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Anaerobic digestion of the solid residue after EPS extraction at haloalkaline conditions

TU Delft

Anaerobic digestion of the solid residue after EPS extraction at haloalkaline conditions

By

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Preface

My own work style and that of the microbial community used in this study, have a few things in common. We both like to work at a deliberate pace and to do multiple things at the same time. When I started in October 2018, we knew that the batch tests with this inoculum were going to be *slow*. At that point, we were not even sure whether the micro-organisms in the inoculum were still active, as they had been in the fridge for two years. So we got the first tests going as soon as possible, waiting for activity to appear. I am not the one that likes to sit around and wait, and I had other projects on my hand as well. The first four months, I was only around part-time, monitoring the micro-organisms and obtaining the first positive test results.

From February onwards, I worked full-time on the project and time went by so fast. Though, as the bugs were still slow, we started various projects in parallel. More enrichments, scale-up of the set-up, a bioreactor; over the length of the project we managed a total of 66 set-ups. In April, Lena Depaz joined the team for her BSc thesis. She delved into the topic of storage polymers and the question of why the micro-organisms were not making more biogas from the available substrate. Altogether, working for 8.5 months on this project was a lot of fun and I hope this thesis will give you some insight into the process.

I would like to conclude with my favourite quote from Jure: ‘many people bare co-responsibility for any value these chapters have, while the errors and lapses are mine alone’. Jure, Dimitry, Gerben, Lena, Mark, and Robbert, thank you for all the valuable discussions and bright ideas; Ralph thank you for taking a seat in the thesis committee.

Valerie

Delft, August 2019

Abstract

Extracellular polymeric substances (EPS) extracted from aerobic granular sludge (AGS) have recently gained commercial interest as a valuable resource. The alkaline EPS extraction yields roughly 25% of soluble EPS, and about 75% of solid alkaline residual sludge. This study aims to give the first outlook to implement treatment of this alkaline residual sludge with anaerobic digestion (AD) at haloalkaline conditions (pH 9.5, Na^+ [0.6 M]). Batch AD enrichment experiments were performed with an inoculum of haloalkaline anaerobic microorganisms, originating from soda lake sediments. The residual sludge was successfully digested and 40-50% of the theoretical production of biogas was obtained. A higher biodegradability was obtained compared to the digestion of AGS at haloalkaline conditions. Due to the alkalinity of the medium, the majority of the CO_2 remained in solution, which yielded a high methane content in the gas phase, on average 98%. Environmental factors such as the cation composition of the medium, the salt concentration and substrate preparation showed a negative influence on the biogas yield. In addition, the first implications for continuous AD operation are discussed.

Contents

1 Introduction

Aerobic granular sludge technology (AGS) offers a compact, efficient, and cheaper design of wastewater treatment compared to conventional activated sludge technology (Pronk 2016). The AGS is a biofilm composed of mixed microbial community that is encased in a matrix of highly hydrated self-produced extracellular polymeric substance (EPS). Recently, considerable scientific efforts have been put forward in the extraction and characterisation of the EPS in the granules (Lin et al. 2010). This EPS, derived from waste sludge streams, has also gained commercial interest as a valuable resource.

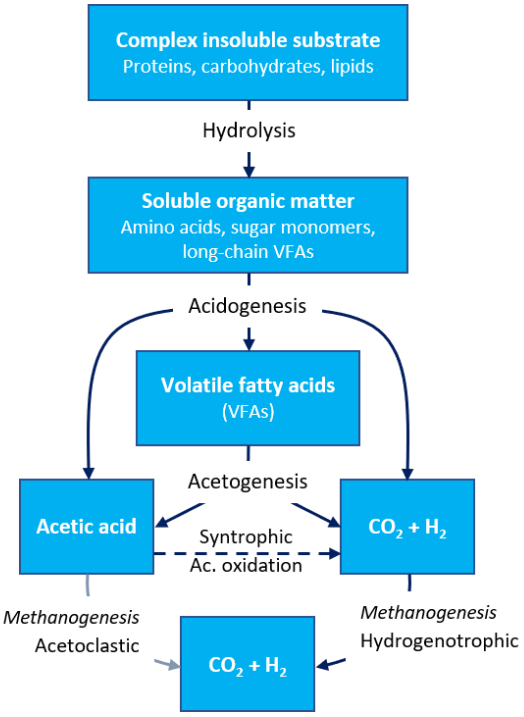

Figure 1 | Anaerobic digestion conversions. At alkaline conditions, acetoclastic conversion is often limited and instead a syntrophic acetate oxidation takes place (Kleerebezem 2014, Timmers et. al. 2018).

The Dutch government has the ambition to have a circular economy in 2050, and water authorities in the Netherlands are taking the initiative to invest and develop in new technologies. Integrated with wastewater treatment, they want to establish a biorefinery that is a source for sustainable energy, resources and clean water (Blankesteijn et al. 2017; Energie en Grondstoffen Fabriek n.d.). One of the main targets for resource recovery is waste sludge, since the disposal of this sludge adds up to more than a quarter of the operational costs of a wastewater treatment plant (WWTP). In the Netherlands, about 350,000 ton of dry weight is produced annually (Koorneef et al. 2005). In the NAOP[1] research program, water authorities, STOWA[1], Royal HaskoningDHV and the Delft University of Technology (TUD) work together to realise the extraction of EPS from waste sludge. At the time of this study, two EPS extraction facilities are under construction. The first one in Zutphen is planned to be operational in October 2019, and will commercialise EPS as Kaumera Nereda® Gum, produced from the Nereda® waste sludge.

The general EPS retrieving process consists of an alkaline extraction step and an acid precipitation step (Felz et al. 2016). The extraction step yields roughly 25% of soluble EPS, and about 75% of alkaline residual sludge (Schaafsma et al. 2016). This solid, alkaline waste stream consists of an inorganic fraction, and an organic fraction of mainly proteins, carbohydrates, and lipids. In the Netherlands, waste sludge is either (i) incinerated, and efforts are made to recover nutrients; or (ii) digested anaerobically, and some additional energy is generated (Wiegant et al. 2005).

By anaerobic digestion (AD), the sludge is degraded, and its components are converted to methane and CO_2 in a series of conversions. These conversions are carried out by a microbial consortium of hydrolytic, acidogenic, acetogenic and methanogenic bacteria and archaea (**Figure 1**) (Kleerebezem 2014). In general, methane is produced via a hydrogenotrophic or an acetoclastic pathway, carried out by distinct types of methanogens. However, literature reports that acetoclastic conversions are often limited at alkaline and/or extreme conditions such as high ammonia concentrations (Schnurer and Nordberg 2008). At these conditions, acetate can be oxidized by syntrophic acetate oxidizing bacteria (SAOB). The produced H_2 and CO_2 is then consumed by hydrogenotrophic methanogens, to produce methane (Timmers et al. 2018). The conversions are given in **Table 1**.

[1] Dutch full name: Nationaal Alginaat Onderzoeksprogramma (NAOP) and Stichting Toegepast Onderzoek Waterbeheer (STOWA).

Table 1 | Methane production and thermodynamic calculations of the hydrogenotrophic, acetoclastic and syntrophic conversions. ΔG^1 is calculated using media concentrations, assuming formate in equilibrium with bicarbonate and hydrogen gas: pH 9.6, ionic strength 0.5 M, P_{H2} 10^{-4} bar, HCO_3^- 0.33 M, P_{CH4} 0.1 bar, CH_3COO^- 10 mM.

Micro-organism	Reaction	ΔG^1 $(kJ\ mol^{-1})$
Hydrogenotrophic methanogens	$HCO_3^- + 4\,H_2 + H^+ \leftrightarrow CH_4 + 3\,H_2O$	- 90
Acetoclastic methanogens	$CH_3COO^- + H_2O \leftrightarrow CH_4 + HCO_3^-$	-90
Syntrophic acetate oxidizing bacteria	$CH_3COO^- + 4\,H_2O \leftrightarrow 2\,HCO_3^- + 4\,H_2 + 1\,H^+$	+4.1
SAOB + hydrogenotrophic	$CH_3COO^- + H_2O \leftrightarrow HCO_3^- + CH_4$	-90

To reduce the total processing costs of the residual sludge after EPS extraction (**Figure 2**), this study gives a first outlook to implement treatment with AD at haloalkaline conditions. It encompasses the possibilities to minimize the organic content and to create value by means of methane production.

Although alkaline pre-treatment of substrates is widely explored as a way to improve its biodegradability (Gonzalez et al. 2018), AD at neutral pH remains the standard. Alkaline extraction of EPS can be regarded as a pre-treatment step, and instead of adjusting the pH back to neutral; direct AD at these haloalkaline conditions could be performed.

It is known that methanogenic archaea are present in alkaline soda lakes (Sorokin et al. 2015). Only a few studies so far, have used a haloalkaline microbial consortia obtained from soda lakes to produce biogas (Dragone 2016; Van Leerdam et al. 2008; Nolla-Ardèvol 2014). The research performed at the TUD in 2016, explored AD of the residual sludge after EPS extraction in a haloalkaline medium (pH 9.6, [Na+] 0.6 M) (**Figure 3**), and was the starting point of this research. There, it was shown that it is possible to anaerobically degrade this residual sludge with the production of biogas. This study aims to further explore the mechanisms of haloalkaline AD and to research the conditions and parameters that influence the process.

One of the main advantages of anaerobic digestion at alkaline conditions is the possibility to produce a biogas rich in methane that can be implemented directly as an energy source with a high calorific value. At neutral pH, ratios of 25-60% CO_2 and 40-75% CH_4 are usually obtained (Ryckebosch et al. 2011). However, the pK value of CO_2/HCO_3^- is 5.2 for the conditions used in this study (**Figure 4**). Thus the fraction of CO_2 is minimal at pH 9.6, and remains in solution as (bi)carbonate. This yields a biogas predominantly composed of methane (>95%).

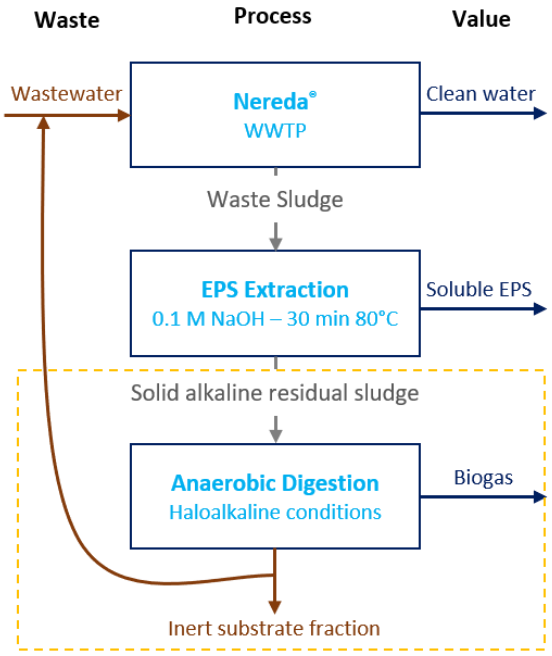

Figure 2 | Process scheme of Nereda® wastewater treatment, EPS extraction and the proposed anaerobic digestion of the alkaline residual sludge.

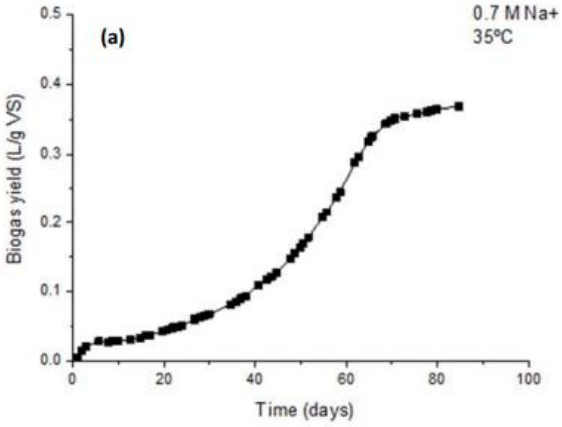

Figure 3 | Preliminary result TUD (Dragone, 2016): Cumulative biogas yield of alkaline AD of residual sludge after EPS extraction of AGS (from lab-scale set-up). Four phases in the batch process can be distinguished: initial (day 0-5); lag (5-25); exponential (25-70); and stationary phase (70-85).

Another advantage of AD at alkaline conditions is that the acidification of the system due to an imbalance in the production and consumption rates of volatile fatty acids (VFAs), is less likely to occur. The pKa values of VFAs are such that at alkaline conditions, most are in the dissociated form. Thus, they cannot passively cross the cell membrane and disrupt the proton balance (**Figure 5**, [5]). The same is true for sulphide compounds, as the main molecules are present as HS^- which also results in lower concentrations of H_2S in the biogas (Sousa et al. 2015).

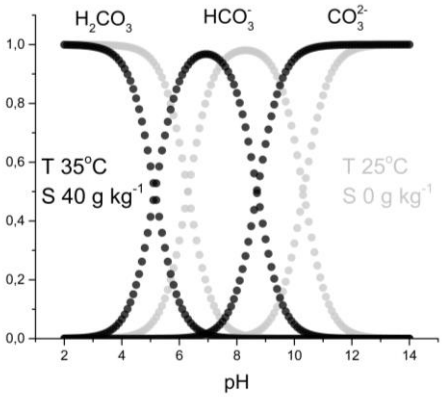

Figure 4 | The shift in dissociation constants due to temperature and salinity. Grey: standard conditions; black: conditions used in this study, T=35°C and salinity (S)=40 g kg^{-1}. At pH 9.6, the fraction of CO_2 approaches zero (Papadimitriou et al. 2018).

The alkaline conditions do introduce a new challenge in the form of NH_3 toxicity. The dissociation constant of NH_4^+/NH_3 is 9.25 at standard conditions. Thus, at the conditions used in this study, pH 9.6, the majority of the molecules will be in the NH_3 form. Therefore, passive diffusion of NH_3 does disrupt the proton balance and H^+ needs to be compensated from catabolic reactions or active transport. This is energy demanding, which cannot be used for methane production or other conversions essential in the AD chain (**Figure 5**, [1-4]) (Sousa et al. 2015). For this study a maximum of 30 mM NH_4-N was used as a guideline, a number obtained for methanogens after adaptation by D. Sorokin (unpublished data).

