Characterisation of storage polymers during anaerobic digestion in haloalkaline conditions

By

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Abstract

The goal of this experiment has been to characterize storage polymers produced during anaerobic digestion under haloalkaline conditions. The project focused on three research questions: the type of storage polymers produced, which conditions selected for the production of storage polymers and which organisms are responsible for the production of the storage polymers. Based on BODIPY® staining of various batch tests with different conditions, it can be concluded that the produced storage polymer is PHA. The innate fluorescence of the methanogens was used to determine that the methanogens are not the micro-organisms producing and storing the PHA. It is unclear which organisms are responsible for this. The characterisation and the staining of different digestions and the set-up of a new batch test which focused on the influence of trace metals, acetate and toxic compounds were unable to determine the exact conditions which selected for storage polymers. Follow-up research is therefore recommended, for example new digestion series with a new inoculum from the hypersaline soda lakes in Russia instead of the mixture of the sediment from the hypersaline soda lakes and biomass from a previous digestion series. It is also recommended to perform a genomics analysis of the microbial community to find a possible PHA producer.

Preface

This has been my first time working extensively on a project and it has been a very interesting and educational. Even though I expected the project to be complicated given the unique conditions of it, this project has taught me that the practice will always be more complicated than I can imagine. As my lab safety supervisor, Jure Zlopasa, often said: 'The difference between practice and theory is bigger in practice than it is in practice.'

Luckily, I was supported and helped by a lot of people in the Environment Biotechnology department. I am especially thankful to Valerie Sels, who was my mentor and lab partner during my whole bachelor thesis. I would also like to thank Jure Zlopasa and Gerben Stouten for helping me find a project that suited my interests on one hand and the many constructive and insightful discussions on the other hand. I would also like to thank Mark van Loosdrecht and Robbert Kleerebezem for agreeing to be my supervisors and taking time out of their busy schedules to tend to my Bachelor thesis.

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1 Introduction

Alkaline extraction of extracellular polymeric substances (EPS) has in recent years gained a place in the spotlight to deal with waste sludge in an environmentally and economically beneficial way. This is proven by recent commercial interest, for example the newly built EPS extraction factory by Royal HaskoningDHV in Zutphen, the Netherlands, using the Nereda® process. However, this process results in up to 70% residue. Since dealing with this residue is the most expensive part of the process, anaerobic digestion of this residue with the goal to produce biogas could prove valuable. Figure 1 shows the general process. By coupling the anaerobic digestion to the (municipal) waste sludge treatment, less waste is generated which results in a more circular economy.

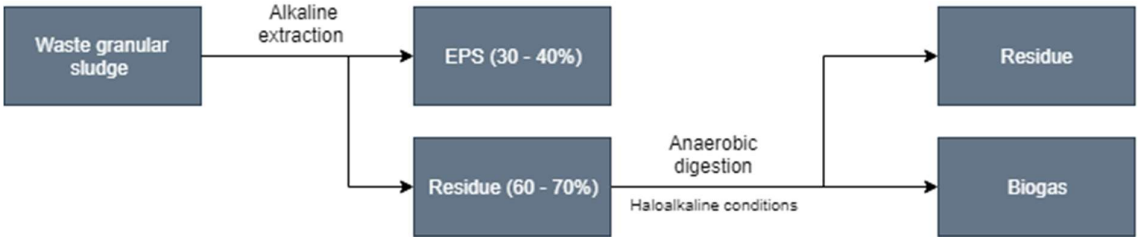

Figure 1. Overview of the overall wastewater treatment using alkaline extraction of EPS and anaerobic digestion under haloalkaline conditions.

1.1 Anaerobic digestion

Anaerobic digestion is a biotechnological process during which biodegradable material is broken down first into water-soluble organic compounds and finally into methane-rich biogas (Kleerebezem, 2015). This process is strictly anaerobic. Anaerobic digestion is preferable for producing biogas because it has a huge intrinsic thermodynamical advantage compared to chemical reactions: in a thermodynamically closed system, the organic carbon will be converted to a compound with the least amount of energy per electron, which is methane. Therefore, there is a huge thermodynamic pressure to produce methane compared to chemical reactions. However, anaerobic digestion is only thermodynamically possible due to the syntropy of bacteria and archaea. This can be seen in Figure 2. Due to the high salinity, ammonium/ ammonia concentration and volatile fatty acid concentration, it is expected that syntrophic acetate oxidation (SAO) is the dominant mechanism for methane production (Schnurer, Zellner, & Svensson, 1999).

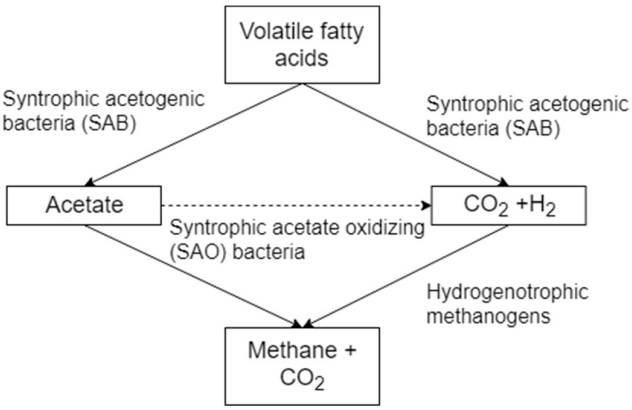

Figure 2. Schematic of the syntropy between the acetate production/ conversion, the hydrogen gas production/ conversion and the methane production

Common volatile fatty acids for the formation of acetate are propionate and butyrate. Formulas 1 to 8 give the most important reaction equations for the anaerobic digestion including their Gibbs free energy at pH 9. The used concentrations were the concentrations at day 82 of digestion 2 with rEPS-LS and Na-medium, namely 1.3804 mM for acetate, 7.27945 mM for propionate and 0 mM for formate. For H_2, a concentration of 0.1 mbar was assumed. The other concentrations were kept at standard concentrations (eQuilibrator, 2019). If no concentrations could be reliably assumed or measured, the Gibbs free energy was not calculated.

(1) $Butyrate^- + 2H_2O \rightarrow 2\,Acetate^- + H^+$ $\quad \Delta_r G'^0 = 71.5 \frac{kJ}{mol}$

(2) $Butyrate^- + 2HCO_3^- \rightarrow 2\,Acetate^- + H^+ + 2\,Formate^-$ $\quad \Delta_r G'^0 = 73.3 \frac{kJ}{mol}$

(3) $Propionate^- + 3H_2O \rightarrow Acetate^- + H^+ + HCO_3^- + 3H_2$ $\quad \Delta_r G'^0 = -43.0 \frac{kJ}{mol}$

(4) $Propionate^- + 2HCO_3^- \rightarrow Acetate^- + H^+ + 3\,Formate^-$ $\quad \Delta_r G'^0 = -43.6 \frac{kJ}{mol}$

(5) $H_2 + \frac{1}{4}HCO_3^- + \frac{1}{4}H^+ \rightarrow \frac{1}{4}CH_4 + \frac{3}{4}H_2O$ $\quad \Delta_r G'^0 = -14.8 \frac{kJ}{mol}$

(6) $Formate^- + \frac{1}{4}H_2O + \frac{1}{4}H^+ \rightarrow \frac{1}{4}CH_4 + \frac{3}{4}HCO_3^-$ $\quad \Delta_r G'^0 = -110.9 \frac{kJ}{mol}$

(7) $Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$

(8) $4H_2 + HCO_3^- \rightarrow CH_4 + 3H_2O$

Anaerobic digestion consists of 4 different stages: the hydrolysis, the acidogenesis, acetogenesis and the methanogenesis. In this reaction, the hydrolysis and the methanogenesis are the rate-limiting steps. Figure 3 shows an overview of the anaerobic digestion.

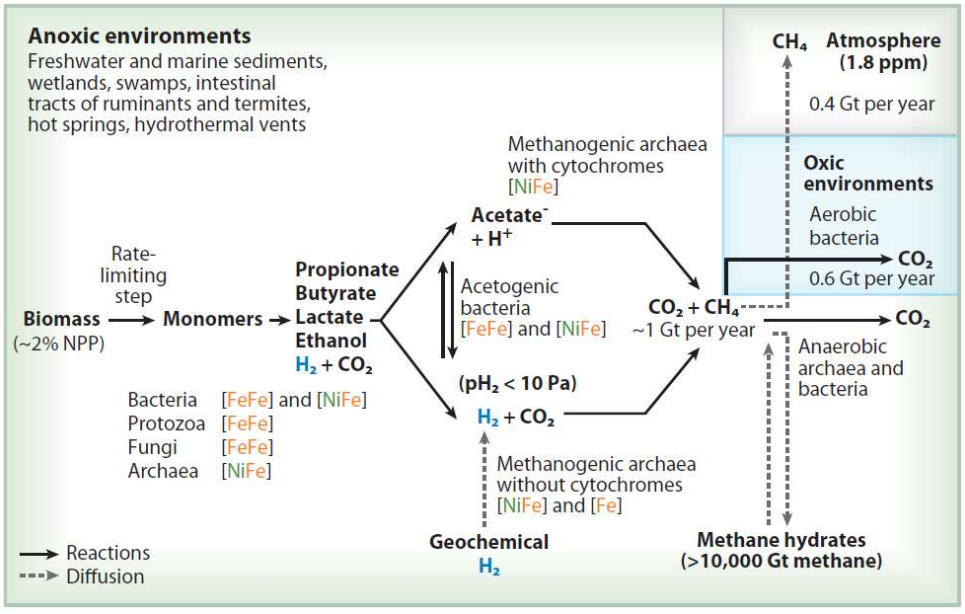

Figure 3.Schematic of the anaerobic digestion. The used hydrogenases for each reaction are indicated by [NiFe], [FeFe] and [Fe]. The required partial hydrogen pressure is also indicated. (Thauer, et al., 2010)

The acetate formation and the methanogenesis are heavily influenced by the partial pressure of hydrogen. The acetogenesis occurs at a hydrogen partial pressure between 1 and 10 Pa, while the methanogenesis by methanogenic archaea with cytochromes from acetate happens

at a hydrogen partial pressure above 10 Pa (Thauer, et al., 2010). The methanogenesis, both by methanogenic bacteria with and without cytochromes, keeps the hydrogen partial pressure low by consuming the hydrogen to produce methane and CO_2.

To use the H_2, it must be activated by hydrogenases. This can be seen in Formula 9. The hydrogenases usually require iron and nickel, but methanogens can use a hydrogenase that functions only on iron in the absence of nickel (Thauer, et al., 2010). The absence of these compounds causes a lack of hydrogen and hydrogen partial pressure, thereby stunting the methane production and causing the need for an electron sink.

(9) $$H_2 \leftrightarrows 2e^- + 2H^+$$

Anaerobic digestion usually takes place at pH 7. However, to minimize the amount of acid needed following the EPS extraction at high pH, it is possible to do the anaerobic digestion above pH 9 and with high salinity. This provides two major advantages and one major disadvantage compared to anaerobic digestion at pH 7. The disadvantage is the potential toxicity of ammonia. Ammonium has an acid dissociation constant (pK_a) of 9.4 which means that at a pH above 9.4, most of it will be in the form of ammonia. Since ammonia is an uncharged and small molecule, it can be passively diffuse over the cell membrane. This interferes with the membrane potential and disrupts the cell homeostasis (Kleerebezem, 2015).

The first advantage of the high pH is the low levels of CO_2 which will be produced. This is beneficial because CO_2 lowers the caloric value of the biogas which means that a bigger volume of the gas is needed to obtain the same amount of energy if there is a higher CO_2 content. By having a low amount of CO_2 in the gas phase, it lowers the downstream processing costs of upgrading the biogas.

