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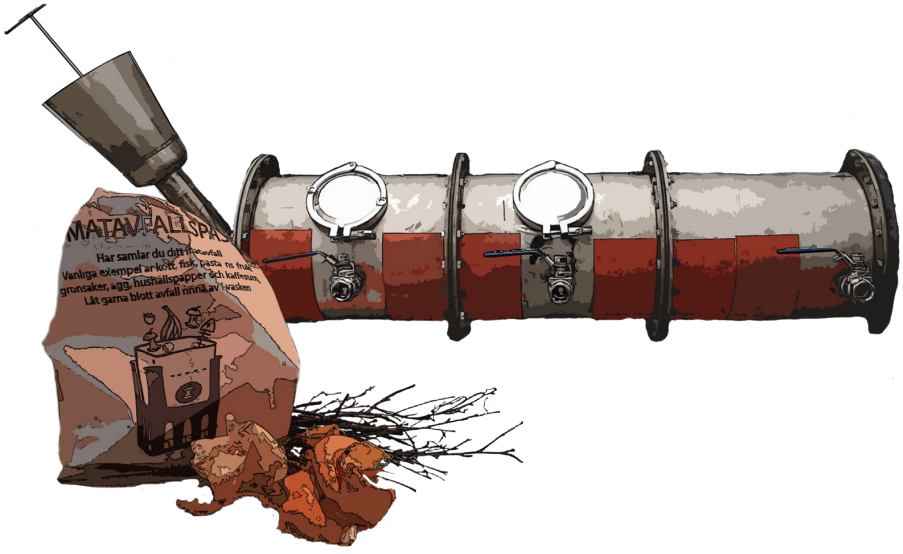
Challenges associated with high-solid anaerobic digestion

Down-scaling, plug-flow behaviour and ammonia inhibition

Ebba Perman



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Cover: Image illustrating a laboratory-scale plug-flow reactor and examples of high-solid substrates (organic household waste and garden waste), created by Edvin Perman.

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Abstract

Biogas production through anaerobic digestion (AD) enables use of resources contained in organic waste streams to produce renewable energy and biofertiliser. For treatment of organic material with high total solids content, high-solid anaerobic digestion (HSD) reduces the need for dilution, but has not been as extensively studied as more conventional wet AD processes. The low dilution rate in HSD involves a high risk of accumulation of toxic substances, such as ammonia, a well-known inhibitor of AD processes. Monitoring of thermophilic HSD systems in this thesis indicated disturbances, with associated accumulation of propionate and changes in microbial population at ammonium-nitrogen concentrations >4 g/L. Continuous HSD processes are typically run in reactors of plug-flow type (PFR), characterised by a concentration gradient of organic material and degradation products from inlet to outlet. This thesis demonstrated challenges in obtaining plug-flow dynamics and phase-separated microbial communities in PFRs both at laboratory and industrial scale. Serial digestion systems, which have characteristics similar to plug-flow behaviour, improved methane yield and protein degradation significantly when food waste was digested in wet conditions. A down-scaling study of an industrial-scale HSD process showed that similar process performance and yield could be obtained in laboratory-scale PFRs, but also that HSD experiments can be carried out in completely stirred-tank reactors (CSTR) usually used for wet materials. Thus the work in this thesis addressed some of the challenges associated with HSD and demonstrated that laboratory-scale studies can be used for future process optimisation trials.

Keywords: Biogas production, serial digestion, high-solid (dry) anaerobic digestion, plug-flow reactors, continuous stirred-tank reactors, microbial community structure, process disturbances, ammonia inhibition, down-scaling

Utmaningar relaterade till torrötning

Nedskalning, plugg-flöde och ammoniakhämning

Sammanfattning

Biogasproduktion via anaerob nedbrytning (rötning) ger möjlighet att ta tillvara på resurser i organiskt avfall för att producera förnybar energi och biogödsel. Tekniken torrötning reducerar behovet av utspädning vid nedbrytning av material med hög torrsubstanshalt. Trots fördelarna med denna teknik så har den inte studerats i lika omfattande grad som den mer konventionella våtrötningstekniken. Den låga utspädningsgraden vid torrötning medför dock hög risk för ackumulering av toxiska substanser, exempelvis ammoniak som är välkänd för att orsaka hämning av rötningsprocesser. Termofila torrötningssystem som studerades i denna avhandling visade tecken på processstörningar, med ackumulering av propionat och förändringar i den mikrobiella populationen som följd, vid ammonium-kvävehalter >4 g/L. Kontinuerliga torrötningsprocesser körs ofta i reaktorer av pluggflödestyp som kännetecknas av koncentrationsgradienter av både organiskt material och nedbrytningsprodukter mellan inlopp och utlopp. Detta arbete demonstrerade dock utmaningar med att åstadkomma pluggflöde och fasseparering av mikrobiomet, i både laboratorie- och industriell skala, vilket indikerar ett behov av fortsatta studier. Seriella rötningssystem, som har egenskaper liknande pluggflöde, visades kunna ge en signifikant ökning av metanutbyte och proteinnedbrytning vid våtrötning av matavfall. En nedskalningsstudie av en industriell torrötningsprocess visade på att liknande förhållanden och processutbyte kunde erhållas i pluggflödesreaktorer i laboratorieskala, men också att torrötningsexperiment kan utföras i totalomblandade reaktorer som vanligtvis används vid våtrötning. Arbetet som presenteras i denna avhandling syftade till att uppmärksamma några av de utmaningar som associeras med torrötning, och också demonstrera att försök i laboratorieskala kan användas för framtida optimeringsstudier.

Keywords: Biogasproduktion, seriell rötning, torrötning, pluggflödesreaktorer, kontinuerligt omrörda reaktorer, mikrobiell sammansättning, processtörningar, ammoniakinhibering, nedskalning

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Perman E, Schnürer A, Björn A, Moestedt J. (2022). Serial anaerobic digestion improves protein degradation and biogas production from mixed food waste. *Biomass and Bioenergy* 161, 106478. https://doi.org/10.1016/j.biombioe.2022.106478.

II. Perman E, Westerholm M, Liu T, Schnürer A. (2024). Comparative study of high-solid anaerobic digestion at laboratory and industrial scale – Process performance and microbial community structure. *Energy Conversion and Management* 300, 117978. https://doi.org/10.1016/j.enconman.2023.117978.

III. Perman E, Karlsson A, Westerholm M, Isaksson S, Schnürer A. Technologies for digestion of high-solid substrates – a comparison of completely stirred and plug-flow reactor systems. (submitted)

IV. Perman E, Bakar Siddique A, Westerholm M, Schnürer A. Metatranscriptomic study of a high-solid anaerobic digester operated at high ammonia levels. (manuscript)

Papers I-II are reproduced with the permission of the publishers.

The contribution of Ebba Perman to the papers included in this thesis was as follows:

I. Part of laboratory work (microbial community analysis), data compilation and analysis, statistical analysis, writing of original manuscript draft.

II. Execution of the majority of laboratory work, data compilation and analysis, statistical analysis, writing of original manuscript draft.

III. Part of laboratory work (sample analyses, microbial community analysis), data compilation and analysis, statistical analysis, writing of original manuscript draft.

IV. Execution of the majority of laboratory work, data compilation and analysis, bioinformatics and statistical analysis, writing of original manuscript draft.

List of figures

Abbreviations

AD	Anaerobic digestion
CSTR	Completely stirred-tank reactor
HRT	Hydraulic retention time
HSD	High-solid digestion
OLR	Organic loading rate
PFR	Plug-flow reactor
RMP	Residual methane potential
RTD	Retention time distribution
SAOB	Syntrophic acetate-oxidising bacteria
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids

1. Introduction

1.1 Biogas production through anaerobic digestion

In the biogas process, organic waste streams are transformed into valuable resources, *i.e*. renewable energy in the form of biomethane (CH_4) and nutrient-rich biofertiliser (IEA, 2020; Baştabak & Koçar, 2020). Production of biomethane is highly interesting in light of the current high demand for sustainable and renewable energy sources, and can help reduce the need for natural gas from fossil sources (EurObserv'ER, 2023; Scarlat *et al*., 2018; REPowerEU, 2022). Due to several advantages, including sustainable waste treatment and production of an organic fertiliser, biogas production can be an important part of a sustainable society.

Methane production through anaerobic digestion (AD) is carried out by communities of microorganisms, a process that occurs naturally in environments with low levels of oxygen. However, only a fraction of the methane emitted to the atmosphere comes from natural processes, while around 60% is estimated to be a result of human activity, mainly agriculture, fossil energy industry and waste (Saunois *et al*., 2020; Lee *et al*., 2017; IEA, 2021). Emissions of methane represent a major environmental problem, since it is a potent greenhouse gas (Saunois *et al*., 2020). In a biogas reactor, AD takes place in a controlled environment, where the biomethane is collected and used as a biofuel, instead of leaking out into the atmosphere. This can help reduce methane emissions from agriculture and waste handling and provide a replacement for fossil fuels (Rama *et al*., 2023; Chadwick *et al*., 2011; Clemens *et al*., 2006; Scarlat *et al*., 2018).

The AD process is generally divided into four steps (Figure 1): hydrolysis, acidogenesis (fermentation), acetogenesis (anaerobic oxidation)

and methanogenesis (Anukam *et al*., 2019; Schnürer & Jarvis, 2018; Sarker *et al*., 2019; Angelidaki *et al*., 2011). In short, AD systems are fed substrates composed of complex organic polymers, mainly proteins, fat and carbohydrates. During hydrolysis, the polymeric molecules are cleaved into monomers by extracellular enzymes excreted by hydrolytic bacteria (Angelidaki *et al*., 2011). In the acidogenesis step, the main hydrolysis products, *i.e*. simple sugars, fatty acids and amino acids, are taken up by the bacteria and fermented into various volatile fatty acids (VFA), alcohols, ammonia (NH_3), hydrogen gas (H_2) and carbon dioxide (CO_2) (Sarker *et al*., 2019). Next, in the anaerobic oxidation step, the fermentation products are converted into acetate, CO_2 and H_2 (Angelidaki *et al*., 2011). During methanogenesis, these molecules are the main substrates for the methane-producing archaea, which in general use either acetate (acetoclastic) or CO_2 and H_2 (hydrogenotrophic) for production of the final products, CH_4 and CO_2 (Anukam *et al*., 2019; Sarker *et al*., 2019).

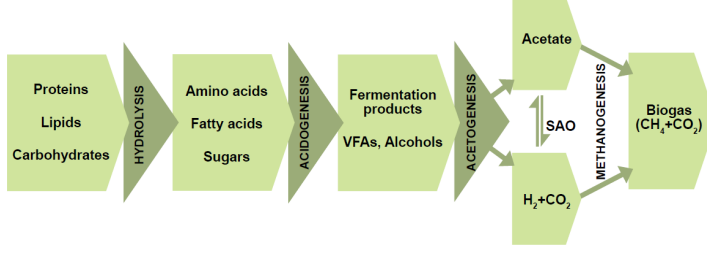

Figure 1. Overview of the anaerobic digestion process. SAO: syntrophic acetate oxidation, VFA: volatile fatty acids.

The microbial community involved in AD consists of taxonomically diverse groups of bacteria and archaea that are responsible for the different degradation steps (Angelidaki *et al*., 2011). During operation, it is of the utmost importance to keep the system in balance, as inhibition of any microbial group leads to the build-up of intermediary products, which can eventually result in process failure. A well-documented example of this, which is also a key issue in many AD processes, is ammonia inhibition (Rajagopal *et al*., 2013). Ammonia is a by-product of protein fermentation and can reach high levels in processes fed with protein-rich substrates, such as food waste (Rajagopal *et al*., 2013; Sarker *et al*., 2019). The methanogens that perform the final methane-producing step are especially sensitive to high levels of ammonia (Rajagopal *et al.*, 2013). Reduced methanogenic activity

in turn affects the microorganisms converting VFA, leading to VFA accumulation and potentially reactor acidification (Westerholm *et al*., 2016; Capson-Tojo *et al*., 2020). However, despite the challenges of process instability and reduced yield, high ammonia level in the process has the advantage that the digestate produced contains a high concentration of ammonia-nitrogen, which is desirable for biofertiliser purposes (Alburquerque *et al*., 2012; Drosg *et al*., 2015).

Common substrate sources are sewage sludge, organic household waste, industrial food waste and agricultural wastes such as crop residues and manure (EurObserv'ER, 2023; IEA, 2020; Sarker *et al*., 2019).The performance and stability of a process are highly dependent on the composition of the substrate and on the organic loading rate (OLR). High OLR can overload the system and result in short hydraulic retention time (HRT), *i.e*. the time required to exchange the reactor volume in a continuous system (Schnürer *et al*., 2016). Short HRT may not permit thorough degradation and could cause washout of slow-glowing organisms, contributing to instability and VFA accumulation (Sarker *et al*., 2019; Ferguson *et al*., 2016). The substrate needs to have a balanced composition, containing all essential macro-nutrients, vitamins and trace elements, in order to support the growth and functions of the microorganisms (Angelidaki *et al*., 2011; Fosua *et al*., 2023). This requirement can be addressed by co-digestion of different substrate fractions to obtain a mixture with a combination of all necessary components (Arelli *et al*., 2018; Zhang *et al*., 2022; Wang *et al*., 2023). Co-digestion can also help balance the content of nitrogen-rich proteins and carbon-rich carbohydrates, and of quickly degradable, such as simple sugars, and more recalcitrant components, such as lignocellulosic materials (Mao *et al*., 2015; Brown & Li, 2013; Lansing *et al*., 2019).

1.2 High-solid anaerobic digestion

In terms of process design, different AD techniques are available, with a key factor in the process selection being the total solids (TS) content present in the substrate. A commonly used type of process is wet digestion, characterised by a TS content typically <15%. An alternative approach, high-solid anaerobic digestion (HSD), generally operates at TS level >15%

(Carlos-Pinedo *et al*., 2019; André *et al*., 2018). In the literature, several advantages of HSD over wet AD are described. The main advantages are:

- Reduced need for fresh water for dilution (Angelonidi & Smith, 2015; Hayyat *et al*., 2024).
- Reduced substrate volume, which lowers reactor size and the energy requirement for heating (Duan *et al*., 2012; Carlos-Pinedo *et al*., 2020).
- Reduced digestate volume and increased concentration of nutrients because of the lower dilution of the material (Fagbohungbe *et al*., 2015; Carlos-Pinedo *et al*., 2020).
- High OLR can be applied while maintaining relatively long HRT (Duan *et al*., 2012; Wang *et al*., 2023).
- High tolerance to bulky material, enabling a wider substrate range with less need of pre-treatment (Angelonidi & Smith, 2015; Akinbomi *et al*., 2022, Colazo *et al.*, 2015).

High-solid digestion is already extensively utilised at industrial scale in Europe (Hayyat *et al*., 2024). According to reviews by Fagbohungbe *et al*. (2015) and Gao *et al*. (2023), >50% of all AD systems in the European Union in 2010 operated under high-solid conditions. In Sweden, however, the first HSD plant was not built until 2013 (Feiz, 2016), and the number of HSD plants is still relatively low. HSD is a suitable technology for treatment of organic material with naturally low water content, *e.g.* various dry fractions of agricultural residues and municipal solid waste fractions including garden waste, food waste and organic household waste (Arelli *et al*., 2021; Carlos-Pinedo *et al*., 2019; Zamri *et al*., 2021; Westerholm *et al*., 2020; Rocamora *et al*., 2020; Kothari *et al*., 2014, Franca & Bassin, 2020). Given the high abundance of municipal solid waste and solid agricultural residues generated within Sweden and Europe (Lindfors & Feiz, 2023; van der Linden & Reichel, 2020), there is great potential for wider utilisation of the HSD technology.

During HSD, careful operation is necessary since these systems run a relatively high risk of suffering from process disturbances, especially during treatment of food waste and other protein-rich substrates (Zamri *et al*., 2021; Bi *et al*., 2020; Shapovalov *et al*., 2020). The combination of low dilution rate and generally high load of organic material leads to high risk of

ammonia-induced disturbances in HSD processes (Wang *et al*., 2023; Bi *et al*., 2020). An additional challenge in treating high-solid substrates is that both substrate and digestate are more difficult to handle compared with wet material, since they can be more bulky and have larger particle size and therefore cannot be pumped (Fagbohungbe *et al*., 2015). Due to the low water content, the reactor material becomes highly viscous, which limits mass transfer and makes it difficult to mix efficiently (Wang *et al*., 2023; Li *et al*., 2023a). Thus, there are several challenges related to this type of process that need to be taken into consideration and numerous studies have pointed out that knowledge about HSD needs to be improved (*e.g*. Akinbomi *et al*., 2022; Carlos-Pinedo *et al*., 2019; Li *et al*., 2023a, Franca & Bassin, 2020).

1.3 Reactor types

For HSD applications, various reactor types exist, each with different geometries and mixing strategies. For exceptionally high TS levels (around 40%), it is common to operate HSD as a batch process (Li *et al*., 2011; Nizami & Murphy, 2010; Zamri *et al*., 2021). This entails filling a reactor with pre-inoculated substrate and allowing it to undergo one retention time without additional feeding, often with minimal stirring. A common type of batch digester is the German garage type, Bekon, which is mixed by recirculation of process liquid (Fagbohungbe *et al*., 2015; Akinbomi *et al*., 2022). Although batch processes are cost-effective and require low energy input (Zamri *et al*., 2021), gas production is not continuous and the labour-intensive task of filling and emptying the digester before and after operation makes them less suitable for larger-scale application. Consequently, batch processes are more commonly used in small-scale biogas production (Gao *et al*., 2023).

Continuous processes are beneficial for industrial-scale production, as this enables continuous substrate treatment and constant and consistent biogas production (Gao *et al*., 2023). Common examples of reactor types for continuous HSD are the Valorga, a vertical reactor mixed by injection of pressurised biogas, the Dranco, another vertical reactor that has no internal mixing system (Fagbohungbe *et al*., 2015; Gao *et al*., 2023; Zamri *et al*., 2021; Elsharkawy *et al*., 2019), and the Kompogas reactor, a continuous horizontal plug-flow type reactor (PFR) with mechanical mixing, developed

for digestion of high-solid substrates with a TS content of 10-40% (Wellinger *et al*., 1993). In Sweden, the most common type of reactor used for HSD is the Kompogas technology (Westerholm *et al*., 2020) and it was therefore the main focus in this thesis work.

A reactor of plug-flow type is tubular in shape, with substrate introduced at one end and digestate extracted from the opposite end. Ideally, the substrate should flow as a plug through the reactor, moving from the inlet to the outlet over one HRT (Panaro *et al*., 2022). In contrast to reactor types typically used for wet digestion processes, *e.g.* the completely stirred-tank reactor (CSTR), the PFR is not completely mixed and is characterised by having no or very limited mixing and diffusion along the horizontal axis of the reactor (Das *et al*., 2016; Toson *et al*., 2019). A disadvantage of continuous processes conducted in CSTRs is that a fraction of the material fed into the reactor exits the system within the first day as a consequence of the complete mixing, a phenomenon referred to as short-circuiting (Boe & Angelidaki, 2009). This issue is avoided in an ideal PFR (Toson *et al*., 2019), which is one of the main advantages of this reactor configuration. In addition, plug-flow operation could theoretically result in division of the AD steps across the reactor, creating a phase-separated process (Panaro *et al*., 2022). This would result in more fresh substrate at the inlet and more thoroughly digested material towards the reactor outlet, a set-up that could enable optimisation of each step separately (Chatterjee & Mazumder, 2019). However, these advantages only apply if plug-flow is obtained within the reactor, highlighting the importance of evaluating plug-flow behaviour as part of the reactor evaluation process.

1.4 Aims of the thesis

The studies described in Papers I-IV in this thesis addressed different aspects of HSD processes, such as reactor types, plug-flow behaviour and phase separation, operating parameters, process stability and down-scaling. All studies also involved evaluation of process yield, degradation efficiency and the microbial communities present. Specific objectives of the work in Papers I-IV were to:

- Study ammonia-driven disturbances in HSD processes and investigate the response in terms of process performance, microbial community structure and microbial activity (II, IV).
- Evaluate whether plug-flow behaviour is actually obtained in reactors of horizontal plug-flow type (II, III) and whether differences in microbial community structure and microbial activity are obtained along the reactor (II, IV).
- Compare the differences between operating an HSD process in a PFR and in a CSTR (III).
- Evaluate effects of down-scaling an industrial-scale system in order to enable laboratory trials of HSD processes (II).

2. Experimental overview and methods

This chapter provides a brief overview and discussion of the experiments and of the methods used in the thesis work. For more detailed information, see the respective methods sections in Papers I-IV.

2.1 Experimental laboratory-scale reactors

Parts of the work reported in this thesis were carried out in two laboratory-scale (active volume 43-47 L) custom-made reactors of horizontal plug-flow type (Figure 2a), which were designed to simulate an industrial-scale Kompogas process under HSD conditions (Paper II). The reactors were constructed by three sections (without dividing walls in between). Substrate was fed into section 1 (S1), passed through the middle section (S2) and digestate was taken out from section 3 (S3). An important feature in the laboratory PFRs was multiple sampling ports, enabling sampling from various points along the reactor length (Figure 2a). This enabled analyses of chemical composition, microbial community structure and process parameters and how they changed along the length of the reactor (Papers II-IV).

In total, the reactors were operated for a period of >770 days, divided into three main experimental phases. In Paper II, the reactors were used to demonstrate the viability of simulating an industrial-scale HSD process at laboratory scale and to examine whether plug-flow or separation of microbial degradation steps was achieved within this type of reactor. In Paper III, digestate from the PFRs was used to inoculate CSTRs operated with the same operating parameters and substrate conditions as those used in the PFRs, in order to compare process yield and operation in the two reactor types. In Paper IV, a controlled process disturbance was induced by additional protein

supplementation in the substrate to study the microbial response to changes in the process parameters. In addition, TS content and OLR was increased in one of the reactors to test the effect on plug-flow behaviour (unpublished data).

Laboratory-scale CSTRs are commercially available and often used for experiments on continuous biogas processes, since they have a simple design and are relatively easy to operate (Schnürer *et al*., 2016; Usack *et al*., 2012). The reactor typically consists of a tank stirred with propeller blades in the middle (Figure 2b), which should enable homogenisation of the material (Usack *et al*., 2012). Within this thesis work, CSTRs were used to evaluate serial digestion of wet material in two or three reactor steps compared with a single-step CSTR (Paper I) and in the comparison of reactor technologies (PFR and CSTR) (Paper III).

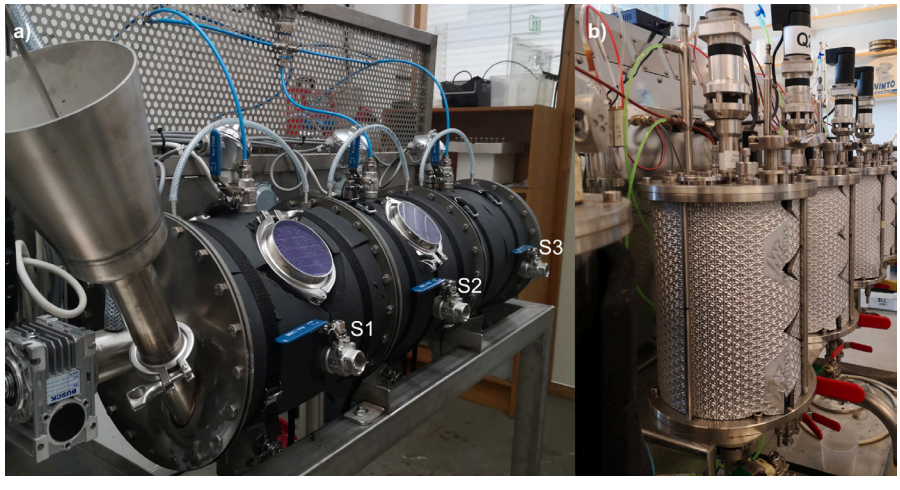

Figure 2. (a) Custom-built laboratory-scale plug-flow reactor (PFR) used in Papers II-IV, where S1, S2 and S3 are sampling ports in reactor sections 1-3. (b) Laboratory-scale completely stirred tank reactor (CSTR) of the type used in Papers I and III.

2.2 Operating parameters

Process temperature, organic loading rate (OLR), hydraulic retention time (HRT), substrate composition and TS/VS level are all key parameters in an AD process (Schnürer *et al*., 2016). For the serial CSTR set-up in Paper I, operating parameters (temperature 41-42 °C, OLR 2.4 g VS/L d, HRT 55 days, VS_{in} 13%) were chosen to mimic a wet AD plant operated with organic

household, slaughterhouse and industrial waste. For the process operated in the laboratory PFRs (Papers II-IV), operating parameters (temperature 52-53 °C, OLR 5.2 g VS/L d, HRT ~42 days, VS_{in} 22%) were initially chosen to simulate an industrial-scale Kompogas process.

Anaerobic digestion processes are generally operated under mesophilic (35-42 °C) or thermophilic conditions (46-60 °C) (Schnürer *et al*., 2016). Mesophilic processes in general have lower energy consumption and higher microbial diversity, while processes at higher temperature have higher degradation rate, but also run a higher risk of experiencing process instability (Guo *et al*., 2014; Labatut *et al*., 2014). Moreover, thermophilic temperature can be used as a method for hygienisation, *i.e.* removal of pathogens, if sufficient residence time of the material can be demonstrated (Jordbruksverket, 2016). Therefore, several continuous HSD processes operated in reactors of plug-flow (Kompogas) type use thermophilic conditions, which offer a practical advantage as no additional hygienisation step of the substrate or digestate is necessary in those cases (Paper II; Westerholm *et al*., 2020; Wellinger *et al*., 1993).

Figure 3. Organic household waste fed to high-solid digestion processes in laboratory- and industrial-scale. (a) Substrate handling at industrial-scale, (b) substrate pre-treatment to reduce particle size in laboratory scale and (c) pre-treated substrate, grinded to particle size of around 10 mm.

In the experiments conducted with PFRs in Papers II-IV, the reactors were fed a substrate mix consisting of food waste, mainly source-sorted municipal organic household waste (~51% of VS) (Figure 3). This is a waste fraction with high methane potential, rich in protein, fat and relatively easily degradable carbohydrates, although the composition and degradability can vary widely depending on the source and waste sorting method (Zhang *et al*.,

2022; Xu *et al*., 2018). The mesophilic, wet AD processes in serial digesters studied in Paper I were operated with similar food waste, but in that case co-digested with slaughterhouse waste, which can contribute a high amount of fat and proteins (Xu *et al*., 2018). The HSD processes in Papers II-IV instead co-digested lignocellulosic waste fractions, in general consisting of garden residues or green wood chips (26% of VS), horse manure (11-22% of VS), crop residues (wheat) (0-6% of VS) and olive cake (0-6% of VS). The high lignocellulose content in these fractions can make the substrate difficult and time-consuming to degrade (Yang *et al*., 2015; Jasińska *et al*., 2023). However, this type of material can also improve process stability when co-digested with nitrogen rich food waste (Borth *et al*., 2022; Perin *et al*., 2020).

The substrate fed to the PFRs was also mixed with digestate (30% of ingoing material) before feeding to recirculate material (Papers II-IV), which is a common approach used in PFRs in order to re-inoculate, *i.e.* return microorganisms, to the first reactor section (Chen *et al*., 2020; Rossi *et al*., 2022a; Li *et al*., 2024).

For most of the experiments conducted in the PFRs (Papers II-IV), the TS level within the reactor was kept at around 15-18% to simulate HSD conditions. However, the cut-off for an HSD process can differ between studies (Angelonidi & Smith, 2015; Kothari *et al*., 2014; Shapovalov *et al*., 2020). In some cases, AD processes are even divided into three types: wet (<10% TS), semi-dry (10-20% TS) and dry or high-solid (20-40% TS) (Abbassi-Guendouz *et al*., 2012). In contrast, Carlos-Pinedo *et al*. (2019) used TS level >15% in substrate to classify processes as high-solid. In this thesis work, a process was considered to operate under HSD conditions at TS >15% within the reactor, while TS in incoming material (TS_{in}) was kept at around 27-28% (Papers II-IV).

2.3 Process monitoring and evaluation

Several different parameters are of importance for monitoring an AD process and monitoring aims at evaluating the stability, yield and efficiency of the process (Drosg *et al*., 2013; Schnürer *et al*., 2016).