The biogas yield that can be obtained for a certain substrate is the starting point of a business case for an anaerobic digester (Angelidaki et al. 2009). An indication of degradability is given by the C:N ratio: for residual sludge these values for a lab- and full-scale installation are 21 and 23, respectively. For aerobic granular sludge, this value is somewhat lower: 16, because it still contains EPS, which is mainly composed of proteins (Felz et al. 2019). Generally, a ratio between 20-30 is considered as suitable for AD (at neutral pH) (Puyuelo et al. 2011).

To explore the possibilities for efficient processing of the alkaline sludge residue, the following research questions were proposed to guide this study:

i. What is the biogas yield of the solid residual sludge substrate, originating from alkaline extraction processes?
ii. What is the calorific value of the produced biogas?
iii. What fraction of the solid residue substrate is biodegradable?
iv. What is (are) the rate-limiting step(s) in the haloalkaline anaerobic digestion process, and can the efficiency of this step be increased with practically feasible adaptations?
v. What is the impact of cation composition of the medium on anaerobic digestion?
vi. Which microbial communities are behind the alkaline anaerobic digestion process?

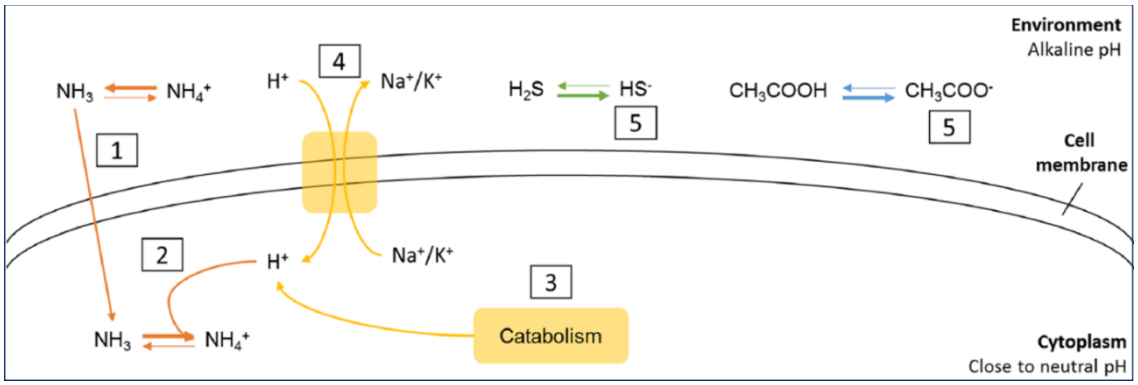

Figure 5 | Effect of ammonia, sulphide, and acetate (model for VFAs) at alkaline pH oh the cell homeostasis of micro-organisms. Picture adapted from (Sousa et al. 2015).

2 Materials and Methods

2.1 Materials

2.1.1 Inoculum

A mixture of sediments originating from hypersaline soda lakes of the Kundula steppe in the Altai province of Russia (Sorokin et al. 2015) and biomass from a preliminary study on haloalkaline anaerobic digestion of solid waste substrate (Dragone 2016) is used as inoculum. The mixture is made anaerobic by flushing with argon in the gas phase and stored in the dark at 4 °C in glass serum bottles.

2.1.2 Substrates

Aerobic granular sludge and its residue after alkaline EPS extraction were used as substrates. For this research, we used AGS from the full-scale (AGS-FS) Nereda® municipal WWTP installation in Vroomshoop, the Netherlands, and AGS from a lab-scale sequencing batch reactor fed with acetate, ammonium, and phosphate, operated at TUD (AGS-LAB). For more details see Pronk et al. (2016; 2015). From these granules, EPS was extracted under alkaline conditions, as previously described by Feltz et al. (2019). 0.1 M NaOH was used at 80 °C for 30 minutes, followed by centrifugation where the solid residue was separated from the supernatant that contains the soluble EPS. In general, the substrates were lyophilised and subsequently ground with mortar and pestle before being sealed and stored at room temperature. For one test series, residual sludge was stored at -20 °C directly after extraction and used as "wet substrate".

A sonicated substrate suspension was used to determine the parameters total nitrogen (TN) and total chemical oxygen demand ($_T$COD), while lyophilised substrate was used to determine volatile solids (VS), total solids (TS) and ash contents (**Table 2**). For details of this analysis and pictures of the substrates see **Appendix 1**.

Table 2 | Substrate characteristics of granular and residual sludge originating from both lab- and full-scale reactors. Data was gathered from multiple extraction batches that were mixed together and analysed 3-4 times over the course of 7 months. Lab-scale granules (AGS-LS) were collected fresh and processed immediately; full-scale granules (AGS-FS) from Vroomshoop were collected before the start of this project and were kept at -20 °C upon processing.

	AGS-LAB*	residue-LAB	AGS-FS	residue-FS
$_T$COD (g gVS^{-1})	1.30	1.44 ± 0.1	1.34 ± 0.3	1.45 ± 0.1
TN (mg gVS^{-1})	92	67.8 ± 18.4	84.4 ± 19.2	62.8 ± 6.7
Ash content (%)	23	14.9 ± 2.0	15.1 ± 5.0	24.9 ± 5.0
VS/TS (%)	78	85.1 ± 2.0	83.8 ± 3.7	75.1 ± 5.0

*Data from Shupan Chen (2019).

The theoretical methane production was calculated as described in Kleerebezem (2014), based on the organic dry matter (gVS gTS^{-1}), $_T$COD and TN content of the substrates. From these measurements, the elemental substrate composition $C_cH_hO_oN_n$ and the stoichiometry of the anaerobic digestion is estimated (**Table 3**). Theoretical methane production was calculated from the stoichiometric CH_4/CO_2 ratio and the biogas production upon full carbon degradation of the substrate:

$$Biogas\ (L) = \frac{organic\ dry\ matter}{12+h+16o+14n}(1-n)\cdot V_m \qquad \text{with } V_m \sim 22.4 \text{ L mol}^{-1}$$

This model does not take into account carbon usage for growth, maintenance, and reduction due to the presence of other electron acceptors, such as sulfate. In the results section of this study, the theoretical methane production is further simplified, based only on the $_{T}$COD measurements of the substrate and the COD value of methane: 4 gCOD/gCH_4.

Table 3 | Theoretical production of methane in mL per gram of volatile substrate (TP_{CH4}) and the expected CH_4 and CO_2 percentages, calculated as described in Kleerebezem (2014).

Substrate	Composition	Degradation	TP_{CH4} (mL gVS^{-1})	CH_4 (%)	CO_2 (%)
residue-lab	$CH_{2.8}O_{0.67}N_{0.12}$	$-1\ CH_{2.8}O_{0.67}N_{0.12} - 0.19\ H_2O$ $+0.64\ CH_4 + 0.24\ CO_2 + 0.12\ NH_4HCO_3$	570	72	28
AGS-FS	$CH_{2.7}O_{0.72}N_{0.13}$	$-1\ CH_{2.7}O_{0.72}N_{0.13} - 0.20\ H_2O$ $+0.61\ CH_4 + 0.26\ CO_2 + 0.13\ NH_4HCO_3$	550	70	30
residue-FS	$CH_{2.8}O_{0.65}N_{0.11}$	$-1\ CH_{2.8}O_{0.65}N_{0.11} - 0.16\ H_2O$ $+0.65\ CH_4 + 0.25\ CO_2 + 0.11\ NH_4HCO_3$	570	72	28

2.1.3 Medium

Two carbonate/bicarbonate buffer solutions were prepared, (i) sodium-based: 15 g L^{-1} Na_2CO_3 and 20 g L^{-1} $NaHCO_3$; and (ii) potassium-based: 19.6 g L^{-1} K_2CO_3 and 23.8 g L^{-1} $KHCO_3$. These solutions were completed with 3 g L^{-1} NaCl, 1 g L^{-1} K_2HPO_4, 0,1 g L^{-1} $MgCl_2$, 0.1 ml L^{-1} of 1 mM Se/W solution, and acidic trace metals (Pfennig and Lippert 1966) (**Table 4**). The solutions were used either as full sodium medium (pH 9.56, Na [0.6 M]); 20/80 potassium/sodium medium (K^{+}_{20}-medium, pH 9.60) or diluted sodium medium with pH adjusted with HCl (pH 9.60, Na [0.3 M]). All buffers were stored at room temperature.

Table 4 | Basic composition of medium solutions added to the sodium or potassium carbonate/bicarbonate buffer.

NaCl (g L^{-1})	**3**	0,05
K_2HPO_4 (g L^{-1})	**1**	0,007 mol
$MgCl_2$ (g L^{-1})	**0.1**	0,001 mol
1 mM Se/W (mL L^{-1})	**0.1**	
Acidic trace metals (mL L^{-1})	**1**	
EDTA (Trilon B) (g L^{-1})	5	
$FeSO_4$ x $7H_2O$ (g L^{-1})	2	0,003
$ZnSO_4$ x $7H_2O$ (g L^{-1})	0.1	
$MnCl_2$ (g L^{-1})	0.03	
H_3BO_3 (g L^{-1})	0.3	
$CoCl_2$ x $6H_2O$ (g L^{-1})	0.2	
$CuCl_2$ (g L^{-1})	10	
$NiCl_2$ x $2H_2O$ (g L^{-1})	20	
Na_2MoO_4 x $2H_2O$ (g L^{-1})	20	

2.2 Methods

2.2.1 Reaction vessels set-up

Multiple reaction vessels of different volumes were operated as batch experiments with anaerobic haloalkaline conditions at 35 °C. Mainly, serum bottles with 50 mL working volume and 67.2±0.3 mL headspace volume were used (**Figure 6**, A). The bottles were closed with rubber stoppers and made anaerobic by flushing the headspace with argon gas (<5 ppm O_2); see full protocol in **Appendix 2.** The addition of one drop dithionite (10% solution in carbonate/bicarbonate buffer) ensured anaerobic conditions in the bottle. The serum bottles were incubated in a 35 °C stove, unstirred. The gas volume was measured directly via a gas-tight 10 mL syringe and a needle through the rubber stopper, as the increased pressure due to gas production in the bottle pushes the plunger of the syringe out. A small overpressure due to the syringe resistance and gas temperature of 35 °C (V_m ~ 25.3 L mol^{-1}) was assumed. As soon as the gas volume reached ~10 mL it was stored for analysis at room temperature.

Also, six reaction vessels with a working volume of 250 mL and a headspace volume of 359±2 mL headspace volume were operated (**Figure 6**, B). Incubation at 35 °C was done in a water bath, placed on a magnetic stirrer and gas production was measured via water displacement. As only the bottom half of the reaction vessel was submerged in the water bath, the gas temperature was assumed to cool down to 25 °C (V_m ~ 24.5 L mol^{-1}). A more detailed description of this set-up can be found in **Appendix 3**. The reaction broth was stirred up to day 62, then until the end of the incubations, the bottles remained unstirred.

Finally, a 2 L stirred tank reactor with a working volume of 250 mL was operated with additional argon (<5 ppm O_2) gas flow through the liquid (**Figure 6**, C). This gas flow provided a 0.2 bar overpressure in the vessel in order to prevent oxygen from leaking in. The flow was set at 10 mL min^{-1} for the first 60 days, and with a mass spectrometer (MS) the off-gas composition (N_2, O_2, H_2, Ar, CO_2, CH_4) was measured once an hour. After 60 days, the gas flow was put to the minimal value of 2 mL min^{-1} to maintain the 0.2 bar overpressure, however, this provided too little off-gas flow for the MS to perform a correct measurement. Then, the gas composition was measured only once a week with a temporary gas flow of 50 mL min^{-1}. In 112 days, 13 samples of ~500 µ reaction broth were taken. pH was monitored continuously with a Metrohm 827 pH probe in the reactor.

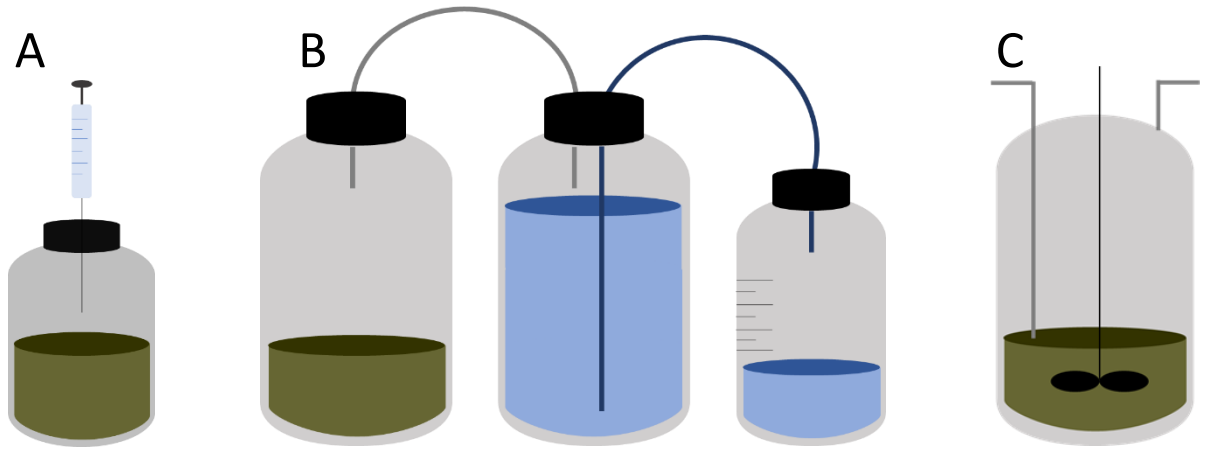

Figure 6 | Schematic overview of reaction vessels: A | serum bottles 50 mL with syringe; B | reaction vessels 250 mL with water displacement; C | bioreactor 250 mL with continuous off-gas mass spectrometry.

