NTNU – Trondheim
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Biogas from Livestock Manure

Microbial Community Analysis of Biogas
Reactors

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Chemical Engineering and Biotechnology
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Declaration

This master thesis is executed independently, and in accordance with the examination regulations at Norwegian University of Science and Technology (NTNU).

Trondheim 16.07.2012

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Preface

This master thesis was created to support the research of PhD candidate Anna Synnøve Røstad Nordgård. It was announced by the Institute of Biotechnology, Norwegian University of Science and Technology, during the fall of 2011, and assigned to the master student in October. It was executed during the spring of 2012 at the Institute of Biotechnology.

I would like to thank everyone who has helped me:

First, and mainly, I would thank PhD candidate and advisor Anna Synnøve Røstad Nordgård for her exceptional support and help. Without her patience and guidance, I would have been lost.

Professor and supervisor Kjetill Østgaard, thank you for the opportunity to work with this thesis. I have learned a lot, and received many helpful advices from you.

Researcher Ingrid Bakke, for practical help and valuable advises on PCR and DGGE.

Researcher Kjell Domaas Josefsen, for letting me use the anaerobe equipment at the SINTEF lab, and helping me change nitrogen tanks when necessary.

I would also thank PhD candidate Wenche Bergland at Telemark University College and farmer Knut Vasdal at Foss farm, for sampling and safe transportation of the samples to Trondheim. Your job has been indispensable, and the extra effort you put in packing the samples made my job much more pleasant.

Trondheim, 16.07.2012

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Ida-Renée Jacobsen Forsberg

Abstract

The aim of this experiment was to monitor the microbial communities in two biogas reactors and evaluate the efficiency of denaturing gradient gel electrophoresis (DGGE) as a technique for visualizing shifts in the microbial compositions. The reactors were followed from September 2011 to May 2012. The first reactor is a pilot scale upflow anaerobic sludge blanket (UASB) reactor situated at Foss farm outside of Porsgrunn, running on cow manure. The second reactor is lab scale and situated at Telemark University College, running on pig manure. Samples were taken from the reactors at regular intervals.

DNA was extracted from the samples and amplified by polymerase chain reaction (PCR). The primers were 338f and 518r, targeting the 16S rDNA sequence. Changes in the microbial diversity were detected by DGGE in both reactors. Some bands appeared and other disappeared during the period. These changes could not be correlated to changes in operating conditions. This was probably because DGGE reflects cell amounts and not microbe activity levels.

DGGE is a highly reproducible and consistently performing fingerprinting technique. It is capable of reflecting long term shifts in the microbial communities and several samples can be compared in one gel. This makes DGGE an effective method for monitoring reactors over time.

Several DGGE bands were excised and sequenced, but the results were either negative, or of too poor quality, for further analysis. The probable cause was insufficient separation of bands leading to multiple sequences in the extracted DNA. This may be overcome by using a more specific primer set to reduce the amount of bands.

Sammendrag

Målet med dette forsøket var å overvåke det mikrobielle samfunnet i to biogassreaktorer og evaluere effektiviteten av «denaturing gradient gel electrophoresis» (DGGE) som metode for å visualisere endringer i den mikrobielle sammensetningen. Reaktorene ble fulgt fra september 2011 til may 1012. Den ene reaktoren var en «upflow anaerobic sludge blanket» (UASB) reaktor i pilotskala på Foss gård utenfor Porsgrunn, basert på kugjødsel. Den andre var en labskala reaktoren på Høyskolen i Telemark, basert på grisegjødsel. Prøver ble tatt fra reaktorene jevnlig.

DNA ble ekstrahert fra prøvene og amplifisert med «polymerase chain reaction» (PCR). Primerne som ble brukt var 338f og 518r, med 16S rRNA som målsekvens . Ved DGGE analyse ble endringer i begge reaktorene påvist. Flere bånd oppsto eller ble borte i løpet av perioden. Disse forandringene kunne ikke knyttes direkte til endringer i driftsbetingelser. Dette var antakelig fordi DGGE reflekterer mengden celler tilstede, og ikke det mikrobielle aktivitetsnivået.

DGGE er en meget reproduserbar metode med gjennomgående gode resultater. Den er i stand til å gjengi langtidsendringer i det mikrobielle samfunnene og flere prøver kan sammenliknes på en gel. Dette gjør DGGE til en effektiv måte å overvåke reaktorer over tid.

Flere DGGE bånd ble klippet ut og sekvensiert, men resultatene var enten misslykket eller av så dårlig kvalitet at videre analyser var umulig. Dette skyldes antaklig for dårlig separering av båndene, noe som førte til flere sekvenser i det ekstraherte DNA. Dette kan løses ved å bruke en mer spesifik primer for å redusere antallet bånd.

Abbreviation

Abbreviation	Explanation
A	Adenine. One of the four DNA bases
APS	Ammonium Persulfate Solution
BL	Blind sample, negative control
BLAST	Basic Local Alignment Tool
BSA	Bovine Serum Albumin
C	Cytosine. One of the four DNA bases
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FISH	Fluorescence *in situ* hybridisation
G	Guanine. One of the four DNA bases
HRT	Hydraulic retention time
OLR	Organic loading rate
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
T	Thymine. One of the four DNA bases
TEMED	Tetrametyletylenadiamin
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acids

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1 Introduction

1.1 The climate challenge

Norway wants to be climate neutral by 2030 (Klima- og forurensningsdirektoratet, 2011), and this will only be possible through reduced emissions. Every part of the society and industry must contribute to reach this objective.

Only 3 % of the area of Norway is used for agriculture, and it is mainly reserved for food production. The potential to reduce total emissions and become more climate neutral is still high in this field. Agriculture can produce, and use, climate neutral energy in the form of biogas from manure. This gas can be used to produce electricity, heat, and fuel. Biogas production from manure is fast growing in e.g. Germany, Sweden, and Denmark, but not yet in Norway. (Det Kongelige Landbruks- og Matdepartementet, 2008 - 2009)

Only a small amount of the manure is used in biogas production I Norway today. Increasing the amount of manure utilized will give a double effect; the emission of methane produced by manure degradation is reduced, and climate neutral energy is produced. The manure can still be used as fertilizer after the production of biogas, so no resources are lost during the process. (Det Kongelige Landbruks- og Matdepartementet, 2008 - 2009)

Stortingsmelding nr. 39 (Det Kongelige Landbruks- og Matdepartementet, 2008 - 2009) states that biogas production is a priority area to be focused on in the following years. The aim is for 30 % of the manure to be used in biogas production by 2030. The technology of small-scale biogas reactors in Norway is still new, and not yet optimized. The high initial investment costs, combined with low electricity prices, makes cost effective production challenging. Some countries have governmental subsidies of biogas reactors, but there is no such initiative in Norway to day.

Based on the requirement for more knowledge in the field, a project named “Biogas Reactor Technology for Norwegian Agriculture” (BIONA) was started in 2011. It is financed by The Research Counsel of Norway and coordinated by Bioforsk. The primary objective is “to make biogas reactor technology cost effective, robust and

well adapted for use in Norwegian agriculture" (Norges Forskningsråd). Analyses of the microbial communities in such reactors are one part of the research needed to achieve the goal of an optimized process.

1.2 Anaerobic fermentation of organic material to biogas

1.2.1 The substrates

Biogas is produced by anaerobic fermentation of organic matter. The organic material can be manure, food waste, plant materials, wastewater etc.

A common problem with some organic substrates such as grass and straw, is that they are not fully degraded during the process. They contain recalcitrant substrates like cellulose, hemicellulose, and lignin. Pre-treatments may increase the microbes accessibility to the recalcitrant substrates and hence the degradation. Examples of pre-treatments are maceration, thermal treatment, alkaline treatment, separation and extrusion. This enhances hydrolysis, and will hence improve biogas production and/or methane yield. (Hjorth et al., 2011)

Manure often contain recalcitrant substrates from the animals feed, but manure presents other challenges as well. Variables such as specie, breed and growth stage of the animal, feed, bedding amount and material, water content, and degradation during the pre-storage will affect the methane production conditions and yield. The theoretical yield of methane per volatile solid (VS) is higher from pig (516 ± 11 L/kg VS) and sow manure (530 ± 6 L/kg VS) than dairy cattle manure (468 ± 6 L/kg VS). (Møller et al., 2004)

The low yield of biogas from cattle manure may be explained by how the cattle digest the food. Ruminants possess a digestive organ called rumen, where microorganisms digest cellulose and other plant polysaccharides. Small food particles are sorted out of the rumen, but large particles are regurgitated, chewed and mixed with saliva before being swallowed and returned to the rumen for further digestion. The food remains in the rumen for 20 – 50 hours. It is transported through the digestive system and into the normal, acidic stomach when it is sufficiently digested. This long

retention time ensures that easily degraded substrates are utilized by the cow, and only heavily digested materials and volatile fatty acids are left. CO_2 and CH_4 produced are released by eructation. (Madigan et al., 2011)

An important factor of the substrate is the carbon to nitrogen ratio (C/N ratio). By degradation of e.g. proteins and nucleic acids, high amounts of nitrate are released in the form of ammonia. Ammonia has been shown to inhibit methane production at high concentrations (5.5 gNH_4^+-N/L), unless the population is given time to adapt (Westerholm et al., 2011). A C/N ratio between 10 and 30 is acceptable in a biogas reactor, but the optimum is between 15 and 25. Manure from cattle lies between 6 and 20, compared with e.g. straw which lies between 50 and 150 (Schnürer and Jarvis, 2009, Bioforsk, 2010). A mixture of more two or more substrates is often used to achieve a robust and stable reaction. (Bioforsk, 2010)

1.2.2 Biogas formation

The fermentation process can hypothetically be divided into four steps, illustrated in Figure 1.1 :

- Hydrolysis
- Acid formation
- Acetic acid formation
- Methane production

A concentration of more than 60 % methane is necessary for direct combustion of the biogas. Glucose as the only substrate will give a theoretical methane concentration of 50 %, but since CO_2 is water soluble, the real concentration will be higher. A more reduced substrate, such as manure, will give higher methane to CO_2 ratio. (Østgaard, 2005)

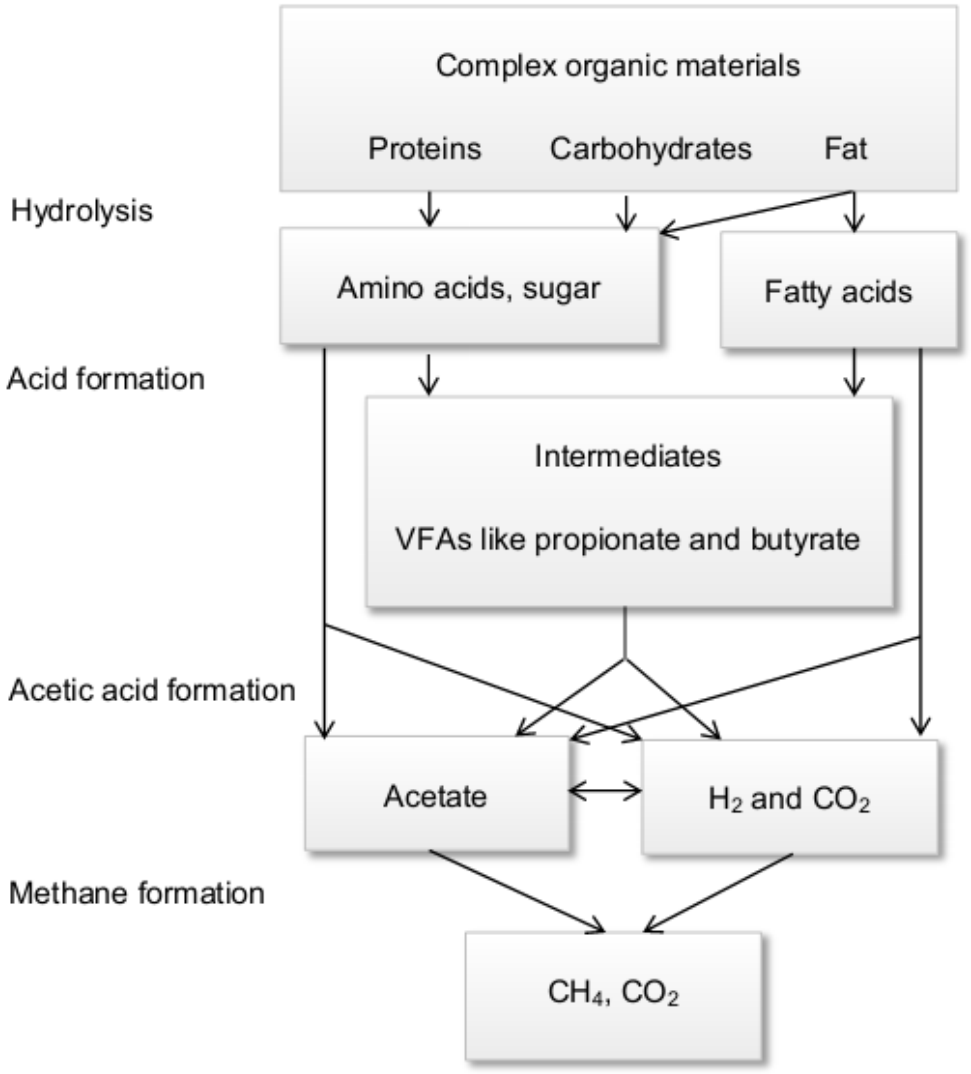

Figure 1.1: Anaerobic fermentation of organic matter to methane and CO_2 (Østgaard, 2005).

The first step is hydrolysis, were organic components such as fat, proteins and carbohydrates decompose to more accessible substrates such as lipids, fatty acids, polypeptides, amino acids and sugars in addition to hydrogen and CO_2. Hydrolysis is a slow process, and normally the rate limiting step.

Hydrolysis does not produce energy for the microbes in itself, and will therefore be coupled with acidogenesis. The main product is volatile fatty acids (VFA) such as acetic acid, propionate and butyrate. CO_2 and H_2 are also potential products. The

formation of acids from e.g. glucose is energetically favourable, since the change in Gibbs energy (∆G°') is negative.

$C_6H_{12}O_6 + H_2 \rightarrow 2\ CH_2COOH + 4\ H_2$ $\Delta G°' = -218$ kJ

All VFAs, such as propionate, can be converted to acetic acid before methane formation. This process has a theoretical positive ∆G°' and is therefore not spontaneous.

$CH_3CH_2COOH + H2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 3\ H_2$ $\Delta G°' = +76$ kJ

Methanogenic archaea will remove the hydrogen and use it for methane production.

$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ $\Delta G°' = -134$ kJ

This removal of hydrogen alters the Gibbs energy in acetate formation to a negative level, making it spontaneous. It is called “interspecies hydrogen transfer” and is a vital element in the methane production. (Østgaard, 2005)

Hydrolysis coupled with acid formation and methane production can be separated into different physical compartments. Fast growing acid producing bacteria will lower pH drastically, unless the acids are used quickly in methane production. Methane producing organisms are slow growing, with an optimum of pH 6.5 – 8.0. It is important to regulate pH, especially in the initiation period of a reactor until the methanogenic archaea population is sufficient. Lowered pH caused by quick acid formation will inhibit and kill the methane producing archaea, eventually stopping all biogas production. A two compartment reactor can prevent this by separating hydrolysis and acid formation into one low pH (≤ 5.5) compartment and the methane production into a higher pH (pH 6.6 - 8.0) compartment. (Østgaard, 2005)

1.2.3 The microbial community

Hydrolysis and acid formation is carried out by a large consortium of bacteria, called acidogenic bacteria (Østgaard, 2005). Some of the genera involved are *Bacteroids*, *Bifidobacteria*, *Clostridium*, *Escherichia*, *Lactobacillus,* and *Proteus* (Gerardi, 2006). Examples of bacteria hydrolyzing cellulose in particular, are *Clostridium thermocellum*, *Acetivibrio cellulolyticus*, *Bacteroides succinogenes*, and *Ruminococcus flavefaciens* (Volfova et al., 1982) .

The acetic acid is formed by acetogenic bacteria such as *Syntrophobacter wolinii (C_3, propionate), Syntrophomonas wolfei (C_4-C_8 fatty acids), Syntrophus gentianae (aromatic compounds) Clostridium bryantii* (alkonic acids < C_{11}), *Desulfovibrio,* and *Acidaminobacter hydrogenoformans* (Lee and Zinder, 1988, Madigan et al., 2011).

Methane is formed by the obligate anaerobic methanogenic archaea in the phylum Euryarchaeota. They are generally divided into three groups based on the substrate, see Table 1.1.

Table 1.1: Overview of methanogenic achaea (Madigan et al., 2011).

Group	Substrates	Example orders
CO_2- type, hydrogenotrophic	CO_2 and H_2	Methanobacteriales, Methancoccales and Methanomicrobiales
Methyl-type	Methyl groups, e.g. methanol	Methanosarcinales
Acetoclastic	Acetic acid	Methanosaeta and Methanosarcina of the order Methanosarcinales

High amount of ammonia has been shown to shift the methane production mechanism from acetoclastic methanogenesis to hydrogenotrophic methanogeneisis and syntrophic acetate oxidation. This is explained by ammonia inhibition of the acetoclastic families *Methanosaetaceae* and *Methanosacinaceae.* Syntrophic acetate oxidizing bacteria (SAOB) increases in activity when the ammonia levels are increased. Few SAOBs are known today, but some examples are *Thermacetogenium phaeum* (Hattaori et al., 2000), *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011), *Syntrophaceticus schinkii* (Westerholm et al., 2010), *Clostridium ultenense* (Schnürer et al., 1996), and *Termotoga lettingae* (Balk et al., 2002).

