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MICROBIAL COMMUNITY DYNAMICS IN ANAEROBIC DIGESTION

BY
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DENMARK

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Preface

This dissertation is submitted in partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy (PhD). Thesis describes the outcomes of the PhD project "Microbial community dynamics in anaerobic digestion", which was carried out from 1 December 2014 to 30 November 2017, at the Department of Chemistry and Bioscience at Aalborg University, under the supervision of Professor Jeppe Lund Nielsen. The results presented here were obtained as part of a work package under the "NomiGas project", funded by the Danish Council of Strategic Research. The thesis consists of an extended introduction that summarises the background and literature relevant to the PhD project, and is supported by seven scientific papers, which are included as appendices.

First and foremost, I would like to express my deepest gratitude to my supervisor, Professor Jeppe Lund Nielsen, for giving me the opportunity to work on this project. His guidance and relentless positivity have been an inspiration in my work, and I wish to thank him for the many discussions we have had over the past three years, scientific or otherwise. The past three years have been an adventure, and I have learned more about myself as a person, and as a scientist than I thought was possible in such short time.

Secondly, I would like to thank my colleagues at the Department of Chemistry and Bioscience, both past and present. Specifically, I wish to thank the members of the Environmental Biotechnology group, they have always made me feel at home, and contributed to a positive and fun working environment. In particular, I would like to thank my office roommates, for always keeping morale high and fun discussions about work and wine. Thank you to Mie, for assisting me with the sequencing of my samples. Thank you to Jane, Marianne and Susanne for your invaluable technical support.

Furthermore, I would like to extend my thanks to all participants in the NomiGas project, especially Åsa Davidsson, Jes la Cour Jansen, Henrik Bjarne Møller, Veronica Moset, Maja Nielsen, Laura Agneessens and Alastair Ward, for great discussions and good collaboration.

Lastly, I would like to extend my thanks to my friends and family. To my parents, thank you for following me on this journey and helping me chase my dreams in academic science. To my friends, academic or otherwise, your support has been invaluable in completing this PhD project. Franzi, Kamilla, Katie, Giulia, Susan, Niels, Daniel and Gerard, you are amazing.

Finally, thank you, reader of this thesis.

I hereby declare that this is my original work.

Nadieh de Jonge

Aalborg, November 2017

English summary

Anaerobic digestion (AD) of organic waste to produce biogas is an important technology for managing waste and for production of renewable energy. Biodegradable waste is reduced through an anaerobic process governed by a complex community of microorganisms of the kingdoms *Bacteria* and *Archaea*. A given AD system can be composed of up to a thousand microorganisms, all performing specific functions that contribute to the different degradation processes. The final product of AD is biogas, consisting of CH_4 (60 %) and CO_2 (40 %), that can either be converted to energy and heat through combustion, or upgraded to biomethane for use as fuel. The reduced biomass is rich in nutrients, and suitable as an organic fertiliser.

In order to utilise AD technology to its fullest potential, a deep understanding of both operational and microbial aspects of the process is paramount. Identification and functional determination of the microorganisms involved in the AD process, as well as studying the microbial community response to operational changes and disturbances will provide insight into the dynamics of AD ecosystems and how to optimise the technology further. This PhD project aimed to contribute to a better understanding of microbial community dynamics in full-scale AD systems. Next generation sequencing technology was used to capture the microorganisms from single point measurements to survey a large number of full-scale digesters. Furthermore, a number of time series measurements were also sampled from digesters of interest. The obtained microbial data was analysed in order to describe the microbial communities involved in AD systems processing animal manures and food waste, investigate microbial and functional stability of full-scale AD systems under stable and unstable conditions, and to identify correlations between the microbial communities and operational characteristics of AD.

The first two studies in the thesis describe a survey of AD systems processing manures and food waste, respectively. For each study, a large number of digesters were subjected to amplicon sequencing of the 16S rRNA and *mcrA* genes to capture the microbial communities associated with these AD substrate types. In the first study, the manure based digesters were observed to have a high degree of microbial community similarity, regardless of reactor temperature and substrate composition. High abundance and diversity of the phylum *Firmicutes* characterised these digesters. The second study observed contrasting results; a clear cluster separation based on temperature and primary substrate components was obtained for the digesters processing food waste as a primary substrate. Furthermore, the microbial communities involved in food waste AD contained a rich presence of syntrophic bacteria and highly redundant methanogenic communities, suggesting a high potential for stability and adaptation.

Obtaining a high resolution of methanogenic community structure with 16S rRNA gene studies can be difficult, due to their low abundance in AD systems, and copy number biases. The third study describes the development of an amplicon sequencing based assay targeting the functional marker *mcrA*, and revealed that it was possible to obtain much higher microbial community data resolution compared to 16S rRNA gene based equivalents.

Understanding which microorganisms are associated with which substrates and operational temperatures, and how well they integrate into mixed AD microbial communities is of great importance for understanding and optimising co-digestion systems. The fourth study applied pairwise statistical comparison of full-scale AD microbial communities showed that it was possible to separate a complex microbial ecosystem into groups of substrate associated organisms and those that are potential generalists. Wastewater sludge was revealed to contain many substrate associated organisms compared to food waste and manures, especially within the phyla *Proteobacteria*, *Actinobacteria* and *Chloroflexi*. The study also revealed potential organisms of interest for further study.

Microbial responses to two perturbation scenarios were investigated by studying time series measurements in the fifth and sixth study. Operational temperature is one of the primary effectors of microbial community structure in AD systems, and it is therefore of high importance to gain a deep understanding of how AD ecosystems handle (natural) variation in temperature. Paper 5 comprises a study of three full-scale plants operating within the mesophilic spectrum. The microbial study revealed that minor temperature changes (± 2 °C) are handled by the microbial community without affecting operational performance. This could likely be attributed to the innate resilience and redundancy of the microbial community. Furthermore, a temperature increase of 4 °C in a mesophilic digester caused a shift in microbial populations, especially within the syntrophic acetate oxidising and methanogenic populations, after which the digester stabilised at the new temperature. The digester retained the new composition through subsequent temperature fluctuation. In the sixth study, an AD system perturbation

in the form of starvation was examined in a realistic lab-scale scenario. After a starvation incident of not receiving substrate, the investigated reactors showed responses similar to those observed under overloading states, likely due to the re-established feeding regimen. Large fluctuations were observed in the microbial communities, and after 45 days of recovery the systems had not yet fully stabilised. The obtained results highlight the importance of regular digester maintenance, and show that while microbial communities in AD systems have the capability to recover from perturbation, there are limitations to their abilities.

Microbial management of AD systems has been suggested as a way to enhance current methods of digester operation. A deep understanding of the dynamics of AD microbial communities is needed in order to work toward microbial engineering of digester systems. The final study of the thesis reflects on the current status of microbial management in AD systems and the tools available for monitoring of AD systems from the operational and microbial standpoint. Practical examples of useful microbial community information that can be gained from studying full-scale AD systems are discussed. In summary, the study found that it is possible to extract information applicable to process optimisation using currently available tools, but further development of existing technologies is needed to achieve true microbial management. In order to gain a deep understanding of microbial dynamics during AD operation, more accurate and convenient methods of (microbial) monitoring are needed; frequent monitoring of full-scale scenario's will assist discovering the indicators of process and microbial response required to optimise AD system management.

In conclusion, the combined results of this thesis highlight of the usefulness of microbial studies in AD systems to obtain a better understanding of the microbiology of AD. Studies of the AD microbial community can deepen the understanding of not only the "workhorses" of the AD process, the microbial community itself, but also their dynamic nature and responses to operational changes, all of which are needed to achieve further optimisation of the technology. This PhD project has yielded new knowledge regarding the microbial community structure of AD system processing food waste and manures as a substrate and microbial responses to common operational disturbances. Furthermore, a promising new assay was developed for the detailed study of methanogenic communities in AD systems. Moreover, by applying microbial community information to the operational process, it will be possible to achieve microbial management; Control and (bio) engineering of digester systems toward optimised usage of the microbial ecosystem.

Dansk Resume

Biogas produktion (metanisering) igennem anaerob udrådning (AU) af organisk affald er en vigtig bæredygtig teknologi indenfor vedvarende energi produktion og affaldshåndtering. Biologisk affald nedbrydes i fravær af ilt ved hjælp af et komplekst mikrobielt økosystem bestående af *Bakterier* og *Arkæer*. Et givent AU system kan indeholde mere end tusind forskellige mikroorganismer, som bidrager til de individuelle nedbrydningsprocesser i specifikke funktioner. Slutproduktet i metanisering er biogas, generelt set bestående af metan (60 %) og kuldioxid (40 %), som kan omdannes til varme og elektricitet ved forbrænding eller opgraderes til brug som brændstof. Den afgassede biomasse er rig på næringsstoffer og velegnet som organisk gødning.

For at kunne udnytte det fulde potentiale i AU-teknologi er det afgørende at opnå en god forståelse af både de operationelle og mikrobielle aspekter af processen. Identifikation og funktionel bestemmelse af mikroorganismer involveret i AU-processen samt undersøgelse hvordan det mikrobielle samfund håndterer operationelle ændringer og forstyrrelser, vil give indsigt i dynamikken af AU økosystemer og hvordan teknologien kan optimeres yderligere. Formålet i dette ph.d.-projekt var at bidrage til at opnå en bedre forståelse af den mikrobielle samfundsdynamik i fuldskala AU-systemer. Ny sekventeringsteknologi blev anvendt til at undersøge mikroorganismerne fra enkeltpunktsmålinger af et stort antal fuldskala-anlæg. Desuden blev en række tidsserier indsamlet fra AU-systemer af særlig interesse. De indsamlede mikrobielle data blev analyseret for at beskrive diversiteten af de mikrobielle samfund involveret i metanisering, med fokus på anlæg som behandler husdyrgødning og madaffald, samt at undersøge mikrobiel og funktionel stabilitet af fuldskala AU-systemer under (u)stabil kørsel. Korrelationer mellem det mikrobielle samfund og operationelle AU egenskaber blev også undersøgt.

De første to studier i denne afhandling beskriver undersøgelser af henholdsvis AU behandling af husdyrgødning og madaffald. I hver undersøgelse blev et stort antal biogasanlæg analyseret ved hjælp af 16S rRNA og *mcrA* gen sekventering for at fange de mikrobielle samfund associeret med disse AU substrat typer. I det første studie blev der observeret stor lighed i de mikrobielle samfund af gødningsbaserede biogasanlæg, uafhængig af reaktortemperatur og substrat sammensætning. Høj abundans og mangfoldighed af det bakterielle phylum *Firmicutes* karakteriserede disse reaktorer. Det andet studie fandt kontrasterende resultater; en klar separation af microbielle økosystmer baseret på temperatur og primære substrater blev observeret i reaktorer, som behandlede madaffald som et primært substrat. Desuden indeholdt de mikrobielle samfund en rig tilstedeværelse af syntrofe bakterier og en høj grad af redundans i det metanogene samfund, hvilket tyder på et stort potentiale for stabilitet og tilpasning.

Mikrobiel analyse af de funktionelle population som metanogener kan være svær at undersøge med tradionelle 16S rRNA gen baserede metoder, på grund af deres lave forekomst i AU systemer og deres variende antal. De tredje studie beskriver udviklingen af et amplikon sekventerings baseret teknologi rettet mod den funktionelle markør *mcrA* (unikt i metanogener), og viste at det var muligt at opnå langt højere mikrobiel samfundsdata opløsning sammenlignet med ækvivalente 16S rRNA baserede teknikker.

Indsigt i hvilke mikroorganismer der er associeret med hvilke substrater og driftstemperaturer, samt deres evne til at immigrere i blandede systemer, er af stor betydning for forståelse og optimering af AU-systemer. Det fjerde studie anvendte parvis statistisk sammenligning af de mikrobielle samfund fra ph.d-projektets samtlige undersøgte AU anlæg. Resultatet viste, at det var muligt adskille et komplekst mikrobiel økosystem i grupper af substrat associerede organismer, samt identifikation af potentielle generalister. Spildevandsslam indeholdt de fleste substratafhængige organismer sammenlignet med madaffald og gødning, især inden for *Proteobacteria*, *Actinobacteria* og *Chloroflexi*. Undersøgelsen afslørede også potentielle nye organismer af interesse for nærmere undersøgelse.

Mikrobielle reaktioner på to forstyrrelsesscenarier blev undersøgt ved at undersøge tidsseriemålinger i femte og sjette studie. Driftstemperaturen er en af de primære effekter af mikrobiel samfundsstruktur i AU-systemer, og det er derfor af stor betydning at få en dyb forståelse for, hvordan AD økosystemer håndterer (naturlig) variation i temperatur. Det femte studie omfatter en undersøgelse af tre fuldskala anlæg, der opererer inden for det mesofile spektrum. Den mikrobielle undersøgelse viste, at mindre temperaturændringer (± 2 ° C) håndteres af det mikrobielle samfund uden at påvirke den operative ydeevne. Dette kunne sandsynligvis tilskrives det mikrobielle samfunds medfødte modstandsdygtighed og redundans. Endvidere forårsagede en temperaturforøgelse på 4 °C i et mesofil biogasanlæg en ændring i de mikrobielle populationer, især inden for de syntrofe acetatoxiderende og

metanogene populationer, hvorefter biogasanlæget stabiliseredes ved den nye temperatur. Reaktoren beholdt den nye sammensætning gennem efterfølgende temperatur-udsving. I den sjette undersøgelse blev en AU-systemforstyrrelse i form af sult undersøgt i en realistisk laboratorieskala opsætning. Efter 60 dages udsultning viste de undersøgte reaktorer tegn som svarende til dem, der blev observeret under overbelastningstilstande, sandsynligvis på grund af det genoprettede foderregime uden ændring. Store udsving blev observeret i de mikrobielle samfund, og efter 45 dages gendannelse var systemerne endnu ikke fuldt stabiliseret. De opnåede resultater fremhæver betydningen af regelmæssig digester vedligeholdelse og viser, at mens mikrobielle samfund i AU-systemer har evnen til at komme sig fra forstyrrelser, er der begrænsninger for deres evner.