Furthermore, the second advantage of the high pH is avoiding the possible toxicity of acetic acid. At low pH, the concentration of acetic acid will be high. Like ammonia, acetic acid can passively diffuse over the cell membrane and interfere with the cell membrane potential. Both at pH 7 and pH 9 there is almost no acetic acid present in the solution. However, given that the acidification step is faster than the methanogenesis, the level of acetic acid can increase if the start position is pH 7.

The anaerobic digestion of activated waste sludge is at the time of writing being reseached by Valerie Sels at the Technical University of Delft. Since this research is the corner stone for this project about storage polymers, it is important to explain the performed experiments and discuss very briefly some important findings. For a more detailed description, please refer to (Sels, 2019).

Valerie Sels' research is based on three separate experiments: batch tests in small bottles (50 mL working volume), batch tests in big bottles (250 mL working volume) and a bioreactor. The batch tests are run using three different substrates and two different media. The small bottle batch tests were used for 2 enrichments of the original inoculum for the rEPS-LS and the rEPS-FS substrate. After the first enrichment of these samples, the samples were combined. Only 1 enrichment has been done for the AGS substrate. After the first digestion, the samples were combined. The enrichment scheme of the small bottles can be seen in Figure 4.

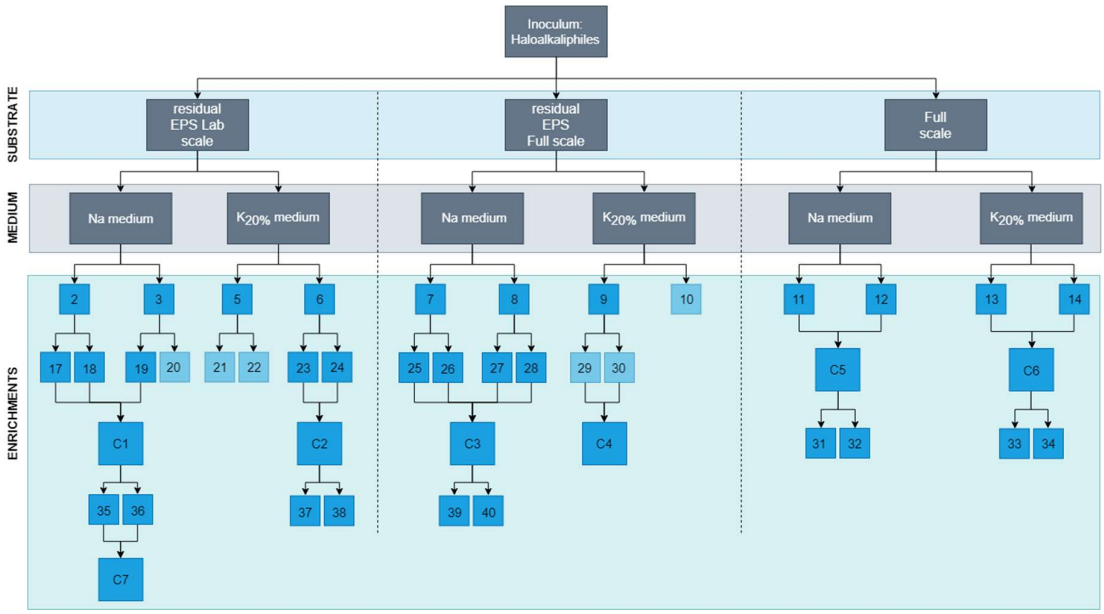

Figure 4. Enrichment scheme of the 25 mL bottles batch tests. Batch 2-11 are part of digestion series 1. Batch 17 - 34 are part of digestion series 2. Batch 35 - 40 are part of digestion series 3. The batch tests indicated in dark blue produced methane. The batch tests in light blue produced no methane. The samples prefaced with 'C' were not a digestion but were the combination of the previous digestion series and the sampling point for the following digestion. Batches 2, 5, 7 and 9 were observed under the microscope. Combination samples C1, C2, C3, C5, C6 and C7 were observed under the microscope.

The combined samples (indicated with a C# in the enrichment scheme) were used to inoculate the big bottles. During the small bottle batch tests, the produced methane was observed and plotted in g_{COD}/g_{COD}. The batch containing rEPS-LS and Na-medium produced the highest methane yield in g_{COD}/g_{COD}, up to 0.5 g_{COD}/g_{COD}. The graph of the methane yield of this batch can be seen in Figure 5. At day 82 of this batch test, the experiment was terminated, and storage polymers were microscopically observed. At the same time, there is still COD and volatile fatty acids available in the sample even though the samples have clearly reached stationary phase.

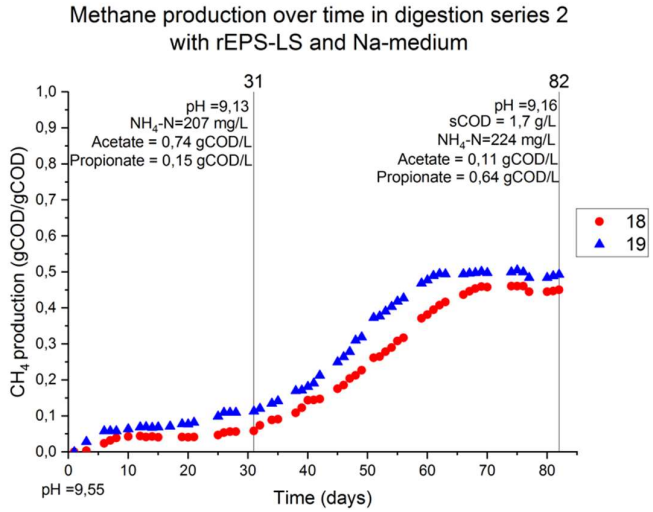

Figure 5. Methane production yield in gCOD/gCOD for the digestion series 2 with Na-medium and rEPS-LS substrate. The important sampling points, day 31 and day 82, are indicated in the graph together with the important characteristics pH, NH_4-N, acetate concentration, propionate concentration and soluble COD (sCOD). The pH at the begin date is indicated below the 0 days data point.

1.2 Storage polymers

During the stationary phase of batch processes of anaerobic digestion, storage polymers were found. Storage polymers are polymers which the cell produces when there is an excess of exogenous compounds and uses when there is a lack of exogenous compounds (Shimada, Zilles, Raskin, & Morgenroth, 2007). The storage polymers provide a biological advantage to the cell. However, the existence of the storage polymers in the sample could mean a loss of biogas production since it is a possible competitor for the substrate use.

Storage polymers can accumulate in the cell because of nutrient limitation while there is an excess of exogenous compounds (Dawes, 1992). These conditions occur at the end of the growth phase. This is essentially an anticipation of starvation. Possible other reasons for producing storage polymers are as a response to osmotic stress or due to a need for reduction of the release of organic acids. A limitation in a terminal electron acceptor can also be a possible reason for the formation of storage polymers (Coats, Loge, Wolcott, Englund, & McDonald, 2007).

The storage polymers themselves can be carbohydrates (Shimada, Zilles, Raskin, & Morgenroth, 2007), lipids or polyesters (van Loosdrecht, Pot, & Heijnen, 1997). In the case of polyesters, it is the most likely that a polyhydroxyalalkanoate (PHA) like polyhydroxybutyrate (PHB) has formed based on literature studies. In the case of carbohydrates, glycogen and glycogen-like compounds like trehalose are the most likely to have formed based on literature studies.

1.3 Aim project

The presence of storage polymers could be a hindrance to the biogas production. Therefore, it is important to characterize the observed storage polymers. This will be done based on three different questions. First, what are the produced storage polymers? Second, what are the environmental conditions that leads to selection of storage polymers? Finally, who are the microorganisms responsible for producing storage polymers?

2 Material and methods

The batch tests were mainly done by Valerie Sels. For a more detailed description of the material and methods of the batch tests, please refer to the unpublished thesis of Valerie Sels (Sels, 2019).

2.1 Inoculum

The inoculum is a mixture of biomass from a preliminary study performed by G.M Dragone to determine the biochemical methane potential of rEPS-LS (Dragone, 2016) and sediments from hypersaline soda lakes of the Kunula steppe in the Altai province of Russia (Sousa, Sorokin, Bijmans, Plugge, & Stams, 2015).

2.2 Substrate, medium and cultivation

The batch tests were conducted using 3 different substrates and 2 different media. The first substrate were granules after alkaline EPS extraction obtained from a lab scale sequencing batch reactor fed with acetate, ammonia and phosphate which has been operated at the Delft University of Technology (rEPS-LS). The second substrate were full granules after alkaline EPS extraction from the full scale Nereda® installation of wwtp Vroomshoop in the Netherlands which is treating municipal sewage (rEPS-FS). The third substrate is aerobic granular sludge that did not undergo alkaline EPS extraction (AGS). The inorganic soluble residues, the rEPS-LS and the rEPS-FS, were extracted from municipal waste granular sludge. The extraction was done using an alkaline extraction using 80°C 0.1M NaOH for 30 minutes (Felz, Al-Zuhairy, Aarstand, van Loosdrecht, & Lin, 2016). The solution was centrifuged for 20 min at 4200 rpm, frozen at -80°C and then freeze-dried. The AGS was frozen at -80°C and subsequently freeze-dried. Table 1 shows the TN, $_T$COD, ash content and the VS/TS of each substrate.

Table 1. TN, $_T$COD, ash content and the VS/TS of the used substrates rEPS-LS, rEPS-FS and AGS. Data is gathered from multiple extraction batches which have been mixed together and analyzed 3-4 times over the course of 7 months. Lab scale granules (AGS-LS) were used to make rEPS-LS and were immediately processed. The AGS from Vroomshop which was used in its entirety as a substrate and used to make rEPS-FS were collected before the start of the project and kept at -20°C upon processing.

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

The 2 different media were a sodium carbonate-bicarbonate alkaline medium and an alkaline medium with 20% potassium. The exact components of each medium can be seen in Table 2. The sodium medium consists of 100% of the general components and the Na medium components. The 20% potassium medium consists of 80% general components and 20% $K_{100\%}$ medium components. The media were stored at room temperature.

Table 2. Components of the two different media. The Na medium consists for 100% of general components and Na-medium components. The $K_{20\%}$-medium consists for 20% of $K_{100\%}$-medium components and 80% general components.

Component	Na medium	$K_{20\%}$ medium
General components		
Trace elements	1 mL/L	1 ml/L
EDTA	5000 mg/L	5000 mg/L
$FeSO_4$ x $7H_2O$	2000 mg/L	2000 mg/L
$ZnSO_4$	100 mg/L	100 mg/L
$MnCl_2$	30 mg/L	30 mg/L
H_3BO_3	300 mg/L	300 mg/L
$CoCl_2$ x $6H_2O$	300 mg/L	300 mg/L
$CuCl_2$	10 mg/L	10 mg/L
$NiCl_2$ x $2H_2O$	20 mg/L	20 mg/L
Na_2MoO_4 x $2H_2O$	20 mg/L	20 mg/L
NaCl	3 g/L	3 g/L
K_2HPO_4	1 g/L	1 g/L
$MgCl_2$	0.10 g/L	0.10 g/L
Se/W	0.1 mL/L	0.1 mL/L
Na medium components		
Na_2CO_3	15 g/L	-
$NaHCO_3$	20 g/L	-
$K_{100\%}$ medium components		
K_2CO_3	-	19.56 g/L
$KHCO_3$	-	23.84 g/L

For the batch tests with bottles with 50 mL working volume (indicated as the 50 mL bottles), each bottle contained 0.375 gTS of one of the substrates and 50 mL of one of the media for a concentration of 7.5 gTS/L. The bottles were made anaerobic using gas exchange by replacing the headpsace with argon gas. The inoculum was added together with 1 mL dithionite, a strong reducing agent which ensures that the residual oxygen is almost completely removed by reducing the oxygen to hydrogen peroxide (Englander, Calhoun, & Englander, 1987). The batch tests were run until they reached stationnary phase for a sufficiently long time. The batches were then used to perform a first and second enrichment. All the digestions were done at 35°C.

