Anaerobic Digester Fluid Rheology and Process Efficiency

Interactions of Substrate Composition,
Trace Element Availability, and Microbial Activity

Luka Šafarič

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Abstract

As the anthropogenic greenhouse gas emissions continue imposing stress on our environment, it is becoming increasingly important to identify and implement new renewable technologies. Biogas production through anaerobic digestion has a great potential, since it links waste treatment with extraction of renewable energy, enabling circular bio-economies that are vital for a sustainable future.

For biogas to have an important role as a renewable energy carrier in society, the scale of its production will need to be increased substantially. New substrates need to be introduced along with raising organic loading rates of the reactors to increase the rate of biogas production. This contributes to challenges in maintaining process stability, thus increasing the risk for process disturbances, including problems that were not commonly encountered before. These difficulties may be particularly pronounced when a broad range of new, largely untested substrates are introduced, leading to an increased heterogeneity of organic material entering the reactors. In the case of currently the most common reactor type; the continuous stirred-tank biogas reactor (CSTBR); such problems may include shifts in rheology (i.e. fluid behaviour) of the anaerobic digester sludge. This may lead to increased energy consumption and decreased digester mixing efficiencies, which in turn may lead to inefficient biogas processes, ultimately decreasing the economic and environmental viability of biogas production. Much is still unknown regarding how rheology shifts happen in biogas reactors, particularly when it comes to what role the substrate plays in rheological dynamics, as compared to the microbial community during varying levels of biogas process stability.

This thesis elucidates the interactions between substrate type, microbial community and its metabolic activity, and anaerobic sludge rheology. A number of sludge samples from mesophilic and thermophilic CSTBRs digesting a broad range of substrates was analysed for their rheology. The specific effects of individual substrate types on CSTBR sludge rheology and the resulting implications for stirring power requirements and mixing efficiency were investigated. In order to also assess to which extent the microbial metabolism affects rheology at different levels of process disturbance, an experiment with a trace-element-induced inhibition of specific metabolic pathways under mesophilic reactor conditions was performed. This was used to identify the sequence of different interactions that occur in the reactor after the process begins to fail, and to evaluate how these interactions link to changes in digester sludge rheology. Finally, a case study of a disturbed thermophilic anaerobic digestion process was performed, including the monitoring of the response of rheology in relation to process stability, which was modified by changing trace element concentrations. The use of artificial substrate without polymeric compounds in both cases allowed for an evaluation of effects of the microbial community and its metabolic products on rheology without including the effects of complex substrates.

The results showed that substrate type has a large effect on how different process parameters correlate with fluid behaviour. This was particularly apparent in the case of total solids and total volatile solids, which correlated well with rheological parameters for samples from

reactors digesting agricultural waste, sewage sludge, paper mill waste, or food waste, but not for mesophilic co-digesters. Among the different substrates investigated, food waste was generally observed to lead to the highest limit viscosities (i.e. apparent viscosities at high shear rates, where it becomes linear and constant) of the anaerobic sludge, while digestion of paper mill waste and thermophilic co-digestion led to some of the lowest. No fluid type could be clearly coupled to a specific substrate, but it could be observed that increased solids content could generally be associated with more complex, non-Newtonian rheological behaviour. The differences in fluid characteristics between reactors corresponded to large differences in modelled stirring power requirements and mixing efficiency. The results indicated that fluids with high values of rheological parameters, such as the consistency index (K) or yield stress (τ_0), would likely require more power or an adapted stirring system to achieve complete mixing. The substrates generally contributed more to the rheology characteristics of the anaerobic sludge than microbial cells on their own. Trace element-induced process disturbance initially led to the inhibition of specific microbial groups among methanogenic archaea or their syntrophic partners, which later escalated to broader inhibition of many microbial groups due to the accumulation of fermentation products. This resulted in microbial cell washout with a corresponding decrease of the contribution of the cells to anaerobic sludge rheology. A recovery of the thermophilic anaerobic digestion process was possible after the supplementation of selenium and tungsten was increased, resulting in increased propionate turnover rates, growing cell densities, and higher viscosity. Major shifts in the methanogenic community were observed, corresponding to the level of process stability. It could be concluded based on these experiments that the specific effect of microbial cells and their activity on sludge rheology were linked to cell density, which corresponded to process stability.

A conceptual scheme was developed based on the studies in this thesis, defining complex interactions between substrate, microbial metabolism, and anaerobic sludge rheology in biogas processes. The possible causes of rheology shifts are visualised and discussed.

Keywords: Anaerobic digestion; biogas; rheology; trace elements; microbiology

Sammanfattning

Med anledning av att antropogena utsläpp av växthusgaser fortsätter att påverka vår miljö negativt, blir det allt viktigare att identifiera och implementera förnybara teknologier. Biogasproduktion, genom anaerob rötning, bidrar till att sammanlänka avfallshantering med förnybar energiomvandling samt möjliggör för cirkulära bioekonomier, avgörande för en hållbar framtid.

För att biogas ska utgöra en central roll som förnybar energibärare i samhället måste omfattningen av dess produktion ökas avsevärt. Nya substrat behöver introduceras, samtidigt som den organiska belastningen i existerande biogasreaktorer ökas, med syfte att öka produktionshastigheten. Detta bidrar emellertid till utmaningar avseende att upprätthålla processtabilitet, med ökad risk för processtörningar, inklusive nya typer av problem. Dessa svårigheter kan vara särskilt uttalade när nya, i stort sett otestade substrat, introduceras, vilket leder till ökad heterogenitet av organiskt material till reaktorerna. I tankreaktorer med omrörning (CSTBR), som i nuläget är den vanligast förekommande reaktortypen, kan sådana processförändringar innebära förändringar i reologiska egenskaper hos rötvätskor. Detta kan i sin tur leda till ökad energiförbrukning och lägre omrörningseffektivitet, vilket kan försämra biogasprocessens effektivitet samt bidra till minskad ekonomisk avkastning och minskade miljömässiga vinster. Det råder fortfarande oklarheter gällande reologiska förändringar av rötammarmaterial i biogasreaktorer, särskilt med avseende på val av substrat i jämförelse med mikroorganismernas roll under varierade betingelser av processtabilitet.

Denna avhandling belyser interaktioner mellan typ av substrat, mikrobiella samhällen och deras metaboliska aktivitet och reologiska egenskaper av rötslam. Reologisk karaktärisering av rötvätskor från mesofila och termofila CSTBRs, som rötade ett brett spektrum av substrat, genomfördes. De specifika effekterna av individuella substrattyper på korresponderande rötvätskors reologiska egenskaper och dess implikationer för omrörning undersöktes. För att även kunna bedöma i vilken utsträckning den mikrobiella metabolismen inverkar på rötvätskors reologi, vid olika nivåer av processtörning, genomfördes en fallstudie med spårelement-inducerad inhibering av specifika metaboliska vägar, under mesofila reaktorbetingelser. Denna studie användes för att identifiera sekvensen av olika interaktioner som uppträder i reaktorn när processtörningar uppstår och för att utvärdera hur dessa interaktioner kopplar till förändringar i slamreologi. Slutligen genomfördes en fallstudie av en termofil biogasprocess innefattande karaktärisering av reaktormaterialets reologi i respons till förändringar i processtabilitet orsakad av förändrade spårelementkoncentrationer. I båda dessa fallstudier, möjliggjorde användningen av ett artificiellt substrat utan komplexa polymerer, att mikrobiella effekter på förändringar i rötvätskans reologi kunde studeras fränkopplat effekter av komplexa substrat.

Resultaten visade att substrattyp har stor inverkan på hur olika processparametrar korrelerar med rötvätskors reologiska egenskaper. Detta var särskilt tydligt med avseende på andelen totala respektive organiska fasta ämnen, vilka korrelerade väl med reologiska parametrar för rötvätskor från reaktorer som rötade jordbruksrester, avloppsslam, avfall från pappersbruk, respektive matavfall, men ej för rötvätskor från mesofila samrötningsanläggningar. Av de undersökta substrattyperna bidrog rötning av matavfall generellt till anaerobt slam med högst uppmätta värdena för gränsviskositet (dvs när viskositeten blir linjär och konstant vid ökade

skjuvhastigheter), medan rötning av avfall från pappersbruk respektive termofil samrötning av olika substrat bidrog till de lägsta värdena för gränsviskoitet. Ingen reologisk vätsketyp kunde tydligt kopplas till en specifik substrattyp, men ökad mängd torrsustans i rötvätskan kunde generellt associeras med komplexa, icke-newtonska, flödesegenskaper. Skillnaderna i flödesegenskaper motsvarade stora skillnader i behov av omrörningskraft och omrörningseffektivitet. Resultaten indikerade att rötvätskor med höga reologiska värden, exempelvis för konsistensindex (K) eller flytgräns (τ_0), sannolikt kräver mer energi eller ett anpassat system för effektivare omrörning. Generellt bidrog substratet mer till rötvätskans reologiska egenskaper än de mikrobiella cellerna på egen hand. Inducerad spårämnesbrist i genomfört reaktorförök ledde i början till en hämning av specifika mikrobiella grupper inom metanogena arkéer och deras syntrofa partners, vilket i sin tur bidrog till en bredare hämning av flera mikrobiella grupper orsakad av ackumulering av olika fermentationsprodukter. Detta resulterade i ursköljning av cellbiomassa vilket i sin tur minskade deras effekt på slammets reologiska egenskaper. En återhämtning av den termofila biogasprocessen var möjlig efter ökade tillsatser av spårelementen selen och volfram, vilket resulterade i snabbare omsättning av propionsyra, förhöjd celldensitet och viskositet. Stora förändringar observerades samtidigt inom det metanogena samhället, vilka var kopplade till olika nivåer av processtabilitet. Den specifika effekten av mikroorganismerna och deras aktivitet med avseende på slammens reologiska egenskaper var kopplad till celldensitet, vilket motsvarade processtabiliteten.

Ett konceptuellt schema utvecklades baserat på resultat av beskrivna studier för att visualisera komplexa interaktioner mellan substrat, mikrobiell metabolism, och reologi av anaerobt slam i biogasprocesser. De möjliga orsakerna till reologiska förändringar visualiseras och diskuteras.

