NTNU
Norwegian University of
Science and Technology

Bioconversion of Heavy oil
Characterizations of Microbial potential to bioconvert Mariner
Maureen-, Peregrino- and Bressay oil

Sandra Steinbakk

Biotechnology

Norwegian University of Science and Technology
Department of Biotechnology

Front cover photo; (Alboudwarej 2006)

Preface

This master project was done as a part of Statoil's heavy oil project; BioTHOR in connection to the master programme at department of Biotechnology, Norwegian University of Science and Technology (NTNU). Experiments were carried out in laboratories at Statoil Centre for Research & Development, Rotvoll and Sintef's laboratories at Gløshaugen in Trondheim, and financed by Statoil ASA.

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   Trondheim /date   Sandra Hoddø Steinbakk

Abstract

70 % of world's oil reservoirs consist of heavy oil, and as the supply of conventional oil decreases, researchers are searching for new technologies to explore and enhance heavy oil recovery. One of the postulated technologies is microbial enhanced oil recovery (MEOR), which is predicted to be a more environmental and economical process for improving oil recovery of heavy oil.

The aim of this Master's project was to give a qualitative indication of three selected consortia's potential to bioconvert Mariner Maureen-, Peregrino- and Bressay oil. The consortia are comprised of microorganisms isolated from oil sand, mud volcano, processing waters from water treatment plants and oil related samples from other locations.

Bioconversion experiments were conducted by inoculating the three selected oils with three consortia. Two terms of different main experimental designs was used; optimal growth temperature of the consortia or the temperature at reservoir conditions, in addition to cultivation with two different growth mediums.

After cultivating aerobically for seven days, the oil was separated from the water phase and analyzed by thin-layer chromatography with flame ionization detection (TLC-FID) to identify possible indication of bioconversion. The results demonstrate a reduction in either one or both saturated hydrocarbons and aromatic hydrocarbons of the oil in several of the oil samples. DNA extracted from the water phase that was analyzed with denaturing gradient gel electrophoresis (DGGE), showed several positive results with indication of high biodiversity.

The overall results indicate that there has been a microbial impact on some of the heavy oil fractions in Mariner Maureen-, Peregrino- and Bressay oil. Additional experiments must be done to identify the specific changes in the oil and prove microbial impact by repeating these experiments. This could potentially lead to identification of microorganisms with the ability to bioconvert heavy oil and the development of MEOR processes.

Abbreviations

Bp	Base pair
BSA	Bovine Serum Albumin
DCM	Dichloromethane
DGGE	Denaturing Gradient Gel electrophoresis
dH_2O	Distilled water
dNTP	Deoxy nucleotide phosphate
HC	Hydrocarbon
HPLC – grade	High-Performance Liquid Chromatography
LC-MS (QTOF)	Liquid Chromatography- Mass Spectrometry Quadrupole Time of Flight
MM	Mineral medium
MMAc	Mineral medium with acetate
PCR	Polymerase Chain Reaction
RMMAc	Enriched mineral medium with acetate
SARA	Saturated hydrocarbons, Aromatic hydrocarbons, Resins and Asphaltenes
TAE	Tris - Acetate/EDTA
TBE	Tris - Borat /EDTA
TEMED	Tetra-methyl Ethylenediamine
TLC-FID	Thin-layer Chromatography with flame ionization detection
T_m	Melting temperature
UV	Ultra violet

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1.   Introduction

The following chapters are a short introduction, background and the aim for this master project.

1.1   Background

Heavy crude oil comprise of four fractions classified in regard to solubility; saturated hydrocarbons (also known as alkanes, paraffin and wax), aromatic hydrocarbons, resins and asphaltenes. In addition the oil also comprises metals and heteroatoms; nitrogen, sulphur and oxygen (NSO) associated with aromatic hydrocarbons. Saturated hydrocarbons are non–polar carbon atoms arranged in cycles or long chains, aromatic hydrocarbons contain one or more aromatic ring structures consisting of many branches of aliphatic parts. Resins and asphaltenes are more polar compounds, consisting of many aromatic structures and a few branches of aliphatic parts. Resins can be trapped within aggregates of asphaltene molecules. Asphaltene is considered to be the largest and most complex of the SARA fractions also with the largest contribution to the high viscosity of the heavy oil (Figure 1). (Leon and Kumar 2005) These components are held together by properties that contributes to the heavy oils appearance; molecular interactions; strong Van der Waals forces and "free radical sites" (associated with condensed polycyclic aromatic structures with highly reactive unpaired electrons). The "free radical sites" are involved in hydrogen bonding, inter– and intra molecular reactions, molecular rearrangements and complexion of metals). These are properties which contribute to the heavy oils appearance. (Leon and Kumar 2005; Seo 2009)
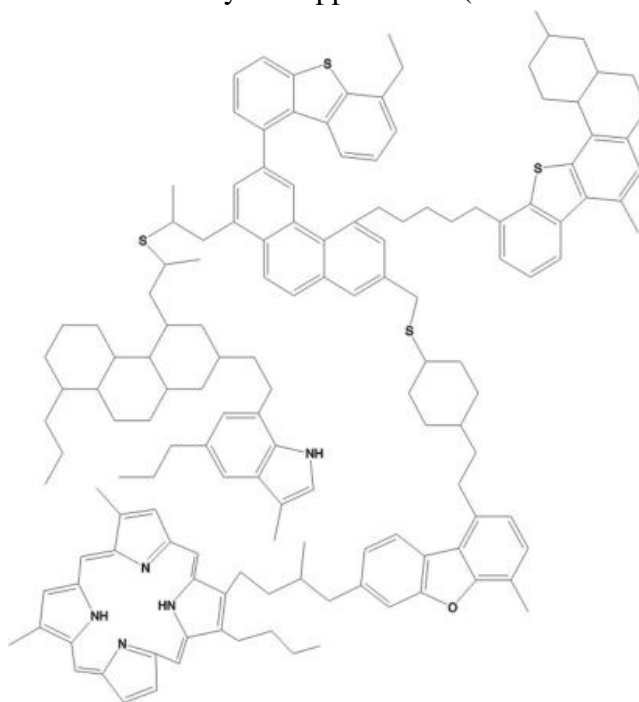
Figure 1. Asphaltene molecule.
Illustrating a common asphaltene molecule with several polycyclic aromatic cores joined together with aliphatic bridges. (Leon and Kumar 2005)

There are many factors limiting oil recovery, examples of this are physiochemical properties of the reservoir (pore-entrance size of the rocks, permeability, surface tension between the oil and the rocks etc). Other concerns like oil viscosity and mobility also affects recovery rate. (Ollivier 2005; Lazar 2007; Brown 2010) Because of this, heavy crude oil also has a lower economical value than conventional oil. The oil's economical value is classified with a °API – value (American Petroleum Institute gravity) which is an expression used for the oils specific weight. °API – value ranges between 10 and 20 for heavy oils and under 10 for extra heavy oil and bitumen (Figure 2). High viscosity oils have a lower °API –number and subsequently lower economical value. (Head, Jones et al. 2003).
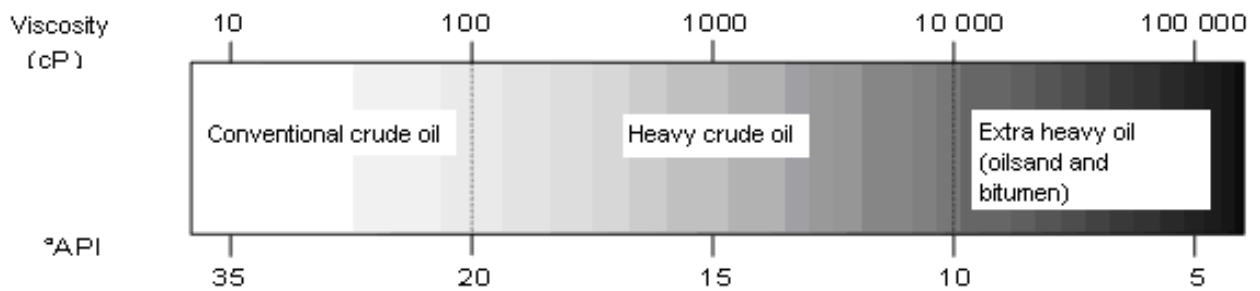
Figure 2. °API – range for crude oil.
Oil is scaled from conventional crude oil to heavy – and extra heavy oil, based on viscosity (cP). The more viscous the oil appears, the lower the °API – value is. (Conaway 1999)

70 % of world's oil reservoirs consist of heavy oil extra heavy oil and bitumen (Figure 3). Researchers are seeking new technologies for increasing the recovery of heavy oil and many different methods of improved recovery are explored.
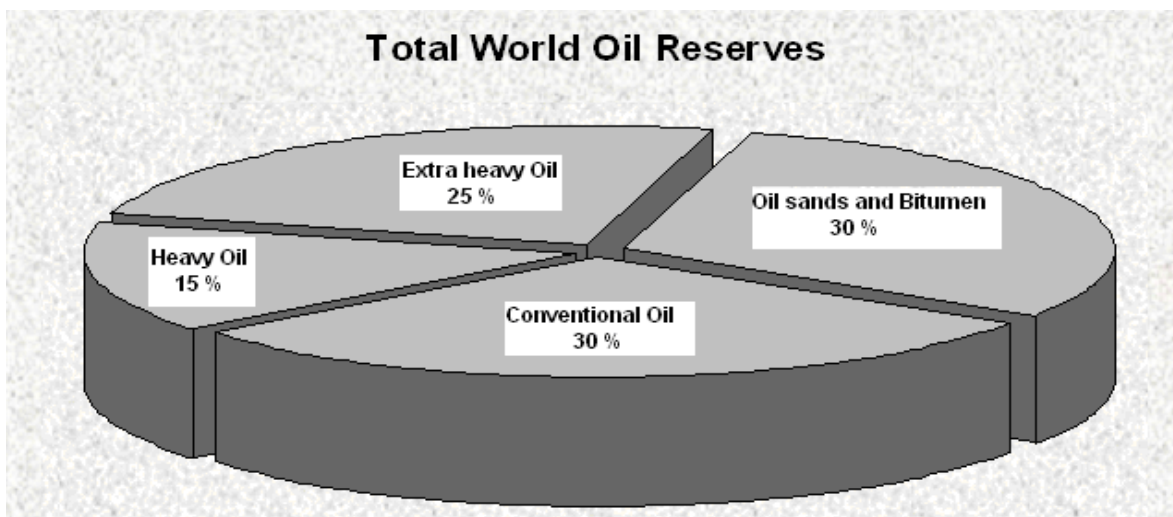
Figure 3. Distribution of the world's oil reserves.
Illustrating that 70 % of the world's petroleum is made up of heavy, extra heavy oil and bitumen. (Alboudwarej 2006)