Estimates based on 16S rDNA analysis made of the microbial community in the rumen, calculates 300 – 400 bacterial species. Many of these may still be present in the manure and hence in the biogas reactor. *Firmicutes* and *Bacteroidetes* dominate the Bacteria, while methanogenic organisms dominate Archaea. *Fibrobacter succinogenes* and *Ruminococcus albus* are the main cellulytic rumen anaerobes, and *Rubinobacter amylophilus* and *Succinomonas amylolytica* dominates when the substrate is starch based. *Lactinospira multipara* digests legume hay which is high in pectin. Secondary fermenters such as *Schwartzia, Selenomonas,* and *Megaspaera* are also present. (Madigan et al., 2011)

1.3 Molecular biological methods for microbial analysis

Most organisms cannot be cultivated in a lab, and direct analysis without cultivation is necessary to get a full picture of the community (Amann et al., 1995). This can be done by analysing the DNA from the environmental sample with molecular biological methods instead of using traditional microbiological methods. Examples of such methods are 16S rDNA sequence analysis by polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) and fluorescence *in situ* hybridization (FISH) (Amann et al., 1995).

The 16S rDNA sequence is commonly used in sequence-based phylogenetic studies because they are universally distributed and has conserved regions useable for

specie comparison. It is large enough to contain sufficient differences in the sequence to separate species, but still small enough to be effective (Amann et al., 1995). By assuming nucleotide changes in DNA accumulate proportional to time, the information from the 16S rDNA sequence can be used to create phylogenetic threes. The Ribosomal Database Project II (Cole et al., 2011) contains a collection of 16S sequences and the database is searchable based on your sequence for comparison (Madigan et al., 2011).

1.3.1 Principle of Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a method used for direct visualization of microbial diversity (Muyzer et al., 1993). Some of the different applications of DGGE are to study community complexity, monitor population shifts, and compare DNA extraction methods.

DGGE separates DNA fragments of the same length based on the GC-content of the sequence. DNA is extracted from the samples and amplified by PCR with a primer containing a GC-clamp. All the fragments will be of the same size. A polyacrylamide gel with a gradient of denaturants (e.g. formamide and urea) is used, starting with a low concentration on the top, near the wells.

Denaturing agents in the gel will separate the double stranded DNA into single strands at a given concentration of denaturing agents. This is called the melting point of the DNA. Melted DNA will be immobilized, and the GC-clamp will prevent a full denaturation.

Samples with a high GC content will denaturize later in the gel at higher concentrations of denaturing agents than fragments with lower GC-content. After staining the gel, bands of DNA with different GC-content can be visualized (Muyzer and Smalla, 1998, Madigan et al., 2011).

1.4 The project objectives

Each anaerobic fermenter is a unique system defined by the substrate material and reactor conditions. The microbial community is correspondingly diverse, and the role of many microorganism involved is still unknown or poorly investigated. This knowledge is important for better understanding the fermentation process and to be able to counteract unfavourable changes in the reactor. (Malin and Illmer, 2008)

The purpose of this master thesis was to further develop molecular biological methods to monitor anaerobic fermentation of manure for biogas production. It was a continuation of the master thesis by Røstad Nordgård (2010) where methanogenic archaea was in focus, and the preliminary investigations made by Forsberg (2011).

The aim was to test primers for PCR and DGGE, and to see if changes in the microbial community were visible through DGGE fingerprinting. The band patterns were compared between the gels, and analysed with respect to operating conditions and gas production results. Different bands from the gels was excised and sequenced. The methods were then evaluated for its use to monitor reactor efficiency.

The fermenters were a reactor running on cow manure situated at Foss Farm outside Porsgrunn, and a lab scale reactor running on pig manure at Telemark University College (TUC) in Porsgrunn. The reactors were sampled about once a month from September 2011 to April 2012.

2 Methods and experimental design

2.1 The biogas reactors

2.1.1 The pilot scale biogas reactor on Foss farm, based on cow manure

The reactor based on cow manure (also called the cow manure reactor) is situated at Foss farm outside Porsgrunn and run by farmer Knut Vasdal. It is an up flow anaerobic sludge blanket (UASB) reactor, and the layout is given in Figure 2.1 together with a picture.

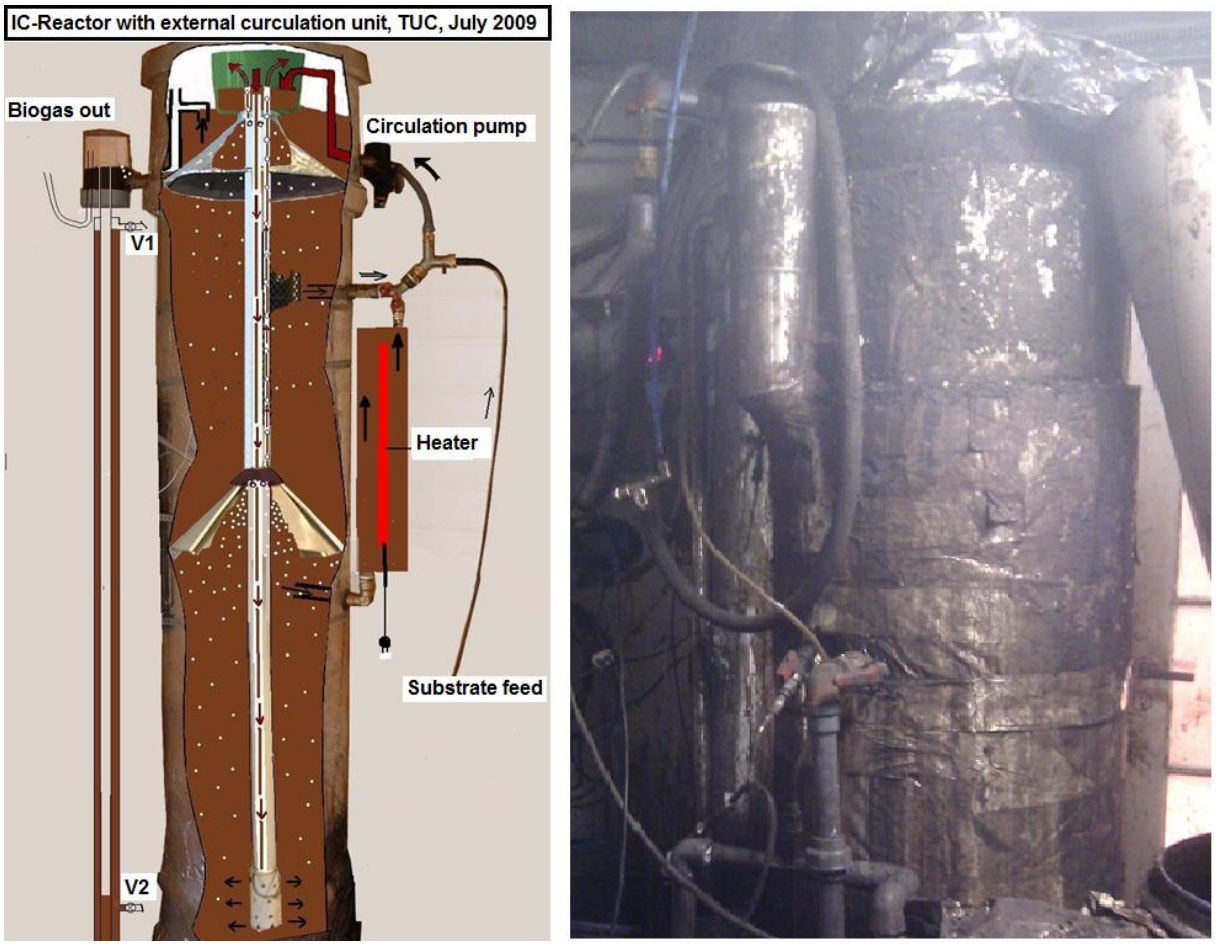

Figure 2.1: Pilot scale reactor based on cow manure at Foss farm outside Porsgrunn. Layout provided by Wenche Bergland, Telemark University College.

The reactor contains 220 L, and the feed rate is between 10 and 50 L/day. The temperature varies between 24 and 35 °C. This is presented in Figure 2.2. The feed is from manure batches changed on September 12th, November 7th, January 30th and March 29th. The organic loading rate, given as gram COD (chemical oxygen demand) per litre feed, is presented in Figure 2.3, and the batch changes are represented by blue, vertical lines. Figure 2.4 presents the biogas production (L/day), which varied from 50 L/day to almost 320 L/day. The primary data is presented in Appendix A. The reactor was exchanged for a new reactor with similar design on April 19th. The content of the old reactor was transferred to the new reactor.

There were problems with the feed rate during a period from November 7th to the 16th. The feed rate fluctuated between 0 and 142 L/day (not visible on Figure 2.2), and this may have caused a washout of biomass. The biogas production decreased rapidly from November 5th, and the feed rate was adjusted from 50 L/day to 10 L/day on November 18th. The lowered production may have been caused by the possible washout, but the steep feed rate adjustment from 25 L/day to 50 L/day on October 28th combined with the earlier temperature increase is the most likely reason since the biogas production rates declined even before the feed rate problems started.

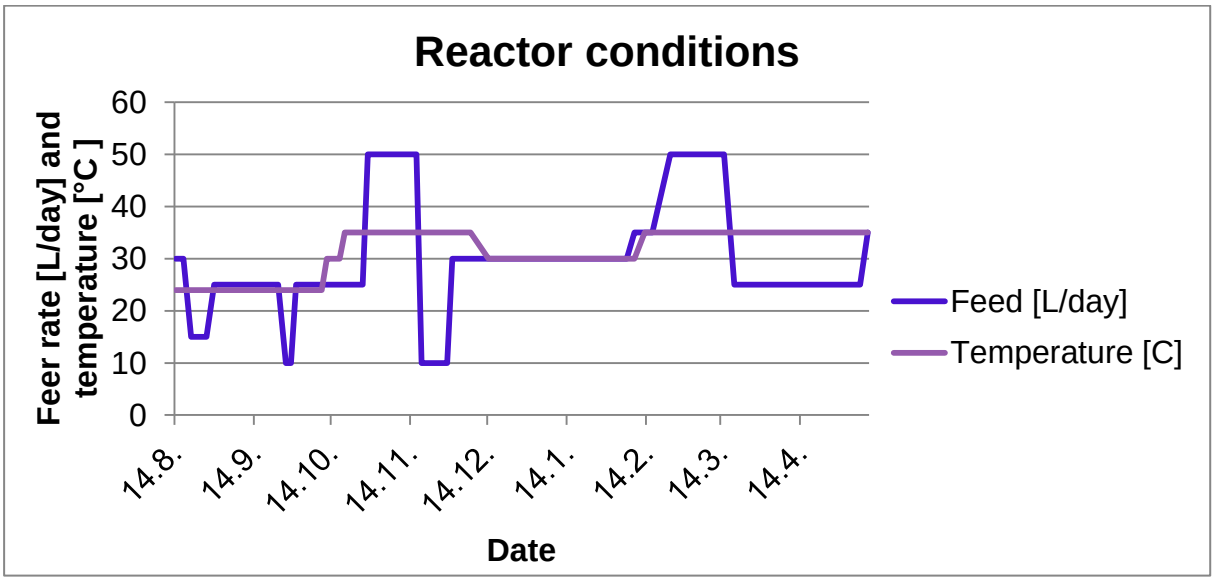

Figure 2.2: The feed rate and temperature for the reactor running on cow manure in the period from August 14th to May 10th.

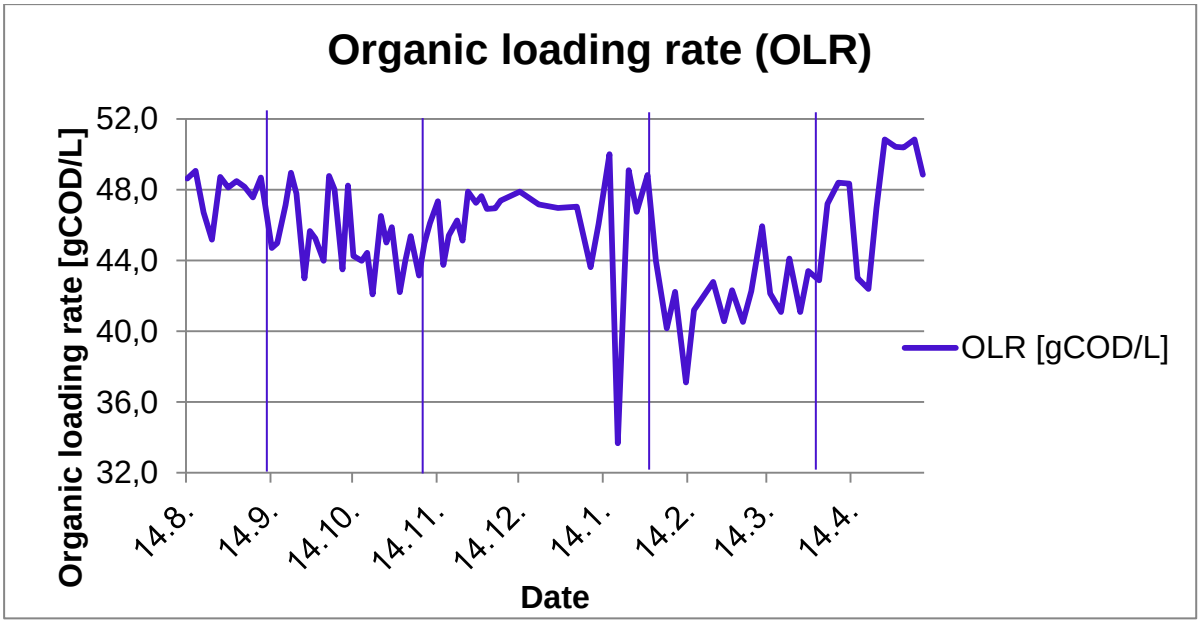

Figure 2.3: The organic loading rate in the reactor running on cow manure, in the period from August 14th to May 10th. The verical blue lines represents the changes in feed batch.

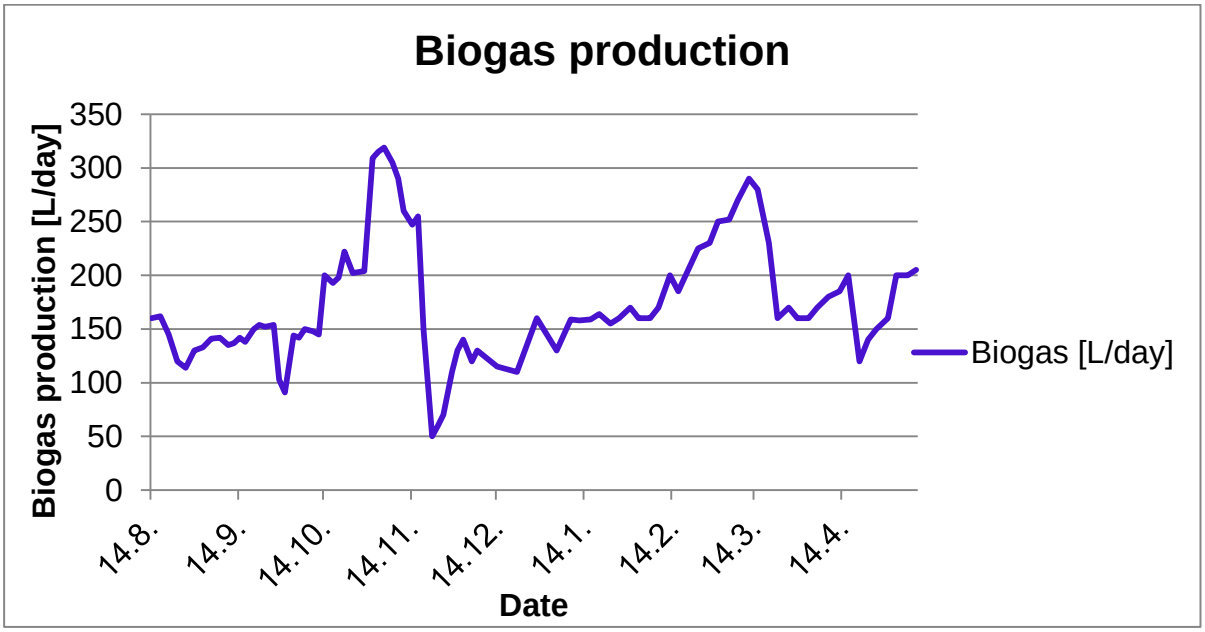

Figure 2.4: Production of biogas in the reactor running on cow manure in the period from August 14th to May 10th.

The manure is first collected in the basement of the barn, flushed down with water. The basement is emptied every 16th – 17th day, and the manure moved to the main storage tank. Approximately every second month, another tank (3 500 L) is filled with manure from the basement and diluted with water. This manure is stored without any heating or isolation, and the decomposition and fermentation will begin. Because of the temperature, the reaction will be less effective during the winter. Batches are taken from this tank and temporarily stored in a small tank (400 L). The liquid phase is fed to the reactor after the solid phase is filtrated out.

The liquid phase is the influent fed to the reactor from the top. Continuous circulation and stirring prevents some of the sedimentation, but most of the granules can be found low in the reactor. The biogas is collected at the top of the reactor, and has a methane concentration between 64 and 78 %

The effluent of the reactor enters a nitrification tank after the AD reactor. The objective of this reactor is to convert ammonia, NH_3, to nitrite, NO_2^-, and nitrate, NO_3^-. Ammonia is for uncertain reasons toxic to most crops and should be removed before the manure is used as fertilizer (Britto and Kronzucker, 2002). All information about the AD reactor is from personal communications with PhD candidate Wenche Bergland, Telemark University College, and the farmer Knut Vasdal.