Mikrobiel styring af AU-systemer er blevet foreslået som en måde til at forbedre nuværende metoder til AU-system operation. En dyb forståelse af dynamikken i de mikrobielle samfund er nødvendig for at kunne arbejde hen imod mikrobiel manipulation af AU-systemer. Den afsluttende undersøgelse af afhandlingen afspejler den nuværende status for mikrobiel styring i AU-systemer og de nuværende værktøjer, der er tilgængelige for overvågning af AU-systemer fra det operationelle og mikrobielle synspunkt. Praktiske eksempler på nyttige mikrobielle samfundsoplysninger, som kan opnås ved at monitorere fuldskala AU-systemer diskuteres. Sammenfattende viste undersøgelsen, at det er muligt at udtrække informationer, der kan anvendes til procesoptimering ved hjælp af de nuværende værktøjer, men at yderligere udvikling af eksisterende teknologier er nødvendige for at opnå ægte mikrobiel styring. For at opnå en dyb forståelse af mikrobiel dynamik under AU-drift er der brug for mere præcise og praktiske metoder til (mikrobiel) overvågning; hyppige undersøgelser af fuldskala scenarier vil hjælpe med at opdage indikatorerne for proces og mikrobiel respons, som er påkrævet for at optimere AU systemstyring.

Samlet set fremhæver de kombinerede resultater af denne afhandling brugen af mikrobielle undersøgelser i AU-systemer for at opnå en bedre forståelse af AU mikrobiologien. Undersøgelser af AU mikrobiologiske samfund kan uddybe forståelsen af ikke kun "arbejdsheste" i AU-processen, selve mikrobielle samfundet, men også deres dynamiske natur og reaktioner på operationelle ændringer, som alle er nødvendige for at opnå yderligere optimering af teknologien. Dette ph.d.-projekt har givet ny viden om den mikrobielle samfundsstruktur af AU-systemer, der behandler madaffald og gødning som substrat og mikrobiel respons på almindelige driftsforstyrrelser. Derudover blev der udviklet et lovende nyt værktøj til den detaljerede undersøgelse af metanogene samfund i AU systemer. Desuden vil det være muligt at opnå mikrobiel styring ved at anvende mikrobiel samfundssinformation til operationsprocessen; Kontrol og (bio) engineering af metaniseringssystemer mod deres optimale mikrobielle konfiguration.

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Objectives of the PhD project

The overall objective of this PhD project was to utilise next-generation molecular technologies (amplicon sequencing) and bioinformatics using multivariate statistics to study and identify the microbial communities involved in anaerobic digestion of organic waste, with focus on manure and food waste based AD systems. Furthermore, the functional and microbial stability of the studied full-scale AD systems were to be investigated, and correlations between operational information and microbial community data were to be identified. Microbial investigations were performed on single point measurements, and time series from full-scale and lab-scale AD digesters.

The aims of the individual studies included in this PhD thesis were as follows:

Paper 1 In order to improve knowledge of microbial communities involved in AD of manure, the microbial communities associated with full-scale anaerobic digesters fed with manure as the primary substrate, as well as potential interactions within the microbiome of manure based AD were investigated.

Paper 2 To investigate the microbial communities associated to food waste AD, and to investigate the influence of food waste on the AD microbiome, the microbial community structure of full-scale anaerobic digesters fed food waste as variable proportions of the substrate composition were characterised.

Paper 3 Development and application an amplicon sequencing based assay for the detailed analysis of methanogenic communities via the *mcrA* gene, and construction of a corresponding taxonomy for *mcrA* gene sequence classification.

Paper 4 In order to investigate how different organic waste types contribute to AD microbiomes, the microbial communities of a large number of full-scale digesters were compared statistically to identify substrate associated microorganisms of manure, food waste and wastewater sludge.

Paper 5 To gain a better understanding of the influence of temperature on the AD microbiome, a detailed microbial investigation of mesophilic full-scale and lab-scale anaerobic digesters experiencing variable degrees of temperature fluctuation was carried out.

Paper 6 To improve the understanding of AD systems experiencing starvation and the subsequent recovery, a detailed microbial investigation of lab-scale thermophilic reactors exposed to prolonged starvation was carried out.

Paper 7 Summarise current tools molecular tools for microbial monitoring and provide a future outlook for application of microbial community data toward microbial management.

Knowledge gathered by the project is to be applied toward obtaining a deeper understanding of the AD process and the microbes involved, and to work towards microbial management of full-scale AD operation. This PhD project was part of a work package under the NomiGas project (funded by the Danish Council for Strategic Research), which was implemented in order to create a new collaborative platform for research in AD technology from the microbiological and operational standpoint.

List of supporting papers

Paper 1:

Microbial network analysis of 28 full-scale biogas digesters processing mixed manures.
Nadieh de Jonge, Maja Nielsen, Henrik Bjarne Møller, Jeppe Lund Nielsen. In preparation.

Paper 2:

Dynamics of food waste based AD ecosystems.
Nadieh de Jonge, Åsa Davidsson, Jes la Cour Jansen, Jeppe Lund Nielsen. In preparation.

Paper 3:

Assessment of high-throughput functional marker analysis of the *mcrA* gene for improved resolution in methanogenic community studies.
Nadieh de Jonge and Jeppe Lund Nielsen. In preparation.

Paper 4:

Dissecting mixed AD systems: Microbial community contributions of major organic waste types.
Nadieh de Jonge, Åsa Davidsson, Henrik Bjarne Møller, Jes la Cour Jansen, Jeppe Lund Nielsen. In preparation.

Paper 5:

Effect of temperature change on the microbial communities in full-scale AD within the mesophilic spectrum.
Nadieh de Jonge, Åsa Davidsson, Jeppe Lund Nielsen. In preparation.

Paper 6:

Microbial population dynamics in continuous anaerobic digester systems during start up, stable conditions and recovery after starvation.
Nadieh de Jonge, Veronica Moset, Henrik Bjarne Møller, Jeppe Lund Nielsen (2017). Published in *Bioresource Technology*, 232, p313-320.

Paper 7:

Towards microbial management in anaerobic digestion.
Nadieh de Jonge, Åsa Davidsson, Jes la Cour Jansen, Jeppe Lund Nielsen. In preparation.

Introduction

1.1 Anaerobic digestion: Technology and analysis

Anaerobic digestion (AD) is a natural process in which organic biomass is degraded through microbial conversion. This process yields reduced biomass and biogas, consisting of 45 – 80 % methane gas (CH_4), 20 - 55 % carbon dioxide (CO_2) and minor fractions of other gases such as nitrogen (N_2), oxygen (O_2), hydrogen (H_2) and hydrogen sulfide (H_2S) (Nizami, 2012). This process naturally occurs in marshes, at landfills and in ruminating animals (Nizami, 2012). Since the beginning of the 20th century, the AD process has been harnessed by humans for production of biogas as a form of renewable energy and reduction of substrates for management of organic waste (Vasco-Correa et al., 2017). As the need for an energy economy based on renewable sources grows, so does the need for research to optimise our existing technologies. Fossil fuels currently service over 85 % of the total world energy demand, and this has had a significant detrimental effect on the environment (IEA, 2015). In order to reduce the output of greenhouse gases (GHG) and the impact of global warming, application and development of renewable technologies such AD and other sustainable technologies need to be expanded and accelerated to achieve the goal of a "green energy economy".

AD for waste management and renewable energy generation

Anaerobic digestion for the production of biogas is considered a very promising technology within the fields of waste management and energy recovery. AD has been used as a way to stabilise or reduce organic waste (waste management) for many years, but in recent years the technology is being used for the production of renewable energy (Vasco-Correa et al., 2017). A large range of organic waste types, including industrial waste(waters), municipal food waste and wastewater, animal manures and energy crops can be subjected to the AD process in order to produce biogas for conversion to heat and electricity, or upgrading to biofuels (Amani et al., 2010; Korres et al., 2013; Ruile et al., 2015). The leftover solid fraction can be used as organic fertiliser and is being used for the production of other biological products, such as fish food (Sawatdeenarunat et al., 2016).

The first industrial application of anaerobic digesters dates back to the first half of the twentieth century. Its popularity grew until around 1950, at which point fossil fuels became difficult to compete with due to their low prices and availability. The use of AD regained some attractiveness as a renewable energy source after the energy crisis in the 1970s, but implementation remained slow due to high cost and limited knowledge of operational strategies (Vasco-Correa et al., 2017). The use of AD technology has increased exponentially in recent years; the amount of organic waste processed grows each year (Appels et al., 2011), and an estimated annual increase in biogas production yield of 11.2 % has been recorded since 2000 (WBA, 2017) (Figure 1a).

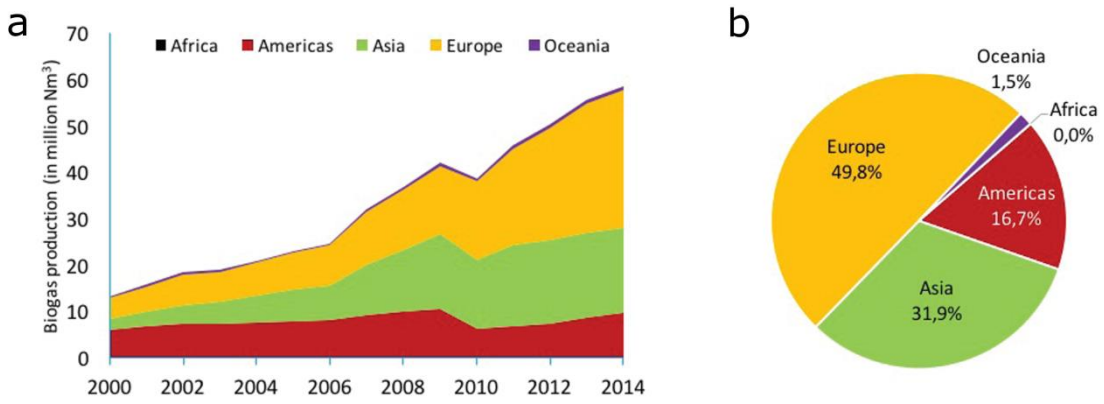

Figure 1: Global production of biogas 2000-2014 (a), and distribution of total biogas production per continent (b). Figure modified from WBA (2017).

In fact, many forms of renewable energy are experiencing a surge in development and application, as a result of the need for change in order to combat global warming. In 2014, a total of 128 gigawatts (GW) of renewable energy was generated using technologies including wind, water, solar and biomass based power from biogas production; this is an increase of over 40 % since 2010 (IEA, 2015), and totals 14.1 % of the total world energy supply (WBA, 2017).

Europe is the world leader in implementation of AD technology and biogas production; almost half of the 59 billion m^3 biogas produced in 2014 was recorded within the European Union (WBA, 2017) (Figure 1b). More than 14,000 biogas plants were operating throughout Europe in 2014, of which over 9,000 are located in Germany (Figure 2). A large number of the European biogas facilities (72 %) process agricultural waste; it is the primary feedstock source of digesters located in Germany and Italy. The primary feedstock component in the United Kingdom and Sweden is sewage sludge, while other countries have a mixed substrate distribution that also includes landfills and other organic waste types such as household waste (EBA, 2014).

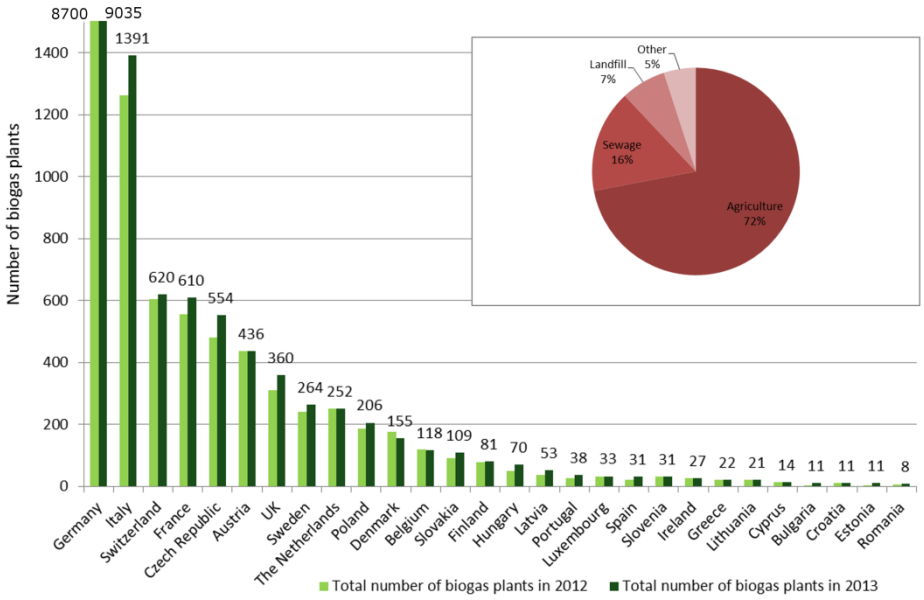

Figure 2: Number of biogas plants in Europe in 2012 and 2013, by country. Insert: Distribution of biogas plant types in Europe in 2013. Modified from EBA (2014).

While developed countries primarily invest in biogas production for the reduction of organic waste and production of green energy, AD has also shown promise in developing countries. In developing countries, biogas is produced at small-scale digesters, often at households and farms, and the produced biogas is used as a low-cost source of clean energy for light and cooking (Bond and Templeton, 2011; Nizami, 2012). The continent of Africa reported the highest percentage energy generated from renewables in 2014; namely 49.6 %, against 12.4 % and 10.3 % in the Americas and Europe, respectively (WBA, 2017). This highlights the suitability of AD as a technology for energy generation at diverse scales and setups.

