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The Effect of Pyrolysis Water on Different Levels of a Reactor for Biological Syngas Methanation

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Abstract

Thermophilic biological syngas methanation has the potential to become an important factor to reduce the usage of fossil fuels and contribute to a resilient energy production in Europe. The technology is built on the complex syntropy of different groups of microbes that together convert syngas (CO, H₂, CO₂, and some CH₄) to CH₄ through a variety of pathways. The pathway for CO conversion to CH₄ has been less studied than the conversion for H₂ and CO₂, but several studies have observed that species that perform hydrogenotrophic methanogenesis and the water-gas shift reaction (WGSR) seem to be dominating in biological syngas methanation in thermophilic temperatures. In future commercial plants for biological syngas methanation, the process will perhaps be disturbed by varying amounts of pyrolysis water, a condensate that might enter the reactor with the syngas. Research Institutes of Sweden (RISE) has conducted an experiment where a trickle bed reactor (TBR) has been exposed to pyrolysis water without seeing any apparent effect on the CH₄ production. However, after conducting a qPCR analysis, targeting the genera *Methanobacterium*, on samples from the TBR, it is possible to conclude that the introduction of the contamination indeed had an effect on the methanogenic community since the population decreased at the top of the reactor, where the contamination was decreased. The reason why this was not apparent on the data gathered from the experiment might be because an inoculum which had been thoughtfully chosen with the diversity in mind had been used, and there were plenty of species that could convert the harmful components. This might have protected the lower parts of the reactor while other methanogenic species than *Methanobacterium* maintained the CH₄ production.

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Går föroreningen upp i rök?

Populärvetenskaplig sammanfattning

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Behovet av fler alternativ för att ersätta fossil bränsle växer för varje dag som går. Biologisk metanisering är en ny teknik som just nu är inne i sista fasen av utveckling, och ser ut att kunna bli ett bra alternativ. Den går ut på att i ett slutet system producera metangas från gaser som koldioxid, kolmonoxid och väte med hjälp av känsliga, anaeroba mikrober som kallas metanogener. De har naturligt förmågan att producera metan i syfte att lagra energi för att överleva. När teknologin för biologisk metanisering är redo, så kommer den att kunna bidra till ett Europa som är mer motståndskraftigt mot oväntade störningar i energiförsörjningen, och mindre beroende av fossila bränslen.

När storskaliga, kommersiella produktioner startar upp i framtiden ser RISE en risk för att varierande mängd kontaminering från den ingående gasblandningen, som ska bygga upp den producerade metanen, följer med in i reaktorn och skadar metanogenerna. Därför är det ett viktigt steg i teknikens utveckling att förstå hur den biologiska processen skulle påverkas av detta. I ett test som RISE har genomfört, där de utsatt en grupp mikrober i en reaktor för föroreningar, ser allting däremot väldigt lovande ut. Nästan lite för bra – de ser inga effekter alls på metangasproduktionen i sina mätningar. Halten av producerad metan är samma som innan, trots att mängden förorening som pumpats in i reaktorn tillsammans med bakteriernas näring uppgår till så mycket som 30%.

Vad händer egentligen med föroreningen i reaktorn? Och hur kommer det sig att metanogenerna, som är kända för att vara extra känsliga, inte påverkas märkbart? Det här är ett mysterium som kan ha fått sitt svar. I en analys som nyligen genomförts syns stor påverkan på en av de viktigaste grupperna av metanogener. I analysen syns också hur bara toppen av reaktorn drabbas – där föroreningen pumpas in – och att botten inte drabbas lika hårt. I vätskan ut från systemet kan man också se att hur föroreningarna minskar ju längre de stannat i systemet. Allting pekar på att föroreningen bryts ner i andra mikrobers metabolism, där slutprodukten i anaeroba miljöer ofta är koldioxid, väte eller metan, som i sådana fall lämnar reaktorn i gasform, kanske man skulle kunna säga att slutsatsen blir att föroreningen faktiskt bokstavligen gick upp i rök. I sådana fall tacklar man bäst en förorening genom att ha en varierad grupp mikrober att förlita sig på, som innehåller grupper som är sugna på allt från kolmonoxid, väte och koldioxid, till skadliga ämnen i en förorening.

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Abbreviations

CH ₃ COOH	Acetic acid
CO ₂	Carbon dioxide
CO	Carbon monoxide
H ₂	Hydrogen gas
CH ₄	Methane
RISE	Research Institutes of Sweden
BES	Sodium 2-bromoethanesulfonate
SLU	Swedish University of Agricultural Sciences
SAO	Syntrophic acetate oxidation
SAOB	Syntrophic acetate oxidating bacteria
TBR	Trickle bed reactor
VFA	Volatile fatty acid

1 Introduction

Biological methanation is a method for producing CH₄ from other gasses, which are converted by microbes inside a closed, anaerobic system (RISE n.d.). The technology has the potential to become an important factor in the shift from fossil fuels to more sustainable options because of its low cost, high capacity for storage, and compatibility with existing infrastructure, like the natural gas grid in Europe (Wegener Kofoed *et al.* 2021). It would also contribute to helping the EU reach its targets for renewable energy, thereby making Europe more energy independent and resilient (European Biogas Association n.d.). The gasses used for biological methanation are either a source of CO₂, for example biogas, with H₂ added separately to the process, or syngas (a mixture of H₂, CO, CO₂, and smaller amount of CH₄), which is a product from gasification of biomass. Methane producing archaea – methanogens – converts the gasses to CH₄ and more CO₂ through a variety of pathways, which often requires a complex syntrophy with other species (Wegener Kofoed *et al.* 2021). The pathway for CO into CH₄ is less studied and more intricate than for H₂ and CO₂ (Grimalt-Alemany *et al.* 2018, Li C *et al.* 2020).

RISE is currently a part of the EU project Biomethaverse (Demonstrating and connecting production innovations in the BIOMETHane uniVERSE) and is constructing a pilot plant for thermophilic biological methanation where syngas, produced through thermal gasification at Cortus AB, will be led directly into a reactor for biological methanation. Future commercial plants for biological syngas methanation will have strategies to cool the gas down before it reaches the reactor to condensate impurities from the gasification process, which produces a liquid of organic compounds in which the water-soluble fraction is called pyrolysis water. Cortus AB produces a particularly clean syngas, so in the Biomethaverse trials it is unlikely that any pyrolysis water ends up in the reactor. However, for other gasification processes it is expected that varying amounts of this impurity will slip through and enter the reactor, which is why the consequences of the contamination are investigated. The pyrolysis water is a liquid which has a low pH in the range of 2-3 and contains aromatic compounds (Andersson *et al.* 2024). These are both factors that have been found to inhibit anaerobic digestion (Jarvis & Schnürer 2009), which is a process that contains the population of microbes needed in a reactor for biological methanation (Wegener Kofoed *et al.* 2021). RISE has conducted a lab-scale experiment with a 5 L trickle bed reactor (TBR) which was fed a contamination of pyrolysis water. During the approximately 630 days of operation, data from various parameters, and samples of the process fluid and carriers were collected. The gathered material from this experiment has been used in this degree project to assess how the microbial community, and consequently the conversion of syngas, in a process for biological methanation is affected by the contamination of pyrolysis water (Andersson *et al.* 2024). This is important to investigate because the microbes are the catalytic element in the conversion, and the methanogens are typically the most sensitive group of microbes in the microbial community (Jarvis & Schnürer 2009). There are several different genera of methanogens with different preferred temperatures and pH; however, in thermophilic syngas methanation it is usually either *Methanothermobacter* or *Methanobacterium* which are the dominating genera (Rachbauer *et al.* 2017, Porté *et al.* 2019, Li Y *et al.* 2022, Cheng *et al.* 2022, Kamravamanesh *et al.* 2023).

1.1 Aim and Objective

The overall aim of the project is to contribute to an increased understanding of the microbial community development in the bioreactors operated at RISE, for future development of the technology. The objective of this degree project is therefore to:

- a) Compare the population of *Methanobacterium* at different levels in a TBR for biological methanation before and after it has been exposed to a contamination of pyrolysis water.
- b) Conduct a preparatory literature study on operational conditions in thermophilic TBRs for biological syngas methanation, with the purpose to provide context to qPCR results and understanding of catabolic pathways for CO conversion to CH₄.

2 Background

This background will describe the societal impact that biological syngas methanation might have in the future, provide the basic knowledge of anaerobic digestion and biological methanation, as well as go more in depth into what conditions the necessary microbial groups thrive in, how they work together in syntrophy to produce CH₄ from syngas, and what catabolic pathways might dominate under different conditions.

2.1 Societal Impacts

Biological methanation is a promising new method for producing biomethane through biological pathways. Generally, the idea in biological syngas methanation is to take lignocellulosic material, like branches, sawdust, and convert the biomass to syngas (a mixture of H₂, CO, CO₂, and small fraction of CH₄) through gasification (Andersson *et al.* 2024). A way of explaining gasification is to think of it as incomplete combustion. Wood, coal, or other solid materials are burnt without enough oxygen to complete the combustion, and without fire. The syngas can be used as an energy carrier for processes where energy is needed (ALL Power Labs 2024). However, by further converting the syngas to CH₄, existing infrastructure can be used. Gas can be stored and transported conveniently in the natural gas grid, which is already in place in Europe and do not require the implementation of any additional infrastructure (Wegener Kofoed *et al.* 2021). The technique could become an important contributor to shift energy production from fossil fuels, help EU reach the targets for renewable energy, and make Europe more energy independent and resilient (European Biogas Association n.d.).

Other benefits of biological methanation, compared to other options under development, is that it operates at atmospheric pressure and low temperatures (37-70 °C). Chemical catalytic methanation, for comparison, has come further in its development than biological methanation, but it is sensitive for impurities, and need high temperature (~300 °C), and pressure (5-20 MPa). Biological methanation has the potential to become cheaper and more readily available given the lower demands on the equipment and conditions (Wegener Kofoed *et al.* 2021).

2.2 Biomethaverse

In the EU project Biomethaverse, RISE are collaborating with the energy producer Cortus AB, and biofuel technology developer Wärtsilä, to construct and run two bioreactors for biological syngas methanation (Borzi 2023). In Biomethaverse, five unique production pathways will be developed in five European countries. By diversifying biomethane production in Europe, Biomethaverse aims to increase the cost-effectiveness, contribute to energy independence, improve the sustainability, and boost the uptake of biomethane technologies. The biological

methanation innovations in Biomethaverse also covers reproducibility of the method, upscaling, and accessibility to the market (Borzi n.d.).

2.3 Anaerobic Digestion

To understand how biological methanation works, it is necessary to start by explaining what anaerobic digestion and biogas is. Biogas forms naturally through anaerobic digestion in decaying organic material and can be produced industrially in biogas digesters. In the absence of oxygen, methane producing archaea – methanogens – produces CH_4 through a variety of pathways in syntrophy with other organisms.

Industrially, this is typically done in an airtight anaerobic digester, or bioreactor, which is fed with a mixture of organic feedstock, such as agricultural waste, municipal waste, manure, wastewater, and food waste. By working together in a complex syntrophy, microbes will digest these substrates into smaller components where the byproduct from one microbe becomes the substrate of another. The different steps of anaerobic digestion are described in *fig. 1*. All steps in the process are performed by a variety of reactions originating from many kinds of microbial species. In the first step – the hydrolysis – complex organic material such as proteins, polysaccharides, and fats are being digested by extracellular enzymes. Most products, except for fatty acids and aromatic structures, from the complex molecules are digested in the next phase, which is the acidogenesis. Here amino acids, simple sugars, and alcohols are broken down into volatile fatty acids (VFAs), alcohols, acetate, H_2 and CO_2 . Then the acetogenesis, during which VFAs, alcohols, and the fatty acids produced during the hydrolysis, becomes oxidized into acetate, and more CO_2 and H_2 . In the last step, CH_4 is finally produced from acetate, or from H_2 and CO_2 through two different pathways: acetoclastic or hydrogenotrophic methanogenesis. There is also a pathway called homoacetogenesis through which, H_2 and CO_2 can be used to produce acetate, which in turn can be used to produce CH_4 , or the acetate can be turned back into H_2 and CO_2 by acetate-oxidizing bacteria.

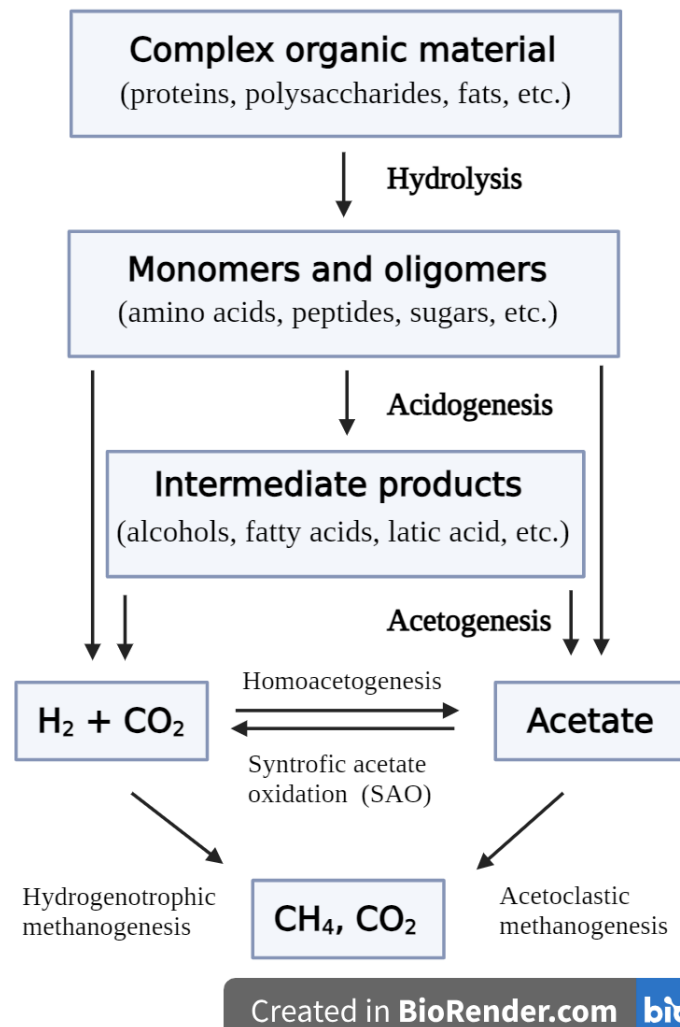


Figure 1: Description of the different steps of anaerobic digestion. The figure is modified from *Microbiology of the biogas process* by Jarvis and Schnurer (2009), and *Biological upgrading of biogas through CO₂ conversion to CH₄* by Wegener Kofoed et al. (2021).

The relevant end products from this process are CH₄ and CO₂, which are the components that make up most of the biogas. The gas is then mainly used for heat and power production, but the pressure of finding substitutes for fuels originating from fossils calls for gas that has a purer content of CH₄. To reach natural gas quality, which would allow it to be injected in the natural gas grid, the raw biogas needs to be purified from contaminants like oxygen, nitrogen, hydrogen, and hydrogen sulphide. The CO₂ also needs to be either removed or further upgraded into CH₄. The product gas from upgraded or purified biogas is called biomethane (Wegener Kofoed *et al.* 2021).

2.4 Biological Syngas Methanation

This master thesis is focused on biological methanation of syngas. However, biological methanation is also used for the process when CO₂ from anaerobic digestion or other CO₂

sources is used together with H₂ generated from electricity. From the perspective of the microbial communities of these processes, biological methanation from CO₂ and H₂, and from syngas are very similar, which is why sources discussing both of these kinds of methanation have been used for some aspects of this report.

In biological syngas methanation, the carbon source is syngas instead of organic material but is otherwise similar to anaerobic digestion. The biomass used to produce the syngas - recalcitrant material like branches, sawdust, and bark – cannot be used to produce biogas because it cannot be decomposed in a traditional anaerobic digester (Andersson *et al.* 2024). Due to the lack of organic substrate, a very limited amount of hydrolysis, acidogenesis, and acetogenesis occurs. This gives an advantage to the methanogens since they do not have to compete with as many other species as they would need to in an anaerobic digester, and the conditions in the reactor can solely be adapted to them, even though the production still is dependent of a complex syntrophy of many species. Digestate – the organic material that remains after an anaerobic digestion process is finished – is often used as inoculum in reactors for biological methanation since it contains the whole microbial consortium necessary for a well-functioning process. However, it is also possible that unwanted reactions are catalysed in the consortium since it is difficult to control all elements of a mixed culture, especially if the process gets disturbed by sudden environmental changes. For example, the production of acetate through homoacetogenesis, and the production of volatile fatty acids (VFAs), such as acetate and propionate, could potentially accumulate, which would create a more acidic environment that is harmful to the methanogens. If the methanogens cease production of CH₄ because they no longer thrive in the environment, this creates a snowball effect where even more acid accumulates (Wegener Kofoed *et al.* 2021).