To maintain process stability, it is essential to keep the pH range optimal for growth and activity of the participating microorganisms (usually 7-8) (Zhai *et al*., 2015; Drosg *et al*., 2013) and to avoid accumulation of ammonia and VFA (Rajagopal *et al*., 2013). Volatile fatty acids are an indicator of

instability and can cause problems, such as reduced pH, at high concentrations, so VFA content is an important parameter to track (Mata-Alvarez *et al*., 2014; Schnürer *et al*., 2016; Boe *et al*., 2010). A relatively quick and simple measure of stability in AD is the ratio between VFA and alkalinity (FOS/TAC) (Drosg *et al*., 2013). The optimal FOS/TAC ratio can differ between different types of processes, but a sudden increase indicates rapid accumulation of VFA or decreased buffering capacity (Martín-González *et al*., 2013). In Paper II, FOS/TAC and pH were used in weekly monitoring to quickly indicate the status of the process. The concentration of different VFA species and total concentration of mineralised nitrogen in the process, referred to as ammonium-nitrogen (NH_4^+-N), were also measured weekly or biweekly, to show process performance (Papers II-IV). Other important indicators of instability are volumetric gas production and gas composition, *e.g*. levels of CH_4, CO_2, H_2 and H_2S in the biogas, where a drop in gas volume or CH_4 or sudden increase in CO_2 or H_2 could be signs of disturbances (Boe *et al*., 2010; Schnürer *et al*., 2016). H_2S is a toxic and corrosive gas, mainly generated during protein degradation, and an increase in H_2S level can indicate high protein content in the substrate (Paper IV, Vu *et al.*, 2022). In some cases H_2S level needs to be regulated, *e.g.* by the addition of iron, to avoid microbial inhibition and damage to equipment (Vu *et al.*, 2022, Zhou *et al.*, 2016).

One of the main objectives in biogas production is to achieve a high yield of methane in relation to the amount of organic material fed into the process, *i.e.* specific methane production (SMP) (Schnürer *et al*., 2016). This is important in order to maximise the outcome of the process and, to the greatest extent possible, avoid potential methane emissions from the outgoing digestate (Ekstrand *et al*., 2022). Thus SMP was one of the main performance parameters used in the experiments in this thesis (Papers I-IV).

Degree of degradation describes how well the process digests organic matter present in the substrate and can be highly variable depending on the type of process and material (Ekstrand *et al*., 2022). Reduction in VS, *i.e.* the fraction of organic material that was converted into gas (Schnürer *et al*., 2016) was evaluated in Papers I-III to estimate degree of degradation. In addition, nitrogen mineralisation, *i.e.* rate of conversion of organic-N into NH_4^+-N, which also gives a measure of degree of protein degradation (Bareha *et al*., 2018; Schnürer *et al*., 2016) was evaluated in Paper III. The efficiency of degradation can also be evaluated based on residual methane

potential (RMP) in the digestate (Ekstrand *et al*., 2022; Ahlberg-Eliasson e*t al*., 2021), which in this thesis was determined through incubation of the digestate in batch mode until gas production stopped (Papers I, II). In Paper II, an additional measure of process efficiency was applied to compare different systems, using a combination of volumetric methane production, RMP and HRT (Rico *et al*., 2015). It has previously been shown to be promising and to accurately represent process performance (Ahlberg-Eliasson *et al*., 2021). In addition, the composition of macromolecules (protein, fat and carbohydrates) in the substrate and digestate can be measured to estimate the reduction of the different substrate components in the process (Papers I, II). This evaluation indicates if a specific type of macromolecule remains to a higher extent in the digestate, thus suggesting whether degradation of a certain fraction needs to be improved (Ekstrand *et al*., 2022). The rate of protein, cellulose and fat degradation in digestate was also evaluated, mainly as a method for comparison of activity in digestate samples from different reactors or reactor sections (Papers I, II). This was performed through measurement of gas production from batch incubation of digestate samples fed with different substrate components (cellulose, albumin and oil).

However, the above mentioned analyses were challenging due to the non-homogeneous nature of the material. Therefore measured values of especially degradation rate, macromolecule concentrations, VS and RMP in digestate samples from the HSD experiments (Papers II, III) showed relatively large variability, indicating one of the challenges of working with high-solid materials compared with wet samples as used in Paper I. Another limitation in the analyses of the high-solid samples was that high TS level was not accounted for when soluble compounds, such as VFA and NH_4^+-N concentration, were measured (Papers II-IV). Pastor-Poquet *et al*. (2018) suggested a model for estimation of concentrations within the liquid fraction of a HSD sample, based on the TS content and density. However, this model was not used in Papers II-IV, since density was difficult to estimate and also since systems with relatively similar TS were compared, which would give similar error in all reactors included in the comparisons.

2.3.1 Tracer tests

A common method for evaluating plug-flow behaviour experimentally is to use a tracer test, where a pulse of an inert tracer (soluble compound or

insoluble particles, representing the substrate added at a certain time-point) is fed into a reactor and the tracer concentration is measured in the outgoing digestate (Nordell *et al*., 2021; Toson *et al*., 2019; Wellinger *et al*., 1993; Hernandez-Shek *et al*., 2023). This gives the retention time distribution (RTD), which shows the probability of material leaving the reactor within a certain period (Toson *et al*., 2019), *i.e.* on average how long the substrate resides within the reactor.

To evaluate plug-flow behaviour in the laboratory PFRs in Paper II, lithium (Li^+) was used as a tracer to illustrate how the soluble material moved through the reactor and was added at a concentration that should be below inhibitory levels in the AD system (Anderson *et al*., 1991). The tracer study was first carried out at a TS level of ~15-18% in the digestate (Paper II) and later the same test was repeated when the TS level in the digestate was ~26% (unpublished data).

2.4 Analysis of microbial communities

Different methods can be used to study the microbial community in reactor samples, such as microscopy, quantitative polymerase chain reaction (qPCR) or a meta-omics approach such as sequencing of the metagenome or metatranscriptome (Cabezas *et al*., 2015; Hashemi *et al*., 2021). A widely used method is sequencing of variable regions within the highly conserved marker gene encoding 16S ribosomal RNA (rRNA) (Cabezas *et al*., 2015). This method gives information about the relative abundance of microorganisms present in the sample, and was used in Papers I-IV. There are many examples of how results from 16S rRNA gene sequencing can be used, for instance to study changes in community structure over time or to compare different operating conditions or substrates (Lv *et al*., 2019; Westerholm *et al*., 2019; Westerholm *et al*., 2015). In this thesis, microbial community structure was studied over time during shifts in process performance (Papers II, IV), to investigate differences between reactors of different type and scale (Papers II, III), and as an indicator of phase separation across a PFR or serial reactor system (Papers I-III). A limitation of this approach is that different microbial groups have different 16S rRNA gene copy numbers per genome. For example, archaea often have a lower gene copy number than bacteria, leading to underestimation of the relative abundance of Archaea when the two groups are compared (Dyksma *et al*.,

2020; Wirth *et al*., 2023). Another limitation is that in Papers II-IV, universal primers (515f/806r) were used for detection of both bacteria and archaea, but these have been shown to be more specific for bacterial groups and have lower coverage for archaea (Merkel *et al*., 2019). In Paper I, primers specific for both domains were used separately, which most likely provided a more accurate view of the species distribution within the archaeal community.

Although data from 16S rRNA sequencing can give valuable information about the most abundant microbial groups within a sample, the function of these microbial groups cannot be directly inferred (Hashemi *et al*., 2021). To gain a better understanding of the function and activity of the microbial community, the genes expressed by the population and quantitative information about gene expression levels can be investigated using metatranscriptomics (Hashemi *et al*., 2021; Wang *et al*., 2022; Gaspari *et al*., 2024). Metatranscriptomic analyses were carried out on samples from one of the laboratory-scale PFRs operated at high ammonia levels in Paper IV, to investigate gene expression in two different reactor sections and before and after a process disturbance. The metatranscriptome is investigated through sequencing of mRNA, which are the transcribed sequences from actively expressed genes. However, in interpretation of the results it is important to consider that this method has several challenges, for instance that mRNA is highly unstable and that the transcription profile can change quickly, which can give results with high variability (Hashemi *et al*., 2021).

3. Process-related challenges in HSD

There are several advantages with using HSD technology, such as reduced freshwater usage, more concentrated digestate and capability for high loading rates. However, since the water content is low and the substrate can be relatively bulky, the material cannot be pumped as in wet AD systems and feedstock handling therefore becomes more difficult (Nizami & Murphy, 2010; Fagbohungbe *et al*., 2015). In addition to the practical aspects, HSD systems also risk developing process disturbances relatively easily, for instance due to high loads of inhibitory substances and limited mass transfer in the material (Shapovalov *et al*., 2020; Wang *et al*., 2023). These aspects are discussed in more detail in this chapter.

3.1 Instability at high ammonia concentration

3.1.1 Mechanism of ammonia inhibition

High ammonia level is one of the most common reasons for instability in anaerobic digestion. Nevertheless, there is a fine balance between too high and too low ammonia concentration, since nitrogen is required for microbial growth (Schnürer & Jarvis, 2018) and is also an important factor for producing a digestate with high biofertiliser value (Arelli *et al*., 2018; Drosg *et al*., 2015). Ammonium-nitrogen (NH_4^+-N) is the total nitrogen concentration in the form of free ammonia (NH_3) and the soluble ammonium ion (NH_4^+). These two forms are in equilibrium, driven towards higher content of ammonia at higher pH and temperature (Astals *et al*., 2018; Rajagopal *et al*., 2013). NH_4^+-N can be inhibiting in its soluble ionic form (Astals *et al*., 2018), but it is mainly free ammonia that is toxic to microorganisms (Rajagopal *et al*., 2013). Therefore, processes operated

under thermophilic conditions are at greater risk of experiencing ammonia inhibition than mesophilic processes.

A theory about the mechanism of ammonia inhibition proposed by Sprott *et al*. (1984) is that ammonia can diffuse freely into cells, where it is converted to NH_4^+ by binding protons, resulting in a pH imbalance in the cell. This is compensated for by active uptake of H^+, leading to loss of potassium (K), since K^+ ions are pumped out of the cell when protons are pumped in. It has been observed in many cases that methanogenic species are the most sensitive to high ammonia levels in an AD system (Rajagopal *et al*., 2013). Related to this, direct inhibition of enzymes involved in methane synthesis has been suggested as another possible mechanism of ammonia toxicity (Sprott & Patel, 1986).

Ammonia toxicity is not limited to the archaeal community and affects all microbial groups to a greater or lesser degree depending on their tolerance (Finn *et al*., 2023). However, when methane production is a rate-limiting step, this affects the entire AD process as there is a risk of accumulation of the substrates used for methanogenesis, *i.e.* acetate and H_2 (Rocamora *et al*., 2023; Pan *et al*., 2021). In turn, accumulation of H_2 reduces the efficiency of VFA oxidation, since this process is energetically unfavourable unless the partial pressure of H_2 is kept below a certain limit (Schink, 1997; Müller *et al*., 2010). Thus, VFA conversion can only occur when H_2 is continuously consumed and a close cooperation, called syntrophy, is necessary between hydrogen-consuming (hydrogenotrophic) methanogens and acid-degrading bacteria (Schink, 1997). Unless the AD system has good buffering capacity, high VFA concentrations can lower the pH, which also can lead to a decrease in methane production rate (Rajagopal *et al*., 2013). In addition, high VFA concentrations are toxic *per se* and can further inhibit the process (Jiang *et al*., 2018; Li *et al*., 2020).

Acetate is one of the main intermediary products in AD, as it is formed in fermentation processes during anaerobic oxidation of longer VFAs and can also be produced from CO_2 by specialist acetogenic bacteria (Pan *et al*., 2021; Müller *et al*., 2010). Acetate degradation is therefore an important step in converting organic matter into CH_4. There are two main pathways for acetate degradation, (acetoclastic) methanogenesis and syntrophic acetate oxidation (SAO), where SAO is carried out by bacteria (SAOB) in cooperation with hydrogenotrophic methanogens (Pan *et al*., 2021). Within the methanogenic community, high ammonia levels are especially toxic to

acetoclastic methanogens, as shown based on both transcriptomic and metagenomics data (Yan *et al*., 2020; Fischer *et al*., 2019; Gaspari *et al*., 2024). This gives the relatively ammonia-tolerant SAOB an advantage at these conditions (Paper II; Westerholm *et al*., 2016; Sun *et al*., 2014; Pan *et al*., 2021).

3.1.2 Operation of thermophilic HSD processes at high ammonia level

Many industrial HSD processes today are operated with organic household waste or food waste as one of the primary substrate components (Carlos-Pinedo *et al*., 2019; Westerholm *et al*., 2020; Wang *et al*., 2023; Angelonidi & Smith, 2015). Since food waste has a high protein content (Xu *et al*., 2018) and HSD processes typically apply high OLR with low dilution rates (Duan *et al*., 2012; Wang *et al*., 2023), HSD processes frequently reach high ammonia concentrations (Paper II; Westerholm *et al*., 2020; Rocamora *et al*., 2020; Shapovalov *et al*., 2020). Ammonia inhibition ultimately decreases methane content in the gas and overall gas production (Paper II; Paper IV; Rossi *et al*., 2022b), reducing the process yield and leading to less efficient substrate utilisation.

As mentioned in section 2.2, many HSD processes in reactors of the Kompogas type operate under thermophilic conditions in order to hygienise the material during digestion. Although this has practical advantages, the high temperature can further increase stress for the microbial community, as thermophilic conditions lead to a higher fraction of free ammonia (Rajagopal *et al.,* 2013). Nevertheless, a microbial community well adapted to high ammonia can function in AD processes under these conditions (Finn *et al*., 2023). Tolerant microbial communities can be obtained through adaptation to high ammonia conditions over time and can be a result of selection of groups with the ability to *e.g.* regulate intracellular pH level and K^+ uptake or block diffusion of ammonia across the cell membrane (Finn *et al*., 2023; Yan *et al*., 2020; Gaspari *et al*., 2024).

Studies examining the methanogenic community of thermophilic HSD processes have in several cases found it to be dominated by the hydrogenotrophic methanogens *Methanoculleus* and *Methanothermobacter* (Papers II-IV, Westerholm *et al*., 2020; Dyksma *et al*., 2020; Rossi *et al*., 2022a, Tang *et al.*, 2011). This indicates that methane production from H_2 and CO_2 was the predominant pathway in those processes, even at ammonia concentrations previously reported to potentially cause severe inhibition of

the process (0.6-1.5 g NH_3-N/L) (Westerholm *et al*., 2016). Previous findings have also indicated that NH_4^+-N concentrations above 2 g/L (0.24 g NH_3-N/L) favour SAO over aceticlastic methanogenesis at thermophilic temperature (Fotidis *et al.*, 2014; Sun *et al*., 2014; Westerholm *et al*., 2016). Methane production through the hydrogenotrophic pathway, coupled to acetate conversion by bacteria with SAO activity, can therefore be an important adaptation strategy to stressful conditions (Westerholm et *al*., 2016).

Thermophilic HSD processes operated with protein-rich substrates have in several cases been observed to exhibit similar bacterial communities, especially dominated by the order MBA03 and/or by the genera *Defluviitoga*, *Halocella*, *Lentimicrobium* and *Proteiniphilum* (Paper II-IV; Rossi *et al.*, 2022a; Dyksma *et al*., 2020; Westerholm *et al*., 2020; Zhang *et al*., 2022). These genera are considered ammonia-tolerant due to their frequently high relative abundances within processes operated at high ammonia loads and are all suggested to be involved in hydrolysis and fermentation of carbohydrates and proteins (Puchol-Royo *et al*., 2023; Ben Hania *et al*., 2012; Maus *et al*., 2016; Sun *et al*., 2016a; Chen & Dong, 2005; Liu *et al*., 2022). The key group, SAOB, can be difficult to identify as these microbes are often present at low relative abundance, but frequently observed SAOB candidates are thermophilic species closely related to *Syntrophaceticus* (Paper II; Paper III; Dyksma *et al*., 2020). Nevertheless, the uncultivated order MBA03, observed at high relative abundance in many AD systems, both thermophilic and mesophilic (Paper II-IV; Otto *et al*., 2023; Westerholm *et al*., 2020), is also suggested to have the ability to perform SAO (Puchol-Royo *et al*., 2023; Dyksma *et al*., 2020; Zheng *et al*., 2019).

The process disturbance that occurred in Paper II resulted in distinct changes in microbial community structure, the most pronounced being an increase in relative abundance of the carbohydrate degrader and acetate producer *Defluviitoga* (Ben Hania *et al*., 2012) in one laboratory-scale reactor (LR1) during VFA accumulation and a simultaneous peak in NH_4^+-N concentration (Figure 4a). However, a similar increase in *Defluviitoga* was not observed in an industrial-scale reactor also exhibiting VFA accumulation (Figure 4b) and high ammonia levels (RK1). Functional characterisation of the microbial community during ammonia accumulation in Paper IV indicated that gene expression of the class Thermotogae, most likely represented by *Defluviitoga*, was relatively unaffected by an increase in

ammonia level. In previous studies, *Defluviitoga* has been found to be positively correlated with high VFA concentration (Guo *et al*., 2014). In addition, Zhang *et al*. (2022) saw a clear increase in the genus when OLR was increased, especially in reactors fed carbohydrate-rich substrate, but could not distinguish a clear trend coupled to VFA or ammonia. Although it can be concluded from the above that the genus *Defluviitoga* can tolerate high ammonia and VFA concentrations, its role during process disturbance remains unclear.

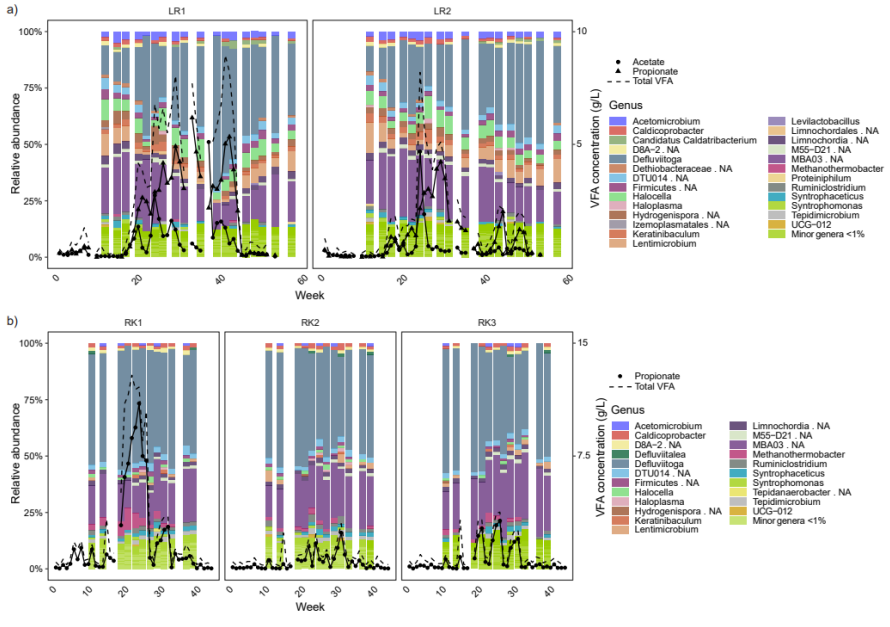

Figure 4. Microbial community structure based on abundance of 16S rRNA gene sequences and acetate, propionate and total volatile fatty acid (VFA) concentrations during process disturbances in (a) two laboratory-scale plug-flow reactors (PFRs) (LR1 and LR2) and (b) three industrial-scale PFRs (RK1, RK2 and RK3). Figure from Paper II, reproduced with permission.

3.1.3 Ammonia-induced disturbances in HSD – monitoring and mitigation strategies

Propionate is one of the VFAs especially prone to accumulation, primarily because anaerobic oxidation of propionate is less energetically favourable than anaerobic oxidation of other VFA types (Stams & Plugge, 2009; Müller *et al*., 2010). Therefore in many cases one consequence of instability,

including instability caused by high ammonia levels, is propionate accumulation, from which it can be difficult to recover (Paper II; Paper IV; Rocamora *et al*., 2023; Moestedt *et al*., 2016; Boe *et al*., 2010; Li *et al*., 2020; Westerholm *et al*., 2020). Thus, finding ways to degrade propionate efficiently can be important to recover quickly from a disturbance.

In an industrial-scale HSD reactor studied in Paper II, fast propionate degradation was observed after a disturbance phase (Figure 4b). This was hypothesised to be related to the simultaneously high relative abundance of *Methanothermobacter* (II), which has been observed previously as an important partner in syntrophic propionate oxidation (Singh *et al*., 2023). However, propionate-degrading taxa, such as *Pelotomaculum*, were low in relative abundance in Paper II. In the metatranscriptome analysis in Paper IV, RNA samples were taken during a phase of accumulation of propionate and NH_4^+-N, and also during the peak in propionate and NH_4^+-N concentration. Gene fragments related to propionate degradation through the most well-studied pathway (the methylmalonyl-CoA pathway) did not show any trend of upregulation or downregulation between the sampling time-points. It was therefore difficult to deduce how the propionate-degrading species were affected by disturbances based on results from Paper IV, and those from Paper II.

As acetate is one of the products of propionate degradation, high levels of acetate can lead to product inhibition and render oxidation of propionate unfavourable (Müller *et al*., 2010). Therefore, the ratio between propionate and acetate is sometimes used as a measure of the risk of suffering a severe disturbance (Westerholm *et al*., 2020; Drosg *et al*., 2013; Boe *et al*., 2010). This was also in line with results from the disturbance phases in Papers II and IV, where propionate was degraded faster when acetate levels were comparatively low.

The HSD systems studied in this thesis work exhibited signs of instability and VFA accumulation in processes that reached NH_4^+-N levels >4 g/L (Papers II & IV). One of the industrial-scale (RK1) and one of the laboratory-scale PFRs (LR2) in Paper II (Figure 4), as well as the reactor studied during protein addition in Paper IV, reached similar NH_4^+-N concentrations of around 4.0-4.2 g/L (0.8-1.6 g NH_3-N/L, with high variation due to pH values), but experienced relatively moderate instability with quick recovery. In contrast, the laboratory-scale reactor (LR1) in Paper II reached a higher NH_4^+-N concentration, 4.5 g/L (2.0 g NH_3-N/L) and experienced prolonged

propionate accumulation (Figure 4a). Potentially the higher NH_4^+-N and NH_3-N level obtained in LR1 (Paper II) resulted in a more severe disturbance that was more difficult to recover from, indicating the importance of trying to address instabilities before they go too far and escalate. Overall, this highlights the importance of monitoring the process to detect disturbance events at an early stage.

High H_2 level is related to poor propionate degradation, and therefore Rocamora *et al*. (2023) suggested that H_2 level is a good measure of process stability in HSD operated with food waste. This was in line with results obtained by Illmer & Gstraunthaler (2009) for an industrial-scale HSD process, and with observations from disturbance phases in Papers II and IV. Additionally, increased CO_2 content in relation to CH_4 in the gas was observed to be a clear indication of perturbation (Papers II & IV). It is also vital to monitor VFA and NH_4^+-N concentrations, but this involves relatively time-consuming analyses. A more instantaneous measure of stability can be obtained by measuring the FOS/TAC ratio (see section 2.3), which has been found to be informative and to serve as an early indicator of process instability (Paper II; Veluchamy *et al*., 2019).

Several different strategies to mitigate ammonia-induced disturbances have been evaluated and described in the literature. For instance, Westerholm *et al*. (2020) evaluated lowering the process temperature of an industrial-scale HSD plant to mesophilic conditions to avoid future process instability. Addition of trace elements is another method to improve process stability and can be necessary for stable digestion of food waste (Banks *et al*., 2012). Trace elements such as iron, nickel, selenium and cobalt are essential components of enzymes involved in *e.g.* syntrophic acid oxidation and other important AD pathways (Banks *et al*., 2012). Addition of trace elements to the process can be especially important under thermophilic conditions, since microorganisms require higher trace element concentrations at higher temperature (Qiang *et al*., 2013; Kang & Ahn, 2024). However, the use of trace elements varies in HSD of food waste (Westerholm *et al*., 2020) and was not used in the industrial-scale process monitored in Paper II.

Operating thermophilic HSD with food waste as the sole substrate fraction, without co-digestion with a more carbon-rich substrate such as garden waste, has been observed to be challenging (Zhang *et al*., 2022). A strategy to overcome this that has proven to be efficient in many cases is to

increase the ratio between total carbon and total nitrogen (C/N) in high-solid substrates (Zhang *et al*., 2022; Huang *et al*., 2016; Rossi *et al*., 2022b; Zeshan *et al*., 2012). In HSD, increasing the C/N ratio in the process through addition of recalcitrant lignocellulosic carbon sources, such as garden waste or straw, that are degraded inefficiently or slowly at anaerobic conditions can be a way of 'diluting' the substrate, *i.e.* lowering the load of easily degradable or nitrogen-rich fractions that are more prone to cause disturbance, while maintaining a high TS level (Shapovalov *et al*., 2020).

However, even during co-digestion of food waste and carbon-rich substrates (horse manure and garden residues), inhibiting ammonia levels were observed in Paper II. A strategy to recover from VFA accumulation used within this thesis work was to temporarily reduce the OLR and consequently increase the HRT (Paper II & IV). This allowed the process to recover and degrade the material at a slower rate and also stopped further accumulation of VFA. However, this strategy might not be optimal for industrial-scale processes that need to treat waste at a certain rate since substrate storage is often limited.

3.2 Influence of high TS content

Relatively high TS levels can be applied in HSD processes, *e.g*. in some cases substrates with TS level up to 40% (Kothari *et al*., 2014). However, there is a limit to the TS level that can be applied without reduced efficiency of microbial activity and substrate availability. Abbassi-Guendouz *et al*. (2012) found that TS level ≥30% led to VFA accumulation in a batch system, while Benbelkacem *et al*. (2015) identified a TS level of >20% as the threshold for efficient degree of degradation. However, evaluations of the optimal or maximum TS content in a HSD system can in some cases be problematic, since this parameter is tightly connected to the OLR in continuous systems, which also has a significant effect on process performance (Chen *et al*., 2014; Wang *et al*., 2023). To distinguish between the effects of overloading the system and the effects of mass transfer limitations, Le Hyaric *et al*. (2011) tested propionate degradation at different TS levels (18-35%) (same load of propionate at all levels). They observed a linear increase in specific methane production with higher moisture content, potentially due to limitations in mass transfer at high TS. This was in line with findings in a study by Xu *et al*. (2014) where a mathematical model was developed to explain the effect

of increased TS level in batch digestion of cellulose. Their results suggested that inhibition caused by hydrolysis products due to poor mass diffusion is the main reason for decreased methane production rate at high TS and that the optimum TS-level in agitated batch systems is 15-20% (Xu *et al*., 2014).

Bollon *et al*. (2013) developed a method to determine diffusion coefficients in digestate with different TS content and observed a sharp decrease in diffusion from 0-15% TS, while diffusion was relatively constant at TS from 15% to 25%. Li *et al*. (2023b) observed decreased diffusion in digestate from co-digested food waste and sewage sludge from 10 to 18% TS. Mass transfer limitation in the material is strongly linked to the viscosity, since diffusion is generally slower in more viscous material. Both TS level and type of waste has been shown to have significant effect on rheological behaviour, *e.g*. shear stress, which has demonstrated increasing trends with higher TS and with higher fraction of green waste in the substrate compared with food waste (Benbelkacem *et al*., 2013). This indicates that both the TS content and substrate composition are important for viscosity characteristics and consequently for mass flow and availability of microorganisms to the substrate (Gao *et al*., 2023).

In addition to the mass transfer limitations at high TS, the water fraction in HSD systems is to a high degree bound to the solid material. For example, Garcia-Bernet *et al*. (2011) estimated that digestate with around 20% TS, originating from municipal waste and green waste, had around 50% of the total water in bound form. This makes the apparent concentration of soluble compounds higher than the total concentration in the system (Pastor-Poquet *et al*., 2018), which is important to keep this in mind when determining substrate composition, since the apparent concentration of *e.g.* ammonia could become higher than expected for a certain load of proteinaceous substrate. Thus, even small variations in substrate composition can have a large impact on the apparent concentrations, making HSD processes sensitive to fluctuations. An example of this was observed in Paper II, where a new substrate batch with slightly higher protein and fat content caused accumulation of ammonia and VFA.