Biogas production in Denmark and Sweden

Denmark is primarily known as a world leader in the wind power industry, however, implementation of other renewable technologies including AD for biogas productions are also considered crucial for achieving Danish climate goals (IEA, 2015). The first biogas facilities in Denmark were built as a response to the oil crisis in the 1970s (Raven and Gregersen, 2007). Located largely at farms, the produced biogas mostly served as a source of heat and as a substitute for oil usage at pig farms (Lybæk and Kjær, 2015). These initial biogas facilities experienced a lot of operational issues due to limited knowledge, and development of the technology progressed slowly (Mæng et al., 1999; Raven and Gregersen, 2007). In 1987, the Danish government instated a plan for the establishment of centralised biogas plants, in order to develop biogas technology as a source of renewable energy in Denmark (Mæng et al., 1999). Since then, production of biogas through AD has experienced a strong development and expansion. In 2013, a total of 154 biogas installations were registered in Denmark (Figure 3a),

divided across centralised facilities processing manure, wastewater treatment plants, junk yards and recycling stations, industrial biogas plants and decentralised farm scale plants (Figure 3b) (Nielsen, 2014).

Centralised plants process mixtures of different organic waste types, including animal manures (80 %), sludge from wastewater treatment (1 %), food industry waste (15 %), and other waste types such as energy crops and household waste (4 %) (Figure 3c) (Lybæk and Kjær, 2015; Mikkelsen et al., 2016). The manure utilised for Danish biogas production is composed of cattle (53 %), pig (38 %), poultry (1 %), mink (4 %) and mixed manures (4 %) (Figure 3d) (Mikkelsen et al., 2016). Biogas produced from centralised plants is primarily used for combined heat and power generation (CHP), and heat distributed locally or regionally, and electricity is sold to the national energy grid (Raven and Gregersen, 2007). Smaller farm scale plants generally process available manures and lignocellulosic agricultural waste such as grasses from the farm itself. The produced heat is used for heating of stables and other farm buildings, and the electricity is sold to the national net (Lybæk and Kjær, 2015).

Biogas production in Denmark is on experiencing a strong development, and is projected to more than double in generated energy output from 4.3 PJ/year in 2013, to approximately 10 PJ/year in 2020. However, the estimated biogas potential in available Danish biomasses is approximately 50 PJ/year (Nielsen, 2014), which highlights that there is still a lot of room for improvement. Denmark's current green energy plan ("Grøn Vækst aftale") states that 50 % of all agricultural waste (primarily manures) is to be treated by AD for biogas production by 2020 (Lybæk and Kjær, 2015). In order to achieve this goal, the production capacity of the existing biogas facilities has to be improved, and new installations added to the network. Primary challenges for expansion of Danish biogas production include biomass acquisition, gas utilisation and strict environmental regulations, all of which complicate establishment of economically viable AD on a large scale. Biogas production purely for the generation of heat is currently not competitive on the Danish energy market, and thus process optimisation through research into both operational and microbial aspects of the AD process are considered a high priority (Møller and Nielsen, 2015; Nielsen, 2014).

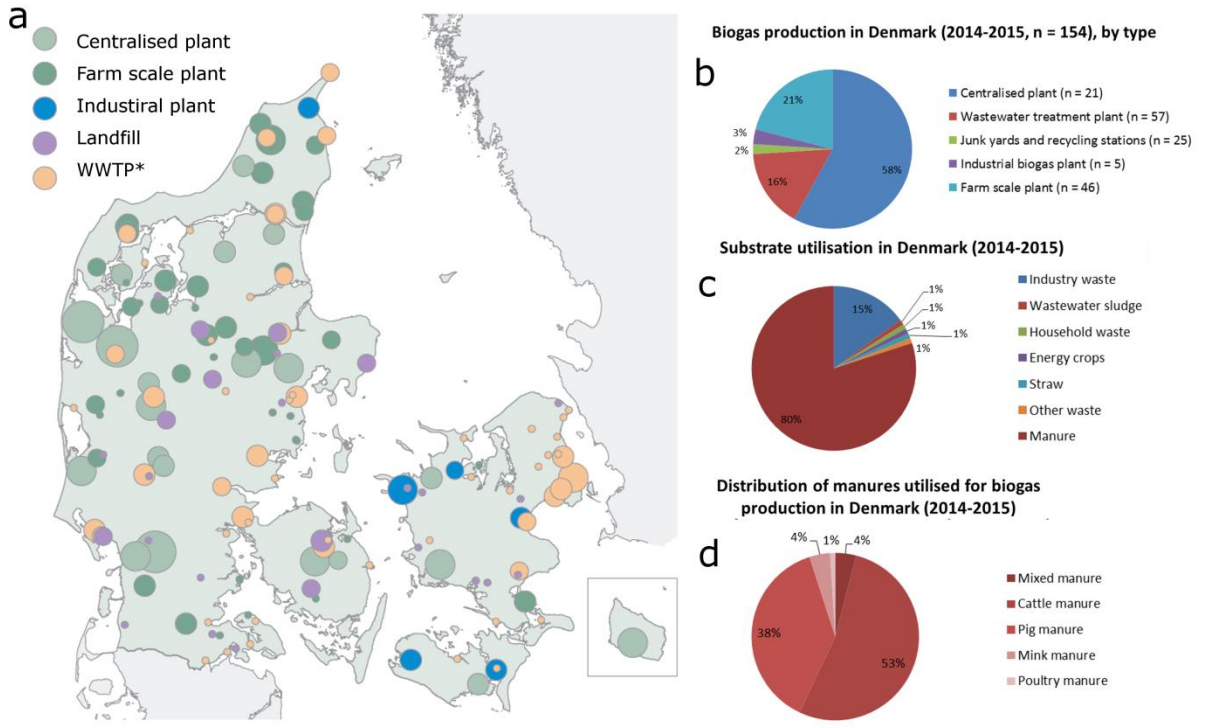

Figure 3: Biogas production in Denmark. Localisation of Danish biogas plants by type, the size of the circles indicates annual biogas production (a), biogas production by plant type (b), substrate utilisation at centralised AD facilities (c) and composition of manure utilised as a substrate for AD (d). *WWTP = Wastewater treatment plant. Modified from Mikkelsen et al. (2016) and Nielsen (2014).

First documented anaerobic digestion applications in Sweden were applied at wastewater treatment plants, with the intention of reducing biomass and sanitation of sludge, in the 1940s. During the developmental years of the

AD technology, biogas produced from organic waste reduction was regarded more as a by-product of AD, rather than a source of energy. As a result, the gas was often used for internal process heating, and the rest was flared (Olsson and Fallde, 2015). Since the 1970s, a diversification of biogas production has occurred in terms of localisation, substrate usage and biogas utilisation. In order to improve the management of waste and environmental impact, biogas facilities expanded into industry and to landfills. It was during this transition that the synergistic relationship between waste management and energy production came into focus (Olsson and Fallde, 2015). Farm-scale AD setups are established in Denmark and Germany (Negro and Hekkert, 2008; Raven and Gregersen, 2007), but experienced a lagged implementation in Sweden, primarily due to financial troubles caused by technological difficulties (Olsson and Fallde, 2015). In recent years, farm-scale facilities have become more attractive for implementation once again, and a significant number (41 in 2016) have since been built (Lantz et al., 2007; Westin and Harrysson, 2015).

In 2016, Sweden collectively produced 7.48 PJ in energy from biogas produced at 279 biogas facilities across the country, an increase of 54 % since 2005 (Westin and Harrysson, 2015). This places Sweden in the top 10 biogas producing countries in Europe, ranked 8th in 2013 (EBA, 2014). As only a small fraction (5 – 10 %) of the total biogas potential is currently utilised, producing biogas as a means of generating renewable energy and biofuels is a focus point in the Swedish energy strategy, and many initiatives throughout the country have been introduced in recent years (Lantz et al., 2007). The primary purpose of the produced gas is upgrading to fuels, 64 % of total gas yield is allocated to upgrading facilities (2016). A prominent product of the upgrading process is the vehicular fuel LBG (liquid biogas). Upgraded biogas is also injected directly into Sweden's natural gas network, for use in households (Lantz et al., 2007; Westin and Harrysson, 2015).

Overview of general biogas plant setup and operation

Biogas production through anaerobic digestion is a complex process that requires specialised and well-designed facilities in order to achieve optimal yield and efficiency. The primary components in a basic AD setup include a pre-mixing tank or substrate storage tank, an anaerobic digester and appropriate storage areas for the produced biogas and the digestate (Figure 4) (Nizami, 2012). Process related peripheral facilities such as pre-treatments, multi-stage AD process steps and post-treatment areas make that the average biogas plant has a complex infrastructure (Pöschl et al., 2010).

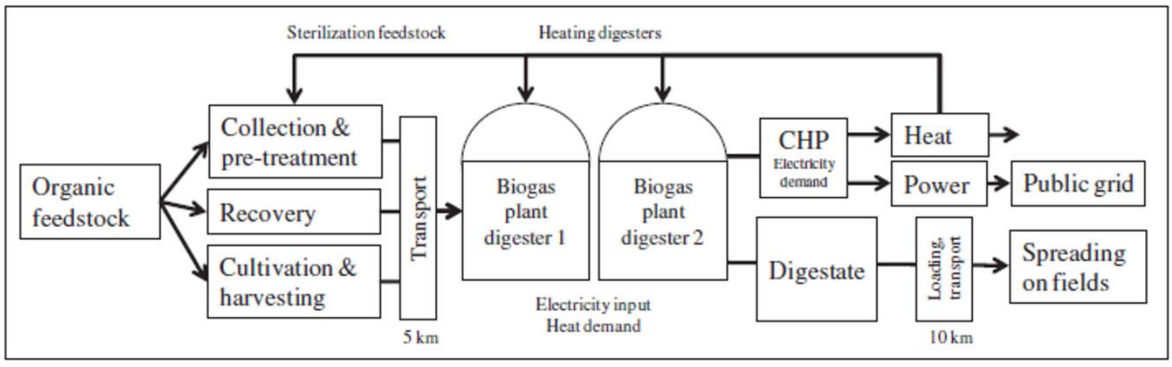

Figure 4: Simplified overview of co-digestion AD facility. Modified from Pöschl et al. (2010).

The performance of an AD process is primarily determined by methane yield and solids reduction, both of which are dependent on the rate of the AD process itself. The rate at which biodegradation progresses is immediately controlled by rate-limitation; for the AD process this is often considered to be the hydrolysis of complex organic matter (Adekunle and Okolie, 2015; Appels et al., 2008; Carlsson et al., 2012). Certain low biodegradable substrates, such as those with a high percentage of lignocellulosic material, can cause severe limitation and even instability of the AD process (Sawatdeenarunat et al., 2015). Ensuring optimal biodegradability of the digester feedstock is highly important for achieving the best possible biogas yield, and a stable process. Therefore, pre-treatment of the substrate in order to improve degradability may be required before efficiency AD can be achieved. Over the last 30 years, a number of pre-treatment types have been implemented successfully, including thermal, chemical, and mechanical treatments (Carlsson et al., 2012).

Thermal hydrolysis pre-treatment (THP) is one of the most commonly applied pre-treatments at full-scale AD facilities, and can be applied to all substrate types (Ariunbaatar et al., 2014), although it is most commonly

observed at wastewater treatment facilities (Barber, 2016). During THP, biomass is heated to low (< 100 °C) or high (> 100 °C) temperatures, often under pressure, in order to improve solubilisation. After treatment, the substrate is cooled down and fed into the digester. This technology has been used successfully in a number of countries since the 1970s (Carlsson et al., 2012). Chemical pre-treatment methods are based on the use of chemicals in order to improve the biodegradability of substrates such as animal slurries (Fangueiro et al., 2015) and lignocellulosic biomass (Sawatdeenarunat et al., 2015). Treatment methods can include oxidation (Carlsson et al., 2012), acidification (Moset et al., 2016) and basification (Sträuber et al., 2015). Pre-treatment of substrates has a significant effect on the biomass itself, but also shapes the microbial community of the digester processing it (Kirkegaard et al., 2017; Pervin et al., 2013).

The most commonly used reactor types for full-scale AD systems include the continuously stirred tank reactor (CSTR) and upflow anaerobic sludge blanket reactor (UASB) (Mao et al., 2015). The choice of reactor type is generally based on the intended operational strategy; factors such as substrate composition, dry or wet process, operational temperature and the complexity of the AD process determine the choice of digester (Nizami, 2012). A CSTR is suitable for AD of many waste types; especially those with a high concentration of suspended solids (SS) during the digestion process, such as animal manures and (industrial) wastewaters. The consistent mixing ensures continuous contact between biomass, substrates and microbes, but uses a sizeable amount of energy. Therefore, a multi-stage CSTR process is becoming more common. The UASB type reactor can process much higher organic loads (OLR) at lower retention times (HRT), but has a greater dependence on the physical shape of the resident microbial community, through granulation (Mao et al., 2015).

Operational choices of process parameters have significant influence on the efficiency and characteristics of the AD process they govern. Major influencing parameters include substrate choice and feeding method, HRT, OLR, reactor temperature, among others (Nizami, 2012) (Table 1). Operational choices and their effects are not limited to the operational side of AD; they also have major impacts on the microbial communities residing in the digester (Amani et al., 2010). The effects of individual operational parameters on the microbial ecosystem of the AD process are discussed in detail in section 1.3.

Table 1: Factors that can affect the design and operation of an anaerobic digester. Modified from Nizami (2012).

