification obtained by the Mcul, Msarc and Msaeta primers. The composition of the mock communities are given in Table 9 while the results are given in Table 10. No corrections for *rrna* operon copy number were made as all three methanogens exhibit one gene copy per genome. The ddPCR with the Msarc primers succeeded in precisely quantifying the relative abundance of Methanosarcina (represented by *M. barkeri*) in the Mock communities. The Msaeta primers performed well with Mock 1, but overestimated the amount of *M. concilii* DNA in Mock 2. ddPCR with the Mcul primers underestimated the amount of *M. bourgensis* DNA in both mock communities. In addition, the total amount of DNA template was the same in Mock 1 and Mock 2, but the results show a 9 % lower amount of copies/µl in Mock 2 than in Mock 1.

Table 9: Mock communities comprised of DNA isolated from *Methanoculleus bourgensis, Methanosarcina barkeri* and *Methanosaeta concilii*. Amount of DNA in one reaction. Relative amounts given in parentheses.

DNA [µl]	M. bourgensis	M. barkeri	M. concilii	Totalt
Mock 1	0.5 (0.083)	0.5 (0.083)	5.0 (0.833)	6.0 (1.0)
Mock 2	1.5 (0.25)	2.5 (0.42)	2.0 (0.33)	6.0 (1.0)

Table 10: Results [copies/µl] of the mock communities amplified with methanogenic genera specific primers. Relative amounts given in parentheses as average of triplicates.

Copies/µl	Mcul	Msarc	Msaeta	Total
Mock 1	120.5 (0.033)	328 (0.090)	3197 (0.877)	3646 (1.0)
Mock 2	370 (0.11)	1324 (0.40)	1623 (0.49)	3317 (1.0)

4.5.2.3 ddPCR assays targeting the archaeal domain

To assess quantitative quality of the general archaeal primers in ddPCR, dilution series of template DNA and a specificity test was conducted for the Ark primers. DNA from the three methanogenic archaea listed in Table 5 was used as specific DNA template, while DNA from all the bacterial strains in the same table was used as non-specific DNA template. Unfortunately, the Ark primers produced a lot of rain, probably due to unspecific amplification, as illustrated in Figure 10A. The results from the dilution series produced a regression line with $R^2 = 0.71$. Threshold was set manually based on the NTC results.

Different ratios of specific and non-specific template were set up in duplicate ddPCR reactions as follows: 2 µL + 0 µL, 2 µL + 2 µL and 2 µL + 4 µL, respectively. The idea was that these ratios should all give the same result despite the increasing amount of non-specific (bacterial) DNA. There were large amounts of rain in all samples. The results were as follows: 751, 882 and 824 copies/µl, respectively, giving an average of 819±66 copies/µl. See Figure 10B. This indicated that the general archaea primers probably amplified some of the bacterial template.

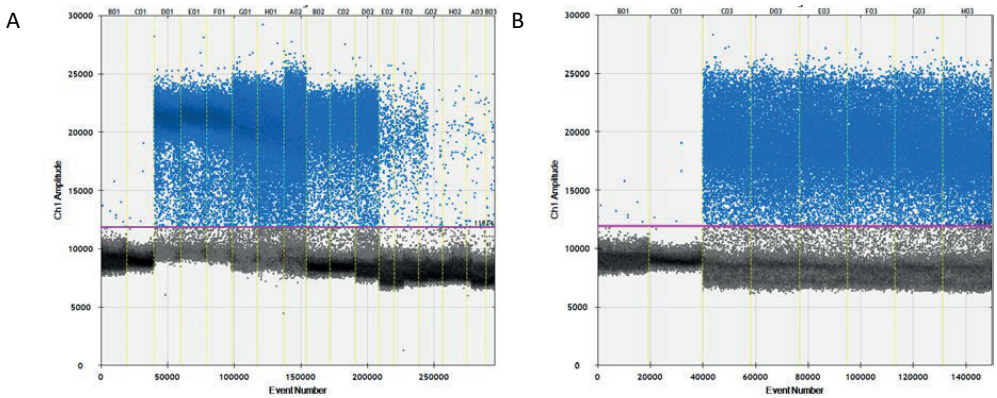

Figure 10: A) Dilution series with Ark primers. The two firs wells represent the NTC, then follows triplicates of 1.0 to 10^{-4} ng/µl specific DNA template. B) NTC and duplicates with ratios of 2 µL + 0 µL, 2 µL + 2 µL and 2 µL + 4 µL of specific and non-specific template, respectively. Rain is seen as droplets with intermediate signal between the negative (black) and positive (blue) droplet bars. The pink line visualizes the manually set global threshold.

4.5.3 Discussion

Droplet digital PCR (ddPCR) system aims at empowering all researchers with a tool that removes the substantial uncertainties associated with using the analogue standard, quantitative real-time PCR (qPCR). Its advertised strength is high precision absolute quantification without needing reference curves. Kim *et al.* (2015) have demonstrated the use of ddPCR for absolute quantification of methanogens linking methanogen communities and operational factors. They found that HRT influence the net abundance of methanogens and activity while temperature governed the composition of the methanogen community. This suggest that it is a promising tool for analyzing the microbial ecology of anaerobic digestion.

Digital droplet PCR standard data analytical approach follows a straight forward framework, but ignores sources of variation from both biological and technical factors. In our case, the proprietary QuantaSoft algorithm often failed to set a threshold and gave the error message “No call”. This was also experienced by Dreo *et al.* (2014). A global manual threshold was therefor set for each primer pair based on the fluorescence level of the NTCs and used for all samples amplified with the corresponding primers. Using manual thresholds leads to more correct results for the selected positive and negative samples as well as reflect upon the behavior of the individual assays (Dreo *et al.* 2014). It also allows for flexibility in the treatment of rain droplets, but choosing a threshold is not straight forward. Jacobs *et al.* (2014) found that sample dilution and the fluorescence cut-off have a substantial impact on reliability, but if they are chosen wisely, they may result in major improvements in target presence detection.

Three primers targeting methanogenic archaea were tested with mock community assays. The Msarc primers performed well in both Mock1 and Mock 2 with a discrepancy of only 0.7 and 1.8 %, respectively, between theoretical and measured abundance (Table 9 and Table 10). The Msaeta primers overestimated the amount of DNA template and had a fairly low discrepancy of 4.4 % for Mock 1, but for Mock 2 it was 15.6 % which was the highest observed. The Mcul primers underestimated the amount of DNA template, but had otherwise similar results as the Msaeta primers with discrepancies of 5 % for Mock 1 and 13.8 % for Mock 2. In general, the discrepancies were low

for the Mock 1 assay where one species was dominating (*M. concilii*) while the Mock 2 assay with a more even community had higher discrepancies. Underestimation might occur due to several reasons; sampling variation, DNA extraction efficiency being <100%, pipetting errors, binding of DNA to tubes, unequal partition size, partition loss and misclassification of droplets (Dreo *et al.* 2014, Jacobs *et al.* 2014).

Assays targeting domains were explored due to the desire to quantify relative amounts of archaea to bacteria, and to enable normalization when genera and species specific primers are employed. We experienced problems with the ddPCR assays targeting domains due to large quantities of rain. Inhibitors from the DNA extraction or slow starting reactions may result in misclassification (positive or negative) of droplets as partitions fail to reach the proper fluorescence threshold even though they contain a target copy. This would result in rain and actual positive droplets could be falsely classified as negative. Universal and general primers need to include degenerate nucleotide positions to be able to span entire domains. This could lead to poor binding to a fraction of the targets. A possible solution to this could be to use a mixture of well designed primers that span smaller portions of a domain, and to increase the primer concentration to ensure that each droplet contain enough copies of every primer. Another suggestion would be to decrease the number of degenerate nucleotide positions and simply accept that some rare taxa will not be covered. Kiss *et al.* (2008) experienced empirically that the number of amplified partitions seemed to increase with the number of amplification cycles. Consequently, rain should be reduced with increasing amplification cycles. The domain targeting primers were not tested beyond 40 cycles in this study, though that could be part of the solution for optimizing such broad range primers.

5 Discussion

Livestock manure amounts to 42 % of the total energy potential for biogas production in Norway (Det Kongelige Landbruks- og Matdepartementet 2008-2009). Only an insignificant fraction of this is exploited today, though it is a goal that 30 % should be treated in biogas facilities within the year 2020.

The aim of this thesis was to contribute to our common knowledge about the microbial diversity of anaerobic digestion of manure liquid fractions using high rate UASB reactors intended for implementation into existing farm infrastructure. In the long run, improved knowledge could be used to enable biogas producers to continuously follow the variation of diversity in their digesters. Carballa *et al.* (2015) has already suggested that a decrease in *Methanosaeta* and/or active archaeal community abundance could serve as a warning indicator. They also suggest using diversity and evenness indices for monitoring the bacterial community. Knowledge about microbial indicators of success and failure would help in optimization and stable operation of biogas plants.

5.1 Efficiency, flexibility and stability of high rate UASB reactors

UASB reactors are the standard of high rate AD, so a small UASB-like sludge bed reactor design was chosen for the present project to test the possibilities of high rate AD slurry treatment. The process capacity and robustness was evaluated by examination of the microbial communities in the reactors and by measuring manure degradation and product formation. The process described here is unique in its utilization of manure supernatant with a particle content well above that which is recommended for use in UASB reactors (Tchobanoglous *et al.* 2003). This is the first time microbial community structure and dynamics in such particle rich high rate UASB processes has been investigated in detail.

Multiple operational parameters were tested, like different influents (dairy cow or pig manure supernatant), a wide range of loading rates (up to 400 g COD L^{-1} reactor d^{-1}), extremely low HRTs (1.7h), high levels of TAN (3.7 ± 0.2 g NH_4-N L^{-1}) and extreme levels of FAN (1.2 ± 0.3 g NH_3-N L^{-1}). Overall, the reactors performed very well under extreme conditions. No foaming, significant drop or increase in pH was observed even without pH control. The reactors showed remarkable stability and adaptation to changes in loading rate. The process did not fail even at the highest OLR tested, implying that supernatant AD is a very robust process.

In paper 2, we compared the performance of UASB reactors fed dairy cow manure filtrate and pig manure supernatant (dairy/pig manure for short). The methane yields obtained at high pig manure OLRs compared to the more particle rich dairy manure indicated that pig manure supernatant was more suitable than dairy manure filtrate as UASB influent. The reactors run on dairy manure needed longer time to stabilize the methane yield, acetate removal and propionate removal, which may be a consequence of the higher fraction of slowly degradable particles in dairy manure. The yield as NL methane L^{-1} influent was, however, quite similar for dairy and pig manure at the end of the high rate experiment (article II). The generally low VFA content in the effluents, especially the low propionate concentration, are signs of a well-functioning AD process on dairy manure (article II). A DGGE gel with samples from the reactors run with dairy cow manure (CA1 and CB1) and pig manure (PA1 and PB1) were run to examine the influence of the influent and granular sludge inoculum on microbial communities in the reactors (Article II, fig. S1). The resulting PcoA plots and comparisons of average Bray-Curtis similarities illustrated that microbial communities in the reactor sludge seemed to be more similar to the communities in the influent than those associated with the granular sludge inoculum. This suggested that the influent had a higher impact on microbial community composition in the

reactors than the granular sludge inoculum. The microbial community in an anaerobic digester is highly influenced by its influent (Ziganshin *et al.* 2013). This involves the influent chemical makeup, *e.g.* fats, proteins and inhibitors and its inherent microbial community as it is continuously introduced to the reactor (Chen *et al.* 2008, Sousa *et al.* 2008, Kovacs *et al.* 2013). An experimental strategy comparing the effects of sterile and nonsterile influents on the reactor communities might be helpful for disentangling the importance of these mechanisms.

Ammonia plays a crucial role in the stability and process performance of reactors treating N-rich influents. It has therefore been important to gain insight into the mechanisms involved in microbial interactions that govern the methane production under such circumstances (Chen *et al.* 2008, Demirel & Scherer 2008, Rajagopal *et al.* 2013, Yenigun & Demirel 2013). The literature is not uniform with respect to ammonia tolerance of different taxa like syntrophic acetate oxidizing bacteria, hydrogenotrophic and aceticlastic methanogens (Wang *et al.* 2015). There are also gaps in our common knowledge on how methanogenic communities respond and adapt to increased ammonia concentrations. Pig manure is rich in nitrogen content and we examined if the communities could be adapted to even higher concentrations (article III and IV). Urea was added to two of the reactors treating pig manure supernatant (HA; high ammonia) in the high ammonia experiment which resulted in a 10-fold drop of the methane yield. Sampling of the microbial communities had to be terminated after 347 days, but the reactors are still running p.t. with constant urea addition to the HA reactors. The first 358 days of the experiment is reported in article III. After approx. 200 days of adaptation, the methane production, COD, propionate and acetate removal increased substantially in the HA reactors. Acetate and propionate removal improved considerably from around day 300, indicating that acetate and propionate oxidation took place at FAN concentrations around 1 g L^{-1}. Thus, ammonia inhibition was much reduced after around 200 days of adaptation, allowing methane production at a rather extreme FAN level. The methane yield, COD_T and COD_S removal of the HA reactors eventually approached the yield and removal from the low ammonia (LA) reactors after 600 days as illustrated by Figure 11 and Figure 12. These improvements cannot be explained by a temperature induced increase in growth rate alone (Henze & Harremoes 1983), indicating an adaptation of the methanogenic consortium to the high ammonia concentration.

Ammonia and sulfate concentrations are generally higher in pig manure than in dairy manure (Chen *et al.* 2008). Sulfate reducing bacteria (SRB) reduce sulfate to sulfide and may compete with methanogens, acetogens, or fermentative microorganisms for available acetate, H_2, propionate, and butyrate in anaerobic systems (Chen *et al.* 2008). Propionate is a key intermediate in anaerobic digestion and a substrate for all SRB. However, SRB was not observed in high abundances in any of the experiments conducted here.

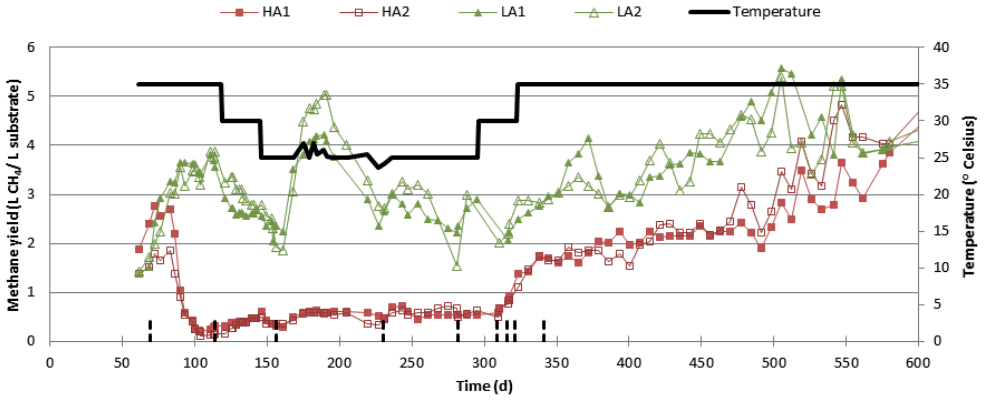

Figure 11: Methane yield and reactor temperature for four UASB reactors fed pig manure supernatant and operated at two ammonia concentrations; approx. 4 and 2 g NH_4^+-N L^{-1} for HA and LA respectively. Operational conditions and results are described in article III. Sampling days for microbial community analysis are indicated by - -. The reactors are still running p.t.

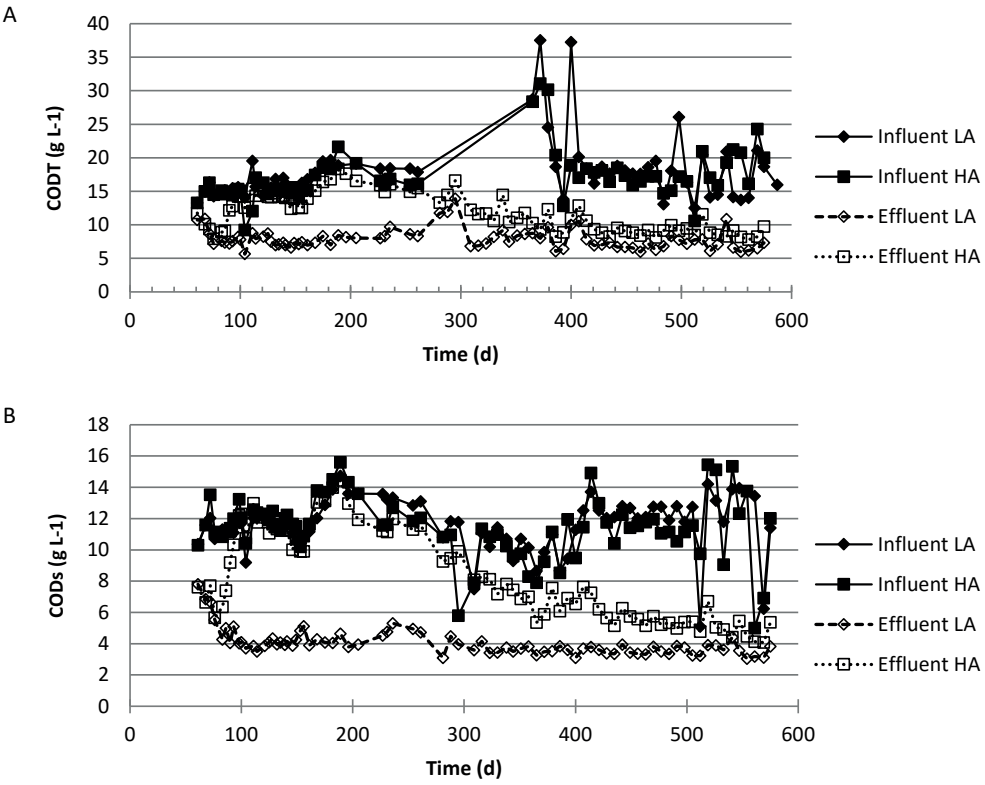

Figure 12: Influent and effluent A) COD_T and B) COD_S for four UASB reactors fed pig manure supernatant and operated at two ammonia concentrations; approx. 4 and 2 g NH_4^+-N L^{-1} for HA and LA respectively. Average of two and two reactors. Operational conditions and results are described in article III.

5.2 Adaptation of reactor sludge and granular communities

Adaptation of reactor sludge and granular communities was a focus point in both the high rate experiment (article I and II) and in the high ammonia experiment (article III and IV). We speculated whether it was possible to adapt granular communities to conditions that were not only different from the conditions they originated from, but also quite extreme in general (*e.g.* HRT <2 h in article I and II, and FAN levels >1 g NH_3-N L^{-1} in article III and IV).

Overall, the results from all the articles illustrate that the granules adapted quickly and efficiently to a wide variety of operational parameters and conditions. The granules originated from a UASB reactor treating pulp and paper process wastewater with nearly no particles, high C/N ratio and a high fraction of easily degradable carbohydrates. These conditions are in stark contrast to the low C/N ratio, particle rich and high ammonia process conditions in this project. In addition, livestock manure contains little easily degradable carbohydrates as the animals have already utilized these. Article I and II determined that the granular sludge adapted to pig manure supernatant in approximately two months and that preadaptation before start-up was not necessary.

Variance partitioning was used in article II to evaluate the influence of OLR on the variation in microbial community composition in the PA and PB reactor sludge of the high rate experiment. We assumed that the relatively small effect of OLR on microbial communities (5 and 2 % for archaeal and bacterial communities, respectively) might be due to the extremely low HRT employed in the high rate experiment (min 1.7h and 4h for PA and PB reactors, respectively). In article II, we argued that the liquid fraction of the reactor sludge would be a mirror image of the influent rushing through the digester and that it was not an active part in the methanogenic communities. This assumption was further examined in article IV, though at constant HRT of 1 day (see chapter 5.4). Due to the low HRT, we hypothesized that most of the biomass was situated in the granules (article II). This assumption was challenged later when we observed that, in the long run, the granules diminished in size (article III). Also, non-granular particles accumulated in the reactors and eventually made up a considerable portion of the solid fraction in the reactors (article IV). The non-granular particles most likely served as both slowly degradable substrate and as carriers for biofilm growth, ensuring stable operation of the reactors. The observation that methane production significantly exceeded the biogas potential of dissolved organics (article I and II) implies that the feed particles were efficiently retained and degraded. The relative importance of the granular and non-granular particles, and interactions between them, are therefore relevant to understand how and why the investigated process is so efficient. The changing size of granules during the course of the biogas production process may have been a result of a combination of microbial growth and decay, granular shear-off, granule-granule collision and granule-wall collisions. The particles in the feed may have been colonized by fragments from the granules and by organisms in the feed that may have originated from the animal gut.

Results from the high ammonia experiment (article IV) clearly show that the microbial communities in the granules changed significantly. The bacterial and archaeal communities adapted into new unique microbiota as a response to the feed composition and elevated concentrations of ammonia with more pronounced changes in the bacterial than the archaeal communities. The altered selection regime caused the abundance of the originally dominating Bacteroidia and Deltaproteobacteria to decrease substantially and the abundance of Clostridia to increase markedly. The archaeal communities had increased abundances of *Methanosarcinaceae* and *Methanobacterium* in the LA reactor granules and of *Methanoculleus* in the HA reactor granules. The abundance of the aceticlastic *Methanosaeta*

decreased during the adaptation period, but was still the dominating archaeal genera in the reactor granules after a year of operation. This contradicts the findings of De Vrieze *et al.* (2012) who reports that TAN levels above 3000 mg TAN L^{-1} together with acetate concentrations above 3000 mg COD L^{-1} would suppress growth of *Methanosaeta* sp. Generally, *Methanosarcina* has been found to be more robust to TAN concentrations up to 4 – 6 g L^{-1} than *Methanosaeta* (Wiegant & Zeeman 1986, Calli *et al.* 2005b, Karakashev *et al.* 2005). This has been explained by the fact that *Methanosarcina* cells form clusters, which may provide protection by reducing the diffusion of ammonia into the cells (Calli *et al.* 2005a), but these clusters disintegrate at FAN levels above 600 mg NH_3-N L^{-1} (Calli *et al.* 2005b) explaining the decrease of *Methanosarcina* in the HA reactors. It is widely accepted that filamentous *Methanosaeta* has an important role in the formation and maintenance of stable anaerobic granules (McHugh *et al.* 2003). Interestingly, here *Methanosaeta* was found in higher abundances in the granules obtained from the HA1 reactor than from the LA1 reactor (article IV). The *Methanosaeta* OTU2 was the second most dominating OTU across all solid fraction reactor samples analyzed in article III and IV. It was identical to the 16S rRNA gene sequence of the type strain of the obligate aceticlastic *Methanosaeta soehngenii* and positively correlated to methane yield in the HA reactors towards the end of the ammonia experiment. Its abundance increased towards the end of the high ammonia experiment which is a strong indication that acetate conversion can be performed by *Methanosaeta* even at FAN level around 1 g NH_3-N L^{-1}. This is ten times higher than the 100 mg NH_3-N L^{-1} Calli *et al.* (2005a) reported to cause loss of filamentous *Methanosaeta* activity. A possible reason for the apparent high ammonia tolerance we observed for *Methanosaeta* in this study could be protection obtained by growing in aggregates with other microbes in the granules and colonized non-granular particles. Previous studies illustrate that keeping a short distance between syntrophic organisms enhance their chance of survival by enabling interspecies electron transfer (Dinamarca *et al.* 2011). The classic example in AD being interspecies hydrogen transfer between SAOB and hydrogenotrophic methanogens. Interestingly, a recent study have demonstrated that *Methanosaeta harundinacea* is able to accept electrons via direct interspecies electron transfer (DIET) for the reduction of CO_2 to methane (Rotaru *et al.* 2014). We hypothesize that there could be some yet-to-be-revealed interactions of this kind in the HA reactors that deminish FAN inhibition and sustains the high abundance of *Methanosaeta*.

The higher abundance of *Methanosarcina* in LA could be due to competitive advantages. The FAN concentration in LA was 0.14±0.10 g NH_3-N L^{-1} which is above the 0.1 g NH_3-N L^{-1} *Methanosaeta* inhibition level but below the cluster disintegrating threshold of 600 mg L^{-1} (Calli *et al.* 2005b, a). In addition, *Methanosarcina* has a higher growth rate than *Methanosaeta* (0.60 versus 0.20 d^{-1}, respectively)(De Vrieze *et al.* 2012). These advantages could be the reason why *Methanosarcina* abundances were higher in LA than in HA.

5.3 Unclassified OTUs could be involved in yet-to-be-identified interactions

We aimed at designing broad coverage 16S rRNA PCR primers for high-throughput sequencing in article III and IV, and apparently succeeded in obtaining a detailed characterization of the microbial communities. A large number of OTUs was only classified as Bacteria, Archaea or not even at the domain level.

New methylotrophic methanogens have recently been discovered in the newly proposed phyla Bathyarchaeota (formerly known as Miscellaneous Crenarchaeota Group; MCG) and *Candidatus* Verstraetearchaeota (Evans *et al.* 2015, Vanwonterghem *et al.* 2016). These phyla are more closely related to Thaumarchaeota than Euryarchaeota. Xiang *et al.* (2017) suggested that Bathyarchaeota may play an important ecological role within archaeal communities via potential symbiotic associations with Methanomicrobia. Members of Methanomicrobia which co-occurred with MCG were affiliated with *Methanosaeta*, *Methanoregula*, *Methanocella*, *Methanoperedens* and uncultured Methanomicrobiaceae. Xiang *et al.* (2017) proposed that acetate may be responsible for this close association between those two groups as it is the primary substrate of *Methanosaeta* and necessary of growth for several hydrogenotrophic genera in *Methanomicrobiaceae.* Some members of MCG possess a gene encoding acetate kinase that may explain the symbiotic associations. Collins *et al.* (2005) have already demonstrated close physical associations between *Methanosaeta* and MCG. Moreover, Crenarchaeotes have been found to coexist with methanogens and were particularly abundant when *Candidatus Methanofastidiosa* was dominant in the digesters (Chouari *et al.* 2015). We speculate that interactions of this sort might contribute to the sustained abundance of *Methanosaeta* in the HA reactor granules and solid fractions. We found that a defined group of OTUs representing archaeal taxa outside the Euryarchaeota were highly abundant in the UASB reactors at high ammonia concentrations. In phylogenetic analysis, these OTUs clustered together in a distinctive group related to, but distinct from, Thaumarchaeota. This indicates a functional role, either directly as undiscovered methanogenic archaea or indirectly through syntrophic associations. Further research is needed for clarifying this issue.

5.4 Liquid fractions and their microbial communities

In article II, we speculated that the relatively small effect of OLR on the microbial communities may have been caused by the low HRTs employed in the high rate experiment (1.7h and 4 hour for PA and PB, respectively). We assumed that when HRT is lower than the maximum growth rate of the microorganisms, the planktonic microbiota may be a reflection of the communities associated with the influent more so than the active part of the microbiota that reside in the solid granular sludge phase. To gain more insight on this, we examined the microbial community structure of the liquid fractions of the reactors in the high ammonia experiment (HRT 1 day, article IV) to uncover differences in community structure between fractions and to elucidate where the active communities were situated. The archaeal communities in the liquid fraction of the reactors resembled those of the liquid fraction of the influent. This suggests that the slow-growing archaeal communities in the liquid fractions of the reactors did not have time to adapt to the new environment due to the HRT being lower than their growth rate. Hence, the archaeal communities in the liquid fraction was strongly influence by the influent community, confirming the assumption above. Hydrolyzing and fermentative bacteria were found in all fractions although different taxa dominated the different locations. Unique bacterial communities evolved in the reactor liquids despite low HRT, shaped by the selection pressure

and conditions in the reactors. These communities might contribute to the degradation of organic matter in the liquid phase through hydrolysis and acidogenesis. These communities are apparently more influenced by the biomass retained as suspended culture in the reactors than the archaeal communities, suggesting that there is a continuous detachment of surplus biomass from the biofilms colonizing the non-granular particles. The detachment is probably a surface erosion since the bacteria carrying out hydrolysis and acidogenesis are expected to constitute the outer layers of the granules and the biofilm covering the non-granular particles.

5.5 Syntropic oxidation of propionate and acetate

The fraction of removed acetate from the influent COD_T remained constant in the PB reactors and up to day 60 in the PA reactors during increasing OLR in the high rate experiment (article I and II). Propionate removal on the other hand was reduced with the load increase and virtually no propionate was removed from the PA reactors at the highest loads. The reduced propionate removal could be explained by low growth rate and inhibition due to increasing levels of acetate and/or hydrogen. High concentrations of these propionate removal products are thermodynamic unfavorable for propionate reduction (Vaccari *et al.* 2005) and can occur during load increase. We speculated that propionate accumulation could be avoided during constant feed operation. This turned out to be correct as we observed in the high ammonia experiment (article III and IV). Here, we observed constant efficient propionate removal in the LA reactors. Propionate accumulated in the HA reactors when urea was added to increase ammonia concentration, but with adaptation of the microbial communities (at constant OLR and HRT), the removal rate improved towards the end of the experiment (Fig. 1, article III).

Propionate has been recommended as state indicator, together with acetate and biogas production, to monitor manure digesters due to the slow growth of propionate degraders (Ahring *et al.* 1995). The putative syntrophic propionate oxidizing bacteria (SPOB) Cloacimonetes (Nobu *et al.* 2015) was the only SPOB detected in the high ammonia experiment at a significant confidence threshold. It had higher average abundances in the LA reactors than in the HA reactors (approx. 3.1 and 0.5%, respectively). We also examined the bacterial OTU table for the presence of previously described syntrophic acetate oxidizing bacteria (SAOBs). *Tepidanaerobacter acetatoxydans* and *Syntrophaceticus schinkii* were detected, but in very low abundances (max 0.16 and 0.09%, respectively). Several putative SAOB have been suggested lately (Frank *et al.* 2016, Mosbæk *et al.* 2016) but none of these were detected here. Hence, we found no indications that previously described SAOBs were associated with the methane production in the HA reactors though we cannot rule out that putative SAOB could be hiding among the numerous unclassified OTUs. In fact, two bacterial OTUs were associated with methane yield in the HA reactors (article III), but these could only be classified at phylum level as Synergistia and Cloacimonetes. Synergistes group 4/PD-UASB-13 has recently been suggested to include SAOB (Ito *et al.* 2011).

6 Concluding remarks and future aspects

Although AD has been used for many decades, the key players and their functions are not well understood. It has been a general problem of bioprocess engineering that design criteria are rarely verified or validated by thorough analysis of the full scale plant during operation.

