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– Impact of increased organic loading and addition of iron

Sara Ericsson

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Sara Ericsson

Supervisor:	Simon Isaksson, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Assistant Supervisor:	Li Sun, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Examiner:	Anna Schnürer, Swedish University of Agricultural Sciences, Department of Molecular Sciences

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Evaluating biogas production from energy rich substrate

Degrading organic material can generate methane, which can be used as a renewable source of energy. The rest product from the process can also be used as a bio fertilizer in agricultural industry. Slaughterhouse waste is a very energy rich material, which in theory can produce a lot of biogas, but the microorganisms degrading slaughterhouse waste can be overwhelmed by this “heavy” substrate, therefor it is quite difficult to produce biogas from slaughterhouse waste. This project was thus aiming to evaluate and optimize biogas production from slaughterhouse waste and swine manure. In the study a mixture of these substrates was added to four laboratory scale digesters out of which two were treated with iron sludge from a water sewage plant. Iron prevents sulphides to accumulate in the gas, which results in a purer gas and better gas quality. Iron also counteract precipitation of trace metals, essential for the microbes degrading the material. The results showed that the digesters treated with iron sludge did perform better than the digesters without any addition of iron sludge.A comparison between three different iron additives were also made to evaluate if there were any differences in how they affect the biogas process, and if one of them is a better alternative to use as additive. The results showed that there were no clear differences between the three iron additives.

Today it is very expensive and hard to maintain a private biogas plant. By optimizing biogas production from agricultural wastes farmers will be able to produce their own energy easily and less expensive. We are facing big environmental pressure on today’s society and biogas is a good renewable alternative for vehicle fuel and electricity production.

Abstract

Anaerobic digestion by microorganisms generate methane which can be used as a renewable source of energy. A complex biological system with a community of microorganisms that work together can be used for generation of methane from substrates such as household wastes and agricultural wastes. The rest product from the process can in turn be used as a bio fertilizer in agricultural industry. In this project four continuously stirred laboratory scale digesters with a working volume of 5L, a temperature of 52˚C (thermophilic) were used to evaluate biogas production from slaughterhouse waste and swine manure and the importance of organic load and iron addition for performance and stability. The performance was evaluated in two digesters using iron sludge as an additive and these digesters were compared to two reference digesters receiving no addition of iron. Iron was used as an additive to reduce the amount of hydrogen sulphide (H_2S) produced by high protein substrates. Differences of the impact of three different iron compounds (iron(III)oxide, iron(II)chloride and iron sludge) were evaluated with samples from an earlier experiment by specifically analyzing the methanogens by qPCR. The specific methane yield for iron treated digesters C2 and D2 were 700-800 ml/gVS*day and 400-500 ml/gVS*day, respectively. The reference digesters C1 and D1, without iron addition, had values between 200-400 ml/gVS*day and 400-500 ml/gVS*day, respectively. The iron treated digesters showed somewhat better methane yield as compared to the references but due to differences between technically identical digesters the results were difficult to evaluate. The content of volatile fatty acids was increasing in the reference digesters without any addition of iron towards the end of the experiment, indicating unstable processes. Hydrogen sulphide levels were lower for the iron treated digesters resulting in better gas quality than for the reference digesters. qPCR analysis shows a slight difference in methanogenic gene abundance during the period where iron(II)chloride were used as an additive, with a comparably lower value during this period. No difference in methanogen gene abundance could be seen for the period where iron(III)oxide and iron sludge were used as additive.

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1 Introduction

1.1 Aim with this project

The aim with this project was to evaluate biogas production and how the processes responded to a high organic loading of slaughterhouse waste combined with swine manure. An additional aim was to evaluate the importance of iron addition using different iron compounds for both process stability as well as abundance of methanogens.

1.2 Background

Biogas is a gas which mainly consist of methane and carbon dioxide. It is produced by microorganisms that degrade organic material in an anaerobic environment. This process occurs naturally in different oxygen free environments but it can also be applied in artificial systems, fort the production of biogas to be used as a renewable energy source (Angelidaki et al., 2011).

Renewable energy is an attractive solution to many problems concerning the climate change that we are facing. To be able to produce energy from our wastes, is a great way to manage our wastes in an environmental friendly way and at the same time contribute the market with renewable energy. The rest product from the digesters can in turn be used as fertilizer in agricultural industry (Angelidaki et al, 2011).

Biogas can be used to generate electricity, for heating of facilities and gas for cooking (Angelidaki et al., 2011). Biogas can also be used as fuel for vehicles, but to be suitable for that purpose the gas needs to be very pure and optimally have a content of H_2S that does not exceed 23ppmv (Moestedt et al., 2013).

1.2.1 Anaerobic digestion

Anaerobic digestion is a process where microorganisms live in an environment without any presence of oxygen. It is a complex biological system which is performed by several steps carried out by different microorganisms. The first step in anaerobic digestion is hydrolysis which is carried out by a large group of bacteria. Carbohydrates, proteins and lipids are broken down to smaller molecules such as amino acids and long chain fatty acids. This is an enzyme dependent process which catalyze the degradation of these compounds. The enzymes involved in this process are for example, lipases, cellulases and proteases (Angelidaki et al., 2011). Fermentative or acidogenic bacteria then transform the smaller molecules generated by hydrolysis into intermediate compounds and acetic acid (Manyi-Loh et al., 2013). The compounds generated by the acidogenic bacteria can then directly be used in methanogenesis which is carried out by methanogens. The methanogens can use acetate, or carbon dioxide and hydrogen to form into methane (Chen et al., 2014, Manyi-Loh et al., 2013; Angelidaki et al., 2011).

1.2.2 The microorganisms

Different bacteria can live in different temperatures, psychrophilic bacteria are able to live in environment below 20 degrees Celsius, mesophilic bacteria perform anaerobic digestion between 25-42 degrees Celsius, which is the most common environment used for controlled biogas production in anaerobic digesters. Thermophilic bacteria can work at with high temperatures between 50-65 degrees Celsius (Schnürer et al., 2016, Angelidaki et al., 2011).

Methanogens are microorganism that classifies as archaea. These organisms are found in several different anaerobic environments and have a vital role in the process of anaerobic digestion, i.e the last step of the anaerobic digestion process (methanogenesis) (Manyi-Loh et al., 2013).

Methanogens are very sensitive microorganisms and will be affected by slight changes of the environment in the digester, such as high content of VFA. Also, the activity of the methanogens is inhibited by low pH in the digester as well as high levels of ammonia (Manyi-Loh et al., 2013).