2.1.2 The lab scale reactor at Telemark University College, based on pig manure

The reactor based on pig manure (also called the pig manure reactor) is a lab scale anaerobic baffled reactor at Telemark University College, operated by master student Fan Yun. A schematic drawing of the reactor setup from September 2011 (Yun, 2011) is presented in Figure 2.5 and a picture taken in October 2011 is shown in Figure 2.6. The reactor was started with continuous operation November 5th 2011, but the experiment started in September with initial operation in 100 mL syringes with 30 mL granules in each. The granules were transferred to the reactor chambers November 5th, a total of 100 mL to each chamber.

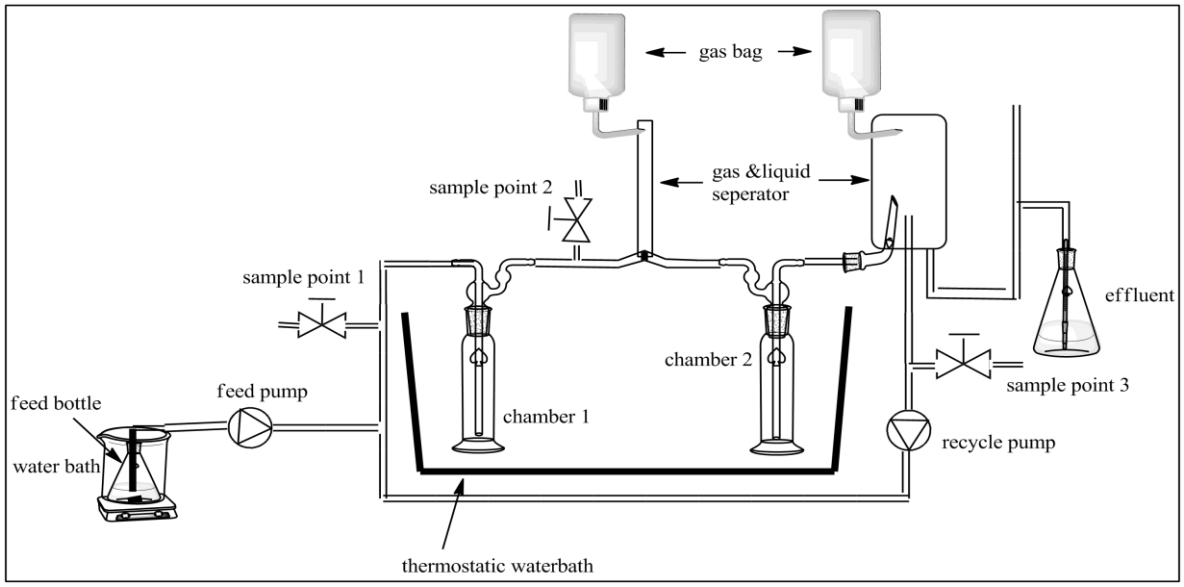

Figure 2.5: Schematic overview of the anaerobic baffle reactor based of pig manure, fall 2011 (Yun, 2011).

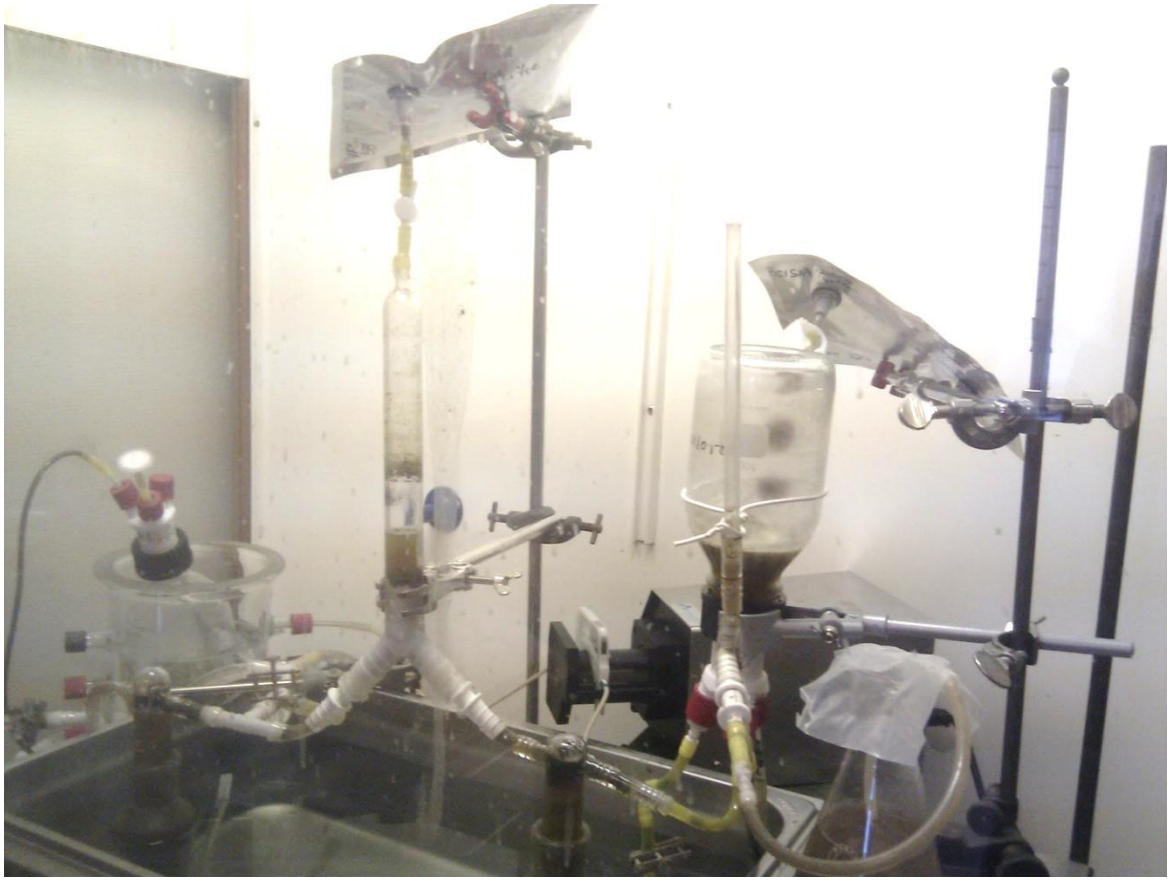

Figure 2.6: A picture of the lab scale reactor based on pig manure, October 2011. It was rebuild in January 2012.

The reactor has two chambers, each with an inner diameter of 5.1 cm and a height of 24 cm. This gives a volume of 350 mL in each. The hydraulic retention time, HRT, and the feed given as organic loading rate, OLR, are respectively decreased and increased, during the operating period from November to April, see Figure 2.7. HRT starts high at 35 days at November 7th to give stable growth conditions for the granules before decreasing rapidly during November to approximately 6 days. It reaches below 1 day around February 25th and ends at approximately 0.5 day at April 15th. OLR increases slowly from 0.6 gram COD/L-day at startup November 7th, to approximately 10 gram COD/L-day on February 7th. The feed is increased more rapidly from February 7th to April 15th up to a level of 52 gram COD/L-day. The reactor operates at 35 °C. The influent is the liquid fraction of stored pig manure, collected from a pig farm outside Porsgrunn.

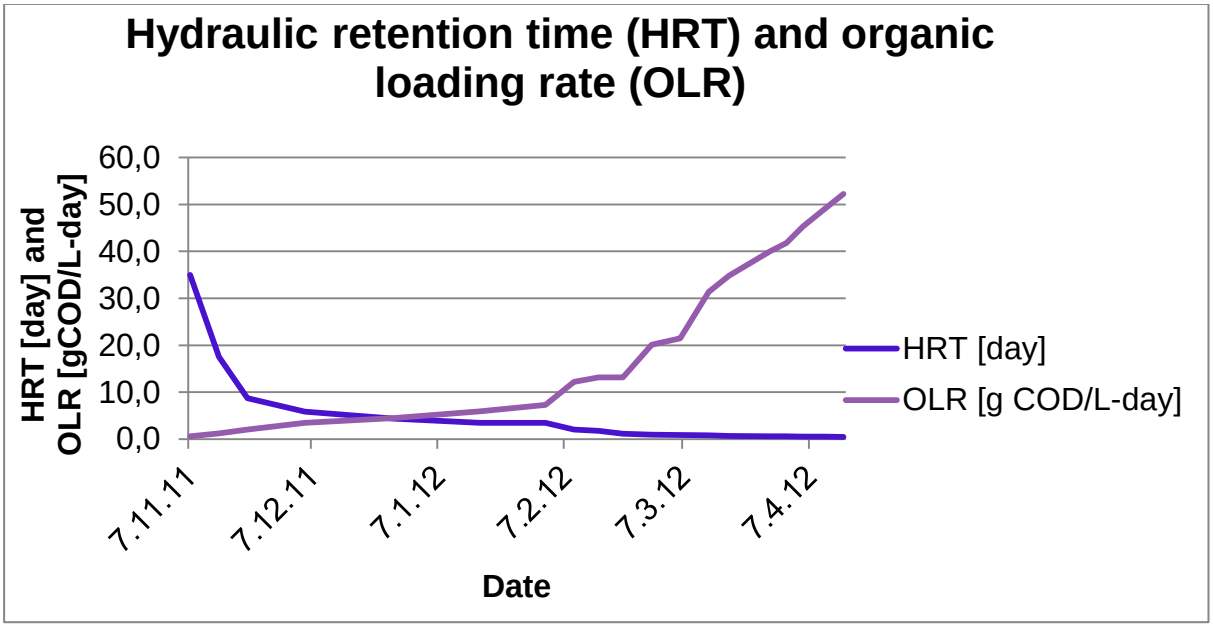

Figure 2.7: Hydraulic retention time (HRT) and organic loading rate (OLR) for the pig manure reactor from November 2011 to April 2012.

The chambers were run in series from November 2011 to February 1st 2012. The total gas production was measured, and is presented in Figure 2.8. The reactor was rebuilt on February 1st, and from February 2nd to April, chamber 1 was run without recirculation. Chamber 2 was run with recirculation from February 2nd to March 24th, and then run without recirculation for the rest of the period. The period from February to April is presented in Figure 2.9. The primary data is presented in Appendix B. The methane concentration was approximately 75 ± 5 %.

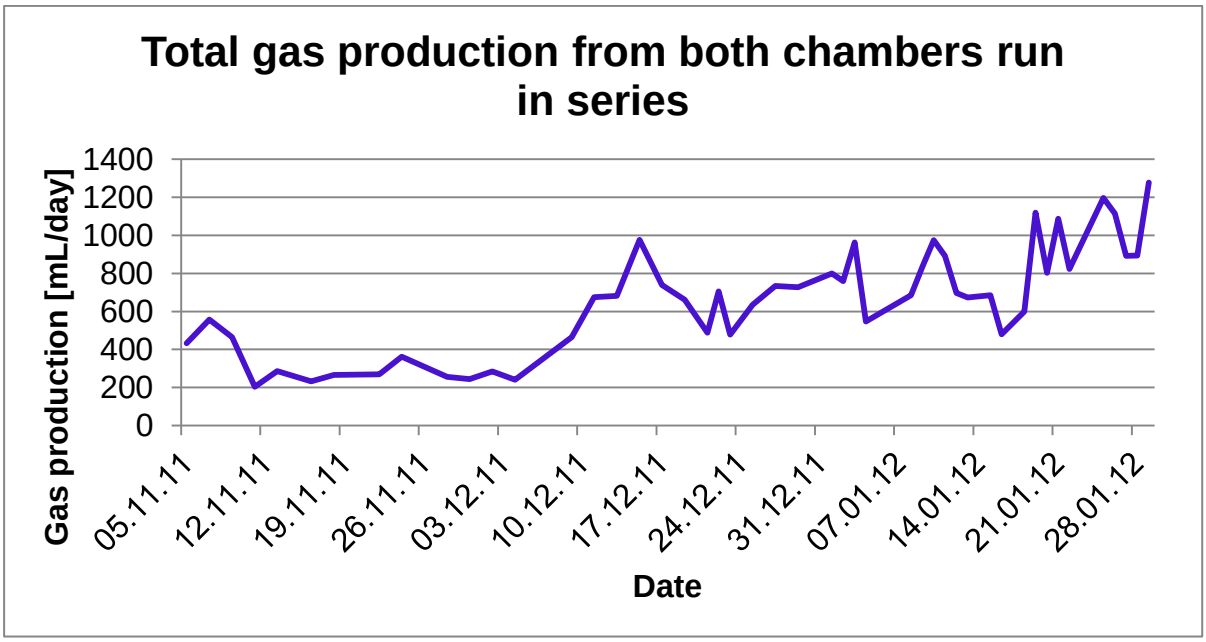

Figure 2.8: Total gas production from the pig manure reactor from November 5th 2011 to January 28th 2012.

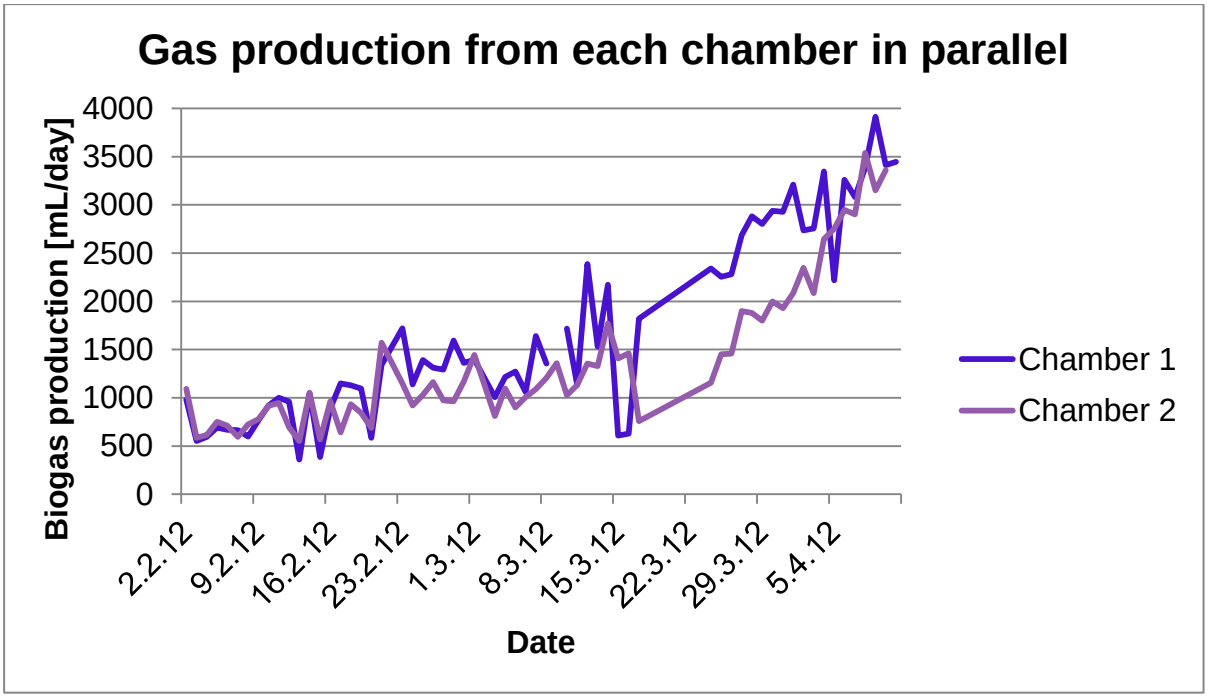

Figure 2.9: Gas production from each pig manure reactor chamber in parallel from February 2nd to April 19th. Chamber 2 was run with recirculation until the 24th of March, and then run without recirculation as chamber 1 for the rest of the period. There was no measurement for chamber 1 on March 10th.

2.2 Samples: Extraction and storage

The samples from the reactor running on cow manure were taken by farmer Knut Vasdal, and the samples from the reactor running on pig manure were taken by master student Fan Yun. The samples were transported by PhD candidate Wenche Bergland to Trondheim by plane, one or two days later, or by mail. The samples and sampling dates are given in Table 2.1

The transportation time from Porsgrunn to Trondheim was about 24 – 48 hours. Upon arrival the samples were transferred to anaerobic bottles and flushed with nitrogen to remove oxygen from the headspace. DNA was extracted as described in section 2.3 for further analysis. The manure samples were stored anaerobically at 4 °C in a refrigerated room.

The main samples received were:

- Raw cow manure before filtration
- Solid cow manure after filtration
- Liquid cow manure after filtration (influent)
- Cow manure samples from the middle of the reactor
- Cow manure samples from the bottom of the reactor
- Cow manure reactor effluent
- Pig manure samples from the pig reactor, effluent and chambers

Raw cow manure before filtration

The diluted manure is stored in an open tank (400 L) right before it is fed to the reactor. The sample is taken from this tank. The consistency is mud-like liquid, with high amounts of dry substance such as grass, hair and other fibres. The sample was called “Cow manure, Raw” (CR).

Solid cow manure after filtration

The manure is filtrated with a vacuum filter, and this sample is taken from the filter cake. It is solid, and looks like fresh manure. The sample was called “Cow manure, Dry” (CD).

Liquid cow manure after filtration, influent

The influent was highly liquid, with only small particles of solid matter. It was called “Cow manure, Influent” (CI).

Samples taken from inside the reactor

The first sample was taken from the middle of the reactor, “Cow manure reactor, Middle” (CM). The sample were liquid, but had more solid matter than the influent. It was suggested that samples taken from the bottom of the reactor might have more microbial activity. Hence the next samples were taken from the bottom and called

“Cow manure reactor, Bottom” (CB). One sample was taken from the reactor top, and named “Cow manure reactor, Top” (CT).