2.5 Catabolic Routes

There are several different catabolic routes that the syngas can take when it is converted to CH₄. They are depicted in *fig. 2*.

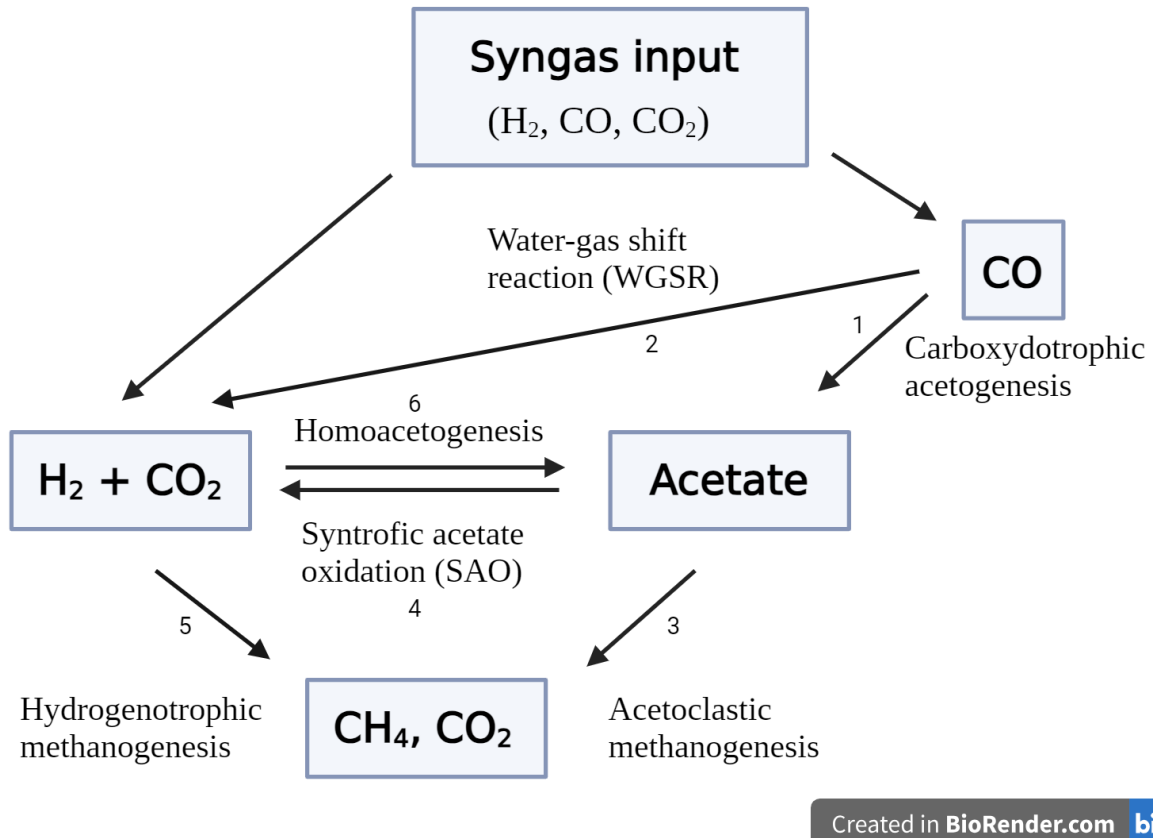
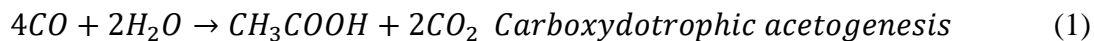


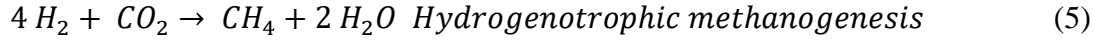
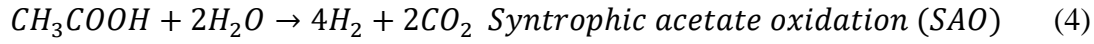
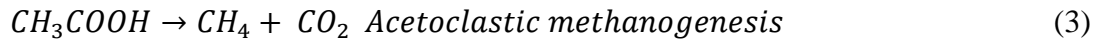
Figure 2: Description of the different catabolic routes the syngas can take when it is converted into CH₄.

The conversion of CO into CH₄ is more intricate than the conversion for H₂ and CO₂ (Grimalt-Alemany *et al.* 2018), and it has also been much less studied (Li C *et al.* 2020, Paniagua *et al.* 2022). Through carboxydrotrophic acetogenesis, CO can be converted into CH₃COOH through reaction 1, but CO can also be turned into H₂ and CO₂ through the water-gas shift reaction (WGSR) through reaction 2 (Grimalt-Alemany *et al.* 2020). It should be noted that acetate, which acetoclastic methanogens convert into CH₄, is the salt or ester form of acetic acid (CH₃COOH), and that they are in equilibrium in solution.

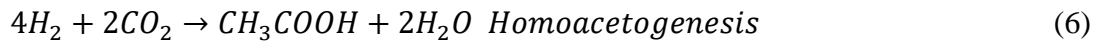


CH₃COOH can in turn be further converted into CH₄ and CO₂ through acetoclastic methanogenesis (reaction 3) or take the route through syntrophic acetate oxidation (SAO)

(reaction 4). It would convert the acetate into H₂ and CO₂, which in turn can be converted into CH₄ through hydrogenotrophic methanogenesis (reaction 5).



The other routes through which H₂ and CO₂ can be converted into CH₄ are through homoacetogenesis (reaction 6), where H₂ and CO₂ react to form CH₃COOH, which can be further converted to CH₄ through reaction 3 by acetoclastic methanogens. Just like CH₃COOH produced from CO (reaction 1), the CH₃COOH can also take the route through SOAs (reaction 4), which would turn it back into H₂ and CO₂ (Grimalt-Alemany *et al.* 2018).



There are also other routes that the syngas can take, but they are of less relevance for this project. Carboxyotrophic methanogenesis – the direct conversion of CO into CH₄ – is possible but has never been observed in a mixed culture. Carboxydotrophic methanogens are inhibited by too low levels of CO, and are outcompeted by hydrogenotrophic methanogens, which grows faster (Grimalt-Alemany *et al.* 2020). The conclusion that carboxydotrophic methanogenesis is negligible in biological syngas methanation has also been made by Sipma *et al.* (2003) and Grimalt-Alemany *et al.* (2017).

2.6 Activity of Catabolic Routes

What catabolic routes, depicted in *fig. 2*, are dominating biological syngas methanation is determined by the environmental conditions of the reactor, which determines thermodynamic limitations and the composition of the microbial community. This was investigated in Grimalt-Alemany *et al.* (2020), in a study where mesophilic and thermophilic (temperature ranges 37-45 °C and 55-70 °C, respectively) enrichment of cultures were performed. These are the two temperature ranges where methanogens are known to thrive. The study was conducted by enriching cultures in batch mode in mesophilic and thermophilic conditions, and testing them with either CO, H₂/CO₂, acetate, or syngas as only substrate. Then the methanogenic community was inhibited by adding sodium 2-bromoethanesulfonate (BES) and observing what intermediate product (H₂/CO₂ or acetate) was accumulated. The mesophilic enriched culture mainly produced acetate as intermediate product when it grew on CO as only substrate, and propionate as secondary product. There are several microbial groups that grow on CO and are reported to produce acetate: carboxydotrophic acetogens, methanogens, hydrogenogens, and sulfate-reducers. Out of them, only carboxydotrophic acetogens have been attributed the properties of acetate production under mesophilic conditions. Furthermore, BES will have inhibited the methanogens in the consortia, and there

was no sulphate in the growth medium, which leaves carboxydrotrophic acetogens as the only group of microbes that could have produced the acetate in the mesophilic experiment. This indicates that the pathway for the mesophilic culture, when CO was used as the only substrate, went through reactions 1 and 3. The same experimental setup with the thermophilic culture and CO used as only substrate showed accumulation of H₂ as the main intermediate product, and acetate as a secondary product. Interestingly, acetate was not observed as a product when the thermophilic enrichment culture was exposed to syngas as substrate, and no acetoclastic activity was recorded in this culture. This indicates that acetate was only produced when CO was used as the only substrate, which means that there was a shift from hydrogenesis to acetogenesis, and the conversion went through reactions 2 and 5, either separately or in series, when syngas was converted by the thermophilic culture.

The enriched cultures did also not convert H₂/CO₂ through the same pathways. In the mesophilic enriched culture, inhibited by BES, acetate was the only intermediate product. In the same experimental setup, without BES inhibition, both acetate and methane were produced. For comparison, the thermophilic enriched culture did not produce any acetate in the same experimental setup, with inhibition by BES. When BES was not added, the H₂/CO₂ was converted strictly to CH₄. The thermophilic enriched culture hence seems to have converted H₂/CO₂ through hydrogenotrophic methanogenesis (reaction 5) only. Meanwhile, the mesophilic enriched culture seemed to have utilized both the pathway through homoacetogenesis (reaction 6) and hydrogenotrophic methanogenesis (reaction 5), which would have competed for substrate.

It was also found that the maximum specific productivity was significantly higher in the thermophilic compared to the mesophilic enrichment, to the point where the difference could not solely be attributed to increased reaction rates due to increased temperature. This could be because the pathways in the mesophilic enrichment were more complex with more groups of microbes involved and the formation of two kinds of intermediate products instead of just one, as in the thermophilic enrichment. Since the pathways used in the thermophilic enrichment had less steps, which would lead to less energy losses, this might explain the higher CH₄ yield. This suggests that the enrichments had a large impact on which catabolic routes that dominated conversion of syngas. The study concluded that the findings can be used to optimizing the operation and increasing productivity in mixed-culture biological syngas methanation (Grimalt-Aleman *et al.* 2020).

In the sections below, it is discussed how the activity of the catabolic routes relates to the temperature in a span of roughly 7-87 °C from a thermodynamic point of view.

2.6.1 Carboxydrotrophic Acetogenesis and the WGSR

The acetogenic microorganisms catalyse the carboxydrotrophic acetogenesis. They are capable of forming acetate and alcohols from CO and H₂O, despite the toxic effect CO has been proven to have on microbes. Some species from genera *Clostridium*, *Acetobacterium*, and *Sporomusa* have been observed to perform this conversion (Paniagua *et al.* 2022). With rising

temperatures, the carboxydrotrophic acetogenesis becomes less thermodynamically favourable (increasing ΔG – less spontaneous reaction) while the second route for CO conversion – WGSR, converting CO and H₂O into H₂ and CO₂ – becomes more favourable (decreasing ΔG – more spontaneous reaction) (Grimalt-Alemany *et al.* 2020). This is in line with other studies that concludes that CO conversion to acetate dominated in mesophilic conditions, and CO conversion to CO₂ and H₂ dominated in thermophilic conditions (Sipma *et al.* 2003, Luo *et al.* 2018). Species belonging to the genera *Rhodospirillum*, *Thermincola*, *Desulfotomaculum*, *Carboxydotherrmus*, *Caboxydocella* and *Moorella* are known to perform the WGSR (Paniagua *et al.* 2022).

2.6.2 Acetoclastic Methanogenesis and SAO

CO taking the route through carboxydrotrophic acetogenesis, forming acetate, can either be converted to CH₄ through acetoclastic methanogenesis, or be converted into H₂ and CO₂ through SOA. Under thermophilic, anaerobic conditions, SOA has been found to be a significant pathway when paired with the syntrophic partners hydrogenotrophic methanogens (Paniagua *et al.* 2022), and are often found in thermophilic anaerobic digestion of biowaste (Kamravamanesh *et al.* 2023). In a paper by Dyksma *et al.* (2020), it was found that SAO dominates over acetoclastic methanogenesis in thermophilic digestion of biowaste when it comes to the competition for acetate, as the syntrophic acetate oxidizing bacteria (SAOB) greatly outnumbered the acetoclastic methanogens. The conclusion of the study is that SOA is an important pathway for methanogenesis (Dyksma *et al.* 2020). A study by Li *et al.* (2020) came to the same conclusion based on the large difference in numbers between SAOB and acetoclastic methanogens. However, thermodynamically; both acetoclastic methanogenesis and SOA become more favourable with rising temperatures (Grimalt-Alemany *et al.* 2020, Dyksma *et al.* 2020). It should be noted that DNA analyses such as sequencing and qPCR cannot be used as evidence for what activity occurs in a reactor. The detected DNA can belong to deceased or inactivated microbes, which means that they currently have no impact on the pathways inside the reactor (Yap *et al.* 2022)

Some well-known SAOB are genera *Tepidanaerobacter*, *Syntrophaceticus*, *Thermotoga*, and *Thermacetogenium* (Dyksma *et al.* 2020). The phylum Firmicutes is also known to have members of SAOB, additionally to having species recorded to hydrolysis and acidogenesis in anaerobic environments (Li C *et al.* 2020). As previously mentioned, only two known acetoclastic genera are known: *Methanosarcina* and *Methanoseata*. They are well-studied in comparison to SAOB, where only a few isolated species are described so far (Dyksma *et al.* 2020).

2.6.3 Homoacetogenesis and Hydrogenotrophic Methanogenesis

Because homoacetogenic microorganisms convert H₂ and CO₂ into acetate, according to reaction 6, they compete with the CH₄ formation by hydrogenotrophic methanogens. However, under mesophilic and thermophilic conditions, homoacetogens generate less energy than the hydrogenotrophic methanogens, and are out-competed (Zabranska & Pokorna 2018,

Grimalt-Alemany *et al.* 2020). However, both the reactions for hydrogenotrophic methanogenesis and homoacetogenesis becomes more thermodynamically favourable with rising temperatures (Grimalt-Alemany *et al.* 2020).

2.7 Methanogens

Methanogens are known to be sensitive and grow slowly. The different species have highly specific environment and require specific substrates, which often makes them the limiting group for the whole process of biological methanation (Zabranska & Pokorna 2018).

Thermophilic methanogens generally thrive in temperatures 50-60 °C, even though some species are reported to do well in conditions up to 70-80 °C. The preferred pH value is typically in the range of 7-8, but there are individual species that prefer pH as low as 6 and as high as 9 (Zabranska & Pokorna 2018). Wegner *et al.* (2021) lists a number of studies on biological methanation and the temperatures of the processes, and among the processes that had been run in thermophilic TBRs, most had been operated at temperatures in the range of 54-65 °C. The growth-rate of methanogens will generally be at its highest closer to 60 °C and decrease greatly in temperatures closer to 65 °C, which makes the time needed for starting up warmer reactor longer than a colder one. However, though it takes longer to start up a warmer process, it has been reported that the productivity continuously increases with the temperature (Dong N *et al.* 2018). At the same rising temperatures, the growth-rate of acid-forming organisms decreases slower than the methanogens, which can cause an accumulation of fatty acids in the process, and consequently a lower pH value.

For a biogas plant, it is recommended that the temperature is kept within a span of +/- 0.5 °C, or potentially within a span of +/- 2-3 °C, if the process is stable in terms of, for example, alkalinity. The microbial community will adapt to whatever temperature is chosen, and seem to do well in the whole thermophilic range of 50-65 °C. An example of the adaptability of microbial community is that a characteristic mesophilic culture can be stepwise transformed into a thermophilic culture if the temperature is changed slowly over time. This requires an inoculate that diverse enough to host a wide range of species (about 10 % of all microorganisms in a mesophilic process for biogas production is estimated to actually be thermophilic species), and a temperature-change at about 1 C°/day. Sudden changes can put the whole synergy between the microorganisms out of balance. This is possible because many microbial species can stay alive in environments that are less than ideal, since it is possible for them to inactivate or slow down their metabolism temporarily until the conditions are favourable again (Jarvis & Schnürer 2009). However, in a paper by Grimalt-Alemany *et al.* (2019), it was observed that a thermophilic enrichment of a mesophilic culture with a drastic change in incubation temperature worked without negative effects. The authors found this striking and comments that this attests to the high adaptability and of mixed cultures when faced with environmental changes.

2.7.1 Different Groups of Methanogens

The formation of CH₄ in a reactor for biological methanation is catalysed by three different groups of methanogens, which categorizes them based on their ability to utilize different substrates. Hydrogenotrophic methanogens use H₂ as electron donor to reduce CO₂ into CH₄. Methylotrophic methanogens use methanol, methylamines and other methylated compounds to reduce CO₂ into CH₄. Acetolactic methanogens use acetate to produce CH₄ and CO₂. However, most methanogens are capable of utilizing H₂ to reduce CO₂ into CH₄, regardless of what group they are classified as (Wegener Kofoed *et al.* 2021).