2.2.2 *Experiments*

Multiple enrichment batch series were performed for all three substrates: (i) residual sludge from lab-scale AGS; (ii) residual sludge from full-scale AGS; and (iii) full-scale AGS. In the prepared alkaline media, 7.5 g L^{-1} substrate was suspended (Dragone 2016). For the first round, 2.5 mL of the combined inoculum was added and for successive enrichments, biomass was transferred with a 1:10 dilution. Biomass was sampled from the reaction liquid, after vigorous shaking and settling of coarse particles. In series 2 and 4 (if applicable), a sample (0.4 µL) was taken at day 30 with a syringe and needle through the rubber stopper. Samples at the end were collected simultaneously with biomass transfer.

Figure 7 summarizes the conducted experiments: three substrates and media, 2-4 enrichment series, lyophilised (standard) and wet substrate, and scale up to 250 mL systems. The replicates that failed in terms of biogas production are faded in the figure. Series 1 was used as an inoculum activity test, and as activity was observed a new round was started. This first series is not considered in the results, as no additional analysis were performed with these bottles.

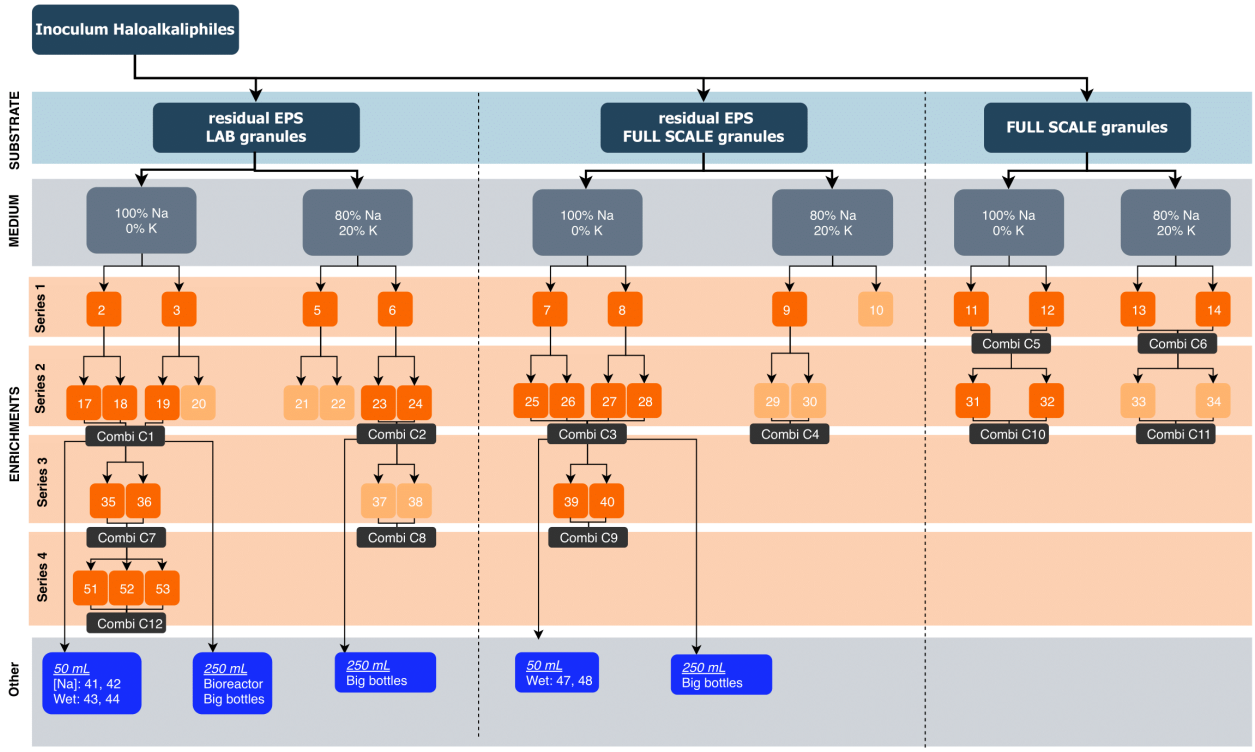

Figure 7 | Overview of conducted experiments. Enrichment broth of positive replicates (■) were combined prior to dilution and transfer to a successive series; non-positive replicates (■). Substrates were lyophilised, except for one test with *wet* substrate. Sodium medium was used as 0.6 M, except for one test with 0.3 M [Na^+].

2.2.3 *Analytical methods*

Next to the continuous measurements of the bioreactor and biogas production monitoring on a daily basis, other parameters were analysed periodically or only at the end of a batch. Biogas from the headspace was sampled and stored in 10 mL glass vials completely filled with a saturated brine solution (NaCl, 26%), which allows for optimal storage due to the low gas solubility. Typically, the brine solution was replaced by 9 mL gas and stored at room temperature until analysis. Biogas composition (N_2/O_2, CH_4, and CO_2) was analysed with a Scion 456-gas chromatograph (Scion Instruments BV, NL) with a porapac column and a TCD detector.

Reaction broth was analysed for total and soluble components (centrifugation and supernatant filtration with a 0.45 µm syringe filter). Total and soluble oxygen demand ($_{T}$COD and $_{S}$COD resp.), total nitrogen (TN), orthophosphate and total phosphate (PO_4-P and TP resp.) were analysed with HACH colorimetric methods (Hach Lange GmbH, Germany). Ammonium (NH_4^+-N) was analysed with colorimetric methods performed by the Gallery™ discrete analyser (ThermoFisher Scientific Inc., USA). pH was measured with a Metrohm pH probe 827 (Metrohm, NL). Volatile fatty acids were analysed with a Waters high-performance liquid chromatograph (HPLC) (Waters Corp., USA) with a BioRad Aminnex HPX-87H column and UV detector (210 nm). The mobile phase was 1.5 mM H_3PO_4 and a flow of 0.6 mL min^{-1} was used. Methylene blue method was used for sulphides determination, as described in Trüper and Schlegel (1964). TS and VS were analysed following the 2540B and 2540A methods of the American Public Health Association (APHA 1995).

At the end of the enrichment batch tests, or during the bioreactor operation, its microbial community was analysed with phase-contrast microscopy with the 'Zeiss Axioplan 2 Imagining' microscope and the Axiovision Release 4.8 software. The metagenome was analysed via 16s-rRNA next-generation sequencing (NGS). From 1 mL of reaction broth, cells were collected by centrifugation, and rRNA was extracted using the DNeasy UltraClean Microbial extraction kit (Qiagen GmbH, Germany) with modifications of the protocol for an alternative lysis: this included a combination of 5 minutes of heat (65 ^{o}C) followed by 5 minutes of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction, the DNA was quantified using a Qubit 4 (Thermo Fisher Scientific, U.S.A.). Samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S-rRNA gene (position 341-806) on an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and OTUs were generated on the base of ≥ 97% identity. Subsequently, microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0). For phylogenetical determination, a most recent SSURef database from SILVA (http://www.arb-silva.de/) was used. As the results of the metagenome analysis came in at the very last minute, these will only be included in the thesis presentation.

3 Results

This study explores the anaerobic digestion at haloalkaline conditions, pH 9.6, [Na^+] 0.6 M, of sludge residue after EPS extraction from aerobic granular sludge (originating from both lab and full-scale reactors). As a comparison, aerobic granular sludge from full-scale reactors itself was also used as a substrate. The biogas production of all three substrates was studied in multiple set-ups, which included analytical measurements and microbial community analysis. In this study a mixed sediment inoculum originated from hypersaline lakes was used.

The main results presented in the following chapter will be of the batch enrichment series of the haloalkaline anaerobic digestion of the three substrates. Also, a first attempt was made to go from batch to semi-continuous process, with the set-up of a bioreactor with continuous gas composition monitoring. For this, the best performing substrate (residual sludge(LAB)) and the inoculum from the enrichment series 2 were chosen and started up as a batch, with the option to add new substrate as soon as the system would reach exponential phase. Unfortunately, this point was never reached and also in this scaled-up system the biogas production never really picked up. These results will be presented at the end of this chapter.

Additional studies can be found in the appendix. **Appendix 4:** A comparative study on the influence of salt concentration and cation composition (Na^+ vs. K^+). **Appendix 5:** A comparative study on the influence of substrate preparation (lyophilised vs. 'wet'). **Appendix 6:** Scale-up: in order to monitor the dynamics in the batch tests more closely, the experiments were also scaled up for the residual sludge substrates. However, within the duration of this thesis, there seemed a problem with the biogas production – it stagnated after a limited production period, and these tests did not yield new insights.

3.1 Substrate degradation at alkaline conditions without inoculum

Prior to the anaerobic digestion, the degradation of the insoluble substrates is studied in the alkaline medium without the addition of the inoculum. This allowed for a differentiation between degradation induced by the alkaline conditions and potential remaining biological activity present in the substrate; against the activity of the haloalkaliphilic microbial community.

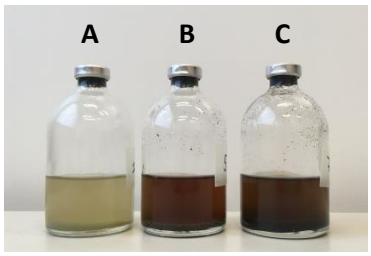

Figure 8 | Substrate suspended in alkaline medium. A | residual sludge(LAB); B | residual sludge(FS); C | granular sludge(FS).

All three substrates were suspended in Na^+-medium (7.5 g L^{-1}), incubated at 35 °C (**Figure 8**), while sampling and the analysis were performed at day 7, 11, 38 and 56 for pH, sCOD, NH_4-N and VFAs (**Figure 9**). Also, gas production was monitored but there was no measurable methane production during the incubation period.

Figure 8 shows the colour difference between the substrates: light colour for lab-scale residual sludge and dark for the full-scale residue and granules. Lab-scale AGS reactors are fed with synthetic wastewater with acetate as C-source and nutrients, resulting in light coloured granules. Full-scale reactors take in more complex wastewater, and the dark colour of those granules is partly caused by the accumulation of the phenolic substances (Felz et al. 2019).

The substrate mainly consists of carbohydrates, lipids and proteins and alkaline hydrolysis will degrade these building blocks into smaller compounds. Proteins will be degraded into peptides and its amino acids, releasing NH_3 in the process (Warner 1942). Hydrolysis of lipids leads to de-esterification and the production of VFAs (Brockerhoff 1962). Formate and acetate are also known to be non-carbohydrate substituents on the exopolysaccharides, which can be de-esterified by the alkaline treatment (Flemming and Wingender 2010; Sutherland 1970).

As expected in this test in alkaline medium, for all substrates, $_T$COD is partially solubilized and the VFA formation causes the pH to drop. The VFAs detected are mainly formate, acetate, propionate, and butyrate. Longer chain VFAs are not included in this result set, as there was a discrepancy in the results of the HPLC (as well as with a check with gas chromatography).

For all substrates, the main degradation takes place within the first ten days (**Figure 9**). Granular sludge(FS) shows the steepest increase in sCOD and NH_4^+-N, as this substrate still contains EPS, which is alkaline soluble. Before comparing the parameter concentrations per substrate, it is important to consider their differences in organic dry matter content[2] (see §2.1.2). For example, the lab-scale residue contains 10% more VS/TS compared to its full-scale equivalent and it is therefore likely that the lab-scale variant will release more C- and N-components.

For residual sludge(LAB) (**Figure 9,** upper graphs), the initial $_T$COD added (9.2 g L^{-1}) was converted to sCOD with a concentration of 4.8 g L^{-1} after 56 days. About 60% of this sCOD is made up by VFAs: 3±0.4 g L^{-1} after 56 days. Acetate was measured as the main VFA, with concentrations from 15 to 30 mM between day 11-56. Formate, propionate, and butyrate were measured all below 2.5 mM. The propionate and butyrate concentrations appear to level off after day 10 and remain unchanged until the end of the experiment. The formate concentration goes from 1 mM at day 10 to zero, which could indicate microbial activity. NH_4^+-N reaches a concentration of 270 mgN L^{-1} after 56 days, which is around 60% of the theoretical N-release upon full degradation of the substrate (based on TN content substrate). The acidification due to the formation of VFAs causes the pH to drop, but as more NH_4^+-N is produced than VFAs (between day 38-56), the pH slightly increases again.

The full-scale equivalent, residual sludge(FS) (**Figure 9,** middle graphs), has significantly less sCOD released: of the initial $_T$COD added (8.2 g L^{-1}), 2.6 g L^{-1} was converted to sCOD after 56 days. About 60% of this sCOD is made up by VFAs: 1.5±0.3 g L^{-1} after 56 days. Here, acetate concentration remains constant after day 11, at 16 mM. sCOD and also NH_4^+-N do not increase significantly after day 11, and NH_4^+-N reaches a final concentration of 130 mgN L^{-1}, about 40% of the theoretical N-release.

Finally, the granular sludge(FS) (**Figure 9,** lower graphs), is comparable to the residual sludge(LAB) in terms of trend and C- and N-release. The initial $_T$COD added (8.4 g L^{-1}) was converted to sCOD, with a concentration of 4.4 g L^{-1} after 56 days. About 55% of this sCOD is made up by VFAs: 2.5 g L^{-1} after 56 days. However, acetate release is lower: 25 mM after 56 days. This granular sludge has a higher N content due to the presence of EPS, that reaches a NH_4^+-N concentration of 340 mgN L^{-1}, which is 65% of the theoretical N-release.

For all substrates, it is observed that VFAs are produced in the first ten days, with formate, butyrate and propionate present and acetate as the major fraction. Also, the main fraction of the N-content of the substrate is released as NH_4^+-N in the first ten days.

The same substrate concentrations and medium are used in the AD enrichment tests, and the results from this section are considered as background activity next to the haloalkaliphilic inoculum activity.

[2] In this study, substrate is added in gTS, as due to the heterogenic nature of the material the VS content can vary per replicate. It would overcomplicate the experiments to calculate each time the exact VS content.