Factors that can affect anaerobic digester design and operation	
Pre-treatment	Co-digestion, Temperature, Physical, Chemical, Biological and Thermal pre-treatment methods, Inoculum, Nutrition addition
Operational strategy	Reactor temperature (RT), pH, Total solids (TS), Volatile solids (VS), Organic loading rate (OLR), hydraulic retention time (HRT), Particle size, Mixing
Post-treatment	Biogas, Biomethane, Fertiliser, By-products

Many organic biomasses can used for AD, but in general substrates can be classified as one of 5 types; wastewater sludge (WWS), (organic) food waste, food industry waste, manures and energy crops (also called agricultural residues) (Carlsson et al., 2012). Before application, new substrate types are generally analysed (waste analysis) in terms of solids content, nutrient composition and biomethane potential (BMP), in order to assess how best to utilise the biomass in full-scale situations (Vasco-Correa et al., 2017). An overview of basic characteristics of commonly used AD substrates is displayed in Table 2.

Efficient and stable AD processes require an appropriate and balanced nutrient availability (often expressed as carbon to nitrogen or C/N ratio) (Adekunle and Okolie, 2015). The nutrient balance of (organic) food waste is difficult to estimate due its dynamic nature, and therefore variable content of carbohydrates, proteins and lipids (Dhamodharan and Kalamdhad, 2014). The high solids content of food waste often causes extra cost in the form of dilution water, which is one of the reasons why it is often used in co-digestion with other waste types (Zhang et al., 2014). Similarly, animal manures posses a high concentration of ammonia, and are generally co-digested with substrates low in nitrogen (Appels et al., 2011). Energy crops and other agricultural residues have the highest theoretical biogas potential, but need either pre-treatment or co-digestion with other substrates such as manures in order to achieve a stable and efficient process, as their lignocellulosic nature makes them recalcitrant (Weiland, 2010). High fat content has the highest methane potential, but require a high retention time due to their poor biodegradability (Weiland, 2010).

Table 2: Characteristics of major AD substrates. Modified from Vasco-Correa et al. (2017).

Characteristics of major AD substrates				
Substrate type	Characteristics	Biogas yield (m^3 / kg VS)	Total solids (%)	C / N ratio
Animal manure	Usually co-digested with bedding material or other high-carbon biomass	0.1 - 0.6	2 – 20	3 - 15
	High buffer capacity			
	Relatively high in ammonia			
	Rich in nutrients and trace elements			
(Municipal) food waste	Needs pre-processing	0.3 - 0.8	5 – 50	15 – 35
	Requires size reduction			
	High variability in composition			
	Highly digestible, may generate inhibition through acidification			
Agricultural residues and energy crops	Abudantly available	0.2 - 0.5	20 – 80	40 – 150
	High (ligno)cellulose content			
	Needs pre-treatment to enhance biodegradability			
	Can be ensiled for storage			
Sewage sludge	Byproduct of wastewater treatment	0.8 - 1.2	20 – 35	40 – 70
	High in solids and nutrients, but also potential for pathogens			
	Low digestibility, pre-treatment or co-digestion may improve it			

Digesters operating under mesophilic and thermophilic temperatures have significantly different characteristics in terms of operation, and differ even more so in microbial community composition (De Vrieze et al., 2015; Moset et al., 2015; de Jonge et al., unpub; Paper 1; de Jonge et al., unpub; Paper 2). Thermophilic temperatures increase the reaction rates of some AD processes, including hydrolysis, allowing for shorter HRTs and higher potential biogas yield (Hagos et al., 2017). Furthermore, thermophilic operation has been shown to be preferable for waste types such as animal manures (Gagliano et al., 2015). RT is therefore considered one of the greatest effectors of AD operation (Amani et al., 2010). In Europe, a large proportion of the anaerobic digesters are operated under mesophilic temperatures (35 – 40 °C). Psychrophilic (15 - 25 °C) and thermophilic (45 – 60 °C) are uncommon, although this is starting to change. In Denmark, more thermophilic digesters are now more abundant than mesophilic ones, and this trend is spreading to other countries (Nizami, 2012).

A digester's retention time refers to the time required to fully breakdown the organic waste inside. Retention time can be described in two different ways; solids retention time (SRT) is the time (in days) that the microbes remain in the digester and the hydraulic retention time (HRT) is the duration the biomass is present in the digester (Mao et al., 2015). The latter is generally used as an operational parameter to monitor the AD process. Full-scale digesters generally operate under a HRT between 15 and 30 days (Mao et al., 2015). HRT needs to be sufficiently long for the microbial community of a digester to degrade the available substrate (Ruile et al., 2015); therefore the substrate composition, HRT, operational temperature and organic loading rate (OLR) need to be well matched in order to maintain a stable process (Hagos et al., 2017). Low HRT can cause operational problems including VFA accumulation (Ruile et al., 2015) and washout of slow growing microbial populations such as acetogens, both of which can destabilise the AD process (Mao et al., 2015).

The organic loading rate of a digester is the rate and volume at which a digester is fed over time. Fluctuations to OLR frequently occur in day to day digester operation, primarily due to the dynamic nature of certain substrates

(such as food waste) (Regueiro et al., 2015). Increases to OLR can lead the digester to go into a state of overloading, which results in process deterioration and can have serious consequences for the microbial communities (Regueiro et al., 2015). Adversely, if the OLR is too low, or fluctuates a lot (discontinuous feeding) a digester can begin to starve, which are highly disruptive to the AD process and require long recovery times (de Jonge et al., 2017; Paper 6). In extreme cases, process failure has also been recorded (Regueiro et al., 2015).

Other effectors of AD operation include pH, ammonia and volatile fatty acid (VFA) concentration, nutrient balance, among others. However, these variables are often a result of other operational parameters mentioned above, and have a greater effect on the microbial communities than the digester operation itself. The effects of these variables are further discussed in section 1.3.

After biomass is treated with AD, a reduced fraction of nutrient-rich material remains, also called the digestate. Based on the composition of the original substrate, the digestate can be used directly as fertiliser, or be processed further in to other bioproducts (Holm-Nielsen et al., 2009). The use of digestate can serve as a replacement for chemical fertilisers produced through fossil fuels, as the high nutrient content is well suited for spreading on farmland. Furthermore, a part of the liquid digestate can be recirculated back into the AD system in order to reduce water usage, and thereby cost (Nizami, 2012). Further processing of digestate into useful products and chemical compounds has gained interest in recent years through the biorefinery concept (Sawatdeenarunat et al., 2016).

In order for biogas to be suitable for utilisation, it needs to be cleaned. A number of toxic compounds can be present in the biogas, including H_2S (hydrogen sulfide). Various methods for scrubbing and cleaning biogas are employed to remove the toxic components before the gas is upgraded (Nizami, 2012). One of these methods is biological desulfurisation, where a small amount of air (2 – 5 %) is injected directly into the raw biogas (Weiland, 2010). The upgrading process for biogas (generally to biomethane) involves removal of unwanted impurities and the removal of CO_2 (Olsson and Fallde, 2015). The most common utilisation of biogas is generation of heat and power, and as such most biogas facilities have combined heat and power conversion process in their infrastructure (Raven and Gregersen, 2007). Biogas is combusted directly in gas engines or turbines, and the generated energy is divided between local use at the plant, and injection to heat and electricity networks (Madsen et al., 2011). Some countries, such as Sweden and the Netherlands also upgrade biogas to vehicular fuel (LBG), as an alternative for fossil fuel based ones like gasoline (Appels et al., 2011; EBA, 2014).

Optimisation of AD technology: challenges and opportunities

Anaerobic digestion is has become an attractive method of producing renewable energy from organic waste, and implementation of the technology increases steadily every year. However, the technology has a number of challenges and unanswered questions that need to be answered in order to further optimise operation and economical viability. Primary areas of opportunity for optimisation include monitoring and (microbial) management of the AD process, substrate utilisation, knowledge of AD microbial communities and utilisation of biogas and degradation products.

The AD process is affected by a complex set of variables from the operational, chemical and microbial aspects of the process. This complicates efficient real-time monitoring and prediction of AD process behaviour (Appels et al., 2011; Hagos et al., 2017). It has therefore been suggested that mathematical modelling can be a useful extension of process management. For this purpose, the Anaerobic Digestion Model (ADM) was developed as a tool for improving operational understanding and process prediction (Batstone et al., 2002). However, this model does not account for the synergistic nature of the microbial communities involved in the process, and can therefore not be applied to prediction of e.g. stability. Modelling approaches need to be combined with a deeper understanding of AD microbial ecosystems in order to allow for better modelling of the AD process (Hagos et al., 2017).

Co-digestion of organic waste types has been suggested as way to overcome drawbacks of individual biomass types, and enhance biogas production yield compared to single substrate AD. An example of this is the recommendation to digest agricultural residues together with animal manures to improve digestion of lignocellulosic biomass, and stabilising the digestion of manures (Hagen et al., 2014; Hagos et al., 2017). In addition to enhancing biogas production and improving digestion efficiency for individual substrates, co-digestion is also suggested to improve process stabilisation, enhance synergistic interactions of the microbial

communities of AD (Hagos et al., 2017; de Jonge et al., unpub; Paper 2; de Jonge et al., unpub; Paper 4) and provide economic advantages as equipment and costs for processing waste types are shared. One of the remaining challenges of co-digestion systems is that balancing the different substrates to obtain an efficient AD process can be difficult due to the sometimes highly different characteristics of the individual substrates, and further work is needed to optimise this type of waste treatment system (Hagos et al., 2017).

The microbial communities involved in the AD process are highly complex and dynamic in nature. Current knowledge of the microbes involved, how they interact and how they react to operational changes is still limited (Carballa et al., 2015). Because of this, unexplained (persistent) operational problems and process instabilities remain a common occurrence during daily operation (Appels et al., 2011; Weiland, 2010). Monitoring of AD systems is primarily performed through measurement of operational variables such as methane production and degradation efficiency, or chemical measurement of intermediate products such as VFA (Madsen et al., 2011). A drawback of these monitoring methods is that they do not provide real time information regards process stability; measurements are always a step behind due to the dynamic nature of the AD process (Carballa et al., 2011). Obtaining a deeper understanding of the microbial communities and their responses to process variation will lead to improved stability and operation of AD systems (Weiland, 2010). Furthermore, microbial studies of realistic operational situation and problems will not only reveal how AD microbes handle change, but may also lead to the identification of indicator species and behaviours to improve monitoring of the AD process in real time. Moreover, microbial knowledge of different AD process aspects will lead to new developments in regards to microbial management; manipulation and engineering of microbial communities through operation to achieve greater stability and performance of a digester (Carballa et al., 2015). The current state and opportunities for microbial management are further discussed in section 1.4.

NomiGas project

The NomiGas project (http://www.en.bio.aau.dk/nomigas) aims to establish a novel microbiological platform for improvement and optimisation of biogas production from organic waste through the use of next generation molecular technologies including (amplicon) sequencing.

The project is divided into four research areas:

- (WP1) Digester survey and microbial community structure
- (WP2) Novel microbiology platform
- (WP3) Optimisation of methane production from animal manures
- (WP4) Optimisation of methane production from sludge from wastewater treatment plants

The total knowledge gathered by this project will contribute to the general knowledge of the AD microbiome, specifically a catalogue of (key) microorganisms identified in AD systems, their potential function and indicator species for process changes. Furthermore, microbial data gathered from single point and time series measurements at full-scale installations will be applied towards microbial ecology questions regarding (functional) stability and operational situations. Moreover, the gathered information may be applied toward improving AD operation, maintenance of stability, solving operational problems and optimisation of AD installation design. Participants in the NomiGas project include universities, research facilities, industry partners and biogas facilities in Denmark and Sweden. Primary substrates of interest were defined as manure, (municipal) food waste and wastewater sludge.

Microbial analysis of the AD process

The study of microbial ecology to understand biological processes has been performed for many years. Traditional microbiological techniques allowed for isolation and study of small numbers of microorganisms (culture dependent studies), but these studies were limited by the fact that only a very small percentage of microbes can be cultivated outside of their natural environment (Hugerth and Andersson, 2017), and studying microbial ecosystems on a large scale was not possible. In 1977, microbiological studies were revolutionised by the discovery of the 16S rRNA gene as a tool for identification of prokaryotes (Woese and Fox, 1977). The 16S rRNA gene is part of the small subunit of the prokaryotic ribosome, a highly conserved gene that is present in all prokaryotes (Figure 5), while containing variable regions that allows for distinction between closely related

organisms (Talbot et al., 2008). The theoretical species threshold for microorganisms has often been set at 97 % 16S rRNA gene sequence similarity as per definition, and results obtained from 16S rRNA gene based studies are therefore often expressed as Operational Taxonomic Units (OTUs).

The discovery of 16S rRNA gene sequence similarity as a method of identification gave rise to a number of new culture independent techniques, generally based on the DNA or RNA of the organisms of interest. The most commonly used type of analysis involves polymerase chain reaction (PCR) or restriction analysis based technologies have been employed combined with sequencing techniques for 16S rRNA gene based identification of microorganisms in complex samples. An example of older molecular techniques applied to study microbes include denaturing gradient gel electrophoresis (DGGE), which is based on sequence and length polymorphisms between species. The primary drawback of these older techniques was throughput, only small numbers of sequences (and thereby organisms) could be identified at the same time (Talbot et al., 2008).

Development of better sequencing technologies has greatly improved throughput and yield of molecular microbial analyses. Sequencing technology developed by Illumina, PACBIO and more recently Nanopore provide multiple options for generating high quality sequencing data (Kchouk et al., 2017). In recent years, the dominant sequencing platform for large scale sequencing analysis has been Illumina. This sequencing technology employs sequencing-by-synthesis; the target DNA is amplified using fluorescently labelled nucleotides, while the DNA templates are immobilised on a flowcell. The fluorescent signals are recorded with high accuracy and on a very large scale, allowing for the generation of millions of sequences in a single run (Quail et al., 2012). The analysis of millions of partial 16S rRNA gene sequences from a single complex microbial sample at the same time is also called amplicon sequencing. This technique has allowed for large scale studies of natural and engineered ecosystems including AD systems (Kirkegaard et al., 2017).