The only two methanogens known to utilize acetate for CH₄ production are the genera *Methanosaeta* (also known as *Methanothrix*) and *Methanosarcina*. However, *Methanoseata* is strictly dependent on acetate, while *Methanosarcina* has the ability to grow on substrates belonging to all three of the groups used for classification of methanogens (Stams *et al.* 2019, Kurade *et al.* 2019).

It has been reported in several studies that acetolactic methanogens are more likely to dominate under mesophilic conditions, and hydrogenotrophic methanogens under thermophilic conditions. This has led to several articles hypothesising that this might be a general rule (Zabranska & Pokorna 2018, Dong N *et al.* 2018, Grimalt-Alemany *et al.* 2020, Li C *et al.* 2020). The explanation for this may lie in the preferred temperature of the acetolactic methanogens, and in the thermodynamics in the methanogenic reaction paths (see [5.4.3 Activity of Catabolic Routes](#)). Zabranska *et al.* (2018) lists a number of species belonging to several different genera, and their optimal temperatures. The items from the list belonging to the two dominating genera *Methanothermobacter* or *Methanobacterium*, and the two acetoclastic genera *Methanosarcina* and *Methanoseata* are listed in Table 1 because they are of higher interest to this project than other methanogenic species.

Table 1: Species of different methanogenic genera listed with their substrate, and preferred temperature and pH ranges (Zebranska et al 2018).

Species	Substrate	Optimal temperature (°C)	Optimal pH
<i>Methanobacterium bryantii</i>	H ₂ /CO ₂	37	6.9-7.2
<i>Methanobacterium formicum</i>	H ₂ /CO ₂ , formate	37-45	6.6-7.8
<i>Methanobacterium thermoalcaliphium</i>	H ₂ /CO ₂	58-62	8.0-8.5
<i>Methanothermobacter thermoautotrophicum</i>	H ₂ /CO ₂	65-70	7.0-8.0
<i>Methanothermobacter wolfeii</i>	H ₂ /CO ₂	55-65	7.0-7.5
<i>Methanosarcina acetivorans</i>	Methanol, acetate	35-40	6.5
<i>Methanosarcina barkeri</i>	H ₂ /CO ₂ , methanol, methylamines, acetate	35-40	5.0-7.0
<i>Methanosarcina mazei</i>	Methanol, methylamines, acetate	30-40	6.0-7.0
<i>Methanosarcina thermophile</i>	H ₂ /CO ₂ , methanol, methylamines, acetate	50	6.0-7.0
<i>Methanosaeta concilii (soehngeni)</i>	Acetate	35-40	7.0-7.5
<i>Methanosaeta thermophila</i>	Acetate	55-60	7.0

2.7.2 Dominating Genera in Biological Methanation

The dominating genera of methanogens in many studies on biological methanation in TBRs, in both mesophilic and thermophilic temperatures, are the hydrogenotrophic methanogens *Methanobacterium* and *Methanothermobacter* (Rachbauer *et al.* 2017, Porté *et al.* 2019, Li Y

et al. 2022, Cheng *et al.* 2022, Kamravamanesh *et al.* 2023). This has also been reported in studies on both *ex situ* and *in situ* setups – the two main reactor configuration in biological methanation – which have utilized H₂ and syngas (Li C *et al.* 2020, Aryal *et al.* 2021, Jiang *et al.* 2021, Braga Nan *et al.* 2022).

2.8 Different Operational Conditions of Biological Methanation

A reactor setup for biological methanation can be categorized as *ex situ*, or *in situ* depending on if the gaseous carbon, for example CO₂, is produced inside the reactor or added to the process from an external source. An *in situ* reactor can be described as an anaerobic digester where biogas (CO₂ and CH₄) is formed from organic material. H₂ is added to the process, which enables upgrading of the CO₂, produced from the anaerobic digestion, further into CH₄. In an *ex situ* setup, both H₂ and CO₂, and possibly other gasses used for the process, are added from an external source, and the reactor does not work as an anaerobic digester, since no organic material is degraded to produce CO₂. Only the microbes that contribute to catalysing the reactions from H₂ and CO₂ to CH₄ are necessary to promote in *ex situ* setups. This means that the conditions inside the reactor can be fully adapted to the methanogens, and no compromises to also sustain a well-functioning anaerobic digestion are needed. This strongly contributes to the fact that the *ex situ* configuration has gathered more interest for potential future applications (Wegener Kofoed *et al.* 2021). Biological syngas methanation is an example of an *ex situ* application, due to the fact that gaseous carbon is added to the process with the syngas and is not produced inside the reactor.

2.8.1 Temperature and Gas-Liquid Mass Transfer

The temperature is an important factor in a reactor for biological methanation since it affects the gas solubility, the gas-liquid mass transfer, and the biological activity of the population of microbes. Since methanogens are exclusively present in wet or moist environments, they can only convert dissolved gasses. Both CO and H₂ are gasses with low solubility, and H₂ has been identified as a limiting factor for the rate of CH₄ production in biological methanation. In comparison with mesophilic conditions, thermophilic conditions lead to decreased gas solubility. However, in the case of H₂ in biological methanation, the increased activity of methanogens at thermophilic conditions will be a strong driving force for the conversion of H₂, which will outweigh the fact that less gas is dissolved (Benjaminsson *et al.*, 2013). Similarly, in processes for biological syngas methanation, the consumption rate for CO is higher in thermophilic than mesophilic conditions, despite the lower solubility (Li C *et al.* 2020). To overcome the issues with low solubility of H₂ and CO, reactor setups have been designed to optimize the gas-liquid mass transfer (see section 5.11 Trickle Bed Reactors). The solubility is also dependent on the partial pressure of the gasses. However, too high partial pressure of H₂ and CO can bring unwanted consequences or activation of the wrong pathways. CO can for example lead to microbial inhibition due to its toxic properties, and it has also been found that $P_{CO} > 1.35$ bar increases the activity of the pathway from acetic acid, which conjugate base is acetate, to ethanol (Paniagua *et al.* 2022). As with other operational

parameters, the best option is to keep the gas composition constant and avoid drastic changes. If a higher CO content is utilized from the start, or increased stepwise, species in the culture with a high tolerance will be selected for, but a sudden increase of CO could inhibit the process of a culture which is adapted to low levels (Grimalt-Alemany *et al.* 2020).

When it comes to the methanogenic reactions, the rates increase with increasing temperatures. This is another factor that might increase the mass transfer rate with increasing temperatures. Thermophilic biological methanation processes are generally reported to produce more CH₄ than mesophilic processes, (Wegener Kofoed *et al.* 2021, Paniagua *et al.* 2022), and have been suggested to be the superior alternative for industrial plants (Grimalt-Alemany *et al.* 2020). However, when designing a reactor for biological methanation, the increased production needs to be weighed against increased costs, such as heating or cooling of the reactor (Wegener Kofoed *et al.* 2021).

2.8.2 pH value

The pH in a reactor for biological methanation should follow the preferred pH for methanogens, which is the same range for which most archaea thrive (6.0-8.0, with optimal growth at 7.0), since bacteria typically can thrive at a wider pH range. As mentioned previously, an accumulation of VFAs could lower the pH in a reactor for biological methanation and could cause the process to be set out of balance since low pH inhibits the methanogens, causing even more VFAs to accumulate (Paniagua *et al.* 2022).

2.8.3 Type of Culture

It is possible to use pure or mixed cultures for *ex situ*, but not for *in situ* biological methanation since the whole process of anaerobic digestion in an *in situ* process is dependent on the syntrophy between many different species. Pure cultures usually only utilize a single strain of a hydrogenotrophic methanogen, but this is a less common and more expensive approach. The hygienisation processes to keep the culture pure is costly, since all ingoing gas and liquid, and the equipment, need to be kept sterile, and the inoculum is not as cheap as an inoculum from digestate (Wegener Kofoed *et al.* 2021). Additionally, a mixed culture is more robust and less susceptible to inhibition (Paniagua *et al.* 2022). For these reasons, most studies have been conducted with mixed cultures, and it is viewed as the more convenient and cheap option for future applications. However, a mixed culture can be difficult to control and can sometimes produce unwanted product (Wegener Kofoed *et al.* 2021).

When using a mixed culture, it is important to have a diverse inocula since it directly affects the performance of biological syngas methanation. In a paper by Li *et al.* (2020), that investigates the effect of the microbial community on biological syngas methanation, it was noted that inocula which is low in different microbial groups greatly affected the products from the methanation. Specifically, it was concluded that no CH₄ was produced from CO when the inoculation culture lacked SAOB and acetoclastic methanogens.

2.9 Trickle Bed Reactors

A Trickle Bed Reactor (TBR) is column shaped and packed with carriers – a small structure that is used to fill up reactors rather than liquid – with a large specific surface area (m^2/m^3) on which a microbial community can be established from an inoculation culture. A nutrient solution, where the culture obtains macronutrients and trace elements, is distributed occasionally in small batches at the top of the reactor, making it trickle down over the carriers on its way down. What sets it apart the most from other reactors that has been researched for biological methanation is that it is filled with gas instead of liquid. The gas is introduced from the bottom or the top of the reactor, and travel through the packed bed to the other end. In comparison to reactors with liquid as the primary phase, a gas filled reactor provides better control of the gas retention time – the time a compound needs to travel through the column – and makes the diffusion of the gasses more efficient because of the large surface area of the interface between the gas and liquid phase (Wegener Kofoed *et al.* 2021). This improves the poor solubility of H_2 . The carriers increase the retention time of the microbes, which favours the methanogens because of their slow growth rate (Jarvis & Schnürer 2009, Wegener Kofoed *et al.* 2021). This is because they become independent of the hydraulic retention time – the average time it takes for liquid to travel through the system – since the majority of them are immobilized and not dissolved in the solution (Wegener Kofoed *et al.* 2021).

2.10 Anaerobic Filters

An anaerobic filter is traditionally a bioreactor for treatment of wastewater from different types of processes (Stanbury *et al.* 2017, Sanitation Techniques in Emergencies 2021). They are liquid filled reactors and can have packing of both natural and synthetic nature depending on their purpose. A variety of process effluents can be treated with this technology; fermentation and pharmaceutical wastes, wastewater from yeast production, and domestic liquid waste (Stanbury *et al.* 2017). As the effluent flows through the filter, the microbial community established on the packing inside the reactor degrades particles of organic material (Sanitation Techniques in Emergencies 2021).

3 Materials and Methods

Chapters [3.1 The TBR Experiment at RISE](#) and [3.2 Data From the TBR Collected by RISE](#) summarizes how RISE operated the TBR and the relevant data that was produced.

Information about the TBR and how it was operated during the experiment has been retrieved from the article published by Andersson *et al.* (2024). This is followed by a description of the samples chosen for analysis, and summarization of extraction of DNA, preparation for sequencing, and upcoming classification, which were conducted as a part of this project.

3.1 The TBR Experiment at RISE

The TBR used to produce the data that has been analysed in this degree project was operated at RISE for almost two years. It was an *ex-situ* lab-scale reactor with an active volume of 5 L (the volume of the reactor filled with carriers), containing the carriers AnoxTMK1 with an active surface of 500 m²/m³. The body of the reactor with active volume was jacketed with hot water, which was controlled with an automatic system to maintain a target temperature of 58-60 °C. The reactor was fed with a synthetic syngas, which mimics the general composition of syngas from gasification. It consisted of approximately 40% H₂, 30% CO, 20% CO₂, and 10% N₂. On a few instances where there were delivery issues with the syngas, other syngases with slightly different composition were used in short periods. Nitrogen gas is not a part of syngas from a real gasification process but was included in the experiment to mimic ingoing CH₄, which is a component of syngas but travels through the system without reacting. The reason to use nitrogen instead was to make it easier to measure the amount of CH₄ that is produced in the methanation process. The syngas used in the experiment was synthetic but had similar composition as a generic syngas from biomass gasification. Liquid (nutrient solution) was trickled over the bed from the top of the reactor, while the gas was introduced from the bottom of the reactor and travelled through the reactor counter current. The set-up also included an anaerobic filter used to treat a portion of the pyrolysis water before it reached the TBR. It was a reactor packed with carriers, but the moving phase was liquid, instead of gas, which circulated from top to bottom. The purpose of the anaerobic filter was to make the pyrolysis water less potent and make sure the TBR would not be completely set off balance when the pyrolysis water was introduced. The anaerobic filter circulated liquid taken from the bottom of the TBR, but also had its own internal circulation. The TBR and anaerobic filter were not coupled in series, instead a portion of the liquid entering the anaerobic filter – ingoing nutrient solution mixed with recirculated process fluid – was redirected and led into the TBR. A schematic figure of the reactor setup can be found in *fig. 3*.

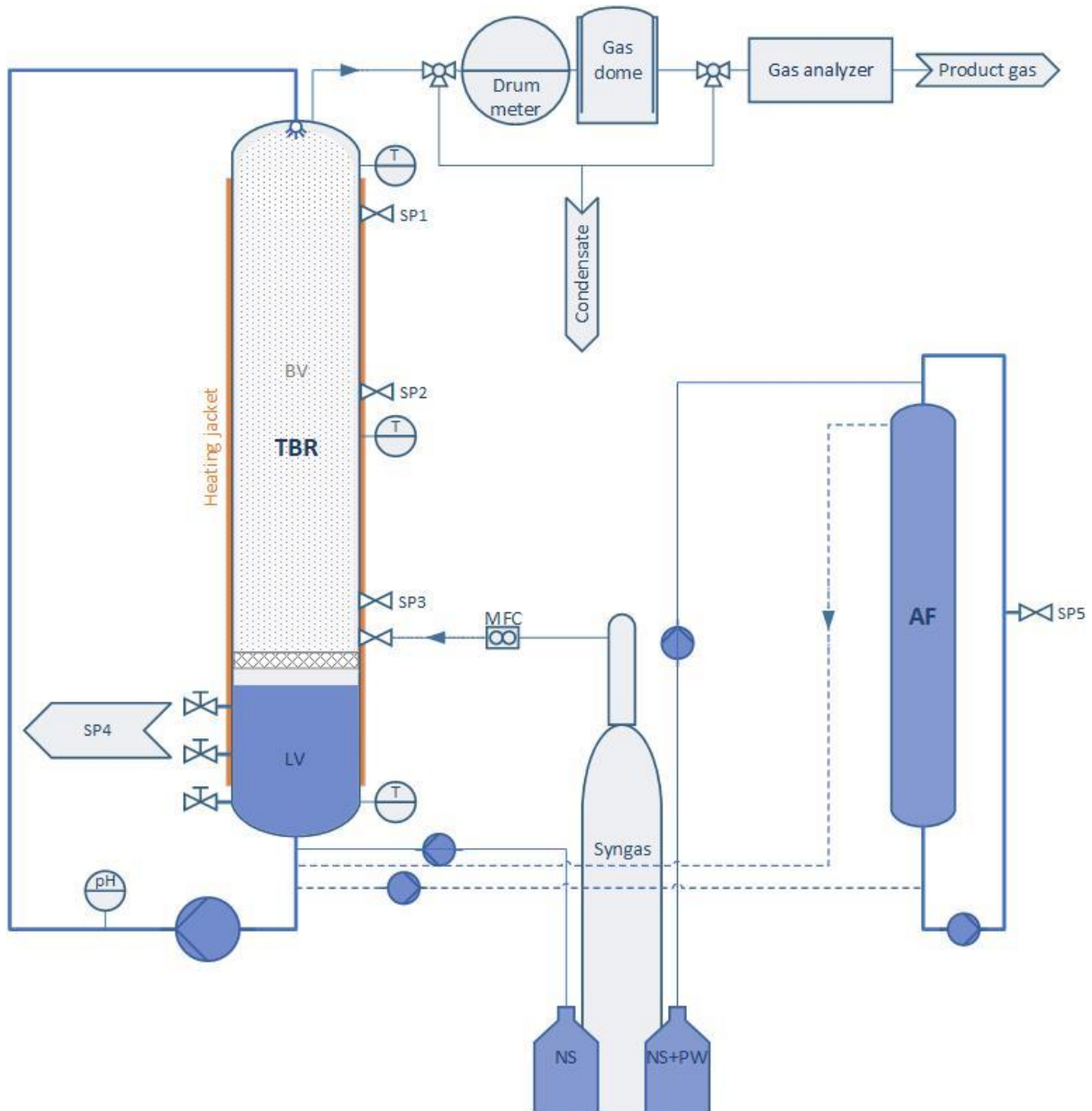


Figure 3: Schematic description of the set-up used in the experiment. Illustration created by Karin Berg at RISE.