Nyckelord: Anaerob rötning, biogas, reologi, spårämnen, mikrobiologi

List of papers

The thesis is based on the following papers, referred to in the text by the corresponding Roman numerals:

- I. Björn A, Šafarič L, Karlsson A, Danielsson Å, Ejlerthsson J, Svensson BH, Shakeri Yekta S (2018). Substrate and operational conditions as regulators of fluid properties in full-scale continuous stirred-tank biogas reactors – implications for rheology-driven power requirements. *Water Science & Technology*, 78 (4): 814-826. DOI: 10.2166/wst.2018.352.
- II. Šafarič L, Shakeri Yekta S, Ejlerthsson J, Safari M, Nadali Najafabadi H, Karlsson A, Ometto F, Svensson BH, Björn A. A comparative study of biogas reactor fluid rheology – implications for mixing profile and power demand. *In review*.
- III. Šafarič L, Shakeri Yekta S, Svensson BH, Schnürer A, Bastviken D, Björn A. Effect of cobalt, nickel, and selenium/tungsten deficiency on mesophilic anaerobic digestion of chemically defined soluble organic compounds. *Manuscript*.
- IV. Šafarič L, Shakeri Yekta S, Liu T, Svensson BH, Schnürer A, Bastviken D, Björn A (2018). Dynamics of a perturbed microbial community during thermophilic anaerobic digestion of chemically defined soluble organic compounds. *Microorganisms*, 6, 105. DOI:10.3390/microorganisms6040105.

Contribution to papers

- I. Participated in analysing the data, performing power demand calculations, and writing the manuscript.
- II. Performed some of the lab work (rheological analyses), analysed the data, drafted the first text, and contributed to writing.
- III. Participated in planning the study, performing all the laboratory work (running the reactors and the analyses), data analysis and manuscript writing.
- IV. Participated in planning the study, performing all the laboratory work (running the reactor and the analyses), data analysis and manuscript writing.

The Author has also contributed to the following publication that is not included in the thesis:

- Ometto F, Berg A, Björn A, Safari L, Svensson BH, Ejlerthsson J (2017). Inclusion of *Saccharina latissima* in conventional anaerobic digestion systems. *Environmental Technology* 39 (5), 628–639. DOI: 10.1080/09593330.2017.1309075

To the reader

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The study was also partially supported by the Biogas Research Center (BRC) hosted by Linköping University, Sweden, with contributions from its partners, Linköping University and the Swedish Energy Agency (grant No 35624-2). BRC is a competence centre, which brings academics and different societal actors together for research, development, and PhD education in the area of biogas. The research focuses on process and technology development in relation to system- and social studies in order to investigate opportunities for development and support implementations of sustainable biogas solutions in society. More information about the BRC is available at: <http://www.biogasresearchcenter.se>.

Acronyms and abbreviations

AD – Anaerobic digestion

AW – Agricultural waste

CD – Co-digestion

CFD – Computational fluid dynamics

CSTBR – Continuous stirred-tank biogas reactor

EPS – Extracellular polymeric substances

FW – Food waste

HRT – Hydraulic retention time

ISHT – Interspecies hydrogen transfer

NMDS – Non-metric multidimensional scaling

OLR – Organic loading rate

PHAD – Post-hydrolysis anaerobic digestion

PM – Paper mill waste

SMP – Soluble microbial products

SS – Sewage sludge

TE – Trace elements

TS – Total solids [% of total mass]

TVS – Total volatile solids [% of total mass]

VFA – Volatile fatty acids

VS – Volatile solids [% of TS]

Table of Contents

<i>Abstract</i>	<i>I</i>
<i>Sammanfattning</i>	<i>III</i>
<i>List of papers</i>	<i>V</i>
<i>Contribution to papers</i>	<i>V</i>
<i>To the reader</i>	<i>VI</i>
<i>Acronyms and abbreviations</i>	<i>VII</i>
1 Introduction	1
2 Background	3
2.1 Anaerobic digestion and its industrial applications	3
2.2 Process disturbance and inhibition in anaerobic digestion	4
2.3 Importance of substrate type for anaerobic digestion performance	5
2.4 Importance of trace element availability for anaerobic digestion	6
2.5 Microbial community structure and activity during anaerobic digestion	8
2.6 Rheology in anaerobic digestion	10
2.7 Future challenges	12
2.8 Aim and research questions	13
3 Comments on the methodology used	15
3.1 Selection of samples for rheology analyses	15
3.2 Rheology analyses	15
3.3 Studies involving CSTBRs with induced TE deficiency	16
3.4 Microbiology analyses	17
3.5 Conceptual scheme of interactions	17
4 Outcomes and reflections	19
4.1 Effects of substrate type, operational, and process parameters on rheological characteristics of anaerobic sludge in CSTBRs	19
4.2 Effect of rheology differences between different CSTBR sludges on power requirements and mixing efficiency	23
4.3 Effects of nutrient deficiency and process stability on sludge rheology	24
4.4 Interactions between substrate composition, microbial community, and sludge rheology	26
5 Implications	31

6 Acknowledgements33

7 References.....35

1 Introduction

With the climate changing at an unprecedented rate due to anthropogenic greenhouse gas emissions, many countries aim to reduce their dependence on fossil fuels by switching to renewable sources of energy. Energy systems are being reviewed and revised in order to minimize their impact on the environment and human health while achieving the agreed-upon goal of limiting the global average temperature increase to less than 2 degrees Celsius above pre-industrial levels and pursuing a limit of less than 1.5 degrees Celsius increase (Gao et al. 2017). For this to be possible, the major sources of greenhouse gas emissions need to be identified, reduced, and/or replaced by new, more climate-neutral solutions.

A range of alternatives to fossil fuels exist, but each of them comes with its own set of challenges that need to be overcome. One of the solutions is the use of biofuels, among which biogas offers a variety of advantages. Biogas is a mixture of mainly methane and carbon dioxide that is produced naturally when organic matter is degraded by a complex microbial community under anaerobic conditions (Deublein and Steinhauser, 2008). Biogas production and utilization as a renewable energy carrier contribute directly to 9 of the 17 United Nations Sustainable Development Goals, including e.g. combating climate change, protecting ecosystems, promoting sustainable agriculture and achieving food security, and allowing for sustainable water management; all while providing affordable, sustainable energy (goals 13, 15, 2, 6, and 7) (World Biogas Association, 2018).

Biogas can complement the production of renewable electricity from wind and solar power by providing power when demand exceeds the supply and potentially even store excess power when demand is low (Pääkkönen et al. 2018). It can also be used in the transport sector directly as a vehicle fuel. Besides the energy-carrying methane in biogas, the anaerobic digestion (AD) process generates nutritious digestate, which can be used as a high-quality fertiliser, substituting the conventional mineral fertilizers. By using organic wastes as substrates for AD, the emissions from landfilling are drastically reduced while recovering energy and the nutrients from the wastes, allowing for sustainable agriculture. Furthermore, organic wastes from industries can be treated to reduce pollution. The resulting biogas may replace fossil fuels in some industrial processes, thus allowing AD to play a key role in the development of biorefinery concepts (Hagman et al., 2018).

Despite the immense range of positive characteristics of AD, we still face challenges when it comes to its implementation. In particular, the costs of producing biogas are still relatively high compared to fossil fuels, indicating that the process needs to be further optimised in order to outcompete the non-renewable energy carriers (Kampman et al., 2016). Furthermore, the scale of biogas production will need to be dramatically expanded if it is to help us meet the increasing demand for renewable energy as fossil fuels are gradually phased out. This is exemplified by the Swedish aim to increase production from their current 2.1 TWh to 15 TWh per year by 2030 (Swedish Gas Association, 2018). Meeting such ambitions is challenging, since new biogas plants will need to be built, while the existing ones are pushed closer to the limit of their capacities. As currently used organic substrates become increasingly utilized, new, as of yet unused substrates will need to be introduced along with new, more efficient technologies for AD, pushing the industry further into uncharted territory (Swedish Gas Association, 2018).

Many factors in the AD process are tightly interconnected, making any change in the substrate or operational conditions (e.g. temperature, solids content, or retention time) cause a ripple effect across the process (Deublein and Steinhauser, 2008), often leading to unpredicted results. The likelihood of this happening is exacerbated when the system is already close to the limit of its capacity or when new, untested substrates are used.

An unbalanced AD system manifests itself as an accumulation of intermediate degradation products, which further amplifies the disturbance, and in the worst case results in complete process failure. More commonly, these disturbances result in decreased biogas production and lower process efficiency. Additionally, unanticipated shifts in fluid rheology or foaming may occur, both of which can be difficult and expensive to rectify (Lindorfer and Demmig, 2016). Achieving a simultaneous high efficiency and stability of the AD process is therefore far from being straightforward, yet it is vital for high production capacities needed to meet our future needs.

This thesis explores the operational aspects of AD, with a focus on importance of substrate characteristics and micronutrient availability for microbial community dynamics, rheology and process efficiency.

The following background provides information on the key parameters of AD and their interconnections, studied in this work. In each subsection, the knowledge gaps that still need to be overcome are identified. The section concludes with a summary of knowledge gaps and research questions, followed by a methods section that motivates the selection of the main experimental and analytical approaches implemented in this thesis. Four studies, described in their respective papers (I-IV), were designed to address the research questions. The importance of substrate and operational conditions for regulation of the rheology of AD processes, as well as implications of rheology characteristics of digester sludge mixing profile and power demand, are explored in Papers I and II. The dynamics of the microbial community structure and activity during varying states of process disturbance and their interconnection with the rheology of AD processes are explored in Papers III and IV. The individual studies of this thesis are combined and evaluated in the context of available knowledge on AD in the Outcomes section. A new conceptual model, which defines the important interactions within the AD process that need to be considered when running AD processes at high efficiency, is proposed. Finally, the Implications section summarizes the main contributions of this work to the field and highlights the future research needs and development possibilities.

2 Background

2.1 Anaerobic digestion and its industrial applications

Anaerobic digestion of organic matter for production of biogas is widely used for organic waste reduction and stabilisation. The process involves a complex community of interdependent microorganisms. Biogas is composed mainly of methane and carbon dioxide, and is produced through four microbial degradation steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis; each carried out by a specific group of microorganisms (Deublein and Steinhauser, 2008). Insoluble polymeric compounds are degraded to soluble monomers and oligomers during hydrolysis. These are then taken up by acidogenic bacteria, which produce fermentation products, including volatile fatty acids (VFA), alcohols, hydrogen and carbon dioxide. During acetogenesis, longer VFA are oxidized to acetate, hydrogen and carbon dioxide, which serve as substrates for the final methanogenic step, performed by methanogenic archaea. Methane is produced predominantly through the hydrogenotrophic and acetoclastic pathways either from carbon dioxide and hydrogen, or acetate, respectively (Deublein and Steinhauser, 2008).