Besides analysis of 16S rRNA genes, amplicon sequencing can also be used to target conserved functional markers in order to capture specific microbial populations of interest. Specific functional populations of interest are generally low abundant in microbial ecosystems and therefore difficult to capture using broad microbial community analyses (Hugerth and Andersson, 2017). Examples of functional groups where amplicon based approaches have successfully been applied include ammonia oxidising archaea (*amoA* gene) (Pester et al., 2012), sulfate reducing bacteria (*dsrAB* genes) (Pelikan et al., 2016) and methanogenic archaea (*mcrA* gene) (de Jonge et al., unpub; Paper 3). Targeted studies of functionally important microbial groups can provide a higher resolution of information regarding their diversity and identity compared to 16S rRNA gene based equivalents.

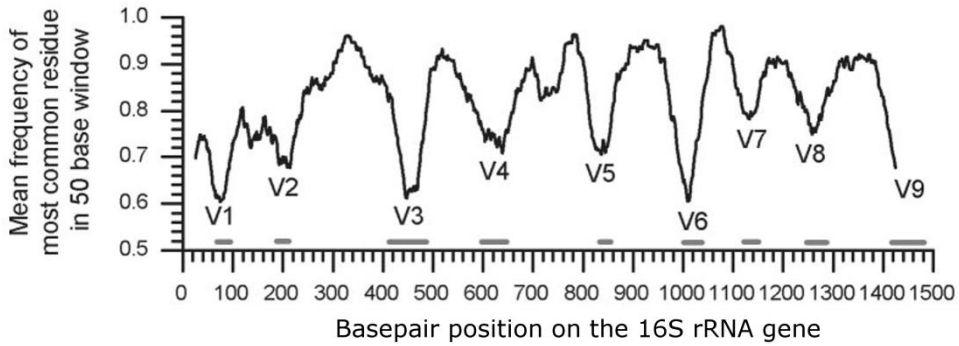

Figure 5: Distribution of conserved and variable regions within the 9 domains of the 16S rRNA gene. Modified from Ashelford et al. (2005).

While amplicon sequences can provide a wealth of information regarding the microbial community composition of the analysed samples, it needs to be considered that this technique is not without drawbacks. Between the steps of sample collection and sequencing, many sources of bias can be introduced into the microbial community profile (Hugerth and Andersson, 2017). Potential sources of bias include method of DNA extraction, choice of 16S rRNA primer target region and person bias. All of these factors can influence the quality and content of the obtained microbial profiles, and limit comparability between datasets (Sinha et al., 2017). Thus, the potential for bias must be considered carefully when working with this technique. Furthermore, microorganisms generally possess between 1 and 12 copies of the 16S rRNA gene, this often referred to as the copy number bias and it varies between genera and species (Stoddard et al., 2014). This makes that data obtained from amplicon based

analysis is to be considered semi-quantitative, and its measurements as a relative reflection of the microbial community composition (Hugerth and Andersson, 2017).

In order to fully understand the dynamics of microbial processes and ecosystems, simply identifying the microorganisms is not sufficient. Determination of activity and interaction are equally important for the description of the microbial community of interest. Analysis of (specific) microbial population activity can be performed through quantitative PCR (qPCR) of functional biomarkers genes including the 16S rRNA gene. This technique is based on the detection of fluorescence during amplification of the target DNA, which allows for accurate and reproducible quantification of the PCR products. For example, the activity of all microorganisms containing the mcrA gene, which catalyses methanogenesis, can be measured at once. However, this generally only allows for limited study designs (Talbot et al., 2008). In recent years, a number of high-throughput technologies to study not only microbial potential and specific activity from genes, but also expression and metabolic activities from other biomolecules have been developed. These techniques are collectively referred to as meta-omics (Vanwonterghem et al., 2014) (Figure 6).

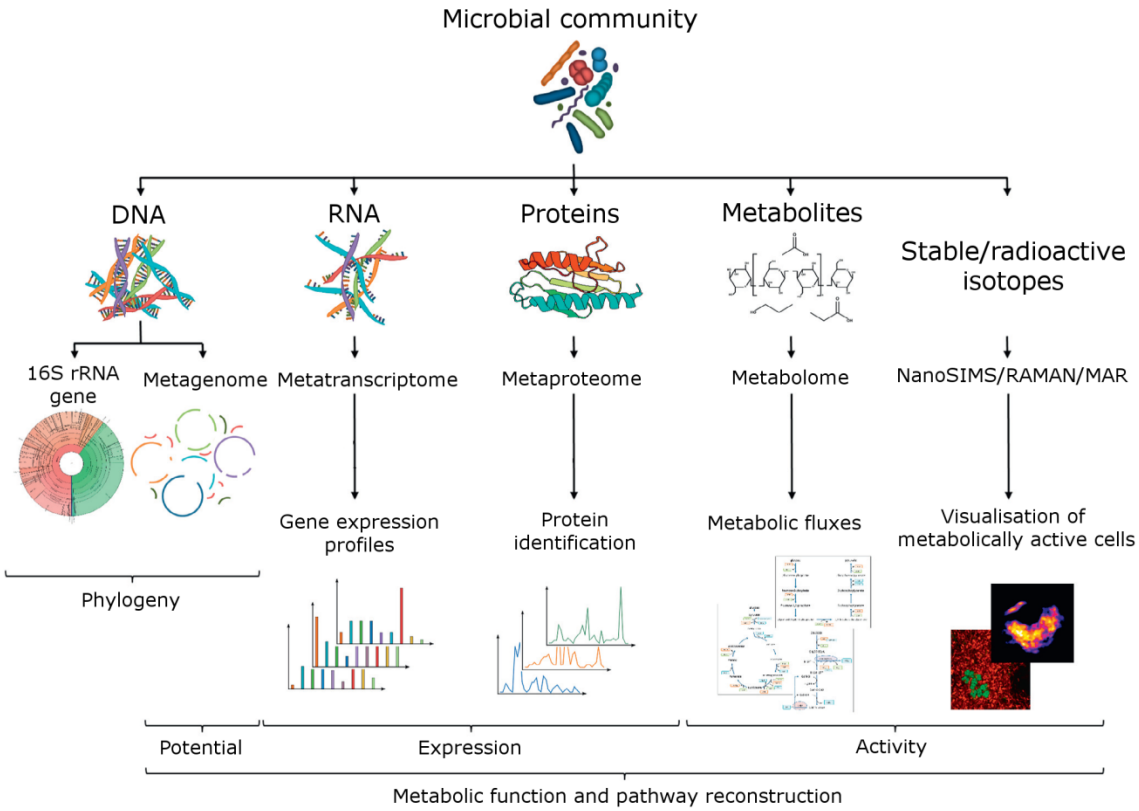

Figure 6: Overview of meta-omics techniques used to microbial community studies. Modified from Vanwonterghem et al. (2014).

Metagenomics refers to the study of microbial diversity, either through amplicon sequencing of short target reads, or generation of metagenomes, where whole microbial communities are amplified at once. Metagenome studies in full-scale AD systems have resulted in the discovery and description of new microbial populations such as the phylum *Fermentibacteria* (Kirkegaard et al., 2016), and large scale collection of amplicon based data resulted in the curated classification database MiDAS (McIlroy et al., 2017), which contains specialised taxonomic information to identify microorganisms in wastewater treatment systems and anaerobic digesters. In order to capture the microbial gene expression profiles of a community, metatranscriptomics can be used to measure transcriptional activity based on RNA. A metatranscriptomics approach has previously been used to profile the metabolically active microbial community members in a production scale biogas plant (Zakrzewski et al., 2012). High-throughput protein expression studies are referred to as (meta) proteomics, and identification of metabolite compositions to study metabolic flux are performed through metabolomics. The degradation of cellulose and associated microorganisms in thermophilic AD systems have previously been studied using a

metaproteomics approach (Lü et al., 2014). Visualisation of microbial activity and interaction can be achieved in two ways; direct visualisation can be achieved through hybridisation of fluorescently tagged DNA probes specific to the target organism(s) (fluorescence *in situ* hybridisation or FISH), and uptake or degradation activity studies can be achieved using targeted incubation with stable or radioactive isotopes. Both method types can be visualised using microscopy based techniques including NanoSIMS, MAR-FISH and RAMAN spectroscopy (Vanwonterghem et al., 2014).

One of the greatest challenges of studying the microbiology of AD has been assigning a function to the identified microbes. Interpretation of metagenomics data with predicted gene annotation is not emough to assign function to a given organism. Combined usage of omics technology (for example metagenomics and proteomics) has improved data resolution, but a practical component is still missing to truly pinpoint specific function. Recent studies have given rise to promising new combinations of technologies in order to make progress on this front. One of these approaches is the combined usage of protein based stable isotope probing (Protein-SIP) and metagenomics (Taubert et al., 2012). A labelled substrate (C^{13} acetate) has been applied in a targeted lab-scale AD study in order to track its uptake throughout the AD pathway, and showed that it was possible to track not only first degree, but also second degree utilisers of the added substrate, identifying both known and putative SAOB (Mosbæk et al., 2016).

Bioinformatics analysis of AD microbiome data

Amplicon sequencing analysis of a complex microbial sample generates millions of short sequencing reads. Before analysis of the data can commence, the sequencing reads have to be subjected to quality control, OTU clustering and taxonomic classification (Hugerth and Andersson, 2017). Several pipelines and softwares have been developed for this purpose , including QIIME (Caporaso et al., 2010), UPARSE (Edgar, 2013) and mothur (Schloss et al., 2009). Taxonomic classification of OTUs is performed using a curated database of 16S rRNA gene sequences such as GreenGenes (DeSantis et al., 2006) and SILVA (Quast et al., 2013), while functional gene classification can be achieved by collecting reference sequences for repositories such as FunGene (Fish et al., 2013). After processing, an amplicon dataset consists of an OTU table which contains reads assignments to the different OTUs per sample and the taxonomic identification of the OTUs. The dataset is then analysed using statistical softwares such as R (R Development Core Team, 2015). Specialised statistical tools to facilitate analysis of amplicon datasets include the R packages ampvis (Albertsen et al., 2015), vegan (Oksanen et al., 2016) and phyloseq (McMurdie and Holmes, 2013), as well as the software STAMP (Parks et al., 2014).

Analysis of (microbial) ecological datasets generated through amplicon sequencing is generally performed using multivariate statistics and diversity indices in order to determine the diversity and abundance of the identified microorganisms, but also to investigate correlations between the obtained microbial community profiles and environmental data (also called metadata) (Ramette, 2007; Vanwonterghem et al., 2014). Descriptive statistical techniques such as hierarchical clustering and ordination are used to compare (groups) of microbial community profiles (beta diversity) (Ramette, 2007). Calculation of distances between samples based on linear relationships (Euclidian) or Bray-Curtis dissimilarity are used to visualise differences between entire samples. Analyses such as Principal Component Analysis (PCA) and Non-Metric Multi-Dimensional Scaling (NMDS) reduce multi-dimensional data to a graphical 2D or 3D representation based on similarity or factors of variation between samples, revealing statistical relationships within the dataset (Hugerth and Andersson, 2017). Similarly, hierarchical clustering may also be performed in order to obtain a dendrogram of microbial similarity between samples (Talbot et al., 2008).

Diversity of a microbial community may be expressed as (estimated) richness, using the observed number of OTUs or diversity indices such as ChaoI (Chao, 1984). Richness describes the number of microorganisms present at a given time, and can be used to monitor changes to diversity over time or to describe differences between groups of samples (Talbot et al., 2008).Calculation of microbial community evenness using the Shannon-Weaver index (Shannon, 1948) provides information regarding the size distribution of the microbial community members, and can provide insight into microbial response in case of disturbance (Talbot et al., 2008).

Hypotheses regarding microbial interactions can be generated through co-occurrence analysis of microbial community data (De Los Reyes et al., 2015). Co-occurrence analysis has also been suggested as a method of detecting keystone species, microorganisms that are suggested to be of importance due to their high microbial

connectivity (Berry and Widder, 2014). Results of co-occurrence analysis are generally displayed as a network of microbial interactions (Hugerth and Andersson, 2017). Network analysis can also be applied to time series data to analyse microbial community relationships that also include a temporal component (Faust et al., 2015).

1.2 Anaerobic digestion: Microbiology and biochemistry

Anaerobic digestion is a complex process, based on a reduction of biomass via a number of biochemical reactions taking place under anoxic conditions. Microorganisms from two kingdoms, Bacteria and Archaea, are responsible for performing the conversion. The AD process can be divided into four distinct phases; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 7) (Weiland, 2010). The individual phases are carried out by different populations of microorganisms with complex relationships within and between groups; this makes a digester a highly complex environment to monitor and study. Initial degradation of biomass is performed by hydrolysing organisms, which break down complex compounds such as polymers to monomers, and produce primarily hydrogen, acetate and VFAs together with the fermentative microorganisms (Adekunle and Okolie, 2015). The microorganisms performing first two stages of AD account for up to 90 % of the microbial community in a digester, while the last two steps are performed by highly specialised organisms (Amani et al., 2010). Because the balance between these two populations is crucial for a stable AD process, the AD process is sometimes described as having two steps as well (Adekunle and Okolie, 2015; Ali Shah et al., 2014). Volatile fatty acids such as propionate are further degraded to acetate, CO_2 and H_2 by acetogenic organisms, all of which are substrates for the methanogens, which perform the last step in the AD process to produce CH_4.