Many different operational variables and their effects on AD have been studied in this thesis; *e.g.* organic loading rate and HRT (article I and II), ammonia and temperature (article III and IV). Process performance indicators like VFA levels and hydrogen concentration are already in use (Pind *et al.* 2003). Unfortunately, there is limited information on benchmark values for microbial community structure associated with optimal reactor performance (*i.e.* microbial indicators) (Carballa *et al.* 2015). The microbial community in an anaerobic digester is highly influenced by its influent (article II). This includes the influent chemical makeup, *e.g.* fats, proteins and inhibitors (Chen *et al.* 2008, Sousa *et al.* 2008, Kovacs *et al.* 2013) and its inherent microbial community as it is continuously introduced to the reactor. Fluctuations in operational variables, such as feeding pattern, HRT, OLR, levels of VFA, pH, ammonium content and temperature, may also influence the reactor microbiota. Each biogas fermenter is therefore a unique system defined by its substrate and process conditions.

Engineers have regarded the microbiome as a black box for decades but with the development of new molecular techniques, it is now possible to move past the black box view. Molecular biology tools enable the study of microbial diversity without a cultivation step and thus overcome the cultivation bias. An efficient approach to investigate microbial communities is to amplify either the 16S rRNA gene or genes specific to the functional microbial group of interest like *mcrA*. The MCR gene has been exploited by researchers for decades but the advantages of metagenomics made it possible to discover a new phylum (Vanwonterghem *et al.* 2016). High-throughput sequencing of 16S rRNA gene amplicons has greatly increased resolution and provided detailed taxonomic information for complex microbial communities (Vanwonterghem *et al.* 2014). Though more research is needed on functionality by *e.g.* metatranscriptomics and –proteomics. Fundamental knowledge about microbial communities, how they behave during disturbances and the need for establishing microbial indicators of good process performance is currently a major knowledge gap (Carballa *et al.* 2015, Goux *et al.* 2015).

Deep sequencing of 16S rRNA gene amplicons has become the golden standard for characterizing the composition of microbial communities. Still, this is an end-point PCR approach, and it is therefore not expected to provide an absolute quantitative presentation of the original community. Metagenomic analysis may provide relative abundances of bacteria and archaea, *e.g.* archaea usually account for <10% of the total abundance (Kovacs *et al.* 2013, Yang *et al.* 2014). Droplet digital PCR with broad-range primers could provide a much simpler, cheaper, and less time-consuming way to obtain quantitative information of this sort.

The granules applied in this project adapted well to new process conditions, but there is still much to learn about their make up and the interactions that take place inside them. It would be interesting to apply the emerging new tools of metagenomics, -transcriptomics and -proteomics to gain a deeper insight into *e.g.* the ammonia tolerance of *Methanosaeta* in granules and the taxonomy and function of the detected non-euryarchaeotal OTUs. Other interesting tecniques could be stable isotope probing of DNA or proteins, perhaps in combination with fluorescence *in situ* hybridization.

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Paper I

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High rate manure supernatant digestion

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ABSTRACT

The study shows that high rate anaerobic digestion may be an efficient way to obtain sustainable energy recovery from slurries such as pig manure. High process capacity and robustness to 5% daily load increases are observed in the 370 mL sludge bed AD reactors investigated. The supernatant from partly settled, stored pig manure was fed at rates giving hydraulic retention times, HRT, gradually decreased from 42 to 1.7 h imposing a maximum organic load of 400 g COD L^{-1} reactor d^{-1}. The reactors reached a biogas production rate of 97 g COD L^{-1} reactor d^{-1} at the highest load at which process stress signs were apparent. The yield was ~0.47 g COD methane g^{-1} COD_T feed at HRT above 17 h, gradually decreasing to 0.24 at the lowest HRT (0.166 NL CH_4 g^{-1} COD_T feed decreasing to 0.086). Reactor pH was innately stable at 8.0 ± 0.1 at all HRTs with alkalinity between 9 and 11 g L^{-1}. The first stress symptom occurred as reduced methane yield when HRT dropped below 17 h. When HRT dropped below 4 h the propionate removal stopped. The yield from acetate removal was constant at 0.17 g COD acetate removed per g COD_T substrate. This robust methanogenesis implies that pig manure supernatant, and probably other similar slurries, can be digested for methane production in compact and effective sludge bed reactors. Denaturing gradient gel electrophoresis (DGGE) analysis indicated a relatively fast adaptation of the microbial communities to manure and implies that non-adapted granular sludge can be used to start such sludge bed bioreactors.

1. Introduction

Governments promote anaerobic digestion (AD) of manure because it can reduce greenhouse gas (GHG) emissions and odors, produce renewable energy as methane and improve fertilizer properties (Masse et al., 2011). The largest potential source of methane by anaerobic digestion (AD) of wet organic waste is manure, e.g. ~40% in Norway, but an insignificant fraction of this is realized (Berglann and Krokann, 2011). The main reason for this is the low energy density of manure, implying low production rates in continuous flow stirred tank reactors (CSTR) currently used for manure AD. Such solutions are not sustainable because the costs of construction and operation of such plants are larger than the value of the methane produced (Berglann and Krokann, 2011). Large scale

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farms may have their own CSTR AD solutions that are economically sustainable (Raven and Gregersen, 2007) but agriculture is dominated by smaller farms where such systems may not be rentable. Manure transport to central AD treatment plants is used to some extent, especially in Germany, but the sustainability of such solutions is questioned due to transport cost of manure with low biogas potential.

More efficient process solutions for AD treatment of manure are therefore required. High rate AD reactors may treat waste in smaller and presumably much cheaper digesters. A high rate AD manure treatment technology that is well integrated with existing farm infrastructure for slurry based manure handling systems, common for cattle and pig farms (Burton and Turner, 2003), is therefore investigated here. Manure from farms using slurry based handling systems has 61% of the total theoretical Norwegian manure energy potential of 2480 GWh/a (Raadal et al., 2008). The situation vary some around the world but it is assumed that the case investigated here is relevant for a large fraction of modern global agriculture, as well as aquaculture and other activities producing organic waste slurries.

Manure storage tanks with 8 months minimum HRT capacity, already installed in cold climate countries (e.g. Norway, to comply with government regulations to avoid use as fertilizer outside the short growth season), may serve as a first step in an AD treatment line and/or be used for effluent storage. It has been observed that manure particles disintegrate and hydrolyze during such storage, thereby improving its quality as AD feed (King et al., 2011; Bergland et al., 2014). In such tanks manure separates spontaneously into a floating layer (straw, wood chips, etc.), a bottom sediment layer and a middle layer with much less suspended solids than the floating and bottom layers (Fig. 1). Potentially suitable high rate AD feed can be taken out from the middle layer at no extra cost. A main issue of the present study is to determine if this middle layer, termed manure supernatant, can be used as feed for high rate AD. The assumption is that, if a sludge blanket high rate AD works well on such feed, this process can become economically feasible.

The original and most extensively used high rate reactor is the UASB (upflow anaerobic sludge blanket), developed by Lettinga et al. (1980). Such sludge blanket reactors are used to treat the liquid fraction of organic waste containing small amounts of suspended solids (Tchobanoglous et al., 2003). The particle content of settled manure (Fig. 1) is higher (>6 g TSS L^{-1}) than recommended for UASB treatment (Tchobanoglous et al., 2003). Alternative high rate AD designs, such as fixed biofilm reactors, have been tested on such wastes but solids build up blocking the void spaces in the filter medium making such alternatives less promising (Bolte et al., 1986). Hybrid UASB (Lo et al., 1994) and a suspended particle-attached growth (SPAG) reactor (Cobb and Hill, 1989), are also available. The UASB is, however, the standard of high rate AD, so a small UASB like sludge bed reactor design was chosen for the present study to test the possibilities of high rate AD slurry treatment.

The objective of this study was to examine the efficiency, flexibility and stability of manure supernatant AD treatment in sludge bed reactors. The process capacity and robustness was evaluated by measuring manure degradation and product formation for a wide range of loading rates, including loads that are much higher than what is expected to be required or optimal. A PCR/DGGE strategy was employed to characterize the microbial communities, and to evaluate the time needed for adaption of the granular inoculum to the conditions in the manure-fed AD reactors. The study is relevant for the development of efficient wet organic waste AD with low energy density and high particulates contents in general (e.g. manure, wastewater treatment plant sludge, aquaculture waste sludge) and it may be decisive for the development of sustainable solutions to recover energy for slurry type manures.

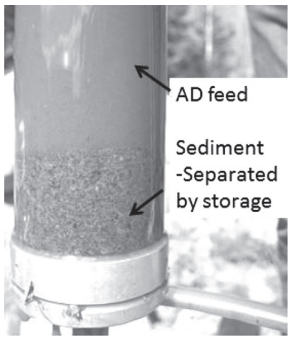

Fig. 1 – Pig manure sample collected near the bottom of a pig manure storage tank.

2. Materials and methods

2.1. *Manure properties and handling*

The process feed was pig manure slurry supernatant regularly collected from a production farm in Porsgrunn, Norway. The manure comes from barns that contains 105 sows, 315 "farrow to finish" and 545 weaners that are fed protein concentrate (14.6% crude protein) added some grass/straw. Wood shavings and straw are used as bedding material. The manure is transported into a storage pit where it is diluted about 30% by wash water from regular barn washing routines. This mixture is what we define as manure slurry, according to Burton and Turner (2003). The HRT of the storage pit varies from 70 to 90 days, which has no significant effects on manure composition (Bergland et al., 2014). The manure separated by gravity in the storage pit into three distinct layers. The top layer is wood shavings and straw. Heavy particles settled to form a bottom layer (Fig. 1). The middle layer, termed the manure slurry supernatant (Table 1), was siphoned and used as feed without any filtering. Fresh manure supernatant was thus collected frequently and stored at 4 °C until use.

2.2. *Reactor design and start up*

The reactor is a simplified UASB (Fig. 2a) made of a 370 mL glass vessel with 345 mL liquid volume, height 130 mm and diameter 60 mm. The substrate inlet is a central tube ending

Table 1 – Properties of the pig manure slurry supernatant used as substrate (Average and Std. Dev.).

Property	Average ± SD
pH	7.3 ± 0.3
COD_T (g L^{-1})	28.1 ± 2.7
COD_S (g L^{-1})	16.0 ± 2.8
COD_{VFA} (g L^{-1})	12.2 ± 1.1
Acetate (g COD L^{-1})	5.7 ± 0.9
Propionate (g COD L^{-1})	2.7 ± 0.6
Butyrate + iso-butyrate (g COD L^{-1})	2.1 ± 0.3
NH_4 – N (g L^{-1})	2.35 ± 0.04
Alkalinity (g L^{-1})	8.7 ± 0.8
TS (g L^{-1})	14.5 ± 1.5
VS (g L^{-1})	7.3 ± 1.5
TSS (g L^{-1})	6.2 ± 2.7
VSS (g L^{-1})	5.1 ± 1.8

10 mm above the reactor bottom, with a horizontal plate at the end to improve distribution of the substrate below the sludge bed. The lab-scale process line is presented in Fig. 2b. Suspended solids are separated inside the reactors to retain biomass while the gas and liquid is separated outside the reactors to ease operation in such small scale reactors. The substrate tank is kept at 4 °C and the four reactors at 35 °C.

Four identical reactors were operated for 68 days. The inoculum was based on granules (70 g L^{-1} VSS) from a UASB reactor treating pulp and paper process wastewater at "Norske Skog Saubrugs" in Halden, Norway. Half of the reactor volumes were filled with granules. Two of the reactors had been fed pig manure for 6 months as an adaption period prior to the experiment. The other two were inoculated using granules without any adaptation (these granules were stored at 11 °C for 6 months with no feed prior to the experiment). The reactors with granules not adapted to pig manure were started at a HRT of 42 h (medium rate) while the reactors with adapted biomass were started at 8.5 h HRT (high rate). Nearly constant HRT was maintained after start up until stable biogas production was established. Then an increase of the feed flow of 5% was imposed every day.

The reactors were fed intermittently, 25 mL each time which is < 1/10 of reactor liquid volume implying >10 feedings for each HRT. It is therefore reasonable to assume continuous flow in the mass balance analysis of the process. Feed flow increases were obtained by increasing the feeding frequency.

2.3. Analysis

Biogas, inflow and outflow liquid samples were collected twice a week. Total chemical oxygen demand (COD_T), soluble COD (COD_S), total solids (TS), volatile solids (VS), total suspended solids (TSS), volatile suspended solids (VSS), pH, alkalinity, NH_4^+–N, VFA's (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, iso-caprionate and caprionate) and gas composition were analyzed.

Gas production (L d^{-1}) and reactor temperature were monitored continuously online. The biogas flow was measured using a volumetric gas meter working according to the same principle as used by Dinamarca and Bakke (2009). The reactors were kept at 35 °C in a water bath.

COD was measured according to US standard 5220D (APHA, 1995). For COD_S determination the samples were first centrifuged at 10,000 rpm for 30 min and then filtered (0.45 μm). Alkalinity was measured by titration according to US standard 2320B (APHA, 1995).

NH_4^+ – N concentration was analyzed on filtered samples (0.2 μm) by ion chromatography using an DX-500 ion chromatographic analyzer equipped with a conductivity detector, a SCS1 cation-exchange column (4 × 250 mm) in combination with a Dionex IonPac PCG1 (4 × 50 mm) guard column. 4 mM methane-sulfonic acid was used as the mobile phase. The oven temperature was kept constant at 35 °C.

VFA's were measured by gas chromatography (Hewlett Packard 6890) with a flame ionization detector and a capillary column (FFAP 30 m, inner diameter 0.250 mm, film 0.5 μm). The oven was programmed to go from 100 °C, hold for 1 min, to 200 °C at a rate of 15 °C min^{-1}, and then to 230 °C at a rate of 100 °C min^{-1}. The carrier gas used was helium at 23 mL min^{-1}. The injector and detector temperatures were set to 200 °C and 250 °C, respectively.

Gas composition (CO_2 and CH_4) was quantified by gas chromatography (Hewlett Packard 5890A) equipped with a thermal conductivity detector and two columns connected in

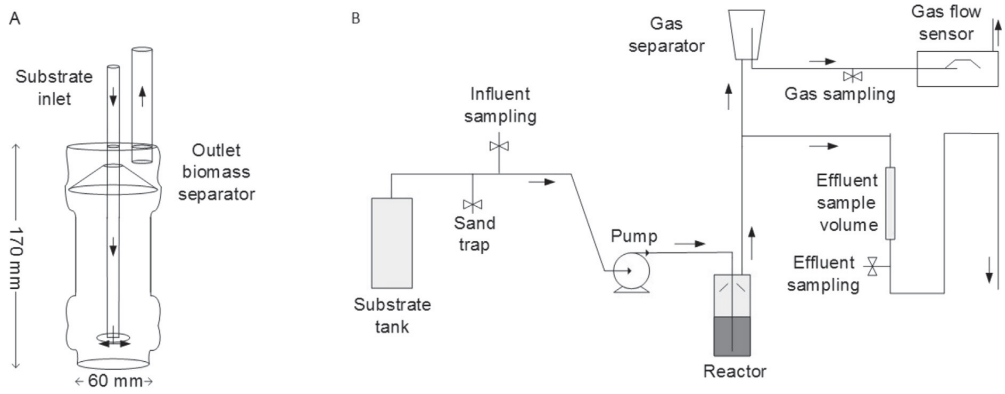

Fig. 2 – A) Sketch of lab-scale AD reactor with central inlet and separator. B) Diagram of lab-scale process line.

parallel: Column 1, CP-Molsieve 5A (10 m × 0.32 mm) and Column 2, CP-PoraBOND Q (50 m × 0.53 mm). The gas carrier was argon at 3.5 bar pressure. The oven temperature was kept constant at 40 °C.

2.4. DNA extraction, PCR, DGGE and statistical analysis

Samples for microbial analysis were taken from the sludge trap of the reactors at days 35, 61 and 68 of the experiment. Total DNA was extracted from the sludge samples by using the PowerFecal DNA Isolation Kit (MoBio) as described by the manufacturers. For bacteria, the v3 region of the 16S rRNA gene was amplified with the primers GC-338F (5′-cgcccgccgcgcgcggcgggcggggcgggggcacgggggg actcctacgggaggcagcag-3′) and 518R (5′-attaccgcggctgctgg-3′) (Muyzer et al., 1993). For methanogenic archaea, PCR primers targeting the 16S rRNA gene were designed. First, conserved regions of the 16S rRNA gene were identified by using alignments of methanogenic archaeal sequences downloaded from the Ribosomal database project (RDP). The Probematch tool of RDP was used for optimization of primer sequences and improving coverage. The resulting primers, GC-624F (5′-cgcccgccgcgcgcggcgggcggggcgggggcacgggggg caccdrtggcgaaggc-3′) and 820R (5′-gccrattcctttaagtttca-3′), was employed to amplify the v5 region of the 16S rRNA gene. PCR reactions were performed using the Taq PCR Core Unit Kit (Qiagen) and 0.3 μM of each primer, and run for 35 cycles of 95 °C for 30 s (s), 53 °C for 30 s, and 72 °C for 60/90 s for bacterial/archaeal PCR products, respectively. The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) with the INGENYphorU DGGE system (Ingeny) and 8% acrylamide gels with a denaturing gradient of 35–55 % for bacterial PCR products and 35–50 % gradient for methanogenic archaeal PCR products, as described in Bakke et al. (2013).

The Gel2K program (Svein Nordland, Department of Microbiology, University of Bergen, Norway) was used for converting band profiles in DGGE images to histograms, where the peaks correspond to DGGE bands. Peak area matrices, reflecting the band intensities, were exported to Excel spread sheets and used for statistical analysis. Individual peak areas were normalized by dividing on the sum of the peak areas for the relevant DGGE profile. Statistical analyses were performed using the program package PAST version 2.17 (Hammer et al., 2001). Bray–Curtis similarities (Bray and Curtis, 1957) were used to compare DGGE profiles, and was calculated based on square root transformed peak areas to reduce the impact of strong bands. Ordination based on Bray–Curtis similarities were performed using non-metric multidimensional scaling (NMDS; Taguchi and Oono, 2005). PERMANOVA was used for testing differences in average Bray–Curtis dissimilarities between groups of samples (Anderson, 2001).

3. Results and discussions

All four reactors produced biogas from day one and stabilized after 35 days of constant hydraulic load. The results are from the subsequent 33 days with 5% daily feed flow increase giving the reactors HRT from 42 to 8.5 h for “medium rate” and from 8.5 to 1.7 h for “high rate”. Biogas production increased with load during the whole experiment with low standard deviations between the parallel reactors.

3.1. Stability

In all the reactors the biogas production was still increasing, due to the increasing load, when the experiment was stopped. No foaming, typically experienced in manure AD (Hill and Bolte, 2000), or significant pH changes were observed. The average effluent pH in all 4 reactors was 8.0 ± 0.1 with influent average pH of 7.3 ± 0.3 and no active pH control. The alkalinity was also stable with similar effluent alkalinities of 10.6 ± 0.8 g L^{-1} (high rate case) and 11.0 ± 0.9 (medium rate case). No visual signs of process failure or instability were observed even at the highest organic load rate (OLR) of 400 g COD L^{-1} reactor d^{-1} tested, implying that pig manure slurry supernatant sludge blanket AD can be a very robust process (chemical signs of process instability are discussed below). The reactors also showed remarkable stability and adaptation to the daily loading rate changes. Stable performance has also been reported for attached growth reactors fed liquid pig manure (Bolte et al., 1986) at loads in the lower range tested here. The observed robustness and process stability is especially important for farm and other small scale AD applications without dedicated process operators.

3.2. Capacity

The methane production rate and yield, VFA and COD results are evaluated to establish process efficiency and capacity of the process. The daily average methane production rate during the daily 5% load increases are given in Fig. 3a with a rate of 58 * $HRT^{-0.79}$ NL CH_4 L^{-1} reactor d^{-1} (HRT in hours; R^2 is 0.99). The highest measured rate was 34 NL CH_4 L^{-1} reactor d^{-1} (= 97 g COD L^{-1} reactor d^{-1}) at HRT 1.7 h. This is about fifty times higher production rate than reported for conventional stirred tank AD processes operated on manure alone (Chynoweth et al., 1999; Summers and Bousfield, 1980). The methane yield on liter basis (Fig. 3b) was 0.75 * ln(HRT) + 2.2 NL CH_4 L^{-1} feed (HRT in hours; R^2 is 0.88) with a maximum of 4.7 NL methane per liter feed at HRT 42–17 h, decreasing to 2.4 NL methane per liter feed for the lowest HRT. This is 0.47 g COD methane g^{-1} COD_T feed at HRT 42–17 h and a decrease to 0.24 at HRT 1.7 h (0.166 NL CH_4 g^{-1} COD_T feed decreasing to 0.086). The biogas methane content was 76–81 % for all HRT. The COD removal, measured as COD_T, COD_S and COD_{VFA}, varied between 24 and 68 %, 38–65 % and 46–90 %, respectively, with increasing effluent concentrations with load (Fig. 4). An observed 49% COD_T reduction at HRT 17 h corresponds well with results from similar cases reported by Kalyuzhnyi et al. (1999) and Kang et al. (2003). No published results are found to compare the highest loads (400 g COD L^{-1} reactor d^{-1}) investigated here but OLR up to 72.5 g COD $L^{-1}d^{-1}$ using cow manure supernatant have been run at steady state obtaining higher yield (Rico et al., 2011). The effluent COD concentrations achieved here are probably not as low as achievable in a steady feed operation. This can be seen in Fig. 4 where the medium rate reactors at the end of the experiment removed significantly less COD_T, COD_S and COD_{VFA}, at

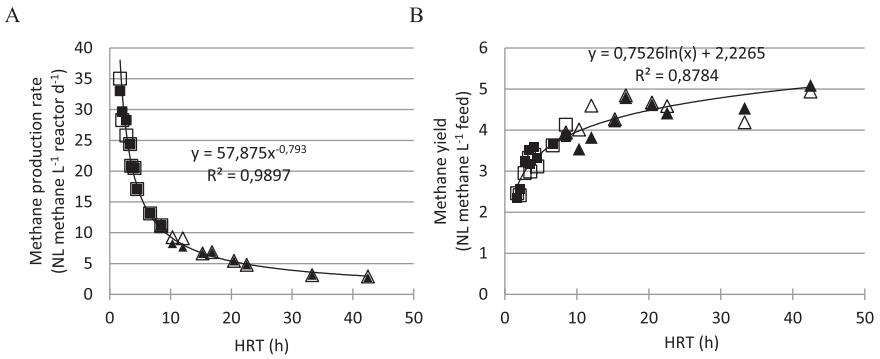

Fig. 3 – Average methane production rate (A) and yield (B) in both medium (Δ ▲) and high (□ ■) rate reactors. One parallel reactor filled symbols and the other one empty.

HRT = 8.5 h than the high rate reactors that started at steady state at this HRT. The daily 5% load increases used here to test the robustness of the reactors are not conducive to maximize transformation efficiency.

3.3. VFA

Process efficiency can be further elucidated from the measured VFA concentrations during the experiment. COD_{VFA} was removed by 86 %–90 % in all the reactors at the start of the load increase and reduced to 46% at the highest load. The effluent acetate concentration (Fig. 4) increased with load but remained quite low during the experiment, implying robust methanogenesis. The reduced methanogenesis with load may be caused by ammonia inhibition, according to the inhibition factors proposed for ADM1 (Batstone et al., 2002) which in this case (measured effluent ammonia = 2.32 ± 0.03 g NH_4–N L^{-1} and pH 8.0) can cause 90% reduction in the acetate removal rate. Such strong effect was, however, not observed, implying that some adaptation to high ammonia (e.g. as explained by Schnürer and Nordberg, 2008; Hattori, 2008) may have occurred. This suggests that inhibited methanogenesis is not the main cause of reduced methane yield with load.

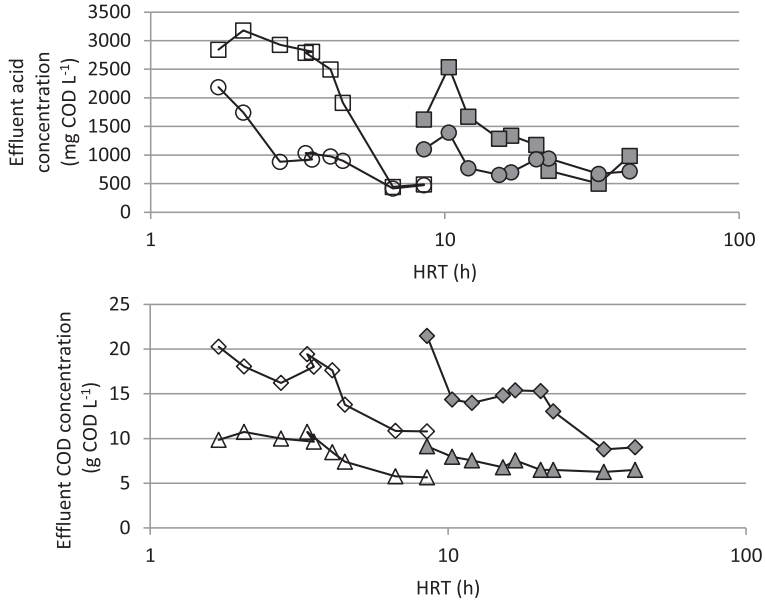

Fig. 4 – Effluent acetate (○), propionate (▫), CODT (◊) and CODS (Δ). Medium rate symbols are filled and high rate symbols are unfilled.

The fraction of removed acetate from the influent COD_T remained constant during the experiment (Fig. 5). Propionate removal on the other hand was reduced with the load increase, but this did not cause other instability symptoms than lowered methane yield (g COD CH_4 g^{-1} COD_T feed) even though virtually no propionate was removed at the highest loads (Figs. 4 and 5). The reduced propionate removal can be explained by low growth rate and inhibition due to high levels of acetate and/or hydrogen. High concentrations of these propionate removal products are thermodynamic unfavorable for propionate reduction (Batstone et al., 2002) and can occur during load increase. During constant feed operation propionate accumulation may be avoided. The increasing feed flow rate used to induce the load increase could also have caused a washout of some dispersed biomass especially at the higher flows, worsening the situation for the slow growing propionate removal organisms.

Propionate has been recommended as state indicator, together with acetate and biogas production, to monitor manure digesters due to the slow growth of propionate degraders (Boe et al., 2010). The observations discussed above confirm that propionate degradation can be an AD rate limiting step and propionate therefore is a useful state indicator.

The reduced conversion efficiency with load, attempted explained by inhibition above, may alternatively have a physical cause. Mass transfer effects on the observed kinetics of substrate uptake have been studied in detail by several authors, as summarized and evaluated for AD by Pavlostathis and Giraldo-Gomez (1991). Given that granular sludge bed processes decouple sludge retention time from HRT they can be mass transfer limited rather than reaction limited. Diffusion of molecules from the liquid phase into the granules and entrapment of small particles may be influenced by hydraulic load: Low HRT allows little time for such mass transfer Contois kinetics proposed to describe substrate uptake AD kinetics predicts effluent substrate concentrations similar to those observed here, typical for mass transfer limited processes (Pavlostathis and Giraldo-Gomez, 1991), but the results are not decisive. Distinguishing mass transfer and reaction limitation in such processes is a challenge for future research.

3.4. *Microbial communities*

The microbial communities in the reactors were compared at three different time points. Non-metric multidimensional scaling of Bray–Curtis similarities indicated that the bacterial and archaeal communities of the reactors differed with respect to the type of granule inoculum used (Fig. 6).

A PERMANOVA test confirmed that there were significant differences in microbial communities between the reactors inoculated with pre-adapted granules and the reactors inoculated with non-adapted granules both for bacteria ($p = 0.003$) and archaea ($p = 0.002$) hence the six months pre-adaptation period of the high rate reactors had a significant impact on the reactor microbial community. The average Bray–Curtis similarities show that the microbial communities in the high rate reactors and the medium rate reactors became more similar with time. The average Bray–Curtis values increased from 0.63 ± 0.03 to 0.77 ± 0.06 from day 35 to day 68 for bacteria and from 0.64 ± 0.04 to 0.75 ± 0.05 for archaea. This implies that a long-lasting adaptation of the granular inoculum from pulp and paper mill UASB wastewater treatment is not needed to make it capable of treating manure. This can perhaps be explained by the diverse microbial community generally found in manure (Hagen et al., 2014; Liu et al., 2009; Barret et al., 2012) such that the AD process is continuously inundated by manure adapted organisms in the feed.

3.5. *Process implications*

The results show that settled pig manure supernatant is a suitable substrate for sludge bed AD in spite of having particulates content above the recommended range for UASB feeds (Tchobanoglous et al., 2003). The manure fraction tested here has similar composition to other slurries, such as wastewater sludge, fish pond aquaculture sludge and other types of manure, encompassing nearly half of all wastes deemed suitable for AD (Berglann and Krokann, 2011). This does not necessarily imply that all such slurries can be treated by high rate AD. Lettinga and Hulshoff Pol (1991) warned that suspended matter can have advers effects.

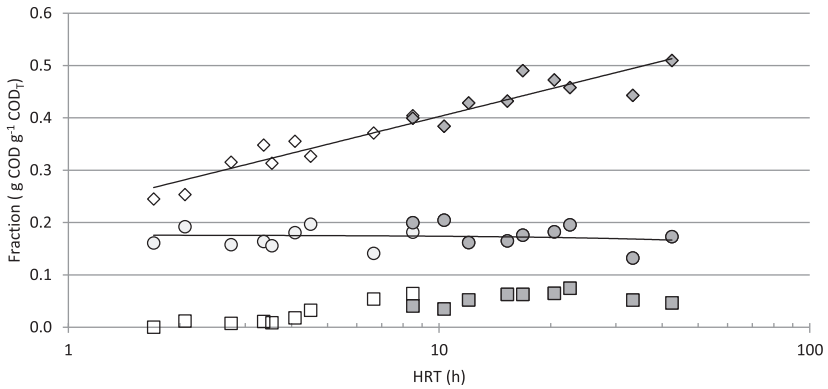

Fig. 5 – Produced biogas (◊), removed acetate (○) and removed propionate (□) as fractions of influent COD_T. Medium rate symbols are filled and high rate symbols are unfilled.