Humans have been taking advantage of AD for the purpose of waste treatment for a long time, but only recently has focus been directed towards optimising the AD processes for maximal biogas production with simultaneous organic waste treatment (Deublein and Steinhauser, 2008). A range of reactor types has been developed for this purpose, among which the continuous stirred-tank biogas reactor (CSTBR) is currently the most common (Kampman et al., 2016). In a CSTBR, the reactor contents, commonly referred to as *anaerobic sludge*, are homogeneously distributed throughout the reactor volume. This is achieved by regular and complete mixing, which homogenises the sludge, facilitates contact between the microorganisms and the substrate, ensures equal temperature distribution, and accelerates the release of biogas (Lindmark et al., 2014; Weiland, 2010; Wu, 2012). Fresh substrate can be added either continuously or semi-continuously with concurrent removal of anaerobic sludge. The sludge contains undigested substrate residues and microorganisms together with their metabolic products, which means that the microbial biomass is constantly being washed out from the reactor. This imposes a requirement for the microorganisms to multiply at a faster rate than they are being removed. This rate is affected by the hydraulic retention time (HRT) of the process, which is the average time a substance stays in the reactor. The selection of this operational parameter is a trade-off between providing microorganisms with the time required for their growth and the CSTBR volume, which determines the capital and running costs of the AD processes.

Reactors are most often operated at either mesophilic (i.e. 35-42°C) or thermophilic (i.e. 50-57°C) conditions. During the latter, higher degradation rates allow for shorter HRT and, thus, smaller reactor volumes, with the downsides of higher energy demands for the reactor operation and a higher sensitivity to process disturbances due to e.g. ammonia toxicity (Braun et al., 1981; Weiland, 2010)

While substantial progress has been achieved in the optimisation of biogas reactor operation, AD plant operators still occasionally experience problems that are difficult to predict and expensive to solve. Examples of this can be severe foaming or drastic shifts in sludge fluid

behaviour (i.e. rheology) in the reactor. With the present plans to substantially increase biogas production, new, less extensively tested substrates will need to be introduced, while the AD processes are made as efficient as possible by producing more biogas per reactor volume and substrate amount. This, however, means that the AD processes will be more often pushed toward their limits, which increases the risks of operational problems associated with organic matter overload.

Since anaerobic sludge is a mixture of material from the substrate matrix in various degrees of degradation and microbial cells, which break it down and utilise its nutrients, it is important to closely evaluate the possible interactions between these components in the anaerobic sludge. In order to minimise the chance of operational problems it is particularly important to understand the interactions between the type of organic material present, the metabolic activity of microorganisms, the nutrients supporting it, and the physical anaerobic sludge characteristics, such as rheology. The knowledge about each of these components and their interconnections are equally important parts of this thesis. Each of them is presented in detail in the upcoming sections, which include what is already known and highlight the knowledge gaps that still need to be addressed.

2.2 Process disturbance and inhibition in anaerobic digestion

The concept of process stability in CSTBR systems hinges on the fact that a sustainable operation of the reactor is possible only if the following criteria are met:

- The metabolic rates between the different microbial degradation steps are balanced so that no intermediate products accumulate or are deficient;
- The growth rate of all the important microorganisms is high enough for them to cope with their continuous washout during sludge removal (i.e. their yields need to be high enough).

These criteria can only be met if the microorganisms are provided with a sufficiently stable environment, free from significant concentrations of inhibitors, and with enough accessible nutrients (Appels et al., 2008; Choong et al., 2016; Deublein and Steinhauser, 2008). Determining how to maintain a stable AD process while still realizing its maximal potential is, however, not clear-cut and needs to be guided by careful monitoring of the process, coupled to implementation of measures to avoid process disturbances.

Monitoring of the AD process often involves regular measurements of process parameters, such as VFA concentrations, pH, methane production, and volatile solid (VS) reduction (% of VS in the substrate that is degraded in the reactor). Unexpected changes in these parameters alert the operator when the reactor performs suboptimally and is possibly heading towards process failure. The usefulness of these tools is, however, limited since they mostly indicate process disturbances after they have already occurred. Thus, an operator would have to abate the process disturbance in addition to solving the problem that led to it. Much research is therefore focused on identifying better indicators for predicting process disturbance before it actually occurs (Kleyböcker et al., 2014).

Process disturbances usually manifest as accumulation of fermentation products, including VFA and hydrogen (Deublein and Steinhauser, 2008). This may eventually affect the pH and lead to further inhibition. Consequently, the VS reduction and methane production may decrease. Occasionally, additional problems occur, such as foaming or shifts in sludge rheology (Kougias et al., 2014). It is currently unclear what happens with sludge rheology characteristics during process disturbance and failure, as well as during its recovery.

There are various reasons for process instability, e.g. overloading with the substrate, lack of specific essential nutrients, inefficient mixing, and/or the presence of inhibitory compounds. One of the most important ways of controlling these potential sources of instability is through ensuring a balanced availability of necessary nutrients for the growth and activity of the microorganisms by careful selection, processing, and dosing of the substrate (Hendriks et al. 2018).

2.3 Importance of substrate type for anaerobic digestion performance

Commonly used substrates in AD include mixtures of primary and activated wastewater sludge, manure, agricultural residues, energy crops, food waste, and different industrial organic wastes (Deremince and Königsberger, 2017; Weiland, 2010). They differ in their chemical compositions as well as their physical properties, such as particle size, water content, and rheology.

A range of factors can affect the efficiency and stability of an AD process while digesting a given substrate. The organic matter content of the substrate serves as an electron source for the microbial community, as well as a source for biomass growth i.e. macronutrients (i.e. C, N, P, K, Na, S, Ca, and Mg) and micronutrients, also known as trace elements (TE; i.e. Co, Ni, Fe, Zn, Mo, W, Se) (Hendriks et al. 2018; Takashima et al. 1990). Macronutrients are chemical elements that the microorganisms use to build their cell material (Hendriks et al. 2018). In order to assure optimum microbial growth and activity, the ratios of these elements in the substrate need to correspond to those required by the cells (Hendriks et al. 2018). Additionally, it is important which kinds of molecules these elements are bound into, with some being more difficult to degrade than others (Deublein and Steinhauser, 2008). Furthermore, certain compounds are inhibitory to some microorganisms and may upset the microbial growth and function when present at high concentrations. These compounds may also become concentrated in the digestate, making it unsuitable for use as biofertilizer. Examples of this can be ammonia, hydrogen sulphide, or heavy metals, the toxicity of which depends on parameters, such as operational temperature or pH (Braun et al., 1981; Oleszkiewicz and Sharma, 1990). Besides the chemical properties of the substrates which affect mainly the AD process itself, it is important to consider their physical characteristics, such as rheology, as well. This can affect how easily a substrate can be handled (e.g. pumping and stirring; Farno et al., 2017) and may lead to inefficient mixing or foaming if the reactor configuration is not built to handle those types of materials.

In conclusion, each specific substrate brings with it a set of challenges that need to be overcome to achieve an efficient and stable AD process. While some information on their effects on the

process is available, it is unclear to which extent the choice of a specific substrate affects the anaerobic sludge characteristics, such as its rheology.

2.4 Importance of trace element availability for anaerobic digestion

In addition to macronutrients, trace elements need to be provided by the substrate or supplemented individually, as reviewed by Takashima et al. (1990). TE are required in relatively low amounts by the cells, but are nevertheless vital for their normal functioning due to their role as cofactors to some of the key enzymes participating in the AD process. They are of particular importance for the methanogenic archaea and their syntrophic acetogens, which rely on a range of metalloenzymes for their metabolisms (Figure 1).

Figure 1: Specific TE requirements in different AD steps (Based on Hendriks et al., 2018, Choong et al., 2016, and Glass & Orphan, 2012). A lack of markings on some pathways does not necessarily mean that no TE are required, since TE requirements of many pathways are so far largely unknown.

A lack of bioavailable TE leads primarily to the accumulation of different fermentation products, such as VFA and hydrogen. This accumulation occurs when the degradation rates for these compounds slow down due to the inability of the microbial community to produce enough functioning enzymes. Examples of these are different hydrogenases and dehydrogenases, methyltransferases, and acetyl-CoA synthase (Hendriks et al., 2018). The supplementation of TE to biogas reactors has been in focus within science and industrial implementation since the 1980s. Particularly iron, cobalt, and nickel have been studied extensively, while other TE received more attention only during recent years. Many studies focused on the effects of individual TE, while only few studies so far investigated their combined effects (Choong et al., 2016).

Furthermore, TE appear to be linked not only to degradation of certain compounds through their effect on enzymatic activities, but also to the production of compounds, such as extracellular polymeric substances (EPS) (Sutherland, 2001) and soluble microbial products (SMP) (Thanh et al., 2015), which have been observed to increase the viscosity of liquids in non-AD systems (Sutherland, 2001; Vlaev et al., 2013). The EPS are key components of biofilms, which represent the normal environment for most microbial cells. Microorganism use this material, for example, to attach to surfaces, aggregate into flocs, form protective barriers, and minimize the diffusion of extracellular enzymes away from the cells (Laspidou and Rittmann, 2002). The production of EPS is regulated by many factors, including temperature, pH, and shear stress (Feng et al., 2015; More et al., 2014). It increases during metabolic stress and in cases of nutrient imbalance. The main components of EPS are carbohydrates, proteins, nucleic acids, and humic substances (Feng et al., 2015; More et al., 2014). Similarly to EPS, SMP are compounds excreted by microorganisms during nutrient imbalance, presence of toxic metals and other stress (Barker and Stuckey, 1999). They can also be released into the medium during cell lysis, diffuse through cell membranes, or are lost during synthesis. The main difference between EPS and SMP lies in their solubility, where EPS are mostly associated with the solid phase, while SMP are completely soluble. SMP can be further divided to substrate-utilization-associated products and biomass-associated products (Barker and Stuckey, 1999). The former are produced during substrate metabolism, while the latter likely form during biomass decay. SMP and EPS exhibit chelating properties, thus affecting how easily the TE are taken up by the microbial cells; i.e. the bioavailability of TE may be affected (Aquino and Stuckey, 2006; Kuo and Parkin, 1996).