4. Plug-flow behaviour and phase-separated digestion

Plug-flow reactors are often used for continuous HSD processes at industrial scale (Hayyat *et al*., 2024; Gao *et al*., 2023). However, the plug-flow behaviour of these reactors is rarely investigated. Plug-flow dynamics can theoretically give rise to phase separation along the reactor, with differences in the microbial community and chemical composition between the inlet and the outlet (Panaro *et al*., 2022). To model and investigate the plug-flow effect, sequential reactors have been used in some cases (Wen *et al*., 2007; Donoso-Bravo *et al*., 2018). Plug-flow behaviour, differences in performance between processes operated in completely stirred reactors (CSTRs) and PFRs, and the effects of sequential digesters are discussed in detail in this chapter.

4.1 Plug-flow behaviour in HSD reactors

In a perfect plug-flow reactor, all material that is fed into the system is retained for exactly one HRT (Das *et al*., 2016). This can be illustrated by the RTD curve that, for ideal plug flow, shows a narrow peak at time τ after tracer addition, equal to the hydraulic retention time (Toson *et al.,* 2019). In contrast, an ideal CSTR has an exponentially decreasing RTD curve, with the highest peak obtained on the first day (Paper I; Toson *et al*., 2019). As new substrate is constantly mixed with the reactor material, part of which is continuously removed from the reactor, short-circuiting is obtained. Therefore, the time available for degradation is more limited in a CSTR compared with a PFR.

One of the main questions regarding the laboratory-scale PFRs used in this thesis was whether plug-flow behaviour could be obtained (Paper II).

However, tracer (Li^+) tests first carried out at a TS level of ~15-18% in the digestate (Paper II), and later repeated with TS ~26% in the digestate (unpublished data), did not yield the RTD expected for ideal plug-flow. Instead, a peak in tracer concentration was seen after 3-5 days at both TS levels (Figure 5). Higher TS level is related to higher viscosity and reduced diffusion within the digestate (Li *et al*., 2023a). It was hypothesised that increased TS could delay the flow of material through the reactor, but this was not the case here (Figure 5). However, as proof of concept of Kompogas reactors, Wellinger *et al*. (1993) showed that the retention time determined by tracer tests of solid material agreed well with the expected retention time, indicating plug-flow behaviour at TS ~27%. In a reactor without an internal stirring mechanism, Hernandez-Shek *et al*. (2023) observed indications of plug-flow behaviour of the solid material in a PFR treating unshredded straw and manure. Nordell *et al*. (2021) demonstrated that obtaining plug-flow characteristics was possible at TS of ~23% in a PFR operated with dewatered digested sewage sludge. In that case, a delay of almost 30 days was obtained before the peak in outgoing tracer was seen, which was just slightly shorter than the retention time (36 days). The features of the process design which enabled plug-flow in the reactor studied by Nordell *et al*. (2021) are unknown, but potential factors are stirring mechanism and reactor length to height ratio, as also discussed in Paper II. Sewage sludge and, in particular, digested sewage sludge are reported to be highly viscous and to have low diffusion coefficient (Zhang *et al*., 2016). For that reason, these materials are difficult to treat under high-solid conditions (Li *et al*., 2018), but could have been beneficial for plug-flow performance in the reactors by Nordell *et al*..

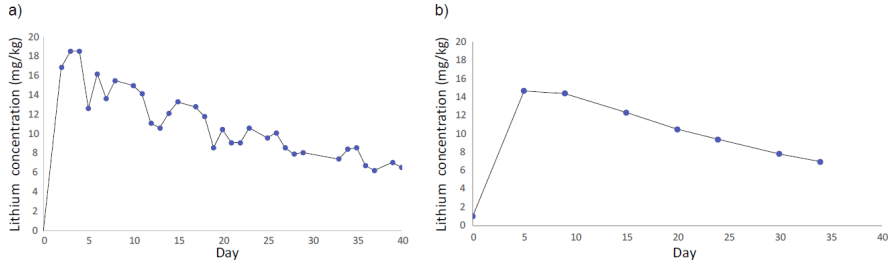

Figure 5. Tracer (lithium) test in laboratory-scale plug-flow reactor. Lithium concentration in outgoing digestate up to 34-40 days after tracer addition during operation at TS level in the reactor of (a) 15-18% (adapted from Paper II) and (b) 26%.

4.2 Phase separation in reactors of PFR type

An effect of plug-flow dynamics within a reactor is, in theory, separation of microbial stages. Fresh substrate is fed into one end of a PFR, which promotes hydrolytic and acidogenic activity of easily degradable matter at the reactor inlet, while the reactor outlet would have higher methanogenic activity and contain more thoroughly degraded material, which could promote digestion of the recalcitrant fractions (Goberna *et al*., 2009; Chatterjee & Mazumder, 2019). This behaviour is revealed by high VFA concentration, high content of VS and low pH in early reactor phases, as well as accumulation of NH_4^+-N towards the outlet (Roy *et al*., 2009; Massé *et al*., 2013; Chatterjee & Mazumder, 2019). Investigation of phase separation was performed in Paper II by measurement of VFA, pH, NH_4^+-N and VS at different ends of the PFRs. In line with the lack of plug-flow behaviour, no clear differences in either chemical composition or microbial community structure were observed between reactor sections in the laboratory-scale PFRs (Paper II). Similar results were obtained by Li *et al*. (2014), who observed no differences in chemical parameters or microbial community structure between three sampling positions along a curved PFR treating manure, although under wet AD conditions (<5% TS) in that case. Similarly, Ren *et al*. (2022) found that the expected division between hydrolysis and methanogenesis within a PFR treating maize straw was not observed.

In contrast, a few previous studies have reported significant separation of microbial community structure (Rossi *et al*., 2022a, Chen *et al*., 2020) and also minor differences in VFA concentration and composition across PFRs (Rossi *et al*., 2022c). Rossi *et al*. (2022a) concluded that VFA production was most active within the middle sections of a PFR treating high-solid organic household waste under thermophilic conditions. As for the microbial community, carbohydrate-hydrolysing genus *Defluviitoga* dominated and had the highest abundance in the first and last sections, while protein-degrading groups (such as *Proteiniphilum*) increased in relative abundance in the middle of the reactor, which was believed to be related to the VFA production (Rossi *et al*., 2022a). Zhou *et al*. (2022) observed a difference in the methanogenesis pathway along a PFR treating food waste, with higher relative abundance of methylotrophic methanogenic taxa towards the reactor outlet. Li *et al*. (2024) studied a pilot-scale PFR operated with solid garden and kitchen waste at increasing OLR and observed significantly higher acetate concentration and FOS/TAC at the inlet section and pH and ammonia

concentration at the outlet. They also observed slight separation of microbial communities between sections, for example with an increase in relative abundance of the cellulose-degrading genus *Halocella* along the reactor at some time-points, although these differences were smaller at higher OLR.

While the reactors studied in Paper II did not exhibit separation in microbial community structure and chemical parameters, there were some indications of section differences in terms of macromolecule degradation rate. Relatively low rate of protein and fat degradation was seen in digestate samples collected towards the reactor outlet (S3) compared with digestate from the first section (S1), while carbohydrate degradation rate was similar in samples from both ends. In addition, the degree of carbohydrate removal increased along the reactor, indicating that there was active carbohydrate degradation also in section S3 (Paper II). This was further supported by the metatranscriptome data, which revealed differences in gene expression between reactor sections (Paper IV) (Figure 6). The results in Paper IV also showed that reactor sections appeared to have larger impact on gene expression than differences in operating parameters, VFA concentration and NH_4^+-N level (Figure 6b). Section comparison showed, in summary, an overall downregulation of genes towards the outlet of the PFR (Figure 6a). The genes that were downregulated in reactor section S3, and effectively upregulated in the feeding end (S1), encompassed several different functional categories, including metabolism, cellular processes and signalling as well as information storage and processing, indicating a more active community in the first reactor compartment (Paper IV). This also suggests phase separation across the reactor, at least temporarily. However, it should be noted that samples used for metatranscriptomic analysis were taken ~4 hours after feeding, to capture a phase with high methane production, which could also be a phase of more clear section separation as the substrate had little time to spread along the reactor. Interestingly, despite the overall downregulation of genes in S3, this section exhibited upregulation of several genes annotated as belonging to Thermotogae, especially within the category carbohydrate metabolism, (Paper IV). As Thermotogae was most likely represented by the genus *Defluviitoga*, this group might be more active in degrading recalcitrant carbohydrate fractions towards the reactor outlet, which could explain the active carbohydrate degradation in S3 observed in Paper II.

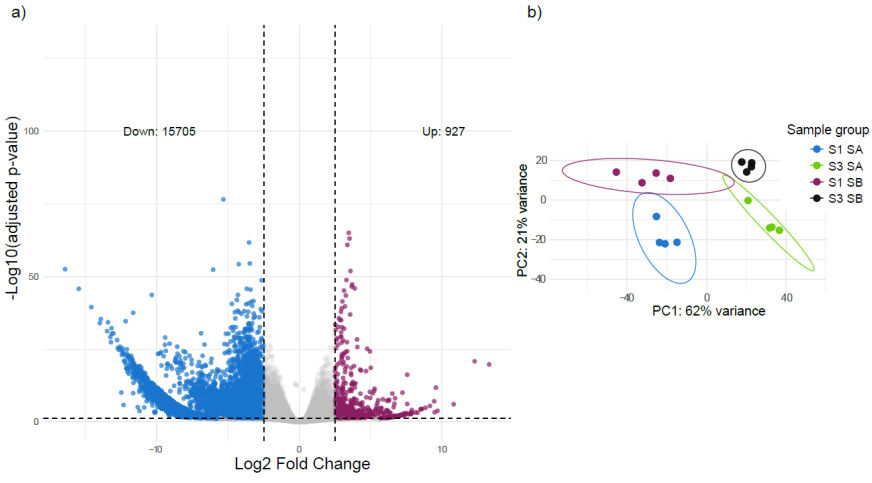

Figure 6. Metatranscriptomic analysis in laboratory-scale plug-flow reactor. (a) Log2-fold change of gene expression in section close to reactor outlet (S3) compared with section close to reactor inlet (S1). Protein-coding genes (open frame reads, ORFs) with log2-fold change <-2.5 or >2.5 and adjusted p-value <0.05 were considered significant. The numbers of significantly upregulated and downregulated ORFs are indicated in the plot. (b) Principal component analysis (PCA) plot of normalised (variance-stabilising transformation) fragment counts in samples taken from reactor sections S1 and S3 during (SA) and after (SB) addition of protein to the substrate. Figures from Paper IV.

A stronger effect of phase separation can be obtained in reactors with compartmentalisation that prevents material from flowing freely within the reactor. An example of this is provided by Varol & Ugurlu (2017), who operated a type of horizontal plug-flow with four separate compartments, where the first two stages acted as acidogenic reactors and the last two stages acted as methanogenic reactors. This design significantly improved methane yield and VS reduction of manure and maize silage compared with a completely stirred reactor, although under wet AD conditions in that study. Veluchamy *et al.* (2019) studied a PFR operated with maize silage (12% TS) that obtained higher VFA, VS and FOS/TAC ratio, and lower pH, in the first reactor compartment compared with the second compartment and the outgoing digestate, and argued that the mixing screw that was running through the reactor helped with compartmentalization. Similar trends for pH and FOS/TAC were seen in a PFR operated with pineapple waste at TS ≤4% when flow was hindered by baffles (Namsree *et al.*, 2012). In that case, methane production mainly occurred within the last reactor compartment (Namsree *et al.*, 2012). In a horizontal tubular reactor operated with cattle

manure, phase separation was enabled by using a U-shaped reactor design (Dong *et al*., 2019). Goberna *et al*. (2009) investigated a thermophilic full-scale PFR, digesting organic household and garden waste, which they claimed did not have any backflow of material. How the reactor was designed to avoid backflow was not described, but significant differences between the reactor parts were observed, with reduced VFA and increased pH at the outlet. Despite this, the microbial community structure remained similar across the reactor, with the exception of increased relative abundance of SAOB towards the outlet (Goberna *et al*., 2009).

Another question relevant for reactors of PFR type is digestate recirculation, which is applied in many cases to maintain an active community and avoid wash-out of microorganisms in the first reactor section (Donoso-Bravo *et al*., 2018). The PFRs studied in Papers II-IV were in most cases recirculated by mixing whole digestate with the substrate at a ratio of 30:70. In an attempt to improve separation between the microbial communities, recirculation was removed from one of the reactors in Paper III. However, irrespective of recirculation, no significant differences between sections were observed in either chemical parameters such as VS, VFA and NH_4^+-N or in the microbial community composition (Paper III). In contrast, previous studies have observed differences between microbial communities in different reactor sections even with 25-60% digestate recirculation, indicating that removal of recirculation in itself does not promote microbial phase separation (Rossi *et al*., 2022a; Chen *et al*., 2020; Li *et al*., 2024). Studies have also shown that recirculation can be beneficial for gas production, conversion of fatty acids and degree of degradation (Namsree *et al*., 2012; Chen *et al*., 2020; Dong *et al*., 2019; Li *et al*., 2024; Gómez *et al*., 2019), and can improve process stability (Nordell *et al*.. 2021). However, in Paper III there was no significant difference in process yield or degree of degradation between the recirculated and the non-recirculated reactor, likely because the reactors had no phase separation.

4.3 Serial anaerobic digestion

Serial CSTRs, where the first reactor is fed substrate, the second is fed digestate from the first reactor and so forth, are frequently used to model the behaviour of reactors of PFR type (Donoso-Bravo *et al*., 2018; Benbelkacem *et al*., 2013; Wen *et al*., 2007). This is also a potential strategy to reduce the

effect of short-circuiting normally obtained in CSTR processes. In theory, this increases the probability of degradation of material before it leaves the reactor (Paper I; Angelidaki *et al*., 2005; Das *et al*., 2016), in the same way as in a phase-separated plug-flow reactor.

Different approaches can be used in serial digestion. One strategy is to create a stage-separated process, with mainly hydrolysis and acidogenesis in the first stage and methanogenesis in the subsequent stage (Roy *et al*., 2009; Massé *et al*., 2013; Moestedt *et al*., 2020; Parawira *et al*., 2008; Chatterjee & Mazumder, 2019). This can be achieved by subjecting the first reactor to overloading through high OLR and short HRT, which leads to production of VFA and consequently low pH. While methanogens are washed out due to the low HRT or inhibited by the low pH, the acidic conditions are beneficial especially for carbohydrate hydrolysis, which helps with efficient solubilisation of carbohydrates before the methanogenic stage (Chatterjee & Mazumder, 2019). Further optimisation can be achieved by using a temperature phased process, with a thermophilic first stage to increase the hydrolysis rate and a subsequent mesophilic stage to reduce the ammonia stress during methanogenesis (Wu *et al*., 2016; Schmit & Ellis, 2001; Amodeo *et al*., 2021). In this way, the stage-separation strategy offers a flexible system where different stages in the AD process can be optimised separately.

An alternative approach is to have a methanogenic first reactor followed by post-digestion to capture remaining methane potential (Paper I; Boe & Angelidaki, 2009; Li *et al*., 2017; Kaparaju *et al*., 2009). This has been shown to improve digestion of lignocellulosic materials (Kaparaju *et al*., 2009; Li *et al*., 2017). Paper I investigated serial reactor systems operated with food waste under wet AD conditions. The systems had a methanogenic first-step reactor followed by one or two post-digesters, which significantly improved the methane yield and reduced the residual methane potential in the digestate compared with a one-step reactor. In addition, protein concentration in the digestate was >20% lower after serial compared with single-step digestion. In line with this, slight enrichment of the proteolytic genera *Proteiniphilum* and *Fastidiosipila* was observed in the post-digesters (Paper I). Previous research has shown that it can be difficult to achieve a good degree of degradation of recalcitrant protein structures (Ekstrand *et al*., 2022) and that protein degradation can be especially inefficient at high carbohydrate concentrations (Breure *et al*., 1986; Tepari *et al*., 2020) and in

acidic conditions (Duong *et al*., 2019). These factors make the hydrolytic reactor in a stage-separated system unsuitable for protein hydrolysis (Moestedt *et al*., 2020). In that regard, a serial set-up could provide a good division between carbohydrate degradation in early reactor steps and protein degradation in later reactor steps, where pH is higher and the main part of carbohydrates have been degraded (Paper I). In addition, since there are reports of reduced cellulose degradation efficiency at high ammonia levels (Ahlberg-Eliasson *et al*., 2023; Sun *et al*., 2016b; Fischer *et al*., 2019), this separation might also be beneficial for cellulose degradation. In comparison with a stage-separated system, an advantage of the serial set-up with post-digestion is that the majority of methane production takes place in the first reactor, where the NH_4^+-N concentration is relatively low (Paper I). Conversely, in a stage-separated system, the main protein degradation takes place in the methanogenic reactor stage (Moestedt *et al*., 2020), posing a greater risk of inhibition of the methanogenesis process due to ammonia accumulation.

Comparing serial AD systems to PFRs, reactors of plug-flow type are unlikely to obtain as clear division of phases and degradation steps as is possible to achieve with serial CSTRs. This was confirmed in Papers II-IV, although with some differences between sections in microbial activity, as mentioned above (Figure 6). However, despite the lack of clear phase separation in the PFR studied by Rossi *et al*. (2022a), they concluded that division of carbohydrate and protein degradation was obtained, based on the microbial community structure. Carbohydrates were assumed to be hydrolysed at the start and very end of the reactor, based on the relative abundance of the main carbohydrate-degrading genus *Defluviitoga*, while the relative abundance of the protein-degrading genus *Proteiniphilum* was higher in the middle section (Rossi *et al*., 2022a). This indicates that PFRs might offer the same advantages of separation between carbohydrate and protein degradation as observed for the serial strategy. Nordell *et al*. (2021) also achieved significant reduction of recalcitrant protein structures in a PFR, potentially enhanced by the plug-flow dynamics, although the mechanism was not completely revealed since the chemical composition in different sections was not investigated. Compartmentalised PFRs could offer an intermediate strategy between a serial digestion system and a PFR. As mentioned above, it has been shown that phase separation can be obtained in such reactors, with acidified first reactor compartments (Veluchamy *et al*.,

2019; Varol & Ugurlu, 2017) and methanogenesis occurring towards the reactor outlet (Ren *et al*., 2022).

4.4 Comparison of reactor types CSTR and PFR

As explained in section 4.1, reactors of the PFR and CSTR types have different characteristics in terms of retention time distribution (RTD). In theory, PFRs outperform CSTRs operated at identical HRT and OLR, because material in the PFR has the potential for complete degradation, while this cannot be achieved in the CSTR because of short-circuiting, an effect that becomes more pronounced with shorter HRT (Das *et al*., 2016).

Whether the impact of extended retention of material in a PFR compared with a CSTR can be demonstrated experimentally was investigated in Paper III, by comparing PFRs and CSTRs operated with the same substrate, HRT, OLR and TS_{in}. The comparison was carried out to assess the effect of the theoretically longer RTD in PFRs, but also to evaluate whether high-solid substrates can be efficiently treated in CSTRs. Thermophilic HSD experiments with similar substrate composition, *i.e.* food waste co-digested with garden waste, have been performed previously (Zhang *et al*., 2022), but CSTRs are more commonly used for wet AD processes (Akinbomi *et al*., 2022; Kothari *et al*., 2014). Problems with high TS content arose in the comparative study in Paper III, with more difficulties obtaining representative samples of TS and VS from the CSTRs than the PFRs. This was potentially due to the dimensions of the feeding and digestate openings in the CSTRs being better optimised for wet materials.

In agreement with the hypothesis that more efficient degradation can be acheived in PFRs, this reactor type exhibited slightly more stable process parameters, higher methane yield (309-321 NL CH_4/kg VS compared with 249-253 NL CH_4/kg VS) and marginally improved mineralisation of organic nitrogen (33-34% compared with 28%) than CSTRs operated at identical operating settings (Paper III). However, the VS reduction was consistent across both reactor types (51-54%) (Paper III). Thus, there was no significant difference in degree of degradation and, since plug-flow behaviour or phase separation was not established in the PFRs (Papers II & III), the reason for the higher methane yield from these reactors was likely not related to the retention time distribution.

A potential challenge when operating CSTRs with dry substrates is that their mixing mechanism is seldom optimised for high TS content in the same way as in PFRs, as pointed out by Veluchamy *et al*. (2019). In line with this, it was concluded that one of the main differences between the reactor types compared in Paper III was the stirring mechanism and speed, which was considerably higher in the CSTRs (70-150 rpm) than in the PFRs (1 rpm). Differences in mixing speed have been demonstrated to significantly affect microbial communities in AD (Singh *et al*., 2020). In general, a more stable and efficient process can be expected at lower stirring speed and frequency (Sekine *et al*., 2022; Zhang *et al*., 2019; Latha *et al*., 2019) and high stirring speed can potentially interfere with microbial interactions, for example between SAOB and hydrogenotrophic methanogens (Singh *et al*., 2020). It can also hinder microbes from attaching to substrates, especially for hydrolysis of cellulose (Kim *et al*., 2017). In line with this, the cellulose-degrading genera *Defluviitoga* and *Halocella* decreased in relative abundance in the more agitated CSTRs. In the CSTRs, the order MBA03 dominated instead and towards the end of the experiment accounted for >60% of the community in the high-solid CSTRs (Paper III). The methanogenic community was also different in the two reactor types, with more *Methanoculleus* in the PFRs and more *Methanothermobacter* and *Methanosarcina* in the CSTRs, potentially relating to the mixing and distribution of H_2, as discussed in Paper III.

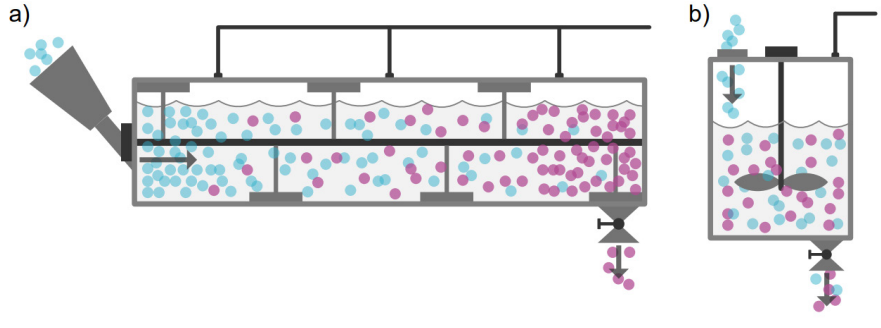

Figure 7. (a) Illustration of concentration gradients of substrate and degradation products along a plug-flow reactor. (b) Illustration of a completely stirred-tank reactor with homogenous reactor content.

When PFR or phase-separated processes are compared with single-stage CSTR in literature, a key question is often the process stability (Park *et al*., 2008; Chatterjee & Mazumder, 2019; Veluchamy *et al*., 2019). Studies by Varol & Ugurlu (2017) and Zhang *et al*. (2017) obtained systems with significantly better stability at increased OLR in phase-separated reactors than in single-stage CSTRs. One of the main differences between PFRs and CSTRs is that material within a CSTR should be completely homogenised, while a PFR should have a gradient of substrate and intermediary products along the reactor (Panaro *et al*., 2022) (Figure 7). This would imply that toxic substances are not necessarily found at inhibitory concentrations within the whole system, thereby keeping parts of the process stable even at high loading rates or during ammonia accumulation. Nordell *et al*. (2021) found that a relatively stable process could be maintained at a NH_3-N level as high as 2 g/L, which was shown to cause severe disturbances in Paper II. They argued that since plug-flow behaviour was demonstrated in their system, it is likely that inhibitory ammonia levels were only obtained in the outgoing digestate, while the level at the inlet was lower (Nordell *et al*., 2021). However, as PFR behaviour was not obtained in Paper III, the potential improvement in stability compared with CSTRs could not be evaluated.

5. Down-scaling of industrial-scale HSD

Industrial-scale HSD plants, especially those operated under thermophilic conditions and with protein-rich substrates, are known to frequently experience disturbances due to ammonia inhibition (Westerholm *et al*., 2020; Illmer & Gstraunthaler, 2009; Zeshan *et al*., 2012). Despite its challenges, HSD is an interesting technology with high potential for industrial-scale biogas production (see section 1.2). For example, according to Angelonidi & Smith (2015), HSD offers advantages over wet AD due to the low water input required and wide range of potential substrates. Also, a modelling study of a thermophilic Kompogas plant by Carlos-Pinedo *et al*. (2020) found that the HSD plant theoretically had 10% higher energy yield than a corresponding wet AD plant.

Given the challenges mentioned in Chapters 3 and 4, such as those relating to reactor design and ammonia inhibition, optimisation experiments are needed to identify strategies that can improve the robustness and performance of HSD processes. An important aspect in this regard is to demonstrate that experiments carried out at laboratory scale are applicable for industrial-scale processes. In particular, comparative studies are important to prove transferability of results and to reveal limitations in terms of up-scaling or down-scaling (Lansing *et al*., 2019). Previous down-scaling studies have demonstrated good agreement between laboratory and industrial reactors (Gallert *et al*., 2003; Lüdtke *et al*., 2017; Bouallagui *et al*., 2010). The aim of these studies has been *e.g*. to identify the OLR limit of the system and to evaluate new substrate pre-treatments, without risking process failure at industrial scale, and they have contributed valuable information (Westerholm *et al*., 2019; Gallert *et al*., 2003). However, these comparative studies were all carried out in wet AD conditions, while there is a lack of research on the agreement between laboratory- and industrial-scale operation

of HSD. There are many examples of HSD experiments at laboratory scale (*e.g*. Rossi *et al*., 2022a; Zeshan *et al*., 2012; Zhang *et al*., 2022; Li *et al*., 2024) and pilot scale (Basinas *et al*., 2021; Carlos-Pinedo *et al*., 2019), but few previous studies have attempted to replicate the conditions in an industrial-scale plant. Carlos-Pinedo *et al*. (2019) reviewed HSD carried out at laboratory, pilot and full scale and found that in general, the laboratory-scale studies achieved higher methane production. However, the mode of operation in the studies reviewed was very different and therefore the scales could not be directly compared. Another observation was that laboratory-scale experiments often studied mono-digestion of single substrate types, while co-digestion was more common in the full-scale cases (Carlos-Pinedo *et al*., 2019), which suggests a lack of laboratory-scale HSD experiments covering common full-scale operating conditions.

One of the main objectives of Paper II was therefore to simulate a thermophilic industrial-scale HSD plant in the laboratory. The plant, treating a mixture of organic household waste, manure and garden waste in three separate Kompogas-type digesters (each with active volume 2100 m^3), was monitored over a period of >40 weeks. During the same period, two laboratory-scale reactors of the same type were inoculated with digestate from the thermophilic plant, operated with the same substrate mixture and monitored during >50 weeks, beginning after a start-up phase.

Some aspects of HSD processes make these more difficult to scale down than wet AD processes. In wet digestion, the same substrate mix can be used in both full- and laboratory-scale operations, making down-scaling more straightforward. Due to the characteristics of high-solid substrates, this type of process is more challenging to operate at small scale and the substrate therefore needs to be mechanically milled to smaller particle size when an HSD process is scaled down (Carlos-Pinedo *et al*., 2019). This theoretically has a significant impact on the potential degree of degradation, as substrate with smaller particle size is degraded more easily (Bong *et al*., 2018; Carlsson *et al*., 2012). In the experiment carried out in Paper II, substrate particle size was reduced from ~60 mm to ~10 mm before the substrate was fed into the laboratory reactors. However, only a small effect on VS reduction was observed when the scales were compared (43% and 41% in the laboratory- and industrial-scale process, respectively) (Paper II). Additionally, in contrast to the VS reduction, SMP was in fact higher in the

industrial-scale system (366 NL CH4/kg VS) compared with the laboratory-scale system (339 NL CH4/kg VS (Figure 8).