Oil recovery methods are staged in three phases; primary, secondary and tertiary (Figure 3), and the methods that is being used depend on each reservoirs' conditions. Primary recovery utilizes the natural pressure drive of the reservoir for production. This method is essentially

not enough for heavy oil extraction and is mainly used for conventional oil recovery. Secondary recovery involves stimulation of the oil flow by injection of gas or water. When handling very viscous and heavy oil, tertiary recovery methods is often used in combination with primary and secondary methods to improve oil recovery (IOR).(Conaway 1999; Ollivier 2005) Tertiary recovery comprises four methods together called enhanced oil recovery (EOR); chemical, miscible, thermal and microorganisms (MEOR). These methods are used to reduce the oil's viscosity and increase its mobility. (Leon and Kumar 2005; Ollivier 2005; Brown 2010) For instance, the addition of polymers to the water phase will increase the sweep efficiency and decrease the water's mobility, which in turn impairs water fingering. (Leon and Kumar 2005) Water fingering is defined as water pushing through the oil phase. (Kotlar 2011)

After the oil has been recovered from the reservoir, pumping through several steps of oil- and water separators prepares the oil for refining and further processing. (Kotlar 2011). Depending on the use, the produced water is treated for different organic and inorganic compounds which are carried from the oil production. Water disposed into sea or rivers is deoiled to a standard acceptable oil concentration for disposed water. Water which is to be re-injected into a reservoir is treated based on compatible criteria within the specific reservoir to avoid plugging, precipitation and corrosion.(Ollivier 2005)

Primary
- Natural pressure drive

Secondary
- Water injection
- Gas injection (Flue gas, N_2, CO_2)
- Miscible (solvent and fluid injection)

Tertiary
- Thermal (Steam flood, combustion)
- Chemical (Polymers, surfactants, alkali)
- Miscible (solvent and fluid inkection)
- MEOR (microorganisms or metabolic products)
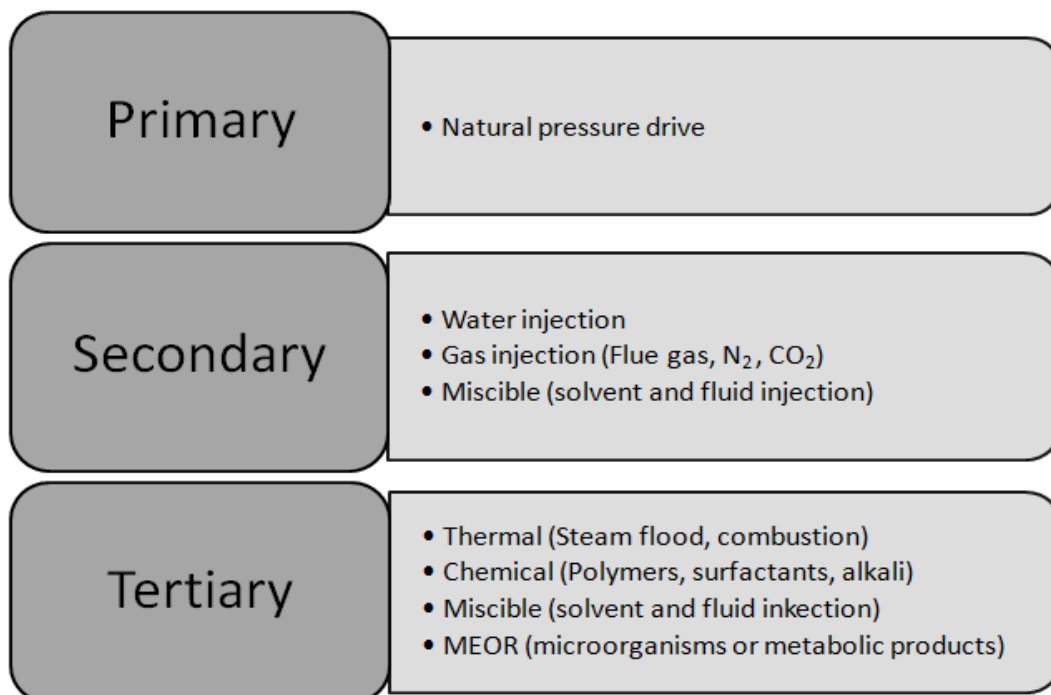
Figure 4. Improved oil recovery.
Schematic overview of the three staged methods for increasing oil recovery. Inspired by; (Sen 2008)

1.2   Microbial enhanced oil recovery (MEOR)

Microbial enhanced oil recovery (MEOR) is defined as; petroleum recovery methods involving the use of a mixed microbial population (indigenous reservoir microorganisms or special, selected microorganisms) and the metabolic products including; bio surfactants, biopolymers, biomass, acids, solvents, gases and enzymes to enhance oil recovery. (Sen 2008). This technology has been postulated since 1926 to be a more environmentally friendly and less expensive method in comparison to conventional recovery methods. The earliest experiments reported, were in 1947 by ZoBell and his co – workers. These experiments have later been reproduced with various modifications by several others. (Lazar 2007). Injection of nutrients to stimulate growth of natural living microbes and *in situ* production of bio surfactants is a good example of successful field work comprising from the first experiments (Lazar 2007; Sen 2008; Brown 2010). Many publications claims the success of using MEOR processes with injection of microorganisms directly into the reservoir, whereas others claim the success of pumping microbial products (solvents, polymers) into the oil-bearing formations. (Sen 2008; Brown 2010). Another suggested method of MEOR technology is to introduce the microorganisms at top side; in one of the first stations after the oil has been pumped up from the ground. (Kotlar 2011)

The fact that microorganisms are able to utilize hydrocarbons in petroleum as a carbon- and energy source is has been stated in many reports(Leon and Kumar 2005; Lazar 2007; Sen 2008; Salehi 2009; Seo 2009), and it has been postulated several ways in which microbial activity could improve the properties surrounding oil recovery (table 1); it might lead to increased oil mobilization by increasing oil permeability in carbonate rocks, or plugging of high – permeability channels by biopolymers and/or microbial cell mass which redirects the water flooding. In addition, microbial activity might reduce the heavy oil fractions which alters the oil's composition and makes the oil less viscous. More specific mechanisms for microbial conversion of heavy oils are relatively unknown, but there has been claimed a general mechanism for bioconversion of conventional oils. These mechanisms comprise of cutting of internal linkages within n-alkanes (saturated hydrocarbons) and oxidation of aromatic ring structures by mono- or deoxygenates. In addition, breaking the asphaltenes by cutting internal linkages (sulphides, ethers and esters) splits the compound into smaller molecules and resins are released. These mechanisms produce molecules with lower molecular weight and lower the oil's viscosity. (Leon and Kumar 2005; Østgaard 2005)

Table 1. Microbial effects.
Problems related to oil recovery and production, and how microbial activity affects these properties.
(Leon and Kumar 2005)

Problem	Molecules responsible	How could microbes improve the property?
Viscosity	Asphaltenes Resins	1. Cutting internal linkages of asphaltenes 2. Oxidation of an aromatic ringstructures 3. Inducing lower average molecular weights 4. Untrapping of resins
Catalyst poisoning, corrosion, environmental poultion	1. NSO compounds in the asphaltene structure and in the crude oil matrix 2. Metals as salts, petrophyrins and other asphaltenic complexes	1. Selective removal of NSO compunds without effecting fuel value 2. Degradation of metalloporphyrines 3. Depolymerization of asphaltenes
Soot formation, poor combustion characteristics of products.	Aromatic hydrocarbons	Catalyzing cleavage of various aromatic hydrocarbons and heterocycles.
Deposition	Asphaltenes, wax (alkanes)	Solubilizing and degradation
Undesirable oil/water emulsions	Amphiphilic molecules, especially resins incl. napthenic acid.	Extra cellular component or whole cell as de-emulsifier

1.3 Advantages, challenges and negative side effects surrounding the use of MEOR technology

There are many advantages using MEOR technologies; injected microorganisms and nutrients are easy to obtain, it is inexpensive and the costs do not dependent on oil prices (as the case is for several of the chemicals used in EOR). The implementation of the MEOR process needs little modifications in the existing field facilities. In addition statistical evaluations done in the US shows that 81 % of all MEOR projects demonstrates a positive and gradually increase in oil production. (Lazar 2007).

Despite all advantages, there are some problematic issues surrounding MEOR technologies. Even when designing a good laboratory experiment and producing sufficient positive results, it is not self-evident that the same results would be reproduced when moving up to field scale. During a bioconversion experiment the microorganism are isolated from external exposure, but after being introduced in a reservoir they will have to compete with the more adapted, indigenous microbes and they are likely to be displaced. (Sen 2008) Another major challenge is the great reservoir heterogeneity and the difference in oil complexities. A method used in one reservoir is not necessary optimal for another oil field. (Sen 2008; Brown 2010)

Some negative side effects may also occur during the use of MEOR technologies. This involves souring of the wells by sulphur-reducing bacteria (SRB), unwanted plugging of pores caused by large microbial cells and corrosion. Some solutions to these problems have been discussed; using nitrate-reducing bacteria to oust SRBs. (Brown 2010). To avoid unwanted plugging, the microorganisms injected should have one tenth of the pore entry diameter. (Kotlar 2011). Side effects like economic and technical issues have also been discussed, regarding for example neighbour production wells. If one owner treats his wells with MEOR, it may also result in increased oil recovery for the neighbouring wells, which could lead to both legal and economic issues (Brown 2010)

Even though many reports claims that microbial activity has an influence on enhancing oil recovery, there are still insufficient results from laboratory trials and field trials; the ultimate oil recovery factor is still too low, and most important: too little is known about the biochemical processes involving live bacteria. (Sen 2008)

1.4 Bioconversion of heavy oil

A bioconversion experiment is designed to create the best possible simulation of the physiochemical forces in regard to the properties of the reservoirs. The oil in the shake flasks are exposed to high temperature and shaking, which may cause additional water-soluble fractions to be eluted from the oil (Kotlar 2011) In addition, an optimal environment is arranged for microbial growth.