The requirement for TE supplementation of a given AD system depends on a range of factors, making the levels needed difficult to predict. The quantity of enzymes required to break down the substrate in a given amount of time depends on the organic loading rate (OLR), which corresponds to the amount of substrate fed per unit of time. As a consequence, increasing the inflow of organic matter into the reactor generally leads to increased TE requirements (Hendriks et al., 2018). They also increase if the process is operated thermophilically (Takashima et al., 2011), likely due to the higher enzyme production rates of thermophilic systems, as well as lower solubility of phosphate and carbonate metal complexes (Hendriks et al. 2018). Poorly soluble metal complexes are believed to be less bioavailable. In addition to temperature, the bioavailability may also be affected by the concentration of metal-binding

compounds, such as sulphides in the anaerobic sludge, which form precipitates with TE (Callander and Barford, 1983; Shakeri Yekta et al., 2016).

TE depletion has been reported to affect rheology or foaming tendencies of CSTBR sludge (Lienen et al., 2014; Moestedt et al., 2015), but the mechanism behind this observation is not known. It is mainly unclear whether a lack of TE would lead primarily to lower substrate degradation rates due to decreased enzymatic activities or to the increased production of compounds, such as EPS or SMP, which would affect the chemical and physical properties of anaerobic sludge. Therefore, it is essential to elucidate the postulated interactions between microbial growth and activity as affected by the indicated interactions with TE and their possible rheological implications.

Since TE availability is essential for microbial metabolism, a decrease in TE concentration is very likely to affect the microbial community composition and activity in a biogas reactor due to an establishment of selective pressure, favouring microorganisms with higher affinity or lower requirements for TE (Feng et al. 2010).

2.5 Microbial community structure and activity during anaerobic digestion

Microbial metabolisms in AD processes are tightly bound, i.e. the products of one group of microorganisms serve as substrates for another. Hydrolysis, the first step in the AD metabolic chain (nr. 1 in Figure 1), is generally performed by a mixture of strict anaerobes and facultative anaerobes mainly from the Firmicutes and Bacteroidetes phyla (Azman et al., 2015). They produce exoenzymes that break down polymers in the substrate to monomers, which can be taken up by the cells. In that way polysaccharides are broken down to oligo- and monosaccharides, proteins to amino acids, and lipids to fatty acids. These monomers are then fermented mostly by the same microorganisms to e.g. H₂, CO₂, alcohols, and VFA. Acetate, H₂, CO₂, formate, methanol, and some methylated compounds can be taken up by methanogenic archaea directly for the production of methane (Ferry, 2011). Other fermentation products, such as propionic and butyric acid, need to be oxidised to acetate, formate, or H₂ and CO₂, which are used as methanogenic substrates. The latter biochemical reactions are thermodynamically unfavourable at standard conditions and are possible only if the concentration of their products (hydrogen or formate) is sufficiently low (Müller et al., 2010; Thauer et al., 1977). This is achieved by syntrophic interactions between the bacteria performing these reactions and methanogenic archaea. These bacteria come from genera such as *Syntrophobacter*, *Syntrophomonas*, *Pelotomaculum*, and *Smithella* (Amani et al., 2010). One of the strategies of the syntrophic partners for achieving a low enough concentration of products is interspecies hydrogen transfer (ISHT). During this process hydrogen can be transferred between the two cells directly and without being dissolved in the medium, allowing for very high transferring efficiencies (Thauer et al., 1977). For this to be possible the cells need to be in physical contact, which may be difficult to achieve if the shear forces in the medium are high (i.e. if the stirring intensity is high) (Lindmark et al., 2014).

Hydrogen serves as an electron carrier in ISHT and allows for the coupling of the metabolisms from both organisms. Formate can be used in a similar way as an alternative electron carrier (Boone et al., 1989; McInerney et al., 2008), or electrons themselves can be transferred by the use of so-called nano-wires (Sure et al., 2016). Propionate is syntrophically oxidised mainly through two pathways; the methylmalonyl-CoA pathway, and the dismutating pathway (i.e. *Smithella* pathway) (Dolfing, 2013; Müller et al., 2010). The former is more common (Li et al., 2012) and involves the oxidation of propionate to acetate, CO₂, and H₂ (nr. 2 in Figure 1), while the latter produces acetate and butyrate from propionate (nr. 3 in Figure 1) and then turns butyrate to acetate by β -oxidation (nr. 4 in Figure 1). Only one mole of hydrogen is produced from a mole of propionate in this pathway as compared to three moles of hydrogen in the methylmalonyl-CoA pathway (Dolfing, 2013). As mentioned above, acetate or CO₂ + H₂, may be consumed directly by methanogens, but they can also be transformed from one to another (i.e. acetate to/from CO₂/formate and H₂) by acetogenic bacteria. The transformation of H₂ and CO₂ or formate to acetate is performed via the Wood-Ljungdahl pathway (WL; nr. 5 in Figure 1) (Schuchmann and Müller, 2014; Wood et al., 1986). The transformation in the opposite direction can be performed by syntrophic acetate oxidizing bacteria either through a reverse WL pathway (Müller et al., 2013) or an alternative pathway, which is mediated by the glycine cleavage system and tetrahydrofolate pathway (Nobu et al., 2015). These bacteria allow AD to function even if acetoclastic methanogenic pathway is inhibited by redirecting the substrate flow to hydrogenotrophic methane formation.

Methanogenic archaea produce methane and CO₂ from a range of substrates as mentioned above. This can be done through three pathways; acetoclastic, hydrogenotrophic, and methylotrophic (nr. 6, 7, and 8 on Figure 1). Archaeal representatives from all orders except *Methanomassiliicoccales* are capable of hydrogenotrophic methanogenesis (Enzmann et al., 2018), where CO₂ or formate is reduced with H₂ to methane. Acetoclastic methanogens are limited to the order of *Methanosarcinales* and transform acetate to methane and CO₂. Finally, methylotrophic methanogenesis; the transformation of methylated compounds (i.e. methanol, methylamines, or methylated thiols) to methane; can be performed by members of orders *Methanomassiliicoccales*, *Methanobacteriales*, and *Methanosarcinales* (Enzmann et al., 2018). The ratios between the groups depend largely on the substrate used. Generally in AD systems there are more substrates available for acetoclastic and hydrogenotrophic methanogens than for methylotrophic ones, which is reflected in the dominance of those two groups. The genera *Methanosarcina* or *Methanoculleus* are often the most common ones in mesophilic AD systems (Enzmann et al., 2018).

All these complex microbial interactions are important for a well-performing AD process. A failure of one reaction can lead to accumulation of intermediates, which can cause feedback effects to other reactions. This may cause substantial changes of the sludge chemistry, which in turn may affect its rheology. While anecdotal observations of such phenomena exist, this has not yet been reported for AD systems and therefore calls for a scientific evaluation.

2.6 Rheology in anaerobic digestion

Rheology is the study of how bodies (i.e. solids, liquids, or gasses) deform under stress (Schramm, 2000). In the context of CSTBRs, it is crucial how the anaerobic sludge behaves under the influence of stress imposed by stirring and pumping. Viscosity is one of the properties of fluids, describing their resistance against irreversible positional change (Schramm, 2000). Because of this resistance, energy must be added continuously to a body of fluid in order to maintain flow. In general, the higher the viscosity, the more power it takes to efficiently stir and induce flow in the fluid. In CSTBRs, pumping and mixing are important for the operation, stability, and efficiency of the AD process, as well as energy consumption of the reactor, which may account for over 50% of the total energy demand of an AD plant (Dachs and Rehm, 2006; Lindkvist et al., 2017).

The term *viscosity* itself often refers to *dynamic viscosity*, which is constant throughout the range of shear rates – in other words, it is not affected by the stirring or pumping intensity. This behaviour is characteristic of so-called *Newtonian fluids*. Most anaerobic sludge fluids, however, are non-Newtonian with variable viscosity depending on the stirring or pumping intensity. Non-Newtonian fluids are characterized by the term *apparent viscosity* (i.e. the viscosity a fluid exhibits at a specific shear rate) (Ratkovich et al., 2013), representing the relationship between shear rate and shear stress (Schramm, 2000). In practical terms, shear rate corresponds to the speed of stirring, while shear stress corresponds to the force acting on the fluid during stirring at a certain shear rate. Rheological models, such as e.g. the Bingham (Eq. 1), Ostwald (Eq. 2), or Herschel-Bulkley (Eq. 3) (Ratkovich et al., 2013), can be used to determine the fluid type by analysing the relationship between shear rate and shear stress.

$$\tau = \tau_0 + K \cdot \dot{\gamma} \quad (\text{Eq. 1})$$

$$\tau = K \cdot \dot{\gamma}^n \quad (\text{Eq. 2})$$

$$\tau = \tau_0 + K \cdot \dot{\gamma}^n \quad (\text{Eq. 3})$$

The symbols τ and $\dot{\gamma}$ represent shear stress [Pa] and shear rate [s^{-1}], respectively, while n and K are flow behaviour [dimensionless] and consistency [Pa·s] indices, respectively. Parameter τ_0 signifies yield stress [Pa], which is defined as the force a fluid must be exposed to in order to start flowing, and reflects the resistance of the structures within the fluid to deformation or breakdown (Spinosa and Lotito, 2003). Yield stress is affected by the physicochemical characteristics of the fluid and impedes flow at relatively low stresses, which might lead to problems like bulking (i.e. the development of a foam-like layer on the sludge surface, which happens when bubble release is impeded; Lindorfer and Demmig, 2016), or uneven distribution of anaerobic sludge in CSTBR systems (Forster, 2002). Furthermore, efficient mixing of yield-stress fluids can be difficult since mixing may be limited to a small area around the stirrer, also known as a *cavern*, with the rest of the reactor remaining unstirred due to insufficient shear being imposed on the fluid for it to flow (Nienow, 2014).