1.2.3 Parameters

A stable biogas process is hard to maintain especially without enough knowledge about the process. There are several parameters that are needed to be taken into consideration for an efficient and stable process (Schnürer et al., 2016). The most important parameters are explained shortly below.

1.2.3.1 pH and alkalinity

The pH in a digester is important to monitor. A drastic change of pH can be caused by an increased concentration of volatile fatty acids (VFA), which indicate that the process is unstable. A steady pH in the digester is desirable for the microorganisms, the optimal pH is around 6.5-7.5, but this depends on the microorganisms and the substrates that are used. It is also of importance that the pH in a digester with high ammonium content stays unchanged, if the pH increases ammonium takes the form of ammonia which is toxic in high concentrations (Schnürer et al., 2016).

1.2.3.2 Temperature

Steady temperature is important for the survival of the microorganisms. A temperature switch can shock the microorganisms and can be a danger to the whole microbial community. The temperature can be changed during the process but then it is of importance that the microorganisms get time to adapt to the new environment. There are several benefits with a thermophilic environment, but it is also a risk for an unstable process. Higher temperatures might give a higher yield of methane and can also reduce the amount of pathogens in the substrates, so that no hygienisation of the substrates is needed. Mesophilic temperatures are however considered to be less sensitive and more a stable temperature for biogas digesters (Schnürer et al., 2016).

1.2.3.3 Volatile fatty acids (VFA)

VFA content in the digesters is an important indicator showing if the biogas process is working correctly. Especially high values of propionate indicate that the microorganisms in the digester are not working properly. High levels of propionate are usually found when the activity of methane producing microorganisms is reduced. HPLC is a common method used to measure the content of VFA in the digester (Schnürer et al., 2016).

1.2.3.4 Total solids/Volatile solids (TS/VS)

VS is a measurement of the organic content in a material and TS is a measurement of the total solids in the material. VS is often used as a measure to determine how much of the content in a substrate that can be used as a source of energy by the microorganisms. It is often applied to determine how much of the substrates that is needed to get the right amount of load per liter digester content (Schnürer et al., 2016).

1.2.3.5 Hydraulic retention time (HRT)

Retention time is the average time for the content in the digester to be completely exchanged. The amount of substrates added each day determines the retention time for the digester. A retention time should not be shorter than the generation time for the microorganisms in the digester, so that the

microorganisms have time to “reproduce” and not being washed out of the digester. Usually the retention time for a digester is around 30 days. To ensure a correct evaluation, the process often needs three retention times to fully see how the process is affected by the different parameters that is applied to the digester (Schnürer et al., 2016).

1.2.3.6 Organic loading rate (OLR)

Organic loading rate is the amount of organic material added in the digester using VS as a measurement. Too high OLR (g VS/L and day) can shock the bacteria and stress them, which can result in a lower yield of methane and also in worst case scenario that the microorganisms might not be able to recover from the stress and the process needs to be restarted. To increase the amount of VS without risking a shock for the microorganisms it has to be done gradually (Schnürer et al., 2016).

1.2.4 Toxicants

Ammonium is used as a nutrient for the microorganisms in an anaerobic digester but in higher concentration, ammonia can act as an inhibitor. When proteins are fermented ammonium-nitrogen will be released. It mostly exists in its ionized form but the toxic unionized form, ammonia, increases when the pH and/or temperature in the digester increase. This is because ammonia has a pKa of 9.3 and will be unionized when the pH increases. That is one of the reasons why it is important that the pH in a digester is not too high (Chen et al., 2014).

Sulphide is released by proteins when degraded, it is originating from the amino acids cysteine and methionine. Sulphides bind to trace metals that are present in the digester, which makes the trace metals unavailable for the microorganisms to use as enzymatic Co-factors. It is also contaminating the biogas and its corrosive properties might damage the equipment (Schnürer et al., 2016).

1.2.5 Substrate

Slaughterhouse waste is a substrate that is energy rich and generates high yield of gas. However, it is a protein rich material which in turn might give rise to toxicants (ammonia and sulphide) making the process less effective, with risk for instability. If a solution to this problem will come, more biogas plants might be able to use slaughterhouse waste as a substrate. This will result in a much lesser impact on the environment (Ek et al., 2011).

Swine manure is a substrate with relatively low VS in comparison with the slaughterhouse waste. It is a substrate with high moisture and also a high content of nitrogen, which as well can lead to increased ammonium and sulphide content in the digester (Nasir et al., 2012).

1.2.6 Iron compounds

Addition of iron in digesters have several benefits. As mentioned, sulphides in digesters have a negative effect on the methanogens, both directly and by “trapping” trace metals of essential importance to the activity of the microorganisms. When added to the digester iron binds to sulphides, the sulphide will then precipitate in the digester instead of accumulating in the gas (Romero-Güiza et al., 2016). Sulphide can also affect the production of methane in the digester by binding other trace metals, making it unavailable for the microorganisms to use as enzymatic co-factors. When iron is present in excess, sulphides will bind to the iron instead of the essential trace metals (Chen et al., 2014). There are several iron compounds to choose between; iron(II)chloride for example have been used to reduce the amount of hydrogen sulphides in digesters since 1998 (Ek et al., 2011). In this study a comparison between iron(III)oxide, iron(II)chloride and iron sludge were made. Iron sludge is a precipitation product from water sewage plants.

2 Materials and methods

2.1 Digester operation

Four stirred tank laboratory-scale digesters containing a liquid volume of 5L were used in this experiment. The digesters were operated with a temperature of 52 degrees and a stirring speed of 90rpm. 6.25g iron sludge was added per kilogram substrate in two of the four digesters, using the other two as references without any addition of iron. The digesters had a HRT of 29 days and were fed a mixture of swine manure and slaughterhouse waste to a total OLR of 2g VS/L*Day. This mixture had an approximate proportion of 80/20 swine manure and slaughterhouse waste. Later on, the substrate ratio was changed to a 50/50 mix of slaughterhouse waste and swine manure, successively increasing the OLR from 1g VS/L*Day to 1.5g VS/L*Day and after a week the final OLR was reached at 2 g VS/L*Day. The volume of the substrate added was in total 200g/day, six days a week. Iron sludge was dissolved in water and 1.25g iron sludge/200g substrate were added to the D2 and C2 digesters. The D1 and C2 digesters received no iron addition. Carbon dioxide measurement for each digester was performed every day, by using a syringe to collect the gas samples and 5 ml of gas was measured in an Einhorn saccharometer containing 7M NaOH. Gas volume were noted every day for each digester, which were measured using sensors on the digesters, as well as plastic bags that collected the gas. 2 ml of digestate was collected from each digester once a week for VFA analysis, an additional 15 ml sample was collected at the same time for future analysis. pH measurement for each digester was performed simultaneously once a week. Collected samples were stored at -20 degrees Celsius. 2 ml and 1 ml gas samples were collected from each digester once a week for methane analysis.