3.1.1 Inoculation

The inoculation cultures for the TBR and the anaerobic filter, which consisted of digestate, had been retrieved fresh from multiple biogas digesters to gain as much diversity to the population of microbes as possible. The digesters ranged from mesophilic to thermophilic (typically run with temperatures close to 37 °C, respective 55 °C) and was based on different proportions of food waste, manure, digested sewage sludge, and agricultural residues as substrates. The TBR and the anaerobic filter were inoculated with different batches of mixed cultures, meaning that they do not have identical microbial communities.

3.1.2 Nutrient Solution

The nutrient solution consisted of digestate, like the inoculation cultures, but was more diluted and did not have the same requirements for freshness and diversity. The purpose of it was to provide both macro- and micronutrients for the microbial community. It was not retrieved with the number of different digesters in mind and has, unlike the inoculation cultures, been stored in the fridge. The exact components of a nutrient solution also varies from batch to batch depending on multiple factors, such as season of the year, how fresh it is, and depending on what kind of waste the biogas plants has processed to produce it.

3.1.3 Pyrolysis Water

The pyrolysis water was introduced into the TBR and the anaerobic filter, after the inoculation and reference period, by diluting the nutrient solution with it in different portions (10, 20, or 30 weight percentages). An analysis of the pyrolysis water in the experiment concluded that the organic compounds in the liquid consisted mainly of ketones, diones, and organic acids, with a small relative amount of phenols and other compounds. An analysis of the nutrient solution diluted to 30% pyrolysis water can be found in Appendix I. The pH of pyrolysis water is typically 2-3.

3.1.4 Operation

In the timeline in *fig. 4*, day 0 is the day of inoculation of the TBR. For the first 120 days, the gas flow was increased gradually to allow the microbial community to establish itself on the carriers. During days 126-233, a reference period with maximal gas flow was established to act as a phase of comparison to the state of the reactor after it has been fed the pyrolysis water. During the inoculation and reference phase, nutrient solution without any pyrolysis water was used to feed the reactor.

The anaerobic filter was inoculated separately on day 105 and was fed a nutrient solution containing 10% pyrolysis water right from the start of inoculation to benefit a population of microbes adapted to the environment. On day 166 the nutrient solution going into the anaerobic filter was diluted to contain 20% pyrolysis water. On day 234, the anaerobic filter was coupled to the TBR, and they both were fed a nutrient solution containing 20% pyrolysis water. On day 267, the nutrient solution was diluted to 30% pyrolysis water. This phase was kept stable until day 491, when the anaerobic filter was disconnected from the TBR. Then, nutrient solution containing 30% pyrolysis water was fed directly into the TBR without being led through the anaerobic filter. From day 552 to the end of the experiment nutrient solution without pyrolysis water was once used again to feed the TBR.

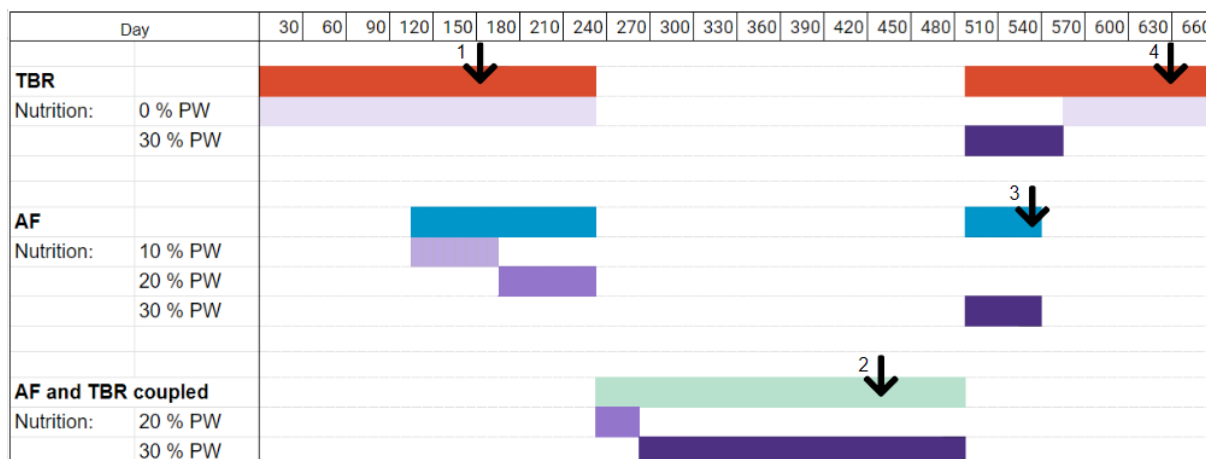


Figure 4: Timeline describing the different operational conditions of the Trickle Bed Reactor (TBR) and the anaerobic filter (AF) during the experiment. The different proportions of pyrolysis water (PW) in the nutrient solution are indicated with darkening shades of purple under the different types of experimental setups. The TBR, AF, and AF and TBR coupled are represented by red, blue, and green lines, respectively. The time-points where samples for analysis of carriers and process fluid have been retrieved from the TBR, or the anaerobic filter, are marked with black, numbered arrows.

3.2 Data From the TBR Collected by RISE

In this section, relevant data that was collected by RISE during the run is presented. The graphs are overlaid with colours to describe when different operational conditions were in place. P1 – reference period with maximal syngas flow rate. P2 – 20% pyrolysis water with the anaerobic filter coupled to the TBR. P3 – 30% pyrolysis water with the anaerobic filter coupled to the TBR. P4 – 30% pyrolysis water directly into the TBR, without the anaerobic filter. P5 – 0% pyrolysis water directly into the TBR, without the anaerobic filter.

One of the most important operational parameters is the syngas flow rate, or gas load (*fig. 5*), which determines the maximum methane yield provided that all CO and H₂ is converted into methane. The production of CH₄ and the conversion rate of CO and H₂ is judged in the context of how much ingoing syngas there was to the TBR at the specific time-point. In this process, the flow rate started low and was increased gradually during the start-up phase. During the reference phase, the process was run with high flow rate. When the pyrolysis water was introduced, the reactor was initially kept as the same syngas flow rate as during the reference phase, but after a period of technical issues with gas availability leading to a longer period with low flow rate, the flow rate had to be increased gradually again. The syngas flow rate was decreased when the process exhibited signs of inhibition (rising levels of CO and H₂ in the outgoing gas) due to temporary disturbances. The maximum stable gas load achieved was 25.9 L/L_{BV}/d (*fig. 5*).

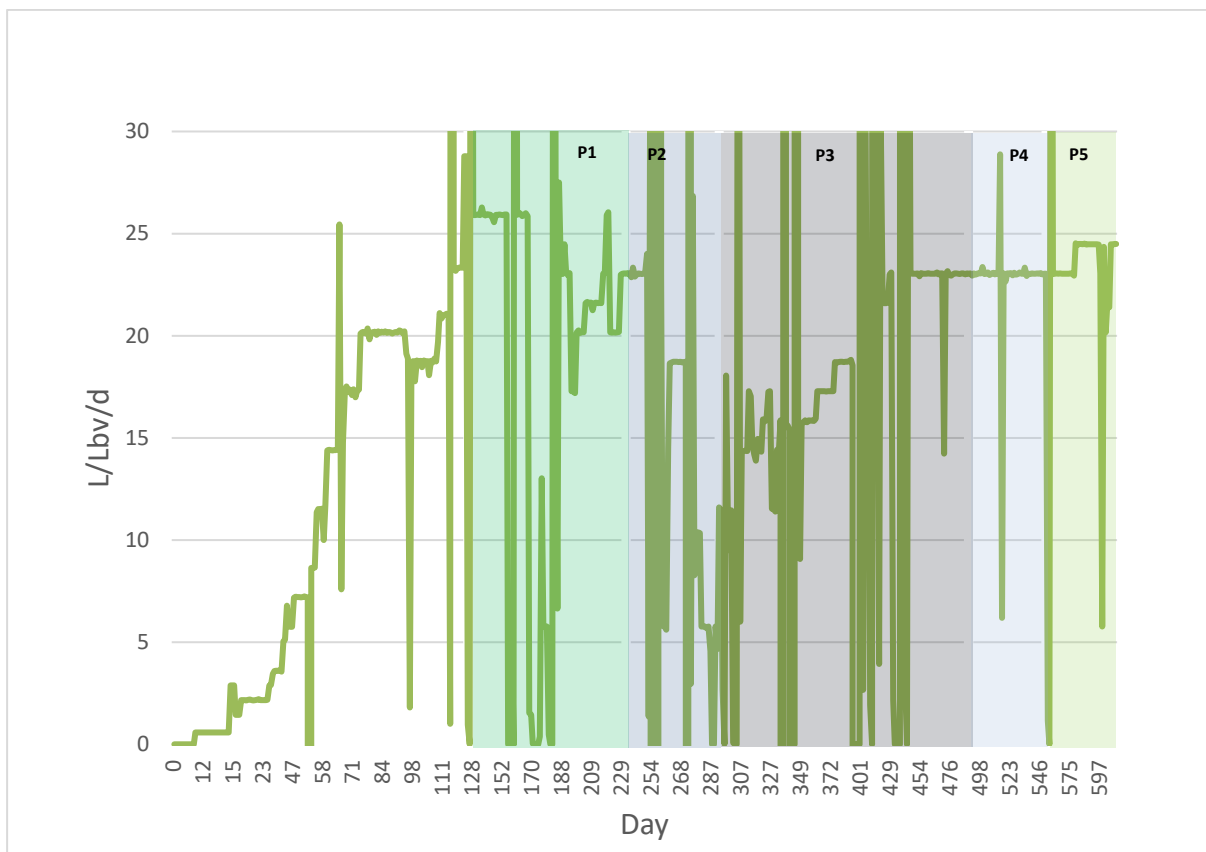


Figure 5: Calculated syngas inflow. Instances where the values are greater than 30 or drops down to 0 suddenly are measuring errors. No data was recorded after day 610.

The production of CH_4 in $\text{L CH}_4/\text{L}_{\text{BV}}/\text{d}$ (volume of produced methane per packed bed volume per day) is plotted below in *fig. 6*. The reference period was from day 126-233, which is the phase P1 in the graphs. Halfway through P1, there was an issue with the delivery of the syngas, which was detected in the data as a dip in the production at day 170. The flow rate of the ingoing syngas was increased gradually when it was in place again. However, before and after the disturbance, a production of approximately $4 \text{ L CH}_4/\text{L}_{\text{BV}}/\text{d}$ was recorded. The production of CH_4 follows the shape of the curve for the flowrate of the ingoing syngas, which is a sign of high yield and a process that have a high conversion efficiency of CO and H_2 . If they were to diverge, it would mean that carbon is accumulating in some shape inside the reactor, possibly as VFAs. That would eventually lower the pH in the reactor, which is harmful to the sensitive methanogens.

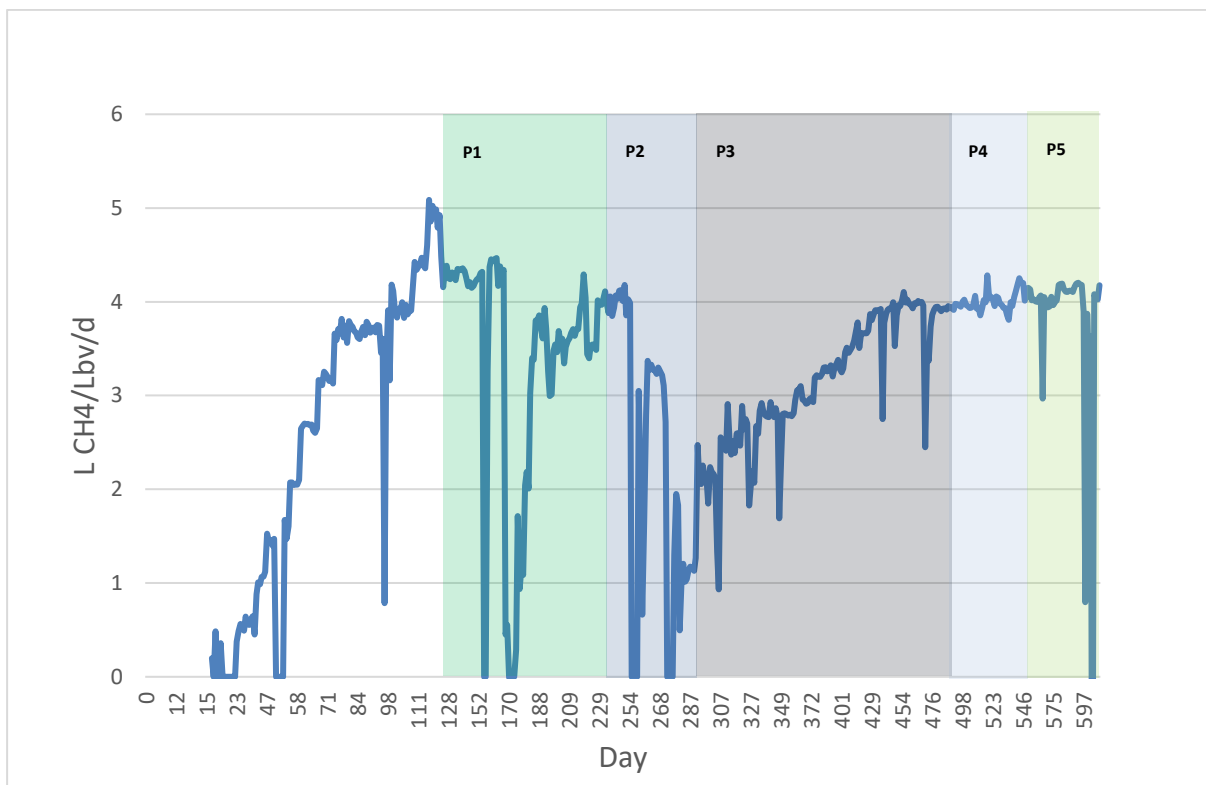


Figure 6: Specific methane production (L CH₄/Lbv/d). No data was recorded prior to day 15, or after day 610.

In the graph depicting the composition of gas outflow (*fig. 7*), there are a few variations in the production. The two spikes in the percentage of outgoing CH₄ (approximately days 106, 294, and 600) took place because another syngas with a different composition was used during these periods, due to issues with availability of the syngas that was supposed to be used. There are also a few dips in outgoing CH₄, all of which can be connected to temporary conditions in the operation of the TBR.

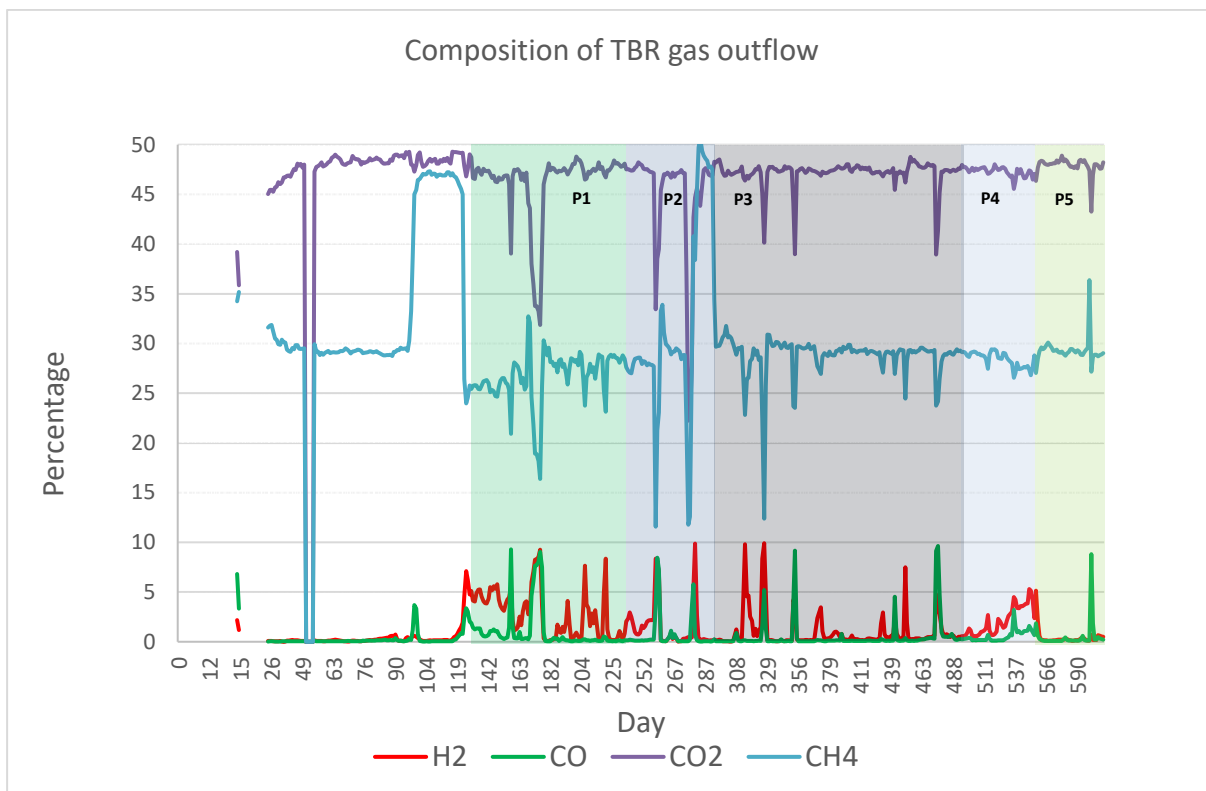


Figure 7: Composition of gas outflow. The measuring equipment were not properly in place before approximately day 60, until the process had stabilized and started running more efficiently after the start-up phase. No data was recorded after day 610.