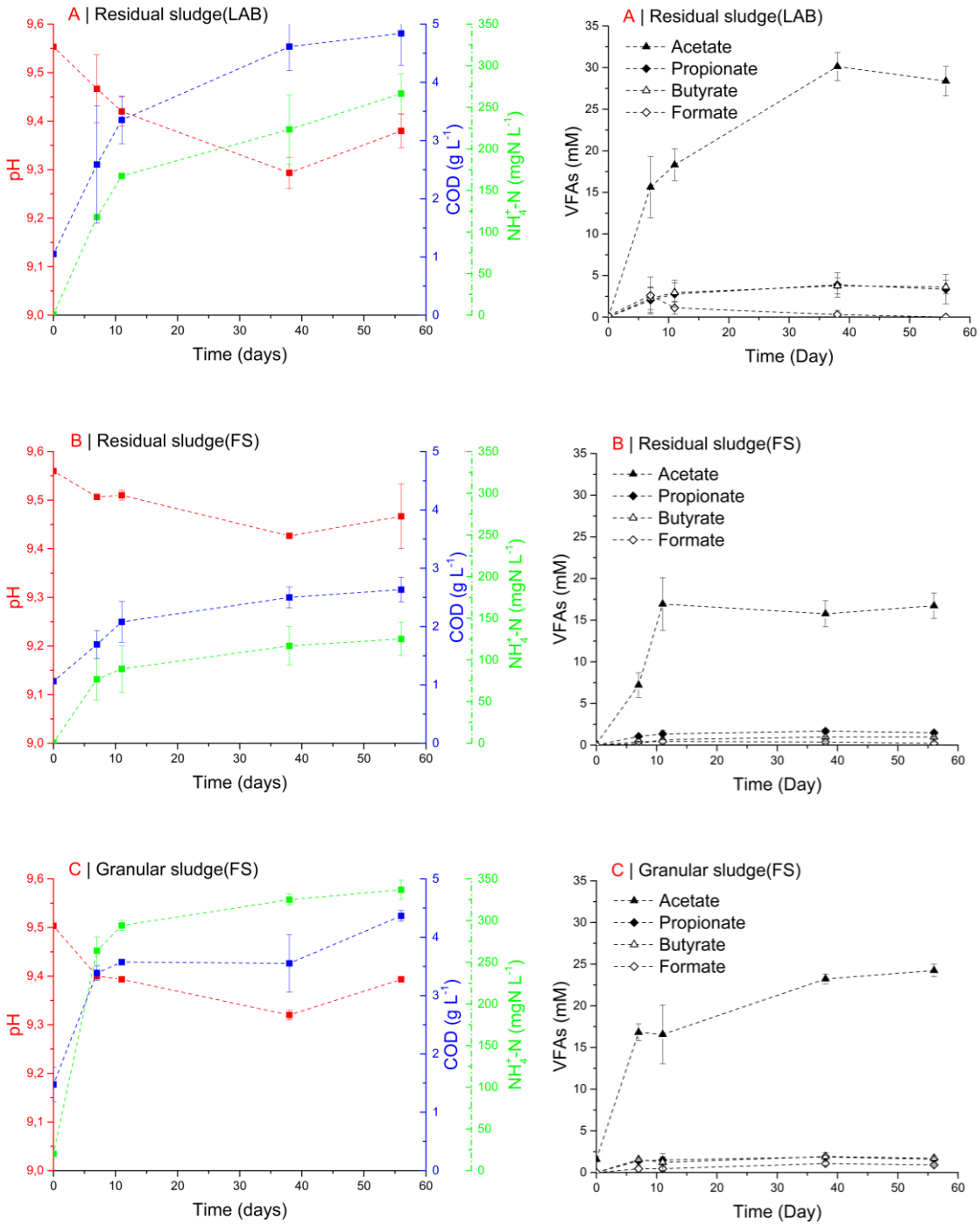

Figure 9 | Substrate degradation in alkaline medium, without inoculum. Tests are performed in triplo for each substrate. Dotted lines are only guidelines. LEFT: (■) pH; (■) sCOD (g L^{-1}); (■) NH_4-N (mgN L^{-1}); RIGHT: VFAs (mM).

3.2 Haloalkaline anaerobic digestion of residual and granular sludge: the enrichments

A preliminary study performed in 2016 showed that anaerobic digestion of residual sludge(LAB) in a haloalkaline medium yielded a biogas rich in methane. In this study, the experimental set-up was repeated in 2-4 enrichment batch series for all substrates with the objective to select for an adapted microbial community. As the first series was just to show inoculum activity, these results are not included in this section. With the assumed simplification that the produced biogas consists of only CH_4 and CO_2, 98% and 2% are obtained respectively; regardless of the substrate. Therefore, the gas production is shown as a methane yield: CH_4 produced over initial substrate $_TCOD$ ($gCOD/gCOD_{in}$). Biogas yields and parameters such as pH, COD and NH_4^+-N [3] varied among the substrates and between enrichment series and will be shown in this section.

3.2.1 Residual sludge(LAB)

For all enrichments of residual sludge(LAB), the $_TCOD$ of the substrate recovered as methane is 40-50%: shown as 0.5 $gCOD\ gCOD_{in}^{-1}$ in **Figure 10**. This corresponds to a cumulative biogas production of 240±30 mL gVS^{-1} at the end of the exponential phase (day 62).

The enrichment series with 2-4 replicates are combined in **Figure 10**, and the analytical parameters are summarized in **Table 5**. Of all 9 tests, one replicate failed and was left out from the results. The biogas production trend from the batch experiments exhibited a very similar trend over the progressive enrichments. First, an initial phase is observed (day 0-10), then a lag phase (day 10-30), followed by an exponential phase (day 30-60), and finally a stationary phase where no significant biogas production occurs.

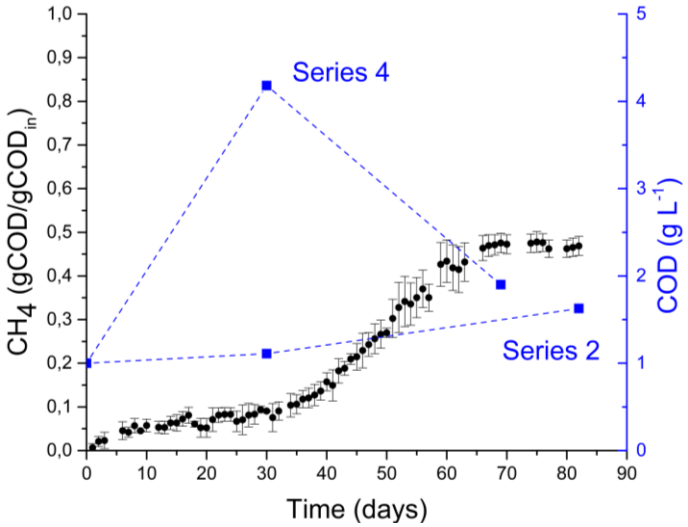

Figure 10 | Anaerobic digestion enrichments of residual sludge(LAB). Cumulative CH_4 production (●) ($gCOD\ gCOD_{in}^{-1}$) of series 2, 3 and 4 combined. sCOD (■) ($g\ L^{-1}$) is shown for series 3 and 4. Results of replicates are averaged, dotted lines are only guidelines.

So far, there is no indication that the microbial community is adapting based on the gas production trend. However, a difference can be observed in the sCOD concentrations between series 2 and 4, indicated in blue in **Figure 10**. At day 30, the sCOD release increased from 1.1 to 4.2 $gCOD\ L^{-1}$ between the successive series. Also, the NH_4-N increases over the progressive enrichments, with the main part released in the first 30 days and a final value of 250 $mgN\ L^{-1}$ (**Table 5**). Both observations are an indication that the hydrolytic microorganisms are adapting to the conditions and are able to hydrolyse the substrate with a higher rate.

Parallel to the sCOD, also the VFA concentrations increase over the progressive enrichments. It is expected that at the end of the exponential phase, the VFAs are consumed. Surprisingly, at the end of enrichment series 4, a surplus of 10.4 mM acetate remains. This is much more compared to the previous two series, and it could be that the digestion was not finished yet. However, it is not clear why in series 2 and 3, the remaining 1.4 mM acetate is not consumed. Also, propionate seems to build up during the batch.

Some additional analysis were performed at the end of enrichment series 2: Ortho-phosphate was measured at 210 $mgP\ L^{-1}$; and sulphur components were only present in small amounts: 66 µm L-1 free sulphides and no FeS was observed.

[3] The colorimetric method to determine NH_4^+-N includes acidification of the sample to obtain the protonated form. However, as the pKa of NH_3 is 9.25, the major fraction will be present as free ammonia in the AD tests.

Table 5 | Anaerobic digestion enrichments of residual sludge(LAB). Parameters measured for series 2, 3 and 4. Results of replicates are averaged and *Zero shows the parameters derived from the substrate degradation (§2.1), at day 0.

	Zero*	**Series 2**		**Series 3**	**Series 4**	
Day	**0**	**31**	**82**	**69**	**29**	**60**
pH	9.6	9.1	9.2	9.1	9.2	9.2
Yield ($gCOD/gCOD_{in}$)	-	0.1	0.5	0.4	0.1	0.4
NH_4^+-N ($mgN\ L^{-1}$)	0	210	220	180	220	250
sCOD ($g\ L^{-1}$)	1.1	1.1	1.6	2.0	4.2	1.9
$_T$VFA ($gCOD\ L^{-1}$)	0	0.9	0.7	1.0	3.3	1.3
Acetate (mM)	0	11.5	1.4	1.3	43.2	10.4
Propionate (mM)	0	1.4	7.7	11.6	6.2	8.3

At the end of the digestion, the microbial community was analysed under the microscope. With autofluorescence, methanogens can be distinguished from other micro-organisms in the sample. According to D. Sorokin, methanogens of the genus *Methanosarcina* can be observed (**Figure 11**), as well as methanogens of the genus *Methanocalculus.* In addition, large aggregated bacteria with storage polymers were observed, which will be discussed in more detail in §3.3.

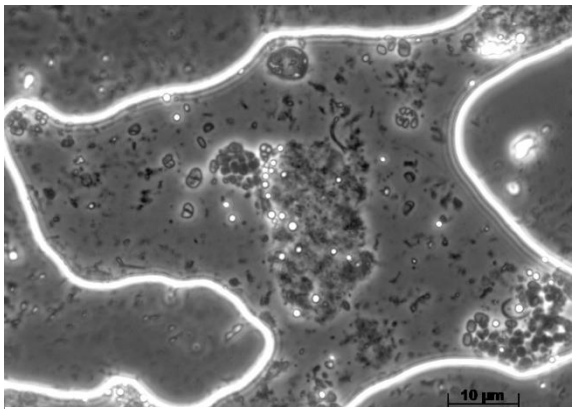

Figure 11 |Phase contrast microscopic image of the biomass from haloalkaline AD enrichment series 2, at t=82 days. Methanosarcina-like aggregates can be observed with F420 type of autofluorescence.

3.2.2 Residual sludge(FS)

The first AD enrichment of residual sludge(FS) did not yield significant biogas production: after 100 days the $_T$COD of the substrate recovered as methane is 10%. This is shown as 0.1 gCOD $gCOD_{in}^{-1}$ in **Figure 12** (in black). The successive enrichment (in white) was more promising, with 40% of $_T$COD recovered as methane. This corresponds to a cumulative biogas production of 200 mL gVS^{-1} at day 80.

The biogas production trend (**Figure 12**) of series 3 shows an initial phase (day 0-10), then a lag phase (day 10-40), followed by an exponential phase (day 40-80). Compared with residual sludge(LAB), both lag and exponential phase are 10 days longer.

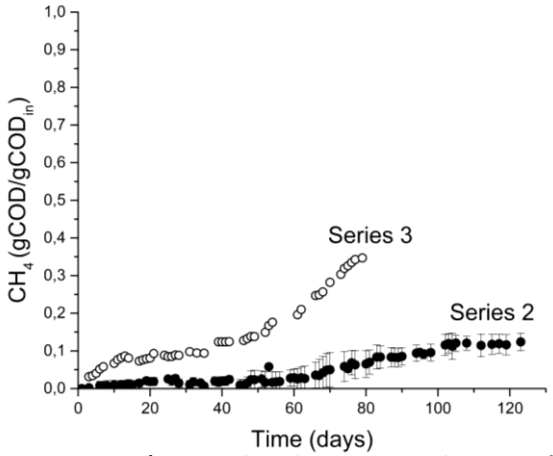

Figure 12 | Anaerobic digestion enrichments of residual sludge(FS). Cumulative CH_4 production ($gCOD/gCOD_{in}$) of series 2 (●) and 3 (○).

The analytical parameters are summarized in **Table 6** with four replicates for series 2 and two replicates for series 3. Li et al. (2019) describe the inhibition of humic acids on hydrolytic bacteria and methanogens. The hydrolytic bacteria appear to have adapted to the conditions as in series 3, more COD is converted to CH_4 and a higher final NH_4^+-N concentration is obtained of 150 $mgN\ L^{-1}$. Compared to residual sludge(LAB), the full-scale variant leaves no measurable VFAs at the end of the digestion and the 0.6 $g\ L^{-1}$ sCOD left-over is of more complex nature and difficult to degrade.

Table 6 | Anaerobic digestion enrichments of residual sludge(FS). Parameters measured for series 2 and 3. Results of replicates are averaged and *Zero shows the parameters derived from the substrate degradation (§2.1), at day 0.

	Zero*	**Series 2**		**Series 3**
Day	**0**	**31**	**123**	**80**
pH	9.6	9.4	9.4	9.3
Yield ($gCOD/gCOD_{in}$)	-	0.01	0.1	0.35
NH_4^+-N ($mgN\ L^{-1}$)	0	70	110	150
sCOD ($g\ L^{-1}$)	1.1	-	0.3	0.6
$_T$VFA ($gCOD\ L^{-1}$)	0	1.0	0	0
Acetate (mM)	0	18.4	0	0
Propionate (mM)	0	1.8	0	0

3.2.3 Granular sludge(FS)

For the two enrichments of granular sludge(FS), the $_T$COD of the substrate recovered as methane is 20-30%: shown as 0.3 $gCOD\ gCOD_{in}^{-1}$ in **Figure 13**. This corresponds to a cumulative biogas production of 130±25 $mL\ gVS^{-1}$ at day 75.