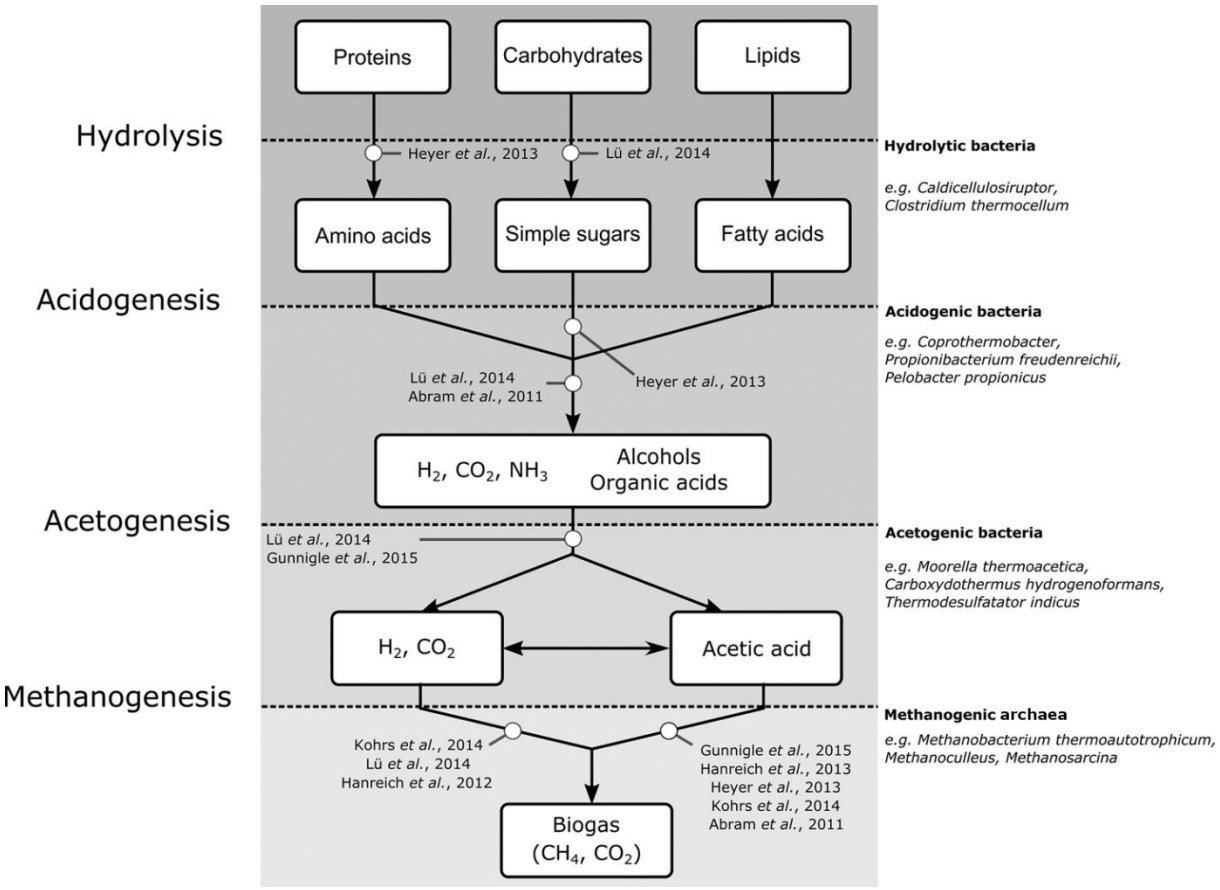

Figure 7: General overview of the anaerobic digestion process and examples of microorganisms of known function within AD. Modified from Herbst et al. (2016).

Hydrolysis

The first step in the AD process is hydrolysis, during which high molecular mass compounds (such as proteins, lipids and polysaccharides) and insoluble organic materials (e.g. lignocellulose) are converted into soluble compounds and smaller molecules, such as monosaccharides, amino acids and simple organic compounds. The products of hydrolysis are suitable for use as a source of energy, and are utilised by microorganisms involved in the subsequent steps of the AD process. Hydrolysis is catalysed by a diverse range of extracellular enzymes,

excreted by the hydrolytic and fermentative bacteria present in the digester (Adekunle and Okolie, 2015; Amani et al., 2010). Hydrolysis is performed by large and diverse microbial populations such as *Clostridia* (Weiland, 2010); examples of microorganisms linked to specific conversions are listed in Table 3. This initial breakdown of complex materials is very important for the progression of the AD process, as many microorganisms cannot utilise the substrate material directly. However, hydrolysis is in many studies considered to be the rate limiting step of the AD process, due to the rapid rate of conversion and resulting production of (volatile) fatty acids (VFA) and other toxic byproducts (Appels et al., 2008; Zhang et al., 2014). The rate of decomposition during hydrolysis depends on parameters such as pH and the characteristics of the substrates fed to the digester; for example, cellulosic biomasses are subject to slower degradation rates compared to other waste types due to their recalcitrant nature (Adekunle and Okolie, 2015; Ali Shah et al., 2014; Amani et al., 2010).

Table 3: Examples of microorganisms involved in hydrolysis, acidogenesis and acetogenesis.

Substrates	**Degradation products**	**Species**	**Reference**
Hydrolysis			
Proteins	Amino acids, sugars	*Clostridium sp.*	(Amani et al., 2010)
		Proteus vulgaris	
		Peptococcus sp.	
		Bacteroides sp.	
		Bacillus sp.	(Divya et al., 2015)
Carbohydrates	Sugars	*Staphylococcus sp.*	(Amani et al., 2010)
		Acetivibrio cellulolyticus	
		Clostridium thermocellum	(Herbst et al., 2016)
		Cellulosiruptor sp.	
Fats	Fatty acids, alcohols, amino acids	*Clostridium sp.*	(Amani et al., 2010)
		Micrococcus sp.	
Acidogenesis			
Amino acids	Volatile fatty acids, alcohols, CO_2, H_2	*Lactobacillus sp.*	(Amani et al., 2010)
		Pseudomonas sp.	
Sugars		*Coprothemobacter sp.*	(Lü et al., 2014)
		Eubacterium sp.	(Henstra et al., 2007)
Fatty acids		*Clostridium sp.*	(Amani et al., 2010)
		Propionibacterium freudenreichii	(Herbst et al., 2016)
Alcohols		*Syntrophomonas wolfei*	(Amani et al., 2010)
		Pelobacter propionicus	
Acetogenesis			
Butyrate	Acetate, Formate, H_2, CO_2	*Syntrophobacter wolinii*	(Merlin Christy et al., 2014)
Propionate		*Syntrophomonas wolfei*	(Li et al., 2012)
		Pelotomaculum thermopropionicum	
		Smithella propionica	
H_2, CO_2		*Clostridium aceticum*	(Amani et al., 2010)
Acetate		*Syntrophaceticus schinkii*	(Westerholm et al., 2010)
		Tepidanaerobacter acetatoxydans	(Westerholm et al., 2011)

Acidogenesis

The monomeric compounds produced by the hydrolysis stage of the AD process are broken down further during acidogenesis. Populations of obligate and facultative anaerobic bacteria convert compounds such as amino acids

and sugars into volatile fatty acids, alcohols, CO_2 and H_2 (Adekunle and Okolie, 2015). The products of acidogenesis serve as substrates for the microorganisms performing the last two steps of the AD process. Other products of acidogenesis include ammonia and H_2S, both of which can be detrimental to the AD process if present in excess (Ali Shah et al., 2014). Examples of acidogenic microorganisms and associated conversions are listed in Table 3.

Acetogenesis

The acetate, H_2 and CO_2 produced during acidogenesis are mostly utilised directly in by methanogenesis, but the other compounds produced during this stage, such as propionate, butyrate and alcohols need to be converted further. This conversion occurs during the acetogenesis stage of the AD process, primarily through oxidation reactions (Ali Shah et al., 2014). Examples of organisms involved in this stage of the AD process are listed in Table 3.

The oxidation of volatile fatty acids during acetogenesis generally occurs in a collaborative fashion with the methanogens, this is also known as syntrophy. This collaborative interaction is based on the partial H_2 pressure in the system; high H_2 pressure is thermodynamically unfavourable for the acetogens, and the methanogens are needed to consume the excess H_2 (Adekunle and Okolie, 2015). In general, these syntrophic relationships are formed with the hydrogenotrophic methanogens (Ali Shah et al., 2014). Examples of these symbiotic relationships include syntrophic propionate oxidation and syntrophic acetate oxidation.

Methanogenesis

The last step of the AD process is methanogenesis, where intermediate products are converted into CH_4 and CO_2. This process is catalysed by a specialised group of Archaea known as methanogens (Adekunle and Okolie, 2015). Methanogenesis is considered a critical step in AD, as it is the slowest biochemical conversion in the process and can therefore be rate limiting. Their highly specific function makes that this microbial population is sensitive to environmental changes such pH, and ammonia, H_2 and VFA concentration in the digester (Ali Shah et al., 2014).

Table 4: Examples of substrates and reactions performed by methanogens during the AD process. Modified from Ali Shah et al. (2014), Liu and Whitman (2008) and Nobu et al. (2016).

Substrate	**Reaction**	**Associated methanogens**
Hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	Most methanogens
Formate	$4HCOOH \rightarrow CH_4 + CO_2 + 2H_2O$	*Methanobrevibacter* *Methanococcus* *Methanospirillum*
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$	*Methanosarcina* *Methanosaeta*
Carbon monoxide	$4CO + 2H_2O \rightarrow CH_4 + 3H_2CO_3$	*Methanothermobacter* *Methanosarcina*
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	*Methanosphaera*
Trimethylamine	$4(CH_3)3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$	*Methanosarcina* *Methanohalophilus* *Methanomethylovorans*
Dimethylamine	$2(CH_3)2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$	
Methylamine	$4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$	
Dimethylsulfide (DMS)	$H_2 + DMS \rightarrow CH_4 + CH_3SH$	*Cand.* Methanofastidiosum

Methane is produced through one of three pathways; acetoclastic methanogenesis primarily utilises acetate, hydrogenotrophic methanogenesis utilises hydrogen as its primary substrate and methylotrophic methanogenesis is driven by methylated compounds such as methylamine (Ali Shah et al., 2014). The most commonly utilised substrates for methanogenesis are H_2 and CO_2, most methanogens can perform this reaction (Liu and Whitman, 2008). Examples of biochemical reactions performed by methanogens and associated methanogenic species are listed in Table 4.

1.3 Microbial ecology of AD systems

The microbial community of AD systems consists of a highly complex network of hundreds of *Bacteria* and *Archaea*. Structure and diversity of the microbial ecosystem are affected by major process parameters such as temperature and choice of substrate, and microbial studies of AD systems in recent years have found large differences in diversity and composition of AD associated microbes.

Microbial communities of full-scale digesters processing manures have been observed to be dominated by the presence of organisms representing the phyla *Firmicutes* and *Bacteroidetes*. Especially the orders *Clostridia* and OPB54 have been reported as diversely present (de Jonge et al., unpub: Paper 1; Moset et al., 2015). Adversely, highly diverse representation of the phyla *Actinobacteria*, *Proteobacteria*, *Chloroflexi* and *Spirochaetes* has been reported in digesters processing wastewater sludge, and microbial communities of thermophilic and mesophilic digesters differ greatly (Kirkegaard et al., 2017; Sundberg et al., 2013). Evidence of shared core communities for digestion of these substrate types has been found (de Jonge et al., unpub; Paper 1; Kirkegaard et al., 2017), in contrast to food waste, where few organisms were found in common between individual digesters. A combination of methanogens capable of utilising different pathways is generally observed in full-scale anaerobic digestion systems, regardless of substrate or temperature (de Jonge et al., unpub; Paper 3).

Process parameters that shape AD microbial communities

Operational temperature is one of the strongest effectors of the microbial communities involved in the AD process (Ho et al., 2014). Previous studies have shown large differences been the diversity and composition of AD microbial communities based on temperature (De Vrieze et al., 2015; Kirkegaard et al., 2017; Sundberg et al., 2013). The most commonly used temperatures for full-scale AD are observed within the mesophilic range (35 - 45 °C).

On the one hand, thermophilic digester operation (50 - 65 °C) has advantages over mesophilic AD in that the capacity for loading rate is much greater, and the rates at which the digestion process occurs is higher. Because of this, the productivity and thereby biogas yield of thermophilic systems is theoretically higher (Mao et al., 2015). Digestion at high temperatures also has drawbacks; The elevated temperature causes the microbial community in the digester become more specialised to their environment, and thereby more susceptible to disturbance, especially from ammonia accumulation and acidification (Mao et al., 2015; Weiland, 2010). On the other hand, mesophilic digesters are generally regarded as more stable due to greater microbial diversity (Hagos et al., 2017), and have been shown to tolerate 2 – 3 °C without noticeable effect to either operation or the microbial community (de Jonge, et al., unpub; Paper 5; Weiland, 2010). However, mesophilic systems exhibit lower methane yields and can experience problems related to the nutrient balance of the substrates (Mao et al., 2015).

Microbial studies of lab-scale digesters operating at different temperatures have previously observed significantly lower microbial richness, especially for bacterial populations, and a shift towards abundance of *Firmicutes* and *Thermotoga* representatives, at the cost of other phyla abundant at mesophilic temperatures such as *Bacteroidetes*, *Proteobacteria* and *Chloroflexi* (Moset et al., 2015). Temperature related changes to the diversity and composition of the methanogenic community of digesters is not always as pronounced as for the bacterial populations. A study focusing on operational temperatures in AD systems processing cattle manure found only minimal changes in abundance of methanogens between mesophilic and thermophilic temperatures (Moset et al., 2015), whereas a similar study involving food waste AD reported almost no similarities between methanogenic communities observed between the two operation types (Zamanzadeh et al., 2016). Diverse representation of methanogens has been observed in full-scale AD systems processing primarily food waste (de Jonge et al., unpub; Paper 2) and manures (de Jonge et al., unpub; Paper 1), while association of *Methanosaeta* and *Methanothermobacter* has previously been reported in mesophilic and thermophilic digestion of wastewater sludge, respectively (Kirkegaard et al., 2017).