Several studies performed at Statoil's Research Centre indicate that microorganisms have an impact on heavy oil composition and that bioconversion of heavy oil leads to a decrease in the relative amount of saturated and aromatic hydrocarbons. (Kotlar 2011)

1.5 Aim

The main aim of this Master's project is to characterize consortia with the ability to bioconvert one or several of the selected oils.

Several microorganisms with the ability to bio convert heavy oil fractions have been screened and identified, but there are still much to learn about the microbial biochemistry. This study uses well-known methods for cultivating microorganisms (which have shown promising results in earlier bioconversion experiments (Kotlar 2011)) at simulated reservoir conditions. Molecular biological tools, in addition to oil analysis are used to gain knowledge about possible bioconverting consortia.

2.   Principles of analytical methods

The following chapters comprise of principles for each analysis that has been used to gain knowledge about the results from each of the bioconversion experiments of this Master's project.

2.1   Thin-layer Chromatography with flame ionization detection (TLC-FID)

The method combines chromatographic separation of the SARA fractions (Saturated hydrocarbons, Aromatic hydrocarbons, Resins and Asphaltenes) in each samples by thin – layer chromatography, with ionization and detection by a flame ionization detector. Samples prepared for chromatographic separation is dissolved in a solvent with low polarity and high volatility, in this project; dichloromethane (DCM). This gives the optimal sample spot as the sample immediately adsorbs to the silica surface, and the solvent diffuses. (Wall 2005)

The separation is based on the fractions' solubility in the mobile phases (organic solvents) and their ability to adsorb to the stationary phase. After the sample is spotted onto the polar silica covered rod, the rack is placed into mobile phases with increasing strength and polarity, each for a definite time. Table 2 shows the solvents used in the method, and which solvent that elutes each fraction respectively. When the mobile phase migrates up the rods by capillary forces, it "competes" with the molecules of the fraction with similar polarity for binding sites on the silica gel. The fractions with less affinity to the stationary phase, than to the mobile phase, will be eluted. This leaves the components in a dynamic equilibrium between being completely dissolved in the solvents and adsorbed to the stationary phase. (Wall 2005).

Table 2. Polarity of the solvents used in this project
The solvent used in this project with respective eluted SARA fraction. (Aylward 2002)

Solvent	Hexane	Toluene	DCM : MeOH (95 : 5)	
Polarity	Unpolar	Less polar	More polar	
Eluting	Saturated HC	Aromatic HC	Resins	Asphaltenes*

*Because of the strong affinity to the silica molecules, the asphaltenes are not eluted and stays on the sample spot area.

After chromatographic separation the rods are burned and ionized in a hydrogen flame. An electrode in the detector is disposed above the hydrogen flame and the ionized gas. Between this electrode and the gas burner there is a high voltage, which generates a positive polarity for the burner, and a negative polarity for the collector electrode. This makes negative and positive ions migrates respectively to the burner and collector electrode. An electric current, proportional to the amount of each separated substance, is detected quantitatively and amplified by a detector electrode surrounding the negative electrode. A data processing unit converts the amplified signal and calculates the percentage area of each peak in the run as a percentage of total area of all peaks, which in turn corresponds to the relative amount of each SARA – fraction. (Agilent 2004; Mitsubishi Kagaku Iatron 2007) The peaks are identified as the different SARA fractions based on a retention time specified for each reference peak in the reference method for heavy oil analysis. (Agilent 2004)

It is important to note that analysis on Iatroscan™ MK-6/6s is a very rough and qualitatively method, which only gives an indication on the relatively changes in the oil at fractional level. Every fraction contains many components and because Iatroscan™ MK-6/6s only detects fractional changes marginal changes will not be detected. Because the software computes the relative amount of each fraction in regard to the total amount of the fractions, a reduction of one will automatically lead to an increase in another. Because it cannot be produced even more of one fraction in an oil sample, the interpretation of the TLC- FID results is based on changes showing a fractional decrease.

The analysis is not run against an internal standard because this has not yet been made for the method at Statoil's research centre. (Kotlar 2011)

2.2 Pre – treatment and DNA – extraction of Gram positive bacteria with "DNeasy Blood & Tissue kit"

Pre – treatment of the DNA samples is based on lysis of the peptidoglycan in bacterial cell wall by lysozyme and degrading of released nucleases with proteinase K.(Pelt-Verkuil 2008) Released DNA is centrifuging using a mini spin column with silica covered membrane, and the nucleic acids adsorb to the membrane as the contaminants and enzyme inhibitors are washed through. Addition of buffer solution enables DNA adsorption to the silica molecules by increasing concentration of chaotropic salt and lowering the pH, this dehydrates the anion effect and overcomes the net electrostatic repulsion between the negative charged nucleic acids and the negative charged silica molecules. (Melzak, Sherwood et al. 1996)

In the end, addition of an elution buffer lowers the salt concentration and increased the pH, which releases the DNA from the silica membrane and the DNA is collected in the collection tube. (Qiagen 2006; Clark 2010)

2.3 Concentration measurement of the purified DNA

The aromatic bases of the nucleic acids absorb UV – light, and the structure decides how much light they absorb. As rings in a double helix are stacked together, they shield each other and less UV –light is absorbed, in contrast to free nucleotides, which therefore absorb more UV –light. (Clark 2010) Aromatic rings of DNA have an absorption maximum at 260 nm, with amount of absorbed light being proportional to the concentration of DNA, as described by Beer - Lambert law. (NanoDrop 2006; Nelson 2008)

The purity of the DNA can be determined by measuring its absorbance at both 260 and 280 nm, and computing the ratio between the two wavelengths. Pure DNA has an A260/A280 ratio of 1.8. A ratio over 1.8 indicates presence of proteins, as they have an absorption maximum at 280 nm (mostly because of aromatic rings of tryptophan). Ratios below 1.8 indicate presence of RNA, which has an absorption maximum at 260 nm. (NanoDrop 2006; Qiagen 2006; Clark 2010)

A secondary calculation of the DNA purity is done by measuring absorbance at 230 nm, and computing the A260/A230 ratio. These values are often higher than the respective A260/A280 values and are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants. (NanoDrop 2006) It is important to note that the generally accepted ratio of 1.8 is a "rule of thumb". The actual ratio will depend on the composition of the nucleic acid. (NanoDrop 2006; Clark 2010)

2.4 Polymerase Chain Reaction - PCR

PCR is used to amplify a desired DNA sequence by repeated cycles of denaturation and replication. Two specially designed primers targets specified areas on the template sequence at either ends of the DNA. Taq polymerase from the heat resistant bacteria; *Thermus aquaticus* which is activated at 95° C, is used to elongate the DNA strand. PCR is executed with a Thermo cycler.

The reaction is divided into three general steps: In step one, the DNA double helix is denaturised by heating the DNA at 95° C. This separates the DNA double helix into two single strands. The reaction proceeds into step two were the hybridization starts. The temperature drops down to 55°C and the primers attach to their complementary bases, at the templates 3'- end, which establish the start point for the DNA polymerase. Step three involves polymerization of DNA. The temperature is increased to 72°C and DNA – polymerase binds to the free 3'-OH end, and reads the template in 3'-5' direction, at the same time as deoxyribonucleotide triphosphates (dNTP) elongates the DNA – strand in 5'-3'- direction.

Two new double helices are now made. The temperature holds 72 °C for a while, until it proceeds into a new cycle, to make copies of the two double helices made from the first round. The cycle is repeated approximately 30 times, before it ends, and temperature is decreased to 8 °C. (Clark 2010)

Because PCR is a very sensitive operation there are certain factors surrounding the DNA purity which could lead to non-specific or inhibition of primer hybridization, degrading of enzymes or inactivation of Taq polymerase; hence interfere with DNA amplification. These factors could be co-purified organic substances (poly cyclic aromatic hydrocarbons; PAH's) and heavy metals from heavy oil. (Fortin 2004) Samples for DNA isolation which further will be amplified by PCR should therefore be at a low volume to reduce the chances of co-purifying inhibitors like; proteins, contaminants and other compounds originating from biological experiment. (Pelt-Verkuil 2008).

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is used in this project as a control to examine the amplification results from the PCR. This is to make sure the primer binding was successful and the right PCR products were made.

A positive and a negative electrode are connected to a high voltage source, and the electric current applied to the gel. This makes the DNA fragments migrate towards the positive electrode, because of the negative charge on each phosphate group of the DNA molecules. As the molecules migrate, they are separated by size due to the meshwork of polymer in the gel. The smaller DNA molecules migrate furthest, while the larger ones get retained in the masks. Thus, the DNA molecules are separated based on charge and size.
A fluorescent dye added to the agarose gel will bind to the DNA fragments and light up when exposed to UV – light. This is because the dye absorbs the light, making its own atoms excite and send out photons with longer wavelength. By comparing the fragments in the gel to a standard, which contain DNA – fragments of known sizes, it is possible to decide the sizes of the amplified DNA fragments from the sample. (Clark 2010)

2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis is based on electrophoretic mobility of partial melted DNA molecules in a polyacrylamide gel with a denaturing gradient. The environment is established with a combination of a uniform temperature at 60 °C and a linear denaturing gradient of urea and formamide. (Muyzer 1993)
The separation of the DNA molecules is based on differences in DNA sequences which results in differential denaturing characteristics of the DNA. Depending on the GC ratio, the DNA will melt into segments called "melting – domains", with sequence specific melting temperatures (Tm). As GC pairing consists of three hydrogen bonds compared to AT pairing, with only two hydrogen bonds, sequences with high amount of GC pairs are more stable. This means that the higher the proportion of GC pair, the higher the melting temperature of the DNA molecule. (Clark 2010)
When the DNA double helix reaches its specific melting temperature, the double DNA strands will partially split up into a Y-shaped molecule, and the migration speed of the molecule will decrease, until it fastens in the meshwork of the gel. Because the DNA sequence is specific for each species, each band that is produced in the gel corresponds to different species. When adding a fluorescent dye, which binds tightly to DNA, the fragments can be visualized by exposing them to UV light. This makes the fluorescent light up.(Clark 2010)

3.   Material and work methods

Chapter 3.1 is an overview of the materials used in all experiments, additional tables of chemicals, kits and equipments used in the experiments are enclosed as appendix B (page 90). Chapter 3.2 explain the experimental designs used in each experiment and an account of the work. Chapter 3.3 explains the methods used in the experiments.