The combination sample of digestion series 2 with rEPS-LS and Na-medium was used to inoculate six bottles with 250 mL working volume (indicated as the 250 mL bottles). Three different conditions were used, namely rEPS-LS with Na-medium, rEPS-LS with $K_{20\%}$-medium and rEPS-FS with Na-medium. The experiments were done in duplicate. The conditions can be seen in Figure 6. The digestions were done at 35°C.

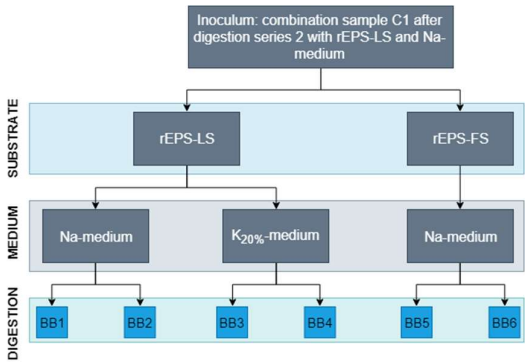

Figure 6. Overview of the digestion scheme of the 250 mL bottles.

A 250 mL sample from C1 was used to inoculate a 2L bioreactor with a working volume of 250 mL. The bioreactor was operated in batch mode, constantly stirred, had an argon gas flow of 10 mL/ min and an overpressure of 0.2 bar to ensure minimal oxygen contamination. The temperature was kept at 35ºC. Table 3 displays when the samples were taken from the bioreactor.

Table 3. The sampling scheme of the bioreactor (abbreviated as BR) with the day of the digestion

Name sample	Day taken
BR0	0
BR1	10
BR2	17
BR3	29
BR4	36
BR5	46
BR6	60
BR7	66
BR8	71
BR9	78

The combination sample of digestion series 3 with rEPS-LS and Na-medium was used to inoculate four batch tests in duplicate. The batch tests were stopped when they reached the stationary phase and then combined. These batch tests were performed to determine more accurately the conditions selecting for the presence of storage polymer
s and to determine whether the production of methane can be resumed by altering the conditions of the system. The descriptions of the batch tests can be seen in Table 4. All the samples have undergone gas exchange and the pH has been measured before the gas exchange.

Table 4. Description of the batch tests using the 10 mL bottles. The batch tests were done in duplicate.

Name	Description
Blank	10 mL of C7
SB1	10 mL of C7 with the addition of acetate to reach a concentration of 11 mM and the addition of 1 mL/L trace metals and 0.1mL/L selenium/tungsten
SB2	10 mL of C7 which has been centrifuged and 5 mL of the supernatant has been replaced by 5 mL fresh sodium buffer solution comprising 15.019 g/L Na_2CO_3 and 20.001 g/L $NaHCO_3$. Acetate is added to reach a concentration of 11 mM. 1 mL/L trace metals and 0.1 mL/L selenium/ tungsten are added.
SB3	10 mL of C7 with the addition of acetate to reach a concentration of 11 mM.

At day 19, 10 µL of 0.121mM $NiCl_2.2H_2O$ and 7.19 mM $FeSO_4.7H_2O$ was added to Blank B and SB3 B.

2.3 Analysis

2.3.1 Headspace analysis

The gasses in the headspace were observed with a gas-tight syringe and a needle through a rubber stopper. The increased pressure due to the gas production will push the plunger of the syringe out. The gas from the headspace was regularly collected in 10 mL glass vials with a saturated brine solution (NaCL, 26%) which allows for correct storing due to the low gas solubility.

2.3.2 Microscopy, fluorescence and staining

The samples were observed under the 'Zeiss Axioplan 2 Imagining' microscope and the Axiovision Release 4.8 software.

To verify the microscopy results, a staining using the fluorescent dye BODIPY® 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) was done. For the BODIPY® staining, 5.065 mg BODIPY® was dissolved in 5 mL DMSO. The solution was kept refrigerated at 4ºC and in the dark. 1 µL of solution was added to a 1 mL sample with a biomass concentration of 1 g/mL. The samples were incubated for 5 min in the dark.

The amount of storage polymers was quantified using gas chromatography with a gas chromatographer from the IHE Delft Institute for Water Education.

2.3.3 Other analytical methods

The volatile fatty acid concentrations were measured for the 50 mL bottles, the 250 mL bottles, the bioreactor and the 10 mL bottles with a Waters HPLC by centrifuging the samples for 5 min at 13 000 rpm and then filtering the supernatant with a 0.45 µm syringe filter. The total and soluble oxygen demand, the tCOD and sCOD respectively, the total nitrogen (TN), orthophosphate and total phosphate (PO_4-P and TP respectively) were analyzed with HACH colorimetric methods (Hach Lange GmbH, Germany). Ammonium (NH_4^+-N) was analysed with colorimetric methods performed by the GalleryTM discrete analyser (ThermoFisher Scientific Inc., USA). pH was measured with a Metrohm pH probe 827 (Metrohm, NL).

3 Results and discussion

The goal of the following experiments is to characterise the storage polymers found during the stationary phase of anaerobic digestion. The characterisation of the storage polymers resolves around the type of storage polymers produced, which conditions selected for the production of storage polymers and which organisms are responsible for the production of the storage polymers.

3.1 Characterisation system

To understand which conditions selected for the production of storage polymers, it is important to characterise the systems which were run in the small bottles, the big bottles and the bioreactor.

3.1.1 50 mL bottles

The first series of batches ran for a very short time to verify whether the inoculum was still viable. Therefore, there is no data available about the gas production of the non-enriched series. Figure 7 shows the gas production of the digestion series 2 of the rEPS-LS (Figures 7-1A and 7-1B) and the rEPS-FS (Figures 7-2A and 7-2B) and the digestion series 1 of the AGS (Figures 7-3A and 73B). Figures 7-A show the series with Na-medium while the Figures 7B show the series with $K_{20\%}$-medium. Two sampling points at day 31 and at day 82, for the systems containing rEPS-LS, at day 123, for the systems containing rEPS-FS, or at day 70, for the systems containing AGS, were indicated with relevant characteristics like pH, NH_4-N concentration, sCOD content, acetate concentration and propionate concentration. For a more detailed table, please refer to Table 5 and Table 6 in the Appendix 1.

The rEPS-LS with the sodium medium obtained the highest methane yield of up to 0.5 g_{COD}/g_{COD}. It should be noted that due to the nature of the test, the presented number could be an underestimation of the actual production due to leakage. The maximum achievable methane production used in this figure is a theoretical value and could therefore be not entirely accurate. Although the methane yield is relatively high compared to BMP values in literature (Kleerebezem, 2015), the COD is not completely converted to methane when the microorganisms reach stationary phase even though according to Formula 8 it is still thermodynamically favorable to do so with a Gibbs free energy of -110.9 kJ/mol. This could be due to the difficulty of digestion of the remaining COD or could indicate that the COD is used for a different purpose than for methane production.

None of the systems had converted all their soluble COD at the stationary phase. Although the system with rEPS-LS and Na-medium obtained the highest methane yield, there were still volatile fatty acids available. This is in contrast with the systems with AGS which had a lower methane yield but did completely convert their volatile fatty acids even though both the AGS system with Na-medium and $K_{20\%}$-medium started off with higher acetate levels (1.9 g_{COD}/L and 1.8 g_{COD}/L versus 0.74 g_{COD}/L respectively). On the other hand, both the AGS systems did not have any propionate at day 31, while the rEPS-LS system with Na-medium had 0.15 g_{COD}/L propionate at day 31.

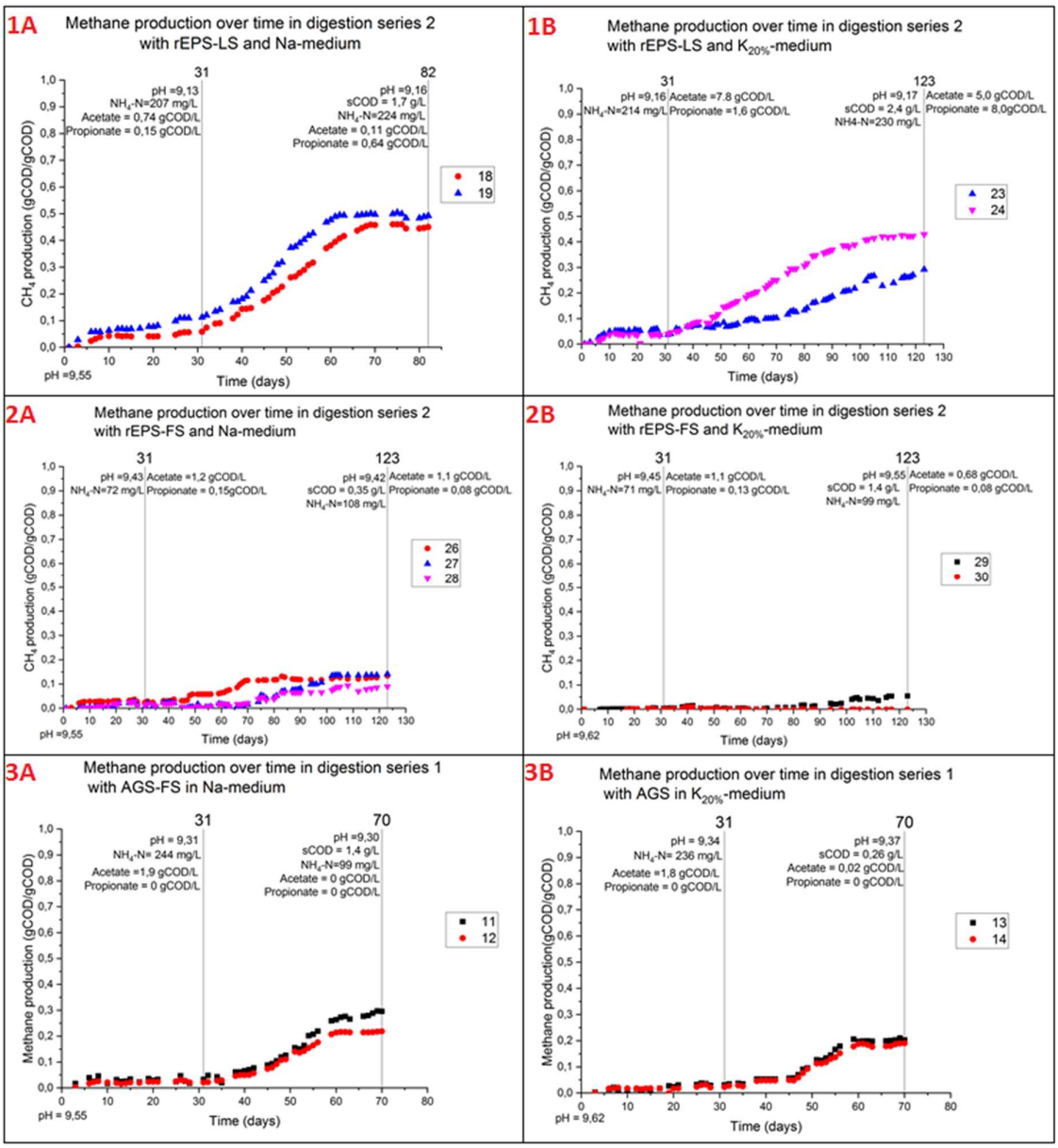

Figure 7. Methane yield over time in the different batch tests. Two measuring points are indicated in each system: one at day 31 and one at the last day of the digestion. For the samples from digestion 2 with rEPS-LS and Na-medium, this is day 82. For the digestion series 2 with rEPS-LS and $K_{20\%}$-medium and the systems containing rEPS-FS, this is day 123. For the systems containing AGS, this is day 70. The data itself is from the individual graphs. The measuring point at day 31 includes pH, NH_4-N, acetate concentration and propionate concentration. The measuring point at the last day of the digestion includes the pH, sCOD, NH_4-N, acetate concentration and propionate concentration. There was no NH_4-N information available for the digestion series 1 with AGS and $K_{20\%}$-medium. 1A: digestion series 2 with rEPS-LS and Na-medium. 1B: digestion series 2 with rEPS-LS and $K_{20\%}$-medium. 2A: digestion series 2 with rEPS-FS and Na-medium. 2B: digestion series 2 with rEPS-FS and $K_{20\%}$-medium. 3A: digestion series 1 with AGS and Na-medium. 3B: digestion series 1 with AGS and $K_{20\%}$-medium.