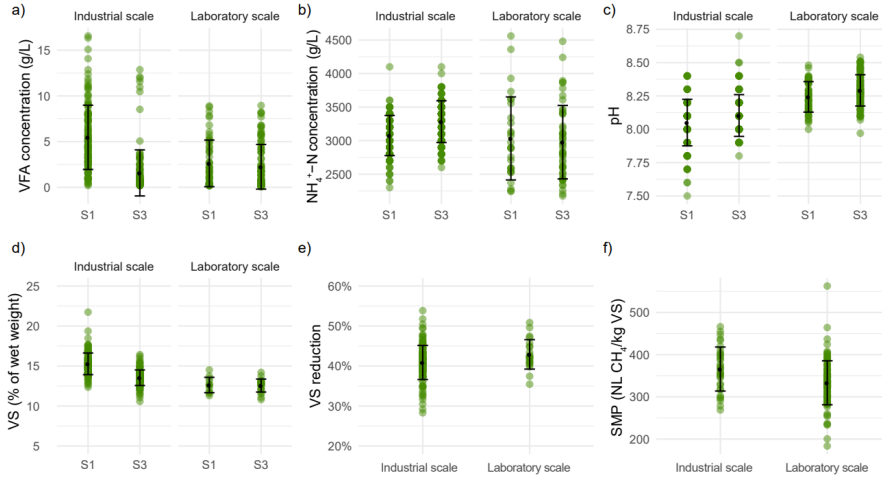

Figure 8. (a) Volatile fatty acid (VFA) concentration, (b) ammonium-nitrogen (NH_4^+-N concentration), (c) pH and (d) volatile solids (VS) level in reactor sections close to inlet (S1) and outlet (S3) in industrial-scale and laboratory-scale plug-flow reactors (PFRs). (e) Volatile solids reduction and (f) specific methane production (SMP) in the laboratory- and industrial-scale PFRs. Figure adapted from Paper II.

At industrial scale, slight phase separation was observed across the plug-flow systems (Figure 8), with significantly higher VFA and VS, and significantly lower pH and NH_4^+-N levels, in the first (S1) compared with the last reactor section (S3). At laboratory scale these trends were much weaker (Figure 8), as also described in section 4.2. Illmer & Gstraunthaler (2009) observed analogous trends while monitoring a very similar HSD plant (treating household waste and green waste in Kompogas reactors), with a clear decrease in VFA and slight increase in average pH and NH_4^+-N concentration along the reactor. It could be argued that, since distances within industrial-scale reactors are greater and therefore homogenisation of material within reactors of this scale likely takes longer, phase separation could potentially be easier to obtain at industrial scale. However, analysis of the microbial population in the industrial-scale reactors in Paper II contradicted any suggestion of true phase separation, since no significant separation of the community structure was observed.

An important factor that will differ at different scales is the practical procedure of reactor feeding. Industrial-scale plants often have automated systems that enable continuous feeding, while feeding in the laboratory is generally performed manually and therefore can only be semi-continuous. In addition, sampling time-point in relation to feeding could affect the results, especially for samples taken from the first reactor section, which contains a mixture of digestate and substrate (Paper II; Illmer & Gstraunthaler, 2009). Thus, a possible explanation for the clearer differences between reactor sections observed in the larger-scale reactors may be related to the sampling time-point, as samples from those reactors were taken during continuous feeding, while samples from the laboratory-scale reactors were taken a day after the previous feeding, giving more time for homogenisation of material at smaller scale (Paper II). Unfortunately, conclusive results on the plug-flow behaviour in industrial-scale reactors could not be obtained in the evaluation in Paper II, since tracer studies at the plant were interrupted 48 hours after tracer addition, only to ensure that requirements for hygienisation were fulfilled.

Although microbial communities were similar along the length of the PFRs at both industrial and laboratory scale, down-scaling had significant influence on the population. A distinct separation of the communities in the two different scales emerged after an initial start-up phase (Paper II). There was in particular a clear decrease in *Defluviitoga*, while groups such as DTU014, *Proteiniphilum*, *Lentimicrobium* and *Halocella* increased as a result of down-scaling. In Paper I, where inocula were taken from industrial-scale wet AD reactors and transferred to laboratory-scale reactors with the same operating parameters, down-scaling effects such as enrichment of *Actinomyces*, *Gallicola* and *Paludibacteraceae* were observed, while *e.g.* MBA03 decreased in abundance. In line with results from Paper II, relative abundance of *Defluviitoga* decreased upon down-scaling also in Paper I. Some of these changes were hypothesised to relate to transition from continuous feeding at full scale to semi-continuous feeding at laboratory scale (Paper I; Mulat *et al*., 2015). Although the reasons for the changes could not be completely revealed, the conclusion was that the overall function of the communities remained similar at both scales (Paper II), which is in line with previous observations (Westerholm *et al*., 2018; Westerholm *et al*., 2019). For example, in Paper II hydrogenotrophic methanogenesis was the main pathway for methane production at both scales, but in industrial

reactors *Methanothermobacter* was the dominant genus, while in laboratory-scale reactors *Methanoculleus* was present in higher relative abundance.

5.1 Summary of challenges and limitations related to down-scaling

The main challenges in comparisons of laboratory and industrial-scale HSD processes that were identified in Paper II were:

- Feeding was carried out more continuously throughout the day at industrial-scale, compared with semi-continuous feeding in laboratory-scale reactors. This likely affected microbial community structure and influenced the results of chemical analyses, especially in samples taken from the reactor section close to the inlet.
- Additional pre-treatment of the substrate was necessary at laboratory scale to reduce the particle size, which potentially affected the degradability of the substrate.
- It was not possible to collect homogeneous substrate samples from the industrial-scale plant. This made evaluation of degree of degradation and OLR difficult and unreliable at industrial scale, as TS and VS values of the different fractions were based on standard values, while TS, VS and chemical composition of each fraction could be more thoroughly analysed at laboratory scale.
- Substrate fractions for laboratory-scale digestion were collected from the industrial-scale plant every 3-4 months and represented the average composition of substrate fractions fed to the full-scale reactors. This made it difficult to simulate fluctuations in substrate composition experienced at industrial-scale. Furthermore, the substrate batches collected for laboratory-scale use might not have accurately reflected the composition of the substrate utilised in the full-scale process.
- It was easier to keep operating parameters consistent at laboratory scale (except during process disturbances), while *e.g.* OLR and substrate composition varied between weeks and over seasons in the industrial-scale process depending on substrate availability.

6. Summary and conclusions

This thesis work identified and explained some important challenges associated with HSD processes, including:

- Ammonia inhibition, especially in HSD operated under thermophilic conditions.
- Difficulty in obtaining plug-flow behaviour of organic material in the tubular reactors designed for HSD processes.
- Digestion of high-solid substrates in laboratory-scale processes and comparison to industrial-scale processes.

High-solid digestion processes are exposed to a high risk of process disturbances due to high loading rate, often with protein-rich substrates, low dilution rate and poor mass transfer. In this thesis, there were cases of ammonia-induced disturbances in thermophilic HSD processes operated with a large fraction of food waste co-digested with manure and garden waste. Disturbances occurred at NH_4^+-N concentrations >4 g/L and led to H_2 and propionate accumulation, and consequently reduced methane yield. The response in terms of microbial community structure varied depending on the severity and time-span of the disturbance and differed between laboratory-scale and industrial-scale reactors. Results from a metatranscriptome study revealed general downregulation of cellular processes at higher ammonia levels in a laboratory-scale PFR.

According to literature, a process operated in a phase-separated PFR has the advantages that the material is retained within the reactor over a longer period than in a completely stirred system, and that process stability and resilience to disturbances are improved. Evaluations of PFR systems in this thesis however showed that it is difficult to obtain plug-flow behaviour and phase separation within tubular reactors, with or without digestate recirculation, at different TS levels and also both at laboratory and industrial

scale. Although functional analysis illustrated some differences in microbial activity across a PFR, no clear phase separation was observed based on chemical parameters or DNA-based microbial analysis. A comparative study between PFRs and CSTRs showed similar process performance and degree of degradation in both reactor types, indicating that the theoretical advantages of a PFR could not be demonstrated in practice due to the absence of plug-flow behaviour. However, slightly better specific methane production and nitrogen mineralisation was obtained in the PFRs, which was partly attributed to the lower mixing speed applied in these reactors. Features similar to a plug-flow system can also be obtained using a set-up of serial completely stirred reactors, which in this thesis was shown to significantly improve methane production and degree of degradation, especially of proteins. This was attributed to degradation of different substrate fractions in different compartments and to a gradient of NH_4^+-N across the systems, with low levels in the main methanogenic compartment.

This thesis showed that high-solid materials can be treated in laboratory-scale reactors, but require additional pre-treatment compared with industrial-scale reactors. A comparative study demonstrated that volatile solids reduction, specific methane production and other performance parameters were within the same ranges in both laboratory and industrial HSD systems, although average values differed in some cases. An effect of down-scaling on microbial community structure was observed, but it did not alter the overall function of the community. Although certain factors, such as feeding strategy and substrate particle size, cannot be kept consistent across different scales, the down-scaling evaluation demonstrated the viability of performing laboratory-scale experiments of HSD processes.

7. Future perspectives

Extensive research has been conducted on biogas processes but there is still much to learn, such as how to avoid and handle process disturbances, utilisation of new substrate types and how to design reactors and choose operating parameters for maximum performance. Optimisation strategies are also important to make the most of existing biogas plants and avoid emissions of residual methane from the digestate. In addition, the complex microbial communities that are the core of AD processes are still largely uncharacterised and consist of plenty of unknown species, often with unknown functions. With better knowledge about the organisms involved in AD processes, optimisation strategies, such as changes in temperature or trace element addition, can be specifically designed in future studies to promote the activity of key species.

7.1 Exploration of microbial communities

A great part of current research on AD revolves around the microbial communities involved, their interactions and functions, and on optimising their potential through different operating strategies. High-throughput sequencing of the 16S rRNA gene is a widely used method for analysis of microbial community composition that can provide large amounts of data with relatively high resolution and enable estimation of diversity within complex communities. However, this method has limitations such as differences in 16S rRNA gene copy numbers and PCR primer specificity, as discussed in section 2.4 of this thesis. In addition, conclusions about the function of the observed microbial groups cannot directly be drawn based on the taxonomic placement, especially since the function of many species remains unknown. Therefore, discussions on how process performance

correlates with microbial community structure are relatively speculative and build on knowledge about known species or metagenomics information for identified taxa, as well as previous experiences from other similar processes.

The activity and function of the microbial community can instead be investigated through isolation and sequencing of mRNA sequences, *i.e.* the metatranscriptome. Even though such studies are challenging, since preparation of RNA samples, sequencing and data analysis are costly and time-consuming, metatranscriptomics can be an important complement to analysis of microbial communities using DNA-based methods. Metatranscriptomic analysis is however only one of the possible methods to study the function of the microbial community more in depth in future studies. For example, combined data from different meta-omics methods, such as metagenomics, metaproteomics and metabolomics, could provide a better understanding of the metabolic pathways within HSD and AD in general, and the activity of microbial groups under different environmental conditions.

7.2 Optimisation of reactor design and operation

The theoretical features of a reactor of plug-flow type offer potential for enhanced process stability and degradation efficiency. This makes it a suitable choice for digestion of high-solid substrates, which may contain protein-rich fractions and recalcitrant material that need long retention time for degradation. However, plug-flow behaviour within a reactor of plug-flow type should not be assumed without performing tracer studies. Identifying operating factors necessary for plug-flow behaviour in a tubular reactor, such as viscosity and substrate composition, is an interesting topic for future research. A potentially rewarding approach in further investigations could be to implement modelling studies to simulate conditions at different TS levels, viscosity and reactor design and identify the optimal parameters for plug-flow dynamics. Such a strategy has been used *e.g.* by Rasouli *et al.* (2018) to investigate mixing within a PFR.

The challenges in obtaining plug-flow behaviour in this thesis indicate that some modifications in reactor design, such as compartmentalisation or altered stirring mechanism, might be necessary to achieve the hypothetical effects and obtain a robust phase-separated system, both in laboratory and industrial scale.

An alternative to PFRs is to use sequential CSTRs. Although CSTRs in most cases are not used for treatment of high-solid substrates, the results obtained in Paper III demonstrate that this is possible, at least at laboratory scale. This makes sequential CSTRs an interesting alternative for evaluation of phase-separated HSD processes, using a relatively simple technology. This set-up could be used to assess to what extent a phase separated process can help reduce the risk of disturbances. Additionally, strategies such as temperature gradient with thermophilic hydrolysis phase and mesophilic methanogenic phase, as well as optimisation of recirculation rate could be evaluated using serial reactors.

The challenges linked to down-scaling identified in this thesis, particularly in HSD processes, suggest that in addition to laboratory-scale analyses, more studies monitoring and evaluating full-scale processes could provide valuable information. This could help estimate the extent to which factors such as substrate particle size and fluctuations in substrate composition affect the process. Such studies could also provide more insights into current challenges during industrial-scale operation and reduce the gap between biogas research and full-scale production.

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Popular science summary

Biogas production plays an important role in contributing to a more sustainable and circular society by utilizing resources in waste products. These resources include energy and nutrients that would otherwise go to waste. Several different types of organic waste, such as sewage sludge, food waste, cow manure and agricultural residues, can be used to produce biogas. A large group of different microorganisms, such as bacteria and archaea, working under oxygen-free conditions, produce the biogas in a decomposition process known as anaerobic digestion. This process has two main products; biomethane and digestate. Biomethane is a renewable energy source that can be used to power cars and buses, or to produce electricity and heat. The digestate contains all the nutrients from the organic waste and can be used as fertilizer in organic farming.

Digestion is a multi-step process that starts with the breakdown of large protein, fat and carbohydrate molecules. After a number of intermediate steps carried out by different bacteria, these complex molecules have finally been converted into acetic acid, carbon dioxide and hydrogen gas, which in turn are the source of energy for specialised archaea that further can convert the molecules into methane. During the various decomposition steps, other products are also formed, for example ammonia, which is produced during protein degradation. The ammonia that is formed is important for the digestate's value as fertilizer. At high concentrations, however, ammonia becomes toxic, especially for the methane-producing archaea.

Many types of waste, for example from households and agriculture, can be relatively dry and usually need to be diluted with large amounts of water before they are used for biogas production. Another method is so-called high-solid digestion that significantly can reduce water consumption. In a high-solid digestion process, the material resembles a viscous porridge,

while in a more conventional wet digestion process, the material is more fluid. This means that special technology and equipment, such as special types of reactors, need to be used to handle the dry and thick material. This thesis focuses on high-solid digestion processes and the unique challenges associated with working with these viscous materials in different types of biogas systems.

One of the challenges is related to toxic levels of ammonia that cause disturbances and reduced biogas production. A combination of the fact that high-solid digestion systems often are fed with protein-rich waste and the low dilution with water means that these processes often suffer from high concentrations of ammonia. In this thesis, disturbances caused by ammonia in different high-solid digestion systems were studied to investigate how the microorganisms reacted and to try finding early warning signs of process inhibition. The results could show that high hydrogen levels and propionic acid concentrations were clear signs of an unbalanced process.

Due to the material's thick consistency and relatively large particle size, high-solid digestion processes are difficult to work with on a laboratory scale. Therefore, high-solid digestion has generally been investigated less than wet digestion which is easier to scale down. In one of the projects within this thesis, special biogas reactors were constructed on a laboratory scale to simulate an industrial high-solid digestion plant. The material fed into the process first needed to be ground before it could be digested in the laboratory. Even so, the processes on laboratory and industrial scale behaved very similarly. When evaluating different ways to improve biogas production, it is a good strategy to first test them on a laboratory scale, before industrial application. One of the conclusions from this thesis is that digestion processes at different scales produce similar results.

The reactors that were constructed for the laboratory-scale experiments are so-called plug-flow reactors. These are built as horizontal tubes. In a plug-flow system, the material is fed to one side of the reactor, moves slowly through the tube where decomposition occurs gradually, and exits at the opposite end as digestate, while the biogas is collected via outlets at the top of the reactor. Unlike completely mixed reactors, which are more often used for wet materials, the contents of a plug-flow reactor should not be evenly distributed. Instead the reactor should have different populations of specialized microorganisms in different parts. In theory, a well-functioning plug-flow system can provide more complete degradation and process

stability compared to a completely mixed reactor. However, achieving this in practice is more difficult, which was also shown in several of the projects within this thesis. To get more optimized high-solid digestion processes in the future, new reactor design can be an interesting strategy.

Populärvetenskaplig sammanfattning

Biogasproduktion spelar en viktig roll för att bidra till ett mer hållbart och cirkulärt samhälle genom att utnyttja resurser i restprodukter. Detta inkluderar energi och näringsämnen som annars skulle gå till spillo. Flera olika typer av organiskt avfall, såsom avloppsslam, matrester, kogödsel och jordbruksrester, kan användas för att producera biogas. En stor grupp av olika mikroorganismer, till exempel bakterier och arkéer, som arbetar under syrefria förhållanden producerar biogasen i en nedbrytningsprocess som kallas anaerob rötning. Denna process har två huvudprodukter; biometan och rötrest. Biometan är en förnybar energikälla som kan användas till att driva bilar och bussar, eller till produktion av el och värme. Rötresten innehåller all näring från det organiska avfallet och kan användas som gödningsmedel i ekologisk odling.

Rötning är en process i flera steg som startar med nedbrytning av stora protein-, fett- och kolhydratmolekyler. Efter ett antal mellansteg som utförs av olika bakterier har dessa komplexa molekyler slutligen omvandlats till ättiksyra, koldioxid och vätgas som i sin tur är energikälla för specialiserade arkéer som vidare kan omvandla molekylerna till metangas. Under de olika nedbrytningsstegen bildas även andra produkter, till exempel ammoniak som produceras vid proteinnedbrytning. Ammoniaken som bildas är viktig för att rötresten ska bli användbar som gödningsmedel. Vid höga koncentrationer blir dock ammoniak giftigt, framförallt för de metanproducerande arkéerna.

Många typer av avfall, exempelvis hushållsavfall och jordbruksavfall, kan vara relativt torra och brukar därför spädas ut med stora mängder vatten innan de används till biogasproduktion. En annan metod är så kallad torrötning, där vattenförbrukningen kraftigt kan reduceras. I en torrötningsprocess liknar materialet en trögflytande gröt, medan materialet i en mer konventionell våtrötningsprocess är mer lättflytande. Det innebär att

speciell teknik och utrustning, som speciella typer av reaktorer, behöver användas för att hantera det torra och tjocka materialet. Den här avhandlingen fokuserar på torrötningsprocesser och de unika utmaningar som är kopplade till att arbeta med dessa trögflytande material i olika typer av biogassystem.

En av utmaningarna är relaterad till giftiga halter av ammoniak som orsakar störningar och minskad biogasproduktion. En kombination av att torrötningssystem ofta matas med proteinrikt avfall och avsaknad av utspädning med vatten gör att dessa processer ofta drabbas av höga koncentrationer av ammoniak. I denna avhandling studerades störningar orsakade av ammoniak i olika torrrötningssystem för att undersöka hur mikroorganismerna reagerade och för att försöka hitta tidiga varningstecken på hämning av metanproduktionen. Resultaten kunde visa att höga vätgashalter och propionsyrakoncentrationer var tydliga tecken på en obalanserad process.

På grund av materialets tjocka konsistens och relativt stora partikelstorlek är torrötningsprocesser svåra att arbeta med i laboratorieskala. Därför har torrötning generellt undersökts mindre än våtrötning som är lättare att skala ner. I ett av projekten inom denna avhandling konstruerades speciella biogasreaktorer i laboratorieskala för att simulera en industriell torrötningsanläggning. Materialet som matades in i processen behövde först malas innan det kunde rötas i laboratoriet. Trots det uppträdde processerna väldigt lika i både laboratorie- och industriell skala. När man utvärderar olika sätt att förbättra biogasproduktion är det en bra strategi att först testa dem i laboratorieskala, innan de appliceras industriellt. En av slutsatserna från denna avhandling är att rötningsprocesser i olika skalor ger liknande resultat.

Reaktorerna som konstruerades för experimenten i laboratorieskala är så kallade pluggflödesreaktorer. Dessa är byggda som horisontella rör. I ett pluggflödessystem matas materialet in på ena sidan av reaktorn, rör sig långsamt genom röret där nedbrytningen sker gradvis och kommer ut i motsatt ände som rötrest, medan biogasen samlas upp via gasutflöden på toppen av reaktorn. Till skillnad från totalomblandade reaktorer, som oftare används för våta material, bör innehållet i en pluggflödesreaktor inte vara jämnt fördelat. Man vill istället ha olika populationer av specialiserade mikroorganismer i olika delar av reaktorn. I teorin kan ett välfungerande pluggflödessystem både ge mer fullständig nedbrytning och processtabilitet jämfört med en totalomblandad reaktor. Att uppnå detta i praktiken är dock

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Serial anaerobic digestion improves protein degradation and biogas production from mixed food waste

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ABSTRACT

Optimization of the biogas generation process is important to achieve efficient degradation and high methane yield, and to reduce methane emissions from the digestate. In this study, serial digester systems with two or three biogas reactors were compared with a single reactor, with the aim of improving degree of degradation and methane yield from food waste and assessing adaptation of microbial communities to different reactor steps. All systems had the same total organic load (2.4 g VS/(L d)) and hydraulic retention time (55 days). Serial systems increased methane yield by >5% compared with the single reactor, with the majority of the methane being obtained from the first-step reactors. Improved protein degradation was also obtained in serial systems, with >20% lower outgoing protein concentration compared with the single reactor and increasing NH_4^+-N concentration with every reactor step. This resulted in separation of high ammonia (>384 mg NH_3-N/L) levels from the main methane production, reducing the risk of methanogen inhibition. *Methanosarcina* dominated the methanogenic community in all reactors, but increases in the hydrogenotrophic genera *Methanoculleus* and *Methanobacterium* were observed at higher ammonia levels. Potential syntrophic acetate-oxidizing bacteria, such as MBA03 and *Dethiobacteraceae*, followed the same trend as the hydrogenotrophic methanogens. Phylum Bacteroidota family *Paludibacteraceae* was highly abundant in the first steps and then decreased abruptly, potentially linked to an observed decrease in degradation in the last-step reactors. Nevertheless, the results indicated a trend of increasing relative abundance of the potentially proteolytic genera *Proteiniphilum* and *Fastidiosipila* with successive reactor steps.

1. Introduction

Production of biogas through anaerobic digestion (AD) is a stepwise degradation process of organic material, carried out by a complex microbial community in an anaerobic environment [1]. Biogas production has several benefits, *e.g.*, it is a sustainable treatment method for organic wastes and the methane in biogas is a renewable energy carrier that can be used as vehicle fuel or for production of electricity and heat [2]. Moreover, during the AD process, mineral nutrients in the wastes are concentrated and the resulting digestate can be used as biofertilizer, thereby contributing to recycling of nutrients and reduced use of artificial fertilizer [3].

In order to reach the full potential of AD in terms of economic and environmental benefits it is important to obtain a high degree of degradation of the organic material. Many previous studies have investigated different strategies to improve degradation and efficiency of various AD processes, including both management and technological approaches. So far, a lot of effort has been put on materials rich in lignocellulose, difficult to degrade and thus typically giving low efficiency of the biogas process [4]. However, several recent publications have reported that also degradation of proteins in AD can be inefficient and result in high levels of residual protein, representing an unexploited potential of methane as well as ammonium-nitrogen (NH_4^+-N), in the outgoing digestate [5–8]. Even so, less effort in the scientific literature has been devoted to understanding conditions giving efficient degradation of proteins.

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Different types of food waste, *e.g.* source sorted organic household waste and residues from food industry, can vary greatly in its chemical composition, but generally has high protein and fat concentrations as well as a relatively low content of complex carbohydrates [9]. Fat and protein are energy-rich, so food waste has high biomethane potential and, owing to a low level of lignocellulose, are relatively easy to degrade [9]. AD of food waste even needs to be managed carefully, as fast degradation can cause accumulation of volatile fatty acids (VFA) and a subsequent drop in pH. Additionally, high levels of protein can lead to inhibitory levels of the fermentation product ammonia (NH_3), which is toxic to the microbial community and especially the methanogenic community [9,10]. Nevertheless, proteins have been shown to represent the major fraction of non-degraded macromolecules in the digestate [8].

Previous studies have evaluated degradation of recalcitrant structures by replacing a single reactor with a main methanogenic reactor followed by a methanogenic post-digester that collects residual gas potential. This has been successful for agricultural residues with high levels of recalcitrant lignocellulosic structures; both for manure [11], and crop residues, where serial reactors improved conversion of cellulose and hemicellulose compared with a single reactor [12]. In the present study we hypothesize that a serial reactor setup, in comparison to a single reactor, could enable improved degradation of food waste, and specifically proteins, in the same way as previous studies on serial digestion have shown an improved degree of degradation of recalcitrant lignocellulosic substrates [11,12]. In line with this hypothesis, Nordell et al. [13] achieved improved protein degradation in sewage sludge upon addition of a post-digester step to the process. During serial digestion, the process can be set up in different ways in regard to hydraulic retention time (HRT) and organic loading rate (OLR). For food waste, previous studies have mainly applied a high load and short retention time in the first reactor with the aim to obtain microbial phase separation [14]. Separation of hydrolysis/acidogenesis and methanogenesis steps can be a way of improving the degradation of food waste and many studies have shown promising results in terms of process stability, high methane yields, volatile solid (VS) reduction, and potential to extract additional products, such as VFA or hydrogen (H_2) gas, from one of the process stages [9,14–17]. However, as mentioned above, serial digestion can also be applied without phase separation and with methanogenesis in all reactors, a setup that remains to be investigated for processes operated with food waste.

In the current study, we are evaluating a setup with serial methanogenic reactors for biogas production from food waste. The main aims of this study were to investigate the possibility for improved process performance and degradation of recalcitrant structures, *e.g.* proteins, compared with a single reactor. If successful, this approach could give both higher methane yield and reduce the risk of methane emissions from the digestate. An additional aim was to investigate the microbial community structure in the different reactor steps. It has been shown that stage separation of the AD process leads to differences in the microbial communities in the different reactor steps, with enrichment of hydrolytic/acidogenic species in the first reactor [15,18]. However, the effect of a serial digester setup on the microbial community structure in different reactor steps has not yet been studied, nor how this is coupled to the degree of degradation of different molecular structures, *i.e.*, proteins, lipids, and carbohydrates. For the study, an industrial-scale biogas plant using food waste from households, in co-digestion with slaughterhouse waste and industrial organic waste, was used as a model for an experimental lab-scale setup. Systems with one, two, and three serial reactors, all with the same total HRT, were operated and compared with regard to efficiency and process performance. The industrial-scale plant has already access to several reactor tanks which, based on the result from this study, easily could be reorganized in a way that optimizes the overall process performance, without need for construction of new digesters.

2. Material and method

2.1. Experimental setup

Three systems of laboratory-scale reactors were set up (see graphical abstract): a one-step system with a single reactor (A1), a serial system with two reactors (B1 and B2), and a serial system with three reactors (C1, C2, and C3). In the two- and three-step systems, the first reactors were fed with substrate, while the other reactor/s were fed the outgoing digestate from the previous reactor step. Inoculum for the reactors was collected from a full-scale co-digestion plant in Linköping, Sweden, that runs a two-step completely-stirred tank reactor (CSTR) process (total active volume 17,100 m^3). The full-scale process is operated at 42 °C and 35 days HRT in the first step and 41 °C and 20 days HRT in the second step. For the laboratory-scale reactors A1, B1, C1, and C2, the inoculum used was collected from the first reactor step of the biogas plant, while the inoculum for B2 and C3 was collected from the second reactor step. The co-digestion plant receives food waste from households (50% of incoming wet weight), organic industrial residues (25%), and slaughterhouse waste (25%) and is fed an average total OLR of 4 kg VS/(m^3 d) to the first-step reactor. The first-step reactors in this lab-scale study (A1, B1, C1) were fed substrate with a VS content of 13.3% retrieved from a hygienization tank at the co-digestion plant (temperature 70 °C). This substrate was collected on a single occasion and frozen in batches of 5 L until thawing and use. A process additive (Kemira Oyj, Helsingborg, Sweden, developed and patented by Tekniska verken i Linköping AB (publ.)) containing iron (Fe^{2+}/Fe^{3+}), cobalt (Co^{2+}), nickel (Ni^{2+}) and hydrochloric acid (HCl, <0.5%) was added in the substrate, as in the full-scale plant. The dose was set to suppress H_2S levels to <50 ppmv in the biogas and supply the reactor microorganisms with extra trace elements (0.5 mg/kg Co^{2+} and 0.2 mg/kg Ni^{2+}).