To summarize, there is no evident influence on the operating data from the nutrient solution containing pyrolysis water, which suggests a robust and insensitive system. If the pyrolysis water had any impact at all, it is small enough to be impossible to tell from the data because the other varying parameters in the experiment – gas inflow, the composition of the syngas, interrupted gas flow, and electrical outage – seems to have affected the system significantly more. This can also be concluded from the graphs depicting pH level, VFAs, and temperature inside the reactor (*fig. 8*). There were no finds in the data that could be directly connected to the introduction of pyrolysis water.

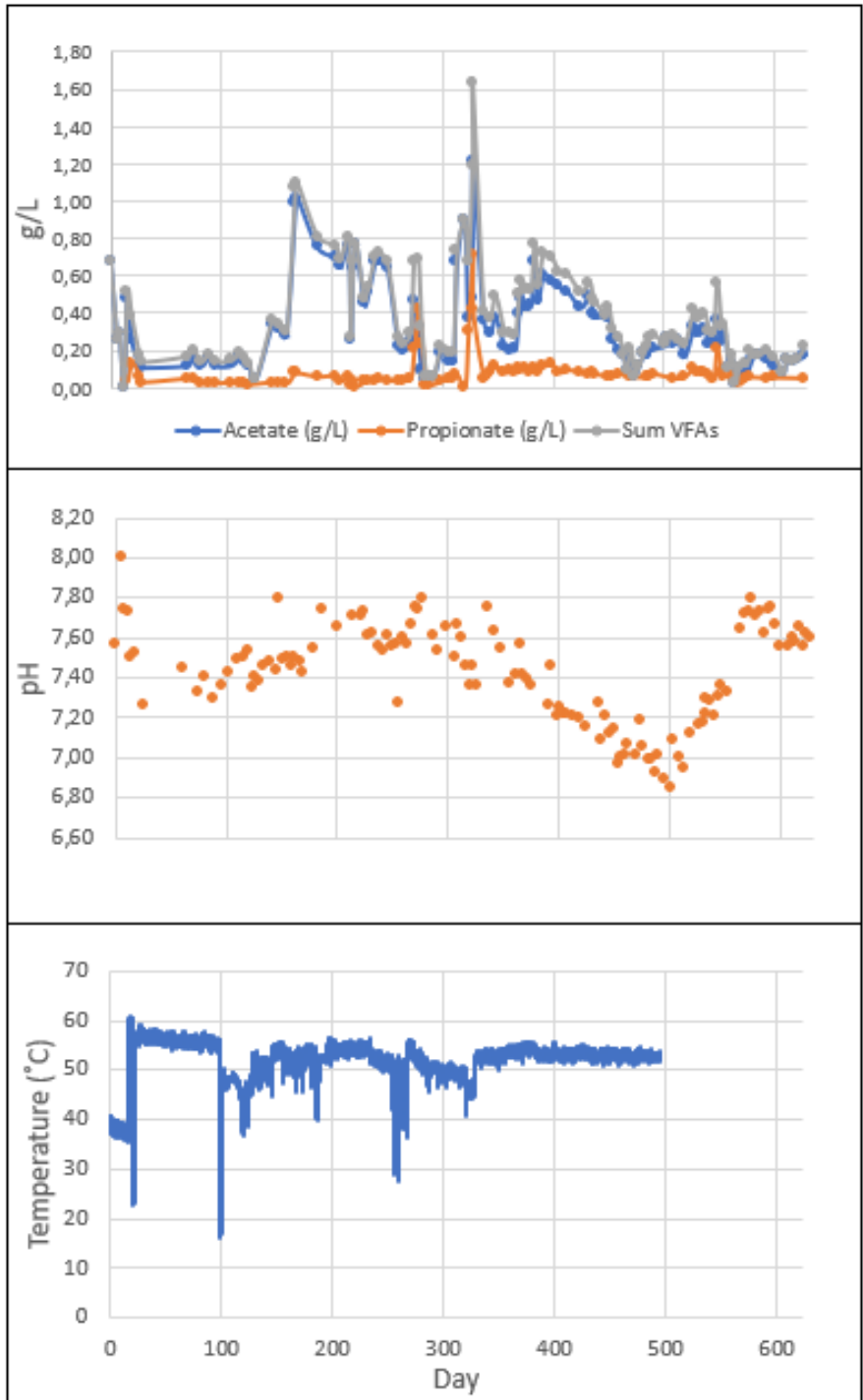


Figure 8: Changes in VFA levels, pH and temperature inside the reactor throughout the experiment. Temperature data after day 500 is missing.

3.3 Samples for Sequencing and qPCR

During the experiment, carrier samples were retrieved from the TBR at three different time-points, from the anaerobic filter at one time-point, and samples of the process fluid from both reactors were taken continuously several times a week. From the ingoing nutrient solution, samples were taken every time a new batch was made. All time-points where the samples, except from the samples of ingoing nutrient solution, analysed in this degree project, were retrieved from are indicated in *fig. 4* with black, numbered arrows. 1 – samples from the reference period of the TBR. 2 – samples from the TBR, which at this point has been coupled to the anaerobic filter and together they have been operated with nutrient solution containing 30% pyrolysis water for approximately 6 months. 3 – samples from the anaerobic filter, which has been disconnected from the TBR and is only operated with internal liquid circulation. 4 – samples from the TBR, which has been operated with nutrient solution without pyrolysis water for approximately two months.

3.3.1 Samples From the TBR

Since the carrier filled volume (the bed volume) make up the majority of the total reactor volume, and since previous experiments at RISE have indicated that the carriers carry the bulk of microbes involved in the production of methane (personal communication Karin Berg, RISE), they are the focus of this degree project. The image in *fig. 9* depicts one carrier from the TBR and is approximately 1 cm in diameter.



Figure 9: Carrier from the TBR before DNA extraction.

One carrier sample from the TBR was retrieved either from the top, the middle, or the bottom of the reactor, and consisted of 3-6 carriers. The positions of the sampling ports for the carriers can be found in *fig. 3*, marked as SP1, SP2, SP3. From each sample, DNA was extracted in three replicates, except for from the middle sample of day 628 where four replicates were made due to a large variation in the subsequent qPCR results. One replicate of a carrier sample is the material obtained from a single carrier. Process fluid samples have been analysed from the same timepoints as the carriers. They were obtained through any of the three ports for the manual liquid levelling of the liquid reservoir, which are marked as SP4 in *fig. 3*. All samples and replicates from the TBR are listed in Table 2.

Table 2: Analysed samples of carriers and process fluid retrieved from the TBR.

Day	Replicates	Context
155 (Timepoint 1)	3 of the top carriers. 3 of the middle carriers. 3 of the bottom carriers. 3 of the process fluid.	The samples have been retrieved during the reference period with maximal gas inflow and nutrient solution without pyrolysis water.
439 (Timepoint 2)	3 of the top carriers. 3 of the middle carriers. 3 of the bottom carriers. 3 of the process fluid.	The reactor had been fed with nutrient solution containing pyrolysis water for approximately 6 months.
628 (Timepoint 4)	3 of the top carriers. 4 of the middle carriers. 3 of the bottom carriers. 3 of the process fluid.	The reactor had once again been fed with nutrient solution without pyrolysis water for approximately one month.

3.3.2 Samples From the Anaerobic Filter

Since the nutrient solution with the pyrolysis water was also led through an anaerobic filter, it was of interest to see how the filter was affected in comparison to the TBR. However, since it was not the focus of the experiment, carriers were only retrieved when the anaerobic filter was disassembled and no longer in use. A sample of process fluid from the anaerobic filter from the same date was also analysed. The sample was obtained from SP5 in *fig. 3*. Instead of analysing triplicates from each level in the reactor, one replicate was made from each level (Table 3). This was because the anaerobic filter was filled with circulating liquid instead of gas, which makes the conditions in reactor more homogenous, and it is expected that the difference between the levels is smaller than the difference between the levels in the TBR. The anaerobic filter was also not the focus of this degree project but was rather included as a complement in case no difference between the samples from the TBR would be recorded. In such a case, it would be interesting to see if the microbial community was affected by the pyrolysis water before it affected the TBR, and thus hypothetically reduced the toxicity in favour for the activity in the TBR.

Table 3: Analysed samples of carriers and process fluid retrieved from the anaerobic filter.

Day	Replicates	Context
Day 537 (Timepoint 3)	1 of the top carriers. 1 of the middle carriers. 1 of the bottom carriers. 3 of the process fluid	The anaerobic filter operated only with internal circulation for two months, after disconnection from the TBR.

3.3.1 Samples From the Nutrient Solution

DNA extracted from the ingoing nutrient solutions was sent for sequencing because the microbial community in the nutrient solution was expected to affect the microbial community in the process fluid, and consequently also in the carriers. This is because the composition of the nutrient solution varies from batch to batch, and therefore there is a possibility that new species were introduced through it. To find out whether a change detected in the microbial community was caused by operational changes or because a new batch of nutrient solution had been used, all batches that have been recorded to have been used during the experiment have been analysed. Data from nutrient solutions introduced on day 119, and 155, have already been obtained by RISE through other projects, but two more batches were analysed as a part of this degree project (Table 4). The samples that were analysed did not yet contain any pyrolysis water.

Table 4: Analysed samples of ingoing nutrient solution.

Day	Replicates	Context
2022-07-08 387	3	The only two nutrient solutions fed to the reactors during the experiment that had not already been analysed by RISE.
2023-02-02 596	3	

3.4 DNA Extraction

The DNA was extracted from the samples using the FastDNA[®] SPIN Kit for Soil by MP Biomedicals. All solutions mentioned were a part of the kit except for the Humic Acid Wash, which was prepared as described below. The protocol used had modifications from the instructions of the manufacturer.

3.4.1 Preparation of Humic Acid Wash

The function of Humic Acid Wash is to remove humic acids, which are negatively charged, stable molecules that forms during long-term degradation of biomass. Humic substances that are not removed during the extraction of DNA could cause problems in subsequent steps, such as PCR reactions, due to their inhibitory properties on enzymes that participates in manipulating DNA (Dong D *et al.* 2006). The Humic Acid Wash used for the extractions was

prepared in advance, since it does not need to be prepared fresh each time. 978 μ l Sodium Phosphate Buffer, 122 μ l MT buffer, and 250 μ l PPS solution were added to an autoclaved 2 ml microcentrifuge tube. 12 tubes were prepared at once to get enough wash for all extractions. The tubes were mixed by shaking them by hand, and then centrifuged at 14 000 g for 5 minutes. The supernatant of all the tubes were pooled in a falcon tube, and a 1:1 volume of 5.5 M guanidine thiocyanate was added. The tube was mixed gently by inverting the tube by hand. Using a syringe and a 0.2 μ m filter the solution was sterile filtered into a new falcon tube.

3.4.2 Extraction Using the FastDNA[®] SPIN Kit for Soil

For most liquid samples, a sample volume of 300 μ l was used for extraction. However, due to low yield of DNA from some samples, there are also material that was extracted from 2 ml samples. For the 2 ml samples, 1 ml was first added to an autoclaved 2 ml microcentrifuge tube and centrifuged at 14 000 g for 5 minutes, then the supernatant was discarded and 1 ml more of sample was added to the pellet. The tube was centrifuged again with the same settings, the supernatant was discarded, and the final pellet was resuspended in 300 μ l Sodium Phosphate Buffer, which was the volume used for the extraction. From a carrier, as much material as possible was scraped off by hand using a tweezer and a spatula, and then the carrier was washed in 300 μ l Sodium Phosphate Buffer. The entire volume of buffer used for washing was then used for the extraction. From each sample, the extractions were performed in at least three replicates. For the carriers, the material from one carrier was regarded as one replica.

The material obtained from a sample (a volume of approximately 300 μ l, regardless if it originated from a liquid sample or a carrier) was added to the Lysing Matrix E tube together with 978 μ l of Sodium Phosphate Buffer, and 122 μ l of MT Buffer Lysis Solution. The mixture was homogenized for 40 s at the speed setting 6.0 m/s in a FastPrep-24 Instrument by MP Biomedicals and centrifuged at 14 000 g for 15 minutes to pellet the debris. For all centrifugations in the protocol, a Centrifuge 5424 R by Eppendorf was used. The supernatant was transferred to a 2 ml autoclaved microcentrifuge tube together with 250 μ l of Protein Precipitation Solution. The solution was mixed by inverting the tube by hand 10 times, and then centrifuged at 14 000 g for 10 minutes to pellet the precipitated proteins. The supernatant was transferred to an autoclaved 5 ml microcentrifuge tube. The Binding Matrix, which binds the DNA and allow contaminants to be washed out (MP Biomedicals n.d. B), was resuspended by inverting the tube by hand and added to the supernatant. The 5 ml tube was then inverted by hand for 2 minutes and left to rest on a rack for 10 minutes, until the silica matrix has settled in the bottom of the tube. 500 μ l of the supernatant was discarded, and then the pellet was resuspended by gently pipetting up and down. 700 μ l of the solution was transferred to a SPIN filter, and then the tube was centrifuged at 14 000 g for 1 minute. If all liquid had not passed through the column during the centrifugation, the flow-through was discarded and the SPIN filter was centrifuged for another minute. 500 μ l of Humic Acid Wash was added to the SPIN filter, and the pellet was resuspended by pipetting up and down. The

filter was centrifuged at 14 000 g for 1 minute, and the flow-through was discarded. 500 µl of SEWS-M (prepared with 100 ml 99.7% ethanol) was added, and the pellet was resuspended by pipetting up and down. This step removes a variety of remaining contaminants (MP Biomedicals n.d. B). The filter was centrifuged at 14 000 g for 1 minute, and the flow-through was discarded. Without addition of any liquid, the filter was centrifuged for two more minutes at 14 000 g, and then the filter tube was removed and put in an autoclaved 1.5 ml Eppendorf tube. The filter was left to air dry in tube for 5 minutes at room temperature. The DNA was eluted by adding 60 µl of DES water (DNAase Free Water) to the pellet, which was resuspended by carefully flicking the tube. The Eppendorf tube with the filter tube was centrifuged at 14 000 g for 1 minute. The filter was discarded, and the eluted DNA was stored in the tube at -20°C.

3.4.3 Measuring Concentration

The concentrations of the extracted DNA samples were determined by using the AccuGreen™ Broad Range dsDNA Quantitation Kit by Biotum and the Qubit® 3.0 Fluorometer by Thermo Fisher Scientific. 190 µl of 1X AccuGreen Buffer was added to a Qubit® test tube together with 2 µl extracted DNA. Two standards were prepared by adding 10 µl of each to separate tubes with 190 µl 1X AccuGreen Buffer. The mixtures were vortexed thoroughly before the measurements.

3.5 Sequencing

All extracted DNA samples were sent for sequencing at Novogene (Novogene n.d.), but the result of the analysis is subject for future research and will not be discussed as a part of this project due to delayed test results.

3.6 qPCR Analysis

Since the data from the sequencing only provides the relative amount of each species found in a sample, all samples, except for the samples of the nutrient solution, were also analysed with quantitative polymerase chain reaction (qPCR) to get an absolute quantification. The samples from the nutrient solution were not included because they are only sequenced to see if any new groups of microbes were introduced through it. The group of microbes quantified was the genus *Methanobacterium*, which is one of the two most common methanogens to dominate reactors for biological methanation (Rachbauer *et al.* 2017, Porté *et al.* 2019, Cheng *et al.* 2022, Kamravamanesh *et al.* 2023). There were four samples analysed from the middle carriers of day 628 (see Table 2); This was because the initial results from the qPCR showed a high variance, and it was desired to confirm the results by extracting more samples.