3.1.2 250 mL bottles

The incubation of the 250 mL bottles has not finished at the time of writing. The characterisation of this experiment is therefore incomplete. For a full characterisation, please refer to Valerie Sels (Sels, 2019).

An overview of the methane yield, pH, soluble COD, NH_4-N and the volatile fatty acid concentration for the 250 mL bottles can be seen in Figure 8. The data is the overage of the duplicates. At the time of writing, there was no data available for the PO_4-P. It is important to note that the dotted line does not indicate a trend line but is just there for ease of interpreting the graph.

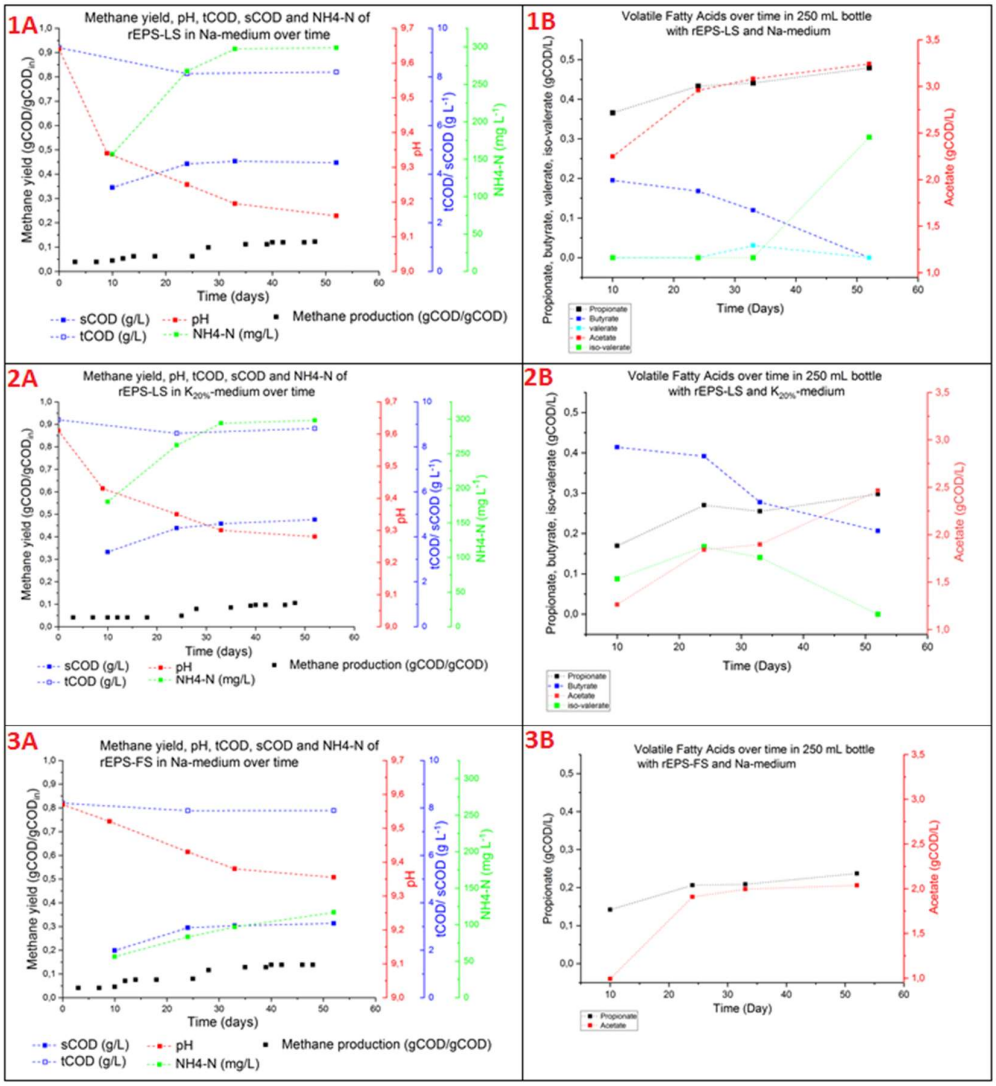

Figure 8. Methane yield, pH, tCOD, sCOD, NH_4-N and volatile fatty acid concentrations in the different systems in the 250 mL bottles. The data is the average of the duplicates when available. Only relevant changes are shown in the graphs. The methane yield can be read on the y-axis on the left. The pH can be read on the y-axis on the right in red. The tCOD and sCOD can be read on the y-axis on the right in blue. The NH_4-N can be read on the y-axis on the right in green. The acetate concentration can be read on the y-axis on the right in red. Other volatile fatty acid concentrations can be read on the y-axis on the left in black. 1A: methane yield, pH, tCOD, sCOD and NH_4-N of systems with rEPS-LS and Na-medium. 1B: volatile fatty acid concentrations of systems with rEPS-LS and Na-medium. 2A: methane yield, pH, tCOD, sCOD and NH_4-N of systems with rEPS-LS and $K_{20\%}$-medium. 2B: volatile fatty acid concentrations of systems with rEPS-LS and $K_{20\%}$-medium. 3A: methane yield, pH, tCOD, sCOD and NH_4-N of systems with rEPS-FS and Na-medium. 3B: volatile fatty acid concentrations of systems with rEPS-FS and Na-medium.

3.1.3 Bioreactor

The bioreactor was regularly sampled for pH, sCOD, tCOD, NH4-N and volatile fatty acids. Figure 9 shows an overview of the pH, sCOD, tCOD and NH4-N over time.

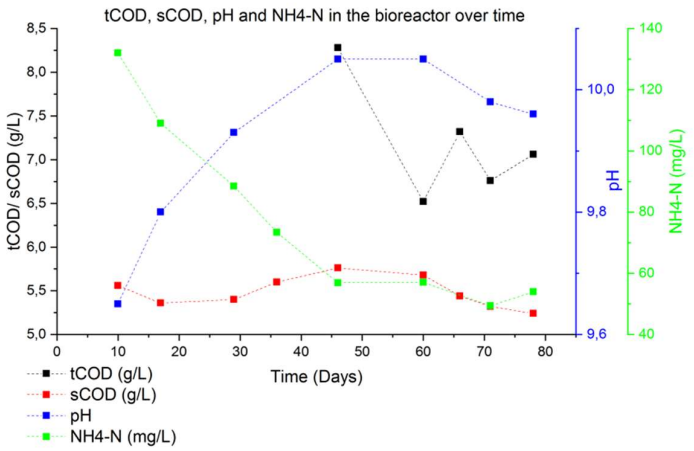

Figure 9. tCOD, sCOD, pH and NH_4-N in the bioreactor over time. The tCOD and sCOD can be read on the y-axis on the left in black. The pH can be read on the y-axis on the right in blue. The NH_4-N can be read on the y-axis on the right in green.

It is important to note that the tCOD determination is not very accurate. It was therefore only used to give a general idea of the magnitude of the amount of tCOD in the sample, but it cannot be used for accurate calculations.

Figure 10 shows an overview of propionate and acetate over time. There were no other volatile fatty acids detected during the experiment at the time of writing.

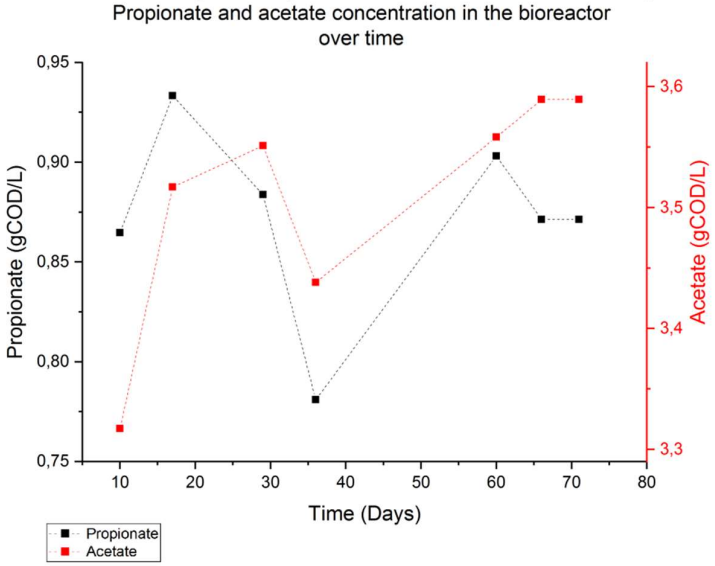

Figure 10. Propionate and acetate concentration in the bioreactor over time. The propionate concentration can be read on the y-axis on the left in black. The acetate concentration can be read on the y-axis on the right in red.

3.2 Staining and fluorescence

A BODIPY® staining was done to verify whether the observed storage polymers are PHA and/or lipids. The BODIPY® 505/515 will localize in hydrophobic environments and will therefore stain PHA and lipid. Even though PHA is not extremely hydrophobic on its own, its high number of hydrophobic groups and flexibility can allow for highly hydrophobic aggregates (Elustondo, Zakharian, & Pavlov, 2012).

It is important to note two things about the handling of the microscopy pictures. First, the pictures have been edited by cropping the pictures to show only relevant information and the scales have been moved to be visible in the final picture. This can sometimes cause discrepancies between the background color of the scale and the rest of the picture. The scale was except being moved not altered in any way, shape or form. Second, the pictures chosen were used to reflect the situation in the sample, but it is possible that this introduces some unintentional bias and/or shows an inaccurate reflection of the conditions in the system itself. The pictures are therefore for reference only and are only used for qualitative analysis. It is not possible to use these pictures for quantitative purposes. It is only possible to verify the presence of PHA.

It is important to note that the PHA will remain fluorescent for a longer time than the lipids and that the level of intensity does not necessarily reflect the amount of storage polymers.
Figure 11 shows a sample taken from the inoculum without staining (Figure 11-A) and with staining (Figure 11-B). Compared to the amount of storage polymers observed in the small bottles and the big bottles, very few storage polymers were observed.