2.2 Analytical methods

VFA analysis of collected samples were performed by using High- Performance liquid chromatography (HPLC), following the procedure described by Westerholm et al., 2012.
The Biogas 5000 (Geotechnical instruments, UK) instrument was used once a week to measure the percentage of methane, carbon dioxide and hydrogen sulphide in the gas from each digester.
Methane analysis was made for all the collected gas samples by using Gas Chromatography (GC), following the procedure described by Westerholm et al., 2012.
All calculations and statistical analyses were made by using excel.

2.3 TS/VS

TS/VS was measured for each substrate by collecting and weighing triplicate samples of the substrate and weighing the samples. The samples were then heated in an oven starting at 85 degrees and then increased to 105 degrees Celsius and was left over night. The samples were weighted and then transferred to an oven and starting a program that finally reached a temperature of 550 degrees Celsius. The samples were again weighted and then discarded. Calculations of TS/VS were made using excel. The equation for VS calculations is shown below.

$$VS = \frac{Dry\ sample - ash}{Wet\ sample}$$

2.4 Biochemical-methane potential test

Iron sludge was weighted to 7.95g and added into flasks containing 195.65g inoculum (3.6gVS) from Uppsala water sewage plant and 196.4g water, to reach a total weight of 400g.

Slaughterhouse waste and swine manure with an organic load of 1.50 gVS/l was weighted to 6.66g and 10.00g respectively to reach a total organic load of 3.0gVS/l. The weighted substrates were added to flasks containing 195.65g inoculum (3.6gVS) and 194.35g water. The flasks were placed in an Automatic Methane Potential Test system (AMPTS II, Bioprocess Control, Sweden) and left for running for approximately 30 days. The BMP test procedure were performed by following the protocol presented in Schnürer et al., 2016.

2.5 DNA extractions

In the earlier digester experiment two digesters (D1 and D2) were fed with 80/20 ratio mixture of swine manure and slaughterhouse waste. The D2 digester was treated with three different iron sources (iron(III)oxide, iron(II)chloride and iron sludge) during different periods of the experiment. The D1 digester was a reference digester without any addition of iron. Digestate samples from this experiment was collected by choosing samples from specific periods of interest (table 1). The DNA from the samples were extracted in triplicates by using MP Biomedicals FastDNA spin kit for soil. The extractions were made according to the manufacturer's protocol but by using 200µl of sample and also adding an extra washing step by using 500µl humic acid wash solution. The humic acid wash solution was prepared by mixing 978 µl Sodium Phosphate Buffer, 122 µl MT Buffer and 250µl Protein Perception Solution.

Concentration analysis of DNA samples were made using Qubit dsDNA BR Assay Kit and the procedure was done according to the protocol, using 2µl of DNA sample and 198µl of BR working solution, 10 µl of standard 1 and 2 were used and 190µl of working solution. Sample concentration was measured using Qubit 3.0 Fluorometer.

Table 1. Samples chosen for extraction and total methanogen analysis by qPCR. The samples were taken from different periods of and the date of sample collection are given I the table as well as the days between samples.

Samples	*Period*	*Date*	*Days of operation*	*Days between samples*
1	Fe(III)Oxide	2015-04-14	70	
2		2015-04-29	85	15
3	Fe(II)Chloride	2015-07-09	156	
4		2015-07-23	170	14
5	Fe(II)Chloride (new substrate)	2015-09-29	238	
6		2015-10-13	252	14
7	Iron sludge	2016-01-21	352	
8		2016-02-09	371	19

2.6 qPCR analysis for total methanogens

The quantification of the methanogens was based on a quantification targeting the 16S rRNA gene. One replicate from each DNA samples were used to investigate presence of any inhibitory compounds, by preparing a 10, 50 and 100-fold dilution of each sample. The 10-fold dilution was prepared by adding 18µl of water in PCR tubes and 2µl of DNA sample, the 50-fold dilution was prepared by adding 20µl water and 5µl of the 10-fold dilution, the 100-fold dilution was prepared by

adding 5µl water and 5µl of the 50-fold dilution. 24 standard samples containing a plasmid solution with an amplicon from the genomic DNA of *M. bourgensis* strain MAB1 were prepared with dilutions ranging from 10^8 to 10^0. A qPCR master mix was prepared by adding 1µl forward primers/ reaction (Met630F GGATTAGATACCCSGGTAGT) and 1µl reverse primers/ reaction (Met803R GTTGARTCCAATTAAACCGCA) with a concentration of 10 pmol/µl, 10 µl of IQ super mix containing SYBR green/ reaction and 5 µl of water/ reaction and 3µl of DNA sample. The samples were loaded in a 96 well qPCR plate by adding the master mix and the DNA sample in each well. The plate was covered with a plastic film and then left for running in the qPCR machine with the following program: Initial denaturation at 95°C for 7 minutes, 40 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 60 s and elongation at 72°C for 40 s, followed by a temperature melt curve (55-95°C, ΔT=0,1°C/s) to monitor any non-specific amplifications or primer dimers.

Agarose gel electrophoresis were run for samples with a 10-fold dilution, preparing a 1.5% agarose gel by weighing 4.5g of agarose and adding 300ml of 0.5X TBE buffer, the mix was heated in a microwave until clear. 5 µl of DNA loading dye containing gel red was added to the samples. 5µl of ladder was loaded in first and last well, and 10 µl of samples were loaded between the ladders, including two positive controls (standard curve). The gel was left for running at 90 V for approximately one hour.

All extracted DNA samples were diluted 10 and 50 times. Two qPCR plates were prepared using the same procedure as for the inhibition test and the plates were left for running in the qPCR machine with the same program as before.

Agarose gel electrophoresis were run for some of the samples with a 10-fold dilution, in total 18 samples including two positive controls (standard curve), following the same procedure as for the earlier agarose gel electrophoresis.