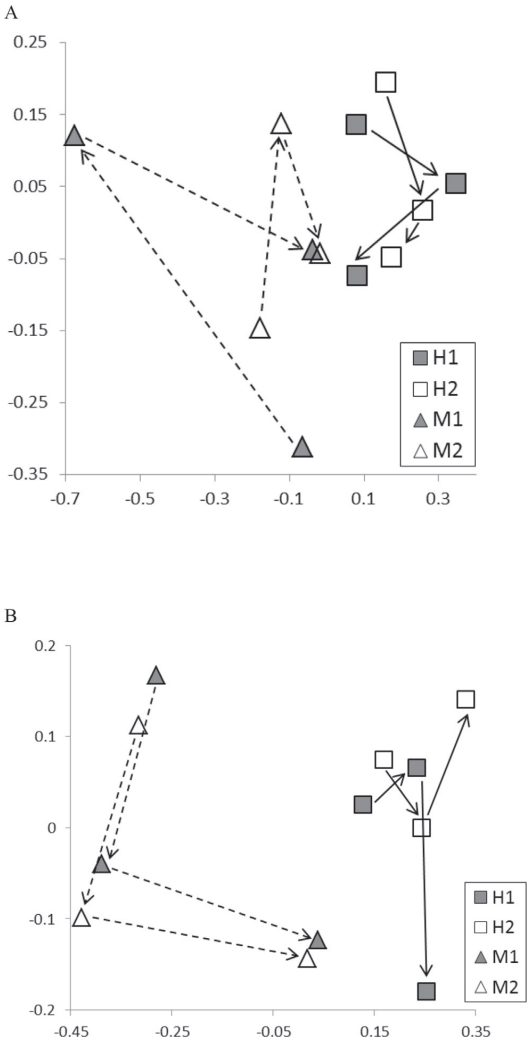

Fig. 6 – NMDS ordination based on Bray–Curtis similarities for comparisons of bacterial (A) and archaeal (B) communities in the high (H) and medium (M) rate reactors at day 35, 61, and 68 of the experiment. The arrows indicate the time course of the samples.

The biogas yield was 0.47 g COD methane g^{-1} COD_T manure from HRT 42 to 17 h, decreasing to 0.24 at HRT 1.7 h (Fig. 5). This implies that HRT > 17 h is adequate to obtain high energy recovery yield and production rates up to 20 g COD methane L^{-1} reactor d^{-1}.

There is a large trade-off between production rate and yield at the highest loads imposed. This can partly be explained by propionate degradation lagging behind in the AD chain reactions. It is likely that this limitation would lessen if steady state was allowed to establish, but some yield loss at high production must be expected. It is still likely that high production during periods of high demand can have greater value than the loss in total production caused by temporary low yield, at least down to HRT = 4 h (Figs. 3 and 5).

Very high and changing loads imposed here did not cause process failure. This suggests that such processes can be operated safely without much monitoring in the whole range tested, up to 400 g COD L^{-1} reactor d^{-1}. The result also demonstrates that it is possible to turn biogas production up and down depending on energy demands, but this must be done with caution. The reduced propionate removal caused by a 5% load increase (Fig. 5) can be seen as a stress symptom, suggesting that faster changes can be risky but achievable.

The microbial communities in the reactors inoculated with pre-adapted granules and non-adapted granules were significantly different with respect to both bacteria and archaea, but became more similar with time. The relatively fast adaptation to manure implies that non-adapted granular sludge may be used to start sludge bed bioreactors for treatment of pig manure supernatant.

Cheap and mechanically simple processes are also required to make manure AD economically sound. The extreme high rate AD obtained here demonstrates that it is possible to treat manure in small and thereby presumably cheap digesters. Mechanical simplicity was achieved by not using recycle flow to fluidize the active biomass (as opposed to standard UASB design). The inflow, controlled with a timer (on/off), hit the reactor bottom in pulses as an alternative way to fluidize the sludge (Fig. 2). The strongest mixing occurred during feeding while it was visually observed that gas production maintained mixing between feedings. It was also observed that the feed flow stirred and mixed well with the lower sludge bed layers during each pulse feed while the upper sludge bed fluidized but was not much stirred. This suggest that the process behaves more like a plug flow than a stirred tank reactor and is thus, in this respect, similar to a conventional UASB. A full scale AD sludge bed reactor without recycle will be tested next. Pulse feeding has been demonstrated to favor the development of efficient granular sludge for wastewater treatment (Franco et al., 2003).

A rather compact sludge bed was observed at the lowest loads while a more expanded bed was observed as the loading increased. The biomass was fluidized to almost fill the whole reactor volume at the highest load, with the potential for biomass washout. This did not occur to any great extent but VFA data suggest a slight loss of biomass with increasing flow, especially at the highest flows, as discussed above.

Expanded beds not fully fluidized could trap organic particulates (Tchobanoglous et al., 2003). This was the case here judging from the removal rate of COD_T (Fig. 7) which is slightly larger than the methane production rate. Particles evidently contributed to the methane production since the COD_S removal rate was less than the methane production rate. This effect appears, however, to be valid for fully fluidized sludge beds also, since the relationships between COD_T, methane and COD_S transformation were the same in the whole range tested.

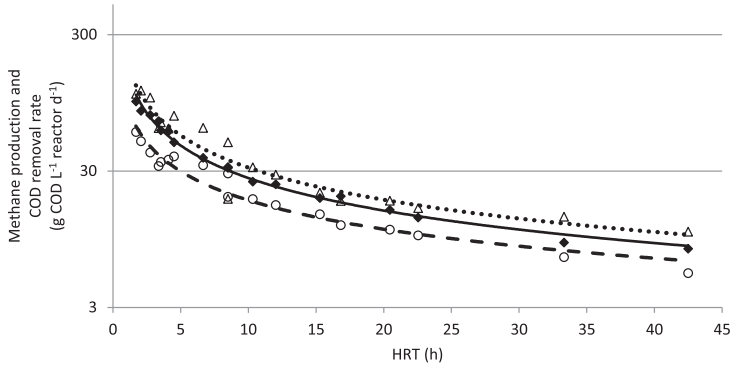

Fig. 7 – Methane production rate (- ◆) compared to the removal rate of CODT (.... Δ) and CODS (- - - ○). All data points are average from the two parallel reactors.

Practical challenges regarding AD feed handling in full scale at the farm will be met through cooperation with farmers, equipment suppliers and agriculture research teams. The two main issues are: 1. How to operate the AD through cycles of manure availability, spreading etc, 2. The high dry matter fraction from bottom and floating layers must regularly be removed to avoid technical problems. When to remove these fractions (and how to do it) depends on a variety of local conditions, especially its final use as fertilizer. Infrequent removal is advantageous for the overall biogas yield as it allows more degradation of particulates compared to shorter storage (Bergland et al., 2014). An AD reactor volume of about 10 m^3 has been identified to be appropriate for the treatment of up to 5000 m^3/y, which covers almost all Norwegian pig farms. Farmers express interest in such solutions to improve their abilities to manage the manure as fertilizer while recovering energy.

4. Conclusion

Sludge bed AD reactors can treat settled pig manure supernatant efficiently.

Biogas production rate of 97 g COD L^{-1} reactor d^{-1} was obtained at the highest load tested (HRT = 1.7 h and OLR = 400 g COD L^{-1} reactor d^{-1}) with no physical signs of process failure.

The process handled 5% daily load increases well with reduced methane yield as the only stress symptom down to HRT = 4 h.

Propionate accumulation was observed at the highest OLRs.

A relatively fast adaptation to manure of the microbial communities implies that non-adapted granular sludge can be used as inoculum for sludge bed pig manure treatment.

High process capacity and robustness in mechanically simple manure supernatant treatment suggests a general potential for sustainable sludge bed slurry treatment.

Acknowledgment

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Paper II

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ORIGINAL ARTICLE

Microbial community dynamics and biogas production from manure fractions in sludge bed anaerobic digestion

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Keywords
biodegradation, denaturing gradient gel electrophoresis, granules, microbial structure, polymerase chain reaction.

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The reactors were operated at Telemark University College, Porsgrunn, Norway, while the microbial analyses were executed at Norwegian University of Science and Technology, Trondheim, Norway.

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Abstract

Aims: To elucidate how granular sludge inoculum and particle-rich organic loading affect the structure of the microbial communities and process performance in upflow anaerobic sludge bed (UASB) reactors.
Methods and Results: We investigated four reactors run on dairy manure filtrate and four on pig manure supernatant for three months achieving similar methane yields. The reactors fed with less particle rich pig manure stabilized faster and had highest capacity. Microbial community dynamics analysed by a PCR/denaturing gradient gel electrophoresis approach showed that influent was a major determinant for the composition of the reactor communities. Comparisons of pre- and non-adapted inoculum in the reactors run on pig manure supernatant showed that the community structure of the nonadapted inoculum adapted in approximately two months. Microbiota variance partitioning analysis revealed that running time, organic loading rate and inoculum together explained 26 and 31% of the variance in bacterial and archaeal communities respectively.
Conclusions: The microbial communities of UASBs adapted to the reactor conditions in treatment of particle rich manure fractions, obtaining high capacity, especially on pig manure supernatant.
Significance and Impact of the Study: These findings provide relevant insight into the microbial community dynamics in startup and operation of sludge bed reactors for methane production from slurry fractions, a major potential source of biogas.

Introduction

Traditional completely mixed reactors are consistently used in agriculture and wastewater sludge treatment at the present. Upflow anaerobic sludge bed (UASB) reactors can be more than 50 times more efficient than these (Lettinga *et al.* 1997; Barber and Stuckey 1999; Tchobanoglous *et al.* 2003; von Sperling and Oliveira 2009). High rate UASB reactors may treat more waste in smaller and presumably much cheaper digesters, but the technology for small-scale biogas facilities is at an early stage. The manure storage may serve as the first step in a treatment line allowing disintegration and hydrolysis of particles and separation of influent supernatant with low content of suspended debris. We recently demonstrated that high rate UASB reactors can be used for anaerobic digestion (AD) of supernatant from pig manure to obtain sustainable energy recovery despite the solids content being well above the levels considered appropriate as UASB reactor influent (Bergland *et al.* 2015). The microbial community structure and dynamics in such high rate processes for AD of manure supernatant has so far not been investigated.

The microbial community in an anaerobic digester is highly influenced by its influent (Ziganshin *et al.* 2013). This involves the influent chemical makeup, e.g. fats, proteins and inhibitors (Chen *et al.* 2008; Sousa *et al.* 2008; Kovacs *et al.* 2013) and its inherent microbial community as it is continuously introduced to the reactor. The microbial community of e.g. municipal solid waste is

different from those found in dairy cow manure (Narihiro and Sekiguchi 2007; Hagen *et al.* 2014). Fluctuations in operational variables may also influence the reactors biota and include feeding pattern, hydraulic retention time (HRT), organic loading rate (OLR), levels of volatile fatty acids (VFA), pH, ammonium content and temperature (Sun *et al.* 2014). Each biogas fermenter is therefore a unique system defined by its substrate and process conditions (Jenicek *et al.* 2010; Krakat *et al.* 2011; Ziganshin *et al.* 2013; Rosa *et al.* 2014). However, the interactions and the roles of the consortia of micro-organisms involved are still poorly understood. Knowledge about how the microbial communities are influenced by reactor design and operational variables is needed to improve design and proper operation of reactors.

Molecular biology tools enable the study of microbial diversity without a cultivation step and thus overcome the cultivation bias. An efficient approach to investigate microbial communities is to amplify either the 16S rRNA gene (Kim *et al.* 2011; Demirel 2014; Madden *et al.* 2014; Tuan *et al.* 2014) or the gene specific to the functional microbial group of interest (Lueders *et al.* 2001; Galand *et al.* 2002; Nettmann *et al.* 2008; Gagnon *et al.* 2011) by PCR, and analyse the PCR products by denaturing gradient gel electrophorese (DGGE). Culture-independent molecular techniques based on 16S rDNA and functional genes have helped linking microbial community structure and dynamics to process performance (Nakasaki *et al.* 2013).

The objective of this study was to examine UASB performance with manure supernatants with high particle contents as influent and to investigate the microbial community dynamics by PCR/DGGE using 16S rRNA gene amplicons. The aim was to elucidate how the influent, granular sludge inoculum and OLR affected the structure of the bacterial and archaeal communities in the biogas reactors.

Experimental procedures

Reactor influent and inoculum

A total of eight laboratory scale reactors were studied. The filtrate of sieved dairy cow manure (named dairy manure for short hereafter) collected from Foss Farm in Skien, Norway, was used as influent for a period of 96 days in four reactors (CA1, CA2, CB1 and CB2). These reactor names are abbreviated C (all reactors run on dairy manure), CA (parallel CA1 and CA2) or CB (parallel CB1 and CB2). The HRT of the CA reactors was decreased towards the end of the experiment while the CB reactors were kept at constant HRT. Dairy manure handling is described in Bergland *et al.* (2014) while the influent properties are in Table 1. The other four reactors (PA1, PA2, PB1 and PB2) were fed pig manure slurry supernatant (named pig manure for short hereafter) for 106 days. These reactor names are abbreviated P (all reactors run on pig manure), PA (parallel PA1 and PA2) or PB (parallel PB1 and PB2). The manure was collected from a production farm in Porsgrunn, Norway. Pig manure handling is described in Bergland *et al.* (2015) while the influent properties are in Table 1. Both manure slurries were stored at 4°C until use.

The granules (70 g VSS l^{-1}) used as inoculum originated from a UASB reactor treating pulp and paper process wastewater at 'Norske Skog Saugbrugs' in Halden, Norway. For six of the reactors, the granular inoculum had been stored for six months at 11°C prior to the experiment. The remaining two reactors (PA1 and PA2) were run on pig manure influent six months prior to the experiment to adapt the granular sludge inoculum. All the reactors were filled half way up with granules at the start of the experiment.

Reactor design and operation

Lab-scale process lines were set up utilizing identical pulse fed 370 ml UASB reactors with a liquid volume of 354 ml. The reactor design and measurements of COD, pH, VFA, NH_4^+-N, gas composition and methane production are described in Bergland *et al.* (2015). The C reactors were all started at HRT 1·77 days together with two of the pig manure fed reactors, PB, while the PA reactors previously adapted to pig manure were started at 0·35 days HRT (Table 2). Effects of load increases were investigated by running periods of reduced HRT, after the biogas production had stabilized in the PA and PB reactors. The HRTs were reduced during days 35–68 by 5% per day. The corresponding OLRs are in Table 2. Production never stabilized in the C reactors, and hence the HRTs were decreased by 5% per day for the CA

Table 1 Properties of the influent for the anaerobic digestion reactors (Average and SD)

Property	Pig manure slurry supernatant	Dairy manure filtrate
pH	7·3 ± 0·3	7·5 ± 0·2
COD_T (g l^{-1})	28·1 ± 2·7	48·3 ± 7·6
COD_S (g l^{-1})	16·0 ± 2·8	13·1 ± 2·2
COD_{VFA} (g l^{-1})	12·2 ± 1·1	5·8 ± 0·9
$COD_{particle}$	12·1	35·2
Acetate (g l^{-1})	5·5 ± 0·8	3·2 ± 0·5
Propionate (g l^{-1})	1·9 ± 0·4	0·8 ± 0·2
NH_4 – N (g l^{-1})	2·35 ± 0·04	0·90 ± 0·14

Table 2 HRT and OLR at the time of sampling for microbial analysis

	HRT (day)				OLR (g COD_T l^{-1} day^{-1})			
	Dairy		Pig		Dairy		Pig	
Time (day)	CA	CB	PA	PB	CA	CB	PA	PB
4	1·77	1·77	0·35	1·8	27	27	79	24
35	1·77	1·77	0·35	1·77	27	27	79	16
61	1·77	1·77	0·12	0·5	27	27	245	56
68	1·77	1·77	0·07	0·35	27	27	397	79
96	0·45	1·77	0·1	0·35	107	27	283	79
103	–	–	0·1	0·21	–	–	283	135
105	–	–	–	0·17	–	–	–	163

HRT, hydraulic retention time; OLR, organic loading rate.

reactors during days 71–96 while HRT of the CB reactors were never changed.

Sampling, DNA extraction, PCR amplification and DGGE

Samples were collected from the effluent line of each reactor on day 35, 68 and 96 for the C reactors and on day 35, 61, 68, 96, 103 (PA only) and 105 (PB only) for the P reactors. The first three samples from the P reactors have been subject to microbial analyses previously (Bergland *et al.* 2015), but are included here for continuance. Samples of both influents were taken on day 35, 68 and 96. The nonadapted granular sludge inoculum was sampled and DNA extracted on two occasions prior to this experiment (six months and one year). Granular sludge inoculum that had been adapted to pig manure was not sampled prior to the experiment start-up.

DNA was extracted using the Power Soil DNA isolation kit from MO BIO Laboratories as described by the manufacturer. PCR was performed using the primer pairs GC-338f (cgcccgccgcgcgcggcgggcggggcgggggcacgggggg actcctacgggaggcagcag) and 518r (attaccgcggctgctgg) amplifying the v3 region of the 16S rRNA gene in bacteria (Muyzer *et al.* 1993) and GC-624f (cgcccgccgcgcgcg-gcgggcggggcgggggcacgggggg caccdrtggcgaaggc) and 820r (gccrattcctttaagtttca) amplifying the v5 region in archaea (Bergland *et al.* 2015). The PCR products were analyzed by DGGE as described by Bakke *et al.* (2013) using 8% acrylamide gels with a denaturing gradient of 35–55% for the bacterial PCR products and 35–50% gradient for the methanogenic archaeal PCR products.

Statistical analysis

The gel pictures were analyzed using Gel2K (Svein Nordland, Department of Microbiology, University of Bergen, Norway). This program converts the band profiles in DGGE images to histograms where the peaks correspond to the intensity in the DGGE bands. The peak area matrices were exported to Excel where the values were normalized and square root transformed to reduce the impact of strong bands. The matrices were then exported to PAST ver. 2.17 (Hammer *et al.* 2001) for statistical and multivariate analysis. Principal coordinate analysis (PcoA) (Davis 1986) was based on ordination of Bray-Curtis similarities (Bray and Curtis 1957). One-way PERMANOVA (Anderson 2001) was employed for testing the differences in average Bray-Curtis similarities between different groups of samples. Variance partitioning (Borcard *et al.* 1992) was carried out with the varpart function in Vegan, a package in R (R Development Core Team 2014), which partitions the variation of the DGGE peak area matrices with respect to two, three, or four explanatory tables (Oksanen *et al.* 2013). Continuous explanatory variables in variance partitioning were transformed to standard normal distribution before analysis.

Results

Reactor performance

The start-up and 5% daily increase periods of the PA and PB reactors are described in Bergland *et al.* (2015). The end of the 5% increase period resulted in a maximum OLR of 397 g COD_T l^{-1} day^{-1} for PA reactors (Fig. 1a) with HRT 0·07 days and a maximum biogas production of 34 NL methane l^{-1} reactor day^{-1} (Fig. 1b). The PB reactors which started at a lower load had a maximum OLR of 163 g COD_T l^{-1} day^{-1} at day 106 with HRT 0·17 days and a biogas production of 16 NL methane l^{-1} reactor day^{-1}. Reduced methane production and propionate removal (Fig. 2) at the highest loads of the PA reactors were signs of stress but the process did not fail and maintained production throughout the experiment, however, with relatively low yield (Fig. 1c). The PB reactors without preadapted inoculum, not exposed to such extreme loads, achieved higher methane yield than PA except for the first two weeks (Fig. 1c).

The C reactors, all inoculated with nonadapted granules, took a long time to reach stable biogas production (Fig. 1). The CB reactors were therefore kept at constant load of 29 g COD_T l^{-1} day^{-1} (Fig. 1a). They slowly increased the methane production yield and showed increasing methane production rate (Fig. 1). From day 71, the CA reactors got 5% daily influent flow increase from HRT 1·77 to 0·45 days and OLR 29 to 107 g COD_T l^{-1} day^{-1} resulting in increased methane production rates up to 3·1 NL methane l^{-1} reactor day^{-1} for CA1 and 4·5 for CA2, introducing the largest devia-

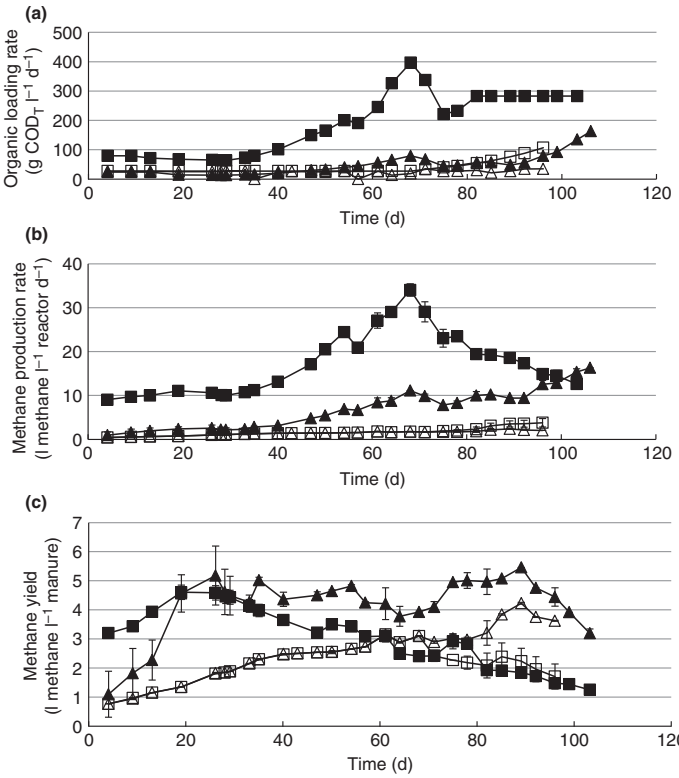

Figure 1 Organic loading rate (a), methane production rate (b) and methane yield (c) for pig manure fed reactors PA (■) and PB (▲), and dairy manure fed reactors CA (□) and CB (△) (average parallel 1 and 2). Error bars represent standard deviation between parallel reactors.

tion between two CA parallel reactors in this study. The standard deviations between effluent samples from parallel reactors are given as error bars in Figs 1 and 2, while often not visible due to similar reactor behaviour. The influent is without error bars, since the reactors receive the same feed.

The yield of the CB reactors increased during the whole test to a maximum of 4·2 l methane l^{-1} manure at 27 g COD_T l^{-1} day^{-1} OLR, never reaching the level of the stable PB reactors of 56 g COD_T l^{-1} day^{-1} OLR with 5 NL methane l^{-1} manure. Methane yield for the CA reactors dropped with increased load to 1·7 NL methane l^{-1} influent at 107 g COD_T l^{-1} day^{-1}. Acetate concentrations for the C reactors (Fig. 2a) shows increasing removal with stabilized 83% acetate removal after day 57. Insignificant propionate removal was observed until day 80 after which 73–92% propionate removal were observed at day 96 for the CB reactors (Fig. 2b). Propionate removal did not occur in the two CA reactors exposed to load increase after day 71. Acetate (Fig. 2c) and propionate (Fig. 2d) removal was considerably higher in the pig manure fed reactors where the propionate levels were low and stable in all cases except for the peak loads.

The COD removal varied between 9–50 and 24–68% for reactors fed with dairy and pig manure respectively. The pH was stable for all the reactor effluents with 7·7–8·3 for pig reactors also at very high loads and 7·5–8·0 for dairy reactors. The methane content was 71–82% for pig manure fed reactors and 75–82% for dairy manure fed reactors.

Microbial community dynamics in reactors fed dairy or pig manure

A DGGE gel with samples from the CA1, CB1, PA1 and PB1 reactors were run to examine the influence of the influent and granular sludge inoculum on microbial communities in the reactors (Fig. S1). Ordination by PcoA based on Bray-Curtis similarities for the microbial communities associated with the influent, the granular inoculum and the reactor slurries (Fig. 3), indicates that the reactor sludge bacterial and archaeal communities

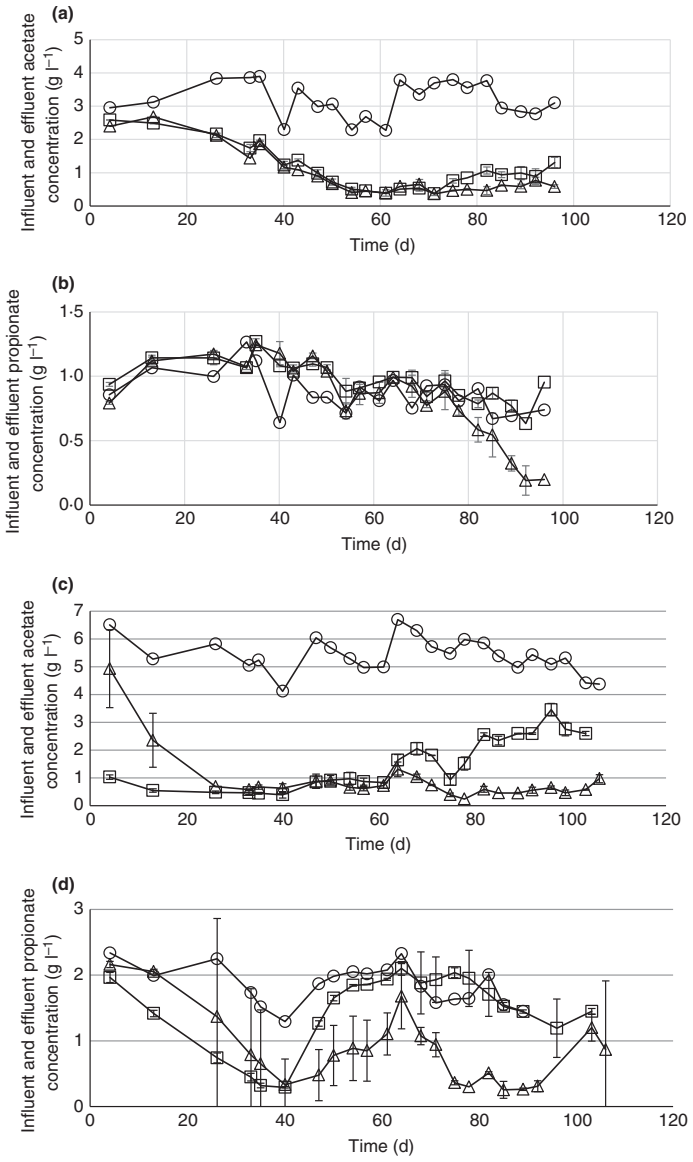

Figure 2 Concentrations and standard deviations in influent (○) and effluent from the dairy manure fed reactors CA (□) and CB (△) and pig manure fed reactors PA (□) and PB (△). Dairy manure acetate (a) and propionate (b), pig manure acetate (c) and propionate (d). Error bars represent standard deviation between parallel reactors.

differed between the reactors run on dairy and pig manure. A PERMANOVA test confirmed this ($P \leq 0{\cdot}0002$).

The microbial communities in the reactor sludge seemed to be more similar to the communities in the influent than those associated with the granular sludge inoculum (Fig. 3). Average Bray-Curtis similarities support this (Table 3). Interestingly, for both bacterial and archaeal communities, the average Bray-Curtis similarities for comparisons of reactor sludge and influent communities were approximately twice those calculated for comparisons of reactor sludge and granular sludge inoculum communities. A *t*-test confirmed that these differences were significant ($P \leq 0{\cdot}0001$). This suggests that the influent had a higher impact on microbial community

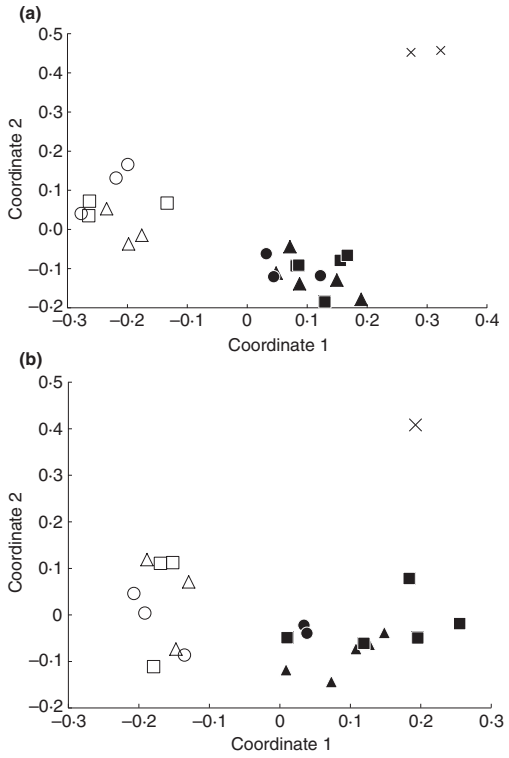

Figure 3 Principal coordinate analysis ordination based on Bray-Curtis similarities for the (a) bacterial and (b) archaeal community profiles of the granular sludge inoculum (×) for the pig manure influent (●) and reactors PA1 (■) and PB1 (▲), and dairy manure influent (○) and reactors CA1 (□) and CB1 (△). Samples from granular inoculum that had been pre-adapted to pig manure (used for the PA reactors only) were not available. Samples were collected from the effluent line of each reactor on day 35, 68 and 96 for the C reactors and on day 35, 61, 68, 96, 103 (PA only) and 105 (PB only) for the P reactors. Samples of both influents were taken on day 35, 68 and 96. The nonadapted granular sludge inoculum was sampled six months and one year prior to the experiment.

composition in the reactors than the granular sludge inoculum, and that the influent is a strong determinant for the reactor microbial communities.

Moreover, the archaeal community profiles were more similar between sample types (influent, reactor sludge and granular inoculum) than the bacterial communities (t-test, $P < 0{\cdot}0001$; Table 3). This was confirmed by determining the average Bray-Curtis similarity for comparisons among all samples, which was found to be significantly higher (t-test, $P = 4^{-28}$) for archaea ($0{\cdot}69 \pm 0{\cdot}11$) than for bacteria ($0{\cdot}56 \pm 0{\cdot}13$). The Shannon's diversity was also more variable across sample types for bacterial than for archaeal communities (data not shown), corroborating the finding that bacterial community structures were more variable than the archaeal ones.

Temporal developments and effects of operational parameters and granular sludge inoculum on the community structure in the reactors running on pig manure supernatant

The PA and PB reactors were run with the same influent under stable and similar conditions except for different granular sludge inoculum (preadapted for PA and nonadapted for PB), and varying OLRs and HRTs (Table 2). To investigate the effect of different granular sludge inoculum and varying OLR on the reactor microbial communities, a DGGE gel (Fig. S2) was run with samples from all the P reactors and all sampling time points. The PcoA ordination based on Bray-Curtis similarities indicate a difference in the microbial community profiles between the PA and PB reactors for both bacteria and archaea (Fig. 4). This was confirmed by a PERMANOVA test ($P < 0{\cdot}01$) and indicates an influence of the granular sludge inoculum on the reactor sludge communities. The PcoA plot further suggests that the bacterial communities (Fig. 4a) of the four reactors were more divergent at the first two sampling dates, but became more similar from day 68 onwards. This trend seemed to be more pronounced for the PB reactors inoculated with nonadapted granules. Average Bray-Curtis similarities for comparisons of bacterial communities between PA and PB reactors were found to be lowest at day 61 ($0{\cdot}52 \pm 0{\cdot}06$) and highest at day 96 ($0{\cdot}78 \pm 0{\cdot}2$). A similar tendency was seen for the archaeal communities (Fig. 4b), with average Bray-Curtis similarities for comparisons of PA and PB communities being lowest at day 61 ($0{\cdot}52 \pm 0{\cdot}01$) and highest at day 96 ($0{\cdot}81 \pm 0{\cdot}1$).