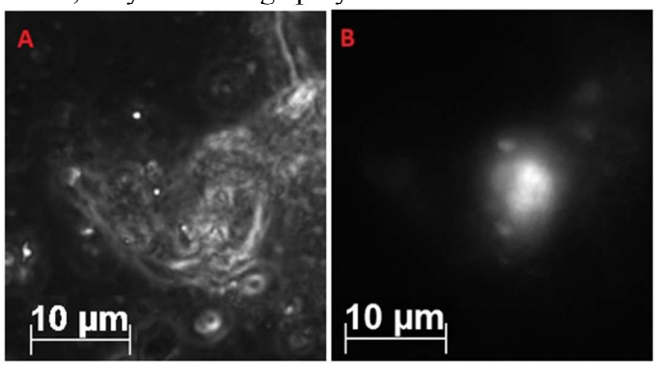

Figure 11. Microscopy picture of the inoculum without (A) and with (B) staining.

In general, the storage polymers are small and in the shape of a circle. It appears that one cell can hold multiple storage polymers. This is true for all the different systems.

A sample of the inoculum was taken and analyzed using a gas chromatographer. There was no PHB or PHV detected in that sample. This can be due to multiple reasons, like the low amount of sample used (31,04 mg), due to inaccuracies with the gas chromatographer or due to a non-representative sample. It is also possible that the BODIPY® staining was a false positive or had indicated lipids instead of PHA.

The substrates and the media were also observed with BODIPY® staining. For the substrates, no storage polymers were observed but there did appear to be some background staining in all the substrates. This is probably due to lipids, water droplets or air bubbles. There were some floating, very small particles which could be micro-organisms but they could also be particulate matter. If they were micro-organisms, it was not clear whether they were alive. A BODIPY® staining of the Na-medium and the $K_{20\%}$-medium did not show any clear staining. There was some fleeting staining which was either background staining or lipid staining.

3.2.1 50 mL bottles

Figure 12 shows the samples from the enrichment series 1 with rEPS-LS and rEPS-FS without staining (Figures 12-A) and with staining (Figures 12-B). Figures 12-1A and 12-1B show the sample with rEPS-LS and Na-medium. Figures 12-2A and 12-2B show the sample with rEPS-LS and $K_{20\%}$-medium. Figures 12-3A and 12-3B show the sample with rEPS-FS

and Na-medium. Figures 12-4A and 12-4B show the sample with rEPS-FS and $K_{20\%}$-medium.

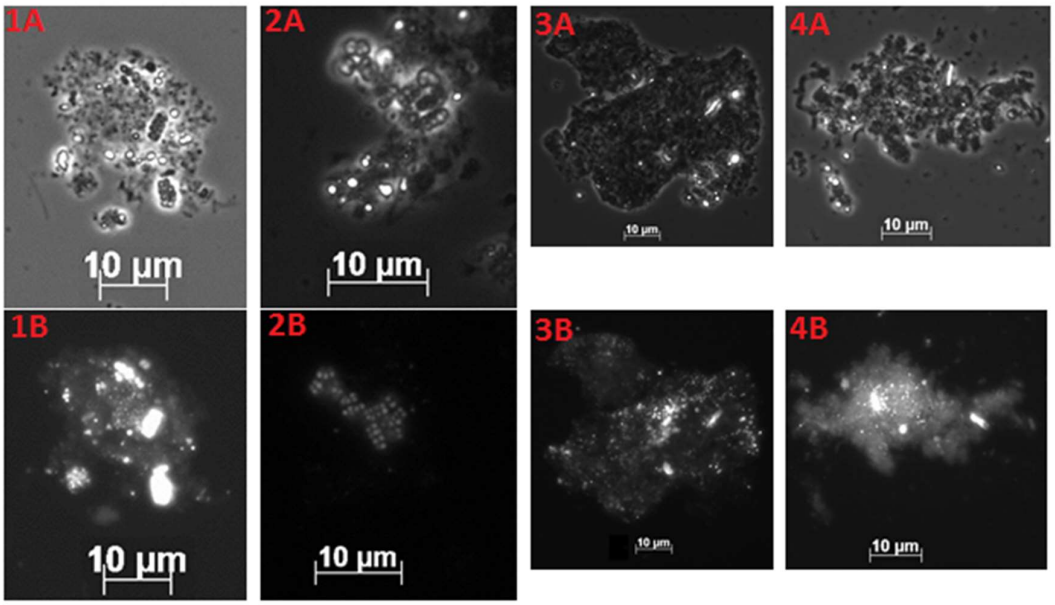

Figure 12. Microscopy pictures of digestion series without (A) and with (B) staining. 1A: digestion series 1 with rEPS-LS and Na-medium without staining. 1B: digestion series 1 with rEPS-LS and Na-medium with staining. 2A: digestion series 1 with rEPS-LS and $K_{20\%}$-medium without staining. 2B: digestion series 1 with rEPS-LS and $K_{20\%}$-medium with staining. 3A: digestion series 1 with rEPS-FS and Na-medium without staining. 3B: digestion series 1 with rEPS-FS and Na-medium with staining. 4A: digestion series 1 with rEPS-FS and $K_{20\%}$-medium without staining. 4B: digestion series 1 with rEPS-FS and $K_{20\%}$-medium with staining.

Figure 13 shows a sample taken from enrichment series 2 with rEPS-LS, with sodium medium (Figures 13-1) and with 20% potassium medium (Figures 13-2), without staining (Figures 12-A) and with staining (Figures 12-B).

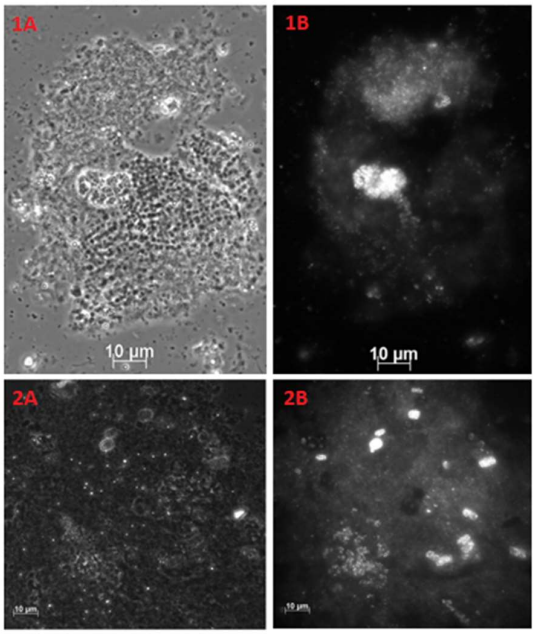

Figure 13. Microscopy pictures of digestion series 2 with rEPS-LS without (A) and with (B) staining. 1A: digestion series 2 with rEPS-LS and Na-medium without staining. 1B: digestion series 2 with rEPS-LS and Na-medium with staining. 2A: digestion series 2 with rEPS-LS and $K_{20\%}$-medium without staining. 2B: digestion series 2 with rEPS-LS and $K_{20\%}$-medium with staining.

There were little to no storage polymers observed in the sample from the digestion series 2 containing rEPS-FS and Na-medium. The pictures taken were therefore not included. The sample from the digestion series 2 containing rEPS-FS and $K_{20\%}$-medium did not produce any methane and was not microscopically observed under the microscope.

Figure 14 shows a sample taken from enrichment series 2 with AGS, with sodium medium (Figures 14-1) and with 20% potassium medium (Figures 14-2), without staining (Figures 14-A) and with staining (Figures 14-B).

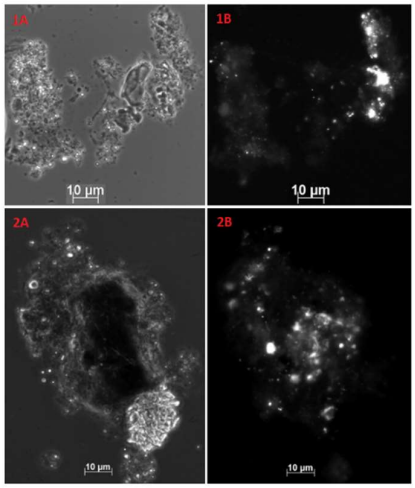

Figure 14. Microscopy pictures of digestion series 2 with AGS without (A) and with (B) staining. 1A: digestion series 2 with AGS and Na-medium without staining. 1B: digestion series 2 with AGS and Na-medium with staining. 2A: digestion series 2 with AGS and $K_{20\%}$-medium without staining. 2B: digestion series 2 with AGS and $K_{20\%}$-medium with staining.

Based on the results of the BODIPY® staining, it can be concluded that the observed storage polymers are PHA since there is fluorescence and it stays longer than it would if it were lipids. This is in line with the expectations based on the literature study. It is not possible to quantify the amount of storage polymers based on the staining. However, notably less storage polymers were observed in the inoculum compared to other samples. It cannot be said with absolute certainty that the observed staining in the inoculum sample is not lipids instead of PHA. Compared to the other samples, the staining seemed to be fainter and there appeared to be more background staining. The samples of the non-enriched batches and the first enrichment batches appeared to contain more storage polymers than the inoculum and the storage polymers themselves were more defined and often more intense. This could be because there are more storage polymers compared to lipids, but it could also be due to difference in staining efficiency or because the samples have lost less of their intensity due to light exposure before the observation under the microscope.

The production of PHA is not uncommon during wastewater treatment. However, the production of PHA is often an (micro)aerobic reaction when aerobic PHA producing bacteria are exposed to air during the treatment process (Pittmann & Steinmetz, 2017) (Coats, Loge, Wolcott, Englund, & McDonald, 2007). Completely anaerobic PHA production has not been studied in great detail.

Based on the innate fluorescence of the methanogens, it seems unlikely that the methanogens are the ones producing and/or storing PHA since the PHA is not found in the methanogens.

Since microscopy cannot be used to quantify the amount of storage polymers, gas chromatography was performed with the samples. The results can be seen in Table 5. Only

the samples that contained detected storage polymers, the combination samples of digestion series 2 containing rEPS-LS, are shown.

Table 5. PHB and PHV content in the combination samples of rEPS-LS with Na-medium (C1) and rEPS-LS with $K_{20\%}$-medium. The PHB and PHV content are displayed in mass and percentage of the total sample. The total amount of PHA in the total suspended solids is indicated with the percentage of PHA/TSS.

Name	Amount of sample (mg)	Amount PHB (mg)	Amount PHB (%)	Amount PHV (mg)	Amount PHV (%)	PHA/TSS (%)
C1	10.1	0.15	1.5	0.45	4.4	6
C2	6.2	0	0	0.14	2.2	2

Only in samples C1, containing rEPS-LS and Na^+ medium, and C2, containing rEPS-LS and $K_{20\%}$ medium, storage polymers were detected. However, the amount of sample was very small and therefore more prone to faults. It is possible that the amount of PHA in the sample was below the detection limit. It is also possible that another PHA was present in the sample that was not detected. It is also possible that the observed storage polymers were in fact lipids. This is not very likely since the staining retained its fluorescence for longer periods of time than would be expected. It is also possible but not very probable that the BODIPY® stained another compound which is not a PHA or a lipid. At present, it seems most likely that the results of the gas chromatographer are not completely trustworthy and should be considered carefully.

During the incubation of the 250 mL bottles, samples were taken twice, once at day 10 and once at day 24, to be observed under the microscope. This was done for all the bottles. The conditions for the 250 mL bottles are very similar to the conditions of the 50 mL bottles and the staining did not yield any outstanding results. For this reason, the pictures of the microscopy and the staining can be found in Appendix 2. The similarity in microscopy pictures suggests that the scaling up of the 50 mL bottles is possible.

A duplicate from the system with rEPS-LS and Na-medium (BB2), both the duplicates from the system containing rEPS-LS and $K_{20\%}$-medium (BB3 and BB4) and a duplicate from the system containing rEPS-FS and Na-medium (BB5) appear to have less storage polymers at day 24 than at day 10. There are a few possible explanations for this observation. If there are less storage polymers, it is possible they have been degraded by the cell. If there are not less storage polymers, it is possible that the staining was not successful or that the samples taken were not representative of the actual situation. The staining could have been unsuccessful due to different handling of the sample during and after the staining, for example due to exposure to light since BODIPY® is light sensitive.