3 Results

3.1 Digester performance

The TS and VS values for substrates used in this experiment are presented in table 2.

Table 2. Calculated TS and VS for each substrate is represented in percent for each replica as well as the average for all replica and the standard deviation between the replicas. TS and VS values are shown in percentage of the wet weight.

Sample	*Replica*	*TS (%)*	*Average*	*STDAV.S (%)*	*VS (%)*	*Average*	*STDAV.S (%)*
Slaughterhouse w	A	9.5	9.7	0.12	8.9	9.0	0.07
	B	9.7			9.0		
	C	9.8			9.1		
Swine manure S2	A	7.1	7.3	0.44	5.8	6.0	0.44
	B	7.0			5.7		
	C	7.8			6.5		
Swine manure S1	A	8.4	8.4	0.07	7.0	7.0	0.06
	B	8.3			7.0		
	C	8.5			7.1		

The specific volume of methane (ml/gVS *Day) obtained for the reference digester C1 and the iron treated digester C2 was, after the stabilization phase, similar and reached 400-500 ml/gVS*Day (figure 1).

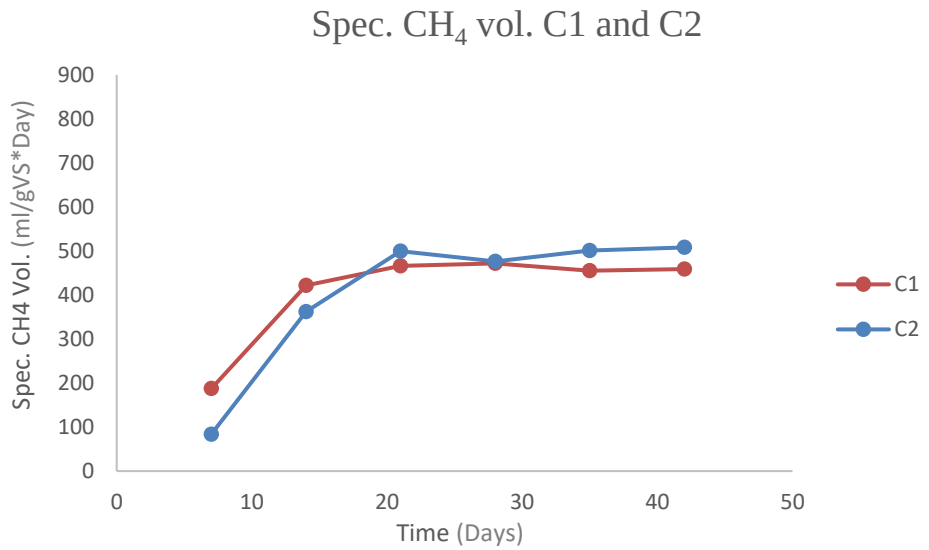

*Figure 1. Specific CH_4 yield (ml/gVS*day) over time in days, in a weekly average. Showing a comparison between reference digester C1 and iron treated digester C2.*

The specific methane yield (ml/gVS*Day) for the iron treated digester D2 was higher than the reference digester D1, with a volume between 700 and 800 ml/gVS*day and 200 and 400

ml/gVS*day, respectively. For D2, a decrease in the production was visible between day 30 and 40 (figure 2).

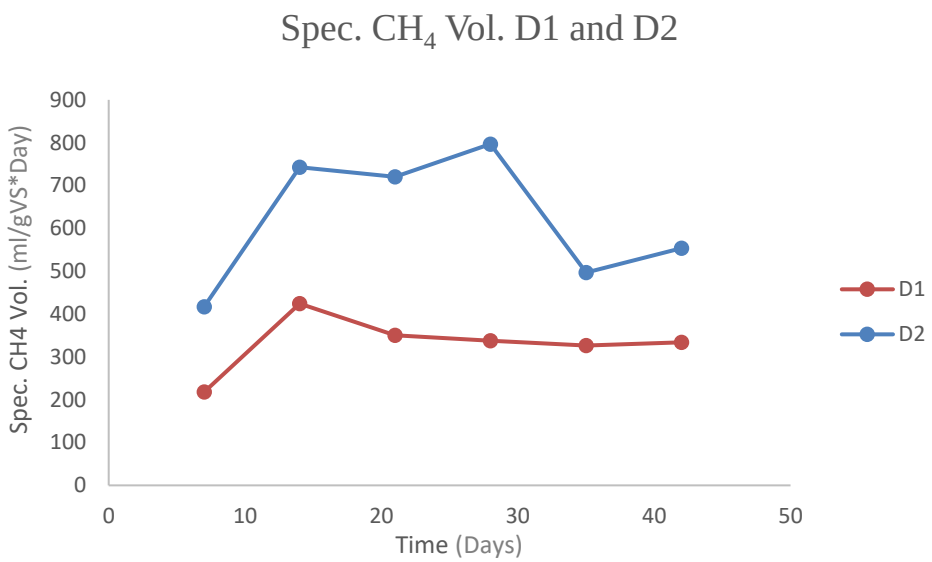

Figure 2. Specific CH_4 yield, average per week. Showing a comparison between reference digester D1 and iron digester D2

Comparison of the two references digesters C1 and D1 showed a difference in methane production between the digesters, with higher methane production in the C1 digester (figure 3).

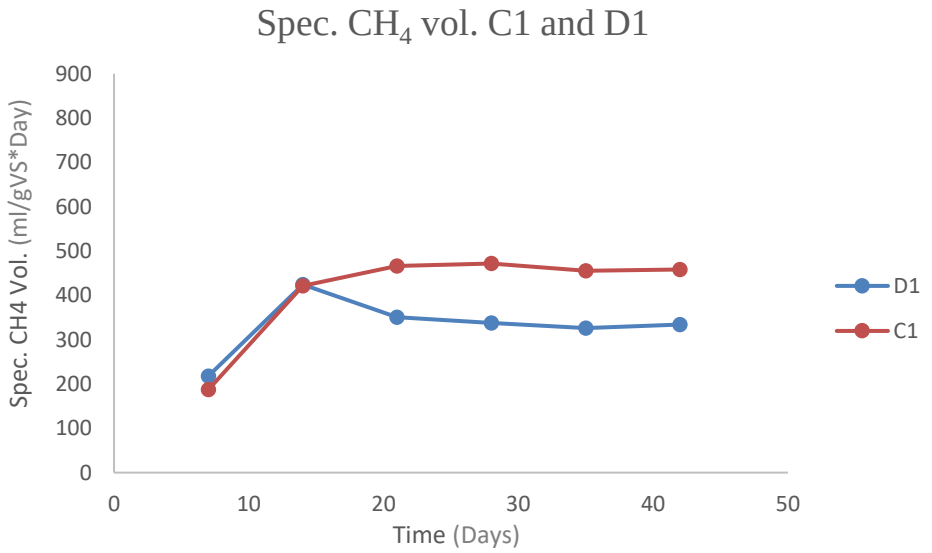

Figure 3. Specific CH_4 volume, average per week. Showing a comparison between reference digester C1 and D1

A comparison between iron treated digesters and their methane production showed that digester D2 have a higher methane production than the C2 digester (figure 4). However, over time the two digesters became more similar.