The digesters at the full-scale co-digestion plant were used as the basis for the experimental design in lab-scale, in terms of relative reactor volumes, HRT, OLR, and process temperatures. The reactor experiment was performed with CSTRs (developed and patented by Tekniska verken [19]) with an active volume of 3.3–9.1 L and operated at 41–42 °C (Table 1). For practical reasons, the initial steps (B1 and C1) were over-dimensioned in comparison with the following steps, in order to allow excess volume for sampling. Initially the total HRT was set to 55 days in all systems, however after 21 days of operation these values had to be slightly changed to compensate for the volume reduction from production of biogas. The mass of gas produced, calculated using the ideal gas law, was estimated to be 11–12% of ingoing substrate volume. Thus, to reach correct volume relationships between the reactors, the HRT of B2 and C2 was adjusted from 20 to 23 days and from 19 to 21 days, respectively (Table 1). In the results, the gas production presented was normalized to the relative volume of the reactors (Table 1). The reactors were fed semi-continuously once per day, 7 days per week, and the volume was adjusted 5 days per week. The reactors were operated for 203 days in total. The process parameters presented here are average values of measurements taken after day 174, unless otherwise stated, after three complete HRTs for all three systems.

Table 1
Process parameters for the laboratory-scale test reactors operating in mono-digestion or in series with two or three reactors.

System	Reactor	HRT (days)	OLR (g VS/(L·d))	Reactor volume (L)	Temperature (°C)	Relative volume (%)
One-step	A1	55	2.43	9.1	42	100
Two-step	B1	35	3.82	9.1	42	64
	B2	23		4	41	36
Three-step	C1	23	5.74	9.1	42	42
	C2	21		6.3	42	35
	C3	12		3.3	41	23

2.2. Analytical methods

Volumetric gas production was measured online with a Ritter milligas counter (MGC-10, Ritter, Waldenbuch, Germany) and methane concentration was determined with a gas sensor (BlueSens, Herten, Germany). Gas volume was normalized for standard temperature and pressure (273.2 K and 1.01325 bar). Gas composition (CH_4, CO_2, H_2S, H_2, O_2) was further analyzed using a Biogas 5000 device (Geotech Instruments, Coventry, UK). The VFA content was analyzed with a Clarus 550 gas chromatograph (PerkinElmer, Waltham, MA, USA) with a packed Elite-FFAP column (PerkinElmer, USA) for acidic compounds [20]. Total NH_4^+-N was analyzed as the sum of NH_4^+-N (aq) + ammonia-nitrogen (NH_3-N) (aq), by distillation (Kjeltec 8200, FOSS in Scandinavia, Sweden) in acidic solution (H_3BO_3). The NH_4^+-N concentration was then determined by titration with HCl (Titro 809, Metrohm, Herisau, Switzerland) according to the Tecator method for Kjeltec ISO 5664. Kjeldahl-nitrogen was determined using the same procedure and equipment as NH_4^+-N, with the exception that the samples were pre-treated with H_2SO_4 and then heated to 410 °C for 1 h. The pH was measured with a potentiometric pH meter at 25 °C, using a Hamilton electrode (WTW Inolab, Houston, TX, USA). Lignin content was measured using standardized method Tappi T 222 (acid hydrolysis/gravimetrical extraction), at MoRE Research Örnsköldsvik AB, Sweden. Bound and complexed sugar concentrations (xylose, mannose, glucose, galactose and arabinose, with measurement uncertainty of 15%, 10%, 5%, 10% and 10% respectively) were measured using SCAN-CM 71:09 (GC-MS), also at MoRE Research Örnsköldsvik AB, Sweden. Concentrations of hemicellulose and cellulose were estimated from the sugar composition. Raw fat concentration was measured using method NMKL 160 mod. (acid hydrolysis/gravimetric extraction) (measurement uncertainty 30%) at Eurofins Food & Feed Testing Sweden, Lidköping, Sweden. Protein concentrations were measured at three time-points (day 148, 169, and 199) and calculated according to equation (1). Total solids (TS) and volatile solids (VS) were measured according to Swedish standard methods (1981, SS028113). Samples for macromolecule analyses (lignin, sugars and fat) were taken twice (day 95 and 193). Since the process was stable, samples were homogenous, and substrate composition was the same throughout the experiment, these samplings were considered to be representative for the processes.

$$\text{Raw protein} = (\textit{Kjeldahl-N} - \textit{NH}_4^+\textit{-N}) \bullet 6.25 \quad (1)$$

2.3. RMP and methane production rate determination

Batch tests were performed to investigate the residual methane potential (RMP) of the different digestates (taken at day 203) and to evaluate the degradation rate of the key substrates: cellulose (crystalline cellulose, Macherey-Nagel GmbH & Co. KG, Germany), fat (refined rapeseed oil, Di Luca & Di Luca AB, Sweden), and protein (gelatin, Haugen-Gruppen AB, Sweden). For this, triplicate 300 mL portions (containing 9.6, 10.2, 8.4, 11.4, 9.0 and 8.4 g VS in A1, B1, B2, C1, C2, and C3, respectively) of each digestate were added to individual bottles (594 mL) and methane production was measured during incubation at 38 °C, using an automatic methane potential test system, AMPTS II (Bioprocess Control, Lund, Sweden). RMP was also measured for digestates without substrate and for digestates with added substrate (cellulose, fat, or protein) to determine how well and at what rate each substrate was digested, using 1.8 g of substrate (6.0 g VS/L). Specific methane production from the substrates was calculated according to Ref. [21]. The experiment was run until gas production leveled off and results collected until day 25 were used. To compare the degradation rates of the added substrates, initial degradation rate was calculated using 50% of the average accumulated methane production at day 25 in digestate from reactor A1 as a cut-off value. Average methane production per day until the time-point where the cut-off value was reached was calculated for each substrate.

2.4. 16S rRNA gene sequencing

DNA extractions were performed on samples from all digestates, taken at day 200, and on the two inocula from the co-digestion plant in Linköping. All samples were stored at −20 °C until extraction. Extraction was done in triplicate, using the FastDNA Spin Kit for Soil (MP Biomedicals Europe) according to manufacturer's instructions, with the exceptions that aliquots of 200 μL of sample were used and an extra wash step was included to remove humic acids as described in Ref. [22]. DNA was eluted using 70 μL of water. DNA concentrations were measured using a Qubit 3.0 Fluorometer with a Qubit dsDNA BR Assay Kit (Invitrogen, Thermo Fisher Science, Waltham, MA, USA). 16S rRNA-gene amplicon libraries for bacteria and archaea were prepared from the DNA samples as described previously [23]. The concentrations of the final PCR products were measured with Qubit 3.0 Fluorometer (Invitrogen, Thermo Fisher Science, Waltham, MA, USA) and 20 ng of DNA from each sample were submitted for sequencing with the Illumina MiSeq platform at SciLifeLab in Uppsala, Sweden.

16S rRNA-gene sequences were processed according to the DADA2 pipeline tutorial (version 1.16) [24], using the DADA2 package (version 1.16.0) in R (version 4.0.2). Primer and adapter sequences were removed from the raw data using Cutadapt [25]. For bacteria, parameters for filtering and trimming the reads to minimize error rates were selected using FIGARO [26]. Trimming sites 236 and 173 for forward and reverse reads, respectively, were selected and maximum number of expected errors was set to 1. For archaea, trimming sites 220 and 200 and maximum number of expected errors of 2 and 5 for forward and reverse reads, respectively, were selected. One replicate of sample C3 in the archaea dataset had few (544) reads, and was therefore removed from the subsequent analyses. Taxonomy was assigned to the sequences using the Silva reference database training set (version 138) [24,27]. The package phyloseq (version 1.32.0) was used to organize the data and visualize relative abundances of the sequences.

Bacterial communities were further analyzed using non-metric multidimensional scaling (NMDS) in R to visualize the similarity between samples from different reactors in terms of bacterial community structure, plotted together with concentrations of macromolecules (protein, fat, sugars, and lignin). To generate the distance matrix, the ordinate function in the package phyloseq was used with the Bray-Curtis calculation method. Plots were generated using the function ggplot in package ggplot2 (version 3.3.3) and macromolecule concentrations were fitted to the data using the function envfit in package vegan (version 2.5.6). Ellipses representing the 50% confidence intervals for the most abundant phyla (represented by more than three data points in the NMDS plot) were included in the NMDS plot showing the phyla, assuming multivariate normal distributions.

2.5. Calculation of retention time distribution

The retention time distribution (RTD) for an ideal CSTR was used here (eq. (A.1)) to indicate how the probability distribution of retention of material in the system changed when CSTRs were connected in series [28]. The RTD for a combination of CSTRs in series, with the HRTs used in this experiment (Table 1), was obtained using eq. (A.2) [28]. The probability of material leaving the system within the total HRT was calculated based on eq. (A.3), using the integrate function in R.

2.6. Data analysis

Statistical analyses on the results were carried out in R programming language (version 4.0.2). One sample *t*-test with confidence level 95% was used for pair-wise comparisons of daily specific methane production between the three different systems and between the three different

first-step reactors. One-way ANOVA with confidence level 95% was used for comparisons of values in the different reactors: macromolecule concentrations, NH_4^+-N- and NH_3-N-concentrations, pH, VS content, VS reduction, RMP, and methane production rates from different substrates. The t.test function was used for t-tests and the linear model (lm) followed by the anova function was used for ANOVA. Pairwise comparisons between reactors were made using the emmeans function in package emmeans (version 1.5.2.1). In case of heteroscedasticity, values were log-transformed before performing the ANOVA.

3. Results

3.1. Effects of serial AD on methane production efficiency

The reactors were operated for 203 days in total and during this time fed semi-continuously with food waste once a day. Measurements presented here were made after all systems had reached three complete HRTs (from day 174), unless otherwise stated. Specific methane production (SMP) for the complete systems was within the range 489–517 L CH_4/kg VS (Table 2, Figure B1). During the course of the experiment the reactors remained stable, both in terms of gas production and process parameters. VFA concentrations were below the detection limit in all reactors throughout the experiment. The average daily SMP was significantly higher ($p < 0.05$) in the serial systems than in the single reactor, reaching 5.7% and 5.2% higher in the two-step and three-step system, respectively (Fig. 1). However, there was no significant difference in SMP between the two serial systems ($p = 0.43$). Most of the methane produced from each serial system was generated in the first-step reactor (96% and 92% in B1 and C1, respectively) and small contributions were obtained from the second and third steps (Table 2). Comparing the SMP in the first-step reactors (Table 2), B1 had significantly higher methane production than both A1 and C1 ($p < 0.05$). However, SMP did not differ significantly between A1 and C1 ($p = 0.56$).

The VS reduction increased with every reactor step and was finally >80% in the two serial systems, which was a significant improvement ($p < 0.05$) in comparison with the single CSTR (79%) (Table 2). In line with this, an increase in both NH_4^+-N and NH_3-N concentrations was observed across the steps in the serial systems (Table 2). The NH_4^+-N and NH_3-N concentrations in the final digestate from the serial systems (B2 and C3) were significantly higher than those in the final digestate from A1 ($p < 0.05$).

Among the reactors, the highest methane content in the gas was obtained in reactor B1 (65%) (Table 2). The highest methane content in the three-stage system was obtained in reactor C2 (64%). Lower methane content, of around 59%, was obtained in the last-step reactors (B2 and C3).

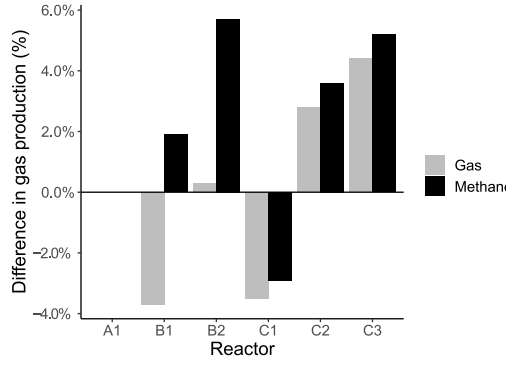

Fig. 1. Difference (%) in average methane and gas production from each serial reactor compared with reactor A1. Reactor A1 was operated as a single reactor, and B1 and B2 and C1, C2, and C3 in two and three steps in series, respectively. The accumulated differences for each system are shown.

3.2. Retention time distributions with serial reactors

To evaluate the probability of material leaving the reactor system within the HRT, the theoretical RTD was calculated for each system. The RTD values obtained illustrated the probability distribution of a pulse of an inert material added at time $t = 0$ to a reactor system with one, two, or three serial ideal CSTRs (Fig. 2). With a single reactor, the probability of material spending less time in the reactor than the HRT was 63%. In the two-step system with the same total HRT this probability was reduced to 60%, while replacing the single reactor with the three-step system reduced the probability further, to 58%. Thus increasing the number of reactors within the system, without changing the total HRT, increased the average time material spent in the reactor system.

3.3. Macromolecule concentrations and RMP in digestate

Concentrations of fat, lignin, and sugars (xylose, mannose, glucose, galactose, and arabinose) were measured in digestate from all reactors (sampled at day 95 and 193) (Figure C1). Measured sugar was assumed

Table 2
Analytical data for the digestate and gas produced from the different test reactors. Reactor A1 was operated as a single reactor, B1 and B2 as a two-step serial system, and C1, C2, and C3 as a three-step serial system. Letters indicate statistical significance (values with different letters differ significantly ($p < 0.05$)). Columns are compared separately.

Reactor	NH_4^+-N (g/kg)*	NH_3-N (mg/kg)*	pH*	VS in digestate (%)*	VS reduction (%)*	Methane content (%)**	SMP complete system (L CH_4/kg VS)**	SMP first-step reactors (L CH_4/kg VS)**	Methane production (%)***
A1	3.0 (0.1)^{a}	287 (9.5)^{a}	7.8 (0.0)^{ab}	3.2 (0.0)^{a}	78.9 (0.3)^{a}	61.8 (0.5)	489 (24)^{a}	489 (24)^{a}	100.0
B1	2.7 (0.1)^{b}	261 (5.6)^{ab}	7.8 (0.0)^{ab}	3.4 (0.0)^{b}	77.3 (0.3)^{b}	65.4 (0.2)	517 (20)^{b}	499 (18)^{b}	96.3
B2	3.4 (0.1)^{ce}	395 (6.4)^{c}	7.9 (0.1)^{ac}	2.8 (0.1)^{c}	81.0 (0.4)^{c}	59.9 (1.1)		ND	3.7
C1	2.4 (0.0)^{d}	200 (24.8)^{b}	7.7 (0.0)^{b}	3.8 (0.1)^{d}	73.4 (0.4)^{d}	62.2 (0.2)	515 (14)^{b}	475 (14)^{a}	92.3
C2	3.3 (0.1)^{c}	384 (6.9)^{c}	8.0 (0.1)^{c}	3.0 (0.0)^{e}	79.1 (0.3)^{a}	64.2 (0.5)		ND	6.2
C3	3.5 (0.1)^{e}	543 (58.5)^{d}	8.0 (0.1)^{c}	2.8 (0.1)^{c}	80.4 (0.7)^{c}	59.3 (1.8)		ND	1.5

*Mean values based on three measurements. Standard deviation within brackets.
**Mean values based on data from day 174–203. Standard deviation within brackets.
***Relative methane production, indicating how much each reactor contributed to the total amount of methane produced from each system.ND = not determined.

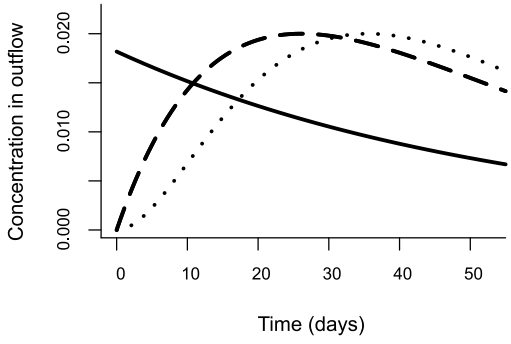

Fig. 2. Retention time distribution (RTD) for the three systems with one (solid line), two (dashed line), or three (dotted line) reactors in series.

to be bound as cellulose and hemicellulose. No significant differences between the reactors in concentrations of fat, lignin, or bound sugar were detected ($p \geq 0.05$). The measured concentrations were within the range 8–14, 3–5 and 2–3 g/kg for lignin, sugar, and fat, respectively (Figure C1). Among the measured macromolecules, the highest concentrations were obtained for protein (12–19 g/kg), with the lowest and highest concentration in reactor C3 and C1, respectively (Fig. 3). Comparison of protein concentrations (measured on day 148, 169, and 199) in digestate from the three different systems showed a significant ($p < 0.05$) improvement in protein degradation with the serial systems (Fig. 3). Overall, 22% and 23% lower protein concentration was obtained in the two- and three-step system, respectively, in comparison with reactor A1.

As an indicator of degradation efficiency, RMP in the digestate was measured, *i.e.*, the accumulated amount of methane produced from digestate during 25 days of incubation (Fig. 4, Figure C2). The highest RMP was obtained for digestate from reactor C1 and the lowest for digestate from C3, although levels in C3 were not significantly different from those in reactor B2 ($p = 0.81$).

Based on the macromolecule analysis, theoretical RMP in the digestate was calculated, assuming that the sugars, protein, and fat (not lignin) contributed to the RMP and assuming theoretical methane potential for these compounds of 415, 496, and 1014 L CH_4/kg

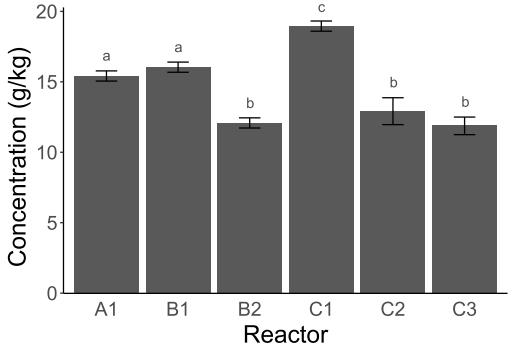

Fig. 3. Protein concentrations in the different reactor systems studied: A1 (single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series). All reactor systems had the same total organic loading rate and hydraulic retention time (values with different letters differ significantly ($p < 0.05$)).

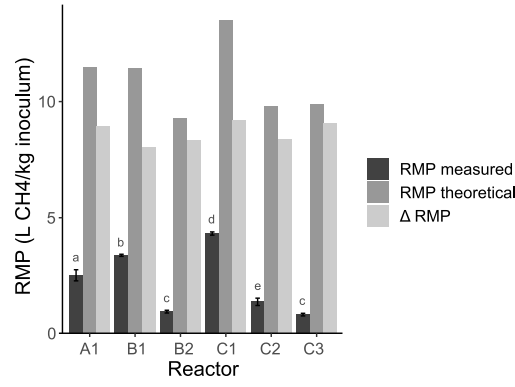

Fig. 4. Residual methane potential (RMP) in digestate from a single reactor (A1), reactors in a two-step system (B1 and B2), and reactors in a three-step system (C1, C2, and C3), after 25 days of incubation, measured in triplicate. Error bars represent standard deviation for the measured RMP. Theoretical RMP is based on sugar, protein, and fat concentrations in the digestates, ΔRMP represents the difference between theoretical and measured RMP. Letters indicate statistical significances (values with different letters differ significantly ($p < 0.05$)).

respectively [29]. A comparison between the theoretical and measured RMPs showed that less than 35% of the theoretical methane potential was reached in all digestates (Fig. 4). The difference between the theoretical and measured RMP was around 8–9 L CH_4/kg digestate for all samples (Fig. 4).

3.4. Conversion of protein, fat, and cellulose in digestate

Methane production from digestate after addition of protein, cellulose, and fat was evaluated in batch tests. To compare the degradation rates in the different reactors, a rate coefficient was calculated using the cut-off values (as described in section 2.3) 167 mL, 155 mL, and 442 mL for protein, cellulose, and fat, respectively (solid line, Figure C3).

For all substrates, the digestate from the first-step serial reactors (B1 and C1) had significantly higher conversion rate than the digestate from the second- and third-step reactors ($p < 0.05$) (Table 3). The most pronounced difference was seen for protein degradation, which was much higher in the tests started with digestate from C1. In general, the rates for fat and protein decreased in the order: C1 > B1 > A1 > C2 > B2 > C3. For cellulose, however, the digestate from A1 had a relatively slow conversion rate and behaved more like that from the second-step reactors (B2 and C2).

Table 3
Methane production rates from fat, protein, and cellulose substrates added to digestate from a single reactor (A1), reactors in a two-step system (B1 and B2), and reactors in a three-step system (C1, C2, and C3). The methane production rates are given as the average volume per day (mL CH_4/(g VS d)) until a cut-off time-point was reached. Methane production rates with different letters differ significantly ($p < 0.05$). Rate coefficients for each substrate are compared separately.

Reactor	Fat	Protein	Cellulose
A1	113.6 (11.6)^{a}	96.2 (3.3)^{a}	35.7 (6.4)^{ab}
B1	136.4 (38.7)^{a}	123.6 (46.4)^{a}	70.7 (2.3)^{c}
B2	45.6 (2.0)^{b}	31.9 (7.0)^{bc}	29.5 (1.6)^{a}
C1	133.9 (37.6)^{a}	208.8 (34.9)^{d}	51.5 (0.4)^{e}
C2	67.5 (6.7)^{b}	50.8 (2.8)^{b}	39.3 (0.6)^{b}
C3	42.1 (3.0)^{b}	21.4 (0.4)^{c}	23.3 (0.4)^{d}

3.5. Microbial community structure

The sequence analysis of 16S rRNA genes indicated that the archaeal community in all reactors was almost entirely dominated by the genus *Methanosarcina* (Fig. 5a). *Methanoculleus* and *Methanobacterium* were also observed at relative abundance >1% in some of the reactors. *Methanobacterium* increased in relative abundance in the second and third reactor steps in comparison with the first step. The bacterial community in the reactors was dominated mainly by the phyla Actinobacteriota, Bacteroidota, Cloacimonadota, and Firmicutes (Fig. 5b). Caldatribacteriota, Synergistota, and Thermotogota were also represented in some of the reactors, but at lower relative abundances.

The relative abundance of Bacteroidota was high in reactors B1 and C1, and decreased abruptly in the next reactor in the system. The most abundant genus within the Bacteroidota was H1, classified as a genus within the family *Paludibacteraceae* (Table 4). H1 had 23% and 31% relative abundance in B1 and C1, respectively, and this decreased to 1% and <1% in B2 and C2, respectively. Other groups showing a trend for slightly decreasing abundance over reactor steps were the family *Rikenellaceae* (phylum Bacteroidota) and genus *Sedimentibacter* (phylum Firmicutes) (Table 4).

For many taxa, a trend for increasing relative abundance over reactor steps was observed (Table 4). A representative of the phylum Bacteroidota, *Proteiniphilum*, was detected in all systems, but in highest relative abundance in reactor A1 (10%). In the serial reactor systems, the relative abundance of this genus was lower, but still with an increasing trend between reactors. A similar trend was seen for *Gallicola*, one of the dominant genera within Firmicutes, which accounted for almost 35% of the sequences in reactor A1. In the serial systems it had lower relative abundance, but it increased between reactors in both the two- and three-step systems. Other groups showing an increasing trend over reactor steps were MBA03 within the class *Limnochordia* (phylum Firmicutes), DTU014 within the class *Incertae Sedis* (phylum Firmicutes), the families *Dethiobacteraceae* and *Erysipelotrichaceae* (phylum Firmicutes), *Fastidiosipila* (phylum Firmicutes), and *Acetomicrobium* (phylum Synergistota).

One of the most highly abundant phyla in all reactor systems was Cloacimonadota, which was represented mainly by two groups; the W5 genus within the family *Cloacimonadaceae* and the W27 family within the order Cloacimonadales. W27 had relative abundance around 25% in both reactors in the two-step system and also in reactor C2. Group W5 was present at lower abundances, with the highest abundance in reactor A1, where it accounted for 4% of the sequences.

Although the two-step system in this study was operated in the same manner (but a down-scaled version) as the co-digestion biogas system used as the source of inocula, these systems were not similar in terms of bacterial community structure, suggesting a downscaling effect. Instead, the single reactor (A1) showed the highest bacterial community similarity with the original inocula from the co-digestion plant (Figure D1). Actinobacteriota was present at high relative abundances in the serial reactors, but at abundances <1% in the original inocula and in the single-step system (Table 4). The genus *Actinomyces* within Actinobacteriota was highly abundant, especially in the three-step system, comprising up to 44% of the sequences in reactor C3. Another effect of downscaling was a clear decrease in relative abundance of Thermotogota, mainly represented by *Defluviitoga tunisiensis* (Table 4).

A NMDS plot showing the bacterial phyla was created to look for clusters in relation to macromolecule concentrations (Fig. 6). The five most abundant phyla were marked with ellipses indicating the multivariate normal distribution with a confidence interval of 50%. A positive correlation was observed between high protein concentration and Actinobacteriota, Bacteroidota, and Firmicutes, and a trend for a correlation was seen between high fat concentration and a high relative abundance of Actinobacteriota and Bacteroidota. Trends for lower protein, fat, and sugar concentrations with higher abundance of Cloacimonadota, and lower lignin and sugar concentrations with higher abundance of Synergistota, were observed (Fig. 6).

4. Discussion

4.1. Degree of degradation and methane production

The SMP from the reactor systems (489–517 L CH_4/kg VS) was in line with previously reported values of biomethane potential for food waste [9,10]. In this study, serial digestion increased methane production by more than 5% in both the two- and three-step systems compared with a single reactor with the same total HRT (Fig. 1). This represents an increase with >25 L CH_4/kg VS, which would give a considerable increase in methane yield on industrial scale. For example, with the system used as the basis for this study design (total volume 17,100 m^3, total OLR 2.43 kg VS/(m^3 d)), a 5% increase in SMP would increase the volumetric production with >1,000 m^3 CH_4/d (corresponding to >9.8 MWh/d [2]). Previous evaluations of serial digestion in comparison with single CSTRs have found increases in gas production of around 8–15% [12,30]. The setup in those studies was similar to that in the present study, but using agricultural waste with high lignocellulose content as substrate, which might benefit more from serial digestion than relatively easily degradable food waste. The improvement in substrate conversion obtained by serial digestion has previously been attributed to the prolonged average

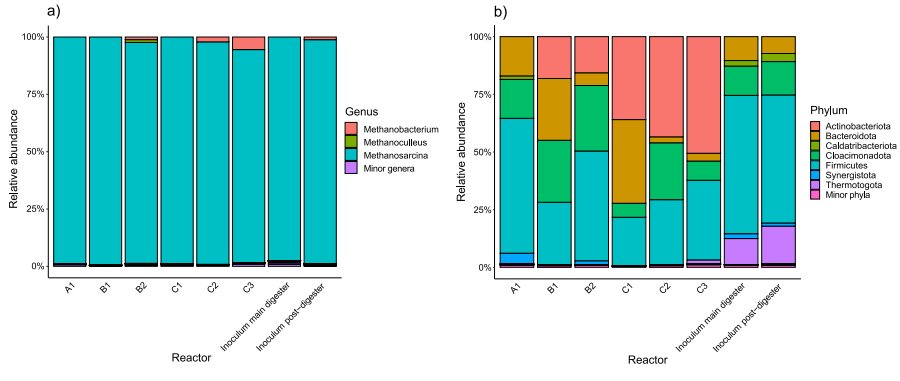

Fig. 5. Relative abundances of a) archaea at genus level and b) bacteria at phylum level, based on 16S rRNA-gene sequences in digestate from reactor A1 (operated as single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series), and in the two inocula. 'Inoculum main digester' was used for inoculation of reactors A1, B1, C1, and C2. 'Inoculum post-digester' was used for inoculation of reactors B2 and C3.

Table 4
Relative abundances (%) of bacterial genera in digestate from the different reactors and the starting inocula. Genera with abundance >1% in at least one of the samples are given. Taxa with abundance <1% are grouped within "Minor taxa". Reactor A1 was operated as a single reactor, and B1 and B2 and C1, C2, and C3 in two and three reactor steps in series, respectively. 'Inoculum main digester' was used for inoculation of reactors A1, B1, C1, and C2. 'Inoculum post-digester' was used for inoculation of reactors B2 and C3.