Reactor effluent

The samples taken from the reactor effluent were the most liquid, with very little solid matter, and no large fibres. They were called “Cow manure reactor, Effluent” (CE).

Samples from the pig manure reactor

The first two samples were taken from the syringes used before the reactor was started. They were named “Pig Syringes” (PS). The first sample from the pig manure reactor was taken from the effluent as to not affect the reaction too much. It was called “Pig manure reactor, Effluent” (PE). Later samples were taken from the reactor chambers and named “Pig manure reactor, Granules” (PG) 1 and 2 after the respective reactor chambers. The first PG samples from 27.11 did however contain very little granules to affect the newly started reactor as little as possible.

An overview of the samples are given in Table 2.1.

Table 2.1: Overview of the samples; the dates they were collected and what they contained. The abbreviations are explained in the text.

#	Date	CR	CD	CI	CT	CM	CB	CE	PS	PE	PG1	PG2
1	26.09.11			X		X		X	X			
2	16.10.11	X	X	X			X	X	X			
3	06.11.11	X	X	X			X	X		X		
4	18.11.11 21.11.11						X	 X				
5	27.11.11	X	X	X			X	X			X	X
6	02.02.12		X	X				X			X	X
7	26.02.12	X	X	X	X			X			X	X
8	22.03.12	X	X	X			X	X			X	X
9	17.04.12 19.04.12	X	X	X		X		X			 X	 X
10	15.05.12	X	X	X	X	X	X	X				

2.3 Extraction of DNA by PowerSoil DNA Isolation Kit

DNA was extracted using a commercial kit; *PowerSoil®DNA Isolation Kit* (MO BIO Laboratories, Inc.). The kit consisted of all solutions and tubes necessary. A himac CT15E centrifuge (HITACHI, Japan) was used during extraction.

PowerSoil®DNA Isolation Kit is made for environmental samples containing a high humic acid concentration including difficult soil types such as compost, sediment, and manure.

The manure sample (0.25 g) was added to a bead beating tube that contained a buffer. The buffer helped disperse the manure particles, dissolve humic acids, and protect nucleic acids from degradation. A lysis solution (C1, 60 µL) was added and the sample was homogenized by a Vortex Genie 2 with a MO BIO Vortex Adapter (max speed, 10 minutes). Hence the cell lysis occurred by both mechanical and chemical methods. The sample was centrifuged (10 000 x g, 30 seconds) and the pellet discarded. Solution C2 (250 µL) was added and the sample incubated (4 °C, 5 minutes) before centrifugation (10 000 x g, 1 minute). The pellet was discarded. The last step was repeated with solution C3 (200 µL). Solutions C2 and C3 were inhibitor removal solutions that precipitated humic substances, cell debris and proteins.

A high concentration salt solution (C4, 1200 µL) was added the supernatant to capture the DNA in a silica membrane spin column (spin filter). The sample was filtrated using a centrifuge (10 000 x g, 1 minute). The DNA captured in the filter was washed with an ethanol based solution (C5, 500 µL) and then released from the membrane by a Tris elution buffer (C6, 100 µL). The complete protocol can be found in Appendix C. (MO-BIO)

Every time DNA was extracted from manure samples, a MilliQ-water sample was added as negative control. This was called a blind sample (BL).

2.4 Amplification of DNA by PCR

The extracted DNA was amplified using polymerase chain reaction (PCR). The primers used are described in section 2.4.1. A thermal cycler from VWR was used. In addition to the DNA samples and the blind sample (BL), there was added a negative, non-template control of MilliQ-water for each round of amplification.

The PCR products were tested by agarose gel electrophoresis (1 % wt/vol, with GelRed by Biotium). PCR product (5 µL) were mixed with 6x “loading dye” (1 µL, Fermentas) and loaded onto the gel. GeneRuler 1 kb DNA Ladder (0.5 µg/µL, Fermentas) was used for size comparison. The gel was run with 100 V for approximately 1 hour. The gel was analysed in a G:BOX (SynGene) with the program GeneSnap (SynGene).

2.4.1 The amplification primers

As the result of testing made by Forsberg (2011) the primer set 338f/518r (215bp, Eurofins MWG Operon, Germany) was used. They are unspecific bacteria primers targeting the 16S rDNA sequence and they have been proven effective for use in DGGE. The forward primer was used with a GC-clamp (338f-GC) designed to stop total denaturation of DNA during DGGE. A version of the primer named 338f-GC-M13 was used when the DNA was to be sequenced. It consists of the M13 sequence followed by the GC-clamp and the standard 338f sequence. The primers and GC-clamp sequences are given in Table 2.2.

Table 2.2: The primer 338f, 518r, M13 and GC-clamp sequences.

Primer name	Sequence (5' – 3')	Reference
338f	ACT-CCT-ACG-GGA-GGC-AGC-AG	(Muyzer et al., 1993)
518r	ATT-ACC-GCG-GCT-GCT-GG	
GC-Clamp	CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-G	
M13 rev (-29)	CAG-GAA-ACA-GCT-ATG-ACC	(Eurofins MWG Operon, 2012)

2.4.2 PCR protocol for amplification of DNA

Master mixes were made based on the *Taq*PCR Core Kit (QIAGEN) and mixed with template in PCR tubes. The composition of the master mix for DNA to be used in DGGE (48 µL master mix, 2 µL template) is given in Table 2.3.

In addition to the TaqPCR Core Kit ingredients, Bovine serum albumin (BSA, New England BioLabs Inc.) was added to minimize inhibition. The primer set used was 338f-GC/518r.

The reamplification of DNA for sequencing required different a master mix composition (24 µL, 1 µL template), given in Table 2.4. The primer set used was 338f-GC-M13/518r. The PCR product was purified using the QIAquick PCR purification Kit (QIAGEN) before it was sent to Eurofins MWG Operon (Germany) for sequencing. The protocol is given in Appendix D. The PCR program is given in Table 2.5.

Table 2.3: Composition of the master mix (50 µL per reaction) used in amplification of DNA for DGGE. The table shows the volumes needed per reaction.

Master mix (48 µL/reaction)	µL per reaction
10 x buffer with 15 mM $MgCl_2$	5
dNTP (10 mM)	1
BSA (10 mg/mL)	2
Forward primer (10 µM)	1,5
Reverse primer (10 µM)	1,5
Taq polymerase	0,25
Sterile water	37

Table 2.4: Master mix (25 µL per reaction) composition used for PCR when DNA was reamplified for sequencing. The table shows the volume needed per reaction.

Master mix	µL per reaction
10 x buffer with 15 mM $MgCl_2$	2,5
dNTP (10 mM)	0,5
$MgCl_2$ (25 mM)	0,5
Forward primer (10 µM)	0,75
Reverse primer (10 µM)	0,75
Taq polymerase	0,125
Sterile water	20

Table 2.5: PCR program for amplification with the primer set 338f-GC/518r.

Temp. [°C]	Time	Comment	Note
95	3 min	Denaturation	
95	1 min[1]	Denaturation	Repeated 30 times
53	30 sec	Annealing (Primer binding)	
72	1 min	Elongation (DNA synthesis)	
72	30 min	Elongation (DNA synthesis)	
4	10 min	Process stop	
15	∞	Storage	

2.5 Fingerprinting by DGGE

Denaturing gradient gel electrophoresis (DGGE) was used to compare and analyse the microbial communities in the reactors. The equipment was from Ingeny (Ingeny, 2011), and is illustrated in Figure 2.10

The DGGE gels are made from 8 % polyacrylamide with a gradient of urea and formamide. The gel solutions of 0 and 80 % (see Table 2.6 for compositions) were made few days in advance, and mixed accordingly to Table 2.7 when the gels were cast. Tetrametylendiamin (TEMED) and ammonium persulfate (APS, 10 %) were added directly prior to gel casting. The finished gel had the lowest denaturing concentration on the top, and the highest in the bottom. The complete protocol is in Appendix E.

[1] The denaturation time for reamplification of DNA for sequencing was 30 seconds.

Table 2.6: Composition of the 0 and 80 % denaturing polyacrylamide solutions made prior to the experiment.

Composition	0 %	80 %
40 % Acrylamide (BioRadLab)	50 mL	50 mL
50 x TAE buffer	2,5 mL	2,5 mL
Urea		84 g
Deionized formamide		80 mL
Distilled water	Up to 250 mL	Up to 250 mL

Table 2.7: Composition of the denaturing gel solutions used to cast the gel.

	0 % (mL)	80 % (mL)	TEMED (µL)	APS (µL)
0 %	8		10	40
25 %	16,5	7,5	16	87
30 %	15	9		
40 %	12	12		
45 %	10,5	13,5		
50 %	9	15		
55 %	7,5	16,5		
60 %	6	18		

PCR product (5 - 15 µL) was loaded together with 6x “loading dye” (2 - 4 µL). The gel was run in 1 x TAE with 100 V for 17 – 18 hours at 60 °C. The gels were stained with

SYBR Gold (3 µL SYBR Gold, 30 mL MilliQ water, 600 µL 50 x TAE) for 1 – 2 hours before pictures were taken in a G:BOX and analysed.

If the bands were to be sequenced, they were excised using sterile pipette tips and transferred to MilliQ water (30 µL). They were then frozen overnight, reamplified by PCR and purified by *QIAquick PCR purification Kit* (QIAGEN), as described in section 2.4.2. The purified DNA was sent for sequencing at Eurofins MWG Operon (Germany).

Full protocol for DGGE can be found in Appendix E.

Figure 2.10: DGGE equipment from Ingeny (Ingeny, 2011).

3 Results

3.1 DGGE analysis of the cow manure reactor

DNA samples obtained from the inside of the reactor running on cow manure were used to visualize changes in the microbial community on a DGGE gel. The samples used were from the start up in September until May, and taken from the top (CT), middle (CM), and bottom (CB) of the reactor. The total DNA extracted from the reactor samples was amplified using the primer set 338f-GC/518r and the protocol described in section 2.4.2 before use in DGGE. The DGGE gel was cast as described in section 2.5 with a 25 - 60 % denaturing gradient, see Figure 3.1.

The CM sample from November 26th was loaded into the well in insufficient amounts due to an error and was therefore significantly weaker than the other bands. When the gel was overexposed to UV light (not shown here), it was clear that the main bands of the November 26th sample are similar to the main bands of the October 16th CB sample. The October CB sample had slightly less PCR product than the other fall samples when tested on an agarose gel, and was therefore loaded in higher amounts (15 µL) than the other fall samples (10 µL) to compensate.

The band patterns on the gel in Figure 3.1 change between November 27th and February 26th. The samples from September to November have weaker bands than the samples from February to May. They also have fewer clear bands.

The band no. 1, 2, 6, and 7, marked in green in Figure 3.1, appear or become stronger after February 27th. The strengthening of these bands is significant even if the general increase in PCR product quality is adjusted for. The strength of band no. 5 is relatively stable through all the samples and can be used as comparison.

Band no. 4 grows weaker with each sample from October 16th to November 27th and it is not present from February to May. Band no. 3 may seem to increase in strength through the whole period from October 16th to May 15th and the change is especially clear from November 6th to November 18th.

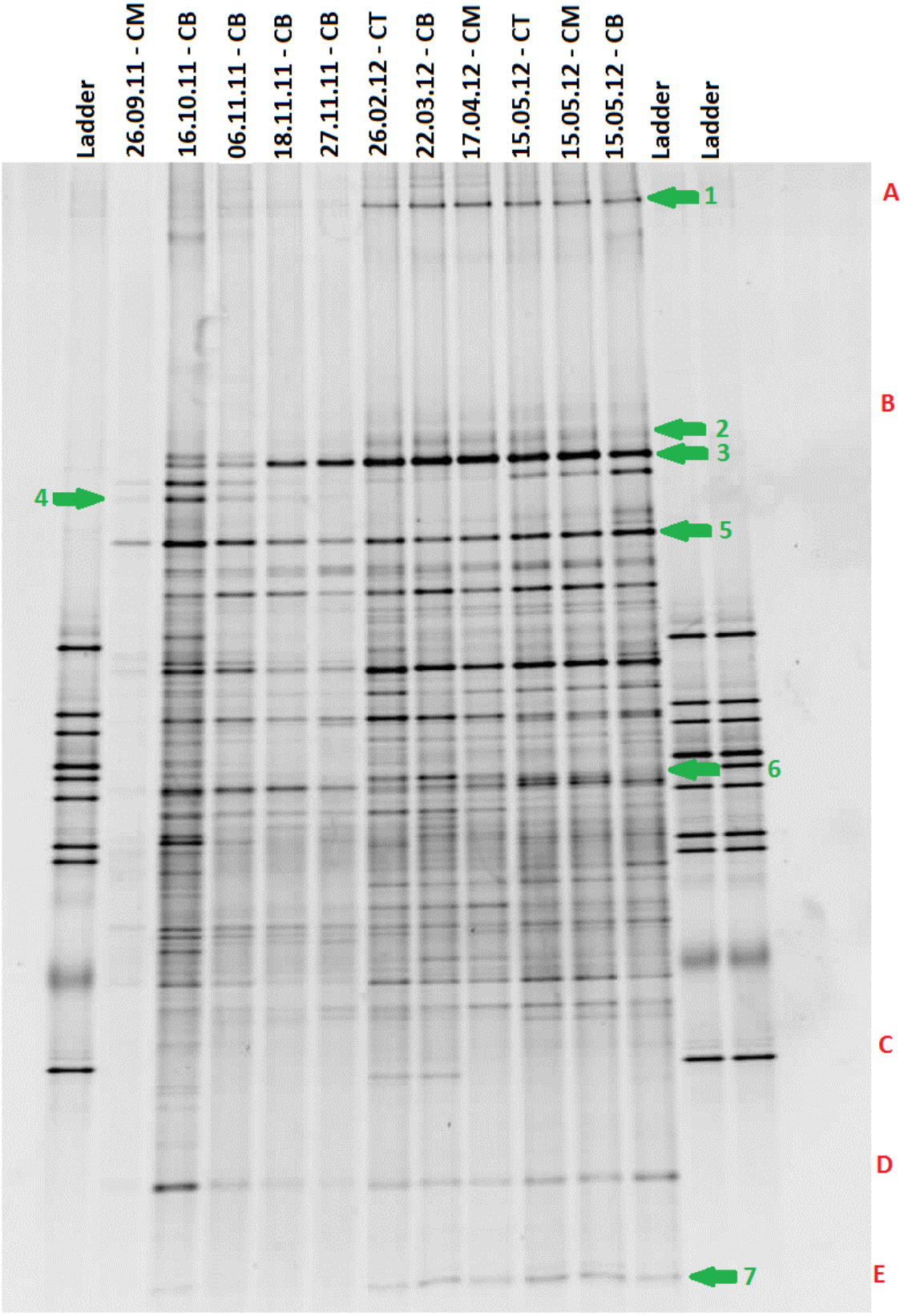

Figure 3.1: DGGE gel showing PCR products amplified with the primer set 338f-GC/518r from the total DNA isolated from samples extracted from the reactor

running on cow manure from September to May. The bands were seperated on a 8 % polyacrylamide gel with a denaturing gradient of 25 - 60 %. The sample are abbreviated Cow manure reactor Top (CT), Middle (CM) and Bottom (CB). Bands and areas of interest are marked with green and red labels.

3.2 DGGE analysis of the pig manure reactor

A DGGE gel was made presenting the changes in the bacterial community from September 2011 to April 2012 in the pig manure reactor. Samples from both chambers were used when available. The total DNA extracted from the samples were amplified using the primer set 338f-GC/518r and protocol described in section 2.4.2, and the DGGE gel was cast as described in section 2.5 with a 25 - 60 % gradient.

The gel is presented in Figure 3.2, and is curved due to an irregularity during casting.

The most prominent change in band pattern is the turning point between November 27th and February 2nd. Several bands visible in the samples from fall 2011 disappear and new bands appear. Four examples are marked with green boxes in Figure 3.2.

The DNA samples extracted from chamber 1 run without recirculation, can be compared with the DNA samples from chamber 2 run with recirculation. There is no significant difference, or change, except in the samples from March 22nd. The bands in the sample from chamber 1 are generally equal or stronger than the bands in the sample from chamber 2. The exceptions are the 6 bands marked in yellow in Figure 3.2. These bands are stronger in the sample from chamber 2 than chamber 1, and they are also generally stronger than the other corresponding spring sample bonds. In the yellow box no. 4, underneath the strong bond in the DNA sample from chamber 2, there is also a bond that is very weak compared to the strong bond in chamber 1 and the other spring samples.