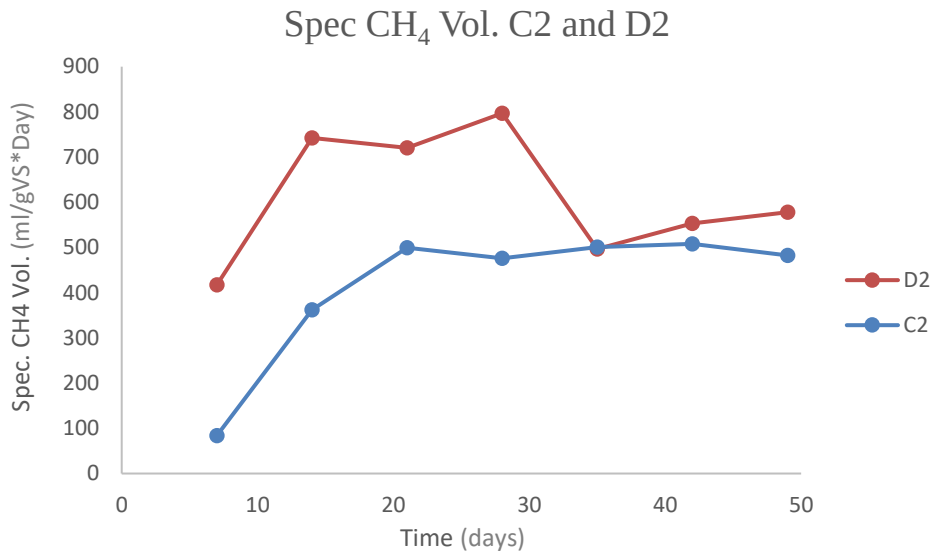

Figure 4. Specific CH_4 volume, average per week. Showing a comparison between iron treated digesters C2 and D2

The methane potential analysis of iron sludge showed no production of methane. The methane potential of the substrate containing slaughterhouse waste and swine manure was shown to be close to 500 ml/gVS*Day (figure 5).

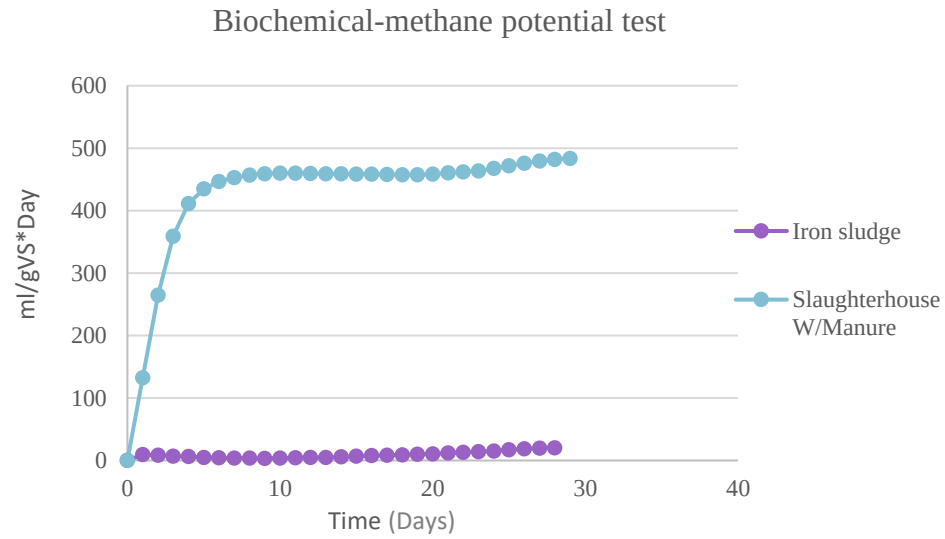

*Figure 5. Bio-chemical methane potential test of iron sludge and substrate containing slaughterhouse waste and swine manure. Specific methane volume (ml/gVS*Day).*

VFA analysis showed high levels of VFA, especially acetate and propionate in the beginning of the experiment. In addition, other VFAs were present in the beginning, but not later, such as valerate, valeriate and butyrate. A decrease of VFA levels is seen in all digesters as well as a rise for all digesters between day 20 and 30. Iron treated digester C2 and D2 do show lower leves of VFA accumulation after day 20, compared to the reference digesters C1 and D1 (figure 6).