Phylum	Class	Order	Family	Genus	A1	B1	B2	C1	C2	C3	Inoculum main digester	Inoculum post-digester
Actinobacteriota	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	0.7	16.7	13.9	32.7	38.5	44.0	0.1	0.2
Actinobacteriota	Actinobacteria	NA	NA	Actinobacteria _cl	0.0	1.4	1.8	2.4	4.9	6.3	0.0	0.0
Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	H1	5.6	23.0	1.3	31.2	0.2	0.4	1.0	0.3
Bacteroidota	Bacteroidia	Bacteroidales	Dysgonomonadaceae	Proteiniphilum	10.1	1.9	2.7	0.5	0.8	1.6	1.5	3.7
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut_group	0.0	0.5	0.2	1.8	0.0	0.1	1.8	0.2
Bacteroidota	Bacteroidia	Bacteroidales	Dysgonomonadaceae	Dysgonomonadaceae _fa	0.1	0.0	0.0	0.2	0.0	0.0	3.8	1.1
Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	Paludibacteraceae _fa	0.7	1.0	0.1	1.7	0.0	0.0	0.2	0.0
Bacteroidota	Bacteroidia	Sphingobacteriales	Lentimicrobiaceae	Lentimicrobium	0.4	0.0	0.5	0.2	1.1	1.2	1.2	1.6
Caldatribacteriota	Caldatribacteriia	Caldatribacteriales	Caldatribacteriaceae	Candidatus_Caldatribacterium	1.4	0.1	0.8	0.0	0.2	0.0	2.4	3.5
Cloacimonadota	Cloacimonadia	Cloacimonadales	Cloacimonadaceae	W5	4.0	1.0	1.2	0.3	0.5	0.0	4.1	4.0
Cloacimonadota	Cloacimonadia	Cloacimonadales	Cloacimonadaceae	Cloacimonadaceae _fa	0.1	0.8	0.7	0.8	1.2	0.0	0.0	0.0
Cloacimonadota	Cloacimonadia	Cloacimonadales	W27	W27 _fa	12.7	25.1	26.5	4.8	22.9	8.2	8.6	10.2
Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Peptostreptococcales-Tissierellales_fa	Alkaliphilus	0.3	0.0	0.0	0.0	0.0	0.0	1.0	1.7
Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Peptostreptococcales-Tissierellales_fa	Gallicola	34.7	5.2	17.3	5.6	9.6	15.8	7.6	11.7
Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Sedimentibacteraceae	Sedimentibacter	0.3	0.8	0.1	0.7	0.1	0.0	5.4	1.9
Firmicutes	Clostridia	Caldicoprobacterales	Caldicoprobacteraceae	Caldicoprobacter	2.0	4.4	3.6	3.4	5.2	1.9	2.5	1.3
Firmicutes	Clostridia	Clostridia_or	Hungateiclostridiaceae	Fastidiosipila	5.9	4.9	6.8	3.0	3.7	3.9	11.8	8.3
Firmicutes	Clostridia	Clostridia_or	Hungateiclostridiaceae	HN-HF0106	0.1	0.3	0.0	0.8	0.0	0.0	2.8	1.2
Firmicutes	Clostridia	Peptostreptococcales-Tissierellales	Anaerovoracaceae	Anaerovoracaceae _fa	0.4	0.4	0.3	0.4	0.0	0.1	1.4	1.2
Firmicutes	Limnochordia	MBA03	NA	MBA03 _or	5.4	5.8	8.2	3.0	4.9	6.3	9.3	8.5
Firmicutes	Limnochordia	NA	NA	Limnochordia _cl	0.2	0.0	0.3	0.7	0.0	0.0	2.3	0.5
Firmicutes	Incertae_Sedis	DTU014	NA	DTU014 _or	4.8	2.9	7.9	1.0	2.7	4.6	8.1	11.2
Firmicutes	Dethiobacteria	Dethiobacterales	Dethiobacteraceae	Dethiobacteraceae _fa	3.0	0.5	1.9	0.1	0.0	0.4	1.3	2.2
Firmicutes	Bacilli	Erysipelotrichales	Erysipelotrichaceae	Erysipelotrichaceae _fa	0.2	0.9	1.0	0.6	0.8	1.1	0.0	0.0
Synergistota	Synergistia	Synergistales	Synergistaceae	Acetomicrobium	4.7	0.7	1.6	0.0	0.1	0.4	2.0	1.2
Thermotogota	Thermotogae	Petrotogales	Petrotogaceae	Defluviitoga	0.5	0.0	0.3	0.0	0.7	1.7	11.3	16.2
Minor taxa					1.6	1.8	0.9	3.7	2.0	2.1	8.8	7.8

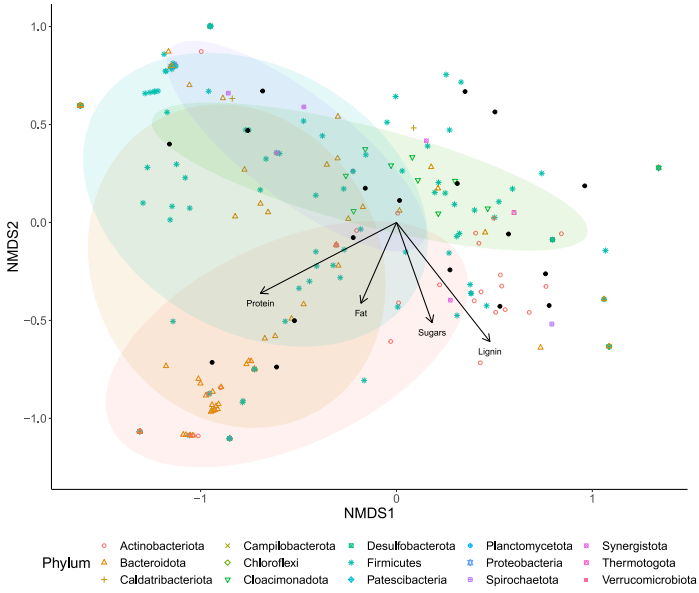

Fig. 6. Non-metric multidimensional scaling (NMDS) plot of bacterial phyla in digestate samples from reactors A1 (single reactor), B1 and B2 (operated in series), and C1, C2, and C3 (operated in series). Arrows indicate concentrations of macromolecules protein, fat, lignin, and bound sugars (xylose, mannose, glucose, galactose, and arabinose) in the reactors. The taxa are subset to not include unidentified phyla. Ellipses mark a multivariate normal distribution with 50% confidence interval for phyla with >3 points in the plot. Stress = 0.097.

retention of organic matter in a serial reactor system compared with a single reactor [30,31]. As shown by the theoretically calculated RTDs (Fig. 2), increasing the number of reactors in series, without changing the total HRT, delayed the exit of material from the system. In theory, a system with an infinite number of serial reactors and a sufficient total HRT could give complete degradation. In line with this, we observed a slightly greater VS reduction in the serial systems than in the single reactor (Table 2). Moreover, protein concentration was more than 20% lower after the serial systems compared with the single reactor (Fig. 3), suggesting that for food waste with recalcitrant protein, a setup with serial reactors is beneficial. Less efficient degradation in the single reactor (A1) was also demonstrated by higher RMP compared with the last steps in the serial systems (B2 and C3). Interestingly, comparisons of theoretical RMP, estimated from the macromolecule concentrations, with measured RMP in the digestates showed that approximately the same amount of undigested material remained in all digestates after the residual methane was removed (Fig. 4). This may indicate that all remaining organic material was microbial biomass that was similar in all reactors. If this was the case, the degradation was complete after the RMP test and the low RMP from B2, C2, and C3 was due to a low amount of remaining substrate, and not inactive microbial communities. However, the comparison between measured and theoretical RMP should be interpreted with care since the theoretical values were based on macromolecule concentrations, measured at a different time point as compared to the RMP. Although serial digestion improved protein degradation and VS reduction, lowered RMP in the digestates, and increased methane production, no significant differences in these parameters were observed when comparing the two serial systems. Thus for the process studied, two reactors in series were sufficient to reach higher productivity.

An important advantage of serial systems is increased average retention time of the material in the total system, but slightly higher methane production was obtained already in the first reactor in the two-step system compared with the single reactor ($p = 0.046$), despite the shorter HRT. Moreover, the SMP in reactor C1 did not differ significantly from that in A1, indicating that, compared with the single reactor, the system could be 'pressured' more by shortening the HRT and increasing the OLR, without causing disturbances and a decrease in methane production. This has previously been shown to be feasible and a way of optimizing the productivity of biogas processes [32]. The increase in OLR and shortened HRT in B1 were even slightly favorable for the SMP, possibly as a result of the lower NH_4^+-N concentration obtained in this reactor compared with A1 (Table 2).

4.2. Hydrolysis of macromolecules and microbial community structure

Methane production rates from different macromolecules indicated significantly faster methane production with inocula from the first-step reactors compared with the subsequent steps (Table 3). The first-step reactors all had the lowest NH_4^+-N relative concentration and were fed with fresh substrate at a higher organic load than subsequent reactors in the series, which might have contributed to more active microbial communities in these digestates. As regards macromolecule concentrations in the digestate, there were no significant differences in the concentrations of sugars, lignin, and fat between the reactors. It is probable that the majority of hydrolysis of these compounds took place already in the first reactor in the serial systems. Slightly lower methane content in the gas from the first-step reactor with the shortest HRT (C1) relative to the subsequent reactor (C2) (Table 2) could also be a sign of higher hydrolytic activity in the first step since this increases the CO_2 content in the gas [14]. The relative abundance of *Paludibacteraceae* H1 decreased notably after the first steps, suggesting involvement in the initial hydrolysis and acidogenesis steps in the first reactor. Members of *Paludibacteraceae* have previously been reported to utilize various sugars [33], starch [23], and potentially cellulose [34]. Although the differences in fat concentrations between the reactors were not statistically significant, an interesting trend was observed for Cloacimonadota in the NMDS plot, with higher relative abundance of this phylum at lower fat concentrations (Fig. 6). Members within this phylum may be involved in digestion of long-chain fatty acids [35], which might explain the comparatively higher relative abundance of Cloacimonadota family W27 in reactors B1, B2, and C2 (Table 4).

One of the most abundant genera in the serial systems was *Actinomyces*. The most abundant sequence classified as *Actinomyces* had 99.6% similarity with *Gleimia europaea* (NCBI, RefSeq Genome database), a sugar-fermenting bacterium [36,37] previously observed in biogas systems processing food waste [38]. In the present study, higher relative abundance of Actinomyces was observed in reactors with shorter HRT. This is in line with Feng et al. [38], who observed a sharp increase in relative abundance of *Actinomyces* upon downscaling from industrial to laboratory scale, suggesting that the genus was favored by the shorter retention time applied in the laboratory-scale reactors. Actinobacteria has been shown to be an important phylum in degradation of lignocellulosic material in compost, expressing a wide range of lignin-degrading enzymes [39]. Jaenicke et al. [40] also observed genes belonging to Actinobacteria in a biogas community degrading agricultural waste. Whether *Actinomyces* is involved in fiber degradation under the conditions maintained in reactors in the present study is unknown, but the NMDS analysis indicated higher abundance of Actinobacteriota with higher lignin and sugar concentrations (Fig. 6).

Defluviitoga, one of the most abundant genera (11–16%) in the inocula, is known for its ability to degrade carbohydrates [41]. It is a thermophilic genus but with the ability to grow at temperatures down to 37 °C [41], which explains its prevalence in the present study. In contrast to genus *Actinomyces,* the relative abundance of *Defluviitoga* decreased (<2%) in all reactors as compared to the inocula, suggesting a downscaling effect (Table 4). Effects of the microbial community by downscaling have been shown before and suggested to be caused by changes in feeding regimes [42,43]. Still, previous work on lab-scale processes have indicated that such changes not necessarily lead to differences in function and performance of the process [32,43].

In previous studies, inefficient protein degradation in AD has been reported at certain conditions, such as low pH [6] and high carbohydrate concentration [44,45]. It has been noted that proteins are not degraded in the acidogenic stage in phase-separated processes [15]. Nevertheless, Breure et al. [44] suggested that a phase-separation reactor setup would be beneficial for protein degradation, since it would allow carbohydrates to be acidified in a separate chamber and protein degradation could take place in the methanogenic chamber. No phase separation was used in the present study, but carbohydrate hydrolysis and acidogenesis still appeared to take place mainly in the first-step reactors, thereby creating a favorable environment for protein degradation in the subsequent reactors, with slightly higher pH and lower carbohydrate concentrations. A stepwise decrease in protein concentration across the serial systems (Fig. 3) and an increase in relative abundance of the potentially proteolytic genera *Proteiniphilum, Fastidiosipila*, and *Acetomicrobium* over reactor steps was observed. *Proteiniphilum,* within Bacteroidetes, has been described to degrade peptides and also complex carbohydrates [46–48]. *Fastidiosipila,* within Firmicutes, has previously been coupled to proteolytic activity in pure cultures and in AD systems [15,49,50]. The type species of *Acetomicrobium* (*Acetomicrobium flavidum*), a genus within Synergistota, can hydrolyze starch, casein, and tributyrin [51]. The relative increases in these groups in the second and third reactors in this study could also be a result of relatively high NH_3 tolerance, *e.g.*, it has been shown that *Acetomicrobium* can grow at high NH_3 levels [52].

Although an important proportion of protein degradation appeared to have occurred by later reactor steps, methane production from protein measured in the substrate conversion test was especially fast in reactors B1 and C1 (Figure C3, Table 3). The protein degradation in these reactors might have been performed by members within the phylum Bacteroidota classified as *Paludibacteraceae* (using the Silva database), which was highly abundant in both reactors. The most abundant of these sequences (relative abundance 20% in B1 and 25% in C1) had 100% sequence similarity with uncultured *Porphyromonadaceae* (NCBI, Nucleotide collection (nr/nt) database), whose type genus *Porphyromonas* is described as being proteolytic [53]. High relative abundance of Bacteroidota was also positively correlated with protein concentration according to the NMDS analysis (Fig. 6), possibly indicating involvement of this phylum in protein degradation in the study reactors.

In summary, one of the main trends in terms of links between microbial community structure and degradation of molecular structures was a sharp decrease in the abundance of family *Paludibacteracea* in the second-step reactors relative to the first steps. This decrease was potentially related to a comparably higher reduction of readily degradable macromolecule structures, both proteins and carbohydrates, in the first as compared to the second steps. On the other hand, there was a slight increase over reactor steps in the abundance of the potentially proteolytic genera *Proteiniphilum, Fastidiosipila*, and *Acetomicrobium*, possibly linked to the step-wise decreasing protein concentrations. Additionally, a link between Cloacimonadota family W27 to fat degradation was indicated.

4.3. Effect of increasing ammonia concentrations over reactor steps

The NH_3-N level is an important parameter in biogas processes, as methanogens are known to be inhibited at concentrations around 200–400 mg/L in mesophilic processes [54]. The toxicity depends on the process parameters, as the equilibrium between NH_3 and NH_4^+ is driven towards higher NH_3 levels at increasing temperature and pH. The NH_4^+-N concentrations were kept at relatively low levels in the first reactors (A1, B1 and C1), constantly diluted with fresh substrate, and accumulation was observed in the later reactor steps as more protein was degraded (Table 2). The pH also increased over reactor steps, creating a more toxic environment for ammonia-sensitive microorganisms in the last reactor steps compared with the first. A great advantage with the serial reactor setup used here was that the majority of the methane from each system was produced in the first reactor step (Table 2), separately from accumulation of NH_3-N. Nevertheless, although the NH_3-N concentrations were at potentially inhibiting levels in the last reactors (Table 2), the VFA concentrations did not increase, which is normally a sign of process disturbance caused by ammonia inhibition [54]. However, the OLR in the reactors with the highest NH_3-N levels was low and the material was represented by slowly degradable recalcitrant material, which might have reduced the risk of VFA accumulation.

As the NH_3-N concentration increased, there was also a slight change in methanogen community structure (Fig. 5a). The relative abundance of the hydrogenotrophic genera *Methanoculleus* and especially *Methanobacterium* increased in the later reactors in the serial systems, where the ammonia levels were higher. In general, hydrogenotrophic methanogens are more tolerant to high ammonia levels than acetoclastic species [54]. Although the abundance of hydrogenotrophic methanogens increased slightly with reactor steps, *Methanosarcina* was still the predominant genus in all reactors. This is a methanogen with a wide substrate range that can produce methane from *e.g.*, acetate, methanol, and CO_2 and H_2 [55]. The *Methanosarcina* genus is also known to be tough and have a high tolerance to stress factors such as high ammonia, low pH or high OLR [56].

The hydrogenotrophic methanogens are important to enable syntrophic oxidations of various organic acids, which is unfavorable at high partial pressure of H_2 [54]. A trend seen in our systems was for potential syntrophic acetate-oxidizing bacteria (SAOB) to increase in relative abundance with reactor steps, in line with the increase in hydrogenotrophic methanogens. This was possibly linked to increasing NH_3-N levels, since the syntrophic acetate oxidation (SAO) pathway for acetate degradation can often compete better with acetoclastic methanogenesis at high NH_3-N concentrations [54]. SAOB are also slow-growing, and could therefore also have been favored by the long total retention time in the last digesters in the serial systems. The orders DTU014 and the NH_3-tolerant MBA03 and family *Dethiobacteraceae* within Firmicutes, groups with suggested SAO activity [34,57,58], followed the trend for increasing abundance over reactor steps.

In summary, although the methanogenic community was dominated by genus *Methanosarcina*, hydrogenotrophic methanogenic genera (*Methanoculleus* and *Methanobacterium*) increased in relative abundance over reactor steps, possibly due to increasing NH_3-N concentration. A similar trend was observed for the suggested SAO bacterial taxa, DTU014, MBA03, and *Dethiobacteraceae*.

5. Conclusions

Although food waste contains relatively easily degradable material that is digested soon after it has been added to the process, this study clearly showed that serial digestion significantly can improve degradation of recalcitrant structures. Analysis of process performance showed that the serial system increased the methane yield by >5% compared with a single reactor, but with no significant differences between the two-step and three-step reactor systems. Serial systems also improved the degree of degradation, indicated by significantly lowered RMP, which both suggested a more efficient substrate utilization and proposes lowered risk for residual methane emission from the digestate.

Among macromolecules, proteins showed the highest reduction, with concentrations >20% lower after the serial systems compared with the single reactor. Microbiological analysis showed adaptations to the changing environment and substrate availability across the serial systems as well as links between the relative abundances of bacterial phyla with suggested hydrolytic and acidogenic function, with macromolecule concentrations and degradation rates. Moreover, the methanogenic community changed in relation to the NH_3-N concentration, also linked to relative abundances of potential SAO bacteria.

The observed positive effects of the serial setups could have derived from comparatively low NH_3-N concentrations in the first reactor step, lowering the risk of ammonia inhibition and associated process disturbances in the reactors where the majority of the methane is produced. The serial setup also had the advantage of retaining recalcitrant structures in the system for a longer time on average, increasing the probability of degradation and thus leading to improved yield and reduced levels of residual methane production from the digestate.

Author contributions

Ebba Perman: Formal analysis, Investigation, Writing – Original Draft, Visualization.

Anna Schnürer: Supervision, Conceptualization, Funding acquisition, Writing - Review & Editing. Annika Björn: Funding acquisition, Investigation, Writing – Review and Editing.

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Jan Moestedt: Conceptualization, Investigation, Resources, Data curation, Funding acquisition, Formal analysis, Supervision, Writing - Review & Editing.

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Data availability

16S rRNA-gene sequence data related to this article can be accessed at BioProject accession number PRJNA781440 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA781440), at Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI).

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Appendix A. Supplementary data

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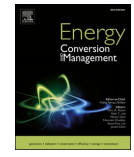

Comparative study of high-solid anaerobic digestion at laboratory and industrial scale – Process performance and microbial community structure

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ABSTRACT

High-solid anaerobic digestion (HSD) for biogas production, compared with wet digestion, is attracting interest due to advantages such as reduced fresh water usage, improved digestate quality and potential for high organic loading rates. However, the underlying processes are not well described and evaluated for HSD. In this study, two laboratory-scale reactors (46 L) of plug-flow type were designed to simulate an industrial-scale HSD process co-digesting food waste, agricultural waste and garden residues under thermophilic conditions. Performance of the laboratory-scale HSD process under stable and disturbed conditions was compared with that in industrial-scale reactors. The results showed that the laboratory- and industrial-scale processes had similar efficiency (93 %) and VS-reduction (43 % and 41 %, respectively) and relatively similar specific methane production (339 and 366 NL CH_4/kg VS, respectively). Results from tracer studies combined with chemical analyses showed no phase-separation or plug-flow behaviour along the horizontal axis in either laboratory- or industrial-scale reactors, indicating a need for further process optimisation. Analyses of microbial community structure showed high similarity between laboratory- and industrial-scale, but with some differences caused by downscaling. During the experiment, the laboratory- and industrial-scale processes both showed signs of disturbance, *i.e.* VFA accumulation at NH_4^+-N levels > 4 g/L, accompanied by a shift in microbial community structure at both scales, with significant increases in relative abundance of *e.g.* genera *Defluviitoga* and *Methanothermobacter*. In conclusion, this study confirmed the validity of simulating HSD at laboratory scale, thus providing valuable insights into biogas production from high-solid substrates, both in laboratory- and industrial-scale processes.

1. Introduction

Anaerobic digestion (AD) is a process in which organic materials are degraded and converted into biogas, a renewable energy source [1,2]. The residual material from the process, *i.e.* the digestate, has a high content of plant-available nutrients and can be used as biofertiliser [3,4]. Industrial-scale AD is widely used for treatment of organic waste streams, *e.g.* sewage sludge, agricultural residues and food waste from households and industries [5]. The most commonly applied AD technology is wet digestion, where the total solids (TS) content is < 15 % [6]. This technology is well-investigated and established at industrial scale. An alternative, less commonly applied technology is high-solid digestion (HSD), which typically operates with TS > 15 % [6]. HSD has several advantages over wet AD, such as lower use of fresh water for substrate dilution and the potential to use relatively high organic loading rate (OLR), and thus smaller reactor volumes in relation to input substrate [7]. Another advantage compared with wet digestion is that the digestate produced has a lower water content and higher nutrient concentration [7]. The dry nature of many agricultural wastes, *e.g.* crop residues and animal solid manure, and of food waste make these substrates suitable for digestion in HSD processes [8]. However, more research is needed to achieve high reliability and profitability at industrial scale [6,9].

Irrespective of the technology used for AD, the substrate is degraded

Abbreviations: AD, anaerobic digestion; VFA, volatile fatty acids; CSTR, continuous stirred-tank reactor; PFR, plug-flow reactor; HSD, high-solid digestion; TS, total solids; VS, volatile solids; OLR, organic loading rate; HRT, hydraulic retention time; SMP, specific methane production; RMP, residual methane production; SAOB, syntrophic acetate-oxidising bacteria.

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by a diverse community of anaerobic microorganisms in four main steps: i) hydrolysis of polymers to monomers, ii) fermentation of monomers to volatile fatty acids (VFAs) and alcohols, iii) anaerobic oxidation of fermentation products to the main methanogenic substrates acetate and H_2/CO_2 and iv) methanogenesis [1]. Conversion of VFAs is strictly dependent on a close syntrophic relationship between bacteria performing anaerobic oxidation and methanogens [1,10]. For an efficient process, all AD steps need to be synchronised. When there is a kinetics imbalance between acid production and consumption rates, VFA accumulation can occur [11].

HSD processes are generally operated at relatively high OLR [8,9,12]. Thus the risk of process disturbances and VFA accumulation is high compared with wet AD, particularly for easily degradable substrates with high protein content, such as food waste [13–16]. During degradation of proteins, ammonium-nitrogen (NH_4^+-N) is released and at high concentrations this can cause inhibition of the microbial community, especially methanogens, resulting in problems with acid conversion [17,18]. Co-digestion with more recalcitrant carbon-rich materials, *e.g.* plant materials, paper or solid manure with high levels of lignocellulose, has been observed to improve process stability [12,14,16,19–21].

In addition to the above-mentioned biological obstacles, HSD also involves some technical challenges relating to use of materials with high TS content, including issues with mixing highly viscous materials with large particle size. To tackle these issues, continuous HSD processes are often run using plug-flow reactors (PFR) [9,22], rather than the continuous stirred tank reactors (CSTR) commonly applied for wet digestion. Horizontal PFRs are fed from one end and digestate is taken out from the other end, and inside the reactor the material is pushed from the feeding inlet to the digestate outlet. In an ideal system there is no horizontal mixing or diffusion, which would create separate reaction zones along the length of the reactor, like a series of CSTRs but within the same reactor [23–25]. Operation at close-to-ideal plug-flow conditions could thereby theoretically give a process similar to multi-phase anaerobic digestion set-up, with phase separation between the hydrolysis/acidification steps at the start of the reactor and methanogenesis towards the end [9,25–27]. Modelling evaluations have suggested that minimal diffusion along the reactor can optimise process efficiency [28], although this could also be problematic in cases of local accumulation of inhibitors [29]. In the ideal case, material resides in the reactor during exactly one hydraulic retention time (HRT) [24], which eliminates short-circuiting of readily accessible organic compounds and thereby optimises substrate utilisation. However, to the best of our knowledge, only one study has previously validated plug-flow behaviour in a lab-scale AD reactor of horizontal PFR type [30]. Thus, the necessary conditions for establishing phase-separated plug-flow in terms of substrate characteristics, process operation and technology remain unclear, which highlights the importance of more investigations on PFRs. Moreover, the microbiology of PFRs and whether distinct separation of the biological steps can be achieved has been investigated in only a few previous studies [31,32].

To maximise efficiency, resource utilisation and economic performance, AD processes must be operated under optimised conditions. New optimisation strategies are best explored using laboratory-scale reactors, thereby avoiding decreases in productivity and risks of process failure in large-scale reactors during the experiments. However, this means that processes at laboratory and industrial scale must be comparable and that the laboratory results can be scaled up. Promising results in this regard were obtained by Gallert *et al.* [33], who optimised OLR at laboratory scale and successfully applied the results in a full-scale AD process. Moreover, Bouallagui *et al.* [34] found laboratory-scale evaluation to be useful for prediction of performance in a full-scale reactor treating sewage sludge and Lüdtke *et al.* [35] observed good agreement in methane production from an industrial-scale process mimicked at laboratory scale. However, these studies were all carried out in CSTR systems and when it comes to HSD systems of plug-flow type there is a lack of comparative studies at laboratory and industrial scale. Moreover, few previous studies have studied the effect of upscaling or downscaling on microbial community structure and links to process performance.

The main aim of this study was thus to investigate whether HSD processes can be operated in the laboratory with the same efficiency and yield as in an industrial-scale system, and thereby serve as a useful model for evaluation of process operation. An additional aim was to investigate plug-flow behaviour and phase separation in laboratory- and industrial-scale reactors. A thermophilic industrial-scale HSD process fed a mixture of food waste, agricultural residues and garden waste was mimicked in the laboratory. The laboratory-scale and industrial-scale reactors were both horizontal PFRs, with three sampling ports along the reactor. Process performance and plug-flow or phase separation between reactor sections were evaluated using chemical and microbiological methods and the processes were assessed under both stable and disturbed conditions.

2. Methods

2.1. High-solid laboratory- and industrial-scale reactors

High-solid digestion of mixed organic waste (see below) was evaluated at laboratory scale for 53 weeks and at industrial scale for 44 weeks. Duplicate laboratory-scale reactors (LR1, LR2) of plug-flow type with horizontal orientation were designed in-house, each with an active volume of 45.7 L (filled to 85 % of reactor height) and a length:width (L:W) ratio of 4.1 (Fig. 1). The industrial-scale system consisted of triplicate reactors of plug-flow type (RK1, RK2, RK3), each with an active volume of 2,100 m^3 (filled to 85 % of reactor height) and L:W ratio of 5.3.

The laboratory-scale reactors were stirred radially at a speed of 1 rpm by six paddle blades scraping the inside walls. Material was added through a feeding funnel and digestate was removed from the other end of the reactor (Fig. 1). The reactors had three sections (S1, S2, S3), each with a separate sampling point, and reactor material flowed freely between the sections. The industrial-scale reactors were constructed similarly, with three sections and radial stirrers (~0.5 rpm), although with slightly higher L:W ratio and different feeding inlets, using a screw instead of a funnel. Due to practical difficulties, section S2 in the industrial-scale reactors was only sampled on one occasion (microbiological analysis in week 33). Sampling was carried out weekly from S1 and S3 of both the laboratory- and industrial-scale reactors.