Variance partitioning was used to evaluate the influence of running time, OLR and type of granular sludge inoculum on the variation in microbial community composition in the PA and PB reactors. Only 26 and 31% of the variation in microbial community structure for bacteria and archaea, respectively, was explained by these three variables. However, most of this variance was explained by interaction effects, and they accounted for 58 and 88% of the explained variance in bacterial and archaeal community composition respectively. Running time alone accounted for 7%, the granular sludge inoculum for 4% and the OLR for 2% of the variance of bacterial community structure. The same variables accounted for only 0, 0 and 5%, respectively, of the variance in the archaeal community structure. These three variables thus had small impacts separately. Running time and OLR, for example, explained together 9% of the variance in the bacterial

Table 3 Average Bray-Curtis similarities and standard deviation for comparison of microbial communities in reactor sludge (average of parallel 1 and 2) to those in the granular sludge inoculum and in the influent

	Bacteria		Archaea	
	Dairy (CA, CB)	Pig (PB)*	Dairy (CA, CB)	Pig (PB)*
Reactor sludge *vs* gr. sl. inoculum	0·29 ± 0·04	0·35 ± 0·03	0·44 ± 0·05	0·46 ± 0·03
Reactor sludge *vs* influent	0·66 ± 0·07	0·62 ± 0·07	0·81 ± 0·05	0·79 ± 0·04

*PA was left out due to lack of samples of the pre-adapted inoculum used for the PA reactors.

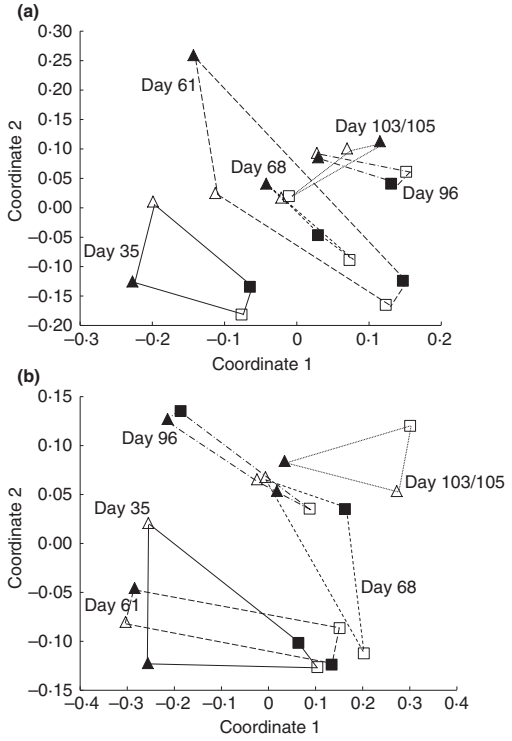

Figure 4 Principal coordinate analysis ordination based on Bray-Curtis similarities for (a) bacterial and (b) archaeal communities in pig manure fed reactors PA1 (■), PA2 (□), PB1 (▲) and PB2 (△).

community while the highest explanatory effect found was on the archaeal community where 21% of the variance was due to the impact of granular sludge inoculum and OLR.

Discussion

The CA and CB reactors needed longer time to stabilize the methane yield, acetate removal and propionate removal than the PA and PB reactors, which may be a consequence of the higher fraction of slowly degradable particles in dairy manure (Table 1). The methane yields obtained at high pig manure OLRs compared to the more particle rich dairy manure also indicates that pig manure supernatant is more suitable than dairy manure filtrate as UASB influent. The yield as NL methane l^{-1} influent is, however, quite similar for dairy and pig manure at the end. The generally low VFA content in the effluents, especially the low propionate concentration, are signs of a well-functioning AD process on dairy manure, even at the relatively high OLR of 27 g COD l^{-1} day^{-1} compared to recommended wastewater UASB loadings of 12–18 g COD l^{-1} day^{-1} (Tchobanoglous *et al.* 2003). Reduced propionate removal, as observed at the highest pig manure loads, can be explained by low growth rate and inhibition due to high levels of acetate and/or hydrogen (Bergland *et al.* 2015). High concentrations of these propionate removal products are thermodynamically unfavourable for propionate reduction (Batstone *et al.* 2002) and can occur during load increase as a stress symptom. The CA performance is comparable to that observed by Rico *et al.* (2011) with 3·1 NL methane l^{-1} dairy manure influent at load 72·5 g COD_T l^{-1} day^{-1}. The CB reactors had the best performance in term of methane yield and propionate removal (Figs 1c and 2b) towards the end of the experiment. This gradual process performance improvement was not reflected as a significant shift in the microbial community, suggesting that there is an alternative explanation. Improved mass transfer in the granular sludge due to morphological changes as an adaptation to particle rich feed is a possibility that cannot be verified the methods applied in this study.

Our findings imply that the influent is a strong determinant for the structure of both bacterial and archaeal reactor communities: First, we found that microbial communities differed significantly between reactors run with dairy and pig manure. Secondly, we saw that the microbial community structure in the reactors were more similar to the community structure in the influent than in the granular sludge inoculum for both bacteria and archaea (Fig. 3, Table 3). The influent can affect the reactor microbial communities both by selection due to its physical and chemical makeup and by its inherent

microbial community that is constantly introduced to the reactors. An experimental strategy comparing the effects of sterile and nonsterile influents on the reactor communities might be helpful for disentangling these two mechanisms. Ziganshin *et al.* (2013) stated that even though the microbial community dynamics may be strongly influenced by the type of inoculum, the influent is still the largest contributor to the microbiota in the reactor sludge, thus corroborating our results.

The bacterial communities in the reactors, influent and granular sludge inoculum were significantly more variable among bacterial samples than archaeal samples (Table 3). In addition, the variations in Shannon's diversity among the microbial communities of the granular sludge inoculum, influent and reactors indicate a more stable community structure for archaea than bacteria. Previous studies of anaerobic digesters report greater diversity within bacterial than archaeal communities in addition to a higher abundance of bacteria (Li *et al.* 2013; Yang *et al.* 2014). This probably reflects that the bacterial communities are characterized by a larger functional redundancy than the archaeal communities. This would allow for more variation in bacterial community structure among samples, as observed in our study. Whole bacterial genera may be substituted when process variables vary, but the niches are not left vacant due to the high diversity of *Bacteria* (Krakat *et al.* 2011; De Schryver and Vadstein 2014). This could explain the higher variability among samples that was observed for bacterial communities (Table 3).

Analysis of the communities in the PA and PB reactors demonstrated that using distinct granular inoculum (pre-adapted or non-adapted) also resulted in significantly different archaeal and bacterial communities. However, the communities developed to become more similar with time (Fig. 4). This development probably reflects adaptation of the microbial communities as a response to similar selective pressure exerted by the influent on the communities in the PA and PB reactors, and indicates a decreasing influence of the original granular sludge inoculum during the first two months of operation. The granular inoculum came from a UASB reactor pulp and paper process wastewater treatment with almost no particles, high C/N ratio and high fraction of easily degradable carbohydrates, which is different from manure. Hence, our results extend the findings in Bergland *et al.* (2015), namely that granular sludge from easily digestible, particle free wastewater treatment can be used for complex, particle rich UASB dairy and pig manure treatment. A longer adaptation period is, however, required for dairy manure than for pig manure.

Even though we found that the inoculum affected the PA and PB reactor microbial communities, particularly at the beginning of the experiment, only a minor part of the variance in community structure among samples throughout the experiment was explained by the inoculum in the variance partitioning analysis. In addition, this analysis showed that only 2 and 5% of the variance in the bacterial and archaeal communities variations observed between samples are explained by the OLR respectively. However, considerable interaction effects were observed, and for the archaeal communities 21% of the variance was explained by granular sludge inoculum and OLR. The OLR affected reactor performance: high OLRs resulted in decreased yield (Fig. 1c) and acetate/propionate removal rates (Fig. 2c, d). The variance partitioning analysis suggests that this is not due to changes in community structure. The relatively small effect of OLR on the microbial communities may be caused by the low HRTs employed in this study. When HRT is lower than the maximum growth rate of the micro-organisms, the planktonic microbiota may be a reflection of the communities associated with the influent more so than the active part of the microbiota that reside in the solid granular sludge phase. Microbes in the granules are expected to be less influenced by the OLR and HRT, although a potential effect on community structure might be mediated by changes in diffusion to the microbes in the granules imposed by the OLR.

Due to the low HRT in our system, most of the biomass in the reactors is present in the granules since planktonic organisms are washed out. The archaea, mainly situated in the deeper layers of granules in UASB reactors (Sekiguchi *et al.* 1999), was expected to be less influenced by OLR than the bacteria which mainly reside in the outer layers, but the opposite was observed. The porosity of biomass aggregates tend to change with OLR (van Loosdrecht *et al.* 2002) so sludge morphology maybe as important as community structure in the adaptation to load changes. This is an interesting topic for further research.

A four years long experiment conducted by Krakat *et al.* (2011) shows that HRT, OLR and the substrate influence the bacterial community but even using beet silage as sole substrate did not give rise to unique community structures linked to process parameters. Fluctuating bacterial communities are rather characteristic to such habitats. Furthermore, Rosa *et al.* (2014) observed differences in the microbial communities in two UASB reactors utilizing a mixture of cassava processing wastewater and glucose that was due to the effect of seed sludge and HRT. The highest explanatory effect found in the present study was the impact of granular sludge inoculum and OLR on the archaeal community while the running time and OLR had the highest impact on the bacterial communities.

HRT was not included in the variance partitioning since the COD concentration in the influent was fairly constant, implying that OLR and HRT are closely coupled variables. The variance partitioning analysis could not explain the majority of the variation in community structure observed throughout the experiment. Temporal changes in the chemical and physical properties of the influent, resulting in altered selective forces in the reactors, could have caused changes in the structure of the microbial communities. Further, stochastic processes, like drift, are assumed to contribute to the structuring of microbial communities (Leibold and McPeek 2006; Hanson *et al.* 2012). Deep sequencing providing information on taxonomy and abundance would contribute to a better understanding of the microbial community dynamics of UASB biogas reactors.

In summary, the UASB fed the more particle rich dairy manure filtrate needed longer time to stabilize than those fed pig manure slurry supernatant. Higher peak production and ability to handle varying loads are additional indications that pig manure slurry supernatant is a more suitable feed for UASB reactors than dairy manure. The yield, measured as methane per litre influent is, however, quite similar for dairy and pig manure at the end, and the generally low VFA content in all reactors at stable loads, imply that UASB treatment is a promising approach for both influents.

The results from the microbial analysis of this study illustrated that the influent is a strong determinant of the microbial community composition in UASB reactors running on dairy manure filtrate or pig manure slurry supernatant. Archaeal communities were found to be less variable over time and between samples than the bacterial communities. The granular inoculum adapted to the manure influents in approximately two months. Non-adapted inoculum may therefore be utilized in the startup of new UASB biogas reactors for dairy or pig manure treatment.

The running time, OLR and granular sludge inoculum explained 26 and 31% of the total variance in the bacterial and archaeal communities respectively. The three parameters had a low impact on the communities separately, but there were considerable interaction effects.

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Conflict of Interest

No conflict of interest is declared.

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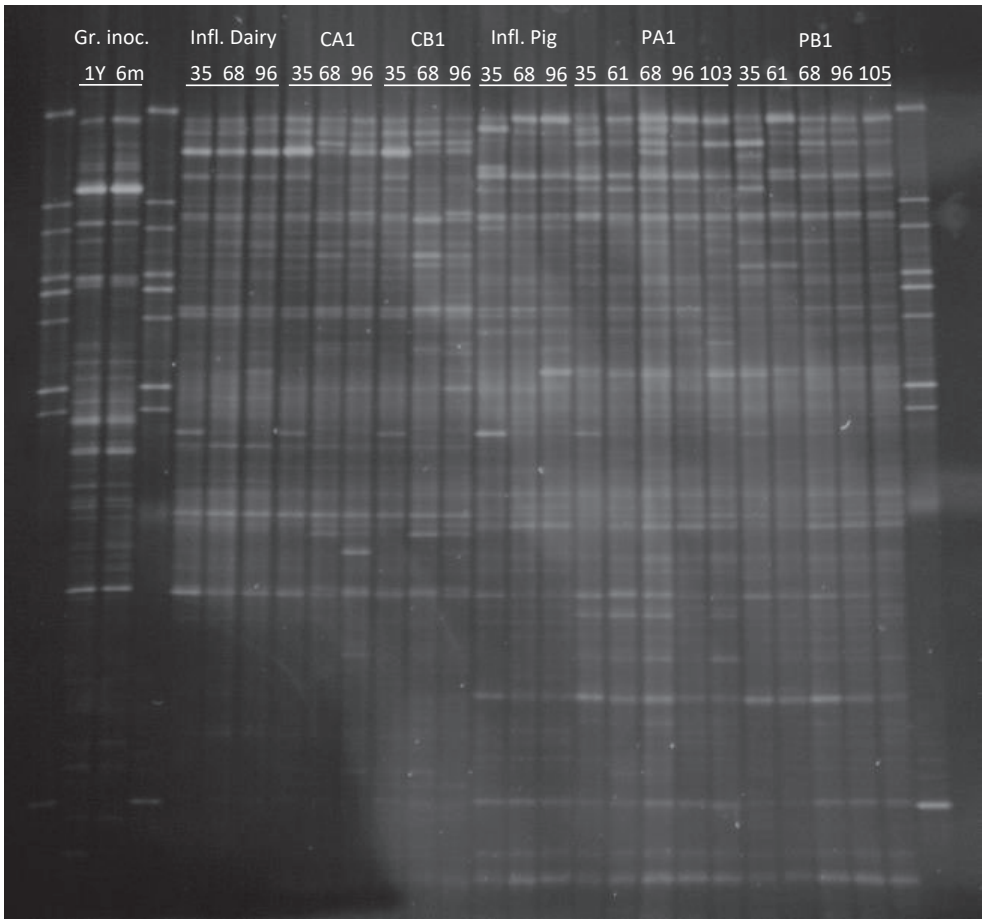
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 DGGE-gel with PCR products obtained with the primer set a) GC-338f/518r targeting bacteria and b) GC-624f/820r targeting archaea.

Figure S2 DGGE-gel with PCR products obtained with the primer set a) GC-338f/518r targeting bacteria and b) GC-624f/820r targeting archaea.

A)


Gr. inoc.
1Y 6m
Infl. Dairy
35 68 96
CA1
35 68 96
CB1
35 68 96
Infl. Pig
35 68 96
PA1
35 61 68 96 103
PB1
35 61 68 96 105

B)

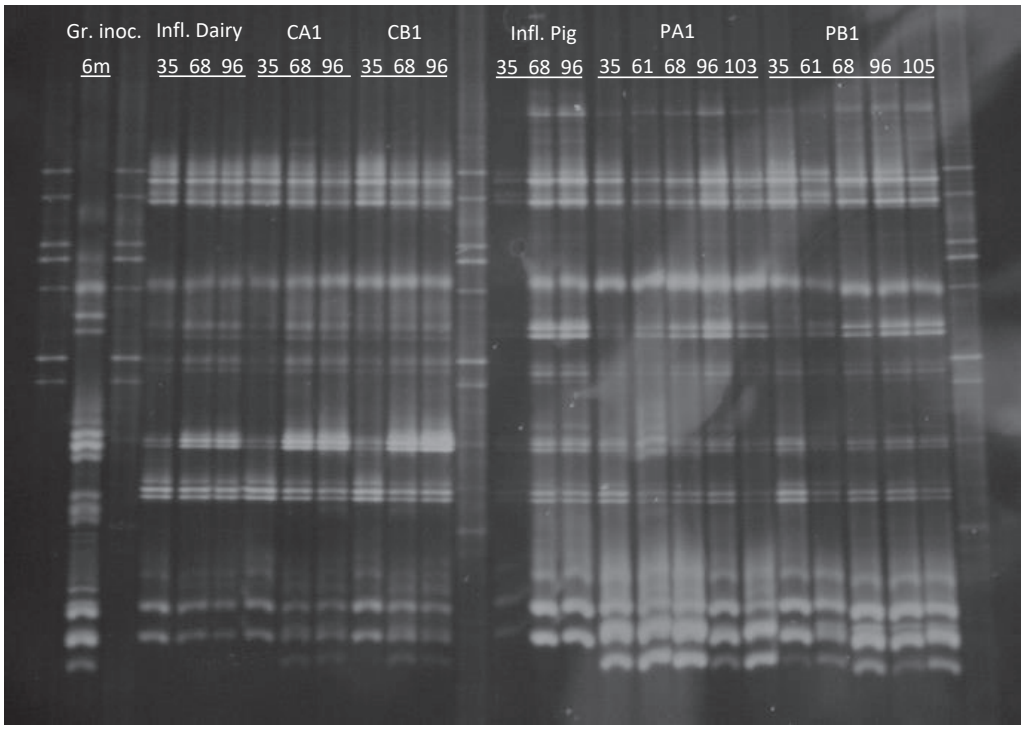
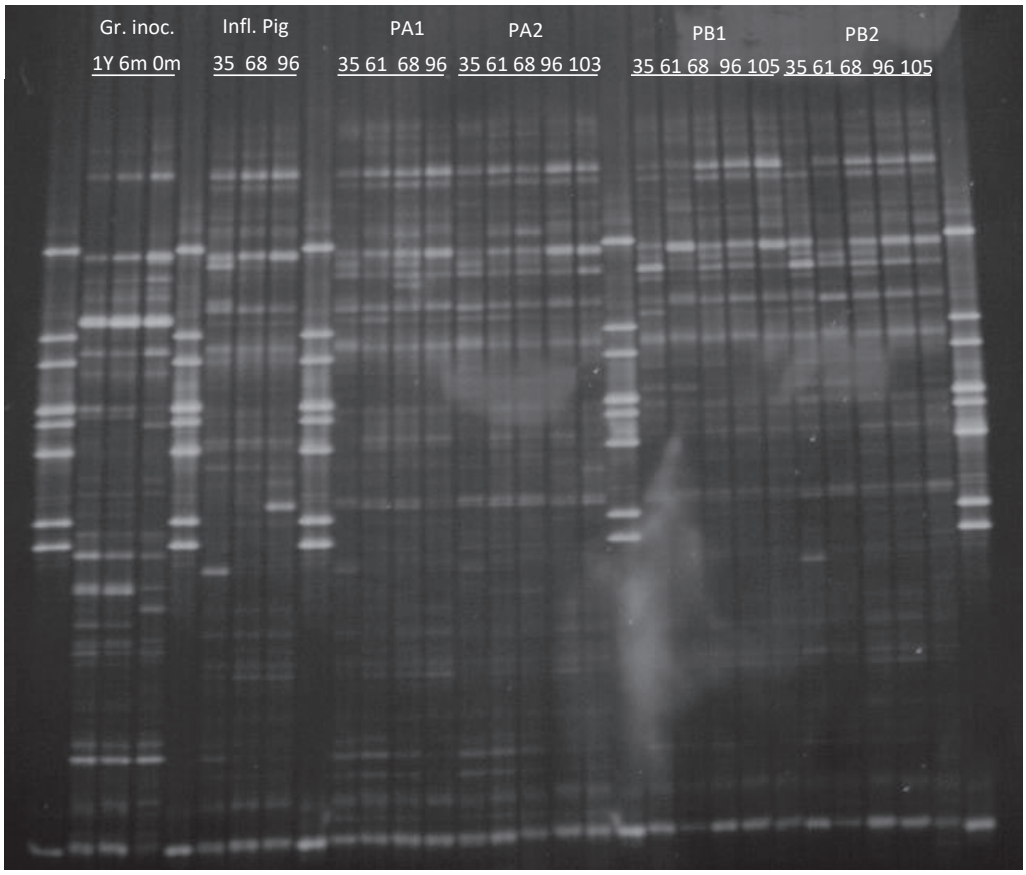


Figure S1: DGGE-gel with PCR products obtained with the primer set a) GC-338f/518r targeting bacteria and b) GC-624f/820r targeting archaea. The samples obtained from the granular sludge inoculum (Gr. inoc) were sampled one year (1Y) and six months (6m) prior to the experiment. The influent of the dairy cow manure filtrate fed reactors (Inf. Dairy), the reactors CA1 and CB1, the influent of the pig manure supernatant fed reactors (Inf. Pig) and the reactors PA1 and PB1 were sampled on day 35, 61, 68, 96, 103 and 105 of the experiment as indicated in the figure.

A)



B)

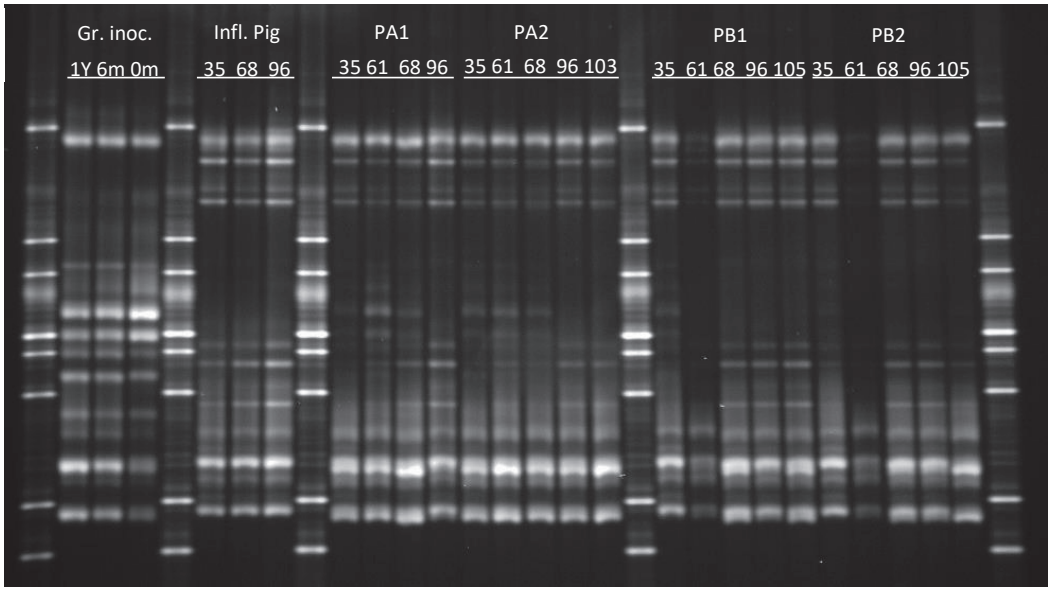


Figure S2: DGGE-gel with PCR products obtained with the primer set a) GC-338f/518r targeting

bacteria and b) GC-624f/820r targeting archaea. The samples obtained from the granular sludge inoculum (Gr. inoc) were sampled one year (1Y) and six months (6m) prior to the experiment and at start up (0m). The influent of the pig manure supernatant fed reactors (Inf. Pig) and the four reactors PA1, 2 and PB1, 2 were sampled on day 35, 61, 68, 96, 103 and 105 of the experiment as indicated in the figure.

Paper III

Anaerobic digestion of pig manure supernatant at high ammonia concentrations characterized by high abundances of *Methanosaeta* and non-euryarchaeotal archaea

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The reactors were operated at University College of Southeast Norway, Porsgrunn, Norway, while the microbial analyses were executed at Norwegian University of Science and Technology, Trondheim, Norway.

Abstract

We examined the effect of ammonium and temperature on methane production in high rate upflow anaerobic sludge bed reactors treating pig manure supernatant. We operated four reactors at two ammonium concentrations ('low' at 1.9, 'high' at 3.7 g L^{-1}, termed LA and HA reactors, respectively) and at variable temperatures over 358 days. Archaeal and bacterial communities were characterized by Illumina sequencing of 16S rRNA amplicons. Ammonium was a major selective factor for bacterial and archaeal community structure. After ~200 days of adaptation to high ammonium levels, acetate and propionate removal and methane production improved substantially in HA reactors. Aceticlastic *Methanosaeta* was abundant and positively correlated to methane yield in the HA reactors, whereas *Methanosarcina* was more abundant in LA reactors. Furthermore, a group of monophyletic OTUs was related to Thaumarchaeota in phylogenetic analysis and highly abundant in the archaeal communities, particularly in the HA reactors. The most abundant bacterial OTU in LA reactors, representing *Syntrophomonadaceae*, was also positively correlated to methane yield in the HA reactors, indicating its importance in methane production under ammonia stress. In conclusion, efficient methane production, involving aceticlastic methanogenesis by *Methanosaeta* took place in the reactors at free ammonia concentrations as high as 1 g L^{-1}.

Introduction

On-farm anaerobic digestion has been suggested as an attractive option for manure treatment, as it reduces greenhouse gas emissions from the agricultural sector, it produces high quality biogas and reduces other environmental impacts such as water pollution and odour emissions [1]. We recently demonstrated that high rate UASB reactors, with hydraulic retention time as low as 2 hours, can be used for anaerobic digestion of supernatant rich in particles from cow and pig manure, and that the process is well suited for on-farm manure treatment [2,3]. Pig manure is ammonia rich. This is a potential problem in anaerobic treatment, as high ammonia concentrations inhibit the methanogenesis and result in poor biogas yields [4,5], even though adaptation of the anaerobic microbial community has been shown to increase the tolerance to ammonia [6,7]. Free ammonia nitrogen (FAN, NH_3) is the component causing the inhibition, and the FAN concentration depends mainly on the total ammonia concentration, pH, and temperature [6]. Acetate is a major intermediate in methanogenesis, and may be converted to methane through two distinct pathways; either to methane and carbon dioxide by the aceticlastic methanogens (*Methanosaeta* and *Methanosarcina*), or to hydrogen and carbon dioxide by syntrophic acetate oxidizing bacteria (SAOB). Hydrogenotrophic methanogens further utilize the hydrogen and carbon dioxide to produce methane [4]. The aceticlastic methanogens are generally considered more sensitive for high ammonia levels than the SAOB and hydrogenotrophic methanogens. At high ammonia concentrations, a switch is therefore expected from aceticlastic methanogenesis to syntrophic acetate oxidation (SAO) and hydrogenotrophic methanogenesis. Recent research indicates that the tolerance to ammonia varies among hydrogenotrophic methanogens and SAOB strains [8]. Also, *Methanosarcina* has

been observed to be rather robust to high ammonia levels in some studies [9-11]. The responses in complex methanogenic consortia to high ammonia concentrations are still poorly understood. Only a few SAOBs have been isolated and characterized so far, including two mesophilic bacteria (*Clostridium ultunense* [12] and *Syntrophaceticus schinkii* [13]), the thermotolerant *Tepidanaerobacter acetatoxydans* [14] and three thermophilic SAOBs (strain AOR [15], *Thermacetogenium phaeum* [16,17] and *Thermotoga lettingae* [18]). The significance of these strains in methane producing anaerobic communities is not well described, and characterization of microbial communities by metagenomic approaches has suggested that also other SAOBs may exist in anaerobic biogas reactors [19,20].

In the present study, we examined how temperature and ammonia concentrations affected the performance of high rate UASB reactors treating pig manure slurry supernatant. We operated four reactors at two ammonia concentrations and at variable temperatures over a period of 358 days, and investigated effects on the methane production. For two of the reactors, the free ammonia concentrations reached as high as 1 g L^{-1}. We characterized the bacterial and archaeal communities using Illumina sequencing of 16S rDNA amplicons, and aimed at identifying key players in methanogenesis at high ammonia concentrations.

Results

Reactor performance

A total of four laboratory scale upflow anaerobic sludge bed (UASB) reactors were studied for 358 days. The granules used as inoculum originated from a UASB reactor treating pulp and paper process wastewater. Whereas two of the reactors (LA1 and LA2; low ammonia concentration reactors) were fed untreated pig manure slurry supernatant throughout the experiment, the other two (HA1 and HA2; high ammonia concentration reactors) received manure slurry supernatant that had been added urea to increase the ammonia concentration in the reactors from day 69. The reactors produced biogas from the first day. Addition of urea to the feed for the HA reactors from day 69 resulted in a 2-fold increase in TAN and 20-fold increase in the FAN concentrations into the HA reactors compared to the LA reactors (Table 1). This, thereby, caused an approximately 2-fold increase in TAN and 6-fold increase in the FAN concentrations in the HA reactors compared to the LA reactors (Table S2). The average TAN concentrations were 3.7±0.2 and 1.9±0.1 g NH_4^+-N L^{-1} for the HA and LA reactors, respectively, whereas average FAN concentrations were 0.8±0.2 and 0.14±0.10 g NH_3-N L^{-1} for the HA and LA reactors, respectively (Table S2). This resulted in a 10-fold drop of the methane yield from the HA reactors around day 100 (Fig. 1B). The yield in the LA reactors increased from 2.0 NL methane L^{-1} substrate to 3.7 during the same period.

The COD_T removal became almost negligible (6%) and acetate accumulated in the HA reactors after urea addition (Fig. 1). Simultaneously, the COD_T removal increased to 49% in the LA reactors (Fig. 1A), and acetate concentration in the effluent decreased (Fig. 1C). The methane yield in the HA reactors increased slightly during the first 77 days of constant urea addition and stabilized at 0.53±0.1 NL methane L^{-1} substrate during the next 150 days (*i.e.* days 146-

296; Fig. 1B). This was accompanied by a slight increase in COD_T removal (from approx. 14 to 18%) and a modest decrease in acetate concentration (Fig. 1). In the LA reactors, the biogas yield was seven times higher during the same 150 days period (3.7±0.8 NL methane L^{-1} substrate), but with fluctuations due to different feed batches collected at the farm. Data from days 161-215 are unreliable because biogas was produced in the feed line due to high ambient temperatures and insufficient feed cooling. COD_T removal was 54±6% and the acetate conversion was almost complete, with a drop in concentration from 0.5 g L^{-1} on day 146 to 0.2 on day 296.

The imposed temperature reductions from 35 to 25°C during the period from day 119 to 146 were accompanied by a 50 % decrease in biogas production in the LA reactors, indicating a positive effect of operation at 35°C. The methane yield did not, however, increase again towards the end of the experiment when the temperature was increased back to 35°C, implying that the observed yield variations in the LA case are independent of temperature. This is supported by the quite constant and low VFA levels (Fig.1C and D) after the start-up phase, independent of temperature levels. The observed yield variations and variations in COD_T removal are therefore attributed to variations in feed composition, to be expected since the feed was fresh pig manure from a barn with mainly farrows and wieners rearing [21].

Towards the end of the experiment, the methane production, COD_T, propionate, and acetate removal increased substantially in the HA reactors, and methane yields of around 1.8 NL methane L^{-1} substrate, and a COD_T removal of almost 60% was obtained (Fig. 1). This coincided with the temperature increase from 30°C to 35°C during days 296-322 (Fig. 1). This increase in methane yield cannot be explained by a temperature induced increase in growth rate alone [22], indicating an adaption of the methanogenic consortium to the high ammonia concentration.