3.2.2 Bioreactor

The samples of day 29, 46 and 71 of the bioreactor were stained and microscopically observed. Figure 15 shows the samples without (Figures 15-A) and with (Figures 15-B) staining. Figures 15-1 show the samples of day 29. Figures 15-2 show the samples of day 46. Figures 15-3 shows the samples of day 71.

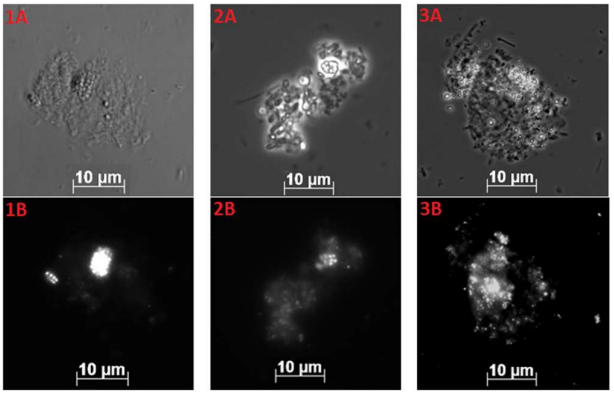

Figure 15. Microscopy pictures of the bioreactor at day 29, 46 and 71 without (A) and with (B) staining. 1A: bioreactor sample at day 29 without staining. 1B: bioreactor sample at day 29 with staining. 2A: bioreactor sample at day 46 without staining. 2B: bioreactor sample at day 46 with staining. 3A: bioreactor sample at day 71 without staining. 3B: bioreactor sample at day 71 with staining.

The staining shows the presence of PHA. There seems to be an increase in storage polymers between day 46 and day 71. As said before, this does not necessarily reflect the situation in the bioreactor and may be a result of differences in staining, use of the microscope or because of the sample.

At day 78, a sample was taken to be analyzed on the gas chromatographer. There were no PHB or PBV detected in that sample on the gas chromatographer. Since only 5.55 mg of dry sample was used, it is possible that the amount of PHB and PHV was below the detectable limit. It is also possible that the staining was false-positive, and that background staining and/or lipid staining had occurred.

3.3 10 mL bottles

To better determine the conditions which led to selection of storage polymers in the system, a batch test was set up with 4 different conditions. One of these was the blank containing 10 mL C7. The second one (SB1) was a batch test containing 10 mL C7 with the addition of acetate and trace metals. The third one (SB2) was a batch test with 10 mL C7 which has been centrifuged and 5 mL supernatant was replaced with fresh buffer and acetate and trace metals were added. The final one (SB3) was a batch test containing 10 mL C7 with the addition of acetate. All the batch tests underwent gas exchange with Argon.

3.3.1 Characterisation system

Table 6 shows the expected changes in the system conditions.

Table 6. Expected changes in the different systems in terms of NH_3, acetate concentration, propionate concentration, trace metals, phosphor content, hydrogen gas, magnesium, salts, biomass, pH and CO_2. A '+' indicates an expected increase compared to the situation at the end of digestion 3. A '-' indicates an expected decrease compared to the situation at the end of digestion 3. A '0' indicates that the condition is expected to stay stable compared to digestion 3.

	NH_3	Acetate	Propionate	Trace metals	P	H_2	Mg^{2+}	Salts	X	pH	CO_2
Blank	0	0	0	0	0	-	0	0	0	0	-
SB1	0	+	0	+	0	-	0	0	0	0	-
SB2	-	+	-	+	0	-	0	0	~0	+	-
SB3	0	+	0	0	0	-	0	0	0	0	-

The blank functioned as a baseline for the other samples. SB1 was used to determine the influence of trace metals when enough food is present. In theory, enough trace metals should be in the media. However, the high pH could make many trace metals not bioavailable (Takac, Szabova, Kozakova, & Benkova, 2009). It is therefore expected that the addition of trace metals will increase the production of methane. SB2 was used to determine influence of potentially toxic compounds (NH_3, propionate) since compared to SB1 the only difference is the amount of NH_3 and propionate. Concentrations of 12 mM of propionate causes serious inhibition of methanogenesis and therefore high concentrations of propionate could be an indicator of anaerobic digestion failure (Franke-Whittle, Walter, Ebner, & Insam, 2014). SB3 was used to determine whether there is a threshold for methane production based on carbon-availability. SB3 was chosen because of the characterization of the system seen Figure 7-1A. At day 30 of the batch tests 35 and 36 containing sodium medium and residual rEPS-LS, 11 mM of acetate was observed. At day 80 of the same system, 1.8 mM of acetate was observed. For this system, day 30 was right before the exponential phase and day 80 was during the stationary phase. It is therefore possible that there is a threshold for methane production related to the acetate content in the sample. By increasing the amount of readily available carbon source acetate, it can be determined whether such a threshold exists. If there is no more methane production in these samples, it can be concluded that either there is no such threshold, the organisms have become inactive due to the stationary phase or another change in the system caused the methanogens to be unproductive, for example the change in headspace and short exposure to oxygen during preparation of the batches.

An overview of the pH, soluble COD, total COD, NH_4-N, acetate concentrations and the propionate concentrations for the batch tests with the 10 mL bottles can be seen in Figure 16. The data for day 0, except for the pH, is obtained from the data from the combination sample C7. The data for each sample is the average of the duplicates. Figures 16-A show the overview of the pH, soluble COD, total COD and NH_4-N. Figures 16-B show the acetate concentration and the propionate concentration. Figures 16-1A and 16-1B show the data for the blank. Figures 16-2A and 16-2B show the data for SB1. Figures 16-3A and 16-3B show the data for SB2. Figures 16-4A and 16-4B show the data for SB3. There was no data available for the sCOD and the tCOD at day 25 for SB2.

In all the batch tests, there is a decrease in sCOD, NH_4-N and pH. The tCOD remained constant, especially considering the larger margin of error for the tCOD test and its big difference between repeated sampling. The decrease in NH_4-N could indicate an increase in biomass and thus growth. This would mean that (part of) the microbial community is actively growing. The decrease in pH could indicate the production of acids, like acetate. This is however countered by the larger decrease in propionate.

In all the batch tests, there is an increase in acetate and a decrease in propionate at day 13 compared to day 0. It is important to note that the samples at day 25 were only run on the HPLC for 25 minutes and there is therefore no data available for the substances with a retention time of longer than 25 minutes.

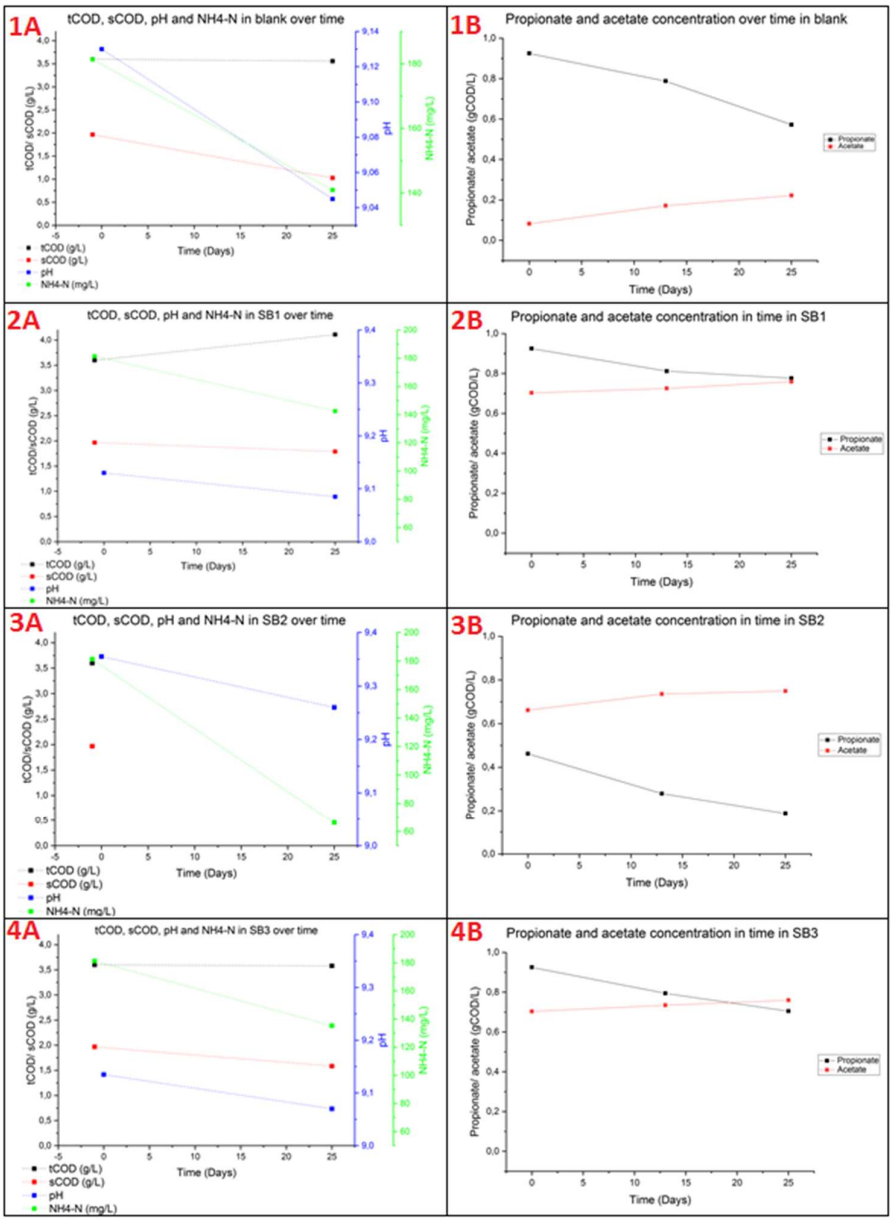

Figure 16. pH, tCOD, sCOD, NH_4-N and volatile fatty acid concentrations in the different systems in the 10 mL bottles.. The data is the average of the duplicates when available. Only relevant changes are shown in the graphs. The tCOD and sCOD can be read on the y-axis on the left in black. The pH can be read on the y-axis on the right in blue. The NH_4-N can be read on the y-axis on the right in green. The propionate concentration can be read on the y-axis on the left in black. The acetate concentration can be read on the y-axis on the right in red. 1A: pH, tCOD, sCOD and NH_4-N of the blanks. 1B: propionate and acetate concentrations of the blanks. 2A: pH, tCOD, sCOD and NH_4-N of SB1. 2B: propionate and acetate concentrations of SB1. 3A: pH, tCOD, sCOD and NH_4-N of SB2. There was no data available for the tCOD and sCOD at day 25. 3B: propionate and acetate concentrations of SB2. 4A: pH, tCOD, sCOD and NH_4-N of SB3. 4B: propionate and acetate concentrations of SB3

The increase in acetate could indicate that hydrolysis of the substrate is still ongoing. This does however not explain the decrease in propionate. It is possible that the propionate is used to make some acetate using Formula 4. However, this reaction would require coupling to methane production using Formula 7 since the reaction itself is energetically not favorable. This is unlikely since no methane production has been observed, as can be seen in Figure 17. Based on this information, it is the most likely that the observed differences are due to the margin of error of the HPLC itself.