Eight standards were prepared from a stock by making a dilution series. The number of copies of the target sequence in each standard were 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 . One PCR reaction contained 10 µl 2X Ora™ SEE qPCR Green ROX L Mix, custom forward and

reverse primer, ordered from Invitrogen by Thermo Fisher Scientific, was added to a final concentration of 0.5 pmol/μl, 3 μl DNA, and sterile and nuclease free water from VWR to a final volume of 20 μl. The extracted DNA and standards were vortexed thoroughly before aliquotation. To ensure exact volumes, the mixtures were loaded using Picus® electronic pipettes by Satorius into a transparent, half skirt, PCR plate which was sealed with tape, both of which were obtained from Sarstedt AG. The plate was centrifuged to get rid of air bubbles before being placed in the QuantStudio™ 3 Real-Time PCR system by Thermo Fisher Scientific. The PCR-programme and primer sequences can be found in Appendix II.

The data was analysed in QuantStudio design & analysis software v.1.5.1. The standards were run in triplicates, and each extracted sample was run in a single replicate since the carrier, process fluid, and nutrient solution samples had already been extracted in triplicates.

3.7 Calculations – pPCR Analysis

By multiplying the concentration of DNA in the extracted samples with the elution volume, the total amount of DNA in the elution volume was obtained. For example: $49 \frac{\mu\text{g}}{\text{ml}} \text{ DNA} \times 60 \mu\text{l} = 2940 \text{ ng DNA}$. The QuantStudio™ 3 Real-Time PCR system calculates the starting quantity (SQ) – the number of detected copies of the target sequences in the sample – with the help of the eight standards that were analysed alongside the samples (see [7.2 Appendix II – qPCR](#)). The SQ of the sample was divided by the total amount of DNA: $\frac{174702288 \text{ copies}}{2940 \text{ ng DNA}} = 59400 \text{ copies / ng DNA}$.

This normalizes the calculation and makes carrier samples comparable to other carrier samples and liquid samples comparable to other liquid samples, in terms of that some extractions might have been more successful than others, and some carriers might have had more biomass on them than others. It is also one of the most conventional ways to prepare the data in similar projects (personal communication Simon Isaksson, SLU). It should be noted that a downside of this method is that the increase or decrease of other species in the samples will also affect the numbers of *copies/ng DNA*. This is because the increase of other species will lead to an increase of the total number of the ng DNA in the sample, meaning that two samples with the same number of copies could still be plotted as bars with different heights, because one of the samples contained other species that had increased or decreased greatly compared to the other sample.

An alternative method to look at the data could have been to plot *copies/carrier*. However, this method does not take into account that different carriers consist of different amounts of biomass, water, particles, and other materials, nor does it take into account that the extractions are done manually by hand, which is likely to result in different success rates. If this method would be used, it would be beneficial to have weighted the material on each carrier, so *copies/g biomass* could be used as unit, which was not done in this project. As mentioned,

the material on each carrier consists of varying amounts of biomass, water and particles, which still makes the measurements uncertain. Instead, the original plan was to also base the report on the sequencing analysis, which might contribute to making the conclusions more reliable. When plotting the data in these two different ways, the unit *copies/ng DNA* gave more consistent and less scattered graphs compared to *copies/carrier*, which can be interpreted as that the deficiencies of that model affects the data less than the deficiencies of *copies/carrier*.

4 Results

In the reference period (*fig. 10*), the graph from the qPCR shows a clear decline in *Methanobacterium* (copies/ng DNA) the further down in the reactor the sample was taken, though the difference is only statistically significant for the top carriers versus the bottom carriers and not for the top/middle carriers or for the middle/bottom carriers. All calculated p-values can be found in Appendix II.

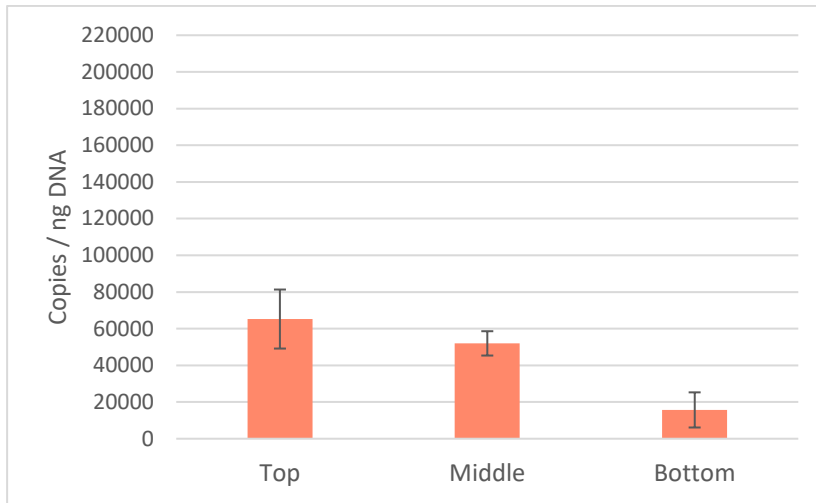


Figure 10: The amount of the *Methanobacterium* target sequence at day 155, in the top, middle, and bottom carriers, during the reference period.

At day 439 (*fig. 11*), after six months of nutrient solution with pyrolysis water, the number of copies per ng DNA from the *Methanobacterium* target sequence has decreased drastically in the top of the reactor, and the difference is statistically compared to day 155. Meanwhile, the number of copies has increased in both the middle and bottom carriers, but the difference is not statistically significant compared to day 155. In this graph (*fig. 11*), the difference between all bars (Top, Middle, and Bottom) is statistically significant.

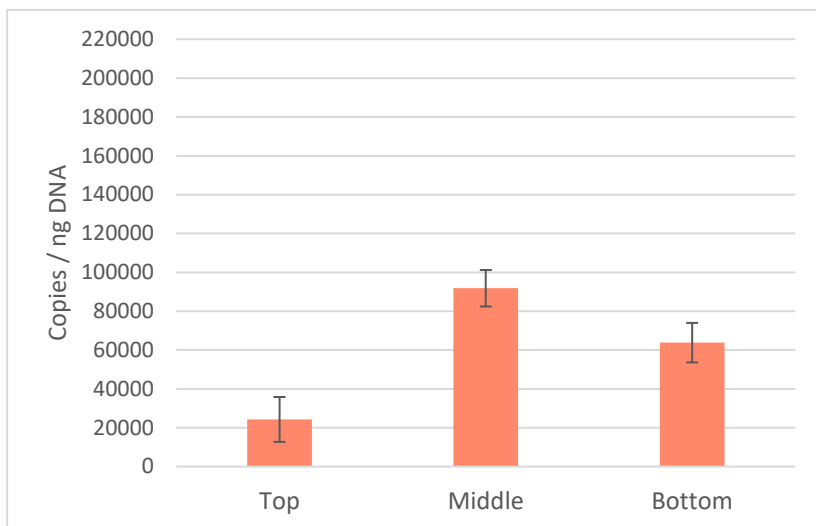


Figure 11: The amount of the *Methanobacterium* target sequence at day 439, in the top, middle, and bottom carriers, after six months of nutrient solution with pyrolysis water.

At day 628, when the TBR had gone back to nutrient solution without any pyrolysis water for 2 months (*fig. 12*), the relative number of copies in the top and middle of the reactor have increased, while the bottom of the reactor is similar to the number on day 439. The variance in the samples is also bigger than before, and none of the bars are statistically significantly different from the other two or compared to the corresponding levels from day 439 or day 155.

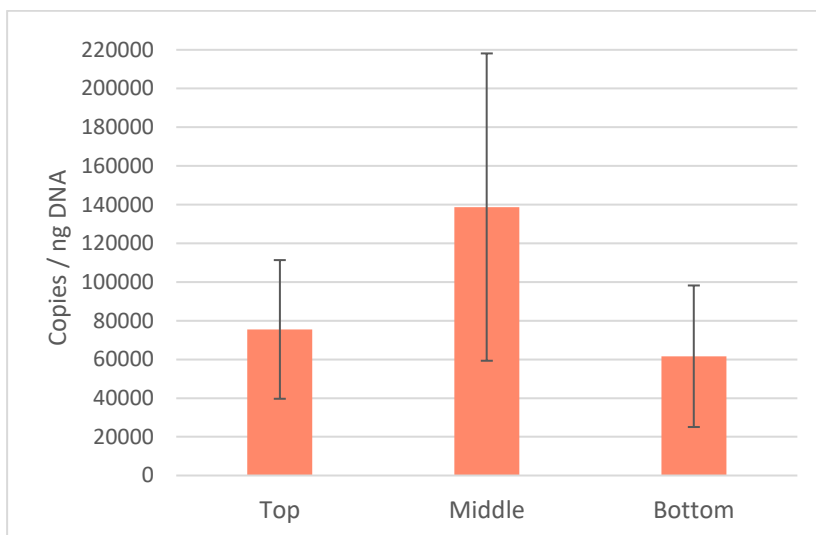


Figure 12: The amount of the *Methanobacterium* target sequence at day 628, in the top, middle, and bottom carriers, after one month back on nutrient solution without pyrolysis water.

Because the process fluid is a completely different environment for the microbes to grow in compared to the carriers, carriers and fluid samples will not be compared to each other. In *fig. 13* below, the qPCR results from the process fluid samples are plotted. In timepoint P1 – the reference period – has many more copies of the target sequence than the others. In

timepoint P3 – the process fluid from the anaerobic filter – the amount is not visible compared to the other bars. qPCR results from carrier samples from the anaerobic filter can be found in Appendix II.

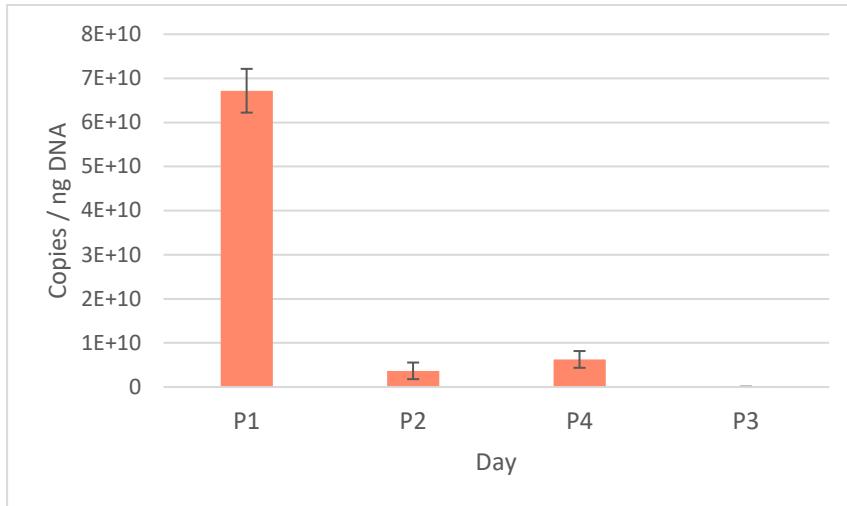


Figure 13: The amount of the *Methanobacterium* target sequence at the different timepoint in the experiment. P1 – reference period (day 155), P2 – 6 months of pyrolysis water (day 439), P4 – the TBR once again fed with nutrient solution without pyrolysis water (day 628), P3 – the anaerobic filter is disassembled (day 537).

5 Discussion

The discussion is divided into sections according to the objectives of the project (literature study regarding the optimal operational conditions, investigation of the catabolic pathway of CO, and comparison of the population of *Methanobacterium* before and after contamination). Future research is the last section and includes thoughts on upcoming sequencing results from the experiment conducted at RISE.

5.1 Operational Conditions

To reach as high methane productivity as possible, it seems that the process should be operated at the highest possible temperature that can be reached without negative effects (Grimalt-Alemany *et al.* 2020, Wegener Kofoed *et al.* 2021). Since microorganisms typically are inactivated before they die from unfavourable conditions, it might be possible to test the limits of a bioreactor by increasing the temperature 1 °C/day, as recommended for anaerobic digesters (Jarvis & Schnürer 2009). For a commercial plant for biological methanation, it would be relevant to investigate the maximal temperature of the method. However, the result of the investigation would only be relevant for reactors with the exact same inoculate and operation, since the species, and the enrichment of species, will be greatly affected by these parameters (Li C *et al.* 2020).

It is possible to theorize that some species might inactivate or die in rising temperatures, even if the CH₄ production is stable, which means that the system might become more inclined to become destabilized when exposed to contamination or other temporary, unfavourable conditions. For example, in the experiment conducted at RISE it is evident that the pyrolysis water is degraded in the reactor (see *fig. 14* and *15*). To perform well, the reactor would therefore also need to have conditions that are favourable for the population of microbes responsible for the degradation.

When the desired temperature is reached, there seems to be important to keep the temperature, and other operational parameters, stable since the key to a stable microbial community is to not expose it to unnecessary environmental changes (Jarvis & Schnürer 2009). This is probably a simpler task in a small-scale batch reactor placed indoors with constant temperature, than it is in a commercial TBR placed outdoors due to its size. The changing outdoor temperature over the course of the day and year, and the eventual temperature difference in freshly fed nutrient solution is two examples of parameters which challenges maintaining a constant temperature. Since the biological methanation process is exothermic (Wegener Kofoed *et al.* 2021), cooling of the reactor is required to achieve a stable temperature in large scale. However, heating is also required during the start-up phase when the internal heat production is not yet large enough. As mentioned in section 2.8.3 Type of Culture, even a process under stable conditions can malfunction if the inoculate is insufficient (Li C *et al.* 2020). A reasonable hypothesis is that a process based on a diverse inoculum will

always have microbes in different functional groups that step up and thrive when the conditions inside the reactor changes. Other microbes, that previously were in charge of managing a pathway, becomes inactive due to environmental changes but do not necessarily die. This logic might also apply to the environmental changes that came with the contamination inside the TBR. A more diverse inoculate is more likely to contain species of microbes capable of digesting the contamination, while a less diverse process would have been more negatively affected than the TBR in the experiment at RISE. It is probably less important for a very diverse mixed culture to have constant operational conditions and few disturbances than it would be for mixed cultures that are less diverse.

5.2 The Pathway of CO in Thermophilic Syngas Biomethanation

The most probable pathway for CO conversion in thermophilic syngas methanation is through the WGSR followed by hydrogenotrophic methanogens converting H_2 and CO_2 into CH_4 . Even though the pathway for acetoclastic methanogenesis becomes more thermodynamically favourable with rising temperatures, it will not play a major role because the pathway that would convert CO into acetate becomes less favourable. This means that the WGSR, which becomes more favourable, prevails in the competition. Similarly, homoacetogens are likely out-competed by hydrogenotrophic methanogens because they have a less efficient energy production due to the thermodynamic conditions (Grimalt-Alemany *et al.* 2020).

5.3 qPCR Results

Fig. 10 from the reference period visualizes that the amount of the target sequence from *Methanobacterium* is significantly higher at the top of the TBR compared to the bottom. This might indicate that the top of the reactor has the most advantageous conditions for microbial growth, or at least for growth of *Methanobacterium*. Since the environment in the reactor is different in the top versus in the bottom of the reactor (the syngas gets gradually consumed as it passes through the reactor), it is possible that other species of methanogens dominates in bottom the of the reactor and is better adapted to that environment than *Methanobacterium* is. However, since the carbon and energy source for the methanogens is syngas, which enters the TBR in the bottom, it is possible to reason that the bottom of the reactor generally should be the most advantageous spot for methanogens. Nevertheless, it is possible that the nutrient solution, which enters from the top, might be the factor that makes the top more beneficial for microbial growth. The purpose of the nutrient solution is to provide the microbes with macro- and micronutrients, but it is possible to theorize that since it consists of diluted, filtered digestate, it also might contain other particles that sediments and helps the microbes to cling onto the carriers. This is a theory that RISE has previously discussed, based on observations in other experiments, that is supported by this result (personal communication Karin Berg, RISE). Another explanation might be that the start-up phase of the reactor might not be completely over. The inoculum enters the reactor through the top, which probably is

advantageous for quick establishment of microbial growth, and it is possible that the growth would have evened out with time if the process would have been undisturbed.

After 6 months of nutrient solution containing pyrolysis water (*fig. 11*), the growth of *Methanobacterium* in the top of the reactor decreased significantly compared to the reference period. The top of the reactor is the most exposed to the pyrolysis water since it is injected into the system with the nutrient solution. Since the population of the *Methanobacterium* in the lower parts of the reactor keeps increasing, the pyrolysis water seems to be digested on its way down. This can also be observed in figures 14 and 15 in Appendix I. It is interesting that no effect on the CH₄ production was observed. There are several explanations to this phenomenon: either other strains of methanogens in the reactor were more resilient to the contamination, or the growth in the lower parts of the reactor made up for the losses in the top. Either way, this also suggests that a diverse inoculum that contains a wide range of methanogens, adapted for a variety of conditions, is useful in a mixed culture for biological methanation.

When the TBR was back in operation on nutrient solution without pyrolysis water (*fig. 12*), the graphs give the impression that the population might be recovering since the bar for the top and the middle carriers are higher than the previous analysis, and the bottom carriers has stayed the same level as on day 439. However, the variance of the samples is very high, and the bars are not statistically significant when compared to each other, or to the bars from day 439, which makes it difficult to draw any reliable conclusions on the status of the reactor at this time point when comparing with previous time points.