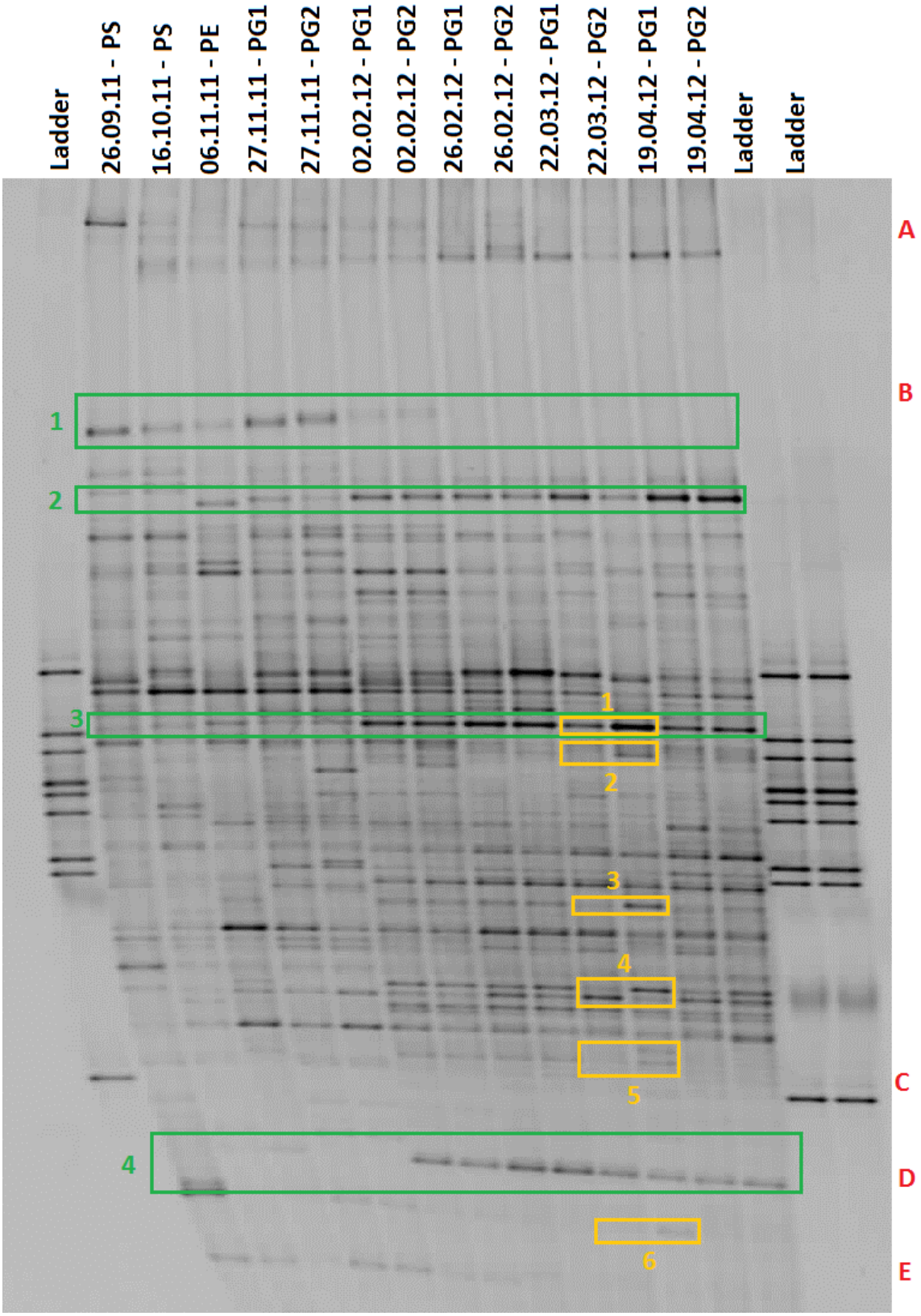

Figure 3.2: DGGE gel showing PCR products amplified with the primer set 338f-GC/518r from the total DNA isolated from samples extracted from the reactor

running on pig manure in the period September to April. The bands were seperated on a 8 % polyacrylamide gel with a denaturing gradient of 25 - 60 %. The sample types are pig manure reactor effluent (PE) and pig manure reactor granules, chamber 1 and 2 (PG1 and PG2). The green boxes show four examples of important changes in the microbial community.

3.3 Sequencing of excised DGGE bands

DGGE gels with DNA samples extracted from both reactors were used in an attempt to develop a method where important DNA bands can be excised, reamplified and purified (see section 2.5) with a high enough quality for sequencing by Eurofins MWG Operon (Germany). The DNA samples were extracted and amplified with the primer set 338f-GC/518r and protocol described in section 2.4.2. The DGGE gels were cast as described in section 2.5

The first gel, Gel A, was cast with a 30 - 55 % gradient, see Figure 3.3 There were several strong bands, and 60 bands from all parts Gel A were excised to be sequenced. The sequence attempt failed for all bands. The sequence analysis of the band marked in red in Figure 3.3 is presented in Figure 3.4.

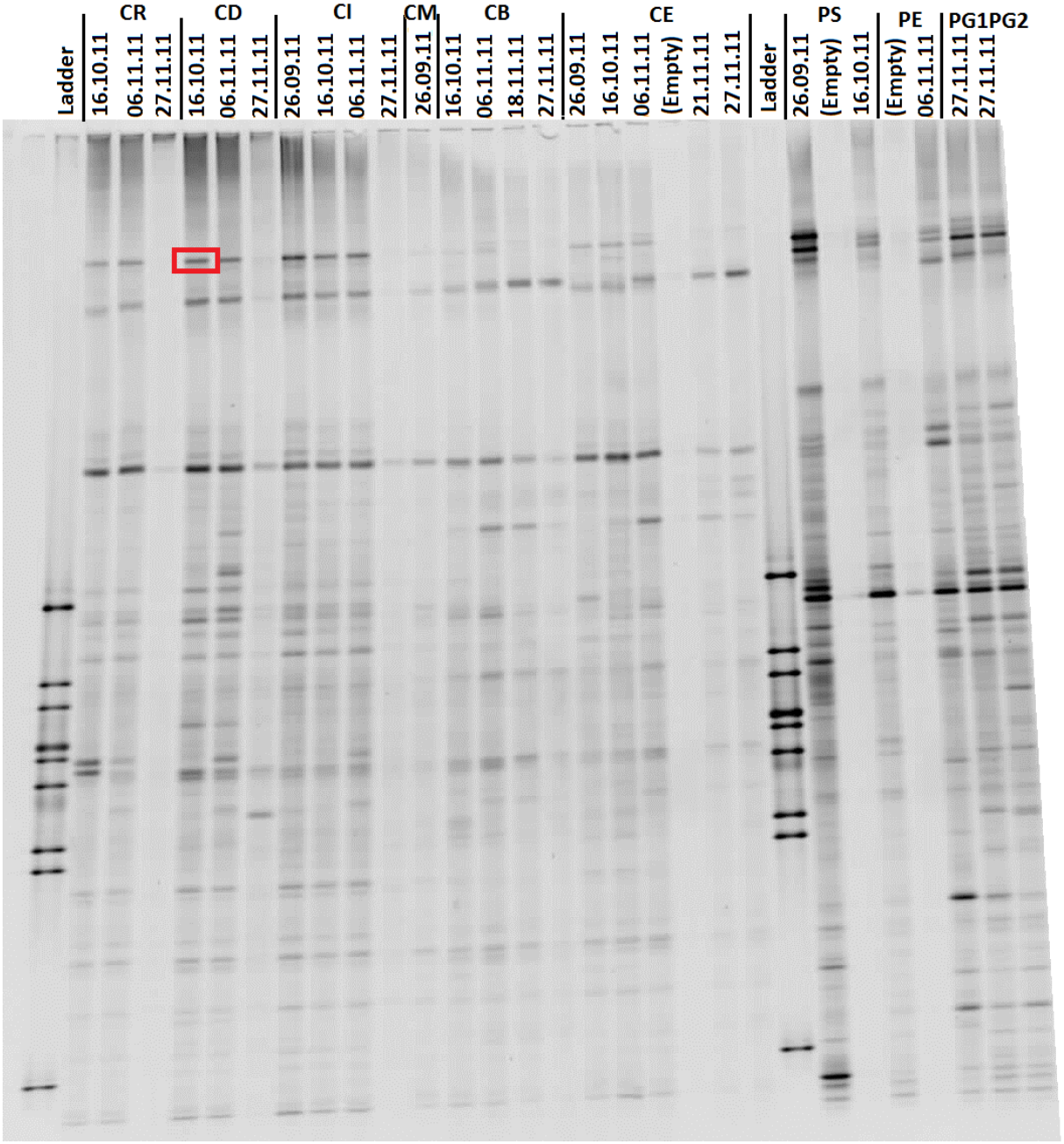

Figure 3.3: Gel A: An 8 % polyacryladmide DGGE gel with a gradient of 30-55 %, where 60 bands including the one marked in red were excised, reamplified and attempted sequenced but failed. The DNA samples were extracted from samples from both reactors from September to November and amplified with the primer set 338f-GC/518r.

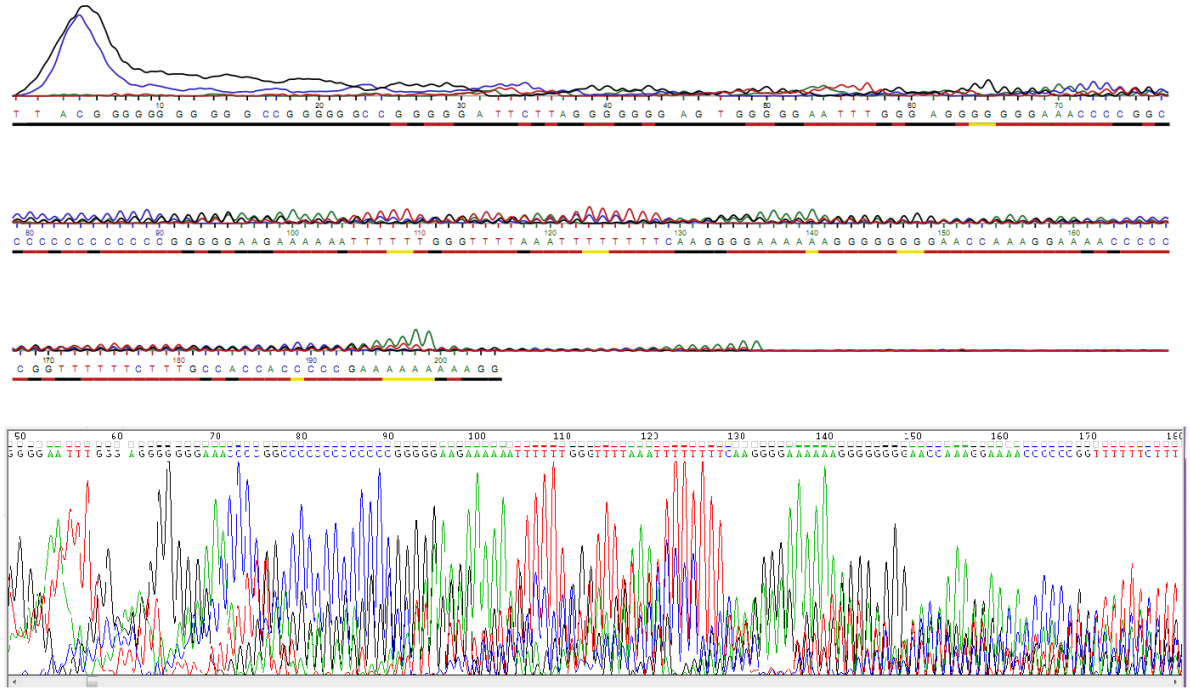

Figure 3.4: The sequence analysis of the band marked in red in Gel A, in Figure 3.3.

The sequence signal presented in the top of Figure 3.4 is of poor quality, and the signal strength is low. By focusing on a part of the sequence (base 50 to 180) in the computer program Chromas (Technelysium Pty Ltd, Australia), it was obvious that the result consists of more than one DNA sequence; see lower part of Figure 3.4. Every peak represents a base signal, and the different bases are represented by colours. Most bases have two or more peaks in different colours, and improbable large sections of the sequence are homologous.

A new DGGE gel, Gel B, with a smaller gradient of 30 - 45 % was made to increase band separation, Figure 3.5. The same DNA samples as in Gel A, Figure 3.3, were used. The separation was increased successfully, easily visible by comparing the area marked in blue in Gel B, Figure 3.5, with the same area in Gel A, Figure 3.3. The number of bands was increased in Gel B. What appeared to be 3 bands in Pig Syringe (PS) from September 26th in Gel A were at least 5 bands Gel B.

The red boxes on Gel B, Figure 3.5, are 16 bands that were excised and sequenced. The sequence result was “failed”, and the most likely reason was still insufficient separation.

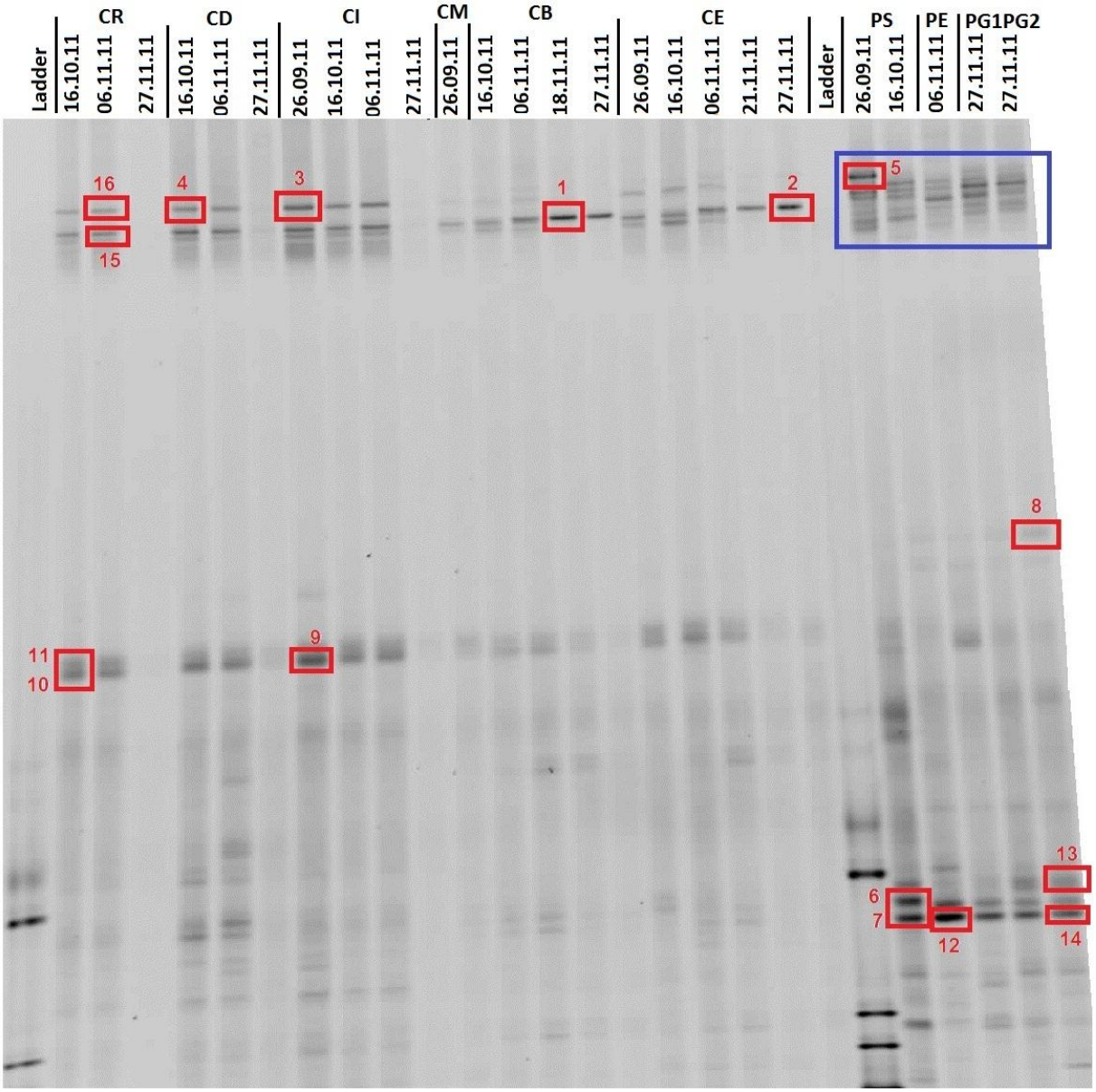

Figure 3.5: Gel B: DGGE gel of 8 % polyacryladmide with a gradient of 30 – 45 %. The 16 bands indicated in red were excised and attempted sequenced but with negative results. The DNA samples applied were extracted from samples from both reactors from September to November and amplified with the primer set 338f-GC/518r.

The 16 excised gel samples from Gel B in Figure 3.5 were frozen in 30 µL MQ-water over night, as described in section 2.5. The eluted DNA was reamplified and applied on a 30 - 40 % gradient DGGE gel, Gel C, shown in Figure 3.6. Each band from Gel B, Figure 3.5, must have contained several different DNA sequences since several additional bands appeared in Gel C, Figure 3.6.

Eighteen bands were excised from Gel C, Figure 3.6. The DNA was reamplified and applied to a 33 - 40 % gel, Gel D, presented in Figure 3.7.