Since fluids can respond to varying amounts of shear in distinctly different ways, they can be classified into different groups based on their behaviour. The first division is to Newtonian (e.g. water or mineral oils; red in Figure 2) and non-Newtonian (most other fluids; Figure 2) fluids, based on the criteria described above. The non-Newtonian fluids are further divided, among others, to pseudoplastic and dilatant with- or without yield stress (Eshtiaghi et al., 2013) (Figure 2), thixotropic, and rheopectic (Schramm, 2000). The non-Newtonian fluids are defined based on how apparent viscosity changes in relation to shear rate and whether a yield stress is observable. Thixotropy and rheopecticity refer to the relationship of viscosity to the time after exposure to shear. For example, a thixotropic fluid will initially behave as a solid, but when exposed to shear, the internal structures of the material will break down, allowing it to flow and resulting in decreased viscosity in relation with time. Once shear is discontinued, the internal structures of the fluid can be re-established, again increasing viscosity. Rheopectic fluid viscosity increases the longer they are exposed to shear. These fluids are, however, extremely rare (Schramm, 2000). Care should be taken when thixotropy is observed since the combination of fluid characteristics, rheometer setup, and the measuring protocol used may lead to artefacts in the results falsely resembling thixotropy (Baudez, 2006).

Figure 2: Relationship between shear rate (s^{-1}) and shear stress (Pa) for five different fluid types: green, Bingham plastic; yellow, pseudoplastic with yield stress; blue, pseudoplastic; red, Newtonian; purple, dilatant.

Increased sludge viscosity and consequently insufficient mixing in CSTBRs have been shown to be connected to process disturbances (Lindorfer and Demmig, 2016). Furthermore, increased viscosity is believed to impede bubble release from the liquid, resulting in bulking or sludge expansion, which may damage the reactor (Lindorfer and Demmig, 2016). Inefficient mixing, on the other hand, leads to flotation of lighter material, sedimentation of heavier particles, and the creation of unstirred *dead zones*. Presence of dead zones may result in organic matter overload of the remaining active volume of the reactor (i.e. the volume where the sludge is still well mixed), leading to process disturbances such as VFA accumulation and foaming (Lienen et al. 2014).

It is thus clear that achieving a higher control over CSTBR sludge viscosity and improving mixing would be beneficial for the biogas industry by lowering energy consumption and improving process stability.

Anaerobic sludge is a suspension of microbial cells, their aggregates, as well as organic and inorganic particles in an aqueous solution with complex physicochemical interactions which determine its fluid behaviour. When it comes to rheology of suspensions in general, the theory states that increasing solid particle concentration will increase the viscosity of a suspension (Seyssiecq et al., 2003). This relationship is described by the *Einstein equation*: $\eta = \eta_0(1 + 2.5\varphi)$, where η represents viscosity, η_0 the viscosity of the fluid phase, and φ the particle volume fraction (Hiemenz and Rajagopalan, 1997).

Of the commonly monitored process parameters during operation of CSTBRs, the total and total volatile solids (TS/TVS) concentrations are the ones that give the closest estimate of the proportion of solid particles suspended in the sludge. The TS/TVS parameter has been extensively analysed in relation to viscosity and is often reported in the literature as the most important process parameter affecting fluid behaviour in CSTBR systems (Aranowski and Hupka, 2010; Baudez et al., 2011; Mbaye et al., 2014). It has been shown that an increase of TS and/or TVS content will lead to increased viscosity and yield stress (Mbaye et al., 2014). Contradictory observations, where no such correlations could be identified between viscosity and TS/TVS, have also been reported (Björn et al., 2012; Moeller and Torres, 1997; Moestedt et al., 2015; Monteiro, 1997). These discrepancies suggest that the fluid behaviour of anaerobic sludge may be regulated by factors other than solid particle concentration.

2.7 Future challenges

While AD is already a relatively widespread technology, it will need to be substantially expanded to fulfil our renewable energy needs. Before we can achieve that, however, we face a range of challenges related to each of the sections described above. In order to reach the increased production levels needed, new substrates will have to be added to the selection of those already used. Due to a lack of systematic studies on AD sludges from CSTBRs digesting different substrates, the extent to which substrates impose their rheological properties on the anaerobic sludge is currently unclear, as is the extent to which these properties are affected by operational (e.g. HRT, OLR, and temperature) and process parameters (e.g. TS, TVS, pH, and VFA). This has important implications for the design and operation of stirring systems in

CSTBRs, which will need to be optimised as much as possible to decrease their high contribution to reactor energy consumption, and more importantly, improve the mixing efficiency and the associated process performance. The lack of data in this field increases the risk for new biogas plants being built with stirring systems that cannot cope with the effects of new substrates, or the existing plants being operated suboptimally.

On the other hand, it is important to understand not only the effects of substrates on anaerobic sludge fluid behaviour, but also the effects of varying levels of process disturbance. The latter will affect not only the rates of biogas production, but may also have important implications for the extent of organic matter degradation, as well as possible production of microbial products, such as EPS and SMP. All of these may have effects on sludge rheology, since they affect its chemical composition. The information on the extent of these interactions is highly relevant for the development of high-performance CSTBRs, since possible shifts of rheology due to process disturbance could increase energy consumption and decrease mixing efficiency, which would have detrimental effects on the reestablishment of process stability.

While it is very likely that process disturbances would affect CSTBR sludge rheology, the relative scale of these effects in comparison with the effects of the substrate have so far not been evaluated. It is also unclear in which ways the substrate type, operational parameters, process stability, and rheology interact inside the reactors.

2.8 Aim and research questions

Based on the background above it is clear that challenges, largely associated with substrate specifications, need to be dealt with for further expansion of biogas production.

Thus, this thesis aims to evaluate to which extent substrate choice and operational conditions affect the rheological properties of anaerobic sludge in CSTBR systems and to which extent this affects mixing efficiency and power demand. Furthermore, it aims to elucidate in which way the metabolic activities of the microbial community affect anaerobic sludge rheology depending on the level of process stability as affected by trace element availability. The major knowledge gaps, identified in the Future challenges section, are addressed based on the following main research questions:

1. How do substrate type, operational, and process parameters affect rheological characteristics of CSTBR sludge? (Papers I and II)
2. How does sludge rheology affect mixing efficiency and power requirements? (Papers I and II)
3. How is trace element depletion associated with microbial structure and metabolic activity, process disturbance, and rheology? (Paper III and Section 4)
4. How does recovery from process disturbance correspond to trace element supplementation and rheological characteristics? (Paper IV and Section 4)
5. Which kinds of interactions connect substrate, microbial community, and sludge rheology in CSTBR systems? (Papers I, II, III, IV, and Section 4)

Each research question was addressed by specific studies described in their respective papers. Outcomes presented in Papers I and II provide insights on the importance of substrate and operational conditions for CSTBR sludge rheology (Figure 3a). The possible effects of the rheology differences on mixing efficiency and power demand are also evaluated. The interactions between rheology, microbial communities, their activity, as well as process disturbance and recovery, are addressed in Papers III and IV (Figure 3b and c).

3 Comments on the methodology used

3.1 Selection of samples for rheology analyses

The first research question was addressed by sampling anaerobic sludge from 8 mesophilic and 4 thermophilic industrial-scale CSTBRs which were digesting a range of different substrates under different operational conditions (Paper I). This was complemented by sampling 21 mesophilic laboratory-scale CSTBRs (Paper II) in order to cover a broader span of the substrate categories used in AD processes. In addition, eleven reactors from both sets were sampled a second time, at least 3 HRT after the first sampling, bringing the total number of samples included in the dataset to 44. The two datasets complement each other by jointly covering a wide range of process- and operational parameters, as well as substrate profiles, including agricultural waste (AW; 7 reactors), co-digestion (CD; 16 reactors), food waste (FW; 4 reactors), paper mill waste (PM; 3 reactors), and sewage sludge (SS, 3 reactors; Figure 3a). The effect of substrate type on sludge rheology, as well as the potential correlations between rheology and different operational conditions (Temperature, TS/TVS, pH, OLR, HRT, and VFA) were evaluated. Finally, samples from 5 reactors operated on a defined medium (described in Section 3.3) were added to the dataset in order to compare how large the effects of microbial community are as opposed to the effects of different substrate types on rheology (*post-hydrolysis anaerobic digestion* - PHAD; 5 reactors).

3.2 Rheology analyses

A consistent rheology measurement approach was used in all studies. This was crucial for the integration of all the data, since rheology data obtained with different rheometers or using different measuring protocols have been shown to be incomparable (Eshtiaghi et al., 2013). A rotational Searle-type rheometer with a concentric cylinder measuring system was used to perform measurements with a three-step protocol adapted from Pevere et al. (2007). This allowed for studying the effects of shear rates on shear stress before and after extensive shearing of the samples. Limit viscosity, defined by Tixier et al. (2003) as apparent viscosity at high shear rates, where it becomes linear and constant (i.e. the extreme right part of the plots in Figure 2, except for dilatant fluids), was chosen as the apparent viscosity value to be compared between samples. In our case, the measuring point at shear rate 800 s^{-1} followed these criteria. This value was used in correlation analyses with operational and process parameters since it has been shown to be a reliable single parameter describing the rheological properties of sludge, as reviewed by Eshtiaghi et al. (2013). Rheological models were fitted to the data with the least-square fitting method, and the most appropriate model chosen among the Bingham, Ostwald, and Herschel-Bulkley models. This provided the rheological parameters that describe the fluid behaviour for each sample.

Pearson correlations between all the measured process parameters and rheological parameters were performed to evaluate which process parameters may help explain the variations in sludge rheology data. This was performed across the entire dataset as well as within each substrate type. More details are available in the methods sections of Papers I and II.

The modelling parameters for the different fluid samples were analysed further to obtain an estimate of their practical implications and thus answer the second research question. This was done through relatively simple calculations in Paper I and through comparatively more advanced computational fluid dynamics (CFD) modelling in Paper II. While both provide an estimate for stirring power requirements, the CFD approach is generally more accurate and provides additional information on mixing efficiency by allowing for the visualisation of the development of unstirred *dead zones*. More details are presented in the methods sections of Papers I and II.

3.3 Studies involving CSTBRs with induced TE deficiency

Despite the high number of possible substrates or their combinations that may impose different characteristics on the AD process, all processes retain a relatively high level of similarity in the main metabolic pathways and intermediates after the hydrolytic step. This step can therefore be thought of as a natural dividing point where AD can be split to the hydrolysis-related part and the post-hydrolysis part, with the latter containing most of the complex microbial interactions that are crucial for the AD system to work.