Richness and diversity of microbial communities

Bacterial and archaeal communities were examined using 16S rDNA primer pairs, and the resulting sequencing data were analyzed separately. A total of 672 907 and 1 318 803 reads were obtained with the bacterial and archaeal primers, respectively, after quality filtering and chimera removal (Table S1). OTU clustering and taxonomy assignment revealed that 22 of 2049 OTUs in the bacterial dataset belonged to Archaea and 820 of 923 OTUs in the archaeal dataset belonged to Bacteria. These were removed, and the OTU tables subsequently yielded 2027 bacterial and 103 archaeal OTUs. The primers were designed to target highly conserved regions of the 16S rRNA bacterial and archaeal gene to maximize coverage and this probably resulted in reduced domain specificity. Comparisons of estimated richness (Chao1) and observed OTUs demonstrated that the sequencing effort across samples on average covered 70% and 92% of the estimated bacterial and archaeal richness, respectively. Thus, despite the high abundance of bacterial sequences in the archaeal data set, the diversity of the archaeal communities was well reflected at the obtained sequencing depth.

The Chao1 richness was around tenfold higher for bacterial than archaeal communities (Fig. 2A). Also, the Shannon's diversity index was considerably higher for bacterial than archaeal communities (Fig. 2B). Increased ammonia concentration appeared to have opposite effect on

the diversity of bacterial and archaeal communities: For bacteria, the Shannon's diversity index was generally highest for the LA reactors, while for archaea, the HA reactor communities were more diverse (Fig. 2B).

Overview of microbial community composition

Firmicutes was the most abundant bacterial phylum in all samples, and accounted for almost half of the sequence reads (44.8±9.1%). A major fraction of these reads was classified as Clostridia at class level (Fig. 3A). In addition, the phyla Bacteroidetes, Cloroflexi, Synergistetes, Aminicenantes, Cloacimonetes, Actinobacteria, and Proteobacteria were observed in all reactor samples (Fig. 3A). A large number of OTUs was only classified as Bacteria or not even at domain level. Some of them were abundant, and they accounted for as much as 19.1±8.2% on average of the reads. The most abundant of these OTUs (OTU_7) was suggested to represent Hydrogenedentes by UTAX and RDP Classifier, although with very low confidence scores (0.12 and 0.21, respectively). This OTU was particularly abundant in LA reactor samples, and accounted for as much as 37% of the reads in reactor LA2 at day 114. Other abundant unclassified OTUs were suggested to represent Clostridiales and Chloroflexi by both UTAX and RDP Classifier, but again with very low confidence scores.

The archaeal communities were generally dominated by Euryarchaeotal taxa such as *Methanosarcinaceae*, *Methanosaeta,* and *Methanobacterium*, but the abundance clearly varied according to the ammonium concentration in the reactors (Fig. 3B). Crenarchaeota was observed in all samples, but never exceeded 1% of the reads in any of the samples. OTUs that could not be taxonomically assigned were highly abundant, particularly in the HA reactor samples (on average approximately 31 and 18% of the reads in HA and LA samples, respectively). Most of these reads were accounted for by 13 OTUs, which were suggested to represent Thaumarchaeota by the Utax classifier and Thermoprotei (Crenarchaeota) by the RDP Classifier, but with very low confidence scores. In sum, these 13 OTUs were abundant, particularly in the HA reactor samples, where they accounted for on average 28 ±6% of the reads (from day 114, Fig. S1). We investigated these OTUs further by maximum likelihood analysis including 16S rDNA sequences classified as Thaumarchaeota, Thermoprotei, and Euryarchaeota (downloaded from the Ribosomal Database project, Fig. 4). The resulting phylogenetic tree indicated that these OTUs constituted a monophyletic clade, closer related to Thaumarchaeota than Thermoprotei (Fig. 4).

Influence of operational conditions on community structure.

A canonical correspondence analyses (CCA) was performed to examine the influence of environmental variables (temperature, TAN, and acetate concentration) on microbial community structure. TAN concentration obviously structured both bacterial and archaeal communities (Fig. 5). The CCA plot further suggested that TAN and acetate concentrations seems to play relatively equal roles in structuring the bacterial and archaeal communities, while temperature had less impact (Fig. 5).

Indeed, both bacterial and archaeal communities differed significantly between LA and HA reactors after day 114 (PERMANOVA, $p<0.0001$). Whereas bacterial communities in LA and

HA reactors appeared to diverge from each other already at day 114 (Fig. 5A), the archaeal HA and LA communities seemed to diverge later (from day 230; Fig. 5B). This indicates a slower response to the increased TAN concentration for the archaeal communities. SIMPER analysis for archaeal communities from day 230 and onwards showed that high abundance of the *Methanosarcinaceae* OTU_1 in LA (49.6% of the reads on average), and high abundance of the *Methanosaeta* OTU_2 in HA reactors (19.8% of the reads on average), explained as much as 46% of the Bray-Curtis dissimilarity between LA and HA archaeal communities (Table 2). The representative sequence of OTU_1 (*Methanosarcinaceae*) was classified as *Methanosarcina* by the RDP Classifier with high confidence, and the representative sequence for OTU_2 (*Methanosaeta*) was 100% identical to the 16S rRNA gene sequence of the type strain *Methanosaeta soehngenii* (S004071134), an obligate aceticlastic archaea. This indicates that *Methanosaeta*, but not *Methanosarcina*, was able to adapt to very high ammonia concentrations. OTU_1 and OTU_2 were the by far most abundant *Methanosarcina* and *Methanosaeta* OTUs, respectively, in the data set. The second most abundant *Methanosaeta* and *Methanosarcina* OTUs did not appear to be affected by the increased TAN concentration, but their abundances were much lower than for OTU_1 and OTU_2 (Fig. S2 and S3). Furthermore, three OTUs included in the non-methanogenic OTU cluster (see above), and two OTUs representing the hydrogenotrophic genera *Methanobacterium* and *Methanoculleus* were more abundant in the HA reactors (SIMPER, Table 2).

The methane yield in the HA reactors were low until it started increasing from around day 300 (Fig. 1A), and the OTUs identified in SIMPER analysis as important differences between the HA and LA archaeal communities were not necessarily responsible for the increased methane production towards the end of the experiment. Spearman correlation analysis, including only HA reactor samples from day 230 to 347, identified a strong negative correlation between acetate concentration and biogas yield in the HA reactors ($p=2\cdot10^{-9}$). Interestingly, the *Methanosaeta* OTU_2 identified in the SIMPER analysis (Table 2) was one of four archaeal OTUs that were positively correlated to methane yield ($p=0.01$). Furthermore, three OTUs representing hydrogenotrophic methanogens (*Methanoculleus* OTU_10, *Methanobacteriaceae* OTU_57, and *Methanobrevibacter* OTU_1142, see Table S3) were positively correlated with methane yield ($p<0.05$). Abundances of these OTUs are given for all reactor samples in Table S3.

The archaeal communities developed differently in the LA1 and LA2 reactors, even though they were operated under the same conditions and had similar methane yields. The most striking difference was the presence of an OTU representing *Methanospirillum* in LA1 in abundances up to 36% (Fig. 2B). In LA2, *Methanospirillum* never exceeded 1.6%, while in the HA reactors, it was barely detected (<0.006%). The increase of *Methanospirillum* in LA1 coincided with a lower abundance of unclassified OTUs compared to the other reactors (Fig. 3B).

For the bacterial communities, an OTUs representing *Syntrophomonadaceae* (OTU_9) was the most abundant one in the LA reactor samples, accounting for on average around 9% of the reads from day 230 and onwards. The second most abundant bacterial OTU (OTU_7; Table 2) in the LA reactors was one of the unclassified OTUs that might represent Hydrogenedents (see

above). This OTU was very rare in the HA reactor communities (Table 2). In the HA reactors, 3 bacterial OTUs, representing *Clostridiaceae_1*, Anaerolinea, and Bacteroidetes (OTU_1, OTU_2, and OTU_3, respectively; see Table 2), accounted for as much as 40% of the bacterial reads on average (from day 230). The RDP Classifier tool classified OTU_3 as *Clostridium sensu stricto* (confidence threshold 0.95). These were the most abundant bacterial OTUs (OTUs 1, 2, 3, 7, and 9), and also contributed most to the Bray-Curtis dissimilarity between the HA and LA reactor bacterial communities (SIMPER, Table 2).

To identify bacterial OTUs associated with the increased methanogenic activity in the HA reactors towards the end of the experiment, we performed a Spearman correlation analysis (including the HA reactor samples from day 230 to 347). Three bacterial OTUs, representing *Syntrophomonadaceae* (OTU 9; Table 2), Synergistia, and Cloacimonetes, were positively correlated to methane yield. The abundance of the *Syntrophomonadaceae* OTU increased in HA reactors towards the end of the experiment, but never reached the same abundance in the HA as in the LA reactor samples (on average 2.5 and 11.4% of the reads for the HA and LA samples, respectively; Table 2). Of these three OTUs, only the Cloacimonetes OTU was more abundant in HA than LA reactors, though it never accounted for more than 0.59% of the reads in any of the HA communities. Abundances for these bacterial OTUs are given for all reactors samples in Table S3.

We examined the bacterial OTU table for the presence of genera including previously described syntrophic acetate oxidizing bacteria (SAOBs). The bacterial OTU table contained four OTUs classified as *Syntrophaceticus* or *Tepidanaerobacter*. These genera include the SAOBs *S. schinkii* and *T. acetatoxydans*. One of the *Tepidanaerobacter* OTUs was highly similar (98% of 402 nucleotide positions) to the *T. acetatoxydans* 16S rDNA sequence (GenBank accession number HF563609). The highest abundance for this OTU was found in the HA1 reactor at day 347, where it accounted for 0.16% of the reads, but it was rare in all other samples (<0.08%). One of the *Syntrophaceticus* OTUs was identical to the *S. schinkii* type strain 16S rDNA sequence (accession number EU386162). However, it was rarely observed in the HA reactor communities (maximum 0.01% of the reads), and was more abundant in reactor LA2, but never exceeded 0.09% of the reads in any of the samples. Furthermore, we searched our data set for OTUs with high similarity to the 16S rRNA sequence of the SAOB identified by Frank et al.[19], but observed maximum 79% sequence identity. Hence, we found no indications that previously described SAOBs were associated with the methane production in the HA reactors.

Discussion

Urea was added to the feed to the HA reactors from experimental day 69 to increase the TAN concentrations. Due to the lack of pH control, the pH increased to around 8.5, resulting in very high concentrations of free ammonia (around 0.83 g L^{-1} in HA reactors, Table S2). After a dramatic decrease in the methane yield on the HA reactors, the methane production stabilized at around 0.53 ± 0.1 NL methane L^{-1} in the period from experimental day 146 to 296 (Fig. 1). This increased methane production coincided with the 10 ℃ reduction in temperature (experimental day 119-146). This may be due to ammonia inhibition being less severe at lower temperatures, as previously described by Rajagopal, et al. [23] and quantified as an inhibition factor in the anaerobic digestion model1 (ADM1) by Batstone, et al. [24]. The NH_3 inhibition factor, using TAN = 3554 g L^{-1} and pH = 8.55 for this case, is 0.03 at 35 °C and 0.05 at 25 °C, where zero is complete inhibition and 1 implies no inhibition, so in this case it should be almost completely inhibited. The difference is a result of the acid dissociation constant K_a changing with temperature. The temperature effect on inhibition is evidently slightly larger than the effect on growth rate since biogas production increased when the temperature decreased (Fig. 1A). Both acetate and propionate removal was poor, and tended to accumulate in the HA reactors in the period from around day 100 to 236, indicating inhibition of acetate and propionate oxidation. Both acetate and propionate accumulations are indicators of process instability [25,26].

The strong increase in biogas production (approximately 200%) observed in the HA reactors towards the end of the experiment coincided with a temperature increase from 25 to 35 °C. However, this large increase in methane yield cannot be explained by a temperature induced growth rate increase alone, according to estimates done by Henze and Harremoes [22]. Acetate and propionate removal improved considerable from around day 300, at FAN concentrations as high as 1 g L^{-1}. Thus, the ammonia inhibition appeared to be much reduced at the end after around 200 days of adaptation, allowing methane production at a rather extreme FAN level.

The LA reactors produced methane at relatively high yields throughout the experiment. Effects of mesophilic temperature variations between 25 and 35 °C on the methane production was examined. Previous studies on this question have shown discordant results; some report a positive correlation between temperature and methane yield, some find no temperature effects, while yet others demonstrated negative correlations, probably due to increased levels of free ammonia [27]. Here, we found a reduction in methane yield accompanying the temperature reduction from 35 and 25 °C in the period from day 119 to 146. However, the temperature increase from day 296 until the end of the experiment did not result in increased methane yield. During this period, because nearly all acetate and propionate was converted, the methane yield was probably close to maximum, and could not be improved further.

Diversity is generally found to be higher for Bacteria than Archaea in anaerobic digestion [28]. Here we found that the OTU richness was as much as 10-fold higher for bacterial compared to archaeal communities. Still, we found relatively high archaeal diversity, with the presence of around 10 relatively abundant methanogenic genera (Fig. 3B). Hagen, et al. [29] found that the

archaeal communities in biogas reactors operated at relatively high FAN concentrations were almost completely dominated by *Methanothermobacter*, with a minor fraction of *Methanosaeta* (around 1% of the reads). Also Ziganshina, et al. [30] found that the archaeal communities in biogas reactors with high TAN concentrations were dominated by a few genera; *Methanosarcina* accounted for around 80-90% of the archaeal reads, while *Methanoculleus* accounted for 10-16%. We aimed at designing broad coverage archaeal 16S rRNA PCR primers, and apparently succeeded in obtaining a detailed characterization of the archaeal communities. It was also interesting to note that the archaeal communities appeared to become more diverse in response to increased TAN concentrations (measured as Shannon's diversity index, Fig. 2B). This is surprising, because environmental disturbances previously has been found to reduce microbial community diversity and functional stability [31].

We identified a cluster of unclassified, non-euryarchaeotal OTUs that were abundant in all samples, but particularly in the HA reactors. Here, they accounted for as much as around 30% of the archaeal reads for most samples. In phylogenetic analysis, these OTUs clustered together in a distinctive group, related to Nitrosopumilales and Nitrososphaerales (Thaumarchaeota, Fig. 4), which include ammonia oxidizing archaea (AOA). These non-euryarchaeal OTUs were obviously ammonia tolerant. However, as part of an anaerobic consortium, they were not likely to oxidize ammonia. The AOAs are generally aerobic, although some appear to thrive at low oxygen concentrations [32,33]. Archaeal taxa that do not represent described methanogens classified as Euryarchaeota have previously been observed in biogas reactors, and has often been referred to as Crenarchaeota [34,35]. Chen and He [36] performed recently a phylogenetic analysis of previously published sequences that had been assumed to represent non-methanogenic archaea in anaerobic reactors. They found that they constituted a single phylogenetic clade related to, but distinct from, Thaumarchaeota, similar to the OTU cluster identified in our study. Chen and He[36] referred to these as non-methanogenic archaea (NMA), and a more detailed analysis suggested that they were placed within the Group I.3 archaeal lineage, together with uncharacterized archaeal populations found in environments like flooded soils and sediments [36]. Chen and He[36] performed batch experiments and demonstrated that acetate promoted growth of NMA (compared to formate, propionate, butyrate, and methanol), but still their relative abundance of NMA was low (around 1%) compared to the aceticlastic methanogens (relative abundances of around 80%). Recently, methanogenic archaea not classified as Euryarchaeota have been identified. Vanwonterghem, et al. [37] described the existence of divergent methylcoenzyme M reductase genes recovered from anoxic environments, and suggested these genomes represented a new phylum, *Candidatus* Verstraetearchaeota. Moreover, Evans, et al. [38] recovered two genomes belonging to the Bathyarchaeota from a deep aquifer containing functional genes needed for methylotrophic methanogenesis. More studies are needed to map the phylogenetic distribution of methanogens among Archaea. We cannot exclude the possibility that the abundant non-euryarchaeal OTU cluster identified in our samples may represent methanogens.

Although we found the highest abundances of the non-euryarchaeotal OTU cluster in the HA reactors, it was generally abundant, accounting for around 15–30% of the reads for most samples (Fig. S1). The noteworthy exceptions were the LA1 samples, which were characterized

by a high abundance of *Methanospirillum* which coincided with a decrease in abundance of the non-euryarchaeotal OTU cluster. Two described *Methanospirillum* species, *M. hungatei* [39] and *M. stamsii* [40], are strict anaerobic, hydrogenotrophic methanogens, utilizing hydrogen or formate and CO_2 for methanogenesis. *M. stamsii* require acetate for carbon supply. One possible interpretation of these observations is that *Methanospirillum* and the non-euryarchaeotal OTU cluster compete in the same niche. The abundances of *Methanospirillum* and the non-euryarchaeotal OTU cluster abundances differed considerably between the LA reactors, although they had been operated at the same conditions and had similar methane yields. This may indicate the existence of functional redundancy and a possible influence of random processes in the methanogenic community assembly.

Previous studies indicate that stressful conditions, such as increased ammonia levels, favor hydrogenotrophic methanogens and a shift from aceticlastic methanogenesis to bacterial syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis [4,28]. However, Fotidis, et al. [41] observed no shift to SAO after exposure of a mesophilic, acclimated methanogenic community to ammonium at high concentrations (TAN of 7 g L^{-1}, but considerable lower FAN concentrations), but found that the aceticlastic *Methanosarcina* was the dominant methanogen. Generally, *Methanosarcina* has been found to be more robust to TAN concentrations up to 4–6 g L^{-1} than *Methanosaeta* [9-11]. This has been explained by the fact that *Methanosarcina* cells form clusters, which may provide protection by reducing the diffusion of ammonia into the cells [42].

Surprisingly, we found that a *Methanosaeta* OTU, with a 16S rRNA gene sequence identical to the type strain of the obligate aceticlastic *Methanosaeta soehngenii*, was positively correlated to methane yield in the HA reactors towards the end of the experiment. It was relatively abundant in the HA reactors throughout the experiment, but increased towards the end (Fig. S2). This is a strong indication that *Methanosaeta* can perform acetate conversion even at FAN level around 1 g L^{-1}. In contrast to the studies referred to above, we found that *Methanosaeta* was more abundant than *Methanosarcina* at high FAN concentrations, and *Methanosarcina* levels dropped markedly, from around 60% to below 10% of the reads from around day 282 (Fig. S3). Calli, et al. [9] observed that the *Methanosarcina* cell clusters disintegrated at FAN levels above 0.6 g L^{-1}. Thus, *Methanosarcina* might not be protected by clustered growth at FAN levels as high as in the HA reactors. Loss of *Methanosaeta* activity at FAN over 100 mg/L has been associated with the loss of filamentous growth [42]. A possible explanation for the apparent high ammonia tolerance we observed for *Methanosaeta* in this study could be protection obtained by growing in aggregates with other microbes in the sludge granules. Zheng, et al. [43] found that *Methanosaeta concilii* was positioned in the core of sludge granules, enclosed by syntrophic consortia, and with filamentous bacteria in the surface layer.

We were not able to identify previously described SAOB in the bacterial communities in our data set, but lately new SAOBs have been identified [19,20] and we cannot exclude the existence of SAOB in the methanogenic communities examined here.

In the LA reactors, the most abundant OTU represented *Syntrophomonadaceae*. It increased in all reactors (both LA and HA) throughout the experiment (Table S3) and was positively

correlated with methane yield in the HA reactors. This indicates that *Syntrophomonadaceae* was probably important for efficient methane production. Members of the *Syntrophomonadaceae* perform β-oxidation of carboxylic acids of four carbons or more, typically butyrate, and produce acetate and H_2 in a reaction which is thermodynamically favorable only when H_2 is maintained at low concentrations by syntrophic partners like hydrogenotrophic methanogens [44]. The second most abundant OTU (OTU_7) in the LA reactors could only be classified at the domain level, but might be related to Hydrogenedentes (see Results). A recent study [45] suggests Hydrogenedentes may be involved in syntrophic degradation of glycerol and lipids in anaerobic consortia, but whether this is relevant for the functional role of OTU_7 is not known. OTUs representing *Clostridiaceae_1* (probably *Clostridium sensu stricto*), Anaerolinae, and Bacteroidetes dominated the bacterial communities in the HA reactors, accounting for almost 40% of the bacterial reads on average after Day 230. *Clostridium* species are known to produce hydrogen through the fermentation of organic substrates [46]. Bacteroidetes are able to ferment polysaccharides, and has been suggested to be involved in hydrolysis of cellulose in anaerobic digestion [47]. Anaerolineae has been found to be abundant in anaerobic digestive communities previously [48,49]. Some Anaerolineae species degrade carbohydrates in cooperation with hydrogenotrophic methanogens, and have been described as "semi-syntrophic" [50]. It was interesting to note that a bacterial OTU representing Cloacimonetes, although at low abundances (OTU_95, Table S3), was positively correlated to methane yield in the HA reactors. The increase in methane coincided with increased propionate removal. Cloacimonetes includes strains able to syntrophically oxidize propionate, such as Candidatus *Cloacamonas acidaminovorans* [51]. We inspected the OTU table for taxa previously known to include syntrophic propionate oxidizing bacteria, such as *Syntrophobacter* and *Desulfobulbus* (Betaproteobacteria), *Pelotomaculum* and *Desulfotomaculum* (*Peptococcaceae*, Clostridia), and Atribacteria [52-55]. However, OTUs representing these taxa were generally rare in all reactor samples. Thus, we were not able to suggest other likely candidates for the increased propionate removal in the HA reactors towards the end of the experiment.

In summary, ammonia proved to be a major selective factor of bacterial and archaeal community structure. TAN concentrations of 3.7 g L^{-1} (FAN around 0.8 g L^{-1}) inhibited methanogenesis in the HA (high ammonia) UASB reactors fed particle rich pig manure supernatant. After almost 200 days of adaptation, acetate and propionate removal and methane production improved substantially. Abundant archaeal and bacterial OTUs, accounting for a large fraction of the reads obtained by Illumina sequencing of 16S rRNA amplicons, could not be taxonomically assigned below domain level. This illustrates that abundant members of anaerobic digestive consortia remain to be described. Phylogenetic analysis suggested that a group of such archaeal OTUs, highly abundant at high TAN concentrations, constituted a monophyletic group related to Thaumarchaeota. Previously described syntrophic acetate oxidizing bacteria were only observed sporadically and at low abundances in the HA reactors. The most abundant bacterial OTU in LA reactors represented *Syntrophomonadaceae*. This OTU was positively correlated to methane yield in the HA reactors, indicating that it was

important for efficient methane production under ammonia stress. *Methanosaeta* was abundant, and was positively correlated to methane yield in the HA reactors, whereas *Methanosarcina* was more abundant in the LA reactors operated at TAN concentrations around 1.9 g L^{-1}. In conclusion, efficient methane production, probably involving aceticlastic methanogenesis mediated by *Methanosaeta*, took place in the UASB reactors at FAN concentrations as high as 1 g L^{-1}.

Material and methods

Reactor influent and inoculum. The manure substrate was collected from a pig production farm in Porsgrunn, Norway. Pig manure handling has been described previously [2] while the substrate properties are given in Table 1. After collection, the manure substrate was stored at 4 °C until use. A total of four laboratory scale reactors were studied for 358 days. Whereas two of the reactors (LA1 and LA2; low ammonia concentration) were fed untreated pig manure slurry supernatant throughout the experiment, the other two (HA1 and HA2; high ammonia concentration) received manure slurry supernatant that had been added urea (4 g L^{-1}) to increase the ammonia concentration in the reactors during experimental days 69-358. Increasing amounts of urea were added during days 69-107, and from day 107 4 g L^{-1} urea was added to the HA substrate for the rest of the experiment. This resulted in increased total ammonia nitrogen (TAN) concentrations in the substrate for HA reactors (3.7±0.2 g NH_4-N L^{-1}) compared to that for the LA reactors (1.9±0.2 g NH_4-N L^{-1}) (Table 1). The pH of the untreated supernatant was 7.6±0.2 during the whole period, while the pH increased to 8.7±0.1 in the feed added urea. Due to the lack of pH control, the addition of urea resulted in an approximately 20 fold increase in the concentration of free ammonia nitrogen (FAN) in the supernatant fed to the HA reactors, reaching as high as 1.2±0.3 g NH_3-N L^{-1} (Table 1).

The granules used as inoculum originated from a UASB reactor treating pulp and paper process wastewater at "Norske Skog Saugbrugs" in Halden, Norway [2]. The granular inoculum had been stored at 11 °C prior to the experiment. The reactors were filled with approximately 180 ml of granules at the start of the experiment. Suspended solids and granules were separated inside the reactors to retain biomass.

Reactor design and operation. Lab-scale process lines were set up utilizing identical pulse fed 370 mL UASB reactors with a liquid volume of 360 mL. The reactor design and measurements of COD_T (total COD), COD_S (soluble COD), pH, VFA, NH_4^+-N, gas composition and methane production has been described previously [2]. The reactors were all operated at HRT 1.0 day. The organic loading rate (OLR) was 16±2 g COD L^{-1} d^{-1} for the entire experiment. The reactors were fed intermittently, 25 mL each time with 14 feedings for each HRT. It is therefore reasonable to assume continuous flow in the mass balance analysis of the process.

The reactors were operated at 35 °C, 30 °C and 25 °C during the following periods: 35 °C from startup; 30 °C during days 119-146; 25 °C at days 146-296; 30 °C at days 296-322 and 35 °C again during days 322-358. Unintentionally the substrate was heated up in the "sand trap" separator before reaching the reactor at days 161-215 due to unusual high summer temperatures. Afterwards this substrate line separator was kept at 5-15 °C by cold water.

Sampling, DNA extraction and PCR amplification. Samples were collected from each reactor on the following days of the experiment; 69, 114, 156, 230, 282, 316, 321, 341 and 347. The reactors were stirred before sampling from the effluent to ensure a homogenous sample. The samples were kept frozen during the experiment and thawed prior to DNA extraction. The liquid phase was removed as follows: Samples were centrifuged at 200 g for 10 minutes, the supernatant was discarded and the pellet resuspended in phosphate buffer saline (PBS, 1x). This was repeated twice before centrifugation at 4000 g for 10 minutes to remove liquid before DNA extraction. Granules were sampled from reactor HA1 and LA1 on day 341 by picking granules with forceps. They were washed with PBS prior to DNA extraction. The granular sludge inoculum was sampled at the start of the experiment.

Total DNA was extracted using the Power Soil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA) as described by the manufacturer. DNA concentration was measured by NanoDrop Spectrometer ND-1000 (NanoDrop Technologies, USA). PCR primers targeting 16S rDNA in both bacteria and archaea were designed to target conserved regions. Coverage was optimized by using alignments of sequences downloaded from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu) and the RDP tool Probematch [56]. The resulting primers amplified the v3-4 region of the 16S rRNA gene; B-338F (5'-CCTACGGGWGGCAGCAG) and B-805R (5'-GACTACNVGGGTATCTAAKCC) amplifying 467 base pairs (bp) for bacterial DNA, A-340F (CCCTAYGGGGYGCASCAG) and A-760R (GGACTACCSGGGTATCTAATCC) for archaeal DNA. Bacterial and archaeal amplicons were generated for all samples and the amplicons were indexed as explained by Goux, et al. [57]. PCR amplifications of the 16S rDNA was performed using Phusion Hot Start DNA polymerase (Thermo Scientific, Lithuania) with 0.6 µM of each primer, approximately 1 ng μl^{-1} DNA template, 0.8 mg ml^{-1} BSA and 2 mM $MgCl_2$ for 30 cycles (98°C 15 s, 50°C 20 s, 72°C 20 s). A second PCR for attachment of index sequences were performed as described by Goux, et al. [57]. PCR clean-up and normalization to obtain equimolar amplicon libraries (1 ng DNA μl^{-1}) of each amplicon was performed using the SequalPrep Normalization Plate (96) Kit (Invitrogen, Maryland, USA) according to the manufacturer's instructions. The bacterial and archaeal amplicons were pooled to generate one library and finally were concentrated to 15 ng μl^{-1} using Amicon Ultra Centrifugal Filter Units (Millipore, Ireland) as specified by the manufacturer. The samples analyzed in this study accounted for 36 of 116 uniquely indexed amplicons constituting the library that was sequenced on a MiSeq lane (Illumina, San Diego, CA) with v3 reagents employing 300 bp paired end reads at the Norwegian Sequencing Centre. PhiX library (Illumina) was blended to 50%. Data was processed using RTA 1.18.54 (Illumina). The resulting Illumina sequencing data were deposited at the European Nucleotide Archive (Study

accession number and sample accession numbers will be provided in a revised version if the manuscript is accepted for publication).

Data analysis and statistics. The Illumina sequencing data were processed with the high performance USEARCH utility (version 8.1.1825) (http://drive5.com/usearch/features.html). The processing was carried out as implemented in the UPARSE pipeline [58]. The major steps in the pipeline included demultiplexing, quality trimming, chimera removal, and clustering to obtain OTU tables at 97% similarity level. The subsequent taxonomy affiliation was based on the Utax script implemented in the UPARSE pipeline with a confidence value threshold of 0.8 and the RDP reference data set (version 15). In practical terms the pipeline was split at six break points where optimization was deemed likely and was supported by seven Perl scripts (available on request) to ease the processing. The Qiime pipeline [59] was used for determining Chao1 richness and relative abundances at different taxonomic levels. The RDP tools Classifier [60] and Sequence Match were used to analyze OTUs of particular interest.

Statistical analyses were performed using the program package PAST version 2.17 [61]. Similarities between community profiles were calculated as Bray-Curtis similarities [62]. Differences in average Bray–Curtis similarities between groups of samples were tested using PERMANOVA [63]. SIMPER (Similarity Percentage) analysis was employed to identify OTUs responsible for differences (measured as Bray-Curtis similarities) between sample groups [64]. Canonical correspondence analysis (CCA) was employed to elucidate the relationship between microbial community composition in the samples and environmental variables [65]. To evaluate correlation between relative abundance of genera and environmental variables we used Spearman's rank-order correlation coefficient to avoid the assumption of linear correlations. The probability of non-zero Spearmans's correlation was computed using a t test. The analyses were performed at the OTU level, and all OTUs with minimum 0.5% abundance in at least one sample were included. A Maximum likelihood tree based on the Tamura-Nei model [66] was inferred using MEGA 7 [67]. Sequences representing Thermoprotei, Thaumarchaeota, and Euryarchaeota were retrieved from the Ribosomal Database Project[56]. Details are given in the legend of Figure 4.

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Author Contributions Statement

Anna Synnøve Røstad Nordgård, MSc, wrote the article, contributed in the planning of the experiment, and performed all biological analyses (DNA extraction, PCR and Illumina library preparation). She also analyzed the sequencing data, results, and performed statistical analysis. This article will be included in her PhD thesis.