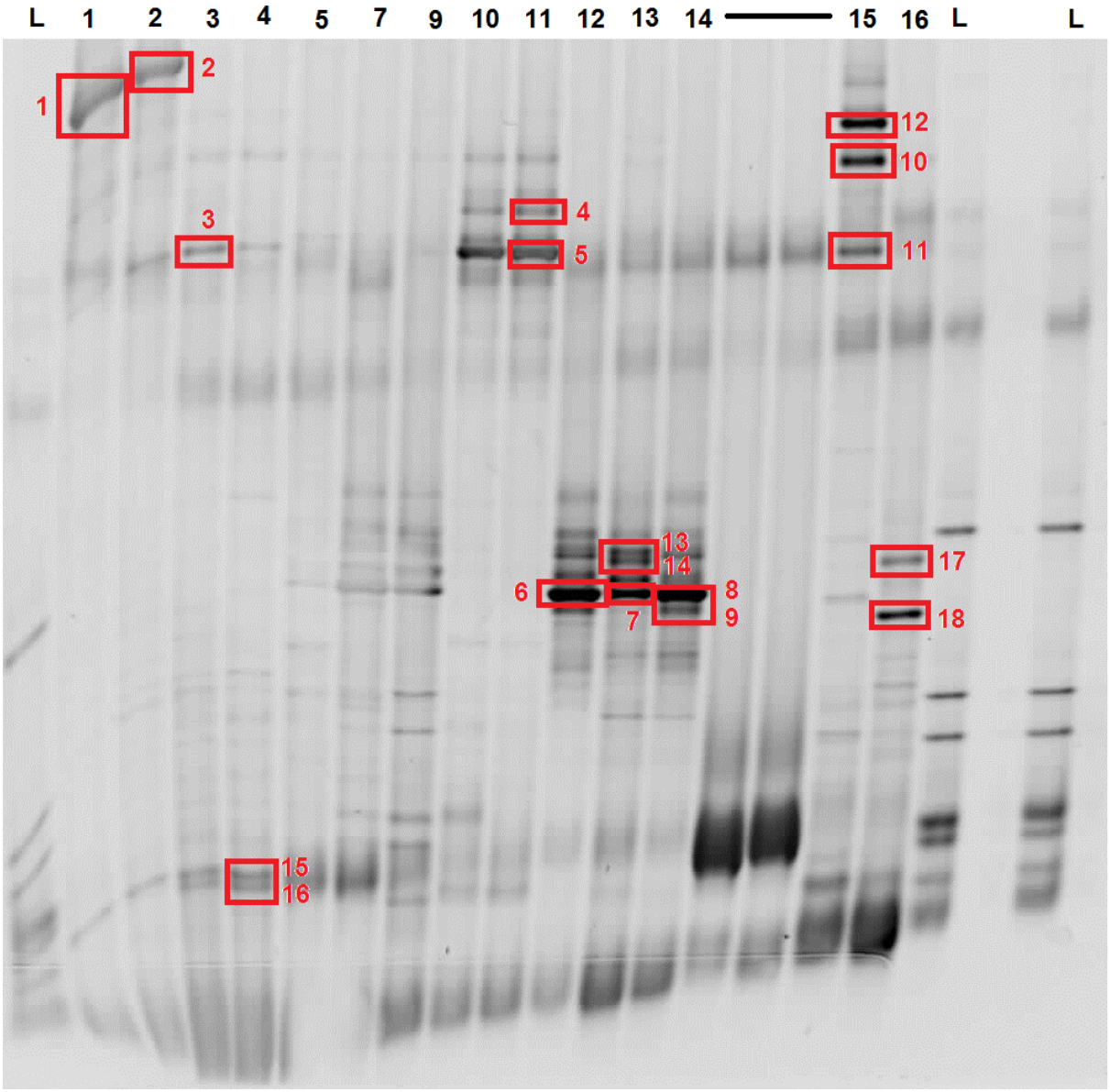

Figure 3.6: Gel C: DGGE gel of 8 % acrylamide with a 30 – 40 % gradient applied with the reamplified DNA products from the 16 bands originating from

Gel B in Figure 3.5. 18 new bands marked in red were excised. The “L” stands for ladder.

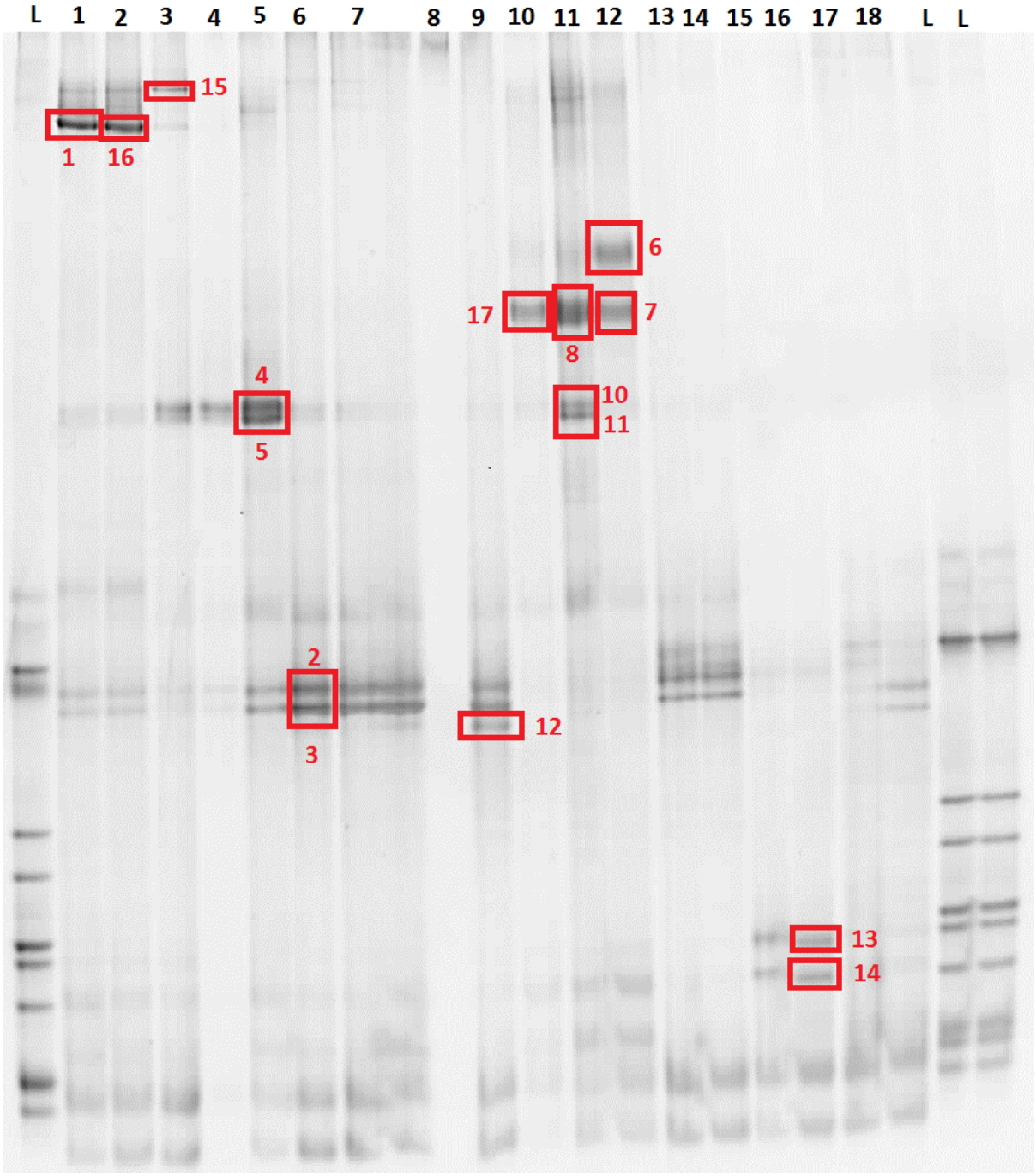

Figure 3.7: Gel D: DGGE gel presenting the 18 excised bands originating from Gel C in Figure 3.6, reamplified and reapplied to an 8 % polyacrylamide DGGE gel with a 33 - 40 % gradient. The new set of 17 bands marked in red was

sequenced successfully but with too poor result for further use. The “L” stands for ladder.

The sequencing of the final 17 bands from Gel D in Figure 3.7 was successful, but the results were of too poor quality for any specie determination or other phylogenetic analysis. Only short sections were of high quality, and they are mostly homologous. Band no. 7 was the longest with 20 accepted base pairs from base pair 81-101. This is the area shaded in white in the top part of Figure 3.8. The chromatogram visualized by the software Chromas (Technelysium Pty Ltd, Australia) is presented in the lower part of Figure 3.8.

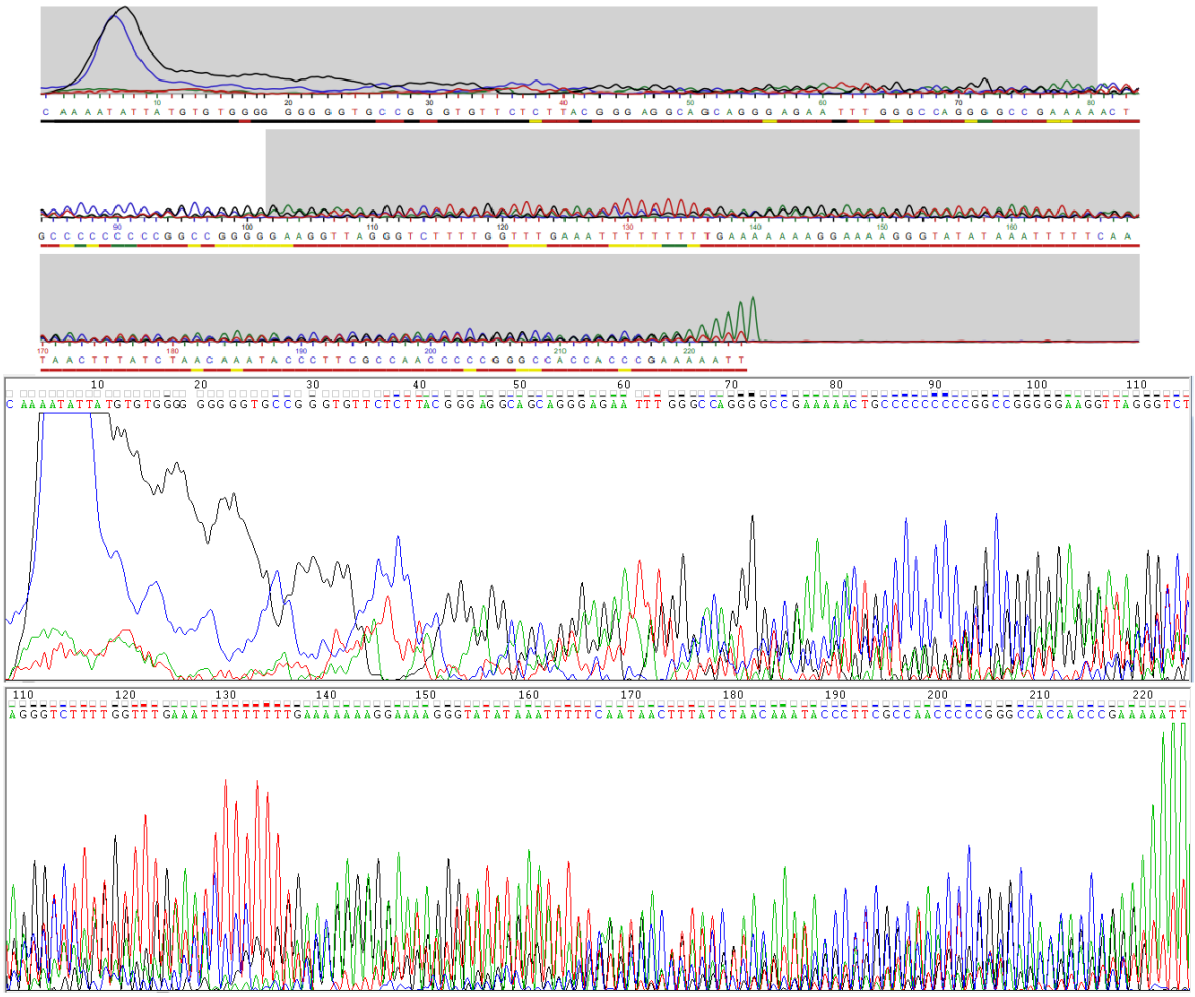

Figure 3.8: The sequencing result of band no. 7 from Figure 3.7. The result was of too poor quality for any further analysis.

4 Discussion

Two anaerobic biogas reactors were followed from September 2011 to May 2012; a pilot scale reactor situated at Foss farm, outside of Porsgrunn, running on cow manure, and a lab scale reactor situated at Telemark University College, running on pig manure. Samples from both reactors were extracted approximately once a month. The total DNA was extracted from the manure samples, amplified by PCR and analysed by DGGE. An assortment of DGGE bands were excised and sequenced.

4.1 DGGE gel analysis

4.1.1 Reactor based on cow manure

The DNA samples extracted from the reactor running on cow manure from September to May were used to analyse changes in the microbial community composition of the reactor and compare them with operating conditions and gas production. The samples were extracted from the reactor top (CT), middle (CM) and bottom (CB). The DNA was extracted as described in section 2.3 and amplified using the primer set 338f-GC/518r and protocol described in section 2.4.2. The amplified DNA samples were applied to an 8 % polyacrylamide DGGE gel with a gradient of 25 – 60 %, see section 2.5. The gel is presented in Figure 3.1.

Changes in the microbial community from September to April

The bands representing the DNA samples from February to May 2012 is generally stronger than the DNA samples from September to November 2011. This may be explained by degeneration in the DNA sample during storage and analysis. The 2011 samples have been frozen and thawed significantly more than the 2012 samples. This may have deteriorated their quality and hence their PCR product and band strength are poorer. Some other changes are visible, and these might be explained by changes in operating conditions.

Changes in the microbial community with respect to operating conditions and biogas production

The feed batches were changed on September 12th, November 7th, January 30th, and March 29th. This gives only 2 – 3 samples per batch, and therefore it is almost impossible to indicate any connection between changes in manure composition following a batch change and the microbial community. Individual differences between samples may just as likely be caused by other operating conditions like feed rate and temperature which varies in the same period, than changes in feed batch.

The most significant example of change in operating conditions occurred between the samples from the 6th and 18th of November. From the 7th to the 16th of November there was a probable washout of biomass caused a problem with the feed rates. The feed rate was lowered to 10 L/day as a response, and in the same time period the biogas production decreased from over 250 L/day to 50 L/day. This is an important and dramatic event, but the samples extracted before and after the event show only minor differences in band strength. These differences are around the band marked as no. 3 in Figure 3.1, and the band patterns are otherwise similar. Band no. 3 increased in strength, but the bands right above and below decreased in strength. Band no. 3 can be a slow growing bacteria either just increasing in amount, or responding to the temperature increase from 24 °C to 35 °C in the middle of October. The general band strength in the DNA sample from November 18th was not weaker than normal, and there were therefore probably no significant consequence of the possible washout.

There is a clear correlation between feed rates, presented in Figure 2.2, and biogas production, presented in Figure 2.4. An increase in feed rate caused an increase in gas production rates. This correlation cannot be found directly reflected by any band in the DGGE gel in Figure 3.1.

The reactor was changed on April 19th. The reactor contents were transferred and the reactor design was not altered. There is no evident difference between the DNA sample from April 17th and May 15th, and the change of reactor did therefore probably not affect the microbial composition for more than a short period of time.

The DNA from the three samples extracted from different parts of the reactor on May 15th appears similar on the DGGE gel presented in Figure 3.1 and the location of the extraction may hence be without importance. This conclusion is supported by Malin (Malin and Illmer, 2008) in a similar experiment, where no visible pattern difference was found between the inlet and outlet samples of an anaerobic biowaste fermenter.

The DGGE gel in Figure 3.1 does not reflect any of the changes in operating conditions mentioned above. This may indicate that only large and long-term adjustments in the reactor are visible by DGGE analysis with this sampling frequency and conditions.

4.1.2 Reactor based on pig manure

The DNA samples extracted from the reactor running on pig manure from September to April were used to analyse changes in the reactor's microbial community and compare them with operating conditions and gas production. The samples from September and October were taken from the initial syringes and the later samples from the reactor effluent (PE) and chambers (PG). The DNA was amplified using the primer set 338f-GC/518r and protocol described in section 2.4.2. The DNA samples were applied to an 8 % polyacrylamide DGGE gel with a gradient of 25 – 60 % cast as described in section 2.5. The gel is presented in Figure 3.2.

Changes in the microbial community from September to April

The DGGE presenting the samples from September to April clearly shows a general increase in both band strength and number through the period. There is also a distinct change between November 27th and February 2nd. Several bands appear or disappear as illustrated by the green boxes in Figure 3.2. The sample extraction method is the main change possibly explaining this. The granules were not present in the manure samples in significant amounts before the February 2nd samples, see section 2.2. Granules permit a more complex microbial composition with a stable surface, variation in living conditions and long retention time compared to pure liquid.

The liquid effluent will hence reflect mainly microbes in the transitory liquid and not the microbes on the retained granules. This will again be reflected in the total DNA extracted and used in analysis. The bands appearing or increasing in strength after February 1st are hence most likely bacteria growing on the granules. Some changes between the effluent sample from November 6th and the chamber samples from November 27th are also likely due to the small granule amounts present in the chamber samples.

Changes in the microbial community with respect to operating conditions and biogas production

The hydraulic retention time, HRT, and the organic loading rate, OLR, are respectively decreased and increased during the operating period from November to April, see Figure 2.7. The difference in the November samples with respect to granule content makes it impossible to examine the possible effect of rapidly decreasing HRT on the microbe community.

The rapidly increased OLR from February 7th to April 15th corresponds with the increase in total gas production in the reactor. The total gas production from both chambers operated in series starts at 300 mL/day on December 9th and increase to 1200 mL/day on January 30th, according to Figure 2.8. There are unfortunately no samples from this period to compare with. The total gas production from both chambers is approximately 1200 mL/day February 7th and 7000 mL/day April 15th. There is no corresponding change in the DGGE gel result in Figure 3.2, except from a possible increase in band strength in the green box no. 2. There is no general change in band number or strength, but microbial activity does not necessary correspond with changes in biomass visible by DGGE.

From February 2nd to March 24th chamber 2 was run with recirculation while chamber 1 was run without. The only significant difference in the chamber profiles was in the DNA samples from March 22nd. Chamber 1 has generally equal or stronger bands than chamber 2, except for the 6 bands marked in yellow in Figure 3.2. These bands may be bacteria utilizing more recalcitrant substrates which will be in relatively higher

amounts in a system with recirculation than without. In the yellow box no. 4 there is also a weaker band compared to both the sample from chamber 1 and the other spring samples. This may be a bacterium utilizing easily available substrates like volatile fatty acids (VFA) which will be in relatively low amounts in a system with recirculation.