In order to address the third research question and study the interactions between microbial activity, fluid behaviour, and general process disturbances, the effects of the substrate needed to be minimized. For this purpose, an artificial medium containing no polymers or particles was applied, as described in Papers III and IV. The artificial substrate composition was chosen to promote post-hydrolysis anaerobic digestion (PHAD) reactions (i.e. fermentation, anaerobic oxidation, and methanogenesis) which occur in all functioning AD processes regardless of substrate origin. A process disturbance was induced in such AD systems by means of specific TE depletion as described in Paper III. Four mesophilic reactors (37°C), designated R_{ctrl} , R_{Co} , R_{Ni} , and R_{SeW} , were fed with the artificial substrate for a stabilisation period of more than 3 HRT before Co, Ni, and Se + W were removed from their respective substrates. Reactor R_{ctrl} served as a control and continued to receive the initial substrate (Figure 3b). This allowed for a controlled induction of gradually increasing process instability. In this way the microbial community structure, metabolism, and sludge rheology could be monitored as the AD process deteriorated. A thermophilic reactor (55°C) that experienced process instabilities after start-up was also used to shed light on TE-related disturbances of AD (Paper IV), as well as to answer the fourth research question. Process performance and rheology dynamics were monitored in conjunction with microbial community composition (Figure 3c) during implementation of measures for process stabilisation. This data was used to complement the data from the study described in Paper III, and provided further insights into how process disturbance influences the rheology characteristics in post-hydrolytic thermophilic AD systems. It also allowed an evaluation of how the recovery of process stability coincides with shifts in rheology. The details on the process monitoring approaches in these studies, including gas production kinetics, TS/TVS, VFA, TE concentrations, and pH, are presented in the methods sections of Papers III and IV. The rheology results from these experiments are reported and discussed in the thesis as a supplement to the information presented in Papers III and IV.

3.4 Microbiology analyses

The microbial community composition was evaluated in papers III and IV by next-generation sequencing of 16S rRNA gene amplicons separately for bacteria and archaea. The details about DNA extraction, amplicon library preparation, and data processing are presented in Papers III and IV. The results were used to evaluate the effects of TE-induced process disturbance on the microbial community and link it to the functioning of the different metabolic pathways presented in Figure 1. In this way it was pinpointed which microbial groups were affected by TE depletion first and how the series of interactions that followed corresponded to shifts in rheology on the way to process failure.

The data was analysed for diversity and evenness by calculating the first order Hill diversity numbers (Lucas et al., 2017), followed by non-metric multidimensional scaling (NMDS) to evaluate when the microbial communities began to change. Clustering analysis was used to compare the different samples and aid data interpretation.

3.5 Conceptual scheme of interactions

The results from all the studies described in this thesis were summarised and connected to published literature in order to address the fifth research question. This placed the findings from the individual papers in a broader context, allowing for a better discussion of their implications. A conceptual scheme was produced based on the results to visualise all the observed interactions in AD reactor systems.

Figure 3: Schematic representation of the different experimental designs used. Designations above each reactor in panel a) signify the individual substrate groups (AW – agricultural waste, CD – Co-digestion of a combination of substrates, FW – Food waste, PM – Paper mill waste, SS – Sewage sludge, PHAD – post-hydrolysis anaerobic digestion). The numbers below the reactors signify how many reactors in total were sampled in each group in studies described in Papers I and II. The plots in panel b) present how supplemented TE concentrations and OLR were changed during the experiment in Paper III. The colours of the lines correspond to the reactor they represent. The line representing several reactors is coloured with a combination of their colours. Black lines represent all reactors. The plots in panel c) show the response of the thermophilic system to the different measures intended to counter the process instabilities as described in Paper IV. The measures are listed in the lower-left corner.

4 Outcomes and reflections

The results of the studies described herein highlight the importance of the many interactions between different components in AD systems that need to be considered when attempting to optimise reactor operation for high efficiency and stability.

4.1 Effects of substrate type, operational, and process parameters on rheological characteristics of anaerobic sludge in CSTBRs

The properties of the substrate entering the CSTBR are arguably some of the most important parameters when it comes to effects on the AD process. Besides the general importance of substrate as a source of macronutrients which shape the microbial community and the general process performance, it also plays important roles in the supply and bioavailability of micronutrients (TE) in the reactor (Gustavsson et al., 2011; Hendriks et al., 2018). There are few studies on the relationship between CSTBR anaerobic sludge rheology and the substrate. Studies based on rheological data from a limited number of anaerobic digesters commonly claim that TS and VS contents are enough to explain the fluid behaviour in CSTBR systems (Mbaye et al., 2014). However, analysis of the anaerobic digesters investigated and presented in Papers I and II, which operated with a considerably broader range of substrate profiles and operational conditions, demonstrated that the rheological fluid properties and process parameters, including TS or TVS contents, correlate best among reactors digesting the same substrate type, but cannot be extrapolated broadly across different substrates. This introduces difficulties in predicting reactor fluid rheology when considering co-digestion, where different substrates are mixed, making the rheological effects of the individual substrates more challenging to follow and anticipate. Co-digestion AD systems account for a large number of industrial biogas plants and an even larger untapped potential for future biogas production (Kampman et al., 2016), signifying the importance of revising the conventional approaches for estimation of sludge rheology based solely on TS and VS for design and operation particularly of mesophilic of co-digesters (Papers I and II).

The rheological characteristics of all the anaerobic sludge samples from industrial and laboratory CSTBRs had a broad range of limit viscosities from below the approximate detection limit (< 3 mPa·s) to 62 ± 1.3 mPa·s (Figure 4). Furthermore, a range of different fluid behaviours (e.g. Newtonian, Bingham plastic, or pseudoplastic) was observed among the digesters (Paper I and II) regardless of substrate type. It could be observed that fluids with higher TVS have an increased chance for more complex rheological behaviour, such as yield stress and/or pseudoplasticity. Furthermore, TVS significantly correlated to the limit viscosity across all samples, but the goodness of fit for a linear model ($R^2 = 0.49$) revealed that only around half of the variability in the viscosity data could be explained by TVS. While the rheological parameters, such as limit viscosity and yield stress, do generally increase with increased TVS values, samples with significantly different behaviour were also observed, where the variations in rheology appeared to be independent of solid contents. In these cases, other parameters besides TVS most likely play an important role. Previously reported candidates that may affect reactor sludge rheology, other than solid content, are particle size distribution, and/or surface charge (Forster, 2002; Pevere et al., 2006; Tixier et al., 2003). These

attributes could in turn be affected by the substrate of each reactor, since substrates of different origins are expected to contain particles with varying characteristics (Tambone et al., 2013). Furthermore, the HRT or OLR have been described before as parameters governing AD rheology (Aranowski and Hupka, 2010; Karlsson et al., 2011; Moeller and Torres, 1997; Monteiro, 1997) and likely affect to what extent particle size and charge of the substrates are affected by the AD system during the degradation processes. The relationship between OLR and rheology was also observed in the study described in Paper II where higher OLR were generally associated with more complex rheological behaviour and increased limit viscosities.

Grouping the samples based on the substrate type revealed differences in the anaerobic sludge rheology characteristics among these groups (Figure 4). An analysis based on limit viscosity revealed that samples from the anaerobic microbial suspension and some samples from AD of paper mill waste (PHAD and PM on Figure 4) had the lowest values, reaching below the approximate detection limit of around 3 mPa.s. They were followed closely by thermophilic co-digestion (CD on the right half of Figure 4), where the lowest value was 3.5 ± 0.3 mPa.s.

Figure 4: Limit viscosities (shear rate 800 s^{-1}) for each substrate group in comparison with microbial suspensions from post-hydrolytic AD systems described in Papers III and IV (marked as PHAD). AW, agricultural waste; CD, co-digestion of two or more substrates; FW, food waste/organic fraction of municipal solid waste; PM, paper mill waste; SS, sewage sludge. The dashed red line marks the approximate lower detection limit of the rheometer (3 mPa.s).

The microbial cell suspension from post-hydrolytic AD reactors behaved similarly to a Newtonian fluid with a limit viscosity of 3.1 ± 0.6 mPa·s for mesophilic, and below 3 mPa·s for thermophilic systems.

The sludge of mesophilic reactors digesting paper mill waste showed the highest variability in limit viscosity values with anaerobic sludge behaving as a Newtonian or Bingham plastic fluid (PM on figure 4 and Paper II). The limit viscosities ranged from < 3 to 56 ± 1.6 mPa·s and corresponded well to differences in TVS between these reactors (Pearson correlation; $r = 1.0$, $p < 0.001$). Care should be taken when interpreting these results, however, since the low sample number resulted in only one reactor sample with a relatively high TVS value and a correspondingly higher limit viscosity. This was also the only sample which behaved as a non-Newtonian fluid, as exemplified by its apparent yield stress.

Sludges from reactors digesting agricultural wastes exhibited limit viscosities ranging from 4.6 ± 0.2 to 37 ± 1.7 mPa·s and behaved mostly Newtonian, except for one sample which was pseudoplastic with yield stress. The latter was also the sample with the highest TS and TVS values, which may explain its non-Newtonian behaviour. A positive correlation of limit viscosity with OLR and TVS was observed (Pearson correlation, $r = 0.90$, $p < 0.001$, and $r = 0.90$, $p < 0.001$, respectively).

The largest group of samples were from co-digestion of combinations of different substrates (CD on Figure 4). Samples from thermophilic CD reactors had relatively low limit viscosities from 3.5 ± 0.3 to 5.3 ± 0.3 mPa·s, while limit viscosities of mesophilic CD reactors ranged from 5.8 ± 0.7 to 25.3 ± 0.6 mPa·s. The sludges from thermophilic CD reactors behaved close to Newtonian fluids, while those from mesophilic systems exhibited a broader range of fluid behaviours, behaving either as Newtonian, Bingham plastic, or pseudoplastic fluids with or without yield stress. The correlation between TVS content and limit viscosity of mesophilic CD reactor fluids resulted in low r values of 0.32 (Pearson correlation, $p < 0.05$), while it was much stronger for thermophilic CD reactors (Pearson correlation, $r = 0.84$, $p < 0.001$).

Sewage sludge digestion (SS on Figure 4) led to a limit viscosity of 5.5 ± 0.1 mPa·s in the only thermophilic SS reactor in this study, and ranged from 4.6 ± 0.1 to 14.4 ± 0.2 mPa·s in the mesophilic reactor samples. The limit viscosities were linearly related to the TVS contents of the sludge (Pearson correlation, $r = 0.99$, $p < 0.001$). All fluids of mesophilic SS reactors with a TS above 3% behaved as pseudoplastic fluids, and the sample with TS values of 2.6% behaved as a Newtonian fluid.