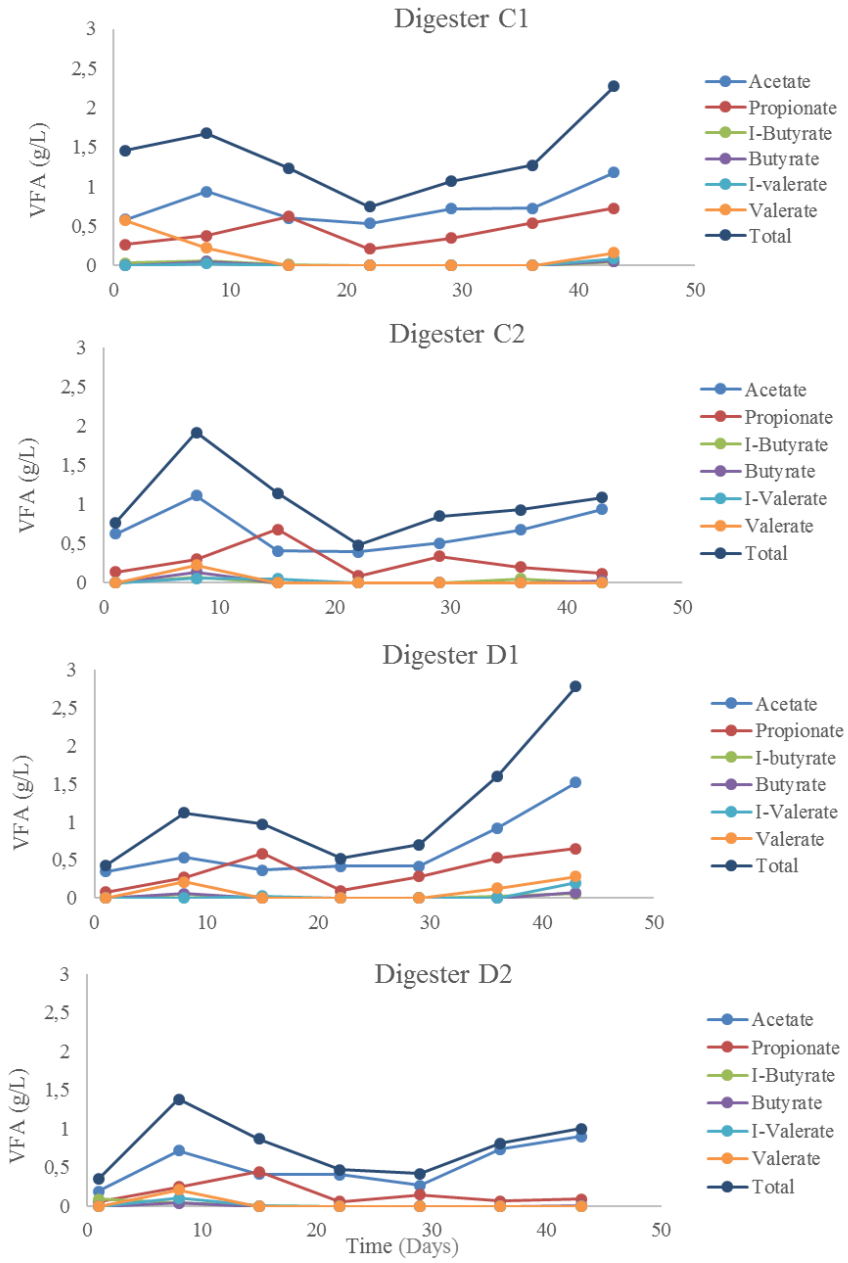

Figure 6. VFA values for digester C1, C2, D1 and D2, showing amount of acetate, propionate, I-butyrat, butyrat, valeriat, valerate and the total amount, over time.

H_2S content in the gas phase of all digesters are represented in figure 7 and 8 below. The digesters treated with iron had a lower H_2S content than the reference digesters. An increase of H_2S occurs over time in all digesters, even after the final OLR was reached.

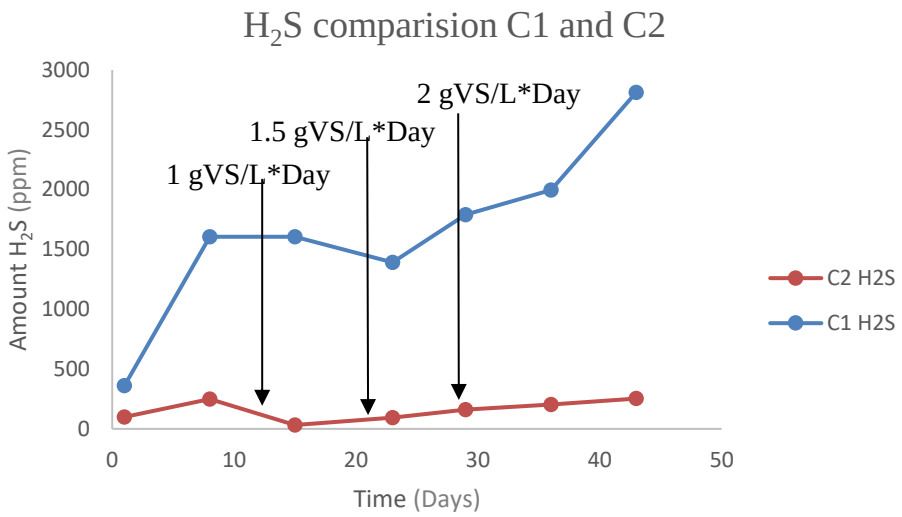

Figure 7. H_2S content in ppm over time, for C1 reference digester and C2 iron treated digester. The arrows show the days of increased OLR.

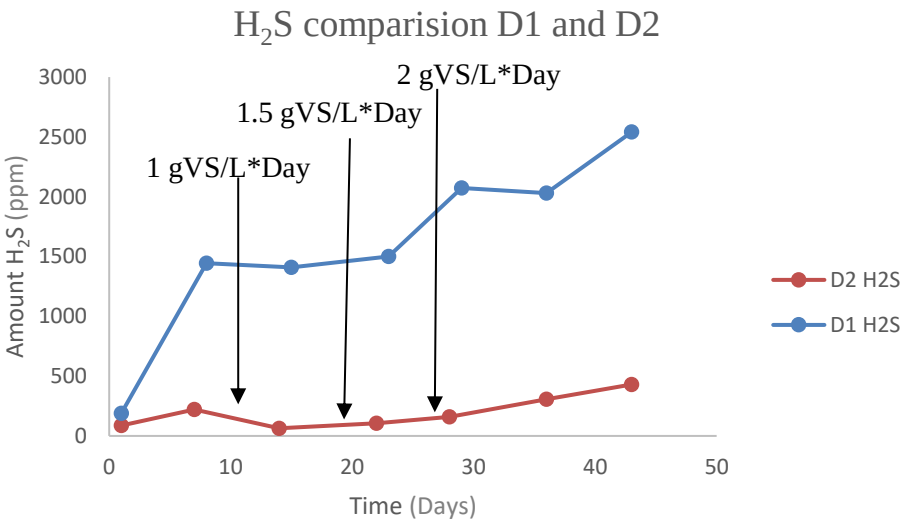

Figure 8. H_2S content in ppm over time in days, for D1 reference digester and D2 iron treated digester. Showing with arrows the days of increased OLR

3.2 Methane yields from earlier experiment

Figure 9 below shows the specific methane yield for the earlier experiment, and for the iron treated digester D2 and reference digester D1, showing the different periods when iron(III)oxide, iron(II)chloride and iron sludge were added. A decrease in methane production is shown after the addition of a new batch of substrate with lower SMP (lower specific methane potential) was introduced. The iron treated digester D2 showed a specific methane yield between 400 and 650 ml/gVS*Day, except for a peak reaching over 700 ml/gVS*Day at the end of the experiment. Reference digester D1 showed a specific methane volume between 400 and 600 ml/gVS*Day.