3.1 Material

Three experiments was executed in this project, the materials used are microorganisms, oil and growth medium. Different chemicals, reagents, kits and equipments were also used during the project, and are listed in the following chapters.

3.1.1 Inoculum

In this project, three inoculums containing a consortium of microorganisms were used; ML, L004 and MMT006 (Table 3). These three were chosen based on earlier experiments producing promising results. (Kotlar 2011) The origin of these consortia and other information about them are strictly confidential (Statoil), however, the microorganism are isolated from samples taken from oil sand, mud volcano, process waters from water treatment plants and oil related samples from other locations.

Both MMT006 and L004 are enriched on mineral medium with acetate and yeast (MMAcYE) and comprise a mixed population of aerobic and facultative anaerobic microorganisms (Kotlar 2011). As for ML, this inoculum was collected as an environmental sample and has been enriched on mineral medium with acetate and yeast (RMMAcYE; Table 4.) and isolated during this Master's project (Chapter 3.3.1).

Table 3. Inoculum.
Each inocula's respective optimal growth temperature (Kjellsen 2010; Kotlar 2011)

Inoculum	Optimal growth temperature
ML	35-36 °C
L004	60 °C
MMT006	60 °C

3.1.2 Medium

Five different mediums both solid and liquid were used in this project (Table 4).

An environmental sample was enriched and isolated using two different solid media; enriched mineral medium with acetate and yeast (RMMAcYE) and mineral medium with acetate (MMAc). Liquid RMMAcYE was used to create a more nutritious environment for the microorganisms during cultivation and preparation of inoculum for usage in bioconversion experiment

Mineral Medium with acetate (MMAc) and MM were used as growth medium for the inoculum incubated with oil in the bioconversion experiments. Medium with acetate was used to give the microorganisms an extra energy source in addition to the oil. This could also open the possibility of co – metabolism of oil components and acetate. (Markussen 2010-2011) Mineral Medium without acetate or other carbon source was used to select for microorganisms which would prefer an innutritious environment and grow on oil as the only carbon – and energy source.

Table 4. Growth media used in this project.
Mediums listed with aim and contents per Litre. Note that TMS, phosphate solution and vitamin solution are made separately. Recipe is found in appendix A, page 88.

Medium	Aim	Contents (per Litre)
Enriched mineral medium w/ acetate (RMMAcYE, solid)	Cultivation and isolation of environmental sample	Ammonium nitrate (Riedel de Häen / Acrõs organics), calcium chloride (Sigma Aldrich / JT. Baker), magnesium sulphate (Sigma Aldrich / JT. Baker), sodium acetate (Riedel de Häen / Acrõs organics), yeast (Oxoid), tryptone (Oxoid), peptone (Oxoid/ ROTH), MQ - water, bacteriological agar (Oxoid), phosphate solution, TMS (1:1:1), vitamin solution (1:100)
Mineral medium w/ acetate (MMAc, solid)	Cultivation and isolation of environmental sample	Ammonium nitrate (Riedel de Häen / Acrõs organics), calcium chloride (Sigma Aldrich / JT. Baker), magnesium sulphate (Sigma Aldrich / JT. Baker), sodium acetate (Riedel de Häen / Acrõs organics), MQ - water, bacteriological agar (Oxoid), phosphate solution, TMS (1:1:1), vitamin solution (1:100)
Enriched mineral medium w/ acetate (RMMAcYE, liquid)	Re inoculation and cultivation	Ammonium nitrate (Riedel de Häen / Acrõs organics), calcium chloride (Sigma Aldrich / JT. Baker), magnesium sulphate (Sigma Aldrich / JT. Baker), sodium acetate (Riedel de Häen / Acrõs organics), yeast (Oxoid), tryptone (Oxoid), peptone (ROTH), MQ - water, phosphate solution, TMS (1:1:1), vitamin solution (1:100)
Mineral medium w/ acetate (MMAc, liquid)	Bio conversion experiment	Ammonium nitrate (Riedel de Häen / Acrõs organics), calcium chloride (Sigma Aldrich / JT. Baker), magnesium sulphate (Sigma Aldrich / JT. Baker), sodium acetate (Riedel de Häen / Acrõs organics), MQ - water, phosphate solution, TMS (1:1:1), vitamin solution (1:100)
Mineral medium (MM, liquid)	Bio conversion experiment	Ammonium nitrate (Riedel de Häen / Acrõs organics), calcium chlorid (Sigma Aldrich / JT. Baker), magnesium sulphate (Sigma Aldrich / JT. Baker), MQ - water, phosphate solution, TMS (1:1:1), vitamin solution (1:100)

3.1.3 The selected heavy oils used in this project

Three different heavy oils were used in this project. Peregrino oil was used in experiment 1, 2 and 3, and Mariner Maureen and Bressay oil in experiments 2 and 3. Peregrino oil is produced offshore Brazil, and Mariner Maureen and Bressay are both produced on British continental shelf, offshore UK. Table 5 shows the oils and their respective characteristics; °API – value, viscosity and reservoir temperature.

Table 5. Heavy oils.
Heavy crude oils used in this project, listed with respective °API, viscosity at reservoir conditions and respective reservoir temperature. Data supplied by Statoil; (Kotlar 2011)

	Peregrino	Mariner Maureen	Bressay
Oil API	14	14	12
Viscosity at reservoir conditions (cp)	163	65	550
Reservoir temperature (°C)	80	46	37

The heavy oils are not sterilized prior to use in bioconversion experiments because of the many volatile components of the oils. Heating the oils over their respective reservoir temperatures could alter the composition of the oil, as volatile components could evaporate and get lost. The oils are only preheated to approximately their respective reservoir temperature (as listed in table 5). Peregrino oil is preheated to 60° C because of high content of volatile components. (Kotlar 2011) This may ease the handling of the oils when adding oil to the shake flasks. Because of this, endogenous microorganisms might still be present in the oil and may contribute to the bioconversion of the oil both in both the negative controls and the tests.

3.2 Experimental design for the main experiments 1, 2 and 3

To establish some clearance; "experiment 1, 2 and 3" refers to the main experiments conducted in this Master's project. Each of these experiments is divided into sub experiments called bioconversion experiments, with the numbers corresponding to their main experiment; bioconversion experiment 1 under main experiment 1 etc... These bioconversion experiments are conducted in concern to either of the two following experimental designs:

<u>Experimental design of main experiment 1</u>

This design is based on cultivating the inocula; ML, L004 and MMT006 at their respective optimal growth temperature with Peregrino oil, and the aim is to get a qualitative indication of the consortia's ability to bioconvert Peregrino oil at 35°C and 60°C (Table 6).

Table 6. Experimental design for experiment 1.
Displays the temperatures of which the inocula were incubated at in series of cultivation with mineral medium (MM) and mineral medium with acetate (MMAc)

	60°C	35°C
Sample id	Neg.control MMAc	Neg.control MMAc
	Neg.control MM	Neg.control MM
	L004 MMAc	ML MMAc
	L004 MM	ML MM
	MMT006 MMAc	
	MMT006 MM	

Experimental design of main experiment 2 and 3

This design is based on cultivating the inocula; MMT006, L004 and ML at the respective reservoir temperatures of Mariner Maureen-, Peregrino- and Bressay oil, and the aim is to get a qualitative indication of the consortia's ability to bioconvert these three oils. (Table 7)

Table 7. Experimental design for experiment two and three.
The oils and their respective reservoir temperatures of which the inoculums were incubated at in series of cultivation with mineral medium (MM) and mineral medium with acetate (MMAc). Peregrino oil was incubated at 78˚C in main experiment 2, and 60˚C in main experiment 3.

	Peregrino (78/60˚C)	Mariner Maureen (46˚C)	Bressay (35˚C)
	Neg.control MM	Neg.control MM	Neg.control MM
	Neg.control MMAc	Neg.control MMAc	Neg.control MMAc
	L004 MM	L004 MM	L004 MM
	L004 MMAc	L004 MMAc	L004 MMAc
Sample Id	MMT006 MM	MMT006 MM	MMT006 MM
	MMT006 MMAc	MMT006 MMAc	MMT006 MMAc
	ML MM	ML MM	ML MM
	ML MMAc	ML MMAc	ML MMAc

Within all bioconversion experiments there are established negative controls which only contain growth medium (MM or MMAc) and oil. They will be used as comparison for the inoculated oil. The inocula are cultivated using both MM and MMAc which makes it possible to compare growth in consideration to co-metabolism of acetate. (Markussen 2010-2011)

All the bioconversion experiments were executed at aerobic conditions as it is more practical and less time consuming. (Kotlar 2011)