4.1.3 Comparison of the DGGE gels from the two reactors

The DGGE gel in Figure 3.1 presents the microbial community in the reactor based on cow manure from September 2011 to May 2012. The gel in Figure 3.2 presents the reactor running on pig manure from September 2011 to April 2012.

Both reactors have the same approximate number of 22 – 28 distinct bands per DNA sample. They also have a similar profile of band clusters with respect to the gradient. A few bands lie in the area on the top of the gels marked with a red A on both figures. The main clusters are between the red B and C marks in the middle of the gels. A few bands are located lower on the gels, below the lowest ladder marker, marked with the red letters D and E. There are areas without band on both gels between the red letters A and B. These similarities in profiles may indicate similarities in the microbial communities. Especially the bands around the red letters A, D, and E are potentially the same bacteria.

It should be noted that the bands marked with the red E in both figures are shoving different development in strength. The band seems to increase in strength in the samples from the reactor based on cow manure, while it is decreasing the samples from the pig manure reactor. This is both interesting and remarkable if the bands are based on the same bacteria.

4.2 Possible weaknesses with the PCR-DGGE technique

4.2.1 PCR-DGGE analysis

Every step in the process of PCR-DGGE analysis has possible weaknesses that may affect the DGGE analysis result. The sample handling procedures must be consistent and adapted to avoid loss or change of microbial diversity, and the DNA extraction must equally favor all organisms to avoid insufficient and preferential disruption of cells. These parameters are believed to be of an acceptable standard after the testing made by Forsberg (2011). The manure samples have been tested and found stable through two months of anaerobic storage at 4 °C, and the *PowerSoil®DNA Isolation Kit* (MO BIO Laboratories, Inc.) is the best of three kits tested.

DNA amplification by PCR may have several weaknesses when applied to environmental samples. Co-extracted contaminants, like humic acids and other humic substances, inhibit DNA modifying enzymes including *Taq* polymerase (Wintzingerode et al., 1997). The contamination is reduced to by the cleaning procedures during DNA extraction and the inhibiting effect is further reduced by adding BSA to the PCR master mix, see section 2.3 and 2.4.2.

Differential amplification can be caused by a variety of factors and should be considered a possible source of error when comparing DNA quantities. All molecules must be equally accessible to primer hybridization, form primer-template hybrids at equal rate and have the same polymerase extension efficiency to avoid uneven amplification rates. This is unrealistic for universal bacteria primers like 338f and 518r with natural variances in affinity with respect to different 16S DNA sequences. An article comparing primer coverage rates (Wang and Qian, 2009) shows that predicted primers covering the bases 338 – 358 (relative to the position in *Escherichia coli*) have an average coverage rate of 97.3 %. The 338f primer used in this experiment is one base shorter than the predicted primer, but has the same sequence covering base 338 – 357. It will therefore probably have a similar coverage rate as the predicted primer. The coverage rate for a known primer covering the bases 334 – 356 is 74.2 %. The 338 – 356 sequence is identical to the 338 primer used here.

DNA sequences with a high GC content is suspected to dissociate with lower efficiency leading to a preferential strand separation of genes with lower GC content (Wintzingerode et al., 1997). It is also been indicated a template threshold of approximately 1 % of the total DNA (Muyzer et al., 1993) making small communities underrepresented in the PCR product even if the total microbe count in the sample is substantial. These are constant biases and should hence not affect comparison between similar samples.

Some differential in DNA amplification of environmental samples are dependent on the choice of primers and number of cycles of replication (Wintzingerode et al., 1997). This is because reannealing of gene products progressively inhibits the formation of template-primer hybrids when primers with high amplification efficiency are used. This may make PCR biases non constant, but in this experiment the same primer and number of replication cycles were used on all DNA samples. The inhibition is likely reduced if the sample is highly diverse since amplification of any gene will less likely produce amplicons in an inhibiting concentration. If non-universal primers were applied the template diversity would decrease significantly and the biases may increase (Wintzingerode et al., 1997).

PCR products can be contaminated by artificial DNA sequences like chimeras made from two DNA sequences with high similarity (Ferris and Ward, 1997), but this will due to small amounts not significantly interfere with DGGE analysis of complex communities (Murray et al., 1996). The PCR product may also be contaminated by alien DNA since universally conserved regions of bacterial genes serve as target sequence. The most likely source of additional bands is still variations in the ribosomal RNA operon copy number and variations in the 16S sequence in each operon (Crosby and Criddle, 2003). The variation in operon copy number will affect the amount of 16S DNA and thus the strength of the DGGE band representing the organism. Each operon may also have distinct 16S sequences, presented as different bands on a DGGE gel. This can make microbial quantity estimation difficult, but comparison of samples is still possible since the biases are constant.

DGGE analysis requires a GC-clamp attached to the primer during PCR. The GC-clamp may cause incomplete strand synthesis leading to multiple and unclear bands

for one template. Dissimilar sequences with similar GC content may co-migrate to the same position in the gel gradient causing bands to be a mixture of more than one sequence. This may interfere with microbe diversity estimations and may be a source of error for DNA sequencing. (Nübel et al., 1996)

4.2.2 Sources of error when sequencing DNA excised from DGGE

Sequencing of DNA excised from a DGGE gel can be difficult due to several possible sources of error. The main source is incomplete separation of strands or contamination of bands. This is clearly the case in Gel C, Figure 3.6, where each PCR product applied is from an excised band from Gel B, Figure 3.5. The PCR products obviously consist of more than one DNA sequence, as Gel C shows several distinct bands for each PCR product. A DGGE band may consist of DNA from more than one sequence due to co-migration of sequences of similar GC content or by incomplete strand synthesis caused by the GC-clamp. The bands can also be contaminated by general traces of the total DNA. The latter is likely since the bands in Gel C, Figure 3.6, are located in all denaturing gradients and not only in the area where the original DNA samples was excised. This is a different result than seen in the master thesis by Røstad Nordgård (2010) were all the different reamplified DNA samples from excised bands became positioned on a horizontal line in the new gel.

The incomplete separation might also be caused by the sheer number of bands produced by a universal primer like 338f/518r. The multitude of bands may prove difficult to adequately separate within the physical limits of a DGGE gel since even the 7 % gradient of gel D in Figure 3.7 was insufficient.

The sequence results presented in Figure 3.4 and Figure 3.8 shows weak signals of poor quality. The weak signal might be caused by low DNA concentrations, but the sample concentrations were tested by NanoDrop measurements and were in the region recommended by Eurofins.

Røstad Nordgård excised 16 DGGE bands and 4 of them were successfully sequenced. They were analyzed and tentatively identified (Røstad, 2010). Røstad

Nordgård used a gene specific primer for methanogenic archaea. This specific primer would have produced fewer bands than the universal 338f/518r primer set, and hence give more separated and distinct bands when applied to a DGGE gel. This would increase the success rate for DNA sequencing and may explain the positive results compared to the negative results in this experiment.

4.3 DGGE as a method for supervising anaerobic fermenters

DGGE is with respect to this experiment's results capable of reflecting community shifts and the gels neatly illustrate the microbial community composition. It is a highly reproducible and consistently performing fingerprinting technique and even the biases are of a constant nature. Band pattern changes do hence reflect actual variations in microbial community composition. However, the band pattern variations is this experiment could not with be directly correlated to either operating conditions or gas production. An increase in sampling frequency could make it possible to connect changes in band patterns to changes in operating condition, but the potential results would probably not be worth the extra work load.

The missing correlation between operating conditions and band pattern could also be explained by the fact that microbial activity does not necessary correspond with changes in biomass that would be visible by DGGE. DGGE bands only indicate the presence of microbes, not their activity levels. This conclusion is supported by Malin and Illmer (2008). DGGE might hence not be first choice of first choice to assess fast changes in fermenter community.

Minor changes in overall DGGE band strength and variations could be considered unreliable due to large uncertainties and variations in the sampling method and sample composition before DNA extraction (e.g. amount of granules relative to liquid).

To increase the advantages of DGGE analysis, DGGE band pattern analysis software like GelCompar II (Applied Maths) or Gel2K (Svein Nordland, University of Bergen) can be used. This will give more reliable and sensitive analysis of the gel pattern variations.

Increased knowledge about the microbes present would give the foundation of further research based on e.g. more specific primers. Sequencing of excised band has been proved difficult with the parameters tested here, and different methods should be evaluated. This will be further discussed in section 4.4. The samples can also be analysed by FISH, to examine the spatial orientation of the microbes.

Measurements of DNA content of the fermenter sludge might be a good parameter to monitor fermenter performance. It has been observed an approximate fivefold increase in DNA content at times with high reactor performance compared to low (Malin and Illmer, 2008).

4.4 Further work

The extracted DNA is eluted in 100µL elution buffer. This may prove to be a small volume if several analyses are needed. DNA extraction should therefore be made with at least two extractions per sample. It would also be relevant to check if the microbial compositions in new DNA extractions from the stored samples are comparable to extractions made when the samples were fresh. This would be a continuation of the storage experiment started by Forsberg (2011).

A better understanding of the changes in microbial composition may give the foundation for optimization of the operating conditions, fewer reactor problems and better biogas yield. Further work should hence focus on optimizing the method for DNA sequencing. The main areas of focus can be choice of primers, see section 4.4.1 or sequencing method, see section 4.4.2. FISH analysis should be performed when suitable probe sequences are established, see section 4.4.3.

4.4.1 Primers

The primers 338f and 518r are probably unsuitable for DNA sequencing, see section 3.3 and 4.2.2, since too many PCR products for sufficient separation on a DGGE gel were produced. More specific primers should hence be tested, even if the microbial diversity is decreased. The universal primer can still be used for monitoring the microbial compositions in the reactors.

Hydrolysis and acid formation is as mentioned in section 1.2.3 carried out by a wide consortium of bacteria. Some of the genera involved are *Bacteroids*, *Bifidobacteria*, *Clostridium*, *Escherichia*, *Lactobacillus,* and *Proteus* (Gerardi, 2006). Acidogens are spread across more than 20 phyla (Kim et al., 2011) indicating that their phylogenetic position and phenotypic function of acid production are not tightly linked. Primer design must hence be even more specific.

In research on wastewater reactors, *Aeromonas* spp. and *Clostridium sticklandii* were identified as common acidogens with Aeromonas the likely major acidogenic group, especially during startup of a reactor. They are both probably significant in both amounts and activity in initiating anaerobic digestion. Two primers presented in Table 4.1 were designed targeting the genus Aeromonas or the species C. sticklandii. Aeromonas have high specie homology (> 97,8 %) for the 16S rDNA sequence, hence the genus target. There were insignificantly few false positives for both primer sets. (Kim et al., 2011)

Some syntrophic acetate oxidizing bacteria has been identified in anaerobic biogas reactors, and suitable primers for identification has been suggested (Westerholm et al., 2011). They are presented in Table 4.1.

Formyltetrahydrofolate synthetase (FTHFS) is a key enzyme in the acetyl-CoA pathway, used for assimilation of CO2 into cell carbon and conversion of energy found in some acetogenic bacteria. The gene coding for FTHFS might hence be used to identify some acetogenic bacteria. (Westerholm et al., 2011)

Table 4.1: Primers targeting bacteria identified in anaerobic fermenters. The primers may be used to identify bacteria in the reactors of this experiment.

Target group	Sequence (5'- 3') Forward/Reverse	Representative target strains	Amplicon size (bp)
Aeromonas[2]	GCCTTGACATGTCTGGAA/ ACTATCGCTAGCTTGCAG	A. caviae A. hydrophila	286
Clostridium sticklandii[2]	CCTCGGGTCGTAAAGCT/ AAGTTCACCAGTTTCAGAG	C. sticklandii	235
Clostridium ultunense[3]	CCTTCGGGTGGAATGATAAA/ TCATGCGATTGCTAAGTTTCA	C. ultunense	127
Syntrophaceticus schinkii[3]	ATCAACCCCATCTGTGCC/ CAGATTTCGCAGGATTGC	S. schinkii	171
Tepidanaerobacter acetatoxydans[3]	AGGTAGTAGAGAGCGGAAAC /TGTCGCCAAGACCATAAA	T. acetatoxydans	237

[2] KIM, J., SHIN, S. G., HAN, G., O'FLAHERTY, V., LEE, C. & HWANG, S. 2011. Common key acidogen populations in anaerobic reactors treating different wastewater: Molecular identification and quantitive monitoring. *Water Research,* 45**,** 10.

[3] WESTERHOLM, M., DOFLING, J., SHERRY, A., GRAY, N. D., HEAD, I. & SCHNÜRER, A. 2011. Quantification of syntrophic acetate-oxidizing microbial communities in biogas production. *Environmental Microbiology Reports,* 3**,** 500-505.

4.4.2 Sequencing methods

The traditional method of sequencing environmental samples is to make and sequence clone libraries. By introducing one 16S rDNA sequence from the environmental sample into each host cell (typically an easy-to-grow, benign, laboratory strain of E. coli bacteria) and plating the host cells, each colony will contain the same alien 16S rDNA sequence in addition to its own genome. DNA can then be extracted from each colony, amplified and sequenced . This method is both time and work consuming, and the number of different sequences possible to process is low compared to other methods for sequencing. (Simmons, 2010)

An alternative to cloning is the 454/Roche FLX pyrosequencing method (F. Hoffmann-La Roche Ltd). This sequencing method enables direct sequencing of several environmental samples at once. The amplified DNA from each sample is marked with a Multiplex Identifier (MID) attached to the primer during PCR to make the sequences traceable (F. Hoffmann-La Roche Ltd, 2011 and 2012). Subsequent analysis will separate the sequence based on MIDs, and estimate phylogenetic diversity. These data can be used to generate taxonomic summaries and phylogenetic threes. The individual sequences can be analyzed by Basic Local Alignment Tool (BLAST).

The system is capable of more than one million reads with a length of 700 high quality bases. A full sequencing run can be completed in 23 hours. There are 132 MIDs available, and 2, 4, 8 and 16 gaskets possible. The gaskets are different physical compartments capable of increasing the number of possible samples significantly. In one experiment, a total of 473 169 sequence reads with an average of 260 bases including MIDs and primer sequences were obtained from 57 samples (Wu et al., 2010).

The sequencing method is available through e.g. Eurofins MWG Operon (Germany) and Norwegian Sequencing Center (NSC). The price for a full sequence at NSC is approximately 100 000 NOK. This gives a total read of 1.2 million sequences. It is possible to separate the run into two or four gaskets, 200 000 sequences per gasket. The price for one gasket is between 22 000 and 25 000 NOK.

4.4.3 FISH

Fluorescence *in situ* hybridization (FISH) is used to identify microbes in their natural environment, *in situ*. Fluorescent probes bind to complementary DNA sequences within the microbe's ribosomal DNA and will hence be visible by a fluorescent microscope. FISH can give information about the relative number of microbes of one type compared to others, and their spatial orientation with respect to each other.

The original three fluorescent colors red, green, and blue can bind to the same organism and together create a new color. Red and green will for example turn yellow when combined. Up to seven different populations can be visualized in the same experiment by combining colors. (Nederlof et al., 1990)

This is a valuable addition to DGGE analysis for microbe analysis of environmental samples and should be used in further analysis of the reactors after species has been identified through sequencing. The sequencing results can be used to design probes relevant to the reactors.

5 Conclusion

Two reactors respectively based on cow and pig manure were followed from September 2011 to May 2012 and sampled regularly. DNA was extracted from the samples and amplified by polymerase chain reaction (PCR) using the universal bacteria primers 338f and 518r. Fingerprinting by denaturing gradient gel electrophoresis (DGGE) was used to visualize the microbial diversity in the reactors. DGGE was capable of detecting shifts in the microbial community, but no correlation was found between changes in band pattern and changes in operating conditions. Changes in the microbial diversity can be slow and DGGE can only present the diversity in relative amounts, not the activity level of said microbes.

DGGE is a highly reproducible and consistently performing fingerprinting technique capable of comparing several samples in one gel. It is hence an adequate technique for monitoring the microbial community of the reactor long term.

An assortment of DGGE gel bands was excised and sequences, but the results were either negative or of too poor quality for further analysis. The main reason is probably insufficient separation or contamination of the DNA bands leading to plural DNA sequences in the same sample.

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Appendices

Appendix A: Primary data for the cow manure reactor

Table A.1 presents operating data for the reactor running on cow manure from August 14th to May 10th received from PhD candidate Wenche Bergland. The data presented is the measuring date, feed batch number, feed rate in L/day, temperature in °C, biogas production in L/day, organic loading rate in gCOD/L-day and the methane concentration in percent. They are the primary data for the figures in section 2.1.1.

Table A.1: Primary data of operating conditions for the reactor running on cow manure, from PhD candidate Wenche Bergland at TUC.