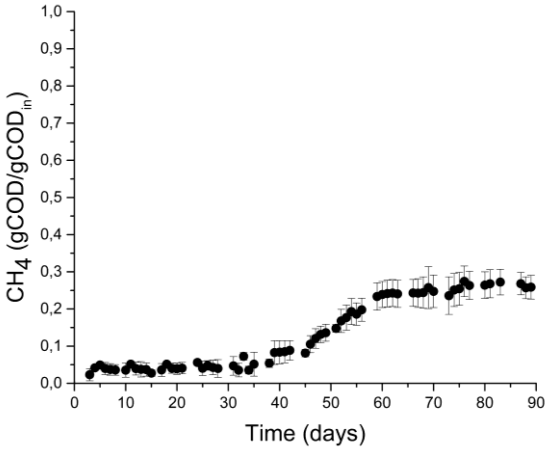

Figure 13 | Anaerobic digestion enrichments of granular sludge(FS). Cumulative CH_4 production ($gCOD/gCOD_{in}$) of the combined series 1 and 2 (●).

Figure 13 combines the results of both enrichment series, with two replicates each. The biogas production trend shows a limited initial jump, then a lag phase until day 40, followed by an exponential phase (day 40-60), and lastly a stationary phase where no significant biogas production occurs. The bottles of series 1 were kept until day 126, in order to see if biogas production would reinitiate. No biogas production was observed, but additional hydrolysis might have occurred. As the sCOD and NH_4^+ are analysed after the 126 days, which could make these results less comparable to the successive enrichment at day 76. These analytical parameters are summarized in **Table 7**. NH_4^+-N concentration immediately increases and does not seem to change much over the batch period, which is in agreement with the results obtained for the substrate degradation experiments (§3.1). All VFAs are consumed, but about 1 $gCOD\ L^{-1}$ is left over at the end of digestion.

Table 7 | Anaerobic digestion enrichments of granular sludge(FS). Parameters for series 1 and 2. Results of replicates are averaged and *Zero shows the parameters derived from the substrate degradation (§2.1), at day 0. **Yield at day 75.

	Zero*	**Series 1**		**Series 2**
Day	**0**	**31**	**126**	**76**
pH	9.6	9.3	9.3	9.2
Yield ($gCOD/gCOD_{in}$)	-	0.05	0.3**	0.2
NH_4^+-N ($mgN\ L^{-1}$)	20	230	240	300
sCOD ($g\ L^{-1}$)	1.5	-	1.4	0.9
VFA ($gCOD\ L^{-1}$)	0	1.9	0	0.02
Acetate (mM)	0	29.1	0	0.2
Propionate (mM)	0	0	0	0

3.3 Production of storage polymers under strict anaerobic conditions

In addition to this study, Lena Depaz wrote her BSc thesis titled 'Characterisation of storage polymers during anaerobic digestion in haloalkaline conditions'. At the end of the AD batch test, storage polymers were observed when analysing the microbial community under the microscope (**Figure 14**, A). Lena characterised these storage polymers as polyhydroxyalkanoates (PHA) with BODIPY® staining (**Figure 14**, B). The occurrence of PHA is highly unexpected in a strict anaerobic system, as it is usually produced aerobically and anaerobic production has not been studied in great detail. Although absolute amounts were difficult to quantify (and were probably minor), most storage polymers were found in the systems with residual sludge(LAB).

Lena tried to find a correlation between the PHA, and the left-over sCOD and VFAs at the end of the batch digestion. These observations point to some kind of inhibition or limitation occurring in the system, that most probably hampers the production and final biogas yield. With additional experiments, an attempt was made to point out the crucial factor for these mechanisms. Adding acetate, trace metals, or diluting out reactants such as NH_4^+ and propionate did not yield conclusive results. All details of this work can be found in the BSc thesis of Lena Depaz. (Depaz 2019)

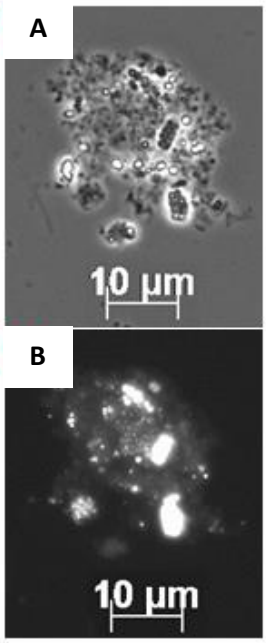

Figure 14 | Storage polymers observed in residual sludge(LAB). A | phase contrast; B | added BODIPY staining to show PHA storage bodies.

3.4 The bioreactor – towards continuous operation

The objective of the bioreactor was to make the first steps from batch to semi-continuous operation. For this, the best performing substrate (residual sludge(LAB)) and the inoculum from enrichment series 2 were chosen and started up as a batch, with the option to add new substrate as soon as the system would reach exponential phase.

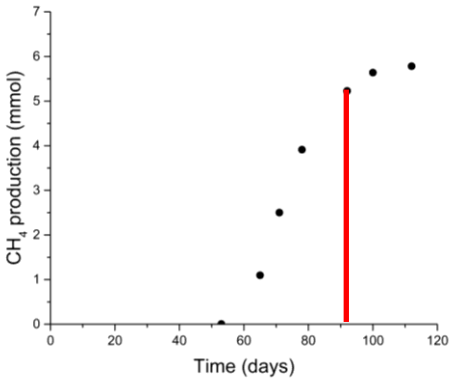

Figure 15| Bioreactor AD: accumulative CH_4 production (mmol) (●).

A 2 L bioreactor was operated with 250 mL working volume, and it was expected that this limited volume would produce a relatively small flow rate of biogas for the mass spectrometer to measure. Therefore, an additional carrier gas flow through the liquid was added (argon < 5 ppm O_2). A minimal flow rate of 10 mL min^{-1} ensured sufficient gas flow to the MS, whilst still containing a detectable amount of CH_4 during the exponential phase. At the same time, NH_4^+-N was effectively stripped out. Unintended, also CO_2 was stripped from the carbonate/bi-carbonate buffer, which caused the pH to increase (**Figure 16**). No measurable methane production was observed even though sCOD and acetate were present, about 5 g L^{-1} and 50 mM respectively. At day 60, the carrier gas flow was lowered to 2 mL min^{-1} as a measure to stimulate biogas production. As a result, an increase in CH_4 production was obtained at day 65 (**Figure 15**), but no long-term consumption of the VFAs was maintained. Methane production was no longer observed at day 112, and a surplus of 35 mM acetate remains. The accumulative methane production is probably underestimated, as before day 60 no measurable amounts were detected by the MS and the limited data points after day 60 make the integration results less accurate. Overall, the obtained biogas yield is about 80 mL gVS^{-1}; much less than the 240 mL gVS^{-1} obtained in the batch enrichment experiments. As a final note, H_2 gas appears in the MS data as soon as CH_4 production levels off.

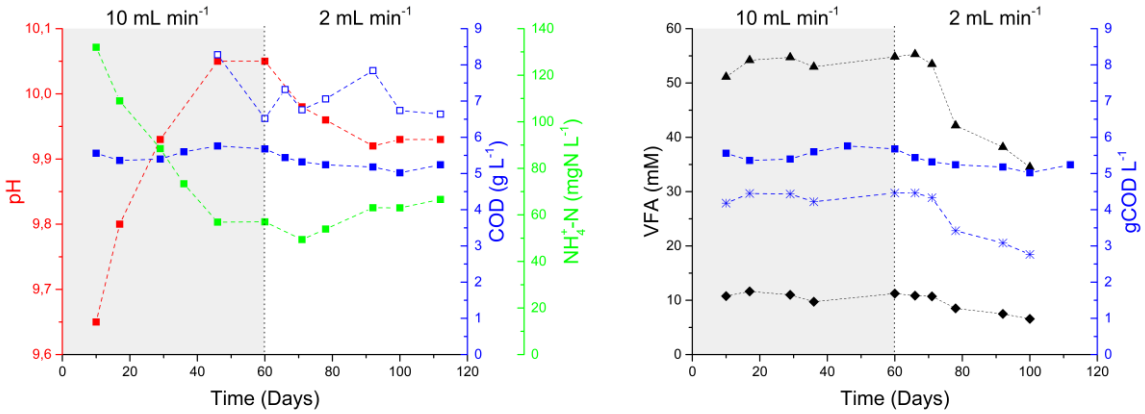

Figure 16 | Bioreactor: anaerobic digestion of residual sludge(LAB). Left: (■) pH; (■) sCOD (g L^{-1}); (□) tCOD (g L^{-1}); and (■) NH_4-N (mgN L^{-1}). Right: VFA production (*) $_T$VFA (g L^{-1}); (■) sCOD (g L^{-1}); (▲) Acetate (mM); (◆) Propionate (mM).

4 Discussion

The aim of this study was to investigate anaerobic digestion of the residual sludge after EPS extraction at haloalkaline conditions. It encompasses the possibilities to minimize the organic content and to create value by means of methane production. This was accomplished with various set-ups, different salt media, and different substrate preparation methods. Three substrates were compared: residual sludge after EPS extraction, originating from both lab and full-scale reactors; and aerobic granular sludge from full-scale reactors itself. The focus of this discussion is on biogas production and hypothesises on the rate-limiting step(s) in the process; biogas composition and the biodegradability of the substrates; and other inhibition factors and bottlenecks.

4.1 Biogas production

Biogas production was achieved for all three substrates used. Due to the alkalinity of the medium, the biogas contains a high methane content: up to 98%. The trends of the AD tests and the possible rate-limiting step(s) will be discussed in this section, as well as a comparison of the biogas yields.

4.1.1 Batch process

Different final yields are obtained for different substrates, but the biogas trend during the batch tests is quite comparable. An initial phase is followed by a long lag phase, then an exponential phase, and finally a stationary phase.

First, an initial jump in biogas production appears within the first 10 days of digestion. This shows the activity of the methanogens and indicates the limited influence of oxygen exposure on these micro-organisms during biomass transfer in between enrichments. The residual sludge(LAB) released 1 mM of formate in the first 10 days of the substrate degradation experiment (§3.1). Formate never appears during AD experiments in the HPLC analysis, thus indicates the presence of easily degradable organic matter that is converted to formate that is directly consumed by methanogens.

After the initial phase, an exceptionally long lag phase of about 20 days, is observed. This period is not shortened over the progressive enrichments. The results from the substrate degradation (§3.1), show that next to formate, at least 15 mM acetate is already available at day 10. In the AD batch test series 4, 40 mM acetate is available at day 30. Therefore, the hydrolysis and acidogenesis do not appear to be the rate-limiting step. It remains unclear why the biogas production does not pick up, even though there is substrate available and the methanogens already showed activity. The systems seem to halt at acetate, and microscopic analysis and studies on other anaerobic digestion systems operating under extreme conditions suggest the absence of acetoclastic methanogens. The literature points to an alternative route for acetate conversion: syntrophic acetate oxidation via the Wood-Lungdjahl pathway (Timmers et al. 2018). This is an energetically difficult pathway and might require very specific conditions before the exponential phase takes off. An additional hypothesis is that these micro-organisms decay during the stationary phase or as a result of oxygen exposure during biomass transfer. Either way, the biomass concentration might be low, and it takes time to grow into a concentration that produces significant biogas production. This is especially true for the SAOBs, as a doubling time of 28 days is reported (Westerholm et al. 2016).

Between day 30-40, the biogas production goes into an exponential phase. For both full-scale originating substrates (residual and granular sludge); all VFAs are consumed at the end of the exponential phase. Some sCOD is left, which was unidentified, but this is probably less easily degradable. For the residual sludge(LAB), VFAs are still present at the end of the exponential phase, most likely caused by inhibition that prevents the last VFAs being consumed. The exponential phase ends abruptly, and no more significant amounts of biogas are produced.

Unfortunately, the results of the metagenome analysis did not arrive in time so the dominant species of the microbial community could not be confirmed. For further research, additional mcrA analysis could be performed to determine the different methanogen species, as described in Sorokin et al. (2015). In parallel to the mcrA analysis, stable isotope fingerprinting could be used to study the activity and metabolic pathways, as described in Nikolausz et al. (2013).

As a final note, the gas production measurement methods were prone to leakage of some gas, along the needle in the rubber stopper. However, this amount is considered insignificant on the total average production of 90 mL during this period.

4.1.2 Biogas yields

The biogas production for the substrates used in this study yielded promising results for the residual sludges (**Table 8**). For residual sludge(LAB), the substrate recovered as methane was about 40-50%, which is in line with the %theoretical production (%TP_{CH4}) based on the model of Kleerebezem (2014). It seemed that over the progressive enrichments, biogas yield got somewhat lower but due to the different digestion times this is difficult to conclude. Comparable with the results from Dragone (2016), the production in mL gVS^{-1} is on the low side. This is surprising, as this study used the inoculum from Dragone and it was expected that biogas production would improve over the progressive enrichments. As Dragone does not include analytical results of the batch experiments, it is difficult to compare and to determine the reason for the different biogas yields. Also, the effect of storage of the inoculum for two years is not known.

At the end of digestion of residual sludge(LAB), acetate is consumed for the largest part, but propionate seems to have accumulated. These left-over amounts of VFAs are surprising compared to the other substrates, about 1 gCOD L^{-1} on average versus none for the full-scale substrates. This can be calculated back to an additional 55 mL CH_4 gVS^{-1} that could have been produced, which is an increase of about 20%.

Biogas production from residual sludge(FS) improved in the last enrichment to 40% substrate recovered as methane. This amount is in line with the calculated %TP_{CH4}. However, this is a lower yield compared to residual sludge(LAB). The difference could be explained by the observations in the substrate degradation experiments (§3.1). There, it is shown that about 50% of the $_T$COD is converted into sCOD for residual sludge(LAB). For residual sludge(FS) this is only 30%. In the AD test, these values would be higher due to the additional microbial hydrolytic activity, but it gives an indication that the $_T$COD of residual sludge(FS) contains more complex organics. Nonetheless, the obtained 40% methane recovery is a promising number and it might be possible to increase it over new enrichments.