3.2.1 Account of the work and experimental design

The same general workflow was used for all main experiments with certain small adjustments which are denoted in chapter 3.3 Methods. Figure 5 shows each stage of workflow numbered with the chapter for the respective method. Both the method- and the result chapter are based on this workflow.
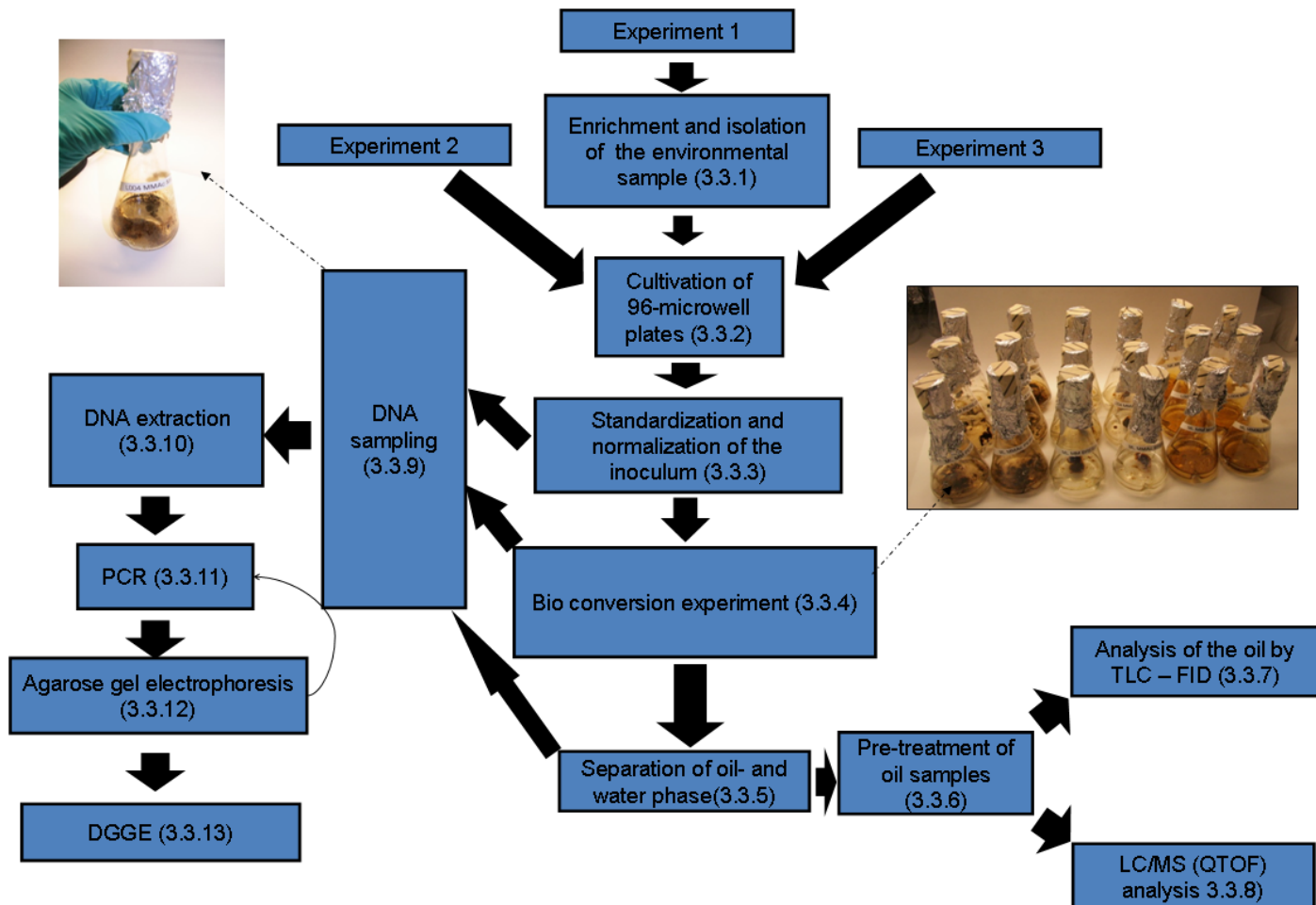
Figure 5. Workflow for each experiment
Showing the extent of each experiment. Each step refers to chapters of method description.

3.3 Methods

Following chapters describe each method used in the bioconversion experiment in addition to the analytical methods which were used for gaining knowledge about the consortia and the compositional changes in the oil. Note that the names and manufacturer for specific equipments used in the following methods are stated only the first time the equipment is mentioned in a section. Table of equipment with respective manufacturers is also enclosed in appendix B, page 90.

3.3.1 Enrichment and isolation of an environmental sample; "ML".

The environmental sample; ML was enriched using an adjusted procedure for isolation of microorganisms developed for use in Statoil and Sintef's BioTHOR project. The procedure with included adjustments denoted A1, A2 etc, is listed below.

<u>Picking colonies into 96-microwell plates</u>

a) Spread extracts from environmental samples onto agar plates of the desired medium. Dilute if necessary. Incubate the plates at 50°C for up to a week. (A1)

b) Study the plates daily. Plates are ready for colony picking as soon as there are enough different types of colonies to pick a full 96-microwell plate. (A2, A6)

c) Label a sterile 96-microwell plate, e.g. MMAcYEL001 HRS/MS ([Type of medium][L (for library)][plate number][your initials/Sidsels initials]). Don't forget to add the new plate to the library list (you find it on the I-disc).

d) Fill each well of the 96-microwell plate with MMAcYE medium (200 µL). (A3)

e) Pick colonies using sterile toothpicks. Try to select colonies with different morphology/from different original samples in order to isolate as many different types as possible. Transfer each colony into one well of the 96-micro well plate by putting the toothpick into the well. Leave the tooth picks in the respective wells until the entire plate is full (this will make it easier to know which wells have already been inoculated).

f) Once the entire 96-microwell plate has been inoculated, remove all the tooth picks with a short stir and put the lid back on the plate. Wrap the plate in paper (blue autoclave paper, this will decrease evaporation) and incubate in a well plate incubator at 50°C, 800 rpm with 85% moisture up to two days. Check the plate after 24 hours. (A4)

g) Replicate the 96-microwell plate by inoculating a new 96-microwell plate containing fresh medium using a 96-microwell plate replicator. This will be the A-plate. Label the new plate e.g. MMAcYE-Loo1A HRS/SM. Incubate the new replica in the same way as the original plate (50oC, 800 rpm with 85% moisture) for 24 hours. (A5)

h) Record growth/no growth, and make a note of which wells of the A-plate that show no growth in the 96-microwell strain library list. Replicate the A-plate by inoculating a new 96-well plate containing fresh medium using a 96-well plate replicator. This will be the B-plate. Label the new plate e.g. MMAcYE-Loo1B HRS/SM. Incubate the new replica, the B-plate, in the same way as the A-plate (50°C, 800 rpm with 85% moisture) for 24 hours. (A5)

i) Add 80 µL glycerol (50%) to each well of the A 96-microwell plate and seal it with sterile plastic film. Freeze the plate at -80°C.

j) Once the strains have shown growth in the B-replica plate, record growth/no growth in the library list and freeze it in the same way as the A-plate. (A7)

Adjustments:

A1. The environmental sample was spread on agar plates of solid RMMAcYE and MMAc, respectively, and incubated at 35˚C as this is the optimal growth temperature for the consortia of ML.

A2. Colonies were picked after incubation for two days and six days, in respective turns to two different 96-microwell plates (Flat bottom, Nunc™, Thermo Fisher Scientific Inc.), which later would serve as master plate no. 1 and 2.

A3. Medium RMMAcYE was used instead of MMAcYE for a more enriched environment.
A4. The 96-microwell master plates were incubated using a reciprocal incubation cabinet (Minitron; Infors AG), at 35 °C, 850 rpm and 60 % humidity for two days.

A5. OD/0.2 mL was measured at 660nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH GmbH) to ensure growth before a robot (Robotic workstation 200, Tecan) was used to re-inoculate two 96-microwell plates (A&B) for each master plate; which further would serve as backup plates. Inoculum (10 μL) was transferred to each backup plates prefilled with RMMAcYE (200 μL). These plates were incubated at the same conditions as the master plate. The master plate was added 80μL glycerol (50 %) and frozen at –80 °C. Records of growth are enclosed in appendix C, page 92.

A6. A third master plate was made by a second round of enrichment using the original agar plates form the first round of enrichment. Colonies were "washed" off by adding MMAc (5mL) to each agar plate, and the cells loosened using a glass rod. The cell suspensions were then diluted in a ten-fold serial dilution (up to10^{-11} dilution). Cell suspensions (0.1 mL) was spread on agar plates (RMMAcYE or MMAc, respectively), further following the same procedure as the other two master plates (no.1 and 2).

A7. Finally, the backup plates showing best measured OD (660nm) were used to manually make a fusion plate of the three master plates, covering all colonies picked. Inoculum (20 μL) was transferred using a automat pipette, from each selected well to a sterile 96-deep well plate (V96 Deep well, MASTERBLOCK, Greiner-Bio-One International GmbH), prefilled with RMMAcYE (800 μL). This plate was incubated over night at 35 °C, 800 rpm and 85 % humidity. Then OD/0.2 mL was measured at 660nm using the same spectrophotometer to ensure growth, and 96-microwell backup plates were made of the fusion plates. This was done by transferring inoculum (150 μL) to new sterile 96-microwell plates prefilled with 75 μL glycerol (60 %). All plates were and frozen at -80 °C. These backup plates were later used in preparation of inoculum for use in the bioconversion experiments.

3.3.2 Cultivation of 96-microwell plates

General procedure

A 96-microwell plate (Nunc™, Thermo Fisher Scientific Inc.) containing the inoculum was thawed. This plate was further shaken up and OD/0.2 ml was measured at 660 nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH GmbH) to ensure growth. Inoculum (10 µL) was transferred to a new 96-microwell plate prefilled with RMMAcYE (200 µL) using a robot (Robotic workstation 200, Tecan). Finally the re-inoculate was incubated using a reciprocal incubation cabinet (Minitron; Infors AG) at 800 rpm and 55-60 % humidity. In addition, a plate containing water was placed under the re inoculate. This was done to ensure the humidity in the system. The incubation temperatures were dependent on optimal growth temperature for each inoculum. Following are some adjustments form the general procedure in consideration to optimal growth temperatures and other experiences acquired during the experiments.

L004 with adjustments

In experiment 1 it was experienced a low yield using the procedure explained previously which resulted in upgrading of the re-inoculum volume to 20 µL into a 96 deep well plate (V96 Deep well, MASTERBLOCK, Greiner-Bio-One International GmbH) prefilled with RMMAcYE (800 µL). The re-inoculate was incubated for four to five days (depending on growth), at conditions mentioned previously and 60°C.

MMT006 with adjustments

Re-inoculation of MMT006 included cultivation on a solid medium because it is difficult to accumulate and experience shows that an agar step gives better growth conditions.

Frozen 24-well plate (Nunc™ polysterene plate, Thermo Fisher Scientific Inc.) containing the sample MMT006 was thawed and inoculum (20 µL) re-inoculated with an automat pipette into a new sterile 24-well plate prefilled with solid RMMAcYE (1.5 mL). After incubating stationary for eight days at 60 °C, the re-inoculate was transferred to a new sterile 24-well plate. This was done by adding a sterile glass ball in each well with RMMAcYE (300 µL) and shaking the cells loose by using a microtiter shaker (IKA microtiter shaker, IKA Works GmbH & C). The cell suspension was transferred to the new sterile 24-well plate. For practical reasons regarding the robot (Robotic workstation 200, Tecan), this plate was used as a reasonable intermediate step as because it was practical for the robot (it cannot transfer cells from agar, only in liquid medium). Inoculum (20 µL) was transferred from each well of the 24- well plate into four wells on a sterile 96-deep well plate (V96 Deep well, MASTERBLOCK, Greiner-Bio-One International GmbH) prefilled with RMMAcYE (800 µL). This was done twice, producing two 96–deep well plates.