Date	Feed Batch no	Feed rate [L/day]	Temperature in reactor [°C]	Biogas production [L/day]	OLR (gCOD/L-day)	% metan in biogas
14.08.2011	20110622	30	24	160	6,63	77,8
17.08.2011	20110622	30	24	162	6,69	78,3
20.08.2011	20110622	15	24	145	3,18	78,5
23.08.2011	20110622	15	24	120	3,08	77,5
26.08.2011	20110622	15	24	114	3,32	76,6
29.08.2011	20110622	25	24	130	5,47	76,3
01.09.2011	20110622	25	24	133	5,51	77,5
04.09.2011	20110622	25	24	141	5,47	78,2
07.09.2011	20110622	25	24	142	5,40	78,2
10.09.2011	20110622	25	24	135	5,53	78
12.09.2011	20110830	25	24	137	5,31	77,3
14.09.2011	20110830	25	24	142	5,08	76,2
16.09.2011	20110830	25	24	138	5,11	75,7

19.09.2011	20110830	25	24	150	5,36	75,5
21.09.2011	20110830	25	24	154	5,56	75,8
23.09.2011	20110830	25	24	152	5,43	76
26.09.2011	20110830	10	24	154	1,95	76,2
28.09.2011	20110830	10	24	103	2,08	75,7
30.09.2011	20110830	25	24	91	5,14	74,5
03.10.2011	20110830	25	24	144	5,00	74,7
05.10.2011	20110830	25	24	142	5,54	75,5
07.10.2011	20110830	25	24	150	5,46	75,7
10.10.2011	20110830	25	24	148	4,94	76
12.10.2011	20110830	25	30	145	5,48	75,9
14.10.2011	20110830	25	30	200	5,03	73,3
17.10.2011	20110830	25	30	193	5,00	72,5
19.10.2011	20110830	25	35	198	5,05	72,7
21.10.2011	20110830	25	35	222	4,78	71,6
24.10.2011	20110830	25	35	202	5,29	72,3
26.10.2011	20110830	25	35	203	5,12	71,6
28.10.2011	20110830	50	35	204	10,43	71,8
31.10.2011	20110830	50	35	309	9,59	73,7
02.11.2011	20110830	50	35	315	9,99	74,6
04.11.2011	20110830	50	35	319	10,31	74,5
07.11.2011	20111107	50	35	305	9,81	74,4
09.11.2011	20111107	50	35	290	10,22	73,6
11.11.2011	20111107	50	35	260	10,48	73,6
14.11.2011	20111107	50	35	247	10,76	71,6

16.11.2011	20111107	50	35	255	9,94	71,2
18.11.2011	20111107	10	35	150	2,06	70
21.11.2011	20111107	10	35	50	2,10	69
23.11.2011	20111107	10	35	60	2,05	67
25.11.2011	20111107	10	35	70	2,18	67
28.11.2011	20111107	10	35	110	2,15	69
30.11.2011	20111107	30	35	130	6,50	69
02.12.2011	20111107	30	35	140	6,40	70
05.12.2011	20111107	30	35	120	6,40	69
07.12.2011	20111107	30	35	130	6,46	70
14.12.2011	20111107	30	30	115	6,53	71
21.12.2011	20111107	30	30	110	6,43	70
28.12.2011	20111107	30	30	160	6,40	71
04.01.2012	20111107	30	30	130	6,41	70,5
09.01.2012	20111107	30	30	159	5,95	70,5
12.01.2012	20111107	30	30	158	6,28	69,8
16.01.2012	20111107	30	30	159	6,82	70,3
19.01.2012	20111107	30	30	164	4,59	69,7
23.01.2012	20111107	30	30	155	6,70	70,2
26.01.2012	20111107	30	30	160	6,37	70
30.01.2012	20120128	30	30	170	6,66	70
02.02.2012	20120128	30	30	160	6,00	69,2
06.02.2012	20120128	30	30	160	5,48	69
09.02.2012	20120128	35	30	170	6,72	69,5
13.02.2012	20120128	35	35	200	5,90	66,5

16.02.2012	20120128	35	35	185	6,55	68
23.02.2012	20120128	50	35	225	9,73	68,8
27.02.2012	20120128	50	35	230	9,22	68,8
01.03.2012	20120128	50	35	250	9,62	69,2
05.03.2012	20120128	50	35	252	9,21	69,3
08.03.2012	20120128	50	35	270	9,61	69
12.03.2012	20120128	50	35	290	10,44	69,8
15.03.2012	20120128	50	35	280	9,58	69,4
19.03.2012	20120128	25	35	230	4,67	70
22.03.2012	20120128	25	35	160	5,01	69,7
26.03.2012	20120128	25	35	170	4,67	69,3
29.03.2012	20120327	25	35	160	4,93	69,2
02.04.2012	20120327	25	35	160	4,87	69,1
05.04.2012	20120327	25	35	170	5,36	69
09.04.2012	20120327	25	35	180	5,50	68,8
13.04.2012	20120327	25	35	185	5,49	69,1
16.04.2012	20120327	25	35	200	4,89	69
20.04.2012	20120327	25	35	120	4,82	
23.04.2012	20120327	25	35	140	5,34	64
26.04.2012	20120327	25	35	150	5,78	65
30.04.2012	20120327	25	35	160	5,73	67,5
03.05.2012	20120327	25	35	200	5,73	68
07.05.2012	20120327	25	35	200	5,78	69,2
10.05.2012	20120327	35	35	205	7,77	69,5

Appendix B: Primary data for the pig manure reactor

Table B.1 and B.2 presents operating data for the reactor running on pig manure from September 11th to April 15th received from PhD candidate Wenche Bergland. Table B.1 presents the hydraulic retention time in days and the organic loading rate given in gCOD/L-day. Table B.2 presents the gas production in the same time period. They are the primary data for the figures in section 2.1.2.

Table B.1: Hydraulic retention time (HRT) and organic loading rate (OLR) for the pig manure reactor from November 2011 to April 2012.

Date	HRT (day)	OLR (gCOD/L-d)
07.11.2011	35,00	0,59
14.11.2011	17,50	1,20
21.11.2011	8,75	2,06
05.12.2011	5,83	3,50
27.12.2011	4,38	4,47
17.01.2012	3,50	5,95
02.02.2012	3,50	7,28
09.02.2012	2,00	12,19
15.02.2012	1,75	13,18
21.02.2012	1,17	13,13

Date	HRT (day)	OLR (gCOD/L-d)
28.02.2012	0,93	20,12
06.03.2012	0,88	21,46
13.03.2012	0,78	31,35
18.03.2012	0,70	34,83
28.03.2012	0,61	40,05
01.04.2012	0,58	41,79
05.04.2012	0,54	45,28
10.04.2012	0,50	48,76
15.04.2012	0,47	52,24

Table B.2: Primary data for the gas production in the reactor running on pig manure, from PhD candidate Wenche Bergland at TUC.

Total gas production from both chambers in series			Without recirc.	With recirc.		Without recirculation	
Date	Gas prod. [mL/day]	Date	Gas prod. Cham.1 [mL/day]	Gas prod. Cham. 2 [mL/day]	Date	Gas prod. Cham. 1 [mL/day]	Gas prod. Cham. 2 [mL/day]
07.11.11	432,0	03.02.12	981	1092	25.03.12	1819	757
09.11.11	557,5	04.02.12	553	583	26.03.12	2340	1157
11.11.11	465,0	05.02.12	596	614	27.03.12	2254	1450
13.11.11	204,0	06.02.12	690	750	28.03.12	2281	1457
16.11.11	286,0	07.02.12	668	711	29.03.12	2686	1900
18.11.11	232,5	08.02.12	663	596	30.03.12	2880	1878
22.11.11	266,0	09.02.12	598	723	31.03.12	2803	1800
24.11.11	270,0	10.02.12	764	778	01.04.12	2937	2000
28.11.11	361,5	11.02.12	920	917	02.04.12	2926	1929
30.11.11	256,5	12.02.12	1000	945	03.04.12	3211	2084
02.12.11	245,0	13.02.12	960	695	04.04.12	2735	2347
04.12.11	285,0	14.02.12	360	552	05.04.12	2756	2085
09.12.11	240,0	15.02.12	1047	1053	06.04.12	3344	2645
11.12.11	465,0	16.02.12	384	568	07.04.12	2219	2751
13.12.11	675,0	17.02.12	889	962	08.04.12	3260	2946
15.12.11	682,4	18.02.12	1148	641	09.04.12	3080	2900

17.12.11	976,3
19.12.11	739,2
21.12.11	662,0
22.12.11	488,1
23.12.11	705,3
25.12.11	478,5
27.12.11	635,3
29.12.11	734,0
01.01.12	727,7
02.01.12	799,0
03.01.12	759,4
04.01.12	963,3
08.01.12	546,8
09.01.12	685,6
10.01.12	836,2
11.01.12	975,3
12.01.12	892,1
13.01.12	696,5
15.01.12	674,0
16.01.12	685,9
18.01.12	480,0
19.01.12	600,0
20.01.12	1120,0
21.01.12	802,8
22.01.12	1088,0

19.02.12	1130	934
20.02.12	1097	844
21.02.12	584	687
23.02.12	1342	1571
24.02.12	1720	1149
25.02.12	1140	920
26.02.12	1390	1030
27.02.12	1311	1164
28.02.12	1293	972
29.02.12	1595	964
01.03.12	1363	1170
03.03.12	1394	1445
04.03.12	1008	810
05.03.12	1217	1095
06.03.12	1271	899
07.03.12	1061	1006
08.03.12	1641	1090
09.03.12	1355	1205
10.03.12		1358
11.03.12	1718	1026
12.03.12	1135	1131
13.03.12	2386	1355
14.03.12	1529	1329
15.03.12	2173	1768
16.03.12	608	1410

10.04.12	3390	3537
11.04.12	3913	3150
12.04.12	3411	3360
13.04.12	3444	

25.01.12	822,9		17.03.12	627	1460			
26.01.12	1197,3							
27.01.12	1113,7							
28.01.12	891,9							
29.01.12	894,3							
30.01.12	1277,8							

Appendix C: Protocol for PowerSoil®DNA Isolation Kit

MO BIO Laboratories, Inc.

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Appendix D: Protocol for QIAquick PCR Purification Kit

QIAquick® PCR Purification Kit

The QIAquick PCR Purification Kit (cat. nos. 28104 and 28106) can be stored at room temperature (15–25°C) for up to 12 months.

For more information, please refer to the *QIAquick Spin Handbook*, *March 2008*, which can be found at: www.qiagen.com/handbooks.

For technical assistance, please call toll-free 00800-22-44-6000, or find regional phone numbers at www.qiagen.com/contact.

Notes before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

2. Place a QIAquick column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.

3. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 30–60 s or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back in the same tube.

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4. To wash, add 0.75 ml Buffer PE to the QIAquick column ▲ centrifuge for 30–60 s or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

Appendix E: Protocol for Ingeny DGGE system

Mounting of glass plates

1. Wash the two glass plates, the spacer, and comb, using Deconex soap and hot tap water. Finally rinse well with water to remove any traces of soap. Polish one side of each glass plate using 96% ethanol and Kimwipe paper.
2. Assemble the glass plates and spacer, and place it all in the gel box. Assure that the spacer is aligned to the lower edge of the glass plates. Tighten the screws.
3. Loosen the two uppermost screws, mount the comb, and then tighten the screws again.

Preparation of DGGE solutions

1. Determine the acrylamide per cent and the denaturing gradient of the gel (for recipes of solutions, -see below).
2. Make acrylamide solutions with the desired denaturing percentages in two 50 ml tubes (total volume in each tube will be 24 ml; see table below for volumes of 0% and 80% denaturing solutions).
3. The 0% denat. acrylamide solution can be added to the 50 ml tubes without sterile filtration. The 80% denat. acrylamide solution needs to be filtered upon addition.
4. Prepare a 50 ml tube with 8 ml 0% denat. acrylamide solution (“stacking gel” for the top part of the gel).
5. When ready to pour the gel, add 16µl TEMED to the 24 ml gel solutions, and 10µl TEMED until the 8 ml ”stacking gel” solution.
6. Directly prior to pouring the gel, add 87µl APS (10% Ammonium persulfate) in both 24 ml gel solutions (for the stacking gel, add 40µl APS, but not until the stacking gel is ready for pouring).

Casting the gel

1. Rinse the gradient mixer and the tubes by pumping MilliQ water through the system.
2. Turn off the pump, close the valve between the chambers of the gradient mixer, and put the gradient mixer on magnetic stirring.
3. Pour the gel solution with low denat. percentage in the “left” chamber. Quickly open and close the valve to remove any air bubbles in the channel between the chambers. Use a pipette to remove the small amounts of gel solution from the “right” chamber.
4. Pour the gel solution with high denat. percentage in the “right” chamber.
5. Start the pump, wait a few seconds until the gel solution from the ”right” chamber has migrated ~7-8 cm out in the tube. Then open the valve between the chambers. Assure stirring in both chambers.
6. Place the syringe between the glass plates (assure that no water from the washing step is left in the tube).
7. When the gel reaches approximately 1 cm below the comb, stop the pump, remove the syringe, and empty leftovers from the mixing chambers and the flexible tubes. Rinse the system with a small amount of MilliQ water.
8. When the mixing chambers are empty from water, close the valve and stop the pump. Add APS to the “stacking gel” solution, mix, and pour into the “right” chamber.
9. Start the pump again. When the glass plates are completely filled with the stacking gel, turn off the pump, and press the comb down to the correct position. Tighten the screws.
10. Leave the gel for polymerization for at least two hours.
11. Pump MilliQ water through the system to avoid gel polymerization in the tubes.

Preparations and addition of samples

1. Make 20 l of 0.5 x TAE (200ml 50 x TAE + 20 l MilliQ) and add appr. 17 l to the buffer tank (the buffer may be used for 3 runs). Turn on the instrument to heat the buffer to 60^0C.
2. Carefully remove the comb from the gel. Loosen all screws, and carefully push down the spacer. Tighten the screws at the sides of the glass plates (the screws at the bottom should be loose throughout the electrophoresis).
3. Place the gel system in the buffer tank. Avoid air bubbles beneath the gel.
4. Attach cables and tube, turn on the recirculation. Use a syringe with buffer to rinse the wells. Turn on the power (100 Volts; should result in approximately 27-35 mA) and let run while preparing the samples.
5. Add 2-4µl loading dye to 5-15 µl PCR sample. When all samples are ready for loading, turn of the recirculation and the push the “low voltage” button. Apply the samples to the wells. Avoid using the 2-3 outermost wells on each side due to ”smiling effects”.

Running the gel

1. Turn on the ”high voltage button”, set the voltage to 100. Run 5-10 min without recirculation.
2. Turn on the recirculation and run for 17-18 hours.

Staining and visualization

1. Turn off the instrument; lift the gel system over to the blue box.
2. Loosen the screws, and lift out the gel. Carefully separate the glass plates (use the small red plastic equipment).
3. Transfer the gel to a plastic foil sheet, and place it in the dark blue box.
4. Prepare the staining solution: 30ml MilliQ + 3µl SYBR Gold + 600µl 50 x TAE in a 50 ml tube.

5. Distribute the staining solution on the gel, put the lid on the box, and leave for 1-2 hours.
6. Carefully take out the gel, rinse with MilliQ water. Carefully let the water run of the gel, use a paper towel at the edges of the gel to remove excess water.
7. Wash the UV plate of the ”gel doc” with distilled water and ethanol. Use Kimwipe paper, and take care to avoid dust and particles on the UV plate (easier to avoid dust if the plate is not allowed to dry). Finally distribute MilliQ water on the plate (this makes it possible to move the gel on the UV plate).
8. Carefully transfer the gel from the plastic foil to the UV plate (by turning the plastic foil “upside down”). Before removing foil, position the gel at the plate.
9. Photograph the gel at different exposures, and save the pictures in original file format, and e.g. pdf or other formats.

Eluation of bands for sequencing

1. Print out a picture of the gel, and number the bands that are to be sequenced.
2. Add 20µl sterile MilliQ water to eppendorf tubes, and number the tubes according to the numbering of bands.
3. Pull out the UV plate, and pull on the UV screen. Cover the wrists to protect from UV radiation. Use the blue 1 ml pipette tips to stick out material from the bands. Take care to avoid touching other bands. Use a pipette to blow out the material in the eppendorf tube with water (it should be possible to see whether there is material in the pipette tip when transferring it to the water).
4. Place the tubes in the fridge overnight.
5. Use 1 µl of the eluate as template in a 25µl PCR reaction.

Recipes

For all solutions, add distilled water to obtain the final volume.

50 x TAE-buffer

Per litre:

- Tris base 242g
- Glacial acetic acid 57,1ml
- 0.5 M EDTA (pH 8,0) 100ml

Autoclave the buffer.

Deionized formamide:

Deionize 200 ml formamide by adding 7,5g DOWEX RESIN AG 501X8, and stir for 1 hour at room temperature.

Acrylamide solution (0% denaturing):

8% acrylamide in 0,5 x TAE (per 250 ml):

- 40% acrylamide solution (BioRadLab Inc., Ca., USA) 50 ml
- 50 x TAE 2.5 ml

Store the solution at 4 ^{0}C, protect from light.

Denaturing acrylamide solution (80% denaturing):

8% acrylamide, 5,6M urea, 32% formamide in 0,5 x TAE (per 250 ml):

- 40% acrylamide solution (BioRadLab Inc., Ca., USA) 50 ml
- 50 x TAE 2,5 ml
- Urea 84 g
- Deionized formamide 80 ml

Store the solution at 4 ^{0}C, protect from light. This solution **must** be sterile filtered before pouring the gel.

Composition of low and high denaturing solutions:

Denaturing %	0%	80%	TEMED + 10% APS	Total volume
15	19,5ml	4,5ml	16µl + 87µl	24ml
25	16,5ml	7,5ml	16µl + 87µl	24ml
30	15 ml	9 ml	16µl + 87µl	24ml
40	12 ml	12 ml	16µl + 87µl	24ml
45	10,5ml	13,5ml	16µl + 87µl	24ml
50	9ml	15ml	16µl + 87µl	24ml
55	7,5ml	16,5ml	16µl + 87µl	24ml
60	6 ml	18 ml	16µl + 87µl	24ml
75	1,5ml	22,5ml	16µl + 87µl	24ml

0% "Stacking gel":

8 ml 0% acrylamide solution, 40µl 10% APS and 10µl TEMED.

10% APS (ammonium persulfate):

10g ammonium persulfate dissolved in 100ml dH_2O

Sterile filter the solution, distribute in eppendorf tubes (250µl in each), and keep frozen.