Changes to the operational temperature during stable operation can have a profound effect on the microbial community in a digester (Mao et al., 2015). In general, small temperature changes within the current temperature range (e.g. mesophilic) are tolerated quite well (de Jonge et al., unpub: Paper 5), but when the digester temperature are altered to outside the optimum range of the microbial ecosystem, significant changes to the

microbial community occur and in some case operation problems arise (Mao et al., 2015). Especially the temperature range of 40 – 44 °C has been reported as highly unstable during AD (Westerholm et al., 2017), although examples of stable operation at these temperatures have also been reported (de Jonge et al, unpub; Paper 5). Other studies reported reactor failure after changing the operational strategy of lab-scale reactors from thermophilic to mesophilic, followed by changes to the loading rate, suggesting that even though the microbial community adapted to the new temperature, its resilience to perturbation had weakened (Westerholm et al., 2017). An increase in temperature has been shown to have the opposite effect; Controlled temperature increases and decreased in a full-scale mesophilic digester led to increase in diversity with the syntrophic and methanogenic populations, and the microbial community retained the adapted community structure throughout subsequent temperature alterations (de Jonge et al., unpub; Paper 5). Syntrophic acetate oxidising bacteria and hydrogenotrophic methanogens increased in abundance in order to adapt the microbial community to the new temperature; this interaction has been observed before in other studies involving operational perturbation including ammonia stress (Westerholm et al., 2015). Microbial community structures of digesters that have experienced a change between the mesophilic and thermophilic spectrum (or vice versa) generally resemble those of similar reactors operating the at the target temperature (Westerholm et al., 2017).

Next to operational temperature, the substrate composition of the organic waste used to feed the digesters has been identified as one of the most powerful determinants of microbial community structure (Ho et al., 2014; Lee et al., 2012). Microbial communities identified in digesters operated on different types of organic waste have been shown to be significantly different from each other in composition and diversity (de Jonge et al., unpub; Paper 1; de Jonge et al., unpub; Paper 2; Kirkegaard et al., 2017; Sundberg et al., 2013). Pre-treatment of substrates also affects microbial community composition in the digester (Carlsson et al., 2012). How the microbial community is shaped during co-digestion and how individual substrates contribute to the collective microbial ecosystem is not yet well-described, however it has been shown that it is possible to determine which microorganisms associate to which substrate types (de Jonge, et al., unpub: Paper 4). Differentiation of microbial communities in lab-scale AD reactors has been reported in a study that tested different types of agricultural residues in co-digestion with cattle manure (Ziganshin et al., 2013). Furthermore, a study focused on changes in feedstock composition showed that microbial communities of AD batch reactors changed significantly based on increases in availability of carbohydrates, proteins and lipids, respectively (Francisci et al., 2015).

The organic loading rate of anaerobic digesters is another influential operational parameter that can change and destabilise microbial communities if not balanced with production. An increased OLR will lead to greater biogas production to a degree, however if it becomes too high it will exceed the microbial community's capability to process the substrate efficiently (Mao et al., 2015). Due to the differences in growth rates between hydrolytic and acidifying microorganisms, and the acetogens and methanogens, an imbalance can occur in the form of VFA accumulation, which can lead to an irreversible acidification of the digester, which ultimately causes reactor failure (Chen et al., 2008). Microbial community studies of AD systems under different loadings have shown that mesophilic digesters generally handle overloading states better than those operating under thermophilic temperatures (Guo et al., 2014; Westerholm et al., 2017). Differentiation in microbial communities in digesters operating under high OLR have been observed for bacterial families associated with syntrophy, such as *Syntrophomonadaceae* and *Thermoanaerobacteraceae* (Hao et al., 2016), and within the methanogens, where especially *Methanosaeta* has been observed to be resilient under stress from increased OLR (Guo et al., 2014; Hao et al., 2016).

Hydraulic retention time determines the length of time that the substrates remain the digester, and is directly related to the substrate composition and OLR (Jang et al., 2013). Operation under an HRT that is either too high or too low, or if consistent fluctuations in HRT occur, the performance of the digester and thereby the microbial community can become destabilised and negatively affect digester operation (Mao et al., 2015). Shorter HRT can increase biogas production, but has also been linked to accumulation of VFA (Regueiro et al., 2015). A shift in the abundance and distribution of organisms representing the phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria* has been observed in lab-scale reactors exposed to hydraulic shocks (Regueiro et al., 2015). Furthermore, microbial communities in AD digesters have also shown a shift in their pathway utilisation for methanogenesis from acetoclastic to hydrogenotrophic organisms during shortened retention times (Vanwonterghem et al., 2015).

Major inhibitors of microbial AD processes

The microbial communities involved in the AD process are complex, and the many microorganisms each have their own optimum conditions and sensitivities to environmental changes. Failure to maintain a balanced AD can cause destabilisation of the microbial ecosystem, potentially leading to process failure. One of the leading causes of operational problems is the presence of inhibitory compounds; substances that negatively affect the growth or activity of the microorganisms they are in contact with. Inhibition of the AD process is usually associated to a decrease in methane production (Chen et al., 2008).

One of the most commonly used indicators of process imbalance in AD processes is the concentration and composition of VFA in the digester (Ahring et al., 1995). During stable operation, the VFA produced during acidogenesis are utilised by the acetogens and the concentration of these acids remains low. However, when a digester experiences a perturbation or general stress, the production of various VFA such as acetate, propionate and butyrate increases, and potentially leads to acidification and process imbalance (Gerardi, 2003). Fermentative populations have a much higher growth rate than methanogenic and syntrophic microorganisms, and an imbalance in the different stages of AD causes accumulation of acids. Acid accumulation leads to low pH, which in turn can destabilise the microbial community severely (Chen et al., 2008). Microorganisms have differing tolerances to changes in pH, and while hydrolytic and acidogenic populations can generally operate in a pH range of 4.0 – 8.5, more specialised populations cannot. Especially methanogens have been shown to be sensitive to pH changes (Amani et al., 2010). VFA accumulation followed by process acidification can be caused by a variety of operational perturbation, including changes in temperature (Regueiro et al., 2014), loading rate (Regueiro et al., 2015), high concentrations of ammonia (Rajagopal et al., 2013) and starvation (de Jonge et al., 2017; Paper 6). Microbial studies of systems affected by accumulations of VFA reported long recovery times, highly unstable methane production and changes to the microbial community structure, especially within the syntrophic and methanogenic populations of the digesters (de Jonge et al., 2017; Paper 6; Regueiro et al., 2014).

Another source of inhibition in AD systems is the concentration of (free) ammonia, the degradation product of nitrogenous biomass. pH changes in digesters can induce a shift in the ratio of free ammonia and the ionised form (NH_4^+), the former of which is toxic to many microorganisms as it passes freely through the cell membrane (Amani et al., 2010). The inhibitory effect of ammonia is closely related to acidification through low pH and VFA accumulation, where process instability due to the former, often leads to the latter (Chen et al., 2008). The interaction between these chemical parameters can lead to what is known as "inhibited steady state", where a digester system operates under stable conditions, but methane production is reduced by up to 30 % (Tsapekos et al., 2017). Microbial community studies focused on AD systems under high ammonia load have found differentiation within the syntrophic acetate oxidising bacteria and methanogens present in the system. A study of AD under extreme ammonia conditions showed that methanogenesis shifted from the acetoclastic toward hydrogenotrophic pathway at higher concentrations with the assistance of SAO populations (Poirier et al., 2016). Increasing abundance of SAO bacteria and hydrogenotrophic methanogens have also been associated with full-scale AD digesters operating under a high ammonia load from industrial wastewaters (Sun et al., 2014).

Stability in complex microbial ecosystems

In order to gain a deep understanding of the microbial communities of AD systems, studying the concept of stability is equally as important as the study of microbial community response to disturbance. In order to begin to predict microbial response to perturbation, it is imperative that the dynamics of stable microbial ecosystems are also understood (Shade et al., 2012). Stability can be defined in several ways; the most commonly observed ones in microbial studies are compositional stability (microbial community does not change over time) and response (or lack thereof) to disturbances (Shade et al., 2012). A number of ecological concepts are useful in describing the stability of microbial ecosystem. Resistance is described as the degree to which microbial communities remain unchanged under the effects of a disturbance. Resilience describes the rate with which a microbial community bounces back to its original composition after a perturbing event (Allison and Martiny, 2008; Shade et al., 2012). It is a combination of resistance and resilience that can be used to define a given ecosystem's responses to disturbance, and thereby its stability. Another relevant parameter to consider is functional redundancy; the measure of redundancy present in a given microbial community. Functional redundancy describes the ability of one microorganism or population to perform the function of another at the same rate under either the same or differing conditions (Allison and Martiny, 2008).

1.4 Microbial management

Currently, anaerobic digesters are primarily operated based on measurable process parameters such as temperature, OLR, HRT and pH (Madsen et al., 2011). In order to maintain stable operation, most AD systems are operated at a longer than needed HRT to avoid incomplete digestion and a lower than optimum OLR to avoid overloading (Mao et al., 2015). Therefore, many digesters are operating under sub-optimal conditions, and this has been estimated to cause up to 30 % of efficiency and thereby biogas yield (Tsapekos et al., 2017). Furthermore, inefficient digester management also increases the risk of operational disturbance events e.g. foaming, overloading and acidosis. Optimisation of digester management is needed to improve operational efficiency and AD system monitoring, one way of achieving this is through simultaneous management of the operational and microbial processes.

Controlling and monitoring the AD process is relatively well understood from the operational viewpoint. However, knowledge regarding the complex microbial ecosystem that governs the AD process is still relatively limited. Furthermore, microbial community responses to operational disturbances are not yet fully understood (Carballa et al., 2015). Microbes are the driving force behind anaerobic digestion and gaining a deep understanding of the microbial community composition and dynamics of AD systems is critical if better management tools are to be achieved.

Process monitoring is performed in accordance with indicators; carefully chosen variables that reflect the status of the AD process. These can be roughly divided in substrate conversion e.g. chemical oxygen demand (COD) and total solids (TS) removals, potential indicators of inhibition such as pH and ammonia, and various metabolites including VFA concentration and CH_4 production (Amani et al., 2010; Madsen et al., 2011). While these parameters provide information on the status of an AD system, they are unable to reflect the state of the digester in real-time; the dynamic nature of the AD process makes that process measurement results are retroactive. Thus, the predictive power of process parameters in regards to performance and stability is rather limited (Carballa et al., 2011).

Table 5: Examples of putative microbial indicators in AD. Modified from de Jonge et al. (unpub; Paper 7).

Scenario or function	**Microorganism**	**Studies**
Generalised ecosystem stress	Shift towards abundance of syntrophic acetate oxidation and hydrogenotrophic methanogenesis	de Jonge et al., unpub; Paper 5 de Jonge et al., unpub; Paper 6
Process acidification	*Tepidimicrobium* *Actinomyces*	Regueiro et al., 2015
VFA accumulation	*Gelria*	Mosbæk et al., 2016
Hydrolysis	*Clostridium thermocellum* *Caldicellulosiruptor*	Ali Shah et al., 2014 Lü et al., 2014 Heyer et al., 2013
Acidogenesis	*Coprothemobacter* *Propionibacterium freudenreichii* *Pelotobacter propionicus*	Abram et al., 2011 Heyer et al., 2013 Lü et al., 2014
Acetogenesis	*Moorella thermoacetica* *Thermosulfatator indica*	Lü et al., 2014
Syntrophic acetate oxidation	*Thermacetogenium phaeum* *Thermotoga lettingae* *Tepidanaerobacter acetatoxydans* *Clostridium ultunense* *Syntrophaceticus schinkii*	Sun et al., 2014
Syntrophic propionate oxidation	*Smithella propionica* *Syntrophobacter wolinii* *Pelotomaculum schinkii*	Li et al., 2012

Direct monitoring of the microbial communities of AD systems has been identified as a useful tool for improving the understanding of AD microbes and their responses to operational change (Carballa et al., 2015). Thereby it will be possible to extract microbial indicators of operational problems and different aspects of the AD process for the purpose system monitoring (Harms et al., 2014). Currently, amplicon sequencing of 16S rRNA gene fragments is the standard for microbial community analysis in full-scale AD systems. Investigations of this type provide information regarding the microbial community profile of a given digester, and how the composition changes over time (Harms et al., 2014). However, knowledge regarding the microbes involved in the AD process and their precise function is still very limited, and a much deeper understanding of these aspects is needed to reveal the details of the microbial community dynamics in AD systems and potential indicators of microbial response. Microbial indicators of stable and unstable AD systems are of high interest as new monitoring tools. The number of suggested microbial associations to different processes and scenario's is very limited, examples of existing putative indicators are shown in Table 5.

Another important microbial aspect for study in AD systems is stability; microbial communities behave dynamically to maintain the stability of their ecosystem where possible (Shade et al., 2012), and studying this process is useful for understanding how to better maintain and potentially enhance stability in a digester. It has been suggested that highly diverse microbial communities have a higher potential for adaptation under changing conditions and are therefore more stable (Erkus et al., 2013), and this concept can also be applied to AD systems. However, diversity measurements provide little more than qualitative information, and their use should be combined with other microbial analyses to determine the resilience and stability of a given ecosystem. The microbial stability of an AD systems can potentially be described by a combination of compositional stability and, resistance and resilience of the microbial communities themselves (Shade et al., 2012). One way of studying the evolution of a microbial ecosystem over time is through network analysis, as a microbial community responds to a change in environmental conditions, so does the network of interaction between populations (Faust et al., 2015). Regular network analysis could potentially reveal operational problems before they are measurable on the operational side, and represents an opportunity to detect indicator organisms and keystone species.