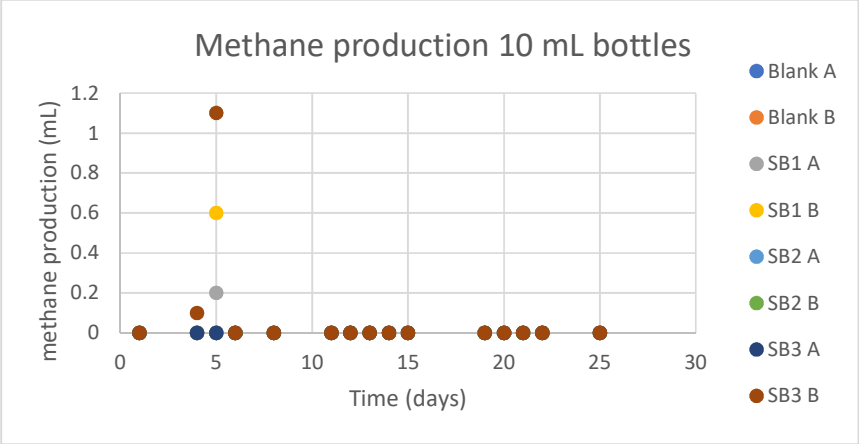

Figure 17. Methane production over time in the different systems in the 10 mL bottles. After day 5, all systems failed to produce any methane.

Due to the absence of methane production at day 13, extra iron and nickel was added to Blank B and to SB3 B. This was done to determine whether the use of EDTA in the trace metal solution had an influence on the methane production. EDTA makes the trace metals more soluble but can potentially decrease the bioavailability (Norvell & Lindsay, 1969). This could be counterproductive to the goal of determining whether trace metals had a positive effect on the methane production. As can be seen in Figure 17, the methane production did not increase after the addition of iron and nickel. It is possible that there was more leakage than gas production given the small volumes and the crude way of measuring the gas production. It is therefore premature to include or exclude the possibility of gas production. The spike at day 5 in the SB1 A, SB1 B and the SB3 B is either due to measurement errors or due to the leakage.

Samples of the overhead space were collected and analyzed using a gas chromatographer. According to that analysis, there was 1% methane gas in the overhead space in every batch. Given the margin of error of the chromatographer and the inaccuracies produced while taking the sample and introducing the sample in the gas chromatographer, it is doubtful that there is any methane at all. The gas chromatographer did reveal a higher than usual CO_2 content across all batch tests. In all batch tests, the CO_2 content was higher than would be expected in the air. It is therefore possible that the microbial community in the batch tests is not inactive but that the methanogenesis is halted due to either unfavorable conditions or some other kind of inhibition.

3.3.1 Staining and fluorescence

The 10 mL bottles were microscopically observed twice: once as part of the combination sample C7 at day zero and once at day 25 of the digestion. Figure 18 shows the sample of day zero without (Figure 18-A) and with (Figure 18-B) staining. `

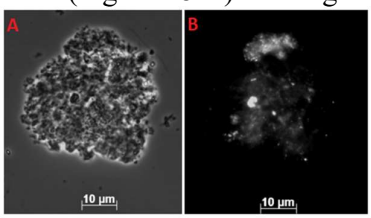

Figure 18. Microscopy picture of the combination sample (C7) of the digestion series 3 with rEPS-LS and Na-medium without (A) and with (B) staining.

Figure 19 shows the blank A and blank B samples of day 25 without (Figures 19-A) and with (Figures 19-B) staining. Figures 19-1A and 19-1B show the sample from the blank A. Figures 19-2A and 19-2B show the sample from the blank B.

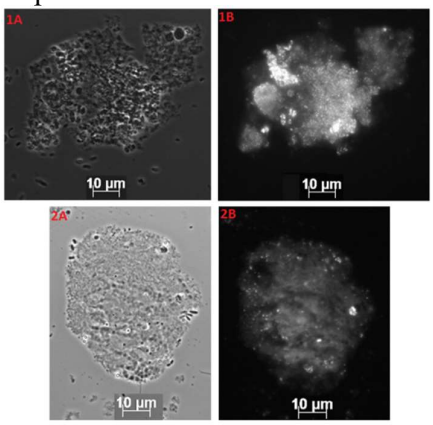

Figure 19. Microscopy pictures of blank samples from 10 mL bottles at day 25 without (A) and with (B) staining. 1A: Blank A without staining. 1B: Blank A with staining (B). 2A: Blank B without staining. 2B: Blank B with staining.

Figure 20 shows the SB1 B samples of day 25 without (Figure 20-A) and with (Figure 20-B) staining. There were no storage polymers or anything else of notice found in sample SB1 A. It was therefore not included.

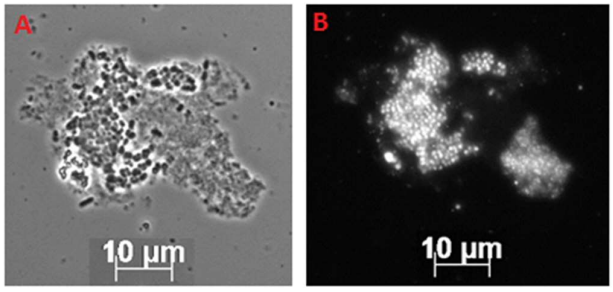

Figure 20. Microscopy pictures of sample SB1 B from 10 mL bottle at day 25 without (A) and with (B) staining.

Figure 21 shows the SB2A and SB2B samples of day 25 without (Figures 21-A) and with (Figures 21-B) staining. Figures 21-1A and 21-1B show the sample from SB2 A. Figures 21-2A and 21-2B show the sample from SB2 B.

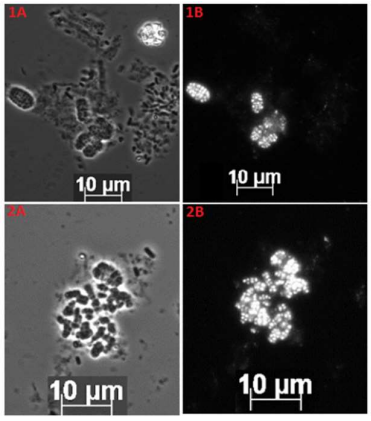

Figure 21. Microscopy pictures of SB2 from 10 mL bottles at day 25 without (A) and with (B) staining. 1A: SB2 A without staining. 1B: SB2 A with staining (B). 2A: SB2 B without staining. 2B: SB2 B with staining.

Figure 22 shows the SB3 A and SB3 B samples of day 25 without (Figures 22-A) and with (Figures 22-B) staining. Figures 22-1A and 22-1B show the sample from SB3 A. Figures 22-2A and 22-2B show the sample from SB3 B.

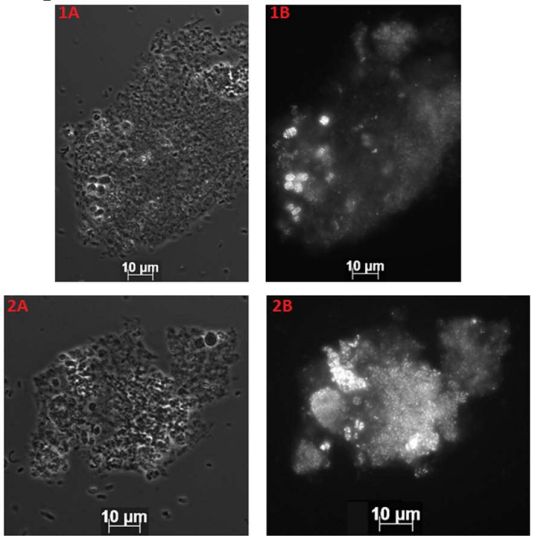

Figure 22. Microscopy pictures of SB3 from 10 mL bottles at day 25 without (A) and with (B) staining. 1A: SB3 A without staining. 1B: SB3 A with staining (B). 2A: SB3 B without staining. 2B: SB3 B with staining.

Except for SB1 A, all the batch tests had storage polymers. It is difficult to say whether there are more storage polymers compared to the combination sample containing rEPS-LS with Na-medium which was used as inoculum.

The complete lack of methane production despite abundance of sCOD present and the availability of a more readily degraded carbon source, namely acetate, indicates that the methanogens must either be limited by a missing compound or be unable to use the present sCOD due to inhibitions in some shape or form. It was expected that the bioavailability of trace metals posed a problem during the previous digestions but the addition of trace metals in the 10 mL bottles did not yield more methane. However, during the transfer of biomass the original headspace was removed and replaced with Argon gas. Since hydrogen is necessary to produce methane gas, it is possible that the absence of the hydrogen gas is interfering with the ability of the methanogens to produce methane even if the addition of trace metals would resume the methane production under normal conditions. It is therefore premature to exclude the potentially beneficial effects of adding trace metals. It is also important to note that the storage polymers have not been completely degraded during the digestion even though the methanogens appear to be inactive. This most likely indicates that the conditions selecting for its production have not been resolved or that the storage polymers never were made as an energy source. It is possible that the storage polymers are produced as a reaction to the osmotic stress. Given the already high osmotic pressure in the broth due to the high salinity, this is possible but not very likely. It is also possible that the storage polymers are produced as an electron sink due to the inability of the microbial community to produce hydrogen gas. In that case, the excess of electrons and a lack of a terminal electron acceptor could be the trigger to produce PHA. The production of PHA in absence of a terminal electron acceptor has been previously observed (Coats, Loge, Wolcott, Englund, & McDonald, 2007). This is at present the most likely theory for the production of the PHA that was observed but further research should be conducted to further support this theory.

4 Conclusion and recommendations

The goal of the following experiments is to characterise the storage polymers found during the stationary phase of anaerobic digestion. The characterisation of the storage polymers resolves around the type of storage polymers produced, which conditions selected for the production of storage polymers and which organisms are responsible for the production of the storage polymers.

Based on a BODIPY® 505/515 staining, it was determined that the observed storage polymers were polyhydroxyalkanoates. This result was not entirely corroborated by gas chromatography. Based on the innate fluorescence of the methanogens, it seems unlikely that the methanogens are the ones storing/ producing PHA. It is unclear which organisms exactly are responsible for the production/ storing of the PHA. The results of the batch tests were inconclusive regarding PHA storage and methane production. Based on the gas chromatography, the difference in PHA storage is centred around the use of rEPS-LS or other substrates. However, this result is not entirely reliable due to the low amounts of product used which could fall below the detection limit.

To further characterise the found storage polymers, it could be interesting to start a digestion series with fresh inoculum from the soda lake in Russia. The inoculum used in this experiment was a mixture of fresh inoculum from the soda lake in Russia and already digested inoculum from previous experiments and storage polymers were detected already in this inoculum mixture, albeit in (visually) very limited amounts. Further experiments could be new digestion series with different conditions regarding amount of trace metals, amount of hydrogen gas, concentrations of volatile fatty acids and potentially toxic and inhibitory compounds. This also allows the opportunity to sample more often and get a more complete image of when the storage polymers are produced and what the conditions of the production were. A better characterisation of the microbial community could also prove interesting by cross-referencing the found micro-organisms with known PHA producers. If the conditions which select for the production of storage polymers are better understood, it is possible to verify whether the storage polymers are possible energy sources by starting a digestion without a carbon source but with storage polymers and without the stressors which selected for the production of storage polymers. Since the stressors are not yet known as of yet, it is difficult to say whether this is doable.

5 Bibliography

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6 Appendix 1: Raw data 50 mL bottles

An overview of the pH, soluble COD, NH_4-N and PO_4-P for the combination samples of the 50 mL bottles can be seen in Table 7.