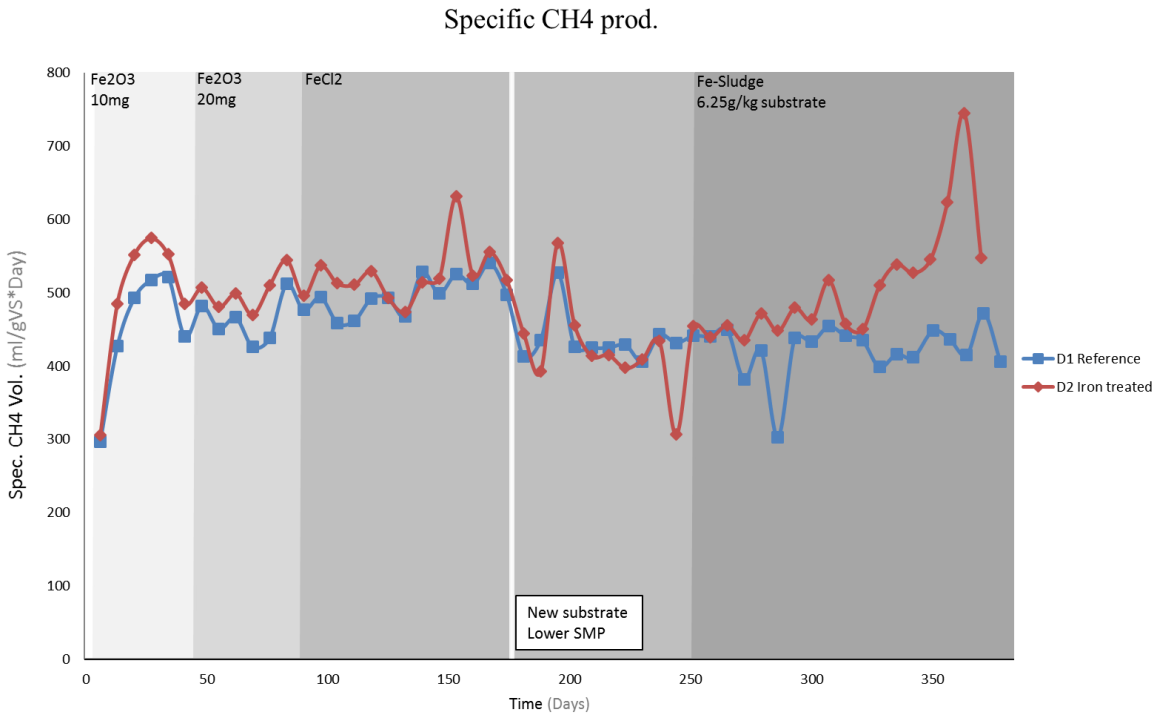

Figure 9. Specific methane volume over time in days obtained in D2, iron treated digester and D1 reference digester, average value per week. Coloured background showing periods of different iron compounds, as well as when a new substrate with lower SMP was used.

3.3 qPCR analysis

A comparison between D1 and D2 qPCR results show that sample 3, 4 and 5 (explained in table 1), reference digester D1 had a slightly higher gene abundance/digester volume than digester D2, as seen in figure 10. The results indicate that when iron(II)chloride (sample 3,4,5 and 6) is used as an iron additive the gene abundance/digester volume is slightly lower. No distinct difference in gene abundance/digester volume can be seen when iron(III)oxide and iron sludge is used.

The efficiency of the qPCR was 93.59 % and the R^2 value was 0.994. The melt curves showed three distinct peeks. Agarose gel electrophoreses control run showed no DNA elsewhere than expected.

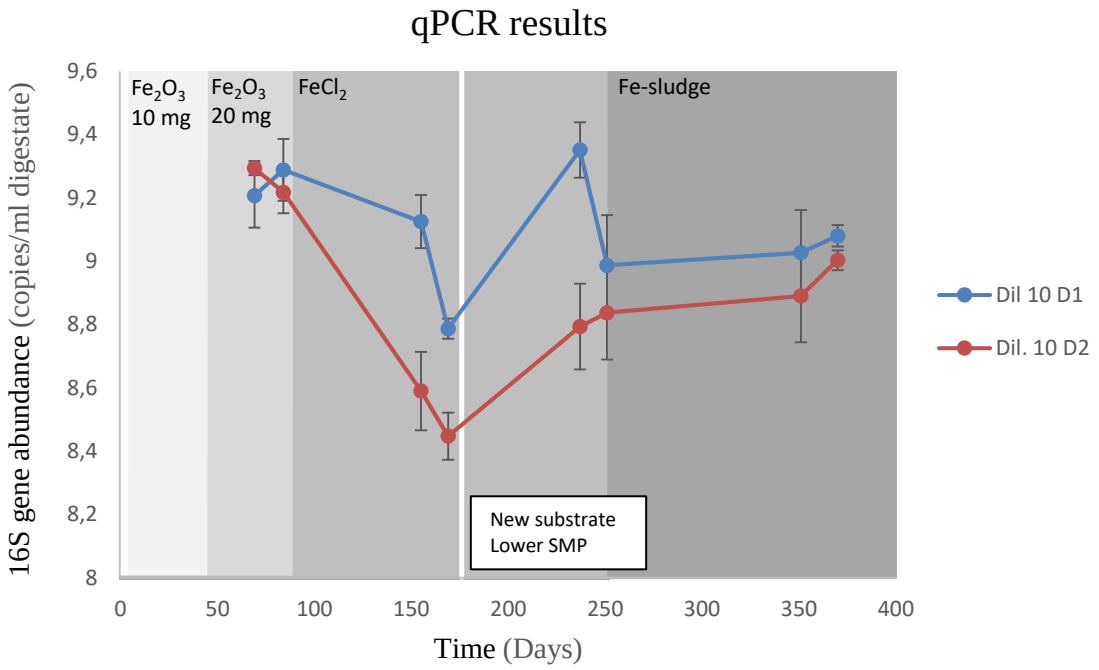

Figure 10. Average log gene abundance/ digester volume over time in days. Reference digester D1 and Iron treated digester D2 is displayed in the graph, as well as standard deviation between the triplicates for each data point. Note that the y-axis starts at 8.00.