The two 96 – deep well plates were incubated at 60 °C, 850 rpm and 50-60 % humidity, reciprocal incubation cabinet (Minitron; Infors AG), and a 96-microwell plate (Flat bottom, Nunc™, Thermo Fisher Scientific Inc.) for five days. Finally, OD/0.2 mL was measured to check the growth by transferring inoculum (200 µL) from the re-inoculated plates to new sterile 96-microwell plates (Flat bottom, Nunc™, Thermo Fisher Scientific Inc), respectively. The rest of the re-inoculum was added (225 µL) to new sterile 96-microwell plates prefilled with 75 µL glycerol (80 %) and frozen at - 80 °C. These plates were used as master plates for preparation of inoculum used in the bioconversion experiments.

ML with adjustments
A master plate containing ML was thawed and re-inoculated (20 µL) into a 96 deep well plate (V96 Deep well, MASTERBLOCK, Greiner-Bio-One International GmbH) prefilled with RMMAcYE (800 µL). The plate was cultivated overnight using reciprocal incubation cabinet (Minitron; Infors AG) under the same conditions as mentioned in the general procedure, but at 60 °C.

3.3.3 Standardization and normalization of the inoculum

<u>Standardization</u>
After the re-inoculate had been incubated at the respective optimal length, inoculum (200 µL) was transferred to a new sterile 96-microwell plate (Flat bottom, Nunc™, Thermo Fisher Scientific Inc.) to measure OD/0.2 ml at 660 nm with a spectrophotometer (FLUOstar Omega, BMG LABTECH GmbH). If it was sufficient growth (meaning values above 0.05 (Markussen 2010-2011)), the robot (Robotic workstation 200, Tecan) was used to transfer a relative amount of inoculum from each well based on the previous measured OD/0.2 ml. This way, each well contributed equally to the standardized inoculum. The inoculum was transferred to eight sterile tubes (ROTH), which was further merged into one single tube (Sarstedt) to be normalized to OD/ ml = 1.

<u>Normalization</u>
Optical density of the standardized inoculum was measured using a 96-microwell plate (Flat bottom, Nunc™, Thermo Fisher Scientific Inc., OD/0.2 ml) to get an indication of the growth. Then the inoculum was centrifuged (Eppendorf 5804; 4000 rpm, 25 minutes). The supernatant was thrown away and the pellet vortexed with Mineral medium (MM, 21.2 mL) and the optical density measured (OD/0.2 ml). The result from the OD measurement was used to calculate the volume of MM which was needed for the dilution of the inoculum to get OD/mL = 1. Calculations are enclosed in appendix D, page 98.

The inoculum was respun at 4000 rpm for 25 minutes, the supernatant was discarded and the pellet was diluted with the calculated volume of MM. OD/0.2 mL was measured after normalization to ensure that OD/mL was 1.

3.3.4 Bioconversion experiment

General procedure

After normalization, inoculum (1 mL) was added to test-shake flasks prefilled with medium; MMAc or MM (48 mL). In addition, the microorganisms in these test-shake flasks were induced with 0.2 % heavy oil (0.1 g). Appendix E, page 103, shows calculations of the percentage oil. Negative controls were also made by adding medium; MM or MMAc (49 mL), in addition to oil (0.1 g). The shake flasks were incubated using a reciprocal incubation cabinet (Incubation shaker; Multitron, Infors AG) at 130 rpm, 55-60 % humidity and temperatures as stated in the experimental design for each experiment in chapter 3.2.

On day three of the experiment, the flasks were added oil up to 2% (1g) and incubated for another four days. Samples (4x1mL) for DNA extraction and backup of whole culture was carried out from the water phase the prior to - (direct from the standardized inoculum) and at the last day of the bioconversion experiment. The samples were centrifuged (Eppendorf 5430 R; 14000 rpm, 20 min), the supernatant was discarded and the pellet was frozen at -80 °C in safe-lock tubes (Eppendorf Biopur®). Samples of whole culture (250 µL) were frozen on 80 % glycerol (750 µL) in cryotubes (Nunc™, Thermo Fisher Scientific Inc.) at -80 °C. Pictures were taken on day three and after the experiment to document the visual appearance of the oil.

Adjustments

During bioconversion experiment in main experiment 1, DNA sampling was only done the first and last day. It was after this experiment that it came to mind that it could be interesting to do a DNA sampling during the bioconversion experiment as well. The same reasoning was made in consideration to the photographs, since no photos were taken before starting bioconversion experiment 1. It was decided to execute this for the following experiments.

Revelation per minute (rpm) was regulated to 110 rpm due to high swirling of the water and oil getting stuck in the cork in experiment 2. It was later discovered that the shake flasks used in experiment 2 had greater baffles than the ones used in the experiment 1.

During experiment 3 there was a lack of reciprocal incubation cabinets, which led to a simple solution of using a shaker platform (Heigar HT) inside a stationary incubation cabinet (BINDER incubator, BINDER, GmBH) in addition to a hybridization oven (ProBlot 125, Labnet International.inc).

3.3.5 Separation of oil- and water phase

After the bioconversion experiment was conducted, the oil and water phase were separated. The water phase was removed using a pipette. More challenging samples containing oil that had emulsified in the water phase were separated using a separatory funnel. The oil left in the shake flasks was further extracted in dichloromethane (DCM), and transferred to beakers.

3.3.6 Pre-treatment of oil samples

Extracted oil was transferred to pre weighed beakers. The beakers containing oil was evaporated in a ventilation cabinet for three days. After the oil had dried out, the beakers were weighed again, and the difference between the beaker with and without oil was calculated, corresponding to the weight of the oil. The oil was then dissolved in DCM, and transferred to volumetric flasks (50 mL). The concentration was calculated as gram oil per millilitre DCM. The oil samples were diluted and prepared for analysis by thin- Layer Chromatography with flame ionization detection (TLC-FID) and LC/MS (Q-TOF), with concentrations of 10 mg/mL and 1 mg/mL, respectively. Table F1, in appendix F, page 105 gives an overview of the weight and the dilution of the oil.

3.3.7 Thin-layer chromatography - flame ionization detection (TLC – FID)

TLC-FID was executed using a standard procedure for analysis of heavy oil fractions (Mitsubishi Kagaku Iatron 2007) as followed;

Oil samples (10 mg/mL) dissolved in DCM (HPLC grade, LabScan), were spotted on Silica Chromarods-SIII (pore diameter 60 Å, particle size 5 μM) using a sample spotter (SES Analysesysteme, GmbH). The sample volume was 1.5 μL which was spotted in repeats of 0.1μL.

Further the samples were eluted in mobile phases with increasing polarity, beginning with n-Hexane (HPLC-grade, Lab-Scan/Merck) for about 20 minutes, until the fluid front reached 100 %. Then in Toluene (HPLC grade, Merck, VWR) for about 10 minutes, until the fluid front reached 60 % and finally in DCM: MeOH (95:5, HPLC grade, Lab-Scan) for about 2 minutes until the fluid front reached 30 %. The rods were air dried for 2 minutes between the solvent exposures.

After chromatographic separation, the Chromarods were turned, and the rack placed into the cabinet of an instrument with flame – ionization detector (Iatroscan™ MK-6/6s, Mitsubishi Kagaku Iatron, Inc.). The samples were scanned using FID hydrogen and air flow rates at 160 mL/min and 2000 mL/min, respectively. Scanning speed was 40 sec/ scan. Samples were separated based on the SARA fractions and the results were processed in a software program specified for chromatographic analysis. (Agilent ChemStation for GC-Systems, Agilent Technologies)

TLC-FID analysis was not run against an internal standard because this has not yet been made for the method at Statoil's research centre. (Kotlar 2011)

3.3.8 LC/MS (QTOF)

Because of a delay at Sintef, the results for LC/MS (Q-TOF) analysis arrived too late for processing. Raw data are enclosed in appendix K, page 126. For method description; contact Anders Brunsvik, Sintef Materials and Chemistry, Trondheim.

3.3.9 DNA sampling

DNA was sampled prior to the bioconversion experiment; from the standardized inoculum (Pre bio) and after ending the bioconversion experiment (Post bio). Samples (1 mL) was centrifuged to a pellet using centrifuge 5430 R (Eppendorf), the supernatant was discharged and the pellet frozen at - 20 °C.

Adjustments;
After running bioconversion experiment 1, it came to mind that it could be interesting to do a DNA sampling during the experiment as well. It was decided to do a DNA sampling day three, before addition of the last amount of oil up to 2%.

3.3.10 DNA extraction

DNA samples were pre-treated for extraction of gram positive bacteria, and purified using the "Animal Tissues Spin column Protocol", from DNeasy® Blood and Tissue kit (Qiagen 2006). See appendix I, page 122 for full procedure.

3.3.11 Measuring DNA – concentration

Concentration was measured for the purified DNA, on a spectrophotometer (NanoDrop® Spectrophotometer, ND – 1000, Thermo Fisher Scientific Inc.) at wavelengths 260 and 280 nm. The ratio between these two wavelengths was calculated by the software program and represented the purity of the DNA.

3.3.12 Polymerase Chain Reaction (PCR)

PCR was executed using primers of 16S rRNA from both archaea and bacteria, in two steps; regular PCR and PCR to DGGE. Primers used for each reaction including product size are listed in table 8. Cycles of the conducted PCR program are shown in figure 11.

Table 8. PCR methods.
Each method is listed with each 16S– rRNA primers used amplifying DNA from either archaea or bacteria in addition to the primer and product size.