Table 7. Overview of the pH, sCOD, NH_4-N and PO_4-P of the combination samples of digestion series 1 and digestion series 2. The combination sample C1 of digestion series 2 with rEPS-LS and Na-medium was terminated at day 82 and this is therefore the last data point. The combination samples C2, C3 and C4 of digestion series 2, with rEPS-LS and $K_{20\%}$-medium, rEPS-FS and Na-medium and rEPS-FS and $K_{20\%}$-medium respectively, were terminated at day 123 and this is therefore the last data point. The combination samples C5 and C6 of digestion series 1, with AGS and Na-medium and AGS and $K_{20\%}$-medium respectively, were terminated at day 70 and this is therefore the last data point.

Sample			pH			sCOD (mg/L)	NH_4-N (mgN/L)		PO_4-P (mgP/L)
Name sample	Sub-strate	media	Day 0	Day 31	Day 82/ 123 /70	Day 82/ 123/ 70	Day 31	Day 82/ 123/ 70	Day 82/ 123/ 70
C1	rEPS-LS	Na^+	9.55	9.13	9.16	1700	207	224.16	212
C2	rEPS-LS	$K_{20\%}$	9.62	9.16	9.17	2400	214	229.52	208
C3	rEPS-FS	Na^+	9.55	9.43	9.42	348	72	108.22	238
C4	rEPS-FS	$K_{20\%}$	9.62	9.45	9.55	1348	71	98.87	236
C5	AGS	Na^+	9.55	9.31	9.30	244	229	-	246
C6	AGS	$K_{20\%}$	9.62	9.34	9.37	264	236	-	262

The volatile acid concentration of the combination samples of the batch tests with the 50 mL bottles can be seen in Table 8. The data for day 30 is the average of the data of the individual batches which have been analysed using the HPLC.

Table 8. Overview of the changed volatile fatty acids in the combination samples of digestion series 1 and 2 at two datapoints: day 30 and the end date. The data at day 30 is the average of the batches that were used to make the combination samples and for which data was available. For C1, this was 17, 18, 19. For C2, this was 23 and 24. For C3, this was 25, 27 and 28. For C4, this was 30. For C5 this was 11 and 12. For C6, this was 13 and 14.

Compound	C1		C2		C3		C4		C5		C6	
Batches	17, 18, 19		23, 24		25, 27, 28		30		11, 12		13, 14	
Day	30	82	30	123	30	123	30	123	30	70	30	70
Formate (mg_{COD}/L)	3	0	0	3	0	0	0	0	0	0	0	0
Acetate (mg_{COD}/L)	742	101	778	501	1177	0	1103	679	1861	0	1846	19
Propionate (mg_{COD}/L)	145	644	163	803	145	0	127	77	0	0	0	0
Iso-Valerate (mg_{COD}/L)	0	0	17	0	0	0	0	0	0	0	0	0
Valerate (mg_{COD}/L)	0	0	0	0	9	0	0	0	0	0	242	0

7 Appendix 2: Microscopy 250 mL bottles

Figure 23 shows the samples containing residual rEPS-LS and Na-medium without (Figures 23-A) and with (Figures 23-B) fluorescence. Figures 23-1 and 23-3 are taken at day 10 and Figures 23-2 and 23-4 are taken at day 24. The samples 23-1 and 23-2 are taken from BB1 and the samples 23-3 and 23-4 are taken from BB2.

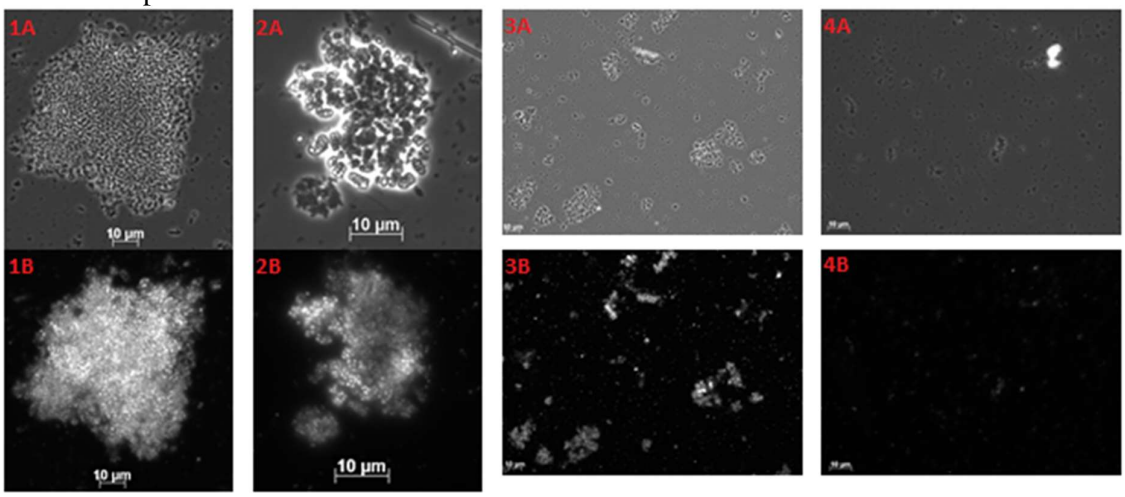

Figure 23. Microscopy pictures of the samples containing rEPS-LS and Na-medium of the 250 mL at day 10 and day 24 without (A) and with (B) fluorescence. 1A: sample BB1 with rEPS-LS with Na-medium at day 10 without staining. 1B: sample BB1 with rEPS-LS with Na-medium at day 10 with staining. 2A: sample BB1 with rEPS-LS with Na-medium at day 24 without staining. 2B: sample BB1 with rEPS-LS with Na-medium at day 24 with staining. 3A: sample BB2 with rEPS-LS with Na-medium at day 10 without staining. 3B: sample BB1 with rEPS-LS with Na-medium at day 10 with staining. 4A: sample BB2 with rEPS-LS with Na-medium at day 24 without staining. 4B: sample BB2 with rEPS-LS with Na-medium at day 24 with staining.

Figure 24 shows the samples containing rEPS-LS and $K_{20\%}$-medium without (Figures 24-A) and with (Figures 24-B) fluorescence. Figures 24-1 and 24-3 are taken at day 10 and Figures 24-2 and 24-4 are taken at day 24. The samples 24-1 and 24-2 are taken from BB3 and the samples 24-3 and 24-4 are taken from BB4.

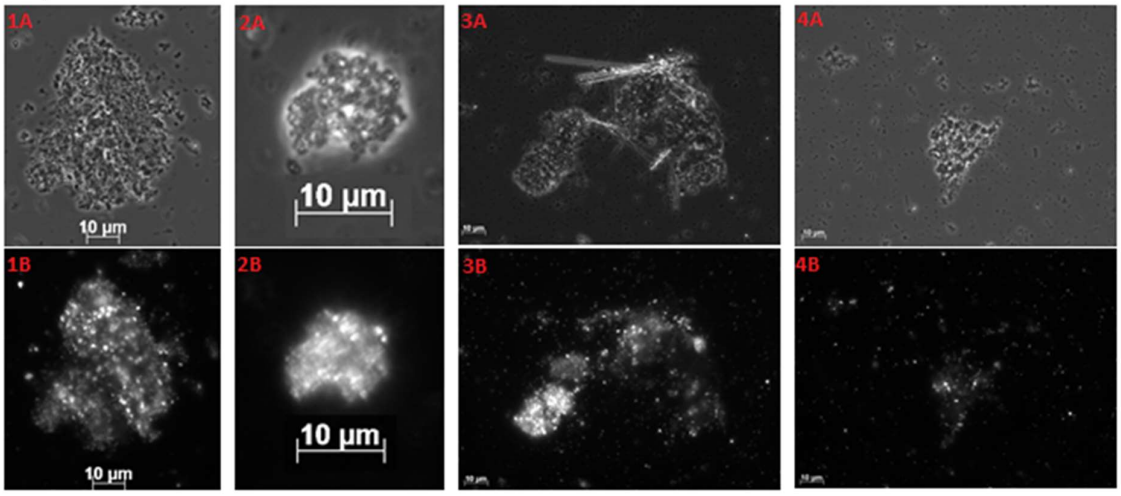

Figure 24. Microscopy pictures of the samples containing rEPS-LS and $K_{20\%}$-medium of the 250 mL at day 10 and day 24 without (A) and with (B) fluorescence. 1A: sample BB3 with rEPS-LS with $K_{20\%}$-medium at day 10 without staining. 1B: sample BB3 with rEPS-LS with $K_{20\%}$-medium at day 10 with staining. 2A: sample BB3 with rEPS-LS with $K_{20\%}$-medium at day 24 without staining. 2B: sample BB3 with rEPS-LS with $K_{20\%}$-medium at day 24 with staining. 3A: sample BB4 with

rEPS-LS with $K_{20\%}$-medium at day 10 without staining. 3B: sample BB4 with rEPS-LS with $K_{20\%}$-medium at day 10 with staining. 4A: sample BB4 with rEPS-LS with $K_{20\%}$-medium at day 24 without staining. 4B: sample BB4 with rEPS-LS with $K_{20\%}$-medium at day 24 with staining.

Figure 25 shows the samples containing rEPS-FS and Na-medium without (Figures 25-A) and with (25-B) fluorescence. Figures 25-1 and 25-3 are taken at day 10 and Figures 25-2 and 25-4 are taken at day 24. The samples 25-1 and 2-2 are taken from BB5 and the samples 25-3 and 25-4 are taken from BB6.

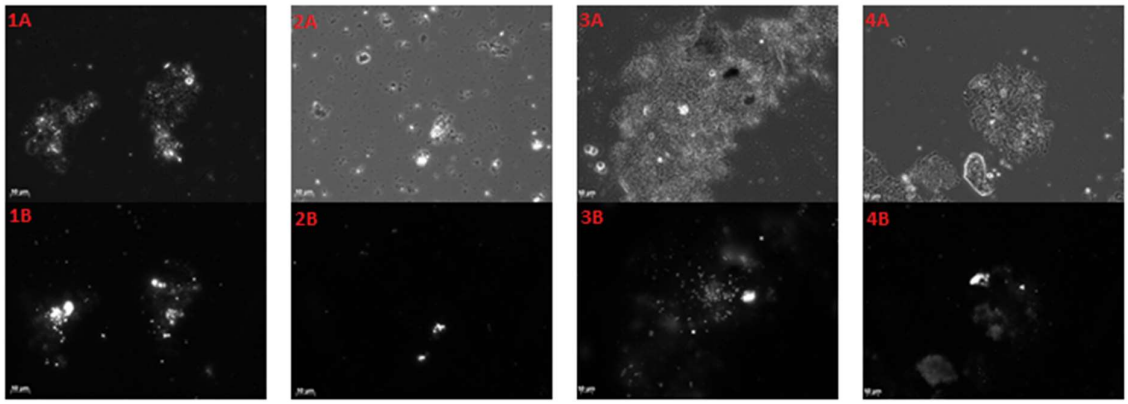

Figure 25. Microscopy pictures of the samples containing rEPS-FS and Na-medium of the 250 mL at day 10 and day 24 without (A) and with (B) fluorescence. 1A: sample BB5 with rEPS-FS with Na-medium at day 10 without staining. 1B: sample BB5 with rEPS-FS with Na-medium at day 10 with staining. 2A: sample BB5 with rEPS-FS with Na-medium at day 24 without staining. 2B: sample BB5 with rEPS-FS with Na-medium at day 24 with staining.3A: sample BB6 with rEPS-FS with Na-medium at day 10 without staining. 3B: sample BB6 with rEPS-FS with Na-medium at day 10 with staining. 4A: sample BB6 with rEPS-FS with Na-medium at day 24 without staining. 4B: sample BB6 with rEPS-FS with Na-medium at day 24 with staining.