Lastly, aerobic granular sludge(FS) yields the lowest biogas production of the three substrates. The 30% biogas yield is in agreement with results obtained at neutral pH for AGS (unpublished results Hongxiao Guo, TUD). In this study, biogas yield did not change much in progressive enrichments. The amounts of sCOD released of granular sludge(FS) is similar to that of residual sludge(LAB), but NH_4^+-N is released faster. Therefore, conditions for granular sludge might be harsher at the start of the AD than for residual sludge due to free NH_3 toxicity.

So far, two other studies are known that have performed haloalkaline AD. Van Leerdam et al. (2008) co-digest methanethiol and methanol, and the results of this N-free substrate are less comparable with this study. Nolla-Ardèvol et al. (2014) digest the micro-algae *Spirulina*, a biomass type of substrate with a major protein fraction. Their obtained results are far from 250 mL CH_4 gVS^{-1}, obtained at mesophilic pH. Also, all three substrates used in this study perform better than *Spirulina*.

Table 8 | Comparison of biogas yields and theoretical production (TP_{CH4}) obtained in this study + literature results.

	residue-LAB	**residue-FS**	**AGS-FS**	**Dragone (2016)[1]**	**Ardèvol (2014)[2] *Spirulina***
Yield ($gCOD_{CH4}$ $gCOD_{in}^{-1}$)	0.5	0.4	0.3	-	-
Production (mL gVS^{-1})	240±30	200	130±25	360	85
TP_{CH4} (mL gVS^{-1})	570	570	550	570	627
%TP_{CH4} produced	42	35	24	63	12.5
%CH_4	98	98	98	98	93
%CO_2	2	2	2	-	0

1: Dragone (2016) used substrate residual sludge(LAB), batch configuration at pH 9.6 and Na+ 0.7 M.
2: Nolla-Ardèvol (2015) used substrate microalga *Spirulina*, batch configuration at pH 10 and Na^+ 2.0 M.

4.1.3 Biogas composition

With the anaerobic digestion of all substrates, biogas composed of 98% CH_4 and only 2% CO_2 was obtained. These results are in agreement with similar research from Dragone and Nolla-Ardèvol (**Table 8**). The biogas composition in this study is calculated based on the assumption that the gas only contains CH_4 and CO_2, and other components usually present in biogas from AD such as small amounts of H_2S, NH_3, water vapor and trace amounts of H_2, N_2 and CO (Ryckebosch et al. 2011) are neglected. All these elements were not analysed, except for H_2 with the biogas set-up, which was not detected. Only small amounts of sulphides were measured in the liquid at the end of an AD batch test. The equilibrium shifts towards HS^- at pH 9, which makes the presence of significant amounts of H_2S gas unlikely. Research with a similar substrate also reports no detection of H_2S (Nolla-Ardevol et al. 2015). NH_3 certainly goes to the gas phase; this is even more pronounced as the content of the bioreactor is mixed or a carrier gas bubbles through, as seen for the significantly decreasing NH_4^+-N concentration in the bioreactor.

The results obtained by Nolla-Ardèvol also suggest that the shift from batch to (semi-)continuous configuration might influence the obtained biogas composition. In batch experiments, 93% CH_4 was obtained (**Table 8**). In their study with a semi-continuous stirred tank reactor, biogas with a 86±5% CH_4 and 4±3% CO_2 composition was obtained. No H_2S gas was detected and the composition of the remaining 10% gas is not reported.

Nonetheless, the methane content of the gas is high and can be directly implemented without the need for post-treatment to remove CO_2. This gives an advantage over conventional AD at pH 7. However, if this research is continued it is advised to study the biogas compositions derived from the substrate used in this study and its variations. Some of the aforementioned gasses, such as H_2S and NH_3, are corrosive for the equipment and can be harmful to both the environment and human health and must be removed in the process.

In conclusion, the high methane content makes this gas suitable to be used as biomethane. It can be used as fuel for vehicles, fed to the national gas supply grids or used for energy production. Assuming the gas contains only methane + inert CO_2, the lower calorific value would be 49 MJ kg^{-1} (Engineering Toolbox 2003). Apart from the energy itself, another advantage is the successful capturing of CO_2 in the liquid that reduces the costs for post-treatment. However, in order to make a real sustainable impact, a destination for the CO_2 has to be found to result in a negative emission.

4.2 Biodegradability improvement

The biodegradability of the substrate is expressed as the percentage of the theoretical biogas production, based on the initial COD, TN and organic content of the substrates. Comparing the full-scale residual and granular sludge, the alkaline pre-treatment improved the biodegradability of the granular sludge. This could be an added incentive for both the implementation of full-scale EPS extraction and anaerobic digestion at haloalkaline conditions.

The remaining $_T$COD of the substrate, which is not converted to CH_4, is distributed over (i) an inert fraction; (ii) sCOD that can consist of VFAs and non-degraded organic material; and (iii) some PHA accumulated in cells. As discussed before, if converted, the VFAs could lead to additional biogas production. It is also possible that the other non-degraded sCOD is biodegradable. This could be studied with an aerobic digestion test. The pH of the reaction broth could be neutralized and inoculated with activated sludge. pH could be controlled if necessary, and sCOD could be monitored for consumption. As it appears that full biodegradability is not reached yet, the final inert fraction of the substrates was not determined.

4.4 Inhibitory compounds and possible bottlenecks

Accumulation of PHA in cells and surplus sCOD and VFAs at the end of a digestion experiment points towards the occurrence of inhibition. For residual sludge(LAB), the $_T$COD recovered as methane is 40-50%. This is quite high, comparing this number to the haloalkaline AD of algae substrates by Nolla-

Ardevòl (**Table 8**). However, at the end of enrichment series 4, a surplus of acetate and propionate are measured. Also, over the progressive enrichments NH_4^+-N keeps increasing. This section will discuss the inhibitory compounds and bottlenecks that might influence the haloalkaline AD process.

4.4.1 Degradation of propionate

Acetate and propionate are the two VFAs present in measurable amounts during the batch AD tests. The major part of acetate is consumed, the hypothesis of the syntrophic pathway is already discussed. Propionate, however, appears to accumulate and is left-over at the end of digestion for residual. Propionate is known to be difficult to degrade anaerobically and a syntrophic partner is needed as propionate to acetate is unfavourable (Mucha et al. 1988). At pH 9.6, I = 0.5 M; propionate 10 mM, acetate 1 mM, bicarbonate 0.33 mM P_{H2} = 10^{-4} bar and P_{CH4} = 0.6 bar:

$$1\ CH_3CH_2COO^- + 3\ H_2O \leftrightarrow 1\ CH_3COO^- + 1\ HCO_3^- + 3\ H_2 + 1\ H^+ \qquad \Delta_r G^1 = -10.8\ kJ\ mol^{-1}$$
$$1\ CH_3CH_2COO^- + 0.75\ H_2O \leftrightarrow 1\ CH_3COO^- + 0.25\ HCO_3^- + 0.75\ CH_4 + 0.25\ H^+ \qquad \Delta_r G^1 = -30.1\ kJ\ mol^{-1}$$

When the propionate degradation is combined with hydrogenotrophic methanogenesis, a bit more energy becomes available. However, this has to be shared over two micro-organisms and is not enough to hydrolyse 1 ATP.

4.4.2 Free ammonia

At pH 9.6, most of the ammonium will be present in the form of free ammonia. This free ammonia is toxic, as it can freely diffuse over the cell membrane and disrupts the cell homeostasis. Many studies report threshold concentrations for inhibition. However, the range of concentration that is reported is rather broad. 1500-7000 mg L^{-1}, which is caused by the many substrates, inoculum, process conditions and adaptation periods used in AD (Rajagopal et al. 2013). For this study, a guideline of 30 mM or 420 mgN L^{-1} was used, obtained by D. Sorokin for adapted pure cultures of methanogens (unpublished data). In the AD set-ups in this study, the N-concentration was always under this value.

For granular sludge(FS), the substrate digestion experiment (§3.1) shows > 250 mgN L^{-1} already released at day 10. Biogas is produced from day 40, and although the final yield is lower than for the residual sludge substrates (with lower C:N ratio), the effect of ammonia on the haloalkaline microbial community is not clear.

By acclimatisation of the methanogens, a higher tolerance could be generated. However, this study shows that also the NH_4^+-N concentration is increasing over the progressive enrichments. This is hypothesised by the adaptation of the hydrolytic bacteria to the environmental conditions, and ideally a longer enrichment period is required to see how these substrates concentrations equilibrate.

To mitigate the risks caused by ammonia inhibition, several options could be considered. In a continuous system, the hydrolytic retention time could be optimized for NH_4^+-N concentration. However, there is a trade-off with maintaining sufficient sCOD and VFA concentration in order to maximize biogas production (Nolla-Ardevol et al. 2015). The substrate concentration could be lowered, either by dilution or by co-digestion of a feedstock with a higher C:N ratio (Jiang et al. 2019). The ammonia can also be removed by stripping, as shown in the bioreactor; or directly removed by a submerged membrane contactor with sulfuric acid (Lauterböck et al. 2012). Ammonia recovery and reuse would be a valuable addition to the total process scheme. For further research, it is recommended to first focus on experiments with an increased and decreased substrate concentration.

4.4.3 The influence of salt

The EPS extraction method will determine the cation composition and concentration of the residual sludge. In this study, 0.1 M NaOH was used at 80 °C for 30 minutes for the extraction step. NaOH can also be replaced for KOH, when potassium is favoured over sodium in the end-product. Therefore, the salt-tolerance to different media of the inoculum is studied. First, from the general 'full' Na^+-medium, 20% Na^+ was replaced by K^+ (K^+_{20}-medium). Second, the full Na^+-medium was diluted from 0.6 M to 0.3 M Na^+.

The most notable result of the batch AD enrichment test with K^{+}_{20}-medium, was a system failure for 9 out of 16 tests (incl. initial activity tests of series 1), i.e. no biogas was produced. sCOD and acetate are available, therefore, the hydrolysis and acidogenesis do not appear to be the rate-limiting step. Some methanogenic activity is present in the initial phase, thus the main bottleneck is once more hypothesised to be with the SAOBs. However, based on the experiments conducted in this study, the exact reason for the absence of biogas production remains unclear and more research is needed. The one batch AD test with 0.3 M Na^{+} yielded a lower biogas production compared to the 0.6 M Na^{+} medium. To determine the exact mechanisms that caused this, more research is needed.

In conclusion, the cation composition and concentration affect the microbial community at work. The SAOBs appear to have the lowest tolerance on the change in salts, but in general, the effects are not clear.

4.4.4 Phenolic compounds

Humic substances are complex molecules with a great variety of chemical structures. Humic substances are present in waste sludges originating from full-scale installations, and Li et al. (2019) describe the effect of humic acids (HAs) on the different AD phases. They demonstrated that the presence of HAs inhibited hydrolytic and methanogenic activity; and promoted acidogenic activity. HAs lowered the activity of associated enzymes, i.e. F_{420}, an important co-factor in the Wolfe-cycle for methane production from H_2 and CO_2 (Thauer 2012). Also, HAs contribute to the sCOD concentration and solubility increases with higher pH, but the compounds are difficult to degrade anaerobically. (Gonzalez et al. 2018).

In this study, a lower yield for the full-scale residue is obtained, compared to its lab-scale equivalent which does contain no or little humic substances. The C:N ratios of these substrates are comparable, and thus inhibition by HAs could be an explanation for this difference. For the residual sludge(FS), the hydrolytic bacteria seem to have adapted to the presence of the HAs over the progressive enrichments, as more sCOD and NH_4^{+}-N is released. The remaining difference between the yield could then be explained by the inhibitory effect on the methanogens, and the fact that the left-over sCOD is probably composed of difficult degradable humic substances. In conclusion, the inhibitory effects of HAs are an important parameter to consider. For instance, a residual sludge from an industrial AGS installation with low HA content might be favourable over a municipal AGS installation.

4.6 Operational challenges

In order to make the first steps towards continuous operation, a bioreactor was set-up. However, scaling up the batch experiments was not straightforward, and this section shortly discusses the operational challenges that were encountered.

4.6.1 Mixing and gas flow

Three different AD set-ups were operated in this study. The first set-up worked well: bottles with a syringe for gas volume determination and static incubation. The second set-up, bottles with water displacement and mixed incubation, only seemed to pick up biogas production after the mixing was shut down. The third set-up, a bioreactor with mixing and continuous gas flow through the liquid, showed minimal biogas production.

One explanation for malfunctioning of the bottles with water displacement is that the set-up was not sufficiently sealed and oxygen was leaking in. Another explanation could be that settling of the biomass and solid substrate formed a micro-environment. This is discussed in more detail in **Appendix 6**.

The bioreactor, however, was operated at an overpressure of 0.2 bar to prevent from oxygen leaking in and was mixed for the whole incubation period. At day 60, the gas flow of only 10 mL min^{-1} of argon (<5 ppm O_2) through the liquid was cut back to 2 mL min^{-1}. From day 65, the bioreactor shows a short period of methanogenic activity. Usually, methanogens take up hydrogen rapidly and the H_2 partial pressure is maintained at a low level (below 10^{-4} atm). This makes the syntrophic oxidation reaction of

acetate favourable. Interestingly, as the biogas production halts, detectable amounts of H_2 are measured. This indicates an inhibition of the methanogens and more extensive research is needed.

4.6.2 Substrate preparation

For easy handling, the substrate was lyophilised and then ground to a dry, powdery form. In practice, the sludge would be fed to a digester directly after centrifugation and separation from the soluble EPS fraction. The comparative study showed that substrate preparation affected the biodegradability of the residual sludge. More research is needed to explain the underlying mechanism, but it is important to consider a lower yield for 'wet' substrates in practice.

4.6.3 Guidelines for future research

The bioreactor operation yielded some valuable lessons and focus points for further research. To answer the remaining questions of this study, it is recommended to pursue continuous operation of a bioreactor. Preferably with a bigger reaction broth volume to produce measurable amounts of biogas. For practical implementations, digestion experiments could be done directly at an EPS extraction facility, as it is important to consider that the composition of AGS depends on operational factors such as solid retention time (SRT); anaerobic feeding times; and the composition of the wastewater.