The limit viscosity of sludges from food waste reactors ranged from 6.2 ± 0.3 to 62 ± 1.3 , which was the highest measured limit viscosity in this dataset. The fluid behaviour ranged from Newtonian at low solids content ($< 2.5\%$ TS), through Bingham plastic as solids increased (TS of 3.8%), to pseudoplastic at TS of 4.9%. Limit viscosity correlated with TVS (Pearson correlation, $r = 0.86$, $p < 0.05$). Among the modelling parameters, pH significantly correlated with the consistency index (K), which decreased as pH became higher (Pearson correlation, $r = -0.96$, $p < 0.05$). Similar correlations were observed before with sewage sludge by Sanin (2002) and Tixier et al. (2003), who argue that the effect of pH on the isoelectric points of flocs in the sludge could be responsible for this observation.

While the correlation coefficients clearly improved when relationships between rheological parameters and solids content were evaluated substrate-wise, the mathematical functions describing these correlations were different for each group. This suggests that while the particle content in sludge does affect its rheological properties, the extent of these effects depends largely on the substrate composition used. It is likely that the chemical compositions of substrates from different substrate groups are vastly different. Since the AD process in CSTBRs is continuous, there are always undegraded substrate components present in the reactor. The concentration of these components depends on the AD process efficiency, and in particular, the VS reduction. It has been shown that substrate type significantly affects the chemical composition of the resulting anaerobic sludge since some of the compounds from the substrate remain in the reactor (Shakeri Yekta et al., 2012; Tambone et al., 2013). This would imply different chemical characteristics of the particles inside CSTBRs digesting different substrates, which together with their physical characteristics (particle concentration, size, and surface charge) may explain the observed differences in how their rheology corresponds to their TS/TVS contents.

It is important to be aware of the limitations associated with the rheology measurement method and instrument used. Specifically, the controlled-rate *Searle-type* rheometers, such as the one used in the described studies, are less suited to measuring samples with low rheological parameters than e.g. controlled-stress *Couette-type* systems performing oscillatory measurements (Schramm, 2000). This implies that the presented values for samples where limit viscosity was near or below 3 mPa·s may carry with them a larger relative error. The same applies for the values of yield stress since the rheometer used is less accurate at low shear rates where yield stress is measured (Schramm, 2000). Nevertheless, the decreased accuracy for these samples does not have significant effects on the conclusions of the studies in this thesis since rheology-associated problems are expected to occur in reactors only at high viscosity and complex (i.e. non-Newtonian) rheological behaviour. The measurements of such samples were in the optimal range of the rheometer and can be considered to be accurate. Additionally, the relative comparison of different samples analysed with the same method provides increased reliability of the results. An inherent difficulty in measuring the rheology of suspensions is the effect of particles on the level of noise in the result. On the one hand particles are one of the key components affecting the rheology of the suspension, but on the other hand they may cause interference in the measurements if their size is too large in relation to the gap size of the rheometer. One common approach to dealing with this is to filter particles above a certain size from the suspension before performing the analysis. While this does decrease the amount of noise, it also decreases the representability of the fluid being sampled. In essence, the choice needs to be made between accepting the risks of noisy, or unrepresentative results. Another approach to abate this problem is to increase the gap size of the rheometer, however, this too is a compromise, since a larger gap increases the risk of only part of the fluid being sheared or turbulent flow being achieved, both of which break important assumptions of measuring rheology with rotational rheometers (Ratkovich et al., 2013). The approach chosen in the studies described in this thesis was to preform measurements on non-filtered samples and removing cases with high noise or variability between replicates from further analysis. This

was a compromise involving keeping the gap size small enough for reliable measurements and not modifying the sample composition beyond removing only the largest individual debris.

4.2 Effect of rheology differences between different CSTBR sludges on power requirements and mixing efficiency

While the substrate-related differences in rheology of the different anaerobic sludges do exist, the question remains what this means in practice when it comes to mixing efficiency and power demand. This was evaluated by calculations of stirring and pumping power demands for all samples in Paper I, and CFD modelling in Paper II, which was applied for three samples chosen to represent i) a case with Newtonian fluid behaviour and low viscosity, ii.) a case with pseudoplastic behaviour with a relatively high consistency index (K), and iii.) the case with the highest yield stress (τ_0) in the dataset. The results revealed large relative differences in power demand across samples in both papers. CFD modelling also revealed large differences in mixing efficiency, where cases ii and iii highlighted that complete mixing would be very difficult to achieve with the assumed reactor setup (Paper II). The implication of these results is that a much simpler stirring setup could be used to operate a reactor on the substrate of case i, while cases ii and iii may experience significant problems if the setup is not adapted to deal with fluids that have high K and τ_0 , or the substrates treated in a way that would reduce their rheological effects. In particular, it could be observed that large non-stirred dead zones would form for fluids with high K and τ_0 in the assumed reactor during the digestion of these substrates, which would likely have negative effects on process performance. This is because no fresh substrate would enter those zones, rendering them useless and effectively decreasing the active volume of the reactor (Saeed et al. 2008). The differences in the stirring power demand would also have important implications since a higher power demand imposes a requirement for stronger, more expensive engines, as well as increased energy consumption. This would be reflected in the energy consumed for stirring and pumping of such a fluid, which account for considerable expenditures for reactor operation. It is therefore safe to assume that the economics of biogas production could be improved through rheology-based optimisation. However, considering that the total price of biogas is currently only to a small percentage dependent on the production-related energy costs (Kampman et al., 2016; Weiland and Schütte, 2010), it could be argued that the improvement of stirring and pumping power consumption would likely not have a substantial effect on the final price of biogas. Nevertheless, it is highly important, since it would indirectly affect the process performance through mixing efficiency, which is linked directly to biogas production, with far larger financial consequences than energy consumption.

It should be noted that the estimates derived from CFD modelling, or even the simpler calculations presented in Paper I, are reactor-geometry-dependent. The absolute estimated values should therefore be considered with that in mind. Nevertheless, the application of the same assumptions to different substrates allows us to compare the specific effects of rheological characteristics of each sample on mixing efficiency and power demand.

4.3 Effects of nutrient deficiency and process stability on sludge rheology

Another way substrate composition may affect the AD process besides rheology is its level of nutrient supply. In particular TE concentration and bioavailability play an important role in AD process stability and efficiency (Murray and Van Den Berg, 1981). As highlighted in the study with the defined medium, described in Paper III, the depletion of TE, in that case Ni or Se + W, primarily led to an inhibition of specific microbial groups. In particular the activities of methanogenic archaea and their syntrophic partners were affected first. As expected, the lack of specific TE slowed down the rates of some metabolic reactions, most likely due to the inability of the microorganisms to synthesize important enzymes for sustaining their growth (Hendriks et al., 2018). This most likely began with hydrogenotrophic methanogenesis pathways and specific steps within the Wood-Ljungdahl pathway, since Ni, Se, and W are required for the functioning of these pathways (5-8 in Figure 1). This was observed to manifest itself first in the methane production kinetics, which indicated a decreased rate of VFA turnover. Primarily the concentrations of propionate were observed to increase in the affected TE-depleted reactors. This implies that the metabolic rates of syntrophic propionate degraders gradually became slower until it took more than 24 hours for them to degrade it. Thus, a corresponding decrease in OLR would be required to keep the system stable. In other words, the depletion of TE led to an overload of the AD system by slowing down the rates of certain metabolic reactions, turning them into bottlenecks for the whole AD process.

Despite similar initial symptoms during Ni and Se + W depletion (i.e. propionate accumulation), the causes of these symptoms most likely stemmed from different metabolic pathways of the process. Since Se and W are needed for formate dehydrogenase as well as hydrogenotrophic methanogenesis (Figure 1 ; De Bok et al., 2003; Schmitz et al., 1992), their depletion likely primarily caused accumulation of formate and/or H₂, which then led to propionate accumulation due to product inhibition. On the other hand, Ni is important for most hydrogenases as well as carbon monoxide dehydrogenase/acetyl-CoA synthase, which are needed by methanogens and acetogens (Funk et al., 2004; Hendriks et al., 2018). Therefore, both groups were likely affected, since an initial accumulation of propionate in combination with a comparatively slower accumulation of acetate was observed. Despite the difference in mechanisms, both cases eventually led to an accumulation of other VFA due to additional microbial groups being inhibited. This was demonstrated by shifts in the bacterial communities, which happened progressively as each new VFA began to accumulate (Paper III). As the VFA concentrations continued to increase, the buffering capacity of the anaerobic sludge was overwhelmed, resulting in a decreasing pH.

When the VFA accumulated and pH decreased further, a simultaneous reduction in methane production and TVS took place, which indicated cell decay or washout, resulting in a decrease of the microbial biomass in the reactor. This was most likely due to VFA imposing increasing inhibition on the microbial community due to the rising VFA concentrations in combination with their increased toxicity as a result of low pH (Mörsche and Jördening, 1999). Consequentially, the growth yields of specific microorganisms decreased, leading to their washout. Significant shifts in bacterial community compositions of these reactors were

observed after the pH drop as compared to before it (Paper III). Regardless of these changes, viscosity remained below the detection limit, implying that the response of the microbial community to TE deficiency did not have any observable effects on the fluid behaviour, e.g. due to formation of EPS and SMP, which could possibly change the rheology (Sutherland, 2001). Instead it appeared the rheology dynamics were mainly associated with cell density, since the viscosity was observed to decrease slightly upon TVS reduction.

The case study, involving the thermophilic reactor operated on a defined medium (Paper IV) revealed similar results regarding the response of rheology to process stability. The anaerobic sludge of the thermophilic CSTBR behaved as a low-viscosity Newtonian fluid. Its viscosity corresponded to process stability, rising above 3 mPa·s only when the process was stable, and decreasing during instability. This indicates that the cell density of the microbial community played an important role in reactor fluid rheology.