4 Discussion

4.1 Digester performance

The specific methane yield in a weekly average in the four digesters seen in figure 1,2,3 and 4 is inconclusive. Digester D2 treated with iron sludge had a higher average specific methane yield compared to reference digester D1, ranging between 700 and 800 ml/gVS*day and 200 and 400 ml/gVS*day, respectively. However, digesters C1 and C2 had similar yields, ranging between 400-500 ml/gVS*Day. Thus, here no conclusion can be draw regarding a higher yield in the iron treated digester compared to the reference digester.

In a study by Cuetos et al., 2008 they evaluated anaerobic digestion of slaughterhouse waste in mesophilic conditions as well as co-digestion of slaughterhouse waste and organic fraction of municipal solid waste (OFMSW). The obtained methane yields from slaughterhouse waste ranged between 600-700 ml/gVS*day and for co-digestion with slaughterhouse waste and OFMSW the methane yield ranged between 400-500 ml/gVS*day (Cuetos et al., 2008). These methane yields are similar the yields in our digesters, co-digesting slaughterhouse waste and swine manure under thermophilic conditions, with exception for iron treated digester D2 that had a slightly higher methane yield.

The production of methane in the C1 digester was clearly higher than in the reference digester D1, even though they were technically identical. This might be due to residues that remain in the digester from earlier experiments. In comparison with the reference digester D1 the iron treated digester C2 had a higher specific methane volume. It is though clear that something is disturbing the C2 process due to the lower specific methane production compared to the parallel reactor D2. In a study by Hagen et al., 2014 a similar outcome was presented. In this study two technically identical digesters

were used, whilst one of them was stable and the other one unstable and eventually failed (Hagen et al., 2014)

4.2 Biochemical-methane potential test

The results from the biochemical-methane potential test for iron sludge showed that the iron sludge did not contribute to the methane production. The VS content in the iron sludge appeared not possible to be used as an energy source for the microorganisms. The slaughterhouse waste and swine manure mix showed a methane potential reaching 500 ml/gVS*Day, which is similar the specific methane production obtained for the C1 and C2 digester. The substrate for the BMP test did not have any addition of iron and neither did the C1 digester, the C2 digester did however have addition of iron. The BMP test was running with a different inoculum than the digesters, this might have had a small impact on the results.
In an article written by Ortner et al., 2014 BMP tests for slaughterhouse waste fractions showed a methane yield around 330-810 ml/gVS*Day (Ortner et al., 2014). In a review by Nasir et al., 2012 a compilation of different articles and their results from swine manure digestion are presented. The methane yield for swine manure usually reach around 300 ml/gVS*Day, depending on the parameters used (Nasir et al., 2012). These findings suggest that the results from the BMP test in this study is reasonable for a 50/50 mix of slaughterhouse waste and swine manure, without addition of iron.

4.3 Process performance

The contents of VFA decreased initially and then increases between day 20 and 30, in all digesters (figure 6). As the organic loading rate increased so did the content VFA, especially for the reference digesters. This is an indicator that the processes are affected by the increased organic loading rate. The pH in the digesters were stable during the experiment and the increase of VFAs did not affect the pH. Mainly acetate and propionate accumulated in the digesters. The ratio of these two compounds can be used as an indicator and the value show if there is a risk of process failure (Schnürer et al., 2016). In an article about mono digestion of slaughterhouse waste written by Ortner et al., 2014, high accumulation of VFA were recorded, reaching around a total amount of 6 g/l. In this study iron addition did not reduce the amount of accumulated VFAs (Ortner et al., 2014). This support the speculation that the rise of VFAs in our digesters probably were due to organic overloading.

It is clearly from the result presented in figure 7 and 8 that the H_2S levels were much higher for the digesters without any addition of iron. A slight increase of the H_2S levels were seen in towards the end of the experiment in all digesters. This might be a result of calibration of the biogas 5000 instrument, this theory can be supported by the increase in both reference digesters and iron treated digesters.
Another more likely possibility is that when increasing the OLR of slaughterhouse waste the added iron was not fully capable of decreasing the amount of H_2S produced during the degradation as when a lower OLR of slaughterhouse waste was used. A rise of H_2S started around 1.5gVS/L*day, and had a steady increasing trend afterwards. The optimal amount of H_2S in a digester should be less than 50 ppm (Schnürer et al., 2016), the iron treated digesters did not exceed 50 ppm. The reference digesters reach a H_2S amount near 3000 ppm, which implies that the gas produced by the digesters without any addition of iron do not have sufficient gas quality.

4.4 DNA extractions

The DNA concentration results did show very similar results for each extraction occasion, the first extraction occasion did have a high DNA concentration, at the second extraction occasion all the samples did have a low DNA concentration. This could have been due to differences in the extraction

efficiency. To see if this was the case, one sample from each period were selected and tested, without making triplicates, to control if the DNA concentrations were extraction dependent. The results from these DNA concentration analyses were very similar to the first extractions of the chosen samples, ruling out that the difference in DNA concentration had anything to do with the specific extraction occasion. It was simply just a very different amount of DNA between the samples.

4.5 qPCR analysis

qPCR analysis for total methanogens were made to investigate any differences in methanogenic abundance during addition of iron(III)oxide, iron(II)chloride and iron sludge. The results from the qPCR analysis did not give any straightforward conclusions regarding the methanogen population in the digesters when the different iron additives were used. What can be seen is that sample 3,4 and 5, from the period when iron(II)chloride were used, show a slightly lower gene abundance between the reference digester D1 and the iron treated digester D2. Sample 3 and 4 from iron treated digester D2 had a lower gene abundance than the reference digester D1. No conclusions that there is a substantial difference in methanogen population between the different iron compounds can be drawn, the only conclusion that can be drawn from these results is that iron in general have an impact on the methanogen population.

Interestingly, sample 3 in the iron treated digester D2 had a comparably lower abundance of methanogens while the methane production for this digester had a peak at the same time. on contrary sample 5 from the same reactor and during the period when iron(II)chloride and a new substrate was showed a lower abundance of methanogens. Sample 5 was collected when a drop in the methane production for digester D2 occurred. Implying a disturbance that might as well have affected the methanogen population at that time.

These speculations do not have enough significant results to be fully supported. By doing further analyses with qPCR by using specific primers for different methanogen groups it could give a better understanding of the impact of the different iron compounds in the digesters.

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