Wenche Hennie Bergland, PhD, contributed in planning of the experiment, the running of the reactors, and performance of the chemical analysis. She also analyzed rector operation and wrote minor parts of the material and methods, results and discussion.

Olav Vadstein, Professor, performed the Spearman correlation analysis, contributed in analysis of the data and writing of the manuscript.

Vladimir Mironov, PhD, contributed by setting up the procedure based on USEARCH and developing Pearl scripts needed to facilitate the processing of numerous data files. He also contributed by writing a minor part of the material and methods section.

Rune Bakke, Professor, contributed significantly to experimental design, data analysis and writing the manuscript.

Kjetill Østgaard, Professor, contributed in planning of the experiment.

Ingrid Bakke, Associate Professor, contributed in planning of the experiment, analysis of sequencing data, analysis of results, statistical analysis and writing of the manuscript.

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Additional information

Competing financial interest

The authors declare no competing financial interest.

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Figures

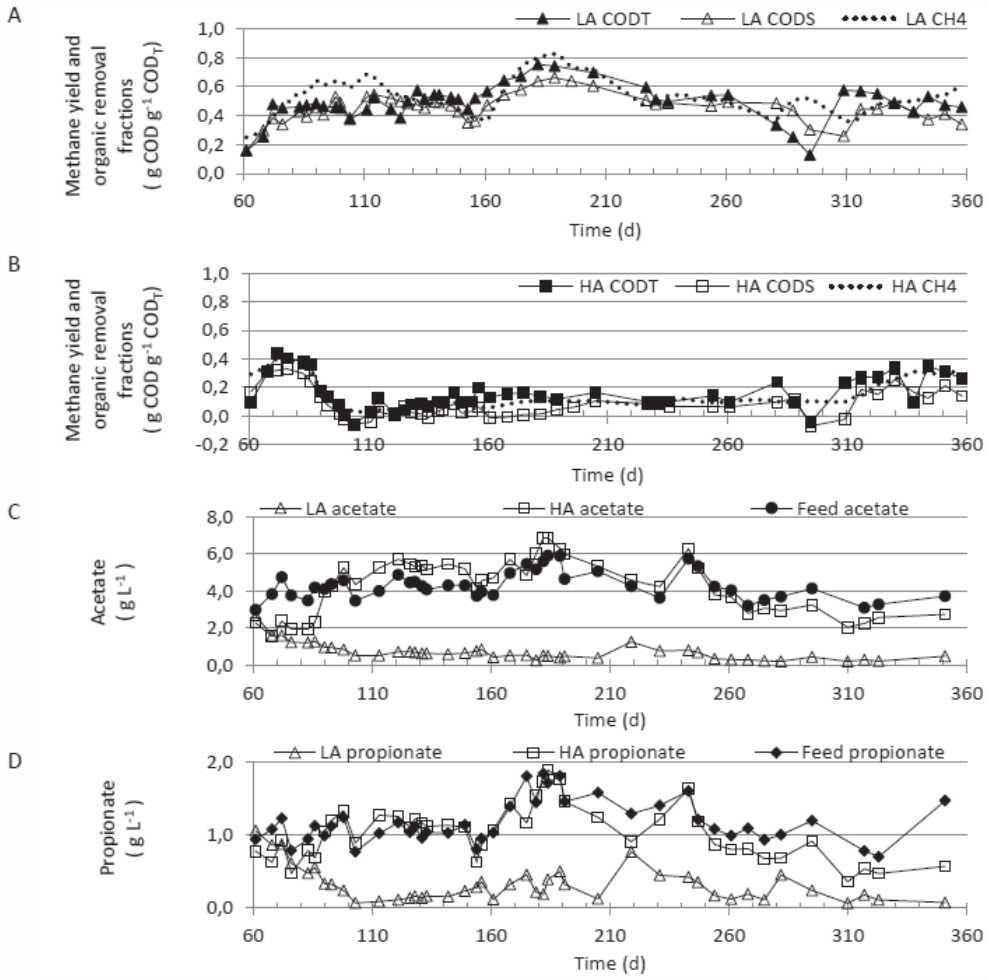
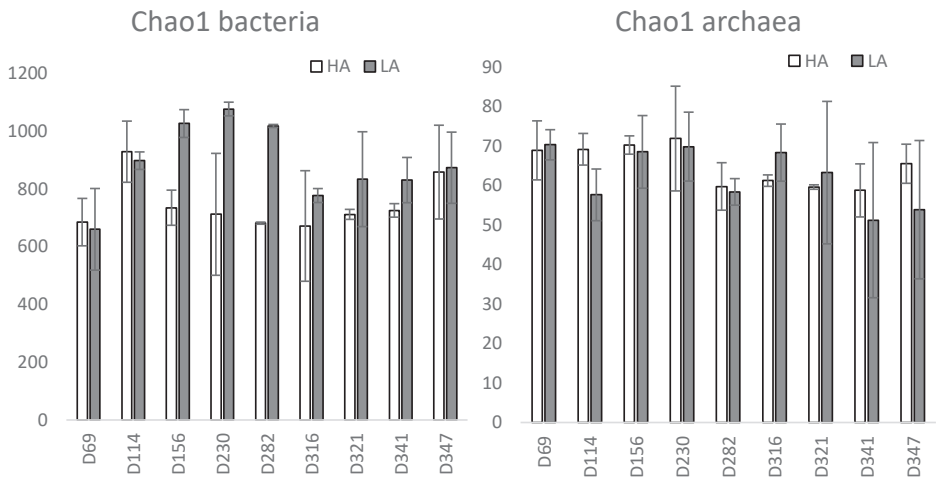


Figure 1: Performance of high ammonia (HA) and low ammonia (LA) rectors over the 358 days experimental period. Methane yield, total and soluble COD removal for A) LA and B) HA reactors, C) acetate concentrations in the feed and effluents, and D) propionate concentrations in the feed and effluents.

A



B

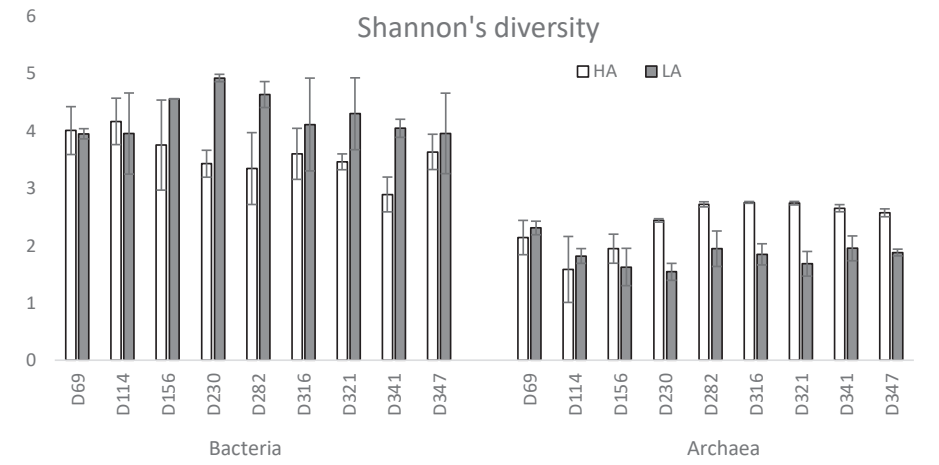
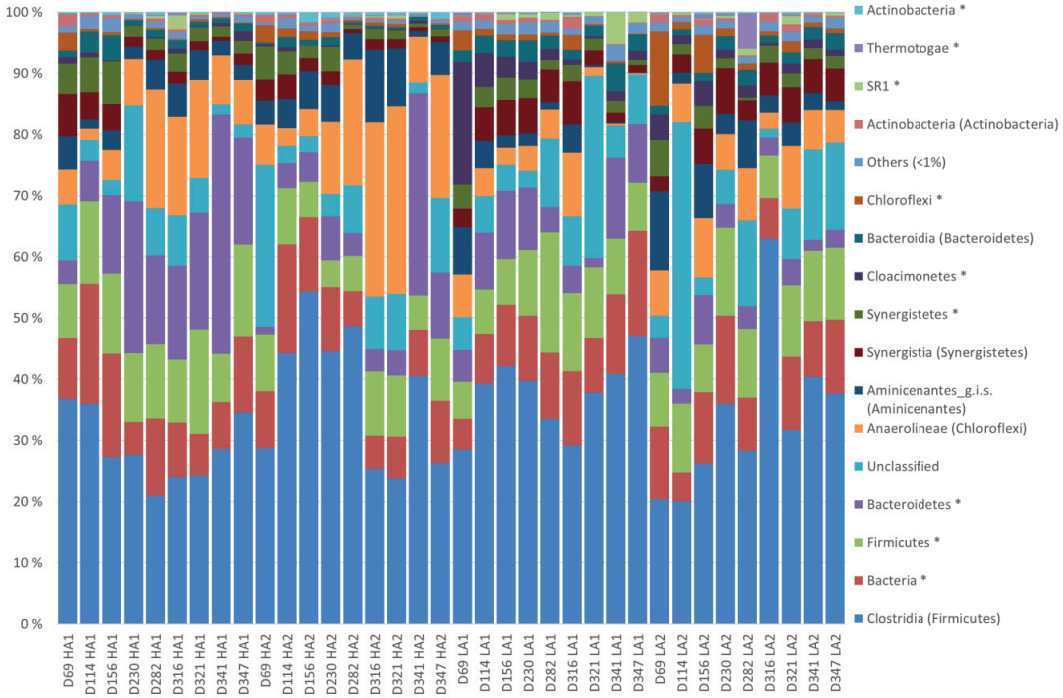
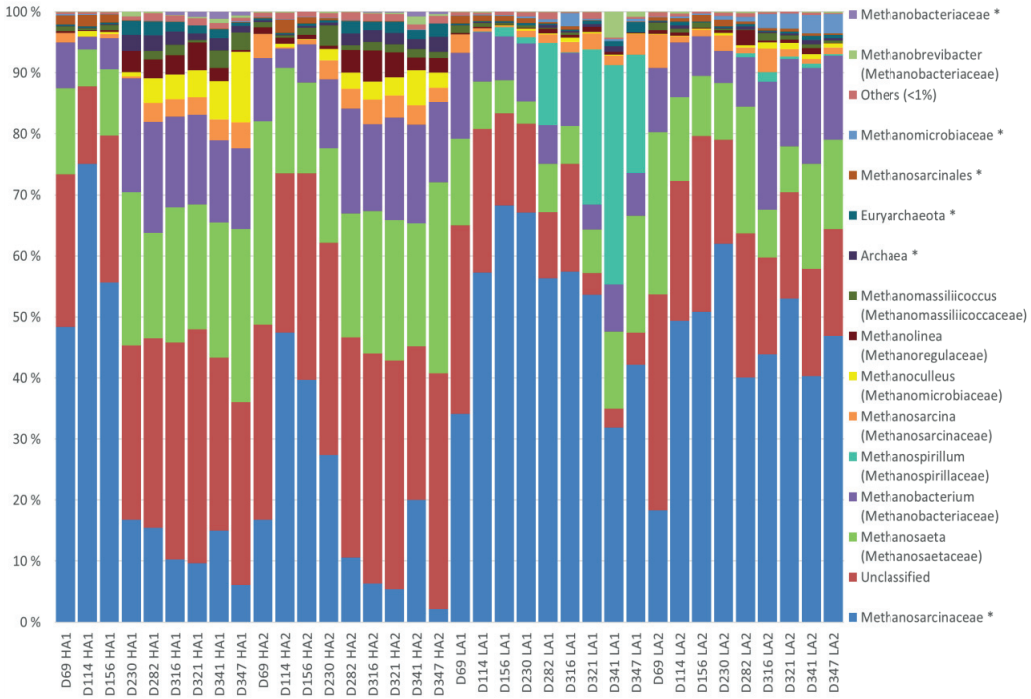


Figure 2. Diversity indices of bacterial and archaeal reactor communities. A) Estimated richness (Chao1) and B) Shannon's diversity index given as average for two replicate reactors. HA and LA refers to the HA and LA reactors, and D refers to experimental day. Error bars represent standard deviation.

A


100 %
90 %
80 %
70 %
60 %
50 %
40 %
30 %
20 %
10 %
0 %
Actinobacteria *
Thermotogae *
SR1 *
Actinobacteria (Actinobacteria)
Others (<1%)
Chloroflexi *
Bacteroidia (Bacteroidetes)
Cloacimonetes *
Synergistetes *
Synergistia (Synergistetes)
Aminicenantes_g.i.s. (Aminicenantes)
Anaerolineae (Chloroflexi)
Unclassified
Bacteroidetes *
Firmicutes *
Bacteria *
Clostridia (Firmicutes)
D69 HA1
D114 HA1
D156 HA1
D230 HA1
D282 HA1
D316 HA1
D321 HA1
D341 HA1
D347 HA1
D69 HA2
D114 HA2
D156 HA2
D230 HA2
D282 HA2
D316 HA2
D321 HA2
D341 HA2
D347 HA2
D69 LA1
D114 LA1
D156 LA1
D230 LA1
D282 LA1
D316 LA1
D321 LA1
D341 LA1
D347 LA1
D69 LA2
D114 LA2
D156 LA2
D230 LA2
D282 LA2
D316 LA2
D321 LA2
D341 LA2
D347 LA2

B


100 %
90 %
80 %
70 %
60 %
50 %
40 %
30 %
20 %
10 %
0 %
Methanobacteriaceae *
Methanobrevibacter (Methanobacteriaceae)
Others (<1%)
Methanomicrobiaceae *
Methanosarcinales *
Euryarchaeota *
Archaea *
Methanomassiliicoccus (Methanomassiliicoccaceae)
Methanolinea (Methanoregulaceae)
Methanoculleus (Methanomicrobiaceae)
Methanosarcina (Methanosarcinaceae)
Methanospirillum (Methanospirillaceae)
Methanobacterium (Methanobacteriaceae)
Methanosaeta (Methanosaetaceae)
Unclassified
Methanosarcinaceae *
D69 HA1
D114 HA1
D156 HA1
D230 HA1
D282 HA1
D316 HA1
D321 HA1
D341 HA1
D347 HA1
D69 HA2
D114 HA2
D156 HA2
D230 HA2
D282 HA2
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D341 HA2
D347 HA2
D69 LA1
D114 LA1
D156 LA1
D230 LA1
D282 LA1
D316 LA1
D321 LA1
D341 LA1
D347 LA1
D69 LA2
D114 LA2
D156 LA2
D230 LA2
D282 LA2
D316 LA2
D321 LA2
D341 LA2
D347 LA2

Figure 3. Relative abundances of bacterial classes (A) and archaeal genera (B) represented in the v3-4 16S rDNA amplicon obtained for individual reactor samples. Each bar represents one sample, and is labelled as follows: D indicates the experimental day; HA1 and HA2 refer to the two reactors operated at 3.7±0.2 g NH_4-N L^{-1}, and LA1, and LA2 refers to the reactors operated at 1.9±0.2 g NH_4-N L^{-1}. OTUs that could not be classified at the domain level are labeled "Unclassified", while OTUs that could not be classified at class or genus level for bacteria and archaea, respectively, are labeled *. Only taxa represented by a portion of ≥ 1% of the sequence reads in at least one of the samples are shown. "Others" include all reads representing the taxa with <1% abundance in all samples.

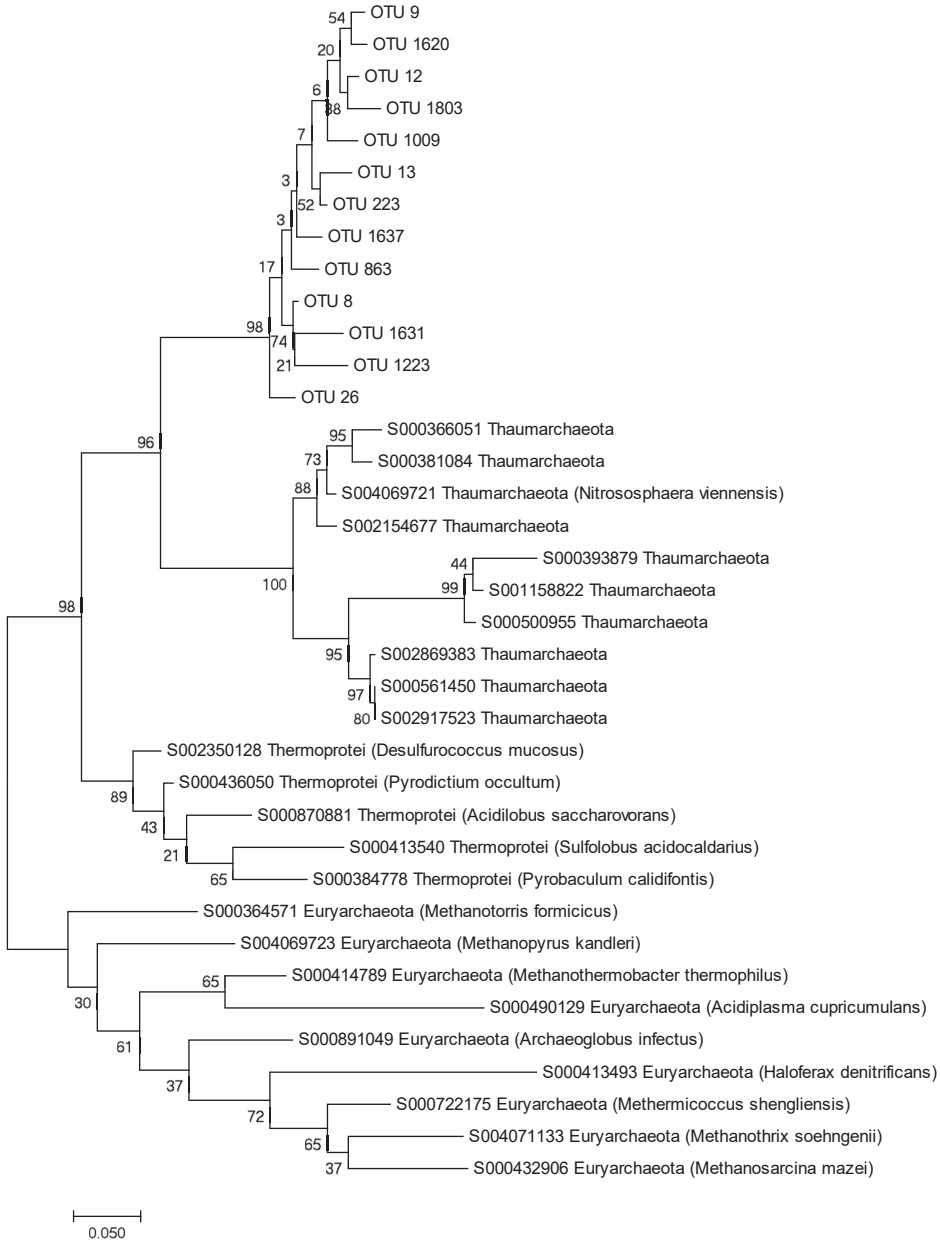

Figure 4. Molecular phylogenetic analysis including 13 OTUs identified in this study as potential relatives of Thaumarchaeota or Thermoprotei, and sequences representing Thermoprotei, Thaumarchaeota, and Euryarchaeota downloaded from the RDP database. For Thermoprotei and Euryarchaeota, "type strains" were selected. "Type strains" were not available for the Thaumarchaeota, but sequences representing both Nitrosopumilales and Nitrososphaerales were included. Number above nodes denote bootstrap percentages of 1000 replicates.

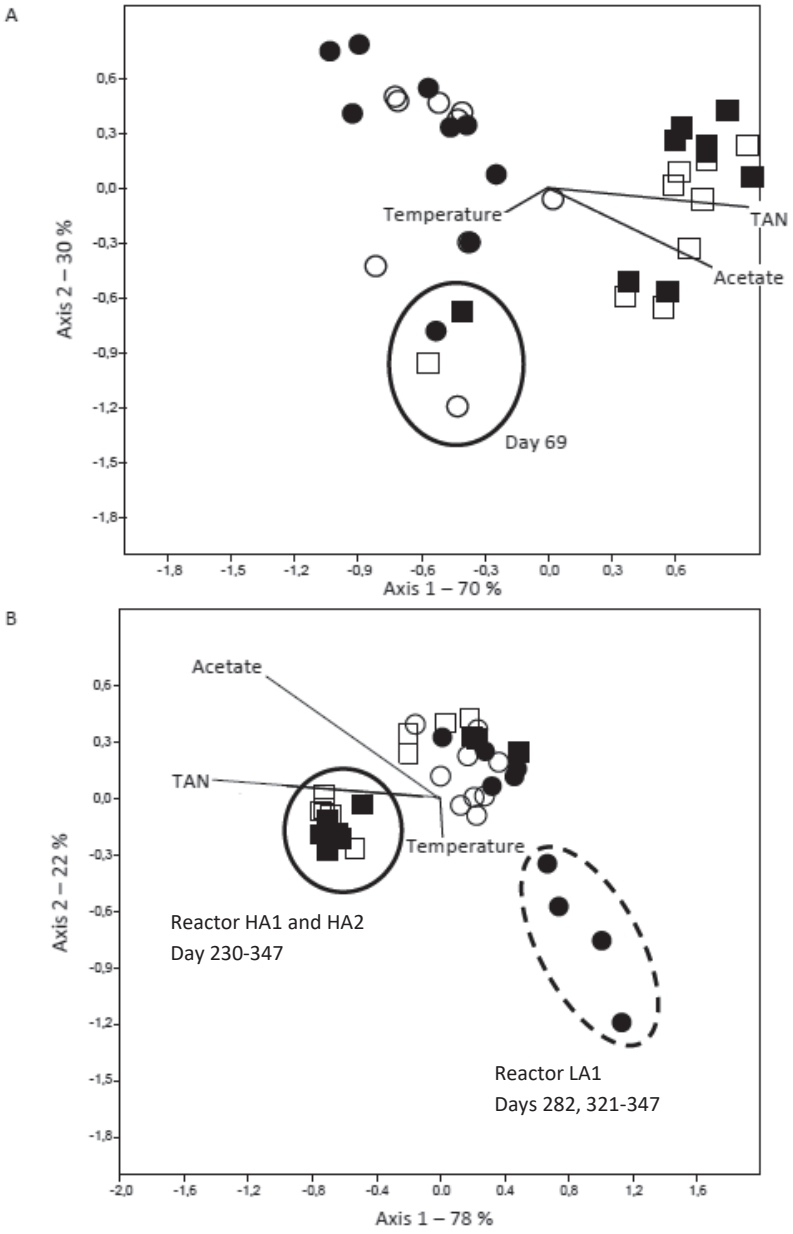

Figure 5: Canonical correspondence analysis with TAN, acetate and temperature as environmental variables, displayed as vectors in the plot, for bacterial (A) and archaeal (B) communities, for samples from reactor HA1 (■), HA2 (□), LA1(●) and LA2 (○).

Tables

Table 1. Properties of the pig manure slurry supernatant used as substrate. COD, acetate and propionate concentrations were the same for both influents. FAN was calculated from day 114 using equations given in Supplementary Information (Eq. S1-2). Total solids and volatile solids have been measured previously [21].

	Average±SD	
Property	**LA influent**	**HA influent**
TAN (g NH_4^+ – N L^{-1})	1.9 ± 0.2	3.7 ± 0.2
FAN (g NH_3-N L^{-1})	0.06 ± 0.02	1.2 ± 0.3
pH	7.6 ± 0.2	8.7 ± 0.1
COD_T (g L^{-1})	16.6 ± 2.6	
COD_S (g L^{-1})	11.7 ± 1.3	
COD_{VFA} (g L^{-1})	8.8 ± 1.6	
Acetate (g L^{-1})	4.3 ± 0.8	
Propionate (g L^{-1})	1.2 ± 0.3	

Table 2. The OTUs contributing most to the Bray-Curtis dissimilarity between LA and HA reactor communities from Day 230 to 347 as identified by SIMPER analysis. The unclassified archaeal OTUs were labeled non-euryarchaeotal archaea (see Fig. 4). The unclassified bacterial OTUs were suggested to be Hydrogenedentes (OTU_7) and Chloroflexi (OTU_12), but with low confidence thesholds.

OTU ID (taxonomy)	Cumulative	Mean abundance (%)	
Archaea	**contribution (%)**	**HA**	**LA**
OTU_1 (*Methanosarcinaceae*)	36.8	12.1	49.6
OTU_2 (*Methanosaeta*)	46.0	18.0	8.9
OTU_14 (*Methanospirillum*)	54.0	0.0018	8.2
OTU_3 (*Methanobacterium*)	59.8	14.7	10.2
OTU_12 (Unclassified)	64.3	5.9	1.4
OTU_9 (Unclassified)	68.7	6.0	1.6
OTU_13 (Unclassified)	72.8	5.3	1.2
OTU_10 (*Methanoculleus*)	76.4	4.1	0.5
Bacteria			
OTU_3 (Bacteroidetes)	10.41	14.9	0.1
OTU_1 (*Clostridiaceae_1*)	18.55	12.4	4.9
OTU_2 (Anaerolineae)	26.40	15.4	4.5
OTU_9 (*Syntrophomonadaceae*)	33.15	2.5	11.4
OTU_7 (Unclassified/Hydrogenedentes)	37.27	0.0016	5.9
OTU_12 (Unclassified/Chloroflexi)	39.78	4.17	0.8
OTU_6 (*Syntrophomonadaceae*)	42.19	4.9	2.5
OTU_19 (Clostridiales)	43.92	2.7	0.2

Supplementary information

Table S1. Number of reads for each sample.

Exp. Day	Bacteria				Archaea			
	HA1	HA2	LA1	LA2	HA1	HA2	LA1	LA2
D69	10498	9544	6697	6502	29021	27159	19375	29302
D114	13826	14097	19166	22362	33338	10454	33946	19725
D156	16931	20696	18045	16553	16378	26183	34419	14172
D230	10867	18075	13388	13514	35544	39937	59669	36248
D282	23593	24431	17596	18544	49475	26240	45905	28285
D316	27045	9909	8653	11454	28431	42880	30555	28233
D321	27553	21213	13641	15852	45634	38311	40409	37590
D341	17891	23001	26136	17361	35470	36607	37843	32789
D347	40816	29858	24563	34537	39812	37466	23900	45895

Table S2. Temperature, total ammonia nitrogen (TAN) and free ammonia nitrogen (FAN) concentrations in the reactor samples.

		Influent HA		Influent LA		HA1		HA2		LA1		LA2	
Day	Temp. [°C]	TAN [mg/L]	FAN [mg/L]	TAN [mg/L]	FAN [mg/L]	TAN [mg/L]	FAN [mg/L]	TAN [mg/L]	FAN [mg/L]	TAN [mg/L]	FAN [mg/L]	TAN [mg/L]	FAN [mg/L]
69	35	2030	153	2030	153	1910	186	1913	190	1845	187	1865	160
114	35	3490	1260	1935	58	3620	1086	3565	1104	1990	189	1935	169
156	25	3305	746	2015	53	3580	631	3450	637	2005	78	1980	84
230	25	3860	927	1950	60	4070	703	3990	690	2010	62	1980	65
282	25	3290	1237	1740	43	3470	600	3440	606	1700	101	1610	176
316	30	3500	990	1790	76	3690	850	3570	1078	1760	143	1920	166
321	30	3620	1315	1750	54	3690	1027	3670	1021	1770	200	1800	225
341	35	3680	1214	1780	76	3580	721	3700	732	1780	162	1800	145
347	35	3600	1557	1750	29	3860	980	3610	826	1810	162	1870	189

Table S3. Abundance of bacterial and archaeal OTUs in all reactor communities, found to be positively correlated to methane yield in HA samples (Spearman, $p<0.05$).

	Bacteria			Archaea			
	OTU_9 Syntrophomonadaceae	OTU_38 Synergistia	OTU_95 Cloacimonetes	OTU_2 Methanosaeta	OTU_10 Methanoculleus	OTU_57 Methanobacteriaceae	OTU_1142 Methanobrevibacter
D69 HA1	1.34	0.10	0.00	11.53	0.01	0.00	0.00
D69 HA2	0.00	0.08	0.00	24.05	0.01	0.00	0.02
D69 LA1	0.78	0.07	0.00	11.54	0.00	0.00	0.02
D69 LA2	0.03	0.06	0.00	21.43	0.01	0.00	0.01
D114 HA1	0.71	1.01	0.00	5.37	0.83	0.00	0.00
D114 HA2	0.04	0.50	0.00	11.69	0.57	0.00	0.00
D114 LA1	2.68	0.38	0.00	5.91	0.05	0.00	0.03
D114 LA2	0.35	0.26	0.00	10.47	0.07	0.00	0.09
D156 HA1	0.02	0.71	0.04	9.80	0.28	0.00	0.00
D156 HA2	0.00	0.21	0.01	13.02	0.05	0.00	0.00
D156 LA1	2.48	1.15	0.00	4.24	0.12	0.00	0.00
D156 LA2	0.40	0.61	0.00	7.05	0.13	0.00	0.00
D230 HA1	0.64	0.22	0.01	16.45	0.71	0.00	0.13
D230 HA2	0.07	0.22	0.19	13.79	1.82	0.01	0.01
D230 LA1	1.37	0.78	0.00	2.74	0.26	0.00	0.02
D230 LA2	2.26	0.75	0.00	6.27	0.41	0.00	0.04
D282 HA1	1.14	0.45	0.06	13.62	4.03	0.03	0.05
D282 HA2	0.77	0.14	0.03	13.46	2.64	0.00	0.03
D282 LA1	6.85	0.73	0.00	6.08	0.21	0.00	0.05
D282 LA2	9.22	0.17	0.01	15.00	0.45	0.00	0.08
D316 HA1	4.71	0.46	0.59	17.76	4.09	0.52	0.03
D316 HA2	0.88	0.23	0.04	17.83	2.98	0.17	0.02
D316 LA1	5.14	0.25	0.02	4.94	0.67	0.00	0.03
D316 LA2	2.20	0.09	0.04	6.59	0.99	0.00	0.02
D321 HA1	7.59	0.24	0.14	16.47	4.35	0.85	0.10
D321 HA2	1.28	0.23	0.13	16.78	2.89	0.19	0.13
D321 LA1	13.46	0.41	0.00	5.23	0.22	0.00	0.04
D321 LA2	5.62	0.28	0.06	6.21	0.98	0.00	0.01
D341 HA1	7.26	0.31	0.20	19.82	6.26	1.18	0.19
D341 HA2	1.51	0.46	0.39	17.51	5.77	0.79	0.86
D341 LA1	22.98	0.29	0.02	10.53	0.16	0.00	2.35
D341 LA2	20.20	0.44	0.01	14.20	0.81	0.00	0.07
D347 HA1	2.61	1.06	0.28	26.27	11.55	0.61	0.06
D347 HA2	1.89	0.41	0.08	26.16	2.48	0.39	0.09
D347 LA1	32.27	0.20	0.02	16.43	0.06	0.00	0.35
D347 LA2	15.18	0.63	0.02	12.04	0.71	0.00	0.04

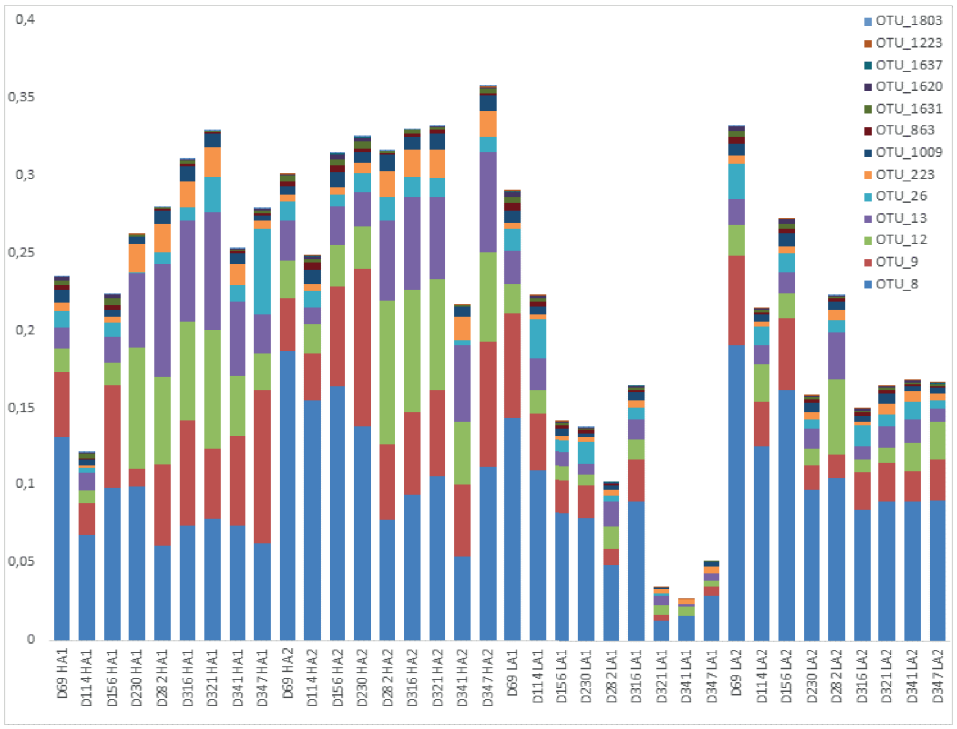

Figure S1. Relative abundances of 13 non-methanogen archaeal OTUs (see Fig. 4) in the reactor communities. D indicates experimental day.