One of the most interesting discoveries was that the process fluid showed a much higher content of *Methanobacterium* in the reference period compared to the other timepoints (*fig. 13*). This might suggest that one of the aspects with utilizing a TBR as a bioreactor concept is that the microbes have the opportunity to grow in a biofilm, which protects the microbial community when exposed to a contamination. The microbes that grow in the fluid will not have the same protection from harmful compounds. However, what speaks against this theory is that the population of *Methanobacterium* does not recover for 2 months when the pyrolysis water is replaced with regular nutrient solution again. It is possible that in the process fluid in the reference period, *Methanobacterium* was boosted by the inoculate, the initial startup liquid consisting of active digestates collected straight from different biogas digesters just before the inoculation. Perhaps it was simply impossible to reach the same high levels again long after the inoculation as the batch-fed nutrient solution was prepared and handled without effort to retain active methanogens.

5.4 Future Research

Based on the literature study in this project, it is expected that the dominating pathway for CO in the TBR in the experiment conducted by RISE is through the WGSR and hydrogenotrophic

methanogenesis. This means that only a small amount of acetoclastic methanogenens is expected to be present in the sequencing results. It seems more likely that genera *Methanosarcina* will be found than *Methanoseata*, if any of them are present. The list in Table 1 is what information that was found in the literature study, but it does not include all species of any of the genera listed, which means that there is still room for many unexpected findings. There was not found any articles in the literature study where *Methanoseata* dominated over *Methanosarcina*, in any type of experiement. This means that it gets more difficult to assess how much of the CO is converted from acetate directly to CH₄ because *Methanosarcina* has the ability to use a variety of substrates. In this case, a tracer study would have to be conducted to prove what pathways *Methanosarcina* utilizes (Grimalt-Alemany *et al.* 2020).

The DNA sequencing analysis sent to an external lab were supposed to be included in the work, but due to delays in the delivery, this had to be omitted. A suggestion for future work is therefore to further analyse these impending DNA sequencing results. When the sequencing result are available, it is expected to show that species, that seem to have appeared after the introduction of pyrolysis water and that is atypical for a reactor for biological methanation or anaerobic digestion, might take part in degrading the contamination. These might be species that are of special interest for inocula for commercial syngas methanation plants, because they might increase the robustness of the process and resilience to contamination. It is also necessary to use the results from the sequencing analysis to re-evaluate the conclusions from the qPCR since comparisons between samples is difficult to do, and the conclusions in this report could become more reliable with access to both analyses.

One of the most novel aspects that the qPCR and sequencing results can potentially contribute with is to monitor the difference in microbial communities at the different levels that the samples are taken from (top, middle and bottom). The literature study did not reveal any information that suggests that this has been investigated before. In future research, it is important to consider investigating more about the differences that might be found in different parts of the reactor. Since there are gradients in the environment throughout the reactor – different gas composition between the input and output gas, and nutrients and contaminants in the process liquid that are being consumed and degraded on its way down through the bed – there might also be a gradient in the growth of different species of microbes. Analyses on gas production and process fluid gives information about the general performance of a TBR, but no insight into what occurs in different parts of the reactor.

6 Conclusion

The main conclusion that came from this project is that the pyrolysis water indeed had an effect on the TBR, even though there was no recorded disturbance in any of the measured parameters from the run that could be connected to the contamination. Given results from the study by Li *et al.* (2020), mentioned in this report, with unstable productions that were connected to inocula missing different groups of microbes, the stability and resilience of the TBR at RISE might be attributed to a diverse inocula.

There was also not the same size of the population of *Methanobacterium* in the top, middle, and bottom of the reactor. It is possible that different groups of methanogens dominate in the different levels in the reactor. For future development of biological methanation of syngas with a TBR, it is important to consider studying the microbial community at different levels (i.e. top, middle and bottom) in the TBR to increase the understanding how gradients in the environmental conditions affect the microbial conversion of syngas. Since the gradient in the environment throughout a TBR logically should cause a gradient in the growth of the microbes throughout the TBR, which the results of this report has indicated. An important conclusion regarding this is that it seems like the top of the reactor might have been more affected by the pyrolysis water than lower levels. The shift in environment might have reduced the number of *Methanobacterium* in the top and instead selected for a population that was able to digest the pyrolysis water, making it less potent before it reached the lower parts of the TBR.

Many studies draw the conclusions that the pathway taken in a thermophilic operation is mainly through hydrogenotrophic methanogenesis. This means that carbon monoxide generally is converted into hydrogen and carbon dioxide before it is converted to methane. Acetoclastic methanogenesis can dominate a biological syngas methanation process but is more likely to do so under mesophilic conditions.

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7 Appendix

7.1 Appendix I – RISE Analysis of Pyrolysis Water

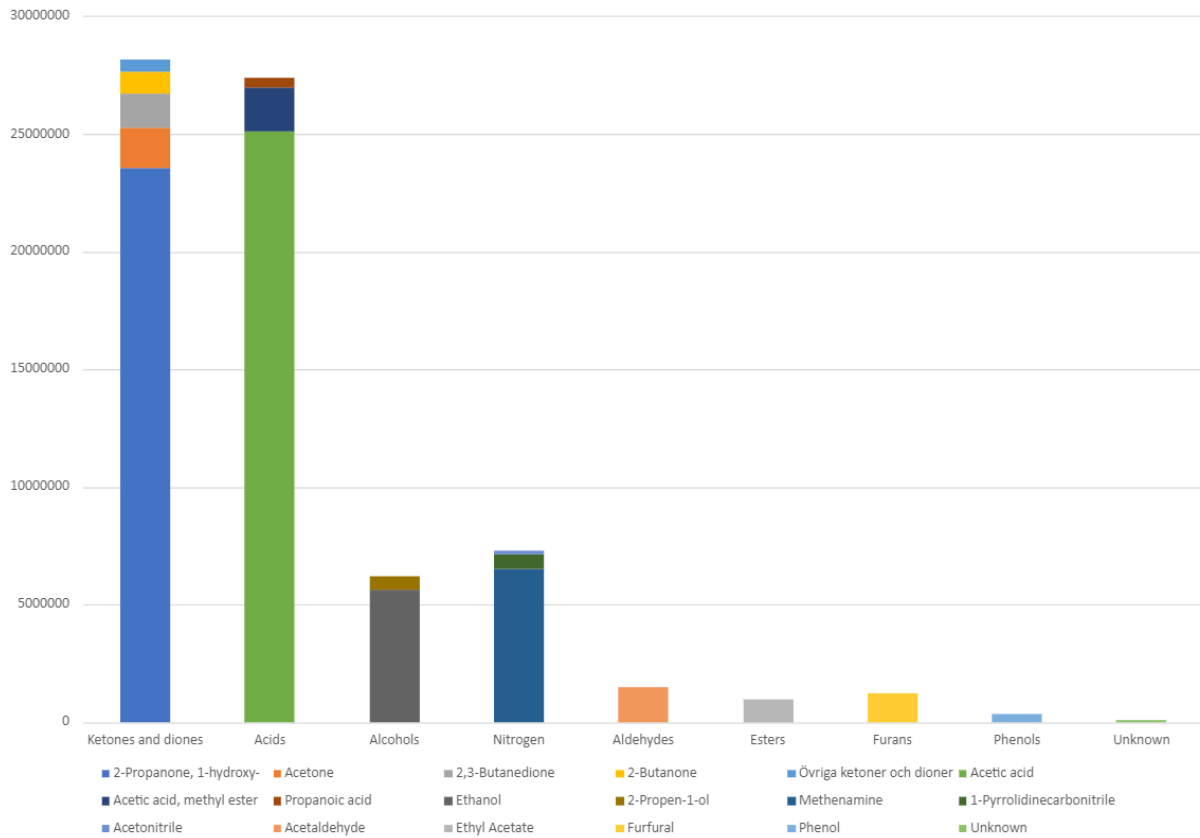


Figure 14: Analysis of content in ingoing nutrient solution diluted with 30% pyrolysis water. Graph retrieved from Andersson *et. al* (2024).

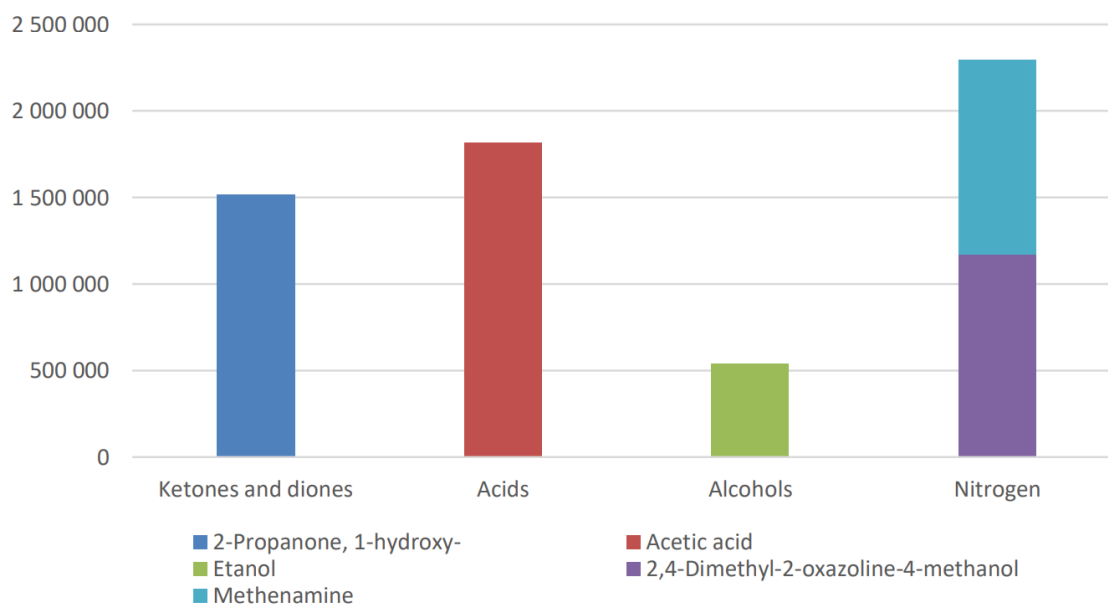


Figure 15: Analysis of content in outgoing process fluid from the anaerobic filter. Graph retrieved from Andersson *et al* (2024).

7.2 Appendix II – qPCR

Two separate qPCR runs were made for the samples. All samples were included in Run 1 (see [10.2.1 Run 1](#)). One sample was run twice to confirm an unlikely result. This was done in Run 2 (see [10.2.1 Run 2](#)). Apart from the standards, which always must be analysed together with the samples this sample was the only one that was included in Run 2.

Instead of including a negative control, it was decided that samples with a Cycle Threshold (CT) value higher than the CT value for the lowest standard (approximately CT = 32 for both runs that were made), would be removed. However, this was not the case for any of the samples. The CT value in quantitative PCR is the cycle where a sample first emits a strong enough signal to reach the threshold to be set apart from the background noise. A high CT-value indicates that it took many cycles before the threshold for the signal was reached, meaning that the sample probably contained a small amount of the target sequence. A low CT-value indicates that it only took a few cycles to reach the threshold, which indicates that the sample contained a high amount of the target sequence (Staff 2022).

The melting curves for standards 10^1 and 10^2 , shows that there was another amplified product than only the amplified target gene (see [fig. 17](#), and [fig. 20](#)). The purpose of the melting curve is to reveal nonspecific binding of the primers to make sure the quantification is not affected by an additional amplification product. However, in this case, the melting temperature of the additional peaks are very low, which suggests they might be due to primer dimers rather than nonspecific binding, and it is only an issue in the standards with the lowest number of copies. It is likely that the extra peaks are due to primer dimers since the primers will be more prone to binding to each other when there are not as much amplified DNA available. If the primers

truly were nonspecific, this would be a problem in all standards, and possibly in the samples as well. It is also clear that the standards with a lower number of copies have higher standard deviation than the other standards (see *fig. 16* and *fig. 19*), which makes sense since the reporting agent SYBR in the OraTM SEE qPCR mix will detect all double stranded DNA and not just the intended product.

Table 5: Sequences of the primers used in the qPCR reaction. The letter W indicates either the base A or T with a 50% probability of each (ThermoFisher Scientific n.d.).

Primer	Sequence (5' → 3')
Forward primer	CGWAGGGAAGCTGTTAAG
Reverse primer	TACCGTCGTCCACTCCTT

Table 6: qPCR protocol.

Stage	Repetitions	Temperature (°C)	Temperature Change (°C /s)	Duration
Hold Stage	None	95.0	1.6	07:00
PCR Stage	45	95.0	1.6	00:40
		58.0	1.6	01:00
		72.0	1.6	00:40
Melt Curve Stage	None	95.0	1.6	00:15
		55.0	1.6	01:00
		95.0	0.15	00:01

7.2.1 Run 1

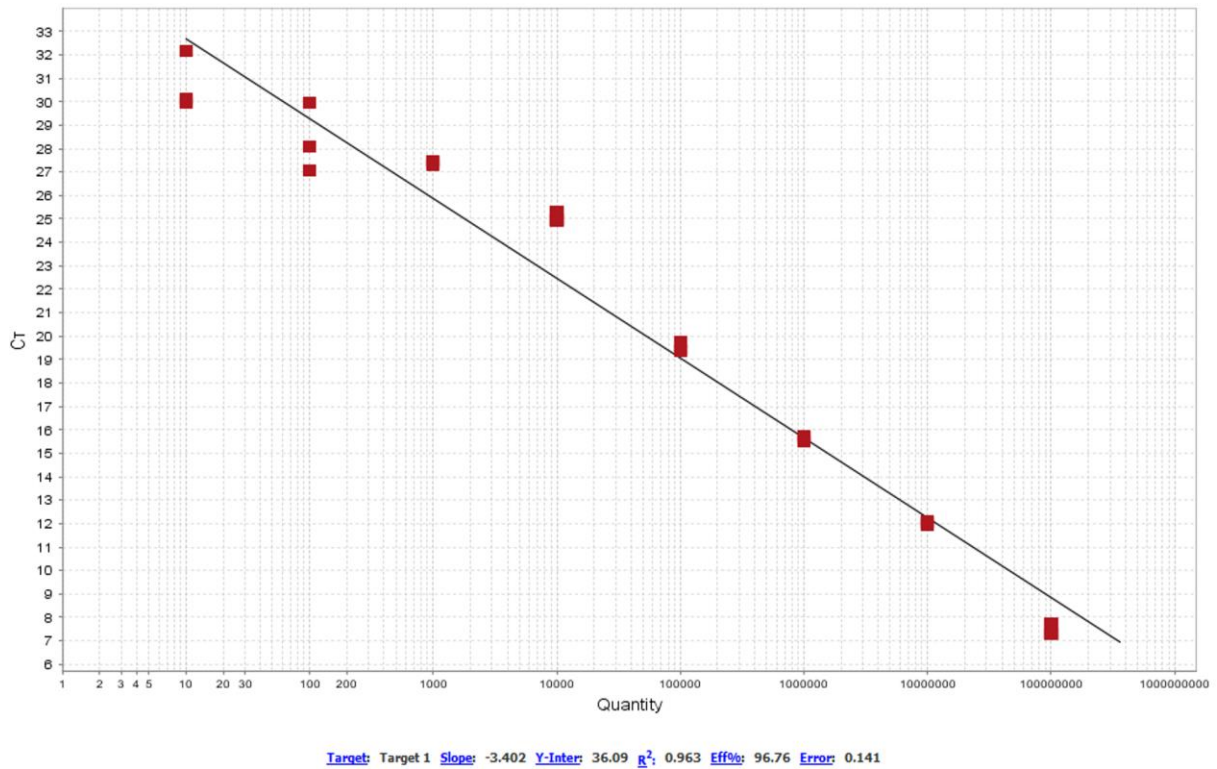


Figure 16: Standard curve for the qPCR, run 1. The eight standards with three replicates each are positioned from the lowest number of copies to the left (10^1) to the highest number of copies to the right (10^8). The cycle threshold value (CT) is the number of the cycle in the PCR reactor where a fluorescent signal has reached the required strength to distinguish itself from the background noise. The CT value is inversely proportional to the amount of DNA in the sample. A sample with a large quantity of DNA will quicker gain a stronger fluorescent signal, which will make it reach the threshold quicker, which gains a low CT-value.