This reactor experienced instability shortly after process start-up. The VFA degradation was completely inhibited as demonstrated by no changes in their concentration when feeding was discontinued. This problem was rectified by increased levels of Se and W supplementation, which led to a rapid consumption of propionic acid. This result suggests that formate dehydrogenase activity was limited in this reactor due to insufficient Se and/or W supply. The OLR could be gradually increased after this was remedied, thus allowing the microbial community to slowly recover as evidenced by increasing TVS concentrations and an increased viscosity up to 3.6 ± 0.6 mPa·s. A dominance of the hydrogenotrophic *Methanoculleus* genus among the methanogens was observed to correspond to periods of process stability, while *Methanosaeta* and *Methanothermobacter* prevailed during process disturbance. This indicated that the studied AD system most likely produced methane mainly through the hydrogenotrophic pathway, with syntrophic acetate oxidizing bacteria transforming the acetate to CO₂ and H₂, and formate dehydrogenase playing a crucial role in the final step of this transformation. The rates of these reactions were, however, still limited since acetate began to gradually accumulate when the OLR was further increased, eventually leading to another period of process disturbance and failure. This once more coincided with a decrease of viscosity below 3 mPa·s.

The results of the experiments described in Paper III and Paper IV therefore revealed that process disturbance and eventual failure manifest themselves primarily as significant shifts in microbial activity with the primary inhibition of a specific microbial group becoming amplified through secondary inhibitions due to intermediate compound accumulation. In the TE-depleted reactors the primary inhibition occurred within the final substrate transformations of the AD process, affecting mainly methanogenic archaea or their syntrophic partners. It was found that close monitoring of the metabolic activity of these microbial groups through methane production rates may allow for an early warning of the overload of the reactions they perform (Paper III). Alternatively, their activity could also be monitored with mRNA analyses instead of DNA analyses performed in the described studies. While that would give additional scientific input to the study, the method is not applicable to routine measurements at industrial-scale due to its complexity and price.

If left unaddressed, the initial disturbance may eventually lead to severe inhibition, coupled to cell washout and decrease in viscosity. This highlights that the effects of microbial activity on rheological behaviour of anaerobic sludge depend on process stability. More specifically, there is a positive correlation between process stability and cell density, which affects the rheological characteristics of the anaerobic sludge in the same way as any particle would according to the *Einstein equation* (Hiemenz and Rajagopalan, 1997). Despite this, an increasing intensity of process disturbances will eventually change the reactor environment to a point where the conditions become suboptimal for hydrolysis and solubilisation of complex structure of substrate organic matter, resulting in an accumulation of substrate polymers and particles (Kim et al., 2003).

4.4 Interactions between substrate composition, microbial community, and sludge rheology

The studies described in Papers I-IV demonstrate that AD rheology depends on a balance between the substrate characteristics and microbial activity through a range of different interactions. These are proposed in the form of a conceptual scheme presented in Figure 5, where the blue arrows mark interactions experimentally confirmed in the studies described in papers I-IV. Solid green arrows are based on interactions confirmed for AD systems by other studies, and dashed green arrows highlight the interactions that were described in systems similar to AD, but which are still to be confirmed for AD systems.

Figure 5: Conceptual model of interactions in AD systems. Blue arrows correspond to interactions evaluated in the studies described in Papers I-IV, green arrows are based on findings in the published literature on AD systems (solid) or non-AD systems (dashed).

A shift in substrate composition may lead to changes in anaerobic sludge rheology as implied by results from Papers I and II (interaction 1 in Figure 5). This is likely regulated by HRT and OLR, which have been previously observed to affect rheology in CSTBR systems (Interactions 2 and 3 in figure 5; Paper II, Aranowski and Hupka, 2010; Battistoni et al., 1993). In addition to that, the new substrate may contain less TE or more compounds that precipitate with TE, decreasing their bioavailability and possibly leading to TE deficiency (interaction 27 in figure 5). The rapid process deterioration in the thermophilic reactor of Paper IV, when it was digesting the same substrate as the mesophilic reactor in Paper III, revealed that operational temperature can affect the TE requirements of a reactor (interaction 28 in Figure 5). Similar observations were reported before by Takashima et al. (2011), who found that thermophilic digestion of an artificial medium required more TE supplementation than mesophilic digestion. This is likely due to a combination of lower bioavailability at higher temperatures and an increased metalloenzyme production (Hendriks et al., 2018). The latter is likely due to increased metabolic rates of thermophilic microorganisms, while the bioavailability, which corresponds to the ease of microbial uptake of a chemical element (Callander and Barford, 1983), likely decreases due to decreased solubility of carbonate and phosphate metal salts at higher temperatures (Hendriks et al., 2018).

If the substrate-induced changes in anaerobic sludge rheology lead to significant increases in rheological parameters, they can substantially increase the power requirements imposed on the stirring systems and pumps of the reactor (Papers I and II). A highly viscous fluid may trap the produced gas bubbles, resulting in bulking of the sludge, which can cause physical damage to the reactor (interaction 4 in Figure 5; Lindorfer and Demmig, 2016). If the stirring system of the reactor is not able to cope with the increased rheology the mixing efficiency may decrease, which has been linked to the creation of unstirred *dead zones*, and increased sedimentation or floatation of material in the reactor (interactions 5 and 7 in Figure 5; Papers I and II). Similar behaviour has been observed in reactors after the breakdown of their stirring systems (interaction 6 in Figure 5; Lienen et al., 2013)). As the mixed *active volume* of the reactor becomes smaller, the chance of substrate overloading increases, causing stress on the AD system (interactions 4 and 11 in Figure 5). On the other hand, the sedimentation of particles increases the solids retention time in the reactor and may lead to increased TS/VS values of the anaerobic sludge if the particles are resuspended. This may close the cycle and lead to further increases of anaerobic sludge rheology (interactions 8 – 10 and 29 in Figure 5).

The process overloading, which may occur either through substrate-induced TE depletion, presence of inhibitors, increased OLR, or decreased reactor active volume, may lead to the accumulation of process intermediates, e.g. different fermentation products (interaction 14 in Figure 5; Papers III and IV). Some of these intermediates have been observed in increased concentrations during foaming events (interaction 25 in Figure 5), but it is so far unclear if they cause foaming or are just a symptom of a disturbed process, which is more likely to experience foaming due to some other mechanisms (Ganidi et al., 2009). Increased concentrations of VFA and hydrogen partial pressures can lead to further inhibition of the AD process (interaction 15 in Figure 5) which gradually leads to complete process failure (Papers III and IV). Increasing inhibition may cause the EPS from the biofilms to be degraded and released in the suspension in the form of shorter SMP molecules (interactions 22 and 23 in Figure 5; Aquino and Stuckey,

2004a). If a substantial part of SMP are proteins, they may accumulate on the surface where they can stabilise the gas bubbles, resulting in foam (interaction 24 in Figure 5; L. Moeller et al., 2012). Foam in ruminants, which are an AD system similar to CSTBR, has been observed to contain higher concentrations of TE, such as Ni and Zn, as compared to the sludge (Harris and Sebba, 1965). This may indicate that foam can possibly play a role in perpetuating TE depletion via phase-separation in AD systems as well (interaction 26 in Figure 5).

Process deterioration also leads to cell decay and washout, which reduces their concentrations in the CSTBR and decreases their effects on anaerobic sludge rheology (interactions 16-18 in Figure 5, Papers III and IV). The inhibition spreads to the point of affecting the substrate degradation, manifesting itself in decreased VS reduction and the accumulation of substrate polymers (interaction 19 in Figure 5). Some of these polymers have been previously shown to cause foaming if they start to accumulate in the reactor (interaction 21 in Figure 5; Kim et al., 2003). At the same time, the substrate particles may accumulate, increasing the TS/VS contents of the anaerobic sludge, which may increase its rheological characteristics (interactions 20 and 10 in Figure 5). Since the substrate-related effects on anaerobic sludge rheology are generally much higher than the microbial community-related effects (Figure 4), it is likely that a process disturbance would lead to increased rheological characteristics of the anaerobic sludge due to the increased effects of the substrate in conjunction with decreased VS reduction (interaction 29 in Figure 5). The extent of this likely depends on the rheology of the substrate, since the anaerobic sludge composition can be expected to approach the composition of the substrate as the microbial activity deteriorates.

5 Implications

This thesis revealed some of the intricacies of how rheological behaviour is affected by the interactions between microbial community structure and activity, the substrate composition, and its nutrient supply for microbial growth. The results have several implications that are relevant for optimisation of AD systems and highlight the potential areas requiring further research.

In summary, the experiments in Papers I and II revealed that substrate type affects the extent to which CSTBR operational and process parameters correspond to anaerobic sludge rheology. This, in turn, has significant implications for mixing efficiency and power requirements. The effects of substrate on fluid behaviour were particularly inconsistent in co-digestion systems where the combinations of substrates likely interact and impose fluid properties that are difficult to predict. The results of experiments in Papers III and IV revealed that as TE limitation affects the activity of specific microbial groups, causing accumulation of intermediate fermentation products and eventual process failure, it can also lead to corresponding shifts in sludge rheology. This relationship between process stability and sludge rheology also has important implications. If a complete digestion of a substrate is achieved, the rheological properties should be entirely determined by the microbial biomass and its products in the same way as in the reactors from Papers III and IV. On the other hand, if the VS reduction were to collapse completely, the fluid characteristics should closely mimic that of the incoming substrate, since the microbial cells and their products would be washed out. These two hypothetical extremes embody all AD processes.

All of these observations imply that more optimal AD of new substrates may be ensured by not only evaluating the substrate from the perspective of its direct effects on process performance, but also on rheology. This would provide a clearer picture on the effects of a given substrate on mixing efficiency and power requirements, which would allow for a better degree of optimisation of reactor design for AD of that specific substrate as well as indicate when possible adjustments to the process are needed. Particularly for substrates with large effects on anaerobic sludge rheology, the use of pre-treatment methods before AD should be considered, since these have been observed to decrease the rheological characteristics of CSTBR sludge (e.g. Tian et al. 2014; Goel et al. 2004; Perez-Elvira et al. 2010). In particular the beneficial effects of pre-treatment methods on power demand for pumping and stirring should be considered just as important as the increased biogas production. Considering the effects of rheology in addition to the effects of the substrate chemical composition would be a more wholesome approach for evaluation of new substrates, which would decrease the likelihood of problems during their implementation.

Continued growth of the AD industry is one of the key ways towards the reduction of fossil fuel consumption and the emissions of greenhouse gasses, while treating organic wastes and enabling sustainable development. Further research is needed in order to ensure this growth happens by developing high-performance AD systems operated on new substrates, thus fulfilling a larger share of our renewable energy requirements. Developments in these directions are important to achieve our sustainability goals.

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