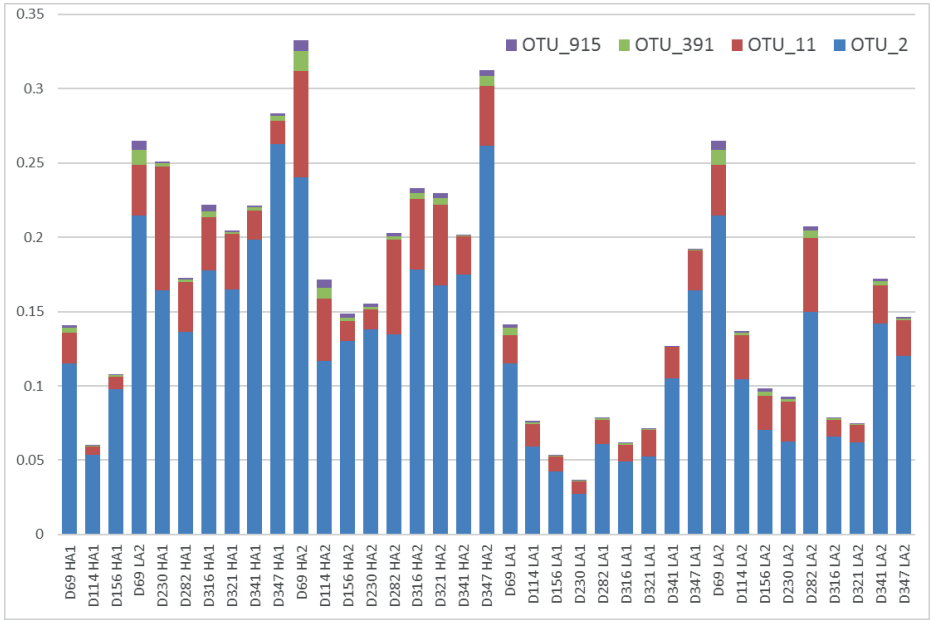

Figure S2. Relative abundance of the four most abundant *Methanosaeta* OTUs in the archaeal reactor communities. D indicate experimental day.

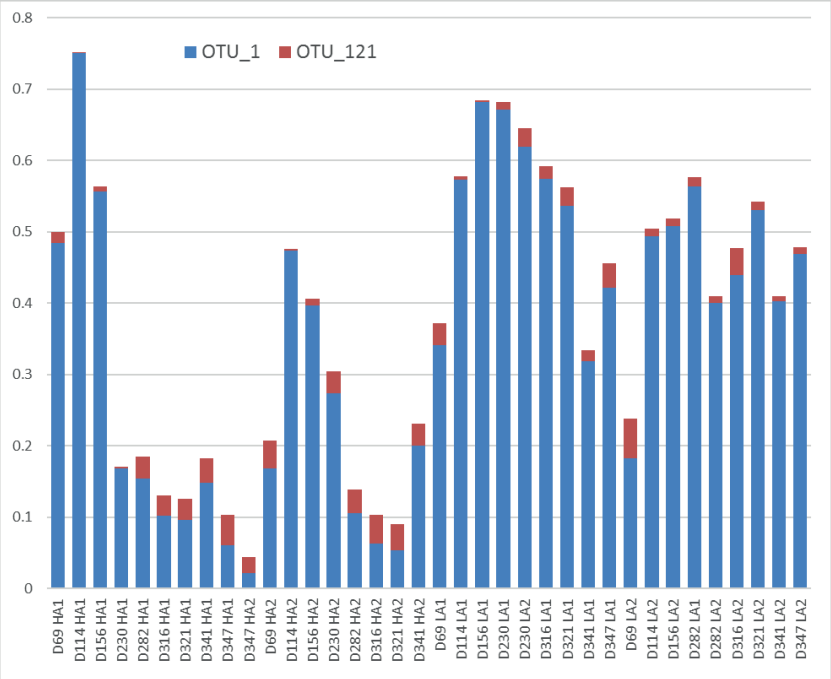

Figure S3. Relative abundance of the two most abundant *Methonosarcina* OTUs in the archaeal reactor communities. D indicate experimental day.

FAN was calculated from day 114 using equations S1 and S2.

$$K_a = \frac{[NH_3][H^+]}{[NH_4^+]} \quad \text{(S1)}$$

$$FAN = \frac{TAN}{1+\frac{10^{-pH}}{K_a}} \quad \text{(S2)}$$

Paper IV

Mapping anaerobic sludge bed community adaptations to manure supernatant in biogas reactors

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The reactors were operated at University College of Southeast Norway, Porsgrunn, Norway, while the microbial analyses were executed at Norwegian University of Science and Technology, Trondheim, Norway.

Abstract

In upflow anaerobic sludge bed (UASB) reactors, biomass is typically present as granules, allowing for long solids retention time without the presence of support materials. In the present study, granules originating from a process treating pulp and paper industrial wastewater were successfully applied as inoculum in UASB reactors treating solid rich pig manure supernatant at high ammonium concentrations. The objective of the present study was to characterize microbial communities using Illumina sequencing of 16S rRNA gene amplicons from both bacterial and archaeal communities that provided a detailed characterization of the microbiota in the inoculum and reactor granules as well as the solid and liquid fractions of the influent and reactors. The granular communities showed high abilities to adapt to changes in selection regimes and to be functional under conditions even highly distinct from those in the process from which they originated. The reactor granules diminished in size and abundance causing the accumulating non-granular solid fraction to play an increasing role, possibly as biofilm carrier. We postulate that microbial communities associated with the solid fraction represented functional important fraction of the methane producing community. The bacterial communities in the liquid fraction of the reactor were unique and contributed to conversion of organic matter.

Introduction

Anaerobic digestion (AD) is considered one of the most promising technologies in the field of renewable energy production and has been used to treat organic wastes such as manure for many decades already. Unfortunately, the low energy density of manure gives relatively low production rates in continuous stirred tank reactors (CSTR) currently used for manure AD. Such tanks without efficient biomass retention will be voluminous and expensive to build and operate for a single small scale farm. Many countries, *e.g.* Germany, transport manure to central AD plants, but this solution is questionable due to the CO_2 release and cost of transport. Hence, an on-farm solution is needed. We have previously shown that high rate upflow anaerobic sludge bed (UASB) reactors can efficiently treat supernatant from pig manure to obtain sustainable energy recovery despite the solids content being well above the levels considered appropriate as UASB reactor influent (Bergland *et al.*, 2015). Granules are one of the main components of such high rate AD. Generally, microorganisms including bacterial acidogens and acetogens and archaeal methanogens, aggregate into granules of 0.5–3 mm size, giving a sedimentation velocity high enough to avoid washout even under high hydraulic load (Lu *et al.*, 2013). In our UASB reactors treating manure supernatant, granules originating from a UASB reactor treating waste water with high carbon to nitrogen ration (C/N) from the pulp and paper industry were successfully applied as inoculum. Suspended solids accumulated in the reactors and formed a solid fraction together with the granules (Bergland *et al.*, 2015, Nordgård *et al.*, 2015). To what extent the accumulating solids played a role in the methanogenesis is not yet clear.

In the present study, the objective was to characterize microbial communities associated with different fractions in UASB reactors after adapting to treatment of pig manure supernatant at high and medium ammonium concentrations. We aimed at answering the following questions: 1) How did the granular communities adapt in response to the particle rich pig manure supernatant used as influent in our reactors? 2) Did the accumulating solids in the reactors contribute in the process? 3) Considering the low HRT, did the liquid fraction of the reactors simply represent the microbial communities introduced by the influent, or did it contribute in the methanogenesis? Illumina

sequencing of 16S rRNA gene amplicons was applied for detailed characterization of bacterial and archaeal communities associated with the solid and liquid fractions of the influents and the reactor sludge. Insight into the microbial community dynamics of the granules, as well as those of the liquid and solid fractions of the sludge, is essential to understand the microbial basis of this process for optimal design and operation.

Experimental procedures

Reactor influent, inoculum and operation

The manure substrate was collected from a pig production farm in Porsgrunn, Norway, and handled as described by Bergland *et al.* (2015). After collection, the manure substrate was stored at 4 °C until use. A total of four laboratory scale reactors (denoted High Ammonia (HA1 and HA2), and Low Ammonia (LA1 and LA2)) were fed pig manure slurry supernatant (hereafter referred to as pig manure) for 358 days. Set up and operation has been described previously (Nordgård *et al.*, submitted). The reactors were operated at hydraulic retention time (HRT) 1.0 day. The organic loading rate (OLR) was 16 ± 2 g COD L^{-1} d^{-1} for the entire experiment. Urea was added to the substrate for two of the reactors (HA1 and HA2) to increase the concentration of total ammonia nitrogen (TAN, *i.e.* NH_4^+ + NH_3) and resulted in 3.7 ± 0.2 g NH_4^+-N L^{-1}. Two reactors (LA1 and LA2) were fed pig manure supernatant as collected, resulting in TAN concentrations of 1.9 ± 0.2 g L^{-1}. Operational variables and properties are summarized in Table 1. Measurements of chemical oxygen demand (COD), pH, volatile fatty acids (VFA), NH_4^+-N, gas composition and methane production were performed as described by Bergland *et al.* (2015).

The granules used as inoculum originated from a UASB reactor treating pulp and paper process wastewater at Norske Skog Saugbrugs in Halden, Norway. These reactors were run with HRT <0.5 days, high C/N ratio, only dissolved organics at OLR 10-20 gCOD L^{-1} d^{-1} and temperature 35 ℃.

Sampling and DNA extraction

Three samples of granular sludge from the pulp and paper industry, representing differential storage periods at 11 °C, were analyzed; first sampling was performed upon arrival from the pulp and paper factory, second sampling after six months and the third after 12 months of storage. The third sampling represents the inoculum. These samples are referred to as the pulp and paper (PP) granules.

Samples were collected from both the HA and LA influents and all four reactors on experimental day 341 (D341) and 347 (D347) of the 358 days long experiment as described by Nordgård *et al.*

(submitted). The samples were then processed into solid and liquid fractions: The solid fraction was collected on both D341 and D347 by centrifuging the sludge samples at 200 g for 10 minutes. The pellet was resuspended in 1x phosphate buffer saline (PBS). These steps were repeated twice before a final centrifugation at 4000 g for 10 minutes to remove liquid.

To collect the liquid fraction (only D347), the samples were added PBS (1x) up to 50 ml and centrifuged at 200 g for 10 minutes. The pellets were discarded, the liquid volume adjusted with PBS, and centrifuged again at the same conditions. The supernatant was transferred to clean tubes and centrifuged at 12 000 g for 20 minutes.

Granules were picked by forceps from reactor HA1 and LA1 (hereafter referred to as reactor granules (Rgr)) from the D341 samples only and rinsed with phosphate buffer saline (PBS, 1x) prior to DNA extraction.

Total DNA was extracted from all samples using the Power Soil DNA isolation kit (Mobio Laboratories Inc., Carlsbad, CA, USA) as described by the manufacturer.

PCR and DNA sequencing

The v3 and v4 region of both the bacterial and archaeal 16S rRNA gene was amplified by PCR as described by Nordgård *et al*. (submitted). Bacterial and archaeal amplicons were generated and indexed for all samples as explained by Nordgård *et al*. (submitted). The amplicons analyzed in this study were part of larger amplicon libraries, and were sequenced on one lane on an Illumina MiSeq Instrument as described by Nordgård *et al*. (submitted). Sequencing data were processed with the USEARCH pipeline (version 8.1.1825; http://drive5.com/usearch/features.html) and two operational taxonomic unit (OTU) tables (Archaea and Bacteria) were generated as described previously (Nordgård *et al.*, submitted).

Statistical analyses

The Illumina Next Generation Sequencing data were processed with the high performance USEARCH utility (version 8.1.1825) (http://drive5.com/usearch/features.html), as described in Nordgård *et al*. (submitted). All statistical analyses were performed using the program package PAST (Hammer *et al.*, 2001). Community structure was compared between samples by calculating Bray-Curtis similarities (Bray & Curtis, 1957). Principal coordinate analysis (PcoA) was performed based on ordinations of Bray-Curtis similarities (Davis, 1986). PERMANOVA was used for testing differences in average Bray–Curtis dissimilarities between groups of samples (Anderson, 2001). SIMPER (Similarity Percentage) analysis was employed to identify taxa primarily responsible for differences between two or more sample groups (Clarke, 1993).

Results

All reactors produced methane during the period examined here, although better methane yields were obtained for LA reactors. High organic loading rate and high methane production were also achieved at high ammonia levels after adaptation. Reactor performance has been analyzed previously (Nordgård *et al*., submitted).

Sequencing effort and microbial diversity

The OTU tables yielded 2446 and 121 OTUs assigned to Bacteria and Archaea, respectively. The number of reads for each sample is given in Table S1. Estimated richness (Chao1) and observed OTUs were compared to illustrate the sequencing effort and resulted in a coverage of 73.2% and 92.4% on average of the estimated bacterial and archaeal richness, respectively (Fig. S1). Richness (Chao1), evenness and Shannon's diversity suggested a considerable increase of diversity for granular bacterial communities after nearly one year in the reactors. A similar trend, although less pronounced, was found for the archaeal communities (Fig. S1). Clostridia (Firmicutes), Bacteriodetes OTUs classified at phylum level only and OTUs classified only as Bacteria were the three most abundant bacterial taxa (Fig. 1). *Methanosaeta* was the most abundant archaeal genus across all samples, then followed OTUs classified only on domain level as Archaea and *Methanocorpusculum* as the second and third most abundant taxa (Fig. 2).

Adaption of granular microbial communities to particle rich pig manure supernatant

The granules used as inoculum originated from a pulp and paper industrial process characterized by a high C/N ratio. They were subjected to fundamentally different selection regimes in the process used here, characterized by low C/N ratio, higher HRT and high content of solids. Here we investigate how the granular microbial communities adapted to this change in process conditions.

The Principal coordinate analysis (PcoA) plot (Fig. 3) based on Bray-Curtis similarities illustrated that the bacterial community profiles in the PP granules were clearly separated from those in the reactor granules after one year of operation. This was corroborated by Bray-Curtis similarities as low as

0.10±0.05 and 0.07±0.02 for comparisons of the PP granules and the HA1 and LA1 granules, respectively (Table 2). It was also interesting to note that only a small fraction of the bacterial OTUs were shared by the PP and reactor granules and as much as 60% of the bacterial PP granule OTUs were unique to the PP granules (Fig. 4). The relative abundance of Bacteroidia and Deltaproteobacteria decreased markedly, from an average of 35±14% and 9±7% in the PP-granules to an average of 1.8±1.6% and 0.5±0.4% in the reactor granules (at D341), respectively. The abundance of Clostridia increased dramatically from 2.4±1.8% to 21±7% on average making it the most abundant bacterial class in the reactor granules (Fig. 1). SIMPER analysis was performed to identify the OTUs contributing most to the difference between the bacterial communities in the PP granules *versus* HA1 and the LA1 reactor granules (Table S2). We found that eight and 12 OTUs, respectively, were responsible for 50% of the Bray-Curtis dissimilarity. OTUs classified as Bacteroidetes and *Syntrophorhabdus* (Deltaproteobacteria) were more abundant in the PP granules, while Anaerolinea (Chloroflexi), Aminicenantes and Firmicutes OTUs dominated the HA1 reactor granules, and Synergistetes, Cloacimonetes and Firmicutes OTUs had higher abundances in the LA1 reactor granules.

Nearly 70 % of the archaeal OTUs were common to all the granules (Fig. 4). Bray-Curtis similarities (Table 2) indicated higher similarities for comparisons between the archaeal communities of the PP and HA1 reactor granules (0.70±0.01) compared to those between the PP and LA1 reactor granules (0.39±0.02). This was corroborated by the low Bray-Curtis similarities when comparing the HA1 and LA1 reactor granules (0.46) and illustrated by the PcoA plot (Fig. 3). Hence, the differences in ammonia concentration in the HA and LA reactors caused the inoculum to adapt into two new unique microbiotas. The archaeal communities in the PP granules were dominated by the aceticlastic genus *Methanosaeta* and OTUs that could not be classified even at domain level, which combined accounted for approximately 75% of the reads on average (Fig. 2). *Methanosaeta* abundance in the granules decreased most notably in the LA reactors (from 40.5 % in the inoculum to 14.4%) and less profoundly in the HA reactors (to 33.4%) in accordance with our previous study (Nordgård *et al*., submitted). There

were seven OTUs classified as *Methanosaeta* in the reactor granules. One of these OTUs accounted for most of the *Methanosaeta* reads (OTU2, 40.5%), both in PP and reactor granules, but in the reactor granules the other *Methanosaeta* OTUs were somewhat more abundant (0.037% in the PP granules and approximately 3% in the reactor granules).

At the end of our experiment, *Methanosaeta, Methanobacterium, Methanosarcinaceae* and Unclassified OTUs combined accounted for approximately 78% on average of the archaeal communities in the HA1 and LA1 reactor granules (Fig. 2). SIMPER analysis showed that only four and three OTUs explained as much as 50% of the Bray-Curtis dissimilarity between the archaeal communities in the PP granules and HA1 granules, and between PP granules and LA1 granules, respectively (Table S3). Variable *Methanosaeta* (OTU2) abundances was among the most important differences between the PP and reactor granules. A *Methanoculleus* OTU (OUT10) had much higher abundance in the HA1 granules (11.9%) than in the LA1 and the PP granules (0.6% and 0.002%, respectively). Similarly, a *Methanosarcinaceae* OTU (OTU1) had much higher abundances in the LA1 granules (19.3%) than in HA1 granules (0.8%) and the PP granules (0.003%). *Methanobacterium* (OTU3) was most abundant in the LA1 granules (25%, 5.7% in PP and 0.6% in HA1 granules).

Analysis of the total solid fraction in the reactors

Visual inspection of the sludge revealed that the granules gradually diminished in size during the experiment, while non-granular particulates accumulated. Hence, non-granular particles made up a substantial part of the solid fraction towards the end of the experiment. We speculated that the solids introduced by the influent manure slurry might play an important role in the process by supplying the methanogenic consortium with an organic, slowly degradable biofilm carrier as substitute for the diminishing granules. We therefore compared the communities associated with the granules with those of the solid fraction (*i.e.* including the granules). First, we compared the communities associated with the solid fraction of the reactors and the influents. Low Bray-Curtis similarities suggested distinct

207 microbial communities for Archaea and Bacteria (Table 2). Thus, the accumulating solids in the reactor
208 did not merely represent the microbes associated with particle-rich manure supernatant influent.

209 PcoA plots and comparisons of Bray-Curtis similarities indicated that the non-granular solid fraction in
210 the reactors represented a distinct methanogenic community. The archaeal communities of the
211 granules and the total solid reactor samples were similar as illustrated by the PcoA plot (Fig. 3). This
212 was corroborated by comparisons of Bray-Curtis similarities that were higher for the archaeal than the
213 bacterial communities (Table 2). OTUs classified only on domain level, *Methanosaeta*, OTUs classified
214 only on family level as *Methanosarcinaceae* and *Methanobacterium* were the most dominant taxa in
215 the reactor granules and the total solid fraction (Fig. 2). *Methanospirillum* and *Methanoculleus* were
216 abundant in the reactor granules and solid fraction of the LA and HA reactors, respectively. The high
217 abundance of these taxa implies an active methanogenic community in the solid fraction, suggesting
218 that influent particles were continuously colonized by methanogens from the granules.

219 There were more distinct differences found between the bacterial communities in the granules and
220 the total solid communities. Relatively low Bray-Curtis similarities (Table 2) indicated that the bacterial
221 communities differed more between these sample types. SIMPER analysis revealed higher
222 abundances of OTUs identified as *Syntrophomonadaceae, Clostridiaceae_1*, and one OTU not
223 classified on domain level in the solid fraction than in the granules. While in the reactor granules, OTUs
224 identified as Anaerolinea, Clostridiales, Synergistetes, *Aminobacterium* and
225 *Aminicenantes_genera_incertae_sedis* were more abundant. These observations suggest that the
226 bacterial niches in the granules and suspended particle biofilms were occupied by different bacteria.

227

228 ## Microbial communities associated with liquid fractions

229 The HRT of this process was as low as one day, which is much less than the generation time of most
230 of the organisms involved. We wanted to investigate whether unique microbial communities

developed in the liquid fractions of the reactors, or whether they were simply a reflection of the microbial communities introduced to the reactors by the influent.

The archaeal communities present in the liquid fractions of the reactor samples were strikingly similar to those of found in the liquid fraction of influent samples (around 0.7, Table 2), as illustrated by PcoA plot and high Bray-Curtis similarities (Fig. 3B, Table 2). Particularly *Methanocorpusculum* and OTUs classified only at phylum level (Euryarchaeota) reached high abundances in the liquid fractions of both influent and reactor samples (Fig.2). Hence, specific archaeal communities probably did not develop in the liquid fraction of the reactors since the hydraulic retention time was much shorter than the generation time of the organisms.

The situation was different for the bacterial communities. The communities associated with the liquid fractions of the reactor and the influent differed considerably (Fig. 3A, Table 2). Bray-Curtis similarities were low (around 0.3, Table 2) for comparisons of the bacterial communities in the liquid fractions of the reactors and the influents. SIMPER analysis revealed that six OTUs were responsible for 50% of the Bray-Curtis dissimilarity. Most strikingly, OTU 11 explained 15 % of the Bray-Curtis dissimilarity between the liquid fractions of the reactors and influents, but could not be classified beyond domain level. Both Utax and RDP Classifier suggested that this OTU represented Clostridiales, but at low confidence thresholds. It amounted to 31.1% on average in the LA reactor liquid communities, but was barely detected in the HA reactors (1.8%) and not at all in the influents. Similarly, OTU 3 explained 14% of the dissimilarity and amounted to 30.5% and 3.4% in the HA and LA reactors, respectively, but was not observed in influent samples. It could not be classified beyond phylum level (Bacteroidetes). Of the six OTUs identified by SIMPER, only one was classified beyond class level. This was a *Pseudomonas* OTU that had high abundances in the influents (Fig. 1, Gammaproteobacteria). Hence, the bacterial liquid fraction of the reactor was not just a mirror image of the influent passing through the digester.

Discussion

Granules are essential for the process efficiency in UASB reactors. Therefore, we investigated the adaption of the granular communities in response to the particle rich pig manure supernatant used as influent in our reactors. Manual inspection of the reactor sludge showed that the granules diminished in size and abundance and the amplicon sequencing analysis illustrated that the bacterial and archaeal communities adapted into new unique microbiotas with more distinct changes in the bacterial than the archaeal communities. We speculated whether the granules would be able to uphold their structure when they undergo major changes in their species inventory. Other studies have reported major changes in granular species inventory due to adaption (Na *et al.*, 2016, Alcantara-Hernandez *et al.*, 2017), but they did not comment on granule size and abundance in the reactors after completing the adaption. In our study, the lab scale UASB reactors were not optimized for upholding granular growth. The granules diminished, but did not disintegrate completely, during the experiment. Thus, it appears that our process selected for a smaller granule size compared to the pulp and paper process from which they originated.

The pulp and paper industrial process was characterized by a high C/N ratio while here they were subjected to low C/N ratio, higher HRT, high content of solids and, particularly in the case of the HA reactors, elevated ammonia concentrations. This altered selection regime clearly made the bacterial communities in the granules adapt as the abundance of the dominating Bacteroidia and Deltaproteobacteria decreased substantially and the abundance of Clostridia increased markedly. The class Deltaproteobacteria comprise a branch of strictly anaerobic genera, which contains most of the known sulfate- and sulfur-reducing bacteria, the well-known species of syntrophic propionate degrading anaerobes *Syntrophobacter* and *Smithella*, and the genera *Syntrophorhabdus* which was found in high abundances in the PP granules (Qiu *et al.*, 2008, Rosenberg *et al.*, 2014). *Syntrophorhabdus* is able to utilize phenol, p-cresol, isophthalate, benzoate, and 4-hydroxybenzoate in syntrophic association with a hydrogenotrophic methanogen (Qiu *et al.*, 2008). This function may

have been more important in the reactors that the PP granules originated from than in our reactors. Bacteroidia and Clostridia are both able to hydrolyze cellulose and produce hydrogen by fermenting carbohydrates (Rosenberg *et al.*, 2014). Apparently, the adaption of the bacterial communities in the granules may be attributed to the loss of a seemingly no longer dominating niche in the case of *Syntrophorhabdus*, and to the potential functional redundancy of Bacteroidia and Clostridia. Vanwonterghem *et al.* (2014) and Carballa *et al.* (2015) have both stated that hydrolytic-fermentative bacteria tend to be functionally redundant.

The archaeal communities in the granules displayed a smaller change than the bacterial communities from the PP granules to the reactor granules. *Methanosarcinaceae* and *Methanobacterium* increased in abundance in the LA granules and *Methanoculleus* increased in the HA granules compared to the PP granules. Increases in these genera during high ammonia concentrations is in accordance with literature (Demirel & Scherer, 2008, Yenigun & Demirel, 2013). Literature also reports decreasing abundances of the obligate aceticlastic *Methanosaeta* with increasing concentrations of ammonia (Chen *et al.*, 2008, Demirel & Scherer, 2008, Rajagopal *et al.*, 2013). Calli *et al.* (2005) suggests that loss of *Methanosaeta* activity with increasing ammonia levels is due to loss of filamentous growth. McHugh *et al.* (2003) states that it is widely accepted that filamentous *Methanosaeta* has an important role in the formation and maintenance of stable anaerobic granules. Interestingly, here *Methanosaeta* was found in higher abundances in the granules obtained from the HA than from LA reactor. This was in accordance with our previous study where we also observed a decrease in methane production with increasing ammonia concentrations (Nordgård *et al*., submitted). Hence, we observed a decease in methane production probably due to inhibition of *Methanosaeta* in the HA reactors, but only a slight decrease in abundance. A possible explanation for the apparent high ammonia tolerance we observed for *Methanosaeta* in this study could be protection obtained by growing in aggregates with other microbes in the sludge granules. One could also speculate that there could also be some yet-to-be-revealed interactions in the HA reactors that sustains the high abundance of *Methanosaeta*. The higher abundance of *Methanosarcina* in LA could be due to a competitive advantage. The FAN

306 concentration in LA was 0.14±0.10 g NH_3-N L^{-1} which is below the cluster disintegrating threshold of
307 600 mg L^{-1} and *Methanosarcina* has a higher growth rate than *Methanosaeta* (0.60 versus 0.20 d^{-1},
308 respectively) (Calli *et al.*, 2005, De Vrieze *et al.*, 2012). These advantages could be the reason why
309 *Methanosarcina* abundances were higher in LA than in HA.

310 In this study, seven *Methanosaeta* OTUs were detected in which six had very low abundances.
311 *Methanosaeta* OTU2 was detected in high abundances in both the PP and reactor granules.The low
312 abundance OTUs increased by a factor of almost 100 during the experiment. Hence, different species
313 and a higher diversity of *Methanosaeta* were selected for in the granules during the current process,
314 possibly reflecting differences in properties such as feed preference and ammonia tolerance.

315

316 We examined the microbial community dynamics of the granular, total solid (*i.e.* non-granular solid
317 fraction plus reactor granules) and liquid fractions of the reactors to uncover differences in community
318 structure between fractions and to elucidate where the active communities were situated. The non-
319 granular solid fraction is especially interesting since the feed used here (settled pig manure) has a
320 particle content much higher than recommended for UASB treatment (Tchobanoglous *et al.*, 2003).
321 Bergland *et al.* (2015) observed that a significant fraction of such influent particles were converted to
322 methane under similar conditions as those tested here. The results presented above suggest that the
323 non-granular solid fraction originated from a combination of influent particles and organisms from the
324 granules. It is envisioned that influent particles served as degradable support for biofilm growth. The
325 increasing importance of such suspended biofilms as the experiment progressed, with decreasing
326 granular size and abundance, can be explained by the high level of suspended compared to dissolved
327 organics in the influent applied here.
328 The archaeal communities were highly similar to each other in the granules and total solid fractions
329 of the reactors (Table 2, Fig. 3). Similar abundances of *Methanosaeta* were found in the reactor
330 granules and the total solid fraction of the reactors (Fig. 2). This strongly suggests that the archaeal

communities present in the accumulating non-granular solid fraction did contribute to the methane production.