For starting up a bioreactor, it is advised to mimic the operation of the small serum bottles: static incubation and no additional gas flow through the reaction broth. As soon as the exponential phase is reached, the effects of mixing and gas flow could be tested, as well as a gradual increase or decrease of potential inhibiting or limiting factors mentioned in this study. Finally, the extension of the exponential phase to a continuous biogas production can be studied.

5 Conclusion

In this study we have shown that it is possible to process the residual sludge obtained after the alkaline EPS extraction of AGS, with anaerobic digestion at haloalkaline conditions. The biodegradability of the substrate is expressed as the percentage of the theoretical biogas production, based on the initial COD, TN and organic content of the substrates. For the residual sludge(LAB), this is 42%; for the residual sludge(FS) 35%; and for the aerobic granular sludge(FS) 24%. For all substrates at the end of the degradation test, surplus sCOD was measured. For residual sludge(LAB), this sCOD includes acetate and propionate, which in theory should be converted to methane. If converted, this leads to an additional increase in biogas production by 20%.

The biogas yield was 240 mL gVS^{-1} for the residual sludge(LAB); 200 mL gVS^{-1} for the residual sludge(FS); and 130 mL gVS^{-1} for the aerobic granular sludge(FS). For all substrates, the methane content of the biogas produced, was around 98%, with 2% CO_2. The remaining CO_2 produced stays in the alkaline medium. This composition makes the obtained biogas suitable for a direct implementation as a fuel or for usage in the national gas grid with minor upgrades, with a lower heating value of 49 MJ kg^{-1}. Also, operating AD directly at alkaline digestion reduces the necessity for further addition of chemicals for pH neutralisation.

In the batch experiments, a long lag phase of 20-30 days was observed, which was not shortened by consecutive enrichments. Interestingly, in this period up to 40 mM of acetate is accumulated that, together with initial methanogenic activity in the first 10 days, could indicate that the syntrophic acetate oxidation is the limiting step.

In addition, at the end of the batch experiments, PHA storage and left-over VFA were observed. This could point towards some inhibitions and/or limitations occurring in the system. Although, the NH_4^+-N concentration increased over progressive enrichments, and with that free ammonia toxicity, no direct correlation was found for ammonia inhibition. The cation composition and concentration of the salt medium has an influence on the biogas yield. The replacement of 20% sodium by potassium frequently caused the AD to fail altogether. The mechanisms behind these observations and possible inhibitors/limitations should be a subject of further research.

In conclusion, the biogas production of residual sludge is promising in terms of biogas yield and composition. The alkaline pre-treatment improved the biodegradability of the waste sludge, which could be an added incentive for both the implementation of full-scale EPS extraction and anaerobic digestion at haloalkaline conditions. More research is needed in order to pursue the successful operation of continuous anaerobic digestion at haloalkaline conditions.

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Appendix

1 Substrates

Analysis of TN and $_{T}$COD is performed on substrate suspensions of 0.5-1 g L^{-1} (total volume ~10 mL) material in demi water that are sonicated for 2-4x 30 seconds. For the TN analysis, the length of the digestion step influenced the final result: it was observed that for three times longer digestion, the TN concentration increased by 20%. As most of the analysis are performed with only one digestion time, these results are corrected with a 1.2 factor.

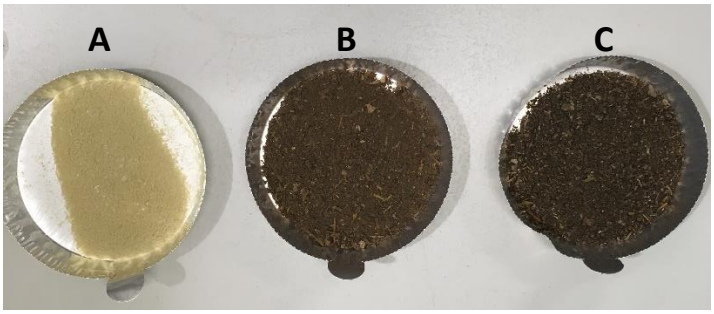


Figure 17 | Lyophilised and grinded substrates. A: residual sludge(LAB); B: residual sludge(FS); C: granular sludge(FS).

2 Gas exchange protocol

2.1 Objective

Correct use of the gas exchange set-up, for both personal safety and to prevent cross-contamination of experiments. Always ask permission to Dimitry Sorokin and indicate the amount and nature of the samples for gas exchange.

2.2 Materials

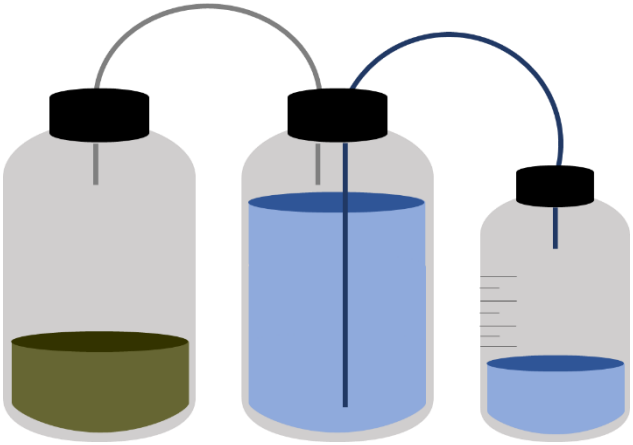
Remove clean needles and double filters of Dimitry, replace with own or used single filters.

- Needles
  - Grey for 100 mL bottles
  - Blue for >100 mL bottles
- Filters red 0.2 µm
- Gas exchanger
  - Make sure are connections are tight
  - 6 out of 8 tubes can be used (Do NOT use 2nd pair from the left side)
  - Close tube clamps

2.3 Methods

1. Argon bottle: open gas flow. Do NOT change pressure settings etc.
2. Switch to Argon flow at gas exchanger. Switch Up: argon, Switch Down: vacuum.
3. Open tube clamps and check gas flow through needles.
4. Insert needles into bottles; under an angle and make sure tip of needle ends up in the middle of the rubber stopper.
5. Switch on vacuum pump and switch gas exchanger to vacuum.
   a. Approx. 5 min for first round. Less for 2nd and 3rd round.
   b. Strike the bottle with Teflon stick on <u>bottom part</u> of bottle, until the sound becomes dull. Make sure to hold bottle at the neck, loosely, with only thumb and index finger.
   c. The solution starts to boil, foam and/or air bubbles appear. If not, remove tube clamp and try again.
   d. Rinse the bottle with the liquid (*O_2 sticks to bottle walls*).
   e. Check bottles one more time with Teflon stick.
6. Switch gas to Argon.
   a. Check to make sure the liquid is moving, max 1 min.
   b. As soon as the liquid of all bottles calms down, switch back to vacuum.
   c. *For large bottles: place needle + syringe to prevent the bottle from exploding
7. Repeat vacuum-argon two more times. No need for using the Teflon stick.
8. Finish off
   a. Shut down vacuum pump, close all clamps and Argon flow.
   b. For storage of bottles: maintain overpressure in bottle, so take needle away and store.
   c. For measurement bottles: detach needle from gas exchanger to level gas in the bottle to atmospheric pressure. Then take needle away from the bottle.

3 Water displacement



The reaction vessel (500 mL) is placed in a water bath of 35 °C with an integrated magnetic stirrer. With norprene tubing, this first vessel is connected to a water bottle (1L). As gas is produced, it flows to the water bottle headspace and pushes the water towards the collection bottle (500 mL). This collection bottle has an additional opening (not pictured), to create a driving force for the produced gas from the first towards the last bottle. For measurements of the displaced water volume, the collection bottle is detached and placed on a balance. Water density of 1 kg L^{-1} is assumed.

The whole system is made anaerobic with argon (<5 ppm O_2) prior to the start of the experiment. The reaction vessel is installed with an overpressure, and this causes an initial water displacement to level out the pressure levels. This water volume is registered and used as zero reference.

Based on the batch test in the 50 mL serum vessels, approximately 500 mL biogas could be produced, and this determined the sizing of the collection and water bottle. It is assumed that the overpressure needed for the initial push of the water is negligible and will not much influence the final biogas yield.

4 The influence of salt

EPS are a complex mixture of polymeric substances and not all components can be extracted by one single method. Therefore, the type of extraction method depends on the type of sludge and EPS of interest. In this study, 0.1 M NaOH was used at 80 °C for 30 minutes for the extraction step. NaOH can can also be replaced for KOH, when potassium is favoured over sodium in the end-product. In practice, the extraction method will determine the cation composition and concentration of the residual sludge. In this experiment, the salt-tolerance of the inoculum is studied. The inoculum is derived originally from soda-lake microbial communities and therefore, it was studied how the community would respond to different media.

Parallel to the batch AD enrichments in 'full' Na^+-medium, enrichments with a medium with 20% Na^+ replaced by K^+ were performed (K^+_{20}-medium). Residual sludge(LAB) showed activity for two replicates and the results of this enrichment will be shown in this section. Residual sludge(FS) failed altogether. Granular sludge had a similar problem as residual sludge(LAB), as the first series worked and the second one failed. These both results will not be discussed further.

Finally, the full Na^+-medium was diluted and [Na^+] concentrations of 0.3 and 0.6 were compared. Here, residual sludge(LAB) was used as inoculum as it yielded the most positive results.

4.1 Residual sludge(LAB) in K^+_{20}-medium

The first series of the batch AD enrichment for residual sludge(LAB) in K^+_{20}-medium showed activity, then biomass was transferred to four replicates. In this second series, two replicates showed activity, both with different rates and final biogas yields (**Figure 18**,black). The two positive replicates were transferred to the final, third series to two replicates. These duplicates did not yield biogas production (**Figure 18**,white). After the second series, biomass was also transferred to the scaled-up set-up. The objective to further investigate the VFA production/consumption rates could not be studied, as one of the duplicates failed and the other did not yield the expected biogas production (**Appendix 6**).

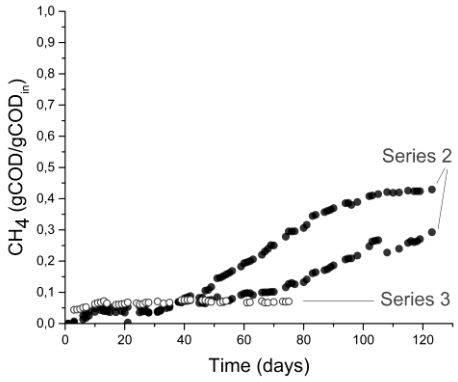


Figure 18 | Anaerobic digestion enrichments of residual sludge(LAB) in K_{20}-medium. Cumulative CH_4 production ($gCOD/gCOD_{in}$) for series 2 (only positive replicates) (●) and 3 (○).

The two positive replicates of series 2 took 123 days to produce 200 mL CH_4 gVS^{-1}. As a comparison, residual sludge(LAB) in full Na^+-medium produced 240 mL CH_4 gVS^{-1} in 70 days. The pH, NH_4^+-N and VFAs are quite similar for both media at day 30 (**Table 9**), and at the end of the digestion of series 2. At day 76 for series 3, 5.1 gCOD L^{-1} and 43 mM acetate is produced and not used for biogas production.

Table 9 | Anaerobic digestion enrichments of residual sludge(LAB) in K_{20}-medium. Parameters measured for series 2 and 3. Results of replicates are averaged and *Zero shows the parameters derived from the substrate degradation (§2.1), at day 0.

	Zero*	**Series 2**		**Series 3**
Day	**0**	**31**	**123**	**76**
pH	9.6	9.2	9.2	9.2
Yield ($gCOD/gCOD_{in}$)	-	0.04	0.4	0.07
NH_4^+-N ($mgN\ L^{-1}$)	0	210	230	330
sCOD ($g\ L^{-1}$)	1.1	1.3	2.4	5.1
VFA ($gCOD\ L^{-1}$)	0	-	1.3	3.6
Acetate (mM)	0	12.1	7.9	43.2
Propionate (mM)	0	2.0	10.1	8.5

4.2 Residual sludge in [Na^+] 0.3 M versus 0.6 M

Biomass from the residual sludge(LAB) enrichment series 2 in the 'full' 0.6 M Na^+-medium was transferred to a 0.3 M Na^+-medium. **Figure 19** shows the biogas production results of the duplicates in 0.3 M Na^+ in black, and the reference production in 0.6 M Na^+ in white. After day 25, in one of the duplicates in 0.3 M Na^+, leakage of O_2 might have occurred after gas collection, as no more gas production is observed.

For the positive replicate a similar lag phase of 30 days is observed, but the exponential phase levels off earlier for the 0.3 M Na^+ variant. Finally, 30% of the substrate $_T$COD is recovered as methane, which corresponds to 175 mL CH_4 gVS^{-1}. sCOD 1.8 g L^{-1} is left-over at the end of digestion. NH_4^+-N release is similar for both salt concentrations. For the 0.3 M Na^+ variant, pH drops to 8.5 at the end of the digestion. With the dilution of the salt medium, also the buffer capacity is decreased.

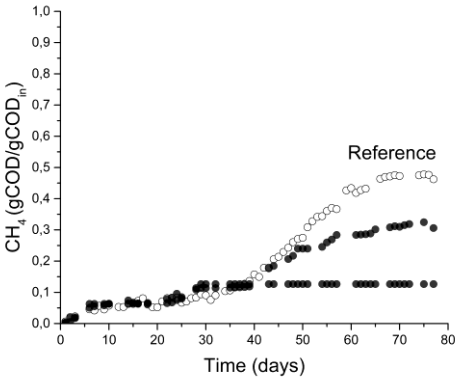


Figure 19 | Anaerobic digestion of residual sludge(LAB) in Na^+[0.3 M]-medium. Cumulative CH_4 production (gCOD/$gCOD_{in}$) duplicates (●), and comparison with substrate in full Na^+-medium (○).

5 The influence of substrate preparation

For easy handling, the substrate was lyophilised and then ground to a dry, powdery form. In practice, the sludge would be fed to a digester directly after centrifugation and separation from the soluble EPS fraction. Therefore, the anaerobic degradation of wet residual sludge from lab and full-scale granules was also performed.