Digestate from the industrial-scale reactors was used as inoculum for the laboratory-scale process. Before inoculation, the digestate was sieved to reduce the particle size to 15 mm and contaminating waste (plastics etc.) was removed. The industrial scale reactors were operated under thermophilic conditions (53–56 °C) to achieve hygienisation during the digestion process and similar conditions were used also in laboratory scale (52–53 °C). Temperature sensors were placed at three positions along all reactors (Fig. 1). At both scales, digestate (without any post-treatment) was recirculated at an average ratio of 30 % of ingoing substrate mass. Recirculation in laboratory scale was carried out manually at each feeding occasion by mixing parts of the discharged digestate with the substrate fed to the feeding inlet. Volumetric HRT was ~ 42 days in laboratory-scale reactors, and HRT and OLR were calculated based on substrate input, excluding the recirculated material. To avoid volume reduction in the laboratory-scale reactors due to gas production, reduction of volatile solids (VS) was estimated to 50 % and was compensated for daily by adjusting the recirculation ratio. The laboratory-scale reactors were fed semi-continuously once per day, six days per week. The industrial-scale reactors were fed semi-continuously for 12 h per day, seven days per week, and the average volumetric HRT was ~ 33 days.

2.2. Substrate characteristics

The substrate consisted of different organic waste fractions: food waste, garden residues, horse manure, olive cake, crop residues (wheat)

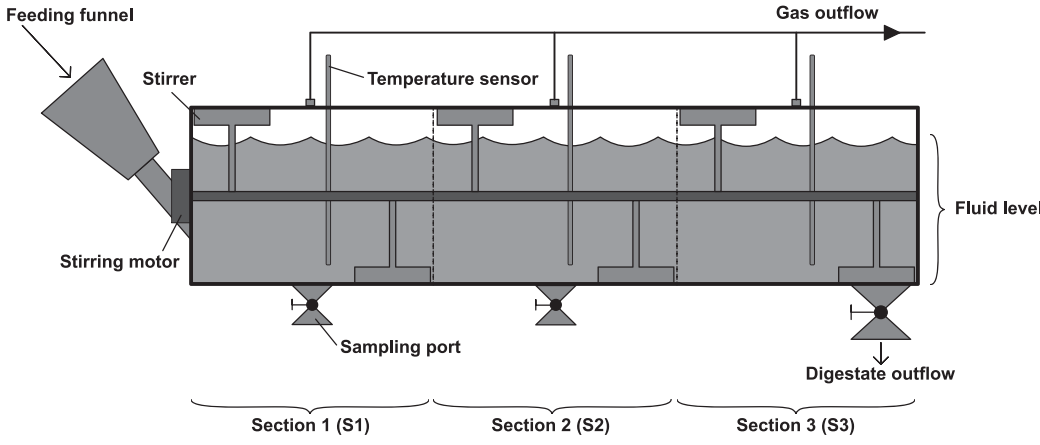

Fig. 1. Schematic illustration of the laboratory-scale high-solid reactor used in the presentstudy.

and potato waste (Table A.1). At industrial scale the substrate was shredded and sieved to particle size ~ 60 mm before being fed into the reactors, with an average VS_{in} of 23 %. Due to variations in the substrate supply chain at the plant, the ratios of different substrate fractions differed somewhat from day to day. Therefore, it was not feasible to collect homogenous substrate samples representing the entire mixture from the industrial-scale reactors. Instead the different substrate fractions were collected sperately and later mixed for use in laboratory scale.

The substrate used in laboratory-scale was mixed to mimick the ratios used in the industrial process (based on the average substrate composition during 24 weeks before the start of the experiment). The substrate fractions were collected at the industrial-scale plant to use the same substrate in both scales. For practical reasons, substrate fractions used in laboratory scale were collected every 3–4 months during the experimental period and stored at 4 °C until use. At laboratory scale, the substrates were pre-treated by grinding to particle size ~ 10 mm. All fractions were analysed for TS and VS, and the final substrate mixes were analysed for total N and C, organic N, NH_4^+-N, and concentration of macromolecules (Table 1). Before feeding the reactors, water was added to the substrate mix to obtain a final VS_{in} of 22 %.

2.3. Analytical methods

The volume of gas produced in the laboratory-scale reactors was measured continuously using RITTER Drum-type meters TG0.5 (RITTER Apparatebau GmbH & Co. KG, Bochum, Germany). Gas composition (CH_4, CO_2, O_2, H_2, H_2S) was measured before every feeding occasion, using an AwiFLEX device (Awite Bioenergie Gmbh, Langenbach).

Concentrations of TS and VS in substrate fractions and digestate samples were analysed in triplicate using standard methods (APHA, 1998). Potential VFA losses during TS analyses were investigated (based on SGC Rapport 2013:273) but found to be negligible, and thus no correction was made for VFA losses.

Organic N (SS-ISO 13 878), NH_4^+-N (FOSS TECATOR, Application Note, AN 5226, based on ISO 11732) and total C (SS-ISO 10 694) in substrate samples were measured by Agrilab AB (Uppsala, Sweden). Biweekly measurements of NH_4^+-N concentration in digestate samples from the laboratory-scale reactors were performed using a LCK 302 Ammonium kit (Hach Lange Gmbh, Düsseldorf, Germany). In brief, digestate samples were sieved using a tea-strainer and then frozen at −20 °C until analysis. Before analysis, samples were thawed and centrifuged at 11500xg for 15 min. The supernatant was removed and diluted in dH_2O, sterile filtered (0.2 μm syringe filter), and then finally added to the test cuvette (200 μL), following the manufacturer's instructions. Absorbance was measured using a DR3900 spectrophotometer (Hach, Germany). Ammonia-nitrogen (NH_3-N) concentration was calculated based on temperature, pH and NH_4^+-N concentration [36].

The concentration of VFA in digestate samples from laboratory-scale reactors was measured by HPLC (Agilent 1100 Series, Agilent Technologies, Waldbronn, Germany), as described previously [37]. Digestate samples were sieved using a tea-strainer and then frozen at −20 °C until sample preparation and analysis. Alkalinity and the ratio between volatile organic acids and total inorganic carbon (FOS/TAC) was measured on fresh, sieved (using tea-strainer) digestate samples from laboratory reactors by titration with 0.1 N H_2SO_4 Standard Solution, using a TitraLab AT1000 Series (Hach Lange Gmbh) according to the manufacturer's instructions, based on the Nordmann method for FOS/TAC measurement.

The protein content in digestate and substrate samples was calculated based on total Kjeldahl-N (EN 13342) and NH_4^+-N (STANDARD METHODS 1998, 4500 mod.) measured by Eurofins Food & Feed Testing Sweden (Lidköping). Carbohydrate content (SLVFS 1993:21) was analysed by Eurofins Environment Testing Sweden AB, and content of raw fat (NMKL 160 mod.) by Eurofins Food & Feed Testing Sweden (Lidköping).

For digestate samples from the industrial-scale reactors, the following analyses were performed: TS (SS-EN 12880:2000), VS (SS-EN 12879:2000), VFA (Clarus 550 gas chromatograph (Perkin Elmer, Waltham, MA, USA) with a packed Elite-FFAP column (Perkin Elmer, USA) for acidic compounds (Jonsson & Borén, 2002)), pH (SS-EN ISO 10523:2012) and NH_4^+-N (ISO 5664:1984). All these analyses were

Table 1
Chemical composition of substrate mixes used in laboratory-scale reactors during different periods of operation.

Substrate period	Week 1–14	Week 14–25	Week 26–53
Tot-N [g/kg]	5.1	6.8	5.1
Org-N [g/kg]	4.7	6.1	5.0
NH_4^+-N [g/kg]	0.4	0.7	0.1
Tot-C [g/kg]	108.5	122.3	90.2
Raw protein [g/kg]	ND	33.4	26.8
Raw fat [g/kg]	ND	35.7	19.6
Carbohydrates [g/kg]	ND	155.7	146.1

ND, not determined.

carried out by Tekniska verken i Linköping AB (Linköping, Sweden).

2.4. Tracer test

Plug-flow behaviour was evaluated in laboratory-scale reactor LR1 with a tracer test using LiCl (Alfa Aesar, ThermoFisher (Kandel) GmbH, Germany), with an average concentration in the reactor of 91 mg Li^+/kg TS [38]. Digestate for recirculation was collected before the tracer was added and was used for recirculation throughout the test. Samples of outgoing digestate were taken on every feeding occasion (6 days/week) for a period of 40 days. Digestate was analysed for Li^+ concentration (SS 028150:1993/SS-EN, ISO 11885:2009) by Eurofins Environment Testing Sweden AB. A tracer test using Li^+ had been performed at the industrial-scale plant, to ensure sufficient residence time for hygienisation. In that test, $LiOH{\cdot}H_2O$ (Helm AG, Hamburg, Germany) was added to one of the reactors to obtain an average Li^+ concentration of 25 mg/kg TS and the Li^+ concentration in the outgoing digestate was measured regularly during the first 48 h. During the test, the reactor was operated under minimum HRT conditions (~25 days), *i.e.* fed the maximum possible substrate amount to simulate a "worst-case scenario". It was estimated that the amount of outgoing digestate during the test was 0.5–0.75 m^3/hour. Analysis of Li^+ concentration in digestate samples was carried out by Agrolab GmbH, Germany.

2.5. Analyses of microbial community

Microbial community structure was analysed by 16S rRNA gene sequencing on two occasions. First, samples from steady-state operation were analysed to compare the different reactor sections (S1-S3) and laboratory- and industrial-scale reactors. Samples at laboratory scale were collected during the start-up phase (before week 1) and in experiment weeks 11 and 14, from all three reactor sections of LR1 and LR2. Samples from S1 and S3 in industrial-scale reactors RK2 and RK3 (RK1 excluded due to process disturbance) were collected in experiment weeks 6, 13, 20, 28 and 33. During week 33, a sample was also taken from S2 in RK3. In the second sequencing round, samples from the period with process disturbances, as observed by VFA accumulation, were collected (18 time points for the two laboratory-scale reactors and 12 time points for the three industrial-scale reactors). DNA extraction was performed as a single replicate (for samples in time-series with ≥ 3 sampling time-points) or three replicates per time-point, using the FastDNA Spin Kit for Soil (MP Biomedicals Europe) as described previously [39]. All samples were stored at −20 °C, both before and after DNA extraction.

Libraries of the 16S rRNA gene were prepared using primers for amplification of the V4 region (515′F/806R). Library preparation and sequencing (Illumina Novaseq platform) was carried out by Novogene (UK) Company Limited, Cambridge, United Kingdom. Raw sequences (with primer and barcode sequences removed) were processed using the DADA2 pipeline v1.16.0 [40]. Optimal trimming sites to minimise error rates were chosen using the tool Figaro [41]. Sequences were annotated with the Silva database v138.1 [42]. Processing of results was carried out in R, using the phyloseq package v1.38.0. Weighted principal coordinate analysis (PCoA) was used to analyse β-diversity in different reactors and sections at steady state. Distances were calculated by the UniFrac method [43] based on a phylogenetic tree generated using neighbour-joining [44], using the phangorn package in R.

The gene copy number of archaeal groups in samples from the steady-state period was investigated by qPCR targeting the 16S rRNA gene. Two different primer pairs were used, designed for detection of orders Methanobacteriales (MBT) and Methanomicrobiales (MMB) [45]. The qPCR protocol and programme were as described previously [37]. Before analysis, sample dilution was tested [46] and the optimal dilution was found to be 100x. The qPCR reaction was run with a QuantStudio 5 Real-Time PCR system (ThermoFisher Scientific), and the raw data were processed using QuantStudio Design & Analysis Software v1.5.2 (ThermoFisher Scientific).

2.6. Degradation efficiency and residual methane production

Residual methane potential (RMP) and degradation rate of protein (egg white powder, Källbergs Industri AB, Töreboda, Sweden), cellulose (microcrystalline cellulose, Alfa Aesar, ThermoFisher GmbH, Kandel, Germany) and fat (rapeseed oil) were measured in digestate samples, taken from both S1 and S3, from laboratory (pooled digestate from LR1 and LR2) and industrial-scale (RK3) , in principle as described previously [47]. Batch tests for analysis of degradation rates were carried out in triplicate with 200 mL digestate and 2 g VS/L of added substrate in bottles with total volume 600 mL. RMP *i.e.,* background methane production, was measured in digestate without substrate addition. Methane production were measured during 28 days incubation at 52 °C in AMPTS II systems (Bioprocess Control, Lund, Sweden).

Volatile solids reduction was calculated as $(VS_{in}\text{-}VS_{out})/VS_{in}$ [32].

Based on RMP, methane production per active reactor volume (MP_V) and HRT, process efficiency (%) was calculated according to Rico *et al.* [48] as: Efficiency (%) = $100*(MP_V*HRT)/(MP_V*HRT + RMP)$.

2.7. Statistical analyses

The process parameters VFA concentration, VS content, VS reduction, pH, specific methane production (SMP), RMP, NH_3-N, and NH_4^+-N concentration were compared between the laboratory-scale and industrial-scale reactors, and also between sections S1 and S3 within each reactor. Sections were compared using paired *t*-test, within reactor and at the same time point. For comparisons between laboratory- and industrial-scale reactors, values from S3 in both systems were compared using Welch's *t*-test assuming unequal variances. All statistical analyses were carried out in R v4.1.2.

3. Results

3.1. Process parameters and performance in the laboratory- and industrial-scale systems

At laboratory scale, mean SMP was 338 ± 57 and 339 ± 47 NL CH_4/kg VS in LR1 and LR2, respectively (Table 2), with no significant difference between the two replicate reactors. Weekly average SMP varied constantly in the laboratory-scale process, but no general descending or

Table 2
Overview of the laboratory-scale and industrial-scale processes, based on measurements on digestate samples taken from the last section (S3) of the reactors. Mean values or minimum–maximum range during the experiment is shown where appropriate.

Parameter	Laboratory-scale	Industrial-scale
OLR [g VS/L day]	5.2[a]	4.2–8.4
$VS_{substrate}$ [%]	22[b]	19–25
TS_{out} [%]	13.6–22.8	15.1–23.9
VS_{out} [%]	10.8–14.2	10.6–16.4
HRT [days]	~42	~33
Recirculation rate [% of ingoing material, ww]	30	30
Weekly average SMP [NL CH_4/kg VS]	200–563 (LR1) 183–464 (LR2)	269–466
Total VFA [g/L]	0.1–9.0	0.2–12.9
NH_4-N (NH_3-N) [g/L]	2.1–4.6 (0.7–2.0)	2.3–4.1 (0.3–1.7)
pH	8.0–8.5	7.5–8.7
Alkalinity [mg $CaCO_3$/L]	14 261–24 284	ND[c]
RMP[d] [NmL CH_4/g inoculum]	5.2 ± 0.4	5.9 ± 0.5

[a] OLR during stable process.
[b] All substrate batches diluted to 22% VS content on ww basis.
[c] Not determined.
[d] Mean value and standard deviation over nine replicates.

increasing trend over time was observed (Fig. 2a). In industrial-scale reactors, SMP was calculated based on the total amount of gas produced in all three reactors per week (Fig. 2b). The mean value over the whole experiment period was 366 ± 52 NL CH_4/kg VS (Table 2).

The OLR was kept constant when the laboratory-scale processes were stable, but had to be lowered to 75 %, 50 % or even 0 % during process disturbance events (Fig. B.1). The process was considered stable when daily volumetric gas production was relatively constant from day-to-day and when there was no significant increase in CO_2 and H_2 content in the gas (Fig. B.1). The FOS/TAC-value was also considered an indicator of process stability, and OLR was decreased when a steep increase in this parameter was observed (Fig. B.1). Changes in process performance, indicating process disturbance, were observed in laboratory-scale reactors around experiment weeks 20–45 in LR1 and 25–35 in LR2. These changes included increasing NH_4^+-N and VFA concentrations, with propionate concentration reaching 6.2 and 4.2 g/L in LR1 and LR2,

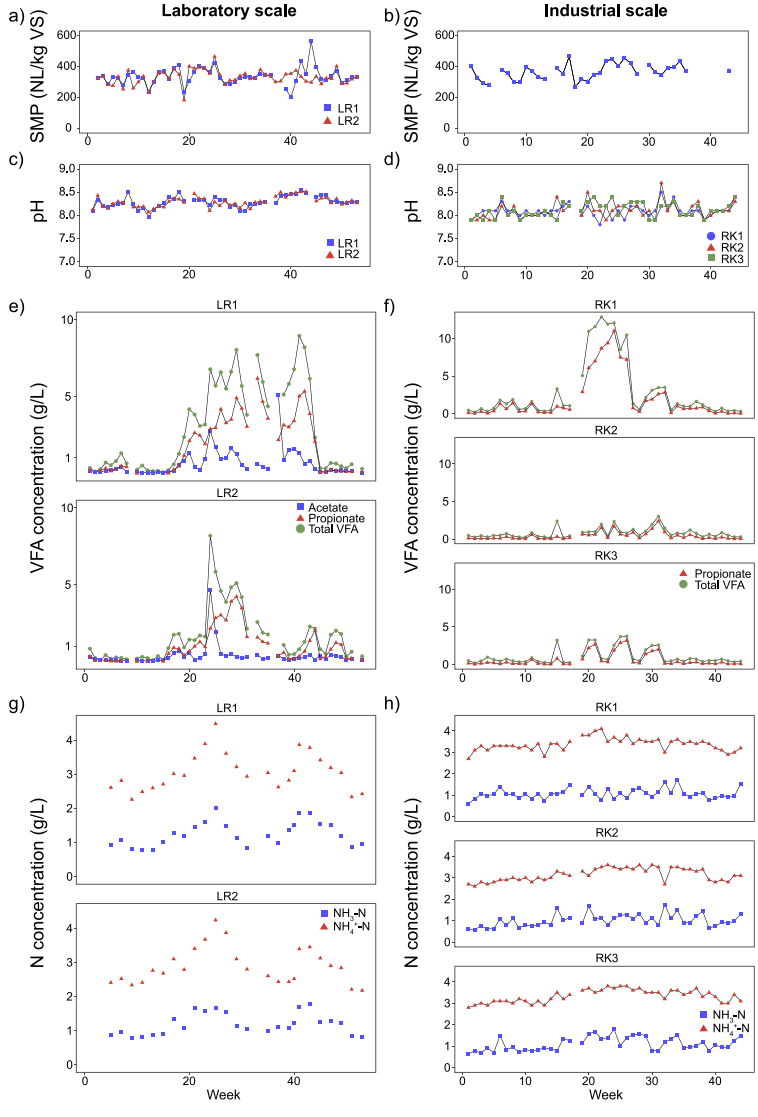

Fig. 2. Process data from (left) laboratory-scale reactors (LR1, LR2) and (right) industrial-scale reactors (RK1, RK2, RK3). Specific methane production at a) laboratory and b) industrial scale (mean production from all three reactors). pH in outgoing digestate (S3) at c) laboratory and d) industrial scale. e) Total VFA, acetate and propionate concentration at laboratory scale and f) total VFA and propionate concentration at industrial scale, all measured in outgoing digestate (S3). Concentration of NH_3-N and NH_4^+-N in outgoing digestate (S3) at g) laboratory and h) industrial scale.

respectively (Fig. 2e, Fig. B.2). Additionally, H_2S and CO_2 content in the gas increased, a sharp peak in H_2 was observed and the CH_4 content dropped slightly (Fig. B.1). However, pH remained relatively stable throughout the entire experimental period (Fig. 2c).

In the industrial-scale reactors, the variation in OLR depended on substrate availability and no adjustments were made based on process performance. During the experimental period, the OLR ranged between 4.2 and 8.4 g VS/L day, with a mean value of 6.9 g VS/L day (Fig. B.1 and Table 2). Process parameters such as pH, NH_4^+-N concentration and gas composition remained relatively stable throughout the experimental period (Fig. 2d, 2 h, Table 2 and Fig. B.1). However, RK1 showed a disturbance during experiment weeks 19–26, with a peak in VFA concentration, mainly represented by propionate reaching 11.0 g/L (Fig. 2f). In reactors RK2 and RK3, the VFA concentration fluctuated during the same weeks but did not reach the same level as in RK1. During the same period, a peak in NH_4^+-N concentration was observed, reaching the highest values in RK1 (Fig. 2h).

3.2. Comparison of process parameters and performance between systems and sections

Comparison of process performance between laboratory and industrial scale revealed several significant differences, with higher VFA concentrations and pH at laboratory scale and higher NH_4^+-N concentrations and VS_{out} at industrial scale (Fig. 3a-d, Table C.1). Mean SMP and RMP were also higher at industrial compared with laboratory scale (Fig. 3f-g). On average, NH_4^+-N levels were higher in industrial-scale reactors, but the relatively high pH in laboratory-scale reactors led to significantly higher levels of free ammonia at laboratory (0.8–2.0 g NH_3-N/L) than industrial scale (0.6–1.8 g NH_3-N/L) ($p = 0.006$).

Comparisons of process parameters were also made between reactor sections S1 and S3 in all reactors, at both laboratory and industrial scale (Fig. 3a-d, 3f, Table C.1). In the laboratory-scale reactors, significant differences between sections were seen in VFA concentration and alkalinity, but not in VS content, pH, NH_4^+-N concentration or RMP. In the industrial-scale reactors, there were significant differences between reactor sections in VFA concentration, pH, NH_4^+-N concentration, VS content and RMP.

To study plug-flow behaviour, a tracer study was carried out using Li^+. At laboratory scale, around 60 % of total Li^+ added to the reactor left the system within 40 days (Fig. D.1). The highest Li^+ concentration in outflowing digestate was seen after 3–4 days (Fig. D.2). A tracer test was also carried out at the industrial-scale plant, for 48 h in total to ensure sufficient residence time for hygienisation. The Li^+ concentration in outgoing digestate from the industrial-scale reactors exceeded the background level ~ 19 h after addition of tracer, but the majority of added Li^+ was not detected during the test period (data not shown).

3.3. Substrate degradation, degradation rates and macromolecule concentrations

VS reduction was measured to evaluate the extent of organic fraction degraded in the reactors. Although VS reduction varied more between time points in the industrial-scale system, the average value was

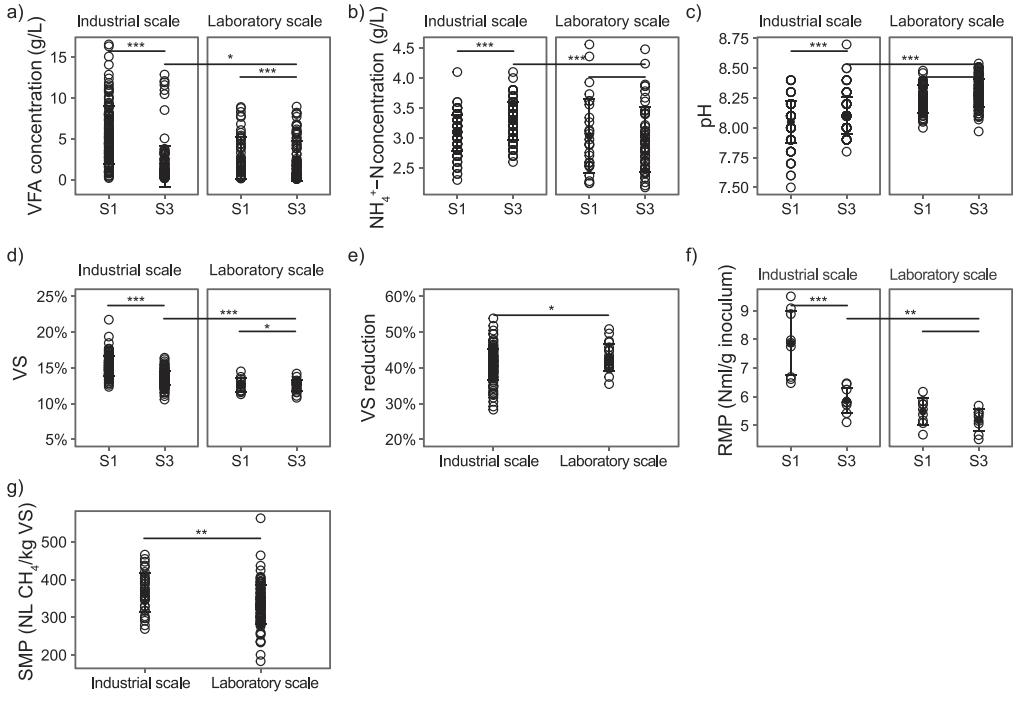

Fig. 3. Process parameters a) total VFA concentration, b) NH_4^+-N concentration, c) pH and d) VS, in industrial-scale and laboratory-scale reactors, with comparison between sections S1 and S3. e) VS-reduction in digestate from S3 relative to substrate VS content (22-23 %). f) RMP in digestate from industrial-scale reactor RK3, sections S1 and S3, and laboratory-scale reactors, sections S1 and S3. g) SMP at industrial and laboratory scale. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

significantly higher at laboratory (43 %) than industrial scale (41 %) (Fig. 3e). RMP, quantifying the remaining gas potential in the digestate, was significantly higher in outgoing digestate from industrial-scale reactors (5.9 NmL/g digestate) compared with laboratory-scale reactors (5.2 NmL/g digestate) (Fig. 3f). Process efficiency, calculated based on HRT, RMP and volumetric methane production, showed similar values at laboratory and industrial scale (92.5 and 93.1 %, respectively).

At both scales, the dry matter fraction in the digestate was dominated by carbohydrates (53–74 % of TS), followed by protein (13–16 % of TS) and fat (2–5 % of TS) (Table 3). The digestate samples from laboratory-scale reactors had similar composition to the substrate mixtures (in terms of percentages of TS), but with a lower fat content and slightly higher protein fraction in digestate samples (Table 3). In all sections of the laboratory-scale reactors, the reduction in comparison with substrate was similar except for carbohydrates which first decreased by 39 % in S1 and then further to 47 % reduction in S3 (Fig. E.1). The most efficient degradation was obtained for raw fat, which decreased by almost 90 % (Fig. E.1). In samples from the industrial-scale reactors, there were no clear differences between digestate samples from sections S1 and S3 (Table 3).

The degradation rate of different components of the substrate was evaluated by determination of gas production from fat, protein and cellulose in digestate from the different reactor sections (S1 compared with S3). The batch tests with digestate from the laboratory-scale reactors revealed similar initial cellulose degradation rate in both sections, whereas degradation of protein and fat was faster in S1 than in S3 (Fig. E.2). Digestate samples from the industrial-scale reactor showed large variations between replicates in the batch tests and therefore no significant differences between sections could be observed (Fig. E.2).

3.4. Microbial community structure

3.4.1. Community during steady-state

Analyses of microbial community structure using 16S rRNA gene sequencing were performed during a period of stable process operation, with the aim of evaluating differences between laboratory- and industrial-scale reactors, and between sections within reactors. All processes were found to be dominated by *Defluviitoga* (phylum Thermotogota), which accounted for 32–39 % in industrial-scale and 10–14 % in laboratory-scale reactors, and the Clostridia group MBA03 (phylum Bacillota), which accounted for 19–26 % at both laboratory and industrial scale when the process was stable. Other genera with high relative abundance were *Halocella* (phylum Halanaerobiaeota), with relative abundance 6–7 % at laboratory scale and 2–5 % at industrial scale, *Lentimicrobium* (phylum Bacteroidota), with relative abundance 10–11 % and 2–3 % at laboratory and industrial scale, respectively, and the group DTU014 within class Incertae Sedis (phylum Bacillota), which accounted for ~ 7 % of the community at laboratory scale and 3–4 % at industrial scale. Within Archaea, the results demonstrated dominance of genus *Methanothermobacter* within the order Methanobacteriales (phylum Euryarchaeota), accounting for ~ 1 % and 3–10 % of the total community at laboratory and industrial scale, respectively. Another dominant methanogen was *Methanoculleus* within the order Methanomicrobiales (phylum Halobacterota), accounting for 3–6 % and 0–2 % of the total community at laboratory and industrial scale, respectively.

Table 3
Concentrations of raw protein, carbohydrates and raw fat in laboratory-scale substrate mixture and in digestate samples from laboratory- and industrial-scale reactors.

		Lab-scale			Industrial-scale	
Macromolecule	Substrate[b]	S1[c]	S2[c]	S3[d]	S1[c]	S3[c]
TS[a] [%]	25	16	16	16	22	19
Raw protein [% of TS]	11	13	13	13 (1)	14	16
Carbohydrates [% of TS]	60	60	61	53 (5)	71	74
Raw fat [% of TS]	8	2	3	3 (0)	4	5

[a] Mean values over the experiment period.
[b] Mean of two substrate mixes used in laboratory-scale reactors, from week 14–25 and week 26–53, respectively.
[c] Mean for reactors LR1 and LR2 at laboratory scale and for reactors RK2 and RK3 at industrial scale (from one time-point each).
[d] Mean values from reactors LR1 and LR2 from two sampling time-points, standard deviation in brackets.