The archaeal communities in the liquid fraction of the reactors resembled those of the liquid fraction of the influent, and showed a high abundance of *Methanocorpusculum*. This suggests that the slow-growing archaeal communities in the liquid fractions of the reactors did not have time to adapt to the new environment due to the HRT being lower than their growth rate. Hence, the archaeal communities in all fractions were strongly influence by the influent community.

Hydrolyzing and fermentative bacteria were found in all fractions although different taxa dominated the different locations (Table 2, Fig. 3). *Syntrophomonadaceae,* Anaerolinea, Clostridiales, Synergistetes and *Aminobacterium* have all been detected in anaerobic digestion previously (Ito *et al.*, 2011, Li *et al.*, 2013, Ho *et al.*, 2014, Na *et al.*, 2016, Hagen *et al.*, 2017) and have functions that contribute to the overall methanogenic process. Interestingly, the reactor liquid fractions were dominated by OTUs that could only be classified on higher levels and that were not detected in the influents. High abundances of unclassified OTUs were also found in a previous study (Nordgård *et al*., submitted) indicating that much remains to be uncovered about microbial interactions and taxonomy. Hence, unique bacterial communities evolved in the reactor liquids despite low HRT, shaped by the selection pressure and conditions in the reactors. These communities might contribute to the degradation of organic matter in the liquid phase through hydrolysis and acidogenesis.

Conclusion. The granular communities showed high abilities to adapt to changes in selection regimes and to be functional under conditions even highly different from those in the process from which they originated. The reactor granules diminished in size and abundance, while accumulating non-granular solid fraction played an increasing role as the experiment progressed possibly as a biofilm utilizing organic feed particles as both substrate and substratum ('biofilm carrier'). Archaeal communities showed high degrees of similarity in the granules and the total solid fractions of the reactors and in

the liquid fractions of the influents and reactors. On the other hand, bacterial communities varied considerably between the granules, total solid fractions and liquid fractions in the reactors. Interestingly, the bacterial liquid fraction of the reactor was not just a mirror image of the influent passing through the digester, but rather a unique community that probably contributed to the overall process.

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441 # Tables and figures

442

443 Table 1: Properties of the influent and reactors given as average ± standard deviation (SD). Total
444 ammonia nitrogen (TAN, *i.e.* $NH_4^+ + NH_3$), free ammonia nitrogen (FAN, *i.e.* NH_3), acetate, COD_T and
445 methane yield are given for day 347.

Property	HA reactors average ± SD	LA reactors average ± SD
pH of the influent	8.7 ± 0.1	7.6 ± 0.2
NH_4 – N in influent	3.7 ± 0.2 g L^{-1}	1.9 ± 0.2 g L^{-1}
TAN in the reactors	3735 mg L^{-1}	1840 mg L^{-1}
FAN in the reactors	903 mg L^{-1}	176 mg L^{-1}
Acetate influent-effluent	3.33 - 2.1 g L^{-1}	4.10 - 0.30 g L^{-1}
COD_T influent-effluent	16.7 - 10.5 g L^{-1}	18.1 - 7.8 g L^{-1}
Methane yield	1.65 NL CH_4 L^{-1} influent	3.00 NL CH_4 L^{-1} influent

446

447

448 Table 2: Average Bray-Curtis similarities given as average with standard deviation for comparisons of
449 community profiles between samples. HA: High ammonia reactor 1 and 2; LA: Low ammonia reactor
450 1 and 2; PP: pulp and paper; D341 and D347: day 341 and 347 of the experiment. Samples for which
451 experimental day is not given were all sampled at day 347.

	Bacteria Average ± SD	**Archaea** Average ± SD
Granules		
PP granules vs Reactor granules HA1	0.10 ± 0.05	0.70 ± 0.01
PP granules vs Reactor granules LA1	0.07 ± 0.02	0.39 ± 0.02
Reactor granules HA1 vs Reactor granules LA1*	0.31	0.46
Solid D341 HA1 vs Reactor granules HA1*	0.36	0.68
Solid D341 LA1 vs Reactor granules LA1*	0.20	0.54
Reactor and influent		
HA reactor liquid vs Influent HA liquid	0.30 ± 0.05	0.72 ± 0.13
LA reactor liquid vs Influent LA liquid	0.27 ± 0.09	0.73 ± 0.01
HA reactor solid vs Influent HA solid	0.21 ± 0.07	0.27 ± 0.04
LA reactor solid vs Influent LA solid	0.14 ± 0.05	0.04 ± 0.03
*Influent samples only**		
Solid D347 HA vs Liquid HA	0.27	0.18
Solid D347 LA vs Liquid LA	0.26	0.49
Reactor samples only		
Solid D347 HA vs Liquid HA	0.28 ± 0.04	0.11 ± 0.05
Solid D347 LA vs Liquid LA	0.26 ± 0.09	0.12 ± 0.11

452

453 * Only one comparison, hence no SD.

454

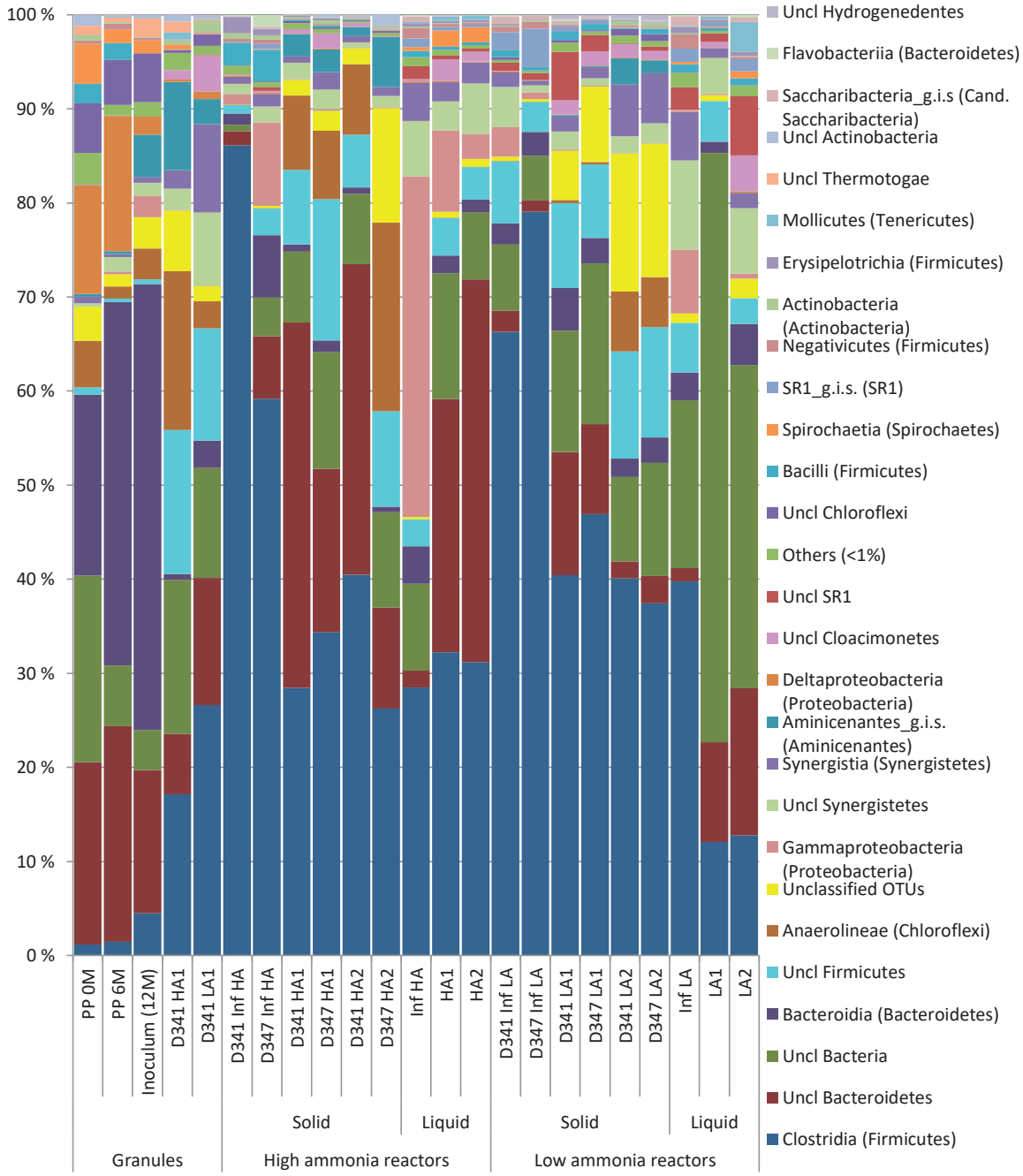

Figure 1: Relative abundances of bacterial classes (phylum) represented in the v3-4 16S rRNA gene amplicons obtained for individual influent, reactor and granular samples. Each bar represents one sample, and is labelled as follows: D indicates the experimental day; PP is short for the pulp and paper granules that were sampled upon arrival and after six and 12 months of storage; Inf HA and Inf LA indicates samples from influent to HA and LA reactors. "*Cand.*" is short for "*Candidatus*" while "*g.i.s.*" is an abbreviation of "*genera_incertae_sedis*". OTUs that could not be classified at the domain level

463 are labeled “Unclassified”, while OTUs that could only be classified above class level are labeled
464 “Uncl”. Only taxa represented by a portion of ≥ 1% of the sequence reads in at least one of the samples
465 are shown. “Others” includes all reads representing the taxa with <1% abundance in all samples.

466

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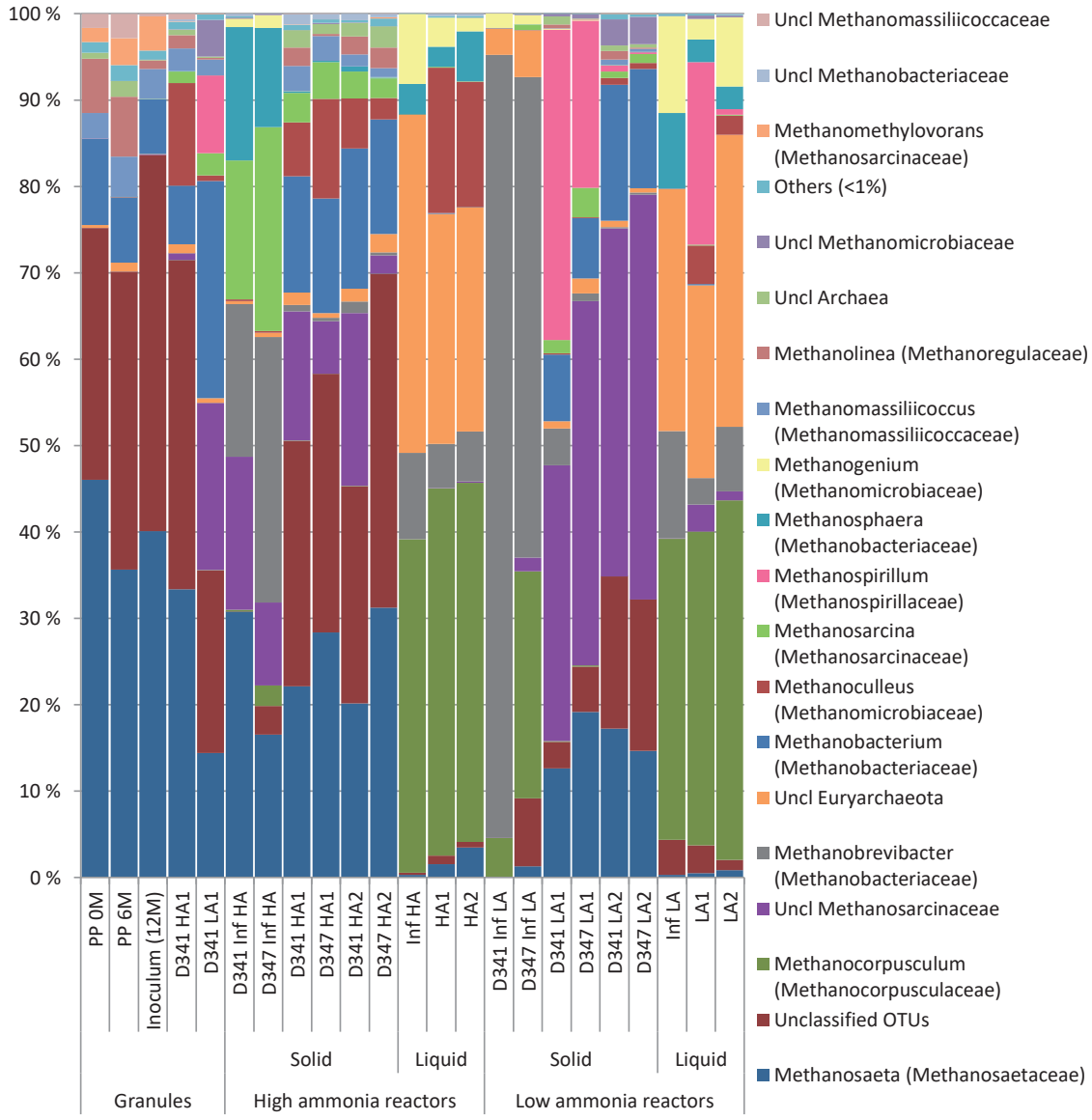

Figure 2: Relative abundances of archaeal genera (family) represented in the v3-4 16S rRNA gene amplicons obtained for individual influent, reactor and granular samples. Each bar represents one sample, and is labelled as follows: D indicates the experimental day; PP is short for the pulp and paper granules that were sampled upon arrival and after six and 12 months of storage; Inf HA and Inf LA indicates samples from influent to HA and LA reactors. OTUs that could not be classified at the domain level are labeled "Unclassified OTUs", while OTUs that could only be classified above family level are labeled "Uncl". Only taxa represented by a portion of ≥ 1% of the sequence reads in at least one of the samples are shown. "Others" includes all reads representing taxa with <1% abundance in all samples.

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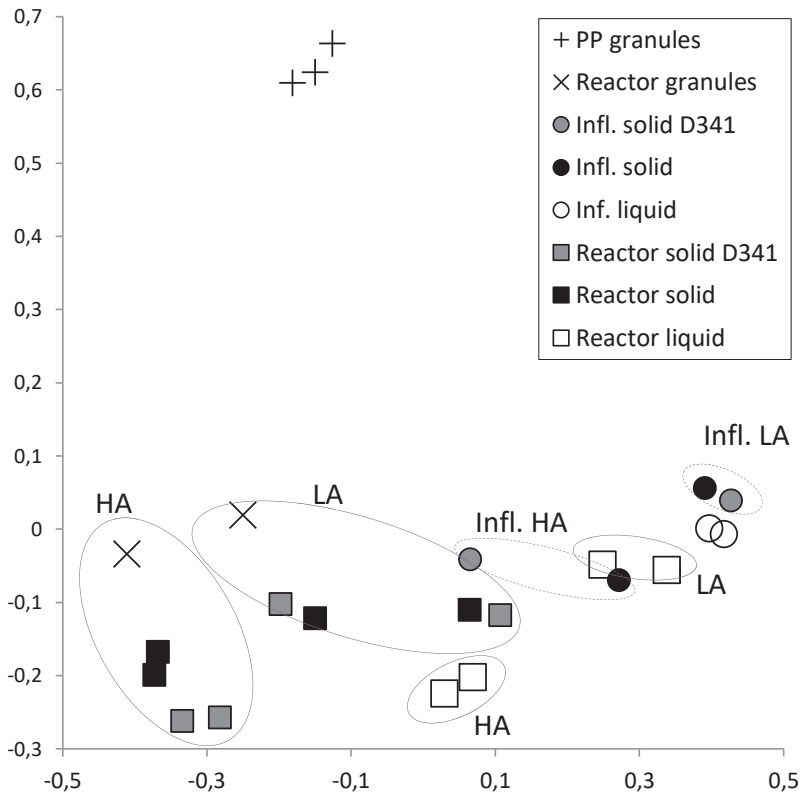
480 A



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B

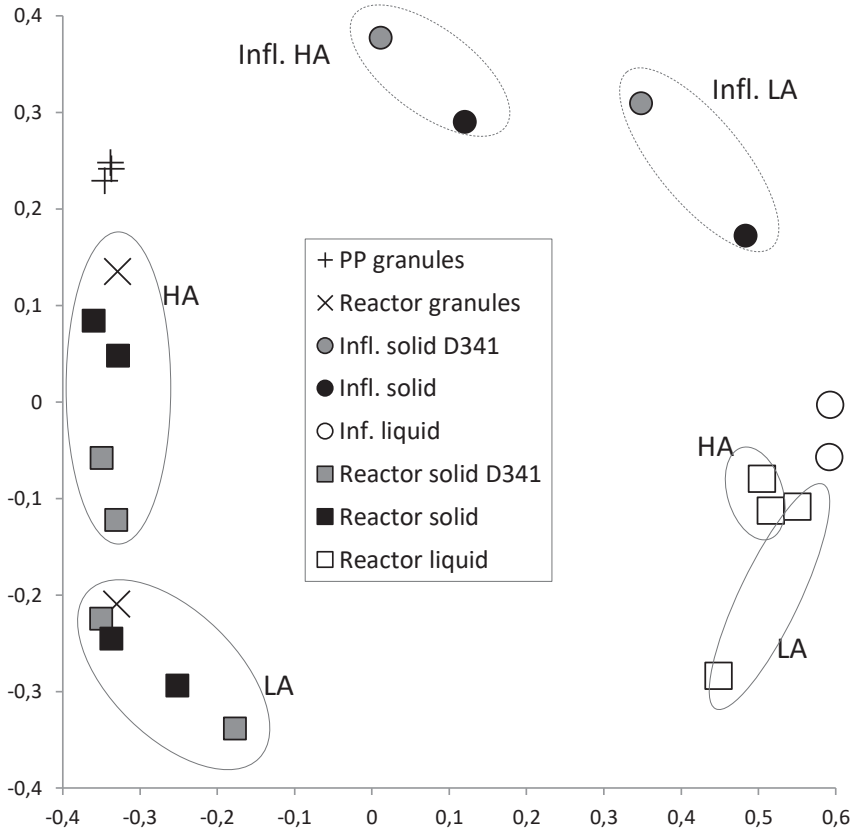
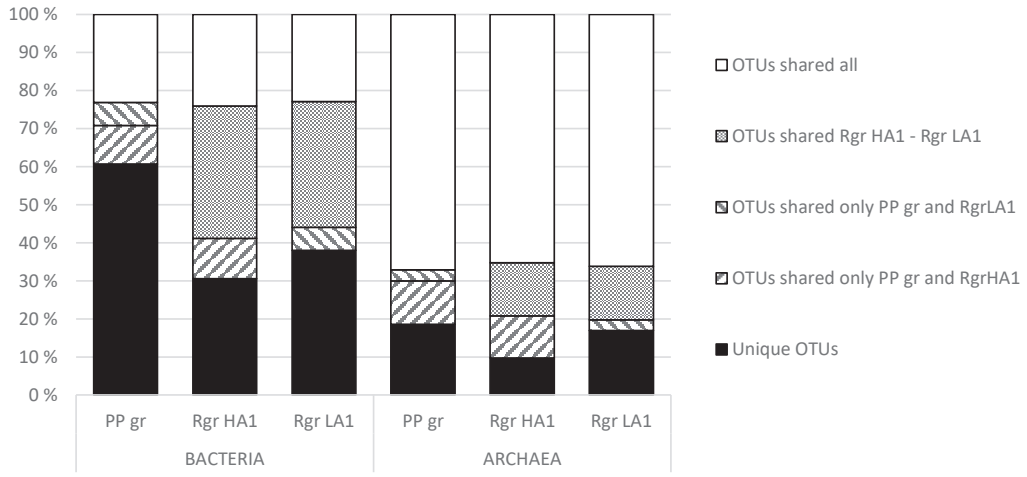


Figure 3: Principal coordinate analysis ordination based on Bray-Curtis similarities for A) bacterial and B) archaeal community profiles associated with granule samples and for liquid and solid fraction of influent and reactor samples. PP granules were sampled 12, 6 and 0 months prior to the experiment. Reactor granules were sampled on experimental day 341 (D341). Other samples were taken at day 347. Solid line circle indicate samples from the reactors while dashed line circles indicate samples from the influents.

492



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494 Figure 4: Bar graph indicating the percentage of unique and shared OTUs between the pulp and paper
495 granules (PP gr) and reactor granules (Rgr).

496

497

Supporting information

Table S1: Number of reads per sample after quality filtering and chimera removal, obtained with the primers B-338F/B-805R amplifying bacterial DNA and A-340F/A-760R amplifying archaeal DNA. The pulp and paper granules (PP gr.) were sampled upon arrival and after 6 and 12 months in storage at 11 °C. The reactor granules were sampled from the HA1 and LA1 reactors at day 341 of the experiment.

Bacteria	D341 Solid	D347 Solid	D347 Liquid	PP granules	Reactor granules
PP gr. 0M				30976	
PP gr. 6M				16226	
Inoculum (12M)				26071	
Inf HA	26050	30931	17078		
Inf LA	18555	27704	14656		
HA1	17891	40816	10066		22139
HA2	23001	29858	12018		
LA1	26136	24563	23800		12517
LA2	17361	34537	28865		

Archaea	D341 Solid	D347 Solid	D347 Liquid	PP granules	Reactor granules
PP gr. 0M				42385	
PP gr. 6M				48644	
Inoculum (12M)				63835	
Inf HA	31317	29005	55028		
Inf LA	21764	20052	40186		
HA1	35470	39857	26827		36836
HA2	36607	37466	28602		
LA1	37887	23916	36572		34678
LA2	32781	45890	37538		

Table S2: OTUs contributing most to the Bray-Curtis dissimilarity between the bacterial communities in the PP granules versus HA1 reactor granules and LA1 reactor granules as identified by SIMPER analysis. Contrib.: contribution; abund.: abundance; p: phylum; c: class; o: order; f: family; g: genus.

PP-granules versus HA1 granules					
Taxon	Contrib. (%)	Cumulative (%)	Mean abund. PP (%)	Mean abund. HA (%)	Taxonomy
OTU 10	19.24	19.24	34.70	0.05	p Bacteroidetes; c Bacteroidia; o Bacteroidales
OTU 2	8.679	27.92	0.40	16.00	p Chloroflexi; c Anaerolineae
OTU 13	4.638	32.55	0.001	8.34	k Bacteria
OTU 27	4.445	37.00	8.31	0.32	p Bacteroidetes
OTU 6	4.252	41.25	1.67	9.31	p Aminicenantes; g *Aminicenantes genera incertae sedis*
OTU 2179	3.152	44.40	0	5.67	p Firmicutes; c Clostridia; o Clostridiales
OTU 49	3.105	47.51	5.61	0.03	p Proteobacteria; c Deltaproteobacteria; f *Syntrophorhabdaceae*; g *Syntrophorhabdus*
OTU 24	2.876	50.38	0	5.17	p Firmicutes
PP granules versus LA1 granules					
Taxon	Contrib. (%)	Cumulative (%)	Mean abund. PP (%)	Mean abund. LA (%)	Taxonomy
OTU 10	18.68	18.68	34.70	0.02	p Bacteroidetes; c Bacteroidia; o Bacteroidales
OTU 33	4.451	23.13	0.47	8.72	p Bacteroidetes
OTU 27	4.209	27.34	8.31	0.51	p Bacteroidetes
OTU 19	3.671	31.01	0.001	6.81	p Firmicutes; c Clostridia; o Clostridiales
OTU 45	3.39	34.40	0.16	6.45	p Synergistetes
OTU 14	3.228	37.63	0.00	5.98	p Synergistetes; c Synergistia; o Synergistales; f *Synergistaceae*; g *Aminobacterium*
OTU 49	3.018	40.65	5.61	0.02	p Proteobacteria; c Deltaproteobacteria; f *Syntrophorhabdaceae*; g *Syntrophorhabdus*
OTU 31	2.292	42.94	4.31	0.06	p Chloroflexi
OTU 24	2.198	45.14	0	4.07	p Firmicutes
OTU 16	2.043	47.18	0	3.79	p Cloacimonetes
OTU 144	1.823	49.00	0	3.38	p Firmicutes; c Clostridia
OTU 107	1.74	50.74	3.23	0.01	p Bacteroidetes

Table S3: OTUs contributing most to the Bray-Curtis dissimilarity between the archaeal communities in the PP granules, HA1 reactor granules and LA1 reactor granules as identified by SIMPER analysis. OUT 8 and 9 could not be classified even at domain level. Contrib.: contribution; abund.: abundance; o: order; f: family; g: genus.

PP granules versus HA1 reactor granules					
Taxon	Contrib. (%)	Cumulative (%)	Mean abund. PP (%)	Mean abund. HA1 (%)	Taxonomy
OTU 10	20.04	20.04	0.002	11.90	o Methanomicrobiales; f *Methanomicrobiaceae*; g *Methanoculleus*
OTU 2	17.2	37.24	40.50	30.40	o Methanosarcinales; f *Methanosaetaceae*; g *Methanosaeta*
OTU 8	7.96	45.2	18.20	16.00	Unclassified on domain level
OTU 16	6.01	51.21	4.72	1.53	o Methanomicrobiales; f *Methanoregulaceae*; g *Methanolinea*
PP granules versus LA1 reactor granules					
Taxon	Contrib. (%)	Cumulative (%)	Mean abund. PP (%)	Mean abund. LA1 (%)	Taxonomy
OTU 2	23.79	23.79	40.50	11.40	o Methanosarcinales; f *Methanosaetaceae*; g *Methanosaeta*
OTU 1	15.8	39.58	0.003	19.30	o Methanosarcinales; f *Methanosarcinaceae*
OTU 3	15.71	55.3	5.74	25.00	o Methanobacteriales; f *Methanobacteriaceae*; g *Methanobacterium*

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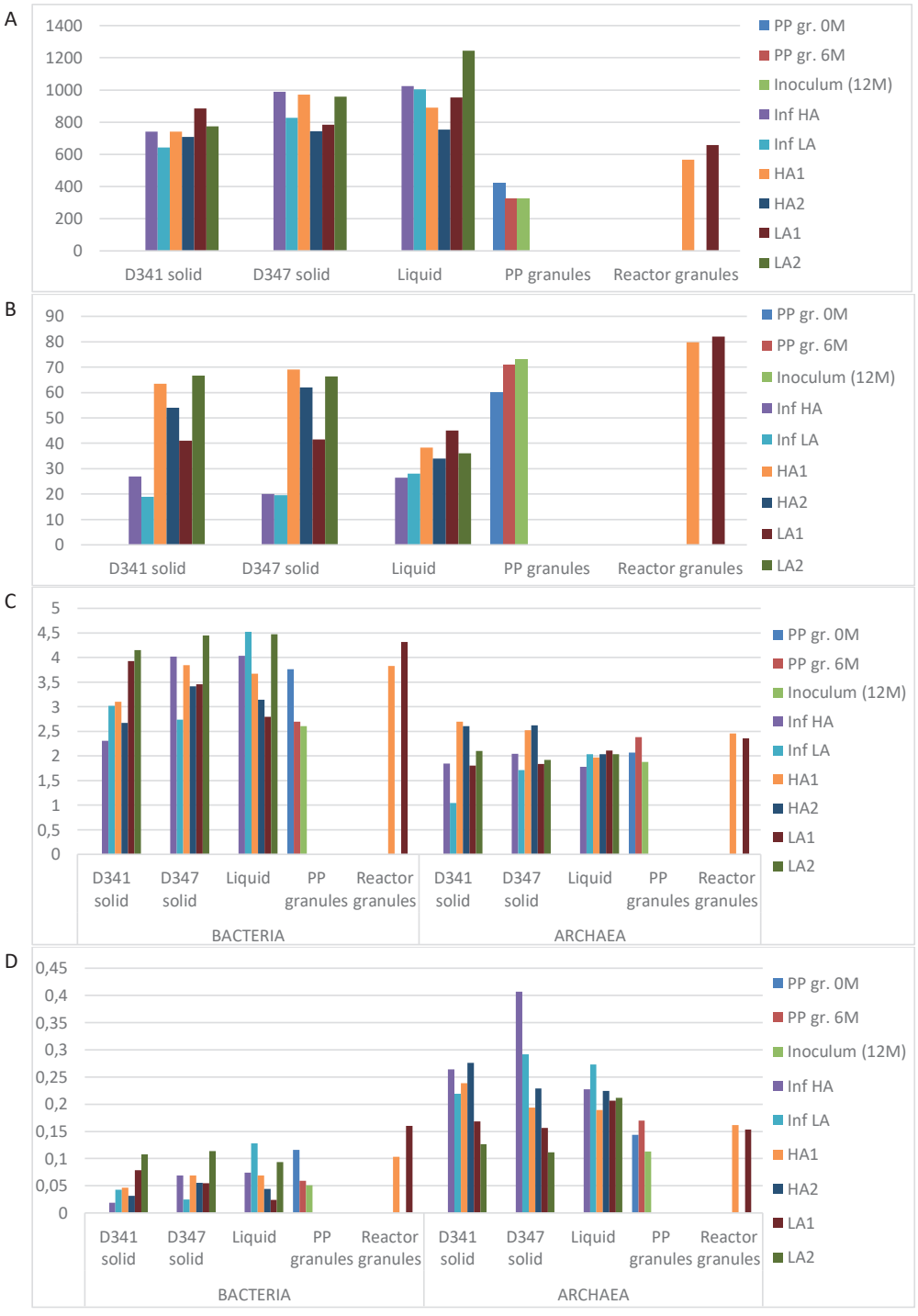
A
1400
1200
1000
800
600
400
200
0
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
PP gr. 0M
PP gr. 6M
Inoculum (12M)
Inf HA
Inf LA
HA1
HA2
LA1
LA2
B
90
80
70
60
50
40
30
20
10
0
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
PP gr. 0M
PP gr. 6M
Inoculum (12M)
Inf HA
Inf LA
HA1
HA2
LA1
LA2
C
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
BACTERIA
ARCHAEA
PP gr. 0M
PP gr. 6M
Inoculum (12M)
Inf HA
Inf LA
HA1
HA2
LA1
LA2
D
0,45
0,4
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0,3
0,25
0,2
0,15
0,1
0,05
0
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
D341 solid
D347 solid
Liquid
PP granules
Reactor granules
BACTERIA
ARCHAEA
PP gr. 0M
PP gr. 6M
Inoculum (12M)
Inf HA
Inf LA
HA1
HA2
LA1
LA2

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Figure S1: Richness and diversity. A) Chao1 for bacterial communities. B) Chao1 for archaeal communities. C) Shannon's diversity for bacterial and archaeal communities. D) Evenness for bacterial and archaeal communities. The pulp and paper granules (PP gr.) were sampled upon arrival and after 6 and 12 months in storage at 11 °C. The reactor granules were sampled from the HA1 and LA1 reactors at day 341 of the experiment. The liquid fractions were sampled only on day 347.