Method	Comment	Forward primer and length (bp)	Reverse primer and length (bp)	Product (bp)
PCR Bacteria	"regular" PCR	341 f Bac (17 bp)	907 r Bac (21 bp)	566
PCR Bacteria to DGGE	Products run with DGGE	341 f Bac clamp (57 bp)	907 r Bac (21 bp)	606
PCR 1st step Archaea	Products used in Archaea 2nd step.	Arch 20 f mod (19 bp)	Arch 958 r mod (19 bp)	938
PCR 2nd step Archaea	Products run with DGGE	PARCH 340clamp f (58 bp)	PARCH 519 r (15 bp)	193

DNA samples were mixed with master mix which was pre-prepared from the recipe described in table 9 and 10. The DNA Mass ladder was used as a standard to qualitatively check the DNA fragments produced. Size markers are shown in figure 6.
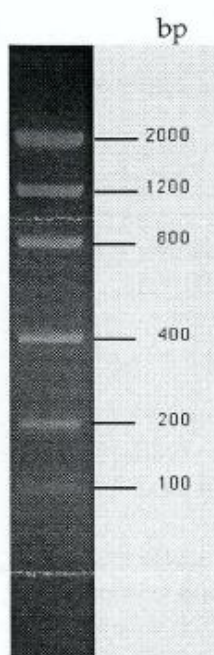
Figure 6. Low DNA Mass Ladder (Invitrogen®).
Showing the molecular weight of the DNA fragments of DNA Mass ladder, when loaded 4μL to a 2% agarosegel. The fragments range from 2000 bp down to 100 bp.

Table 9. Master mix bacteria.
Standard recipe one sample, for regular PCR

Bacteria	
Reagens	**Volume**
PCR buffer	5 µL
dNTP (10mM)	1 µL
Primer F (50µM)	0.5 µL
Primer R (50µM)	0.5 µL
HotStarTaq Polymerase	0.25 µL
dH$_2$O	40.75 µL
Total volume	48 µL

Table 10. Master mix archaea
Standard recipe one sample, for regular PCR

Archaea	
Reagens	**Volume**
PCR buffer	5 µL
dNTP (10mM)	1 µL
Primer F (50µM)	0.5 µL
Primer R (50µM)	0.5 µL
HotStarTaq Polymerase	0.25 µL
Bovine Serume Albumin (BSA)*	2.5 µL
dH$_2$O	38.25 µL
Total volume	48 µL

Regular method
48 µL master mix was added to DNA samples (2 µL) and PCR was run on a Thermo cycler (Eppendorf), using the program Hotstart Regular (table 11).

Adjustments
Some adjustments were made because of poor result when conducting regular PCR. Adjustments were based on three different theories; 1. Too little PCR product applied to the gel, 2; too low DNA concentration or 3; inhibiting factors blocking primer binding.
The solutions that was tried were; 1.doubling of the PCR product, 2; upgrading of the DNA concentration by addition of master mix (45 µL) to DNA (5 µL) Volume dH$_2$O used for master mix preparation was in this case reduced to 38.25 µL.
The last solution that was tried was 3; dilution of the original DNA samples 1:10 or 1:100 in dH$_2$O. DNA (2 µL) was added to master mix (48 µL).

Table 11. Cycles of PCR.
Illustrates the cycles of PCR used to amplify bacterial and archaeal DNA. Steps 1-5 are repeated 30 times, before moving into step 6.

Step	Temperature	Time (min)
1: Denaturation	95˚C	4 min
2: Denaturation	95˚C	1 min
3: Annealing	55˚C	1 min
4: Extension	72˚C	3 min
5: Extension	72˚C	7 min
6: Cooling	8˚C	∞

3.3.13   Agarose gel electrophoresis

Agarose gel electrophoresis was used as a control to ensure that the right PCR products were made with fragments of the expected length.

Amplified PCR product and a standard (Low DNA Mass Ladder, Invitrogen, Life Technologies)was added gel loading buffer, and loaded on a agarose gel (2 %, Carl ROTH GmbH) stained with a fluoridised dye (SYBR®Safe DNA gel Stain, 10 000 X, Invitrogen, Life Technologies). Then the gel was placed into a gel container with TBE buffer (0.5 X, Prime), and coupled to an electroporator (Consort E132, PowerPac™Basic, Bio-Rad Laboratories.Inc). The agarose gel was conducted with 150 V, for 1.5 hours.
Finally the gel was exposed to UV – light, in a photo system (Bio imaging systeme, Gel-Doc, Syngene, Synoptics Ltd.), and the fragments' size in the gel was compared to the known fragments of the standard.

3.3.14   Denaturing gradient gel electrophoresis (DGGE).

DGGE was used to analyze the biodiversity in the DNA samples isolated from the bioconversion experiments. Procedure is supplied by Statoil, and is as followed;

Procedure
DGGE for Bacteria was performed with 6% (w/v) polyacrylamide gels in TAE buffer (1X; Tris-Acetate (40 mM), pH 7.5; acetate (20mM), EDTA (1mM)) with a 20-70 % gradient of the denaturing agents; urea and formamide in a DCode™ Universal Mutation system (BioRad).

The method was divided into two days work. Denaturing solutions of concentrations 20 % (w/v) and 70 % (w/v) was prepared day one (Appendix B, page 90), simultaneously as the perpendicular gel sandwich was assembled. A gradient delivery system (Model 475, Bio-Rad Laboratories Inc.) was used to mould the gel in the gel sandwich, in a gradient ranging from 20-70 %. Combs were inserted, and the gels wrapped in plastic to prevent desiccating of the gel. The gel container (DCODE™System) was filled with (1X) TAE (7 L, Eppendorf), and ready for use the next day.

On day two, the gels were assembled in the gel holder, simultaneously as the gel container (DCODE™System) was preheated to 60 ˚C. 350 mL TAE buffer (1X) was transferred from the gel container to the gel holder and the gel holder was placed inside the container. PCR samples (10 µL) were mixed with DNA gel loading buffer (10 µL, Invitrogen) and the DNA mass Ladder (1 µL, Invitrogen) was mixed with 19 µL DNA gel loading buffer (10X, Invitrogen). The wells were washed with TAE buffer (1X, Eppendorf) and prepared samples (20 µL) were loaded to the gel in addition to the DNA Mass Ladder (20 µL, Invitrogen). The gel was coupled to an electroporator, and conducted for four hours and fifteen minutes with 130 V. Then, the gels were stained using a nucleic gel stain (SYBR®Gold nucleic acid stain, Invitrogen) for forty minutes and the DNA fragments visualized in a Bio imaging system (Gel-Doc, Syngene).

4.   Results

Following are the results produced from each of the main experiments. Results from each analysis are separated based on the main experiments to make it more understandable. The chapters follows the work flow introduced in chapter 3.2.1, figure 5.

At the end of each bioconversion experiment, a visual inspection based on oils appearance in the water phase was conducted. The graduation is based on a scale illustrated in table 12.

It should be noted that is a crude and subjective method, and will only serve as an indication of bioconversion.

Table 12. Visual inspection of heavy crude oil.
Explains the visual gradation criteria for each phase.

Gradadion of oil	The oil's appearance in the water
Negative	Oil and water separated in phases
1+	Smaller breakage of the oil slick
2+	Splitting of the oil into even smaller oil lumps
3+	Formation of minor grains in the water
4+	Total emulsification in the water

4.1   Main experiment 1

The aim of experiment 1 was to give a qualitative indication of bioconversion of Peregrino oil by inocula; ML, L004 and MMT006. Following are each stage of the main experiment.

4.1.1 Enrichment and isolation of environmental sample "ML".

After enrichment and isolation of environmental sample ML, optic density (OD/0.2mL) was found to be sufficient for all of the three master plates, backup plates and the fusion plate. This was based on a "rule of thumb" which indicates that readings over 0.5 indicate growth and measurements between 0.05 and 0.5 indicates poor growth. Results lower than 0.05 are encountered to be back ground noise. (Markussen 2010-2011) OD measurements are enclosed in appendix C, page 92.

4.1.2 Cultivation, standardization and normalization of the inoculum

Optic density (OD/0.2mL) was considered sufficient for the produced inoculum, based on that most of the readings were over 0.05. Variance in the growth is impossible to avoid because the inoculum is a consortium of microorganisms. OD measurements are given in appendix C, page 92.

Optic density (OD/0.2mL) measured before normalization was very poor for ML compared to the other two inocula. Because of this and poor total volume yield, standardization was executed twice for ML. This did not work and it was decided to decrease OD/mL to 0.9 instead of 1, to ensure enough inocula for the bioconversion experiment. After normalization, measurements of optic density (OD0.2/mL) were too low for ML and there were a possibility that there had been a personal misreading and calculation fail when diluting the inoculum. Measurements were considered sufficient for MMT006 and L004. OD measurements are enclosed in appendix C, page 92.

4.1.3 Bioconversion experiment

Each shake flask of the bioconversion experiment was added 0.1 g oil (corresponding 0. 2 % oil) and later increased to total 2 % oil. This was done to create an even starting point for the shake flasks. Because of the high viscosity it was challenging to transfer the correct amount of the oil to the shake flasks, which resulted in giving some of the shake flasks an uneven starting point compared to the rest. Even so, the total amount of oil was close to 2 % for all of the shake flasks after addition of the rest of the oil. Table and calculation of percentage oil added to each flask is enclosed in appendix E, page 103.

4.1.4 Separation of the oil- and water phase and preparation of oil samples

After ending the bioconversion experiment the oil was visually inspected and graded based on its appearance in the water phase (Table 13). Visual inspection criteria are shown in table 11. The most promising results, based on visual inspection are shown in figures 7 – 10. Pictures were unfortunately not taken before starting the experiment, thus there are no pictures showing the oil's appearance in the medium before bioconversion.

Table 13. Visual inspection of Peregrino oil from bioconversion experiment 1
Oil in water is graded for both negative controls and tests with mediums (MM and MMAc). Gradation is based on visual inspection criteria in table 12.

	Set up	Gradation
35°C	Neg.control MMAc	Neg
	Neg.control MM	Neg
	ML MMAc	1+
	ML MM	Neg
60°C	Neg.control MMAc	Neg
	Neg.control MM	Neg
	L004 MMAc	1+
	L004 MM	Neg
	MMT006 MMAc	Neg/1+
	MMT006 MM	Neg/1+

As can be read from the table, there is little visual evidence of bioconversion in either of the tests or negative controls.