For both replicates of residual sludge(LAB) (**Figure 20**, left), a lower biogas production of the wet substrate was observed compared to the lyophilised form. The lag phase seems to take about 10 days longer, and there is a significant difference between the yield of the two replicates. The difference between the two replicates of residual sludge(FS), is also significant (**Figure 20**, right). One of the replicates even obtained a higher biogas yield than its lyophilised equivalent.

The final ammonium concentration is comparable to that of the lyophilised references. For the lab-scale substrate, this is also true for sCOD and VFA concentrations. For the full-scale substrate, > 3 gCOD L^{-1} is left, of which 0.7 mM acetate and 2.2 mM propionate for the best performing replicate. Looking at the flat trend from day 65 onwards, it could be that the digestion was not finished yet and some leakage occurred when changing the syringe.

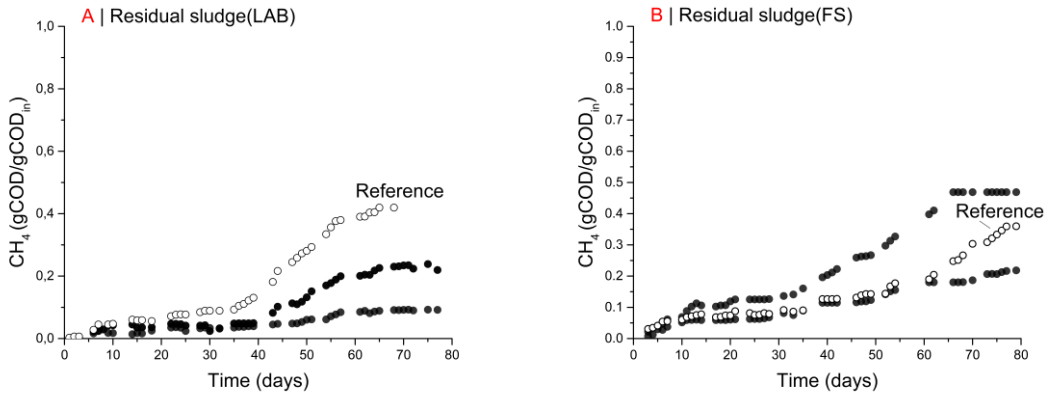


Figure 20 | Anaerobic digestion of 'wet' substrate, compared to its freeze dried equivalent (§3.2). A | residual sludge(LAB); B | residual sludge(FS) .Cumulative CH_4 production ($gCOD/gCOD_{in}$) duplicates (●), and freeze dried substrate (○).

The 'wet' substrate contains clumps of substrate clogged together. Contrary to the dry substrate powder, the clumped wet substrate has a lower surface area that makes the substrate less available for hydrolytic bacteria. This could explain the lower yield obtained for the wet substrate, for both lab and full-scale residual sludge. Furthermore, the effect of lyophilisation on the substrate is not known.

Also, full-scale AGS has a very heterogenic composition, and also contains seeds, twigs and other suspended particles submerged in the granules (see figure **Appendix 1**). The heterogeneity can be somewhat homogenized by the lyophilisation and grinding process, therefore the replicates that used this substrate preparation are comparable. The wet full-scale residual sludge however, shows a significant deviation between the yields of the two replicates. Although in a scaled-up process these differences probably become insignificant due to the volumes used, it is important to consider the possibility of a somewhat lower yield.

Obtaining a lower yield for 'wet' substrates seems contradictory to the results obtained by Nolla-Ardèvol (2014). They report that the percentage of the theoretical biodegradability almost doubled for the 'wet' substrate. Freeze dried *Spirulina* and fresh or 'wet' algal of another origin were compared. However, the 'wet' substrate appears to have a higher biomethane potential at mesophilic pH and has a lower protein content that causes much lower final NH_3 concentrations. This makes it difficult to compare the 'wet' and lyophilised substrate one on one.

6 Scale up anaerobic digestion

The objective of scaling up AD batch test was to obtain more insight in the changes that triggered the transition to a new phase during the batch period. The serum bottles with 50 mL working volume did not allow for much sampling, as this would disturb the process too much. It was expected to obtain similar biogas production trends as in the serum bottles, and to be able to take samples along the different phases (initial, lag, exponential, stationary) of the batch. However, such similar biogas production was never obtained. After a promising initial phase and increase in biogas production between day 20-30, biogas production as well as sCOD and NH_4^+-N production/consumption stagnated.

Three experiments in duplicate were set up with 250 mL working volume, mixed reaction vessels and water displacement gas measurement: (i) residual sludge(LAB) in 'full' Na^+-medium; (ii) same substrate in K^+_{20}-medium; and (iii) residual sludge(FS) in full Na^+-medium. Mixing was added as usually as some moderate mixing is usually applied in continuous systems as well. For 4 out of 5 set ups (one replicate of (ii) failed), an initial gas production was observed, as well as a promising production between day 20-30. Thereafter, production stagnated for all replicates. At day 62, the mixing by magnetic stirrers was stopped, as it was suggested that this might cause the difference compared to the static incubation of the small serum bottles. Then, for the residual sludge(LAB) in full Na^+-medium production seemed to pick up again. Unfortunately, this was not further investigated due to the duration of this thesis.

One explanation for malfunctioning of the bottles with water displacement is that the set-up was not sufficiently sealed and oxygen was leaking in. The bottleneck seems to be with the methanogenesis, as the first steps in the AD process – hydrolysis, acido- and acetogenesis, do not appear to be the rate limiting step. For example, acetate at day 30 is 47 mM and sCOD 4.5 g L^{-1} for residual sludge(LAB). If oxygen is leaking in, the mixing makes the diffusion to the liquid faster. As soon as the mixing is shut down, it could be that the methanogens are slowly regenerating. Another explanation could be that settling of the biomass and solid substrate formed a micro-environment in which diffusion limitations, Donnan equilibrium and stratification play a role in the AD conversions. Han et al. (2016), describe the pH drop in an alkaline sediment can be up to 2 units compared to the bulk liquid. In this study, lower pH could be beneficial for NH_3 toxicity, as the fraction of free form ammonia will be lower at lower pH. A difference between the sediments of residual sludge(LAB) can be observed, that could point to the formation of such a micro-environment (**Figure 24**). Some activity was observed for the inoculum in Na^+-medium, and the sediments show a darker and lighter shade with a clear interface. This colour difference is absent for the inactive sediment with K_{20}^+-medium.

Residual sludge(FS) in 'Full' Na^+-medium

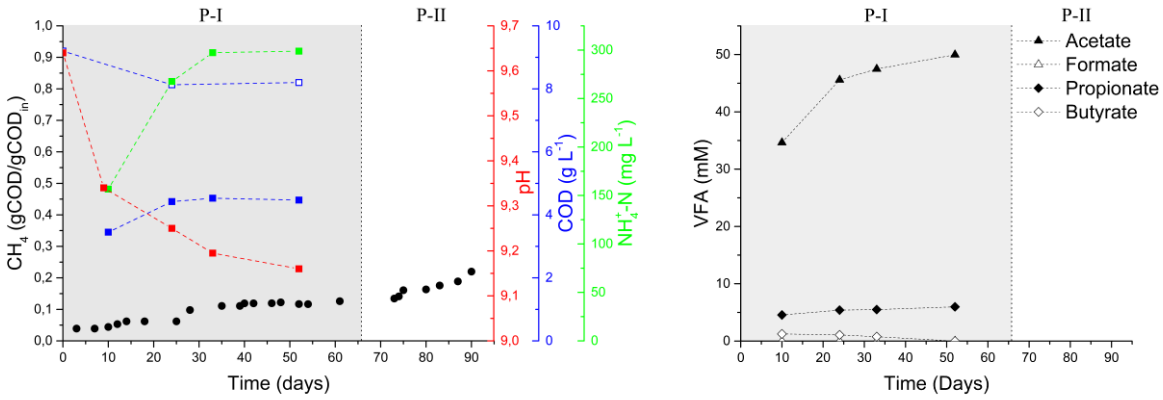


Figure 21 |Anaerobic digestion of residual sludge(LAB) in Na^+-medium: scale up parallel to enrichment series 3. Left: CH_4 production (●) (gCOD/$gCOD_{in}$); (■) pH; (■) sCOD (g L^{-1}); (□) tCOD (g L^{-1}); and (■) NH_4-N (mgN L^{-1}). Right: VFA production. Results of the duplicates are averaged. Dotted lines are only guidelines.

Residual sludge(FS) in 'Full' Na^+-medium

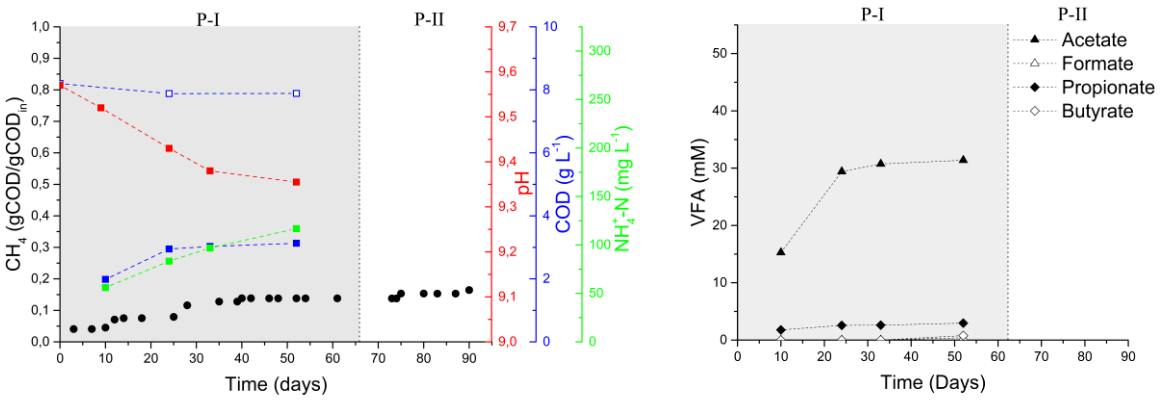


Figure 22 | Anaerobic digestion of residual sludge(FS) in Na^+-medium: scale up parallel to enrichment series 3. Left: CH_4 production (●) ($gCOD/gCOD_{in}$); (■) pH; (■) sCOD ($g\ L^{-1}$); (□) tCOD ($g\ L^{-1}$); and (■) NH_4-N ($mgN\ L^{-1}$). Right: VFA production. Results of the duplicates are averaged. Dotted lines are only guidelines.

Residual sludge(LAB) in K^+_{20} -medium

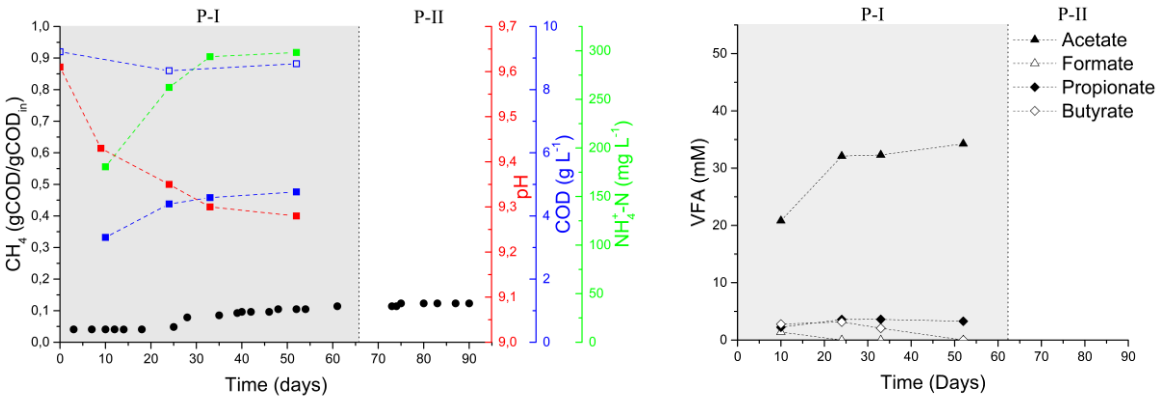


Figure 23 | Anaerobic digestion of residual sludge(LAB) in K^+_{20}-medium: scale up parallel to enrichment series 3. Left: CH_4 production (●) ($gCOD/gCOD_{in}$); (■) pH; (■) sCOD ($g\ L^{-1}$); (□) tCOD ($g\ L^{-1}$); and (■) NH_4-N ($mgN\ L^{-1}$). Right: VFA production. Only results of positive replicate is shown. Dotted lines are only guidelines.

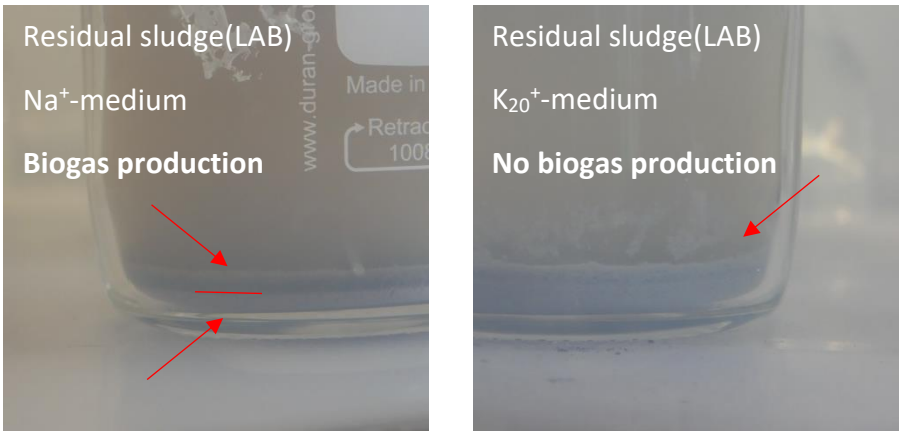


Figure 24 | Sediments of residual sludge(LAB) in full Na^+-medium (left) and K_{20}^+-medium (right). Some activity was observed for the inoculum in Na^+-medium, and the sediments show a darker and lighter shade with a clear interface. This colour difference is absent for the inactive sediment with K_{20}^+-medium.