A number of factors have been shown to strongly affect the microbial community composition in full-scale AD systems, and it can be theorised that these parameters can be used to engineer the AD microbiome (Carballa et al., 2015). Examples of potentially useful parameters for microbial community shaping include temperature (de Jonge et al., unpub; Paper 5), OLR and HRT (Regueiro et al., 2015). Changes to the microbiome could potentially be induced through controlled perturbations of digesters (Briones and Raskin, 2003). Another suggested method of microbial management is through optimisation of the microbial community diversity through substrate administration. An example of this is the suggested enhancing effect of food waste on lesser degradable waste types such as wastewater treatment sludge (Yun et al., 2015).

Advances in methods for microbial and process monitoring are needed to move towards online measurement of AD process performance, and by extension microbial management. Recent developments have provided a potential opportunity in the form of near infrared spectroscopy (NIR), a non-destructive technology capable of detecting a multitude of different compounds, and capable of doing so in real-time (Bruni et al., 2013). Modelling of process parameters such as different VFA (acetate and propionate) has been achieved through measurements with NIR systems (Nordberg et al., 2000). Real-time process performance measurements need to be combined with newly developed sequencing technologies that make regular on-site microbial measurements feasible and convenient in order to move towards online process measurement. Furthermore, efforts in elucidating the identity and function of the microorganisms involved in the AD process need to continue, and be ideally be expanded with a higher frequency of targeted studies of realistic operational scenarios.

Conclusions and perspectives

Limited knowledge of the microbial communities involved in anaerobic digestion is one of the greatest bottlenecks for optimisation of AD technology in terms of process efficiency and operational management. As the process of anaerobic digestion is a microbial one, understanding the microbes involved and their responses to environmental changes is critical for system maintenance. The overall objective of this PhD project was to investigate the microbial communities of full-scale anaerobic digesters in order to contribute to the understanding of microbial community dynamics in AD systems. High-throughput molecular techniques (amplicon sequencing) were utilised in order to capture the microbial diversity of digester samples on a large scale.

Over the course of the PhD project, detailed studies of the microbes involved in food waste and manure based AD systems were carried out (Paper 1 and Paper 2), as well as optimisation of a specialised sequencing approach for studying methanogenic populations at high resolution (Paper 3). All of the gathered single-point measurements were combined to investigate to which degree it is possible to extract information regarding substrate associated microorganisms and potential new indicator species using conventional multivariate statistical methods (Paper 4). Microbial community dynamics of AD systems were investigated in two realistic operational scenarios; variable temperature fluctuations (Paper 5) and recovery from a starvation incident (Paper 6). Finally, the current status and opportunities of available molecular tools for study of microbial community aspects of importance to the development of microbial management of AD systems were summarised (Paper 7).

The microbial communities and potential network of interaction of 28 full-scale AD systems processing manures were investigated in detail in the first study of this thesis (de Jonge et al., unpub; Paper 1). Amplicon sequencing of the 16S rRNA gene region V1-V3 and the *mcrA* gene was performed in order to capture the respective bacterial and methanogenic communities of the sampled digesters at high resolution. The study found that the bacterial composition of manure based digesters is affected by operational parameters including temperature, total solids content, ammonium concentration and different substrate components. This has been observed in many other microbial studies of AD systems working under diverse operational configurations, and was as expected. However, the relationship between temperature and substrates was not as pronounced as those previously observed for wastewater sludge based systems and digesters processing food waste (de Jonge, et al., unpub; Paper 2). The microbial communities associated to animal manure contain microorganisms stemming from the digestive system of the animal of origin, and a number of those are likely retained in the digester, due to the similarity in environments. It can therefore be suggested that manure is governed by a proportion of generalistic microbes that can survive under diverging conditions. This is also supported by the result of the fourth study (de Jonge et al., unpub; Paper 4) that showed only few microbial groupings to be specifically associated to manure as a substrate. The methanogenic communities appeared to be redundant and adapted to the conditions in the individual digesters, and no major trends were observed in the occurrence and abundance of the individual methanogens, even though a small effect of ammonium concentration was detected. Methanogens are highly specialised organisms, and are therefore likely not governed by specific species, as this would leave the population vulnerable to disturbance. Functional redundancy within this population allows the methanogens to change from one pathway to another should their environment change.

Microbial co-occurrence analysis of the manure based microbial communities revealed the potential presence of a core community of hydrolytic and fermentative organisms. As manure based systems are often operated as co-digestion setups also receiving other waste types such as agricultural residues, it is not unthinkable that a diverse and stable population of microorganism that govern the first steps of AD process is ubiquitously present to ensure efficient degradation of all available biomass. Furthermore, a secondary cluster consisting of syntrophic and methanogenic organisms was also identified, highlighting the close relationships that have previously been observed between these populations. Co-digestion AD systems are considered a promising opportunity for the optimisation and expansion of biogas technology, and lot of useful information could be extracted from existing co-digestion digesters, including those processing manures. Further studies are needed to determine how and which organisms can be integrated into existing systems through substrate administration, but controlled amendment of microbial diversity and composition of digester systems could be an important step towards microbial management of AD systems.

The microbial characteristics of digesters processing food waste were also investigated (de Jonge et al., unpub; Paper 2). A survey of 18 full-scale AD systems revealed a strong relationship between operational temperature

and primary substrate components, and the microbial community composition of the digesters. Microbial community composition of the individual digesters differed greatly and only few organisms associated primarily to food waste, compared to other substrates process by the surveyed reactors including wastewater sludge. This can potentially be explained by the easily biodegradable nature of food waste; specialised populations are not needed for degradation of the biomass, which promotes a higher abundance of generalistic organisms. In contrast to the characterised manure based digesters (de Jonge et al., unpub; Paper 1), the presence of other substrate types such as wastewater sludge did affect the microbial community composition significantly. However, this may be related to the large number of substrate associated organisms to this waste type (de Jonge et al., unpub; Paper 4), which will likely displace some of the existing microbiota if added to an existing digester system. Furthermore, digesters processing either a substantial percentage of food waste or several waste types including food waste were characterised by a more diverse and more abundant presence of syntrophic microorganisms, such as SAOB. This functional population has been linked to systems recovering from a disturbance or operating under extreme conditions e.g. high ammonia concentration. It can therefore be theorised that food waste is a suitable substrate for stabilisation of lesser degradable waste types, as the theoretical enrichment of the syntrophic populations will improve digestion efficiency and microbial community resilience. Enhancement of biogas production from wastewater sludge through addition of food waste has previously been suggested and tested successfully.

The results of the manure and food waste based AD surveys (de Jonge et al., unpub; Paper 1; de Jonge et al., unpub; Paper 2) contrast each other partially, and highlight the importance of studying the microbial communities of individual substrates as well potential combinations of waste types. Both studies also support the suggested benefit of improved diversity and resilience of microbial communities in co-digestion systems. Surveying the microbial communities of full-scale AD systems also serves as reference material for studies of operational scenarios. However, individual microbial studies are often not comparable due to differences in the methods employed e.g. choice of 16S rRNA gene region target. Furthermore, low abundance of functional microbial populations of interest to the AD process, such as methanogens, complicate detailed analysis through conventional broad amplicon sequencing approaches. A viable alternative for studying methanogens is found in the functional target *mcrA*, a gene universal but unique to methanogenic populations, which can be targeted in the same way as the 16S rRNA gene.

An amplicon sequencing based assay targeting the methanogens was developed based on a well-described degenerate primer set targeting the *mcrA* gene (de Jonge et al., unpub; Paper 3). Application of the assay to a set of 46 anaerobic digester systems showed that it was possible to obtain a much higher resolution of data regarding the composition and diversity of methanogenic populations in AD systems, compared to equivalent 16S rRNA gene based approaches. While studying functional genes provides valuable information regarding the diversity of the targeted population, it only provides knowledge regarding the potential of the identified microorganisms, a drawback that all sequence based techniques have in common. Methods that reveal identity as well as activity such as protein-SIP or RAMAN spectroscopy are needed if the function of the organisms is to be studied. In order to classify the obtained amplicon, a dedicated database of representative *mcrA* gene sequences belonging to 66 described methanogenic species was constructed. New methanogenic species are still regularly discovered and described, which is the likely reason that not all of the sequences generated by the mcrA assay were received a classification beyond the genus level. However, as more representative sequences are added to the taxonomy when they become available, the classification will improve with time, and the results of Paper 3 showed that the taxonomy is a solid basis for identification of *mcrA* sequences.

Many digester systems are operated on multiple substrate types, either in planned co-digestion or by convenience. The influence of individual substrates not well-described, but it is an important topic for study in regards to how microbial communities in AD systems are shaped. Statistical comparison of microbial community profiles of 55 full-scale digesters processing primarily manure, food waste or wastewater sludge, and those operated on mixed substrates showed that it is possible to extract substrate-associated microbes, and to identify potential generalists and keystone species of interest for further study (de Jonge et al., unpub; Paper 4). Dissecting the microbial communities of mixed substrate digesters is the first step towards understanding how different substrates contribute to the shared microbiome, and perhaps how substrate related microbes can be used to engineering systems towards more desirable compositions.

Microbial monitoring of operational changes is an important way to study the responses and dynamics of the microbial communities involved in AD. Three mesophilic full-scale biogas plants experiencing differing temperature fluctuations and a lab-scale experiment investigating temperature increase within the mesophilic

spectrum were studied in detail to characterise the microbial community responses to temperature change (Paper 5). A microbial investigation of the microbial communities in the full-scale digesters over a period of 6 months revealed that temperature changes of up to approximately 2 °C within the mesophilic temperature range induced only very small changes to the microbial community, and this change was not noticeable on the operational level. One of the studied digesters was subjected to a temperature increase from 37 to 41 °C, which caused a shift in microbial community structure, specifically within the syntrophic and methanogenic populations present in the digester. After a brief transition period, the reactor re-stabilised and became resistant to further temperature change, indicating that the temperature disturbance had permanently altered the microbial community composition. This supports previous observations that the relationship between syntrophic microorganisms and methanogenic *Archaea* plays a very important role in the maintenance of stability in AD systems. Furthermore, the induced temperature disturbance had a positive effect on the resilience of the microbial community, and it could be suggested that controlled disturbances may be used as tools to alter the microbial ecosystem of an AD system toward a more desirable composition. However, this does require a deep understanding of how microbial communities respond to different operational changes, and further (targeted) studies are needed to achieve this.

The microbial responses of thermophilic lab scale reactors operating under stable conditions, which were subsequently exposed to a prolonged starvation event and recovery period were also studied in detail (de Jonge et al., 2017; Paper 6). Accumulations of VFA, ammonia and H_2S were observed as a direct result of the starvation event, causing large fluctuations in the production of CH_4, and a likely decrease in microbial ecosystem stability. The microbial community was also affected by the disturbance; large shifts in abundant organisms were observed throughout the microbial community structure. It is likely that only the microorganisms capable of tolerating the more extreme conditions during recovery were able to thrive and survive. After 45 days, full recovery to a stable state had not yet been achieved, and it is debatable whether or not full recovery was possible. The results of this study highlight the limitations of microbial ecosystems; while recovery and adaptation is possible from many altered states and instability, AD systems can become permanently destabilised and even experience process failure if not properly maintained. While prolonged starvation is an unlikely scenario in day to day digester operation, it does draw attention to the importance of regular feeding regimes as an important factor in digester stability.

The currently available molecular tools for microbial monitoring and applications of microbial studies to obtain useful data for microbial management were summarised and discussed in the final study of the thesis (de Jonge et al.; unpub; Paper 7). Practical examples taken primarily from the other studies in this PhD thesis (de Jonge et al., unpub; Paper 2; de Jonge et al., unpub; Paper 4, de Jonge et al., unpub; Paper 5, de Jonge et al., unpub; Paper 6) showed that is possible to extract useful information from microbial studies generated with currently available sequencing techniques. Knowledge regarding the diversity, drivers, composition and microbial interactions of AD microbial communities is vital for achieving microbial management, and further development of methods to monitor the operational and microbial side of the AD process is needed to improve process management. However, current monitoring methods are sufficient to collect that data needed to serve as the framework for targeted microbial investigations of operational scenarios. Furthermore, microbial studies of full-scale AD systems will also reveal microbial indicators, microorganisms or behaviours that can be directly linked to the stability or operation of the digester. Microbial indicators have great potential in serving as "early warning signs", as the microbial community will generally react to changes in their environment before the current methods of operational monitoring can detect them. Beyond identification of microorganisms and mapping of microbial response to operational change, studying the function of AD associated microbes is equally as important. Utilisation of activity based techniques such as protein-SIP and visualisation with e.g. RAMAN is needed to assign a function to the individual microbes. Advancing all of these microbial community study aspects at once is needed to obtain the deep understanding of the AD process required for achieving true microbial management.

In conclusion, the outcomes of this PhD project contribute to the goal of obtaining a better knowledge of the microbial communities involved in anaerobic digestion of organic waste. Investigation of digesters utilising manure and food waste as their primary substrate produced contrasting results, highlighting the importance of studying individual substrates and the associated microbes. Detailed studies of microbial communities and selected functional populations of interest will contribute to a deeper understanding regarding what drives and shapes AD microbiomes. Studies of operational scenario's such as temperature change yielded important information that can be put towards better management of AD systems, and could potentially yield microbial indicators for use in microbial management. However, further targeted studies are needed to attain this goal.

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