Analysis of β-diversity by weighted PCoA indicated significant differences between industrial and laboratory scale, but no significant differences in community structure between sections (Fig. 4a), sampling time-points or parallel reactors (Fig. F.1). Samples from the inoculum and start-up phase clustered together with the industrial-scale samples (Fig. 4a). Genera that distinctly increased in relative abundance upon downscaling were: *Lentimicrobium* and *Proteiniphilum* from phylum Bacteroidota, *Halocella*, *Acetomicrobium* (phylum Synergistota) and groups within the phylum Bacillota (Firmicutes); Hydrogenispora (order level), DTU014 (order level), *Dethiobacteraceae* (family level) and *Keratinibaculum*. Genera that distinctly decreased in relative abundance upon downscaling were *Defluviitoga* and *Tepidimicrobium* (phylum Bacillota) (Fig. 4b). For Archaea, a shift in the two dominant groups was observed. Higher abundance of *Methanoculleus* in the laboratory-scale reactors and in the inoculum was confirmed by qPCR analysis targeting the two dominant archaeal orders Methanomicrobiales (*i.e. Methanoculleus*) and Methanobacteriales (*i.e. Methanothermobacter*) (Fig. G.1). However, the dominance of Methanobacteriales over Methanomicrobiales in industrial-scale reactors observed in 16S rRNA analysis was not clearly confirmed by qPCR analysis.

3.4.2. Community during process disturbance

To study microbial community dynamics during process disturbances, digestate samples were collected at several time-points during the period of VFA accumulation (Fig. 2e, 2f). These samples were all taken from S3 (*i.e.* no section comparison). In both laboratory-scale reactors (LR1, LR2), there was an increase in relative abundance of *Defluviitoga* and decrease in relative abundance of MBA03 during the disturbance phase, and in LR1 this change was associated with the increase in VFA concentration (Fig. 5a). At the point when VFA concentration decreased in LR1 (around week 45), microbial community structure returned to the original proportions. Industrial-scale reactor RK1 also showed a decrease in relative abundance of MBA03 during the disturbance period, together with a peak in relative abundance of *Methanothermobacter* (Fig. 5b). In both laboratory-scale reactors and in RK1, the genera *Halocella*, *Keratinibaculum* and *Tepidimicrobium* increased in relative abundance simultaneously with the highest VFA concentrations, while there was a decrease in *Lentimicrobium* at the same time-points (Fig. 5a, 5b).

4. Discussion

4.1. Process performance, downscaling effects and link to the microbial community in laboratory- and industrial-scale reactors

4.1.1. Methane production and substrate degradation in laboratory- and industrial-scale reactors

During the experimental period, mean SMP in the laboratory- and industrial-scale reactors was 338 and 366 NL CH_4/kg VS respectively, which is within the range reported previously for thermophilic co-digestion of food waste (~50 % of VS) and vegetable, straw or garden waste (330–520 NL CH_4/kg VS) [13,14,49,50]. The values obtained were also within the wide range of SMP values (160–420 NL CH_4/kg VS) reported in previous studies of thermophilic high-solid digestion systems treating food waste (80–100 % of VS) [15,32,51]. The differences between studies are likely caused by large differences in operating parameters, such as substrate characteristics, OLR and HRT. The overall VS-reduction was similar in industrial- and laboratory-scale reactors

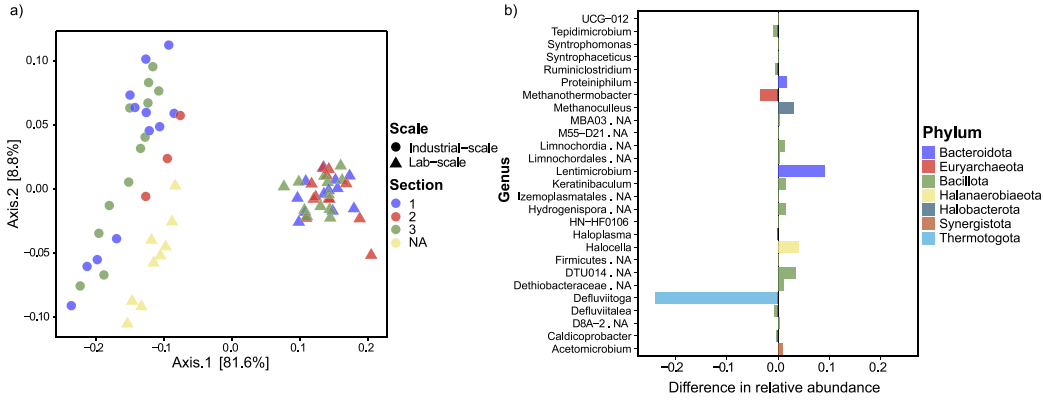

Fig. 4. a) Weighted PCoA plot, with colours indicating samples from different reactor sections at laboratory and industrial scale. Yellow triangles (reactor section not applicable) indicate inoculum and samples from the start-up phase at laboratory scale. Blue, red and green symbols indicate sections S1, S2 and S3, respectively. b) Difference in relative abundance of bacteria and archaea at genus level (colours indicate phyla) in laboratory-scale compared to industrial-scale reactors. Average relative abundances over replicates, time-points, sections and reactors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

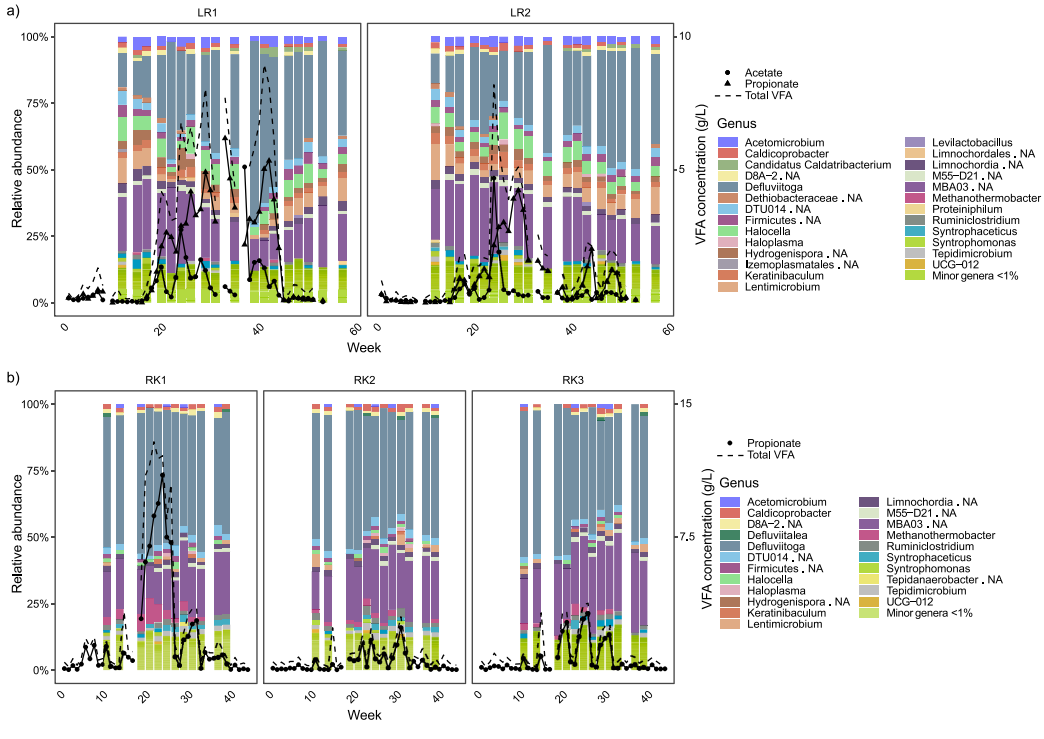

Fig. 5. Relative abundance of bacteria and archaea at genus level in a) laboratory-scale reactors LR1 and LR2, where total VFA concentration in the reactors is indicated by dashed line, acetate and propionate concentrations by circles and triangles, respectively, and b) industrial-scale reactors RK1, RK2 and RK3, where total VFA concentration in the reactors is indicated by dashed line and propionate concentration by circles.

(41–43 %), but at the lower end of the range reported for thermophilic HSD processes digesting food waste (40–70 %) [8,12,15,32]. This result could be expected since the substrate mix used in this study contained only around 50 % food waste and the additional fractions consisted of more recalcitrant lignocellulosic material (*i.e.* horse manure, garden residues and crop residues).

4.1.2. Scale comparison and downscaling effects on overall process efficiency and degree of degradation

When simulating an industrial-scale HSD process at laboratory scale, there are several factors to consider. One is particle size of the substrate and inoculum, which needs to be smaller in a laboratory-scale system. In the present study, the inoculum used for the laboratory-scale reactors was sieved and the substrate was grinded, while rougher shredding and sieving of the substrate was applied at the industrial-scale plant. In addition, the feeding strategy, *i.e.* semi-continuously every day at industrial scale and once per day, six days per week, at laboratory scale, differed for practical reasons. Another difference was substrate composition, which was more consistent in the laboratory and only represented a few samples of the industrial-scale substrate mix. Furthermore, average OLR and HRT differed between the scales. These parameters were initially set according to the operating conditions at the industrial-scale plant, but during the time of the experiment, parameters were changed slightly at the plant.

Despite the above-mentioned differences, process performance was relatively similar at laboratory and industrial scale (Fig. 3). Additionally, overall process efficiency estimated based on volumetric methane production, HRT and RMP [48] was similar for laboratory- and industrial-scale reactors (93 %). However, some small differences were observed, such as slightly higher VS-reduction at laboratory scale (Fig. 3e). This difference could be a result of the additional substrate pretreatment at laboratory scale, potentially increasing substrate availability to microorganisms, and consequently biodegradability and methane production [5,52]. Higher degree of substrate degradation in laboratory-scale reactors was also supported by lower RMP compared with industrial-scale reactors. Additionally, the concentrations of macromolecules (% of TS) in digestate samples were generally slightly higher in industrial-scale (Table 3). Interestingly, in contrast to VS-reduction, SMP was higher (8 %) at industrial scale. Both VS-reduction and RMP pointed towards a higher degree of degradation in laboratory-scale reactors, but the higher level of NH_4^+-N in outgoing digestate from industrial-scale reactors (Fig. 3b, 3e and 3f) instead indicated more efficient protein degradation at the larger scale. However, this difference could also have been caused by greater variability and, on average, higher protein content in the substrate mix at industrial scale. Since proteins in general have higher biomethane potential than *e.g.* lignocellulosic substrates [53], this could potentially explain the higher SMP in the industrial-scale reactors. The larger weekly fluctuations in gas production at laboratory compared with industrial scale (Fig. 2a-b) could also have influenced mean SMP at laboratory scale in a negative way. A similar observation was made in a laboratory-/industrial-scale comparison by Lüdtke *et al.* [35], who speculated that smaller fluctuations in industrial-scale reactors could be a result of more accurate automated feeding and measurement procedures compared with manual operation at laboratory scale. In addition, gas production in industrial-scale reactors in the present study was calculated as a mean value for all three reactors, providing seemingly more stable results compared to separate measurements of gas production from each reactor, which was the procedure in laboratory-scale. Another factor that could have affected average methane production was process disturbances, which were more severe at laboratory scale and only arose in one of the three industrial-scale reactors, which might have lowered average SMP at laboratory scale in comparison with the industrial-scale process.

4.1.3. Microbial community structure in laboratory- and industrial-scale reactors and downscaling effects

In line with previous studies on CSTR reactors [47,54], a downscaling effect was observed on comparing microbial community structure in the laboratory- and industrial-scale processes (Fig. 4a). One cause of this difference may be the different feeding regimes, as previous findings have indicated that feeding frequency has a significant effect on microbial community structure [55]. However, differences in parameters such as OLR, pH, VFA and temperature could also have had an impact (Table 2). Nevertheless, the laboratory- and industrial-scale communities shared strong similarities, *e.g.* dominance of the bacterial group MBA03 and genus *Defluviitoga*. Both these groups have previously been shown to be highly abundant in dry thermophilic AD processes [13,15,56]. The function of MBA03 is not yet known, although it has been suggested to be linked to syntrophic acetate oxidation (SAO) [57] or involved in carbohydrate fermentation [56], while *Defluviitoga* is suggested to be very important for hydrolysis of complex carbohydrates in thermophilic processes [13,56,58]. Another highly abundant genus was *Halocella* which, like *Defluviitoga*, is cellulose-degrading and halophilic [59] and previously has been observed in dry thermophilic digestion processes [15,60]. Another highly abundant genus was *Lentimicrobium*, which is proposed to be involved in acidogenesis in the AD process [61], with the type species growing on starch and simple sugars [62]. However, members of this genus have also been enriched in both acetate- and propionate-fed reactors, and may be involved in acetate degradation [57,63].

Analysis of the carbohydrate-hydrolysing microbial groups upon downscaling revealed a clear decrease in relative abundance of *Defluviitoga*, whereas *Halocella* generally had higher relative abundance in the laboratory-scale than the industrial-scale reactors. Other notable changes were increases in the relative abundance of two genera belonging to phylum Bacteroidota (*Lentimicrobium* and *Proteiniphilum*). In the reactor communities, mainly three genera known to possess proteolytic abilities (*Proteiniphilum* [64], *Keratinibaculum* [65] and the closely related *Tepidimicrobium* [66]) were identified. The relative abundance of *Proteiniphilum* and *Keratinibaculum* increased upon downscaling, while that of *Tepidimicrobium* decreased. These changes in the microbial community indicated shifts in both the proteolytic and saccharolytic groups at laboratory scale compared with the industrial-scale process, but these shifts did not seem to affect the degree of degradation of the corresponding substrates.

For the predominant methanogenic genera, there was a clear shift in dominance by *Methanothermobacter* at industrial scale towards *Methanoculleus* at laboratory scale. Species belonging to *Methanothermobacter* have a growth optimum at 55–65 °C [67], so the slightly higher average temperature in the industrial-scale systems may have favoured growth of this genus. The reason for the increase in *Methanoculleus* relative to *Methanothermobacter* upon downscaling in the present study (Fig. G.1) is not clear, but could be related to the generally lower H_2 level in laboratory-scale than industrial-scale reactors (Fig. B.1). Previous findings suggest that *Methanoculleus* is enriched and has competitive advantage at low levels of H_2 [68,69]. However, no firm conclusions can be drawn on whether the downscaling effect on the methanogenic community influenced overall methane production efficiency.

4.2. Ammonia and VFA levels coupled to microbiology under stable conditions and process disturbance

4.2.1. VFA accumulation in response to high levels of NH_4^+-N

During the entire experimental period, both the laboratory- and industrial-scale processes had high NH_4^+-N levels which, combined with thermophilic conditions and relatively high pH, led to high ammonia concentrations (0.6–2.0 g NH_3-N/L), exceeding levels previously reported to inhibit microorganisms, cause VFA accumulation and decrease methane production in thermophilic systems (0.6–1.5 g NH_3-N/L) [18,70]. In line with this, both laboratory-scale reactors (LR1, LR2) and

one industrial-scale reactor (RK1) showed rapid accumulation of VFA, particularly propionate, when the NH_4^+-N level increased slightly and reached 4.2–4.5 g/L (1.6–2.0 g NH_3-N/L) and 4.1 g/L (0.8 g NH_3-N/L) respectively (Fig. 2g-h, Fig. B.1, Fig. B.2). In laboratory-scale reactors, the increase in NH_3-N was observed after introducing the second batch of substrate mix, which had a relatively high content of fat and protein (Table 1), indicating that the overall high ammonia level made the reactors sensitive to small changes in substrate composition. More severe disturbance and maintained higher propionate concentration were observed in LR1 compared with LR2. This process imbalance could have been caused by technical issues in LR1, such as difficulty maintaining reactor volume and thereby sporadic substrate overloading, in addition to the substrate composition change. Previous studies of thermophilic HSD operating with food waste have also observed signs of disturbance, *e.g.* high propionate levels relative to acetate, upon an increase in OLR [32,71], and in processes with 1.2–1.9 g NH_3-N/L [15]. Accumulation of propionate is considered an important indicator of ammonia inhibition and process disturbance, but also an inhibitor in itself, especially since propionate degradation often has a long lag-phase and elevated levels can persist over a long period [72].

4.2.2. Microbial community structure and links to NH_3-N and process instability

The microbial communities in both laboratory- and industrial-scale reactors were generally dominated by bacterial groups known to be ammonia-tolerant, such as the abundant MBA03 and *Defluviitoga* [15,73]. *Lentimicrobium* has also been observed previously at high NH_4^+-N concentrations in thermophilic conditions [63], as have *Halocella* and DTU014 [15]. Similarly, both dominating methanogenic genera, *Methanothermobacter* and *Methanoculleus,* are known to include members that are ammonia-tolerant. As an example, in bioaugmentation experiments, members of these genera have been shown to significantly improve methane production in ammonia-stressed systems [74,75]. The dominance of these hydrogenotrophic methanogens strongly indicates that, in line with previous findings on thermophilic HSD [15,56,60], the hydrogenotrophic methanogenic pathway was favoured over the acetoclastic in our reactors. Under these conditions, acetate is degraded by syntrophic acetate-oxidising bacteria (SAOB) in cooperation with hydrogenotrophic methanogens [70]. Potential candidate SAOB in the community were MBA03 [57], *Syntrophaceticus* (phylum Bacillota) and *Caldicoprobacter* (phylum Bacillota) [56]. The *Syntrophaceticus* sequence identified in the present study was shown to be identical to the 16S rRNA gene sequence of a novel SAOB candidate that cooperates with *Methanothermobacter* and/or *Methanoculleus* in thermophilic and high-ammonia conditions [76,77]. Also, this genus has previously been shown to comprise a mesophilic SAOB species that cooperates with *Methanoculleus* to degrade acetate [78].

Microbial community structure was studied over time during process disturbance, to reveal the microbial response to high ammonia and VFA levels at both scales. In agreement with Lv *et al.* [79], who observed different changes in community structure in parallel reactors upon ammonia inhibition, all three disturbed reactors (LR1, LR2, RK1) in the present study showed slightly different responses (Fig. 5a-b). Interestingly, after the decrease in relative abundance of *Defluviitoga* upon downscaling, this genus increased again in laboratory-scale reactor LR1 to a peak where it accounted for > 60 % of the total microbial community, which coincided with the NH_4^+-N and VFA peaks. There was also a slow increase in *Defluviitoga* in reactor LR2 during the course of the experiment, but a similar peak in relative abundance was not seen in this reactor. At industrial scale, no difference in relative abundance of *Defluviitoga* was seen between reactors or over time, even in RK1 during the disturbance phase. Thus the link between this genus and reactor instability is not clear. However, the type species, *Defluviitoga tunisiensis*, is known to produce acetate, CO_2 and H_2 [58] and thus the high relative abundance of *Defluviitoga* in LR1 may have contributed to the relatively high acetate levels in that reactor.

Interestingly, in contrast to the laboratory-reactors, the degradation of propionate in RK1 was relatively rapid, which was possibly explained by the lower level of free ammonia in industrial-scale compared with laboratory-scale reactors (Fig. 2e-h, Table 2). An alternative explanation could be the pronounced change in relative abundance of *Methanothermobacter* in reactor RK1, which peaked at > 9 % of the total community during the disturbance period. Singh *et al.* [77] observed that efficient propionate degradation at thermophilic temperature is strongly linked to activity of *Methanothermobacter*. Thus, the high levels of this methanogen in industrial-scale compared with laboratory-scale reactors suggests that *Methanothermobacter* could have played a role in achieving efficient propionate oxidation in the former. A similar pronounced change in relative abundance of methanogens was not observed at laboratory scale. Looking specifically at possible propionate-degrading bacteria, sequences here assigned to *Pelotomaculum* were found to be identical to the potential thermophilic and ammonia-tolerant syntrophic propionate-oxidising bacteria '*Candidatus* Thermosyntrophopropionicum ammoniitolerans' [77]. That species showed low relative abundance (<0.2 %) at all time-points but could still have contributed to propionate degradation, as syntrophic VFA degraders can be present in very low abundance but still be essential for a stable process [80]. Similarly, the potential syntrophic acetate oxidisers *Syntrophaceticus* and *Caldicoprobacter* increased only slightly in relative abundance as a response to peaks in acetate concentration in the laboratory-scale reactors. Relative abundance of MBA03 instead declined during the VFA and ammonia peak, but it was still one of the dominant groups in all reactors. Thus, no clear correlation between VFA level and the relative abundance of potential VFA oxidising groups could be observed.

4.3. Evaluation of plug-flow behaviour and phase separation

The occurrence of phase separation or plug-flow behaviour in laboratory-scale reactors of plug-flow type has been investigated in some previous studies. For instance, Rossi *et al.* [32] studied microbial communities and VFA production in different sections of a thermophilic laboratory-scale plug-flow reactor digesting organic household waste. Plug-flow was also confirmed by Nordell *et al.* [30] in a tracer test in a laboratory-scale reactor operating with dewatered digestate of sewage sludge. Here, the industrial-scale reactors showed indications of phase separation across the reactor, with higher VFA concentration and VS in the first section and higher pH and NH_4^+-N concentration in the outgoing digestate (Fig. 3a-d, Table C.1). If the PFR is assumed to work as a serial digester system, the first step is expected to have a higher hydrolysis and acidification activity, which would lead to higher VFA levels and lower pH [47,81]. In contrast, the last step of a serial system is expected to have more complete degradation and thereby lower VS and VFA levels, but also accumulation of NH_4^+-N [47,82]. However, in the laboratory-scale system of the present study, the levels of NH_4^+-N, pH, VFA, and VS were relatively similar in different sections (Fig. 3a-d, Table C.1), indicating absence of phase-separation, and the behaviour was instead more similar to a single CSTR. The slight phase separation observed in industrial-scale reactors could be a sign of plug-flow behaviour but could possibly also be explained by the sampling and feeding strategy. In laboratory-scale reactors, samples were always taken a day after the previous feeding, while in industrial-scale reactors, samples were taken during continuous feeding, when theoretically *e.g.* the VFA levels are higher [55,83]. Also, this means that samples taken in the first section in the industrial-scale process most likely included some newly fed substrate, which could have contributed to the higher VS level in this section.

At laboratory scale, some differences were still observed between the reactor sections, *e.g.* slightly faster protein and fat degradation in S1 compared with S3 (Fig. E.2). Furthermore, lower carbohydrate concentration towards the end of the laboratory-scale reactors (Table 3, Fig. E.1) indicated slightly better degree of degradation in the last

section. Unfortunately, it could not be confirmed whether the same trends were obtained in the industrial scale reactors since the results from the batch tests showed high variation between replicates (Fig. E.2), probably due to the inhomogenous nature of the digestate samples from the larger scale reactors. Also, there were no clear differences in macromolecule concentrations between S1 and S3 in industrial scale (Table 3). Based on microbial community structure and the results of PCoA analysis (Fig. 4a), there was no significant difference between the sections in either laboratory- or industrial-scale reactors. It is routine practice at the industrial-scale plant to reinoculate the first section of the plug-flow reactor with digestate, which has been observed to increase process stability [30]. Such recirculation of digestate might have contributed to homogenize the microbial community across the sections, potentially hindering separation of the AD steps in the reactor. However, Rossi *et al.* [32] observed significantly different microbial communities in different sections of a laboratory-scale plug-flow reactor, with higher relative abundances of *Defluviitoga* in the first and last section, and higher relative abundance of protein-degrading genera in the middle. This was achieved using a digestate recirculation ratio of 45 %, indicating that recirculation alone does not explain the lack of phase separation. In line with this, Chen *et al.* [31] obtained a significantly different microbial community at the inlet of a plug-flow reactor than in the middle and last sections, even with recirculation ratio as high as 50–60 %. There are several factors that potentially contributed to better phase separation in those studies, such as substrate characteristics, different recirculation ratios and mixer properties. Another important factor in reactor design is length:width (L:W) ratio, which was 4.1 in our laboratory-scale reactors (5.3 in the industrial-scale reactors), while in other studies using laboratory-scale plug-flow type reactors it has typically been slightly higher, *e.g.* 4.4 [84], 6 [30], 10.8 [85], 12 [31], 15.7 [86] or 30 [87]. Unfortunately, of the above mentioned studies, only Nordell *et al.* [30] and Chen *et al.* [31] investigated plug-flow behaviour or phase separation, which makes it difficult to draw conclusions about which conditions are key factors for obtaining a plug-flow. Phase separation can be achieved, and short-circuiting reduced, if the PFR is compartmentalised or the design in some way hinders material from flowing freely through the reactor [87–89]. More research is needed to fully understand the extent to which this can affect yield and process efficiency.

The tracer tests carried out both in laboratory- and industrial-scale confirmed that there was no plug-flow behaviour in the reactors. Theoretically, in an ideal plug-flow system the peak in outgoing tracer would come after one HRT, but the Li^+-concentration curve in the laboratory-scale (Fig. D.1 and D.2) instead resembled that of a completely stirred reactor, with the peak in tracer appearing within the first few days after addition [24]. Although the duration of the tracer test in industrial-scale reactors was not long enough to obtain a tracer curve for an entire HRT, the results indicated short-circuiting also in this system and ingoing material started to flow out already within the first 24 h.

5. Conclusions

This comparison of a laboratory- and industrial-scale process highlighted some of the difficulties that can arise in direct comparisons of systems at different scales. The main differences were the substrate mix (more non-homogeneous, larger particle size and more varying in industrial- compared with laboratory-scale), digestate characteristics (particle size) and feeding strategy. However, the differences in process parameters, efficiency and yield were relatively small, indicating that the laboratory-scale system represented a good approximation of the industrial-scale system over time.

Within the microbial community, pairs or groups of genera with similar suggested functions in the AD process were identified, where one genus decreased upon downscaling while the other increased. Examples were the saccharolytic genera *Halocella* and *Defluviitoga*, the proteolytic genera *Proteiniphilum*, *Keratinibaculum* and *Tepidimicrobium* and the hydrogenotrophic methanogens *Methanoculleus* and *Methanothermobacter*. Thus these might have replaced each other to adapt to the slightly altered conditions in the laboratory compared with the industrial-scale process, without significant loss of efficiency and productivity by the overall microbial community.

Process disturbances occurred in laboratory- and industrial-scale reactors when the NH_4^+-N level reached > 4 g/L and led to accumulation of VFA, especially propionate. Both processes were run at relatively high NH_4^+-N levels throughout the experimental period, which likely increased the risk of even small changes in *e.g.* substrate composition inducing process disturbance, highlighting the importance of continuous process monitoring. Additionally, a potential link between high relative abundance of *Methanothermobacter* and propionate degradation was observed during the disturbance phase.

The results obtained at both laboratory and industrial scale indicated no plug-flow behaviour, suggesting that high-solid digestion in reactors of plug-flow type is perhaps not utilized to its full potential. Theoretically, a true plug-flow system has several advantages over CSTR processes, such as reduced short-circuiting and the possibility to obtain phase separation. To conclude, more investigation is needed to determine how to operate an HSD process to achive plug-flow and better exploit its potential advantages.

CRediT authorship contribution statement

Ebba Perman: Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Maria Westerholm:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Tong Liu:** Supervision. **Anna Schnürer:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

16S rRNA-gene sequence data can be accessed at BioProject accession numbers PRJNA1011979 and PRJNA1012582 at Sequence Read Archive (SRA), National Center for Biotechnology Information (NCBI).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enconman.2023.117978.

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This thesis addressed some challenges associated with high-solid anaerobic digestion, a method for biogas and biofertiliser production from waste materials with high total solids content. The results demonstrated the feasibility of down-scaling high-solid anaerobic digestion to laboratory-scale to simulate the conditions at an industrial-scale plant. However, the results indicated high risk of disturbances due to ammonia inhibition in these processes. Additionally, plug-flow behaviour and phase-separation could not be established in horizontal tubular reactors at either laboratory or industrial scale.

Ebba Perman received her graduate education at the Department of Molecular Sciences, SLU, Uppsala. She received her M.Sc. Eng. in Molecular Biotechnology at Uppsala University, Sweden.

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