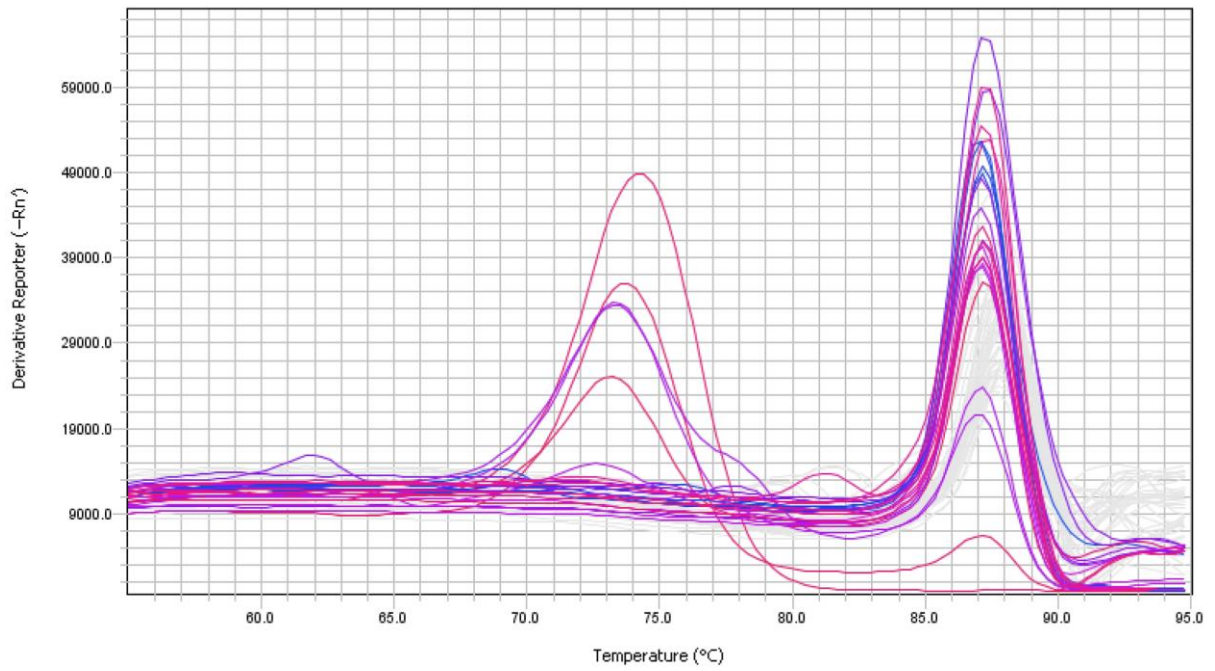


Figure 17: Melt curve plot for standards in the qPCR, run 1.

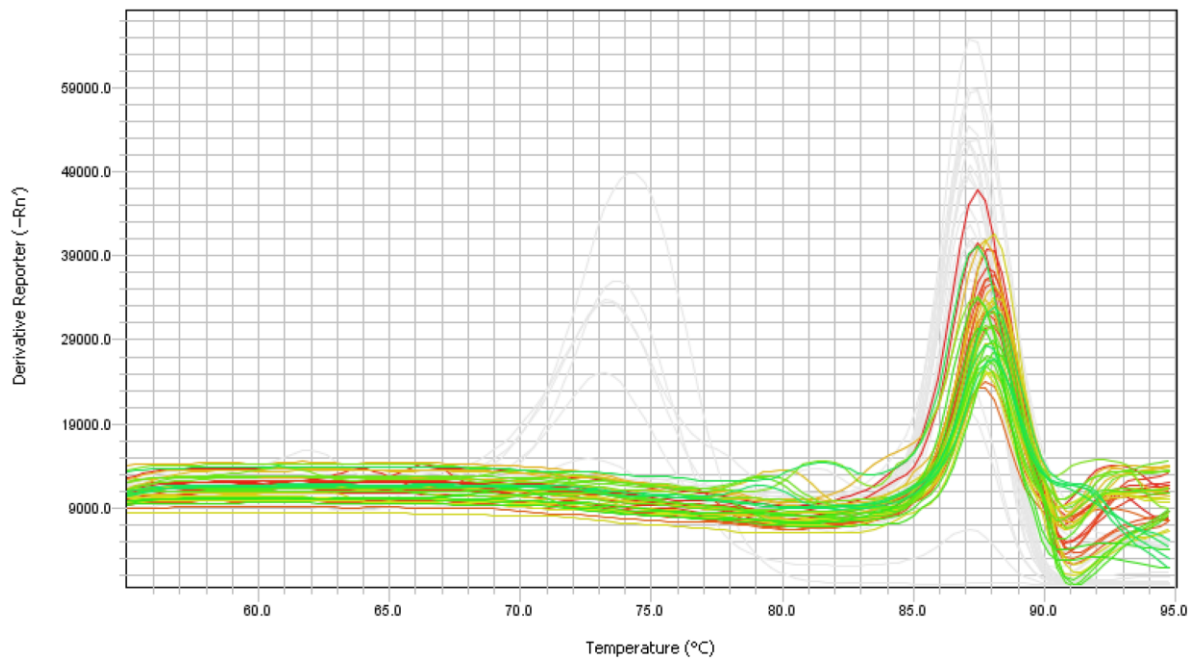


Figure 18: Melt curve plot for samples in the qPCR, run 1.

7.2.2 Run 2

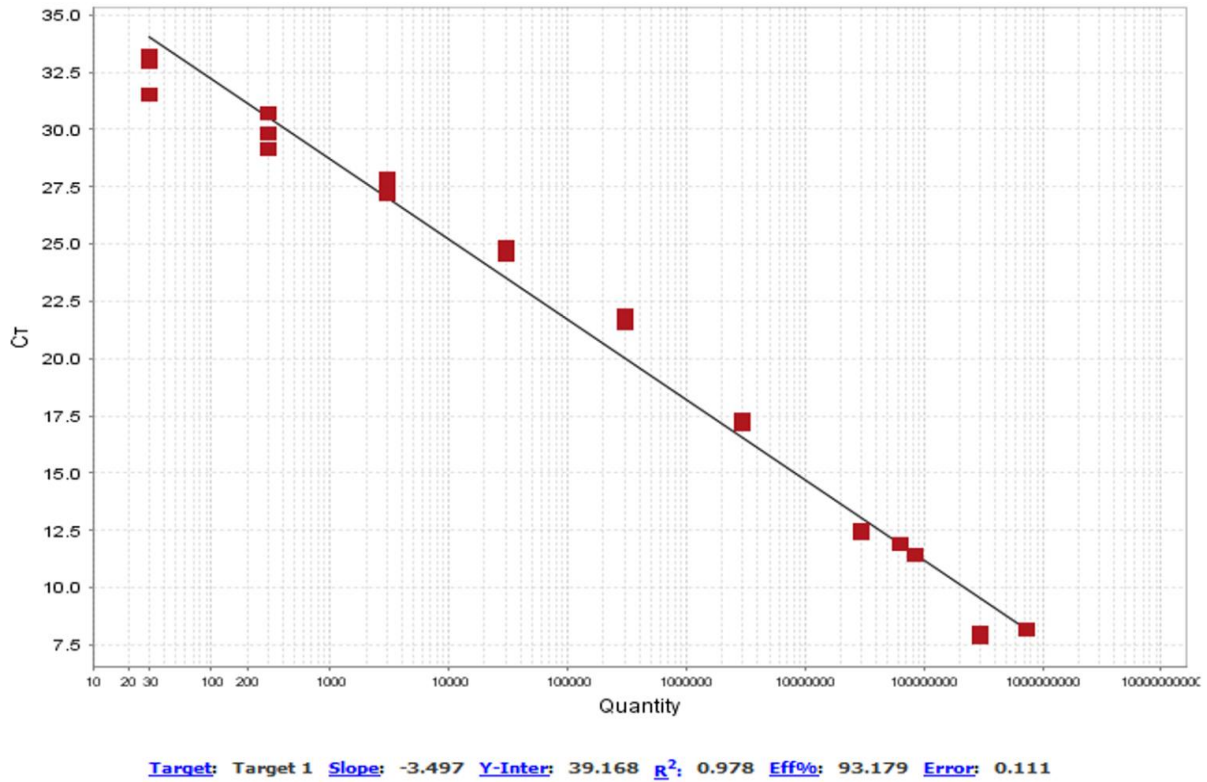


Figure 19: Standard curve for the qPCR, run 2. The eight standards with three replicates each are positioned from the lowest number of copies to the left (10^1) to the highest number of copies to the right (10^8). The cycle threshold value (CT) is the number of the cycle in the PCR reactor where a fluorescent signal from the sample has reached the required strength to distinguish itself from the background noise. The value is inversely proportional to the amount of DNA in the sample. A sample with a large amount of DNA will quicker gain a stronger fluorescent signal, which will make it reach the threshold quicker, which gains a low CT-value.

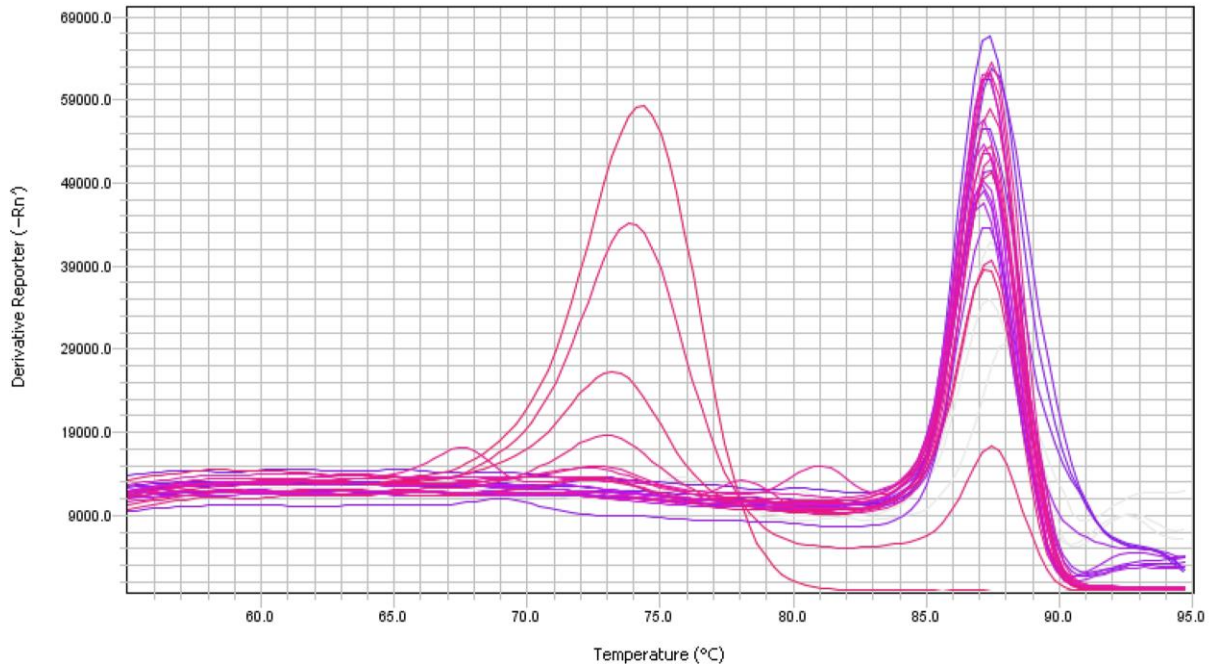


Figure 20: Melt curve plot for standards in the qPCR run 2.

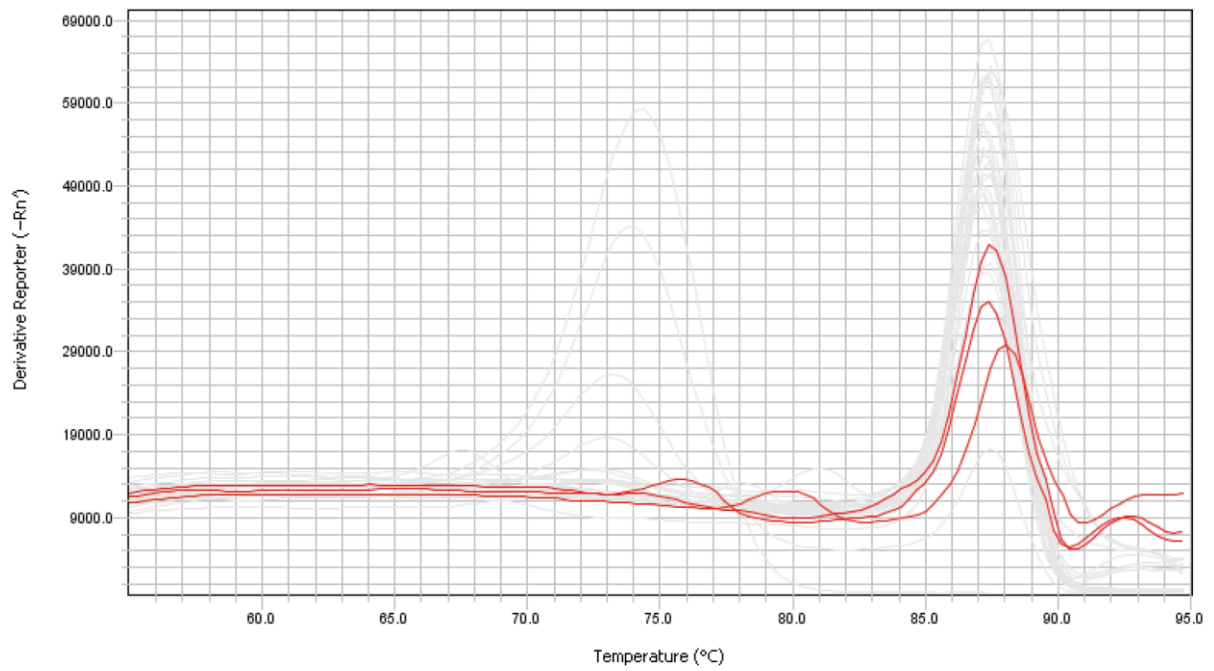


Figure 21: Melt curve plot for samples in the qPCR, run 2.

7.2.3 P-values for qPCR datasets

Statistical significances were determined by the function TTEST in excel, which produces a p-value. The test was performed assuming two-tailed distribution and the test type used was Two-sample unequal variance (different participants and unequal variance is assumed). P-values < 0.05 are marked in green in the tables.

Table 7: P-values for qPCR data comparing the different levels from which the carriers were retrieved from in TBR4.

	Day 155	Day 439	Day 628
Top vs Middle	0.37	0.0035	0.29
Top vs Bottom	0.029	0.023	0.72
Middle vs Bottom	0.015	0.046	0.21

Table 8: P-values for qPCR data comparing the difference between the timepoints in different positions in the TBR.

	Top	Middle	Bottom
Day 155 vs 439	0.048	0.010	0.0083
Day 155 vs 628	0.74	0.15	0.21
Day 439 vs 628	0.17	0.38	0.94

7.2.4 Additional qPCR results

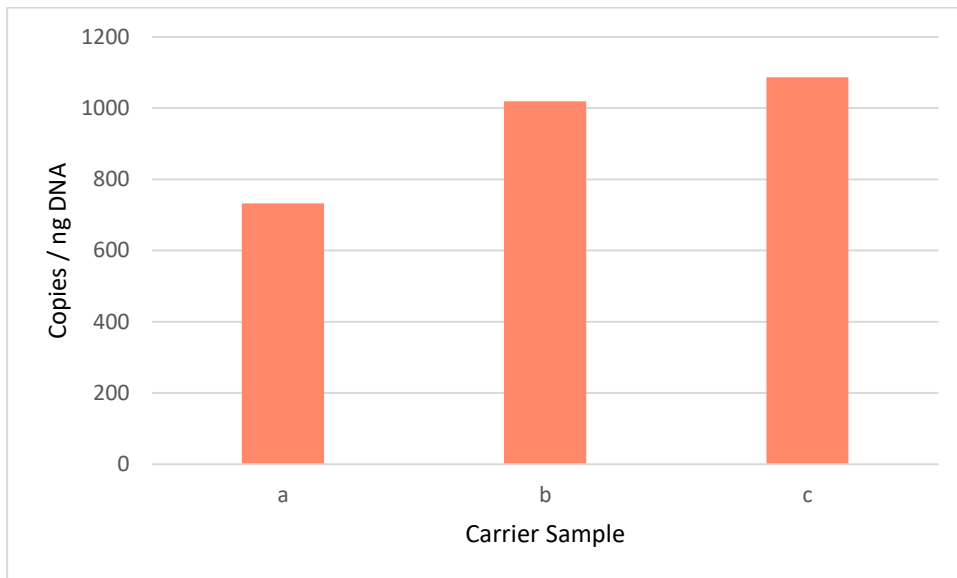


Figure 22: The amount of the *Methanobacterium* target sequence at day 537, in 3 different carrier samples (a, b, and c) in the anaerobic filter.