Bioconversion experiment with Peregrino oil and MMAc at 35˚C
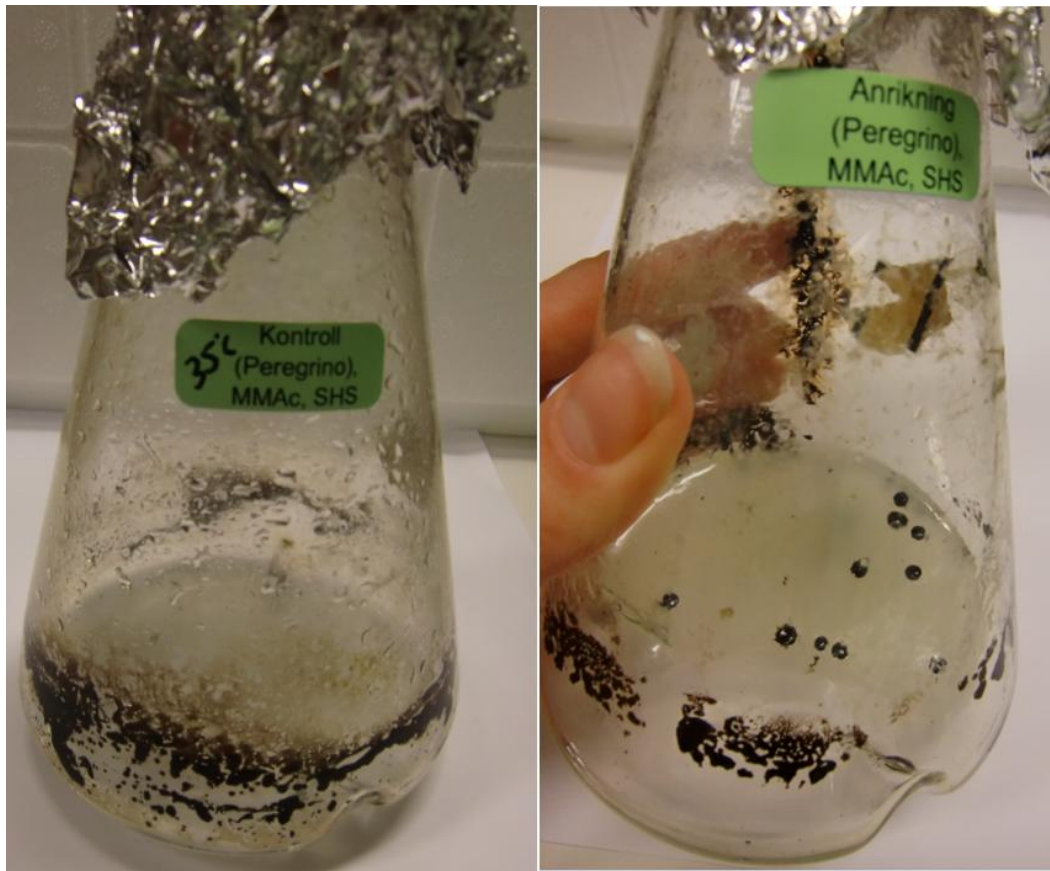
Figure 7. Bioconversion experiment with Peregrino oil and MMAc at 35˚C, day 3.
Showing negative control MMAc to the left and ML MMAc to the right, day three of incubation

Results shown smaller changes in the test ML (MMAc).
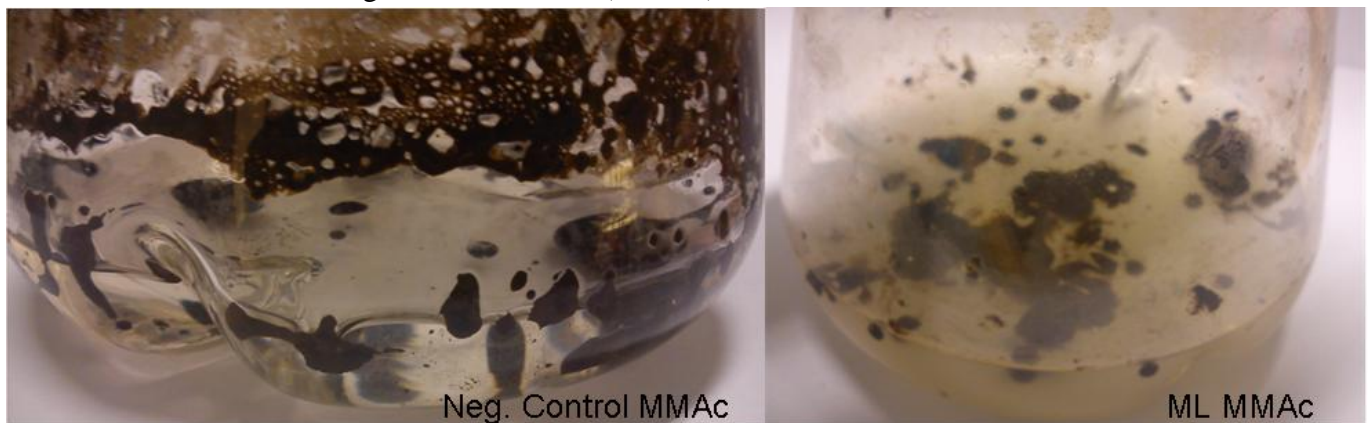
Figure 8. Bioconversion with Peregrino oil and MMAc at 35˚C, day 7.
Showing negative control MMAc to the left and ML MMAc to the right, day three of incubation

Pictures show that there are minor indications of growth in the test ML (MMAc).

Bioconversion experiment with Peregrino oil and MMAc at 60˚C
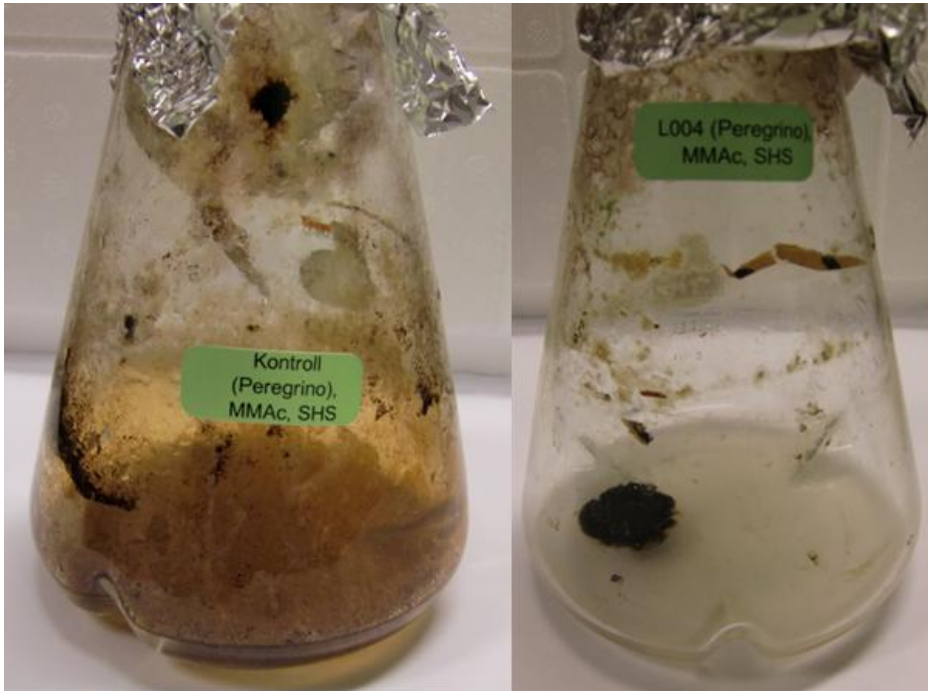
Figure 9. Bioconversion experiment with Peregrino oil and MMAc at 60˚C, day 3.
Showing negative control MMAc to the left and L004 MMAc to the right, day three of incubation

Smaller differences are observable between the negative control and the test L004 (MMAc).
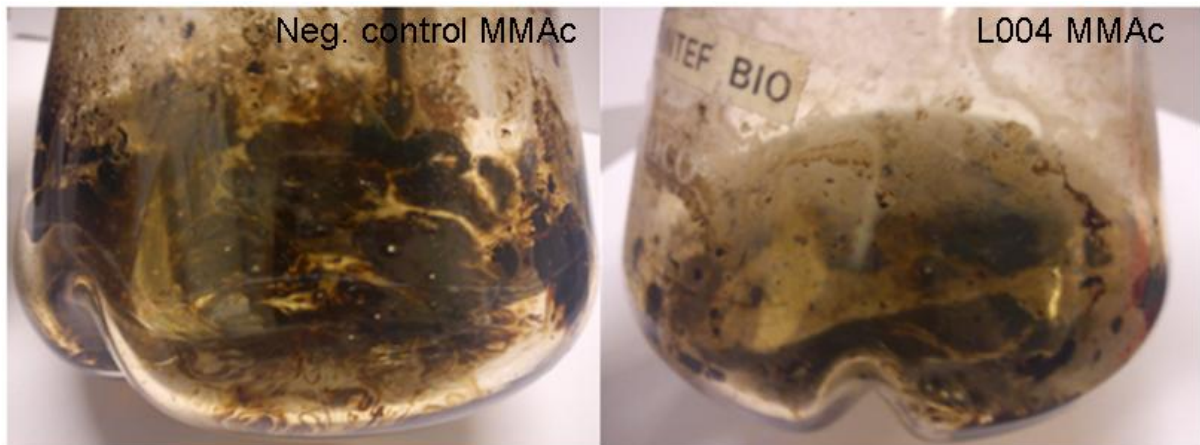
Figure 10. Bioconversion experiment with Peregrino oil at 60˚C, day 7.
Showing negative control MMAc to the left and L004 MMAc to the right, day seven after ending of incubation

Comparison between the oil in the negative control and the test L004 (MMAc) shows little variation.

4.1.5 Thin-layer chromatography with flame ionization detection (TLC-FID)

The oil from the bioconversion experiment was analyzed by thin–layer chromatography with flame ionization detection to give an indication of bioconversion. Comparison was done between the results from native oil and negative control, whereas the tests were compared to the negative control. The results, computed and processed, are illustrated in figures 11-14. Calculation of standard deviation (SD) for each sample conduced with TLC-FID is enclosed in appendix G, page 108.

Bioconversion experiment with Peregrino oil and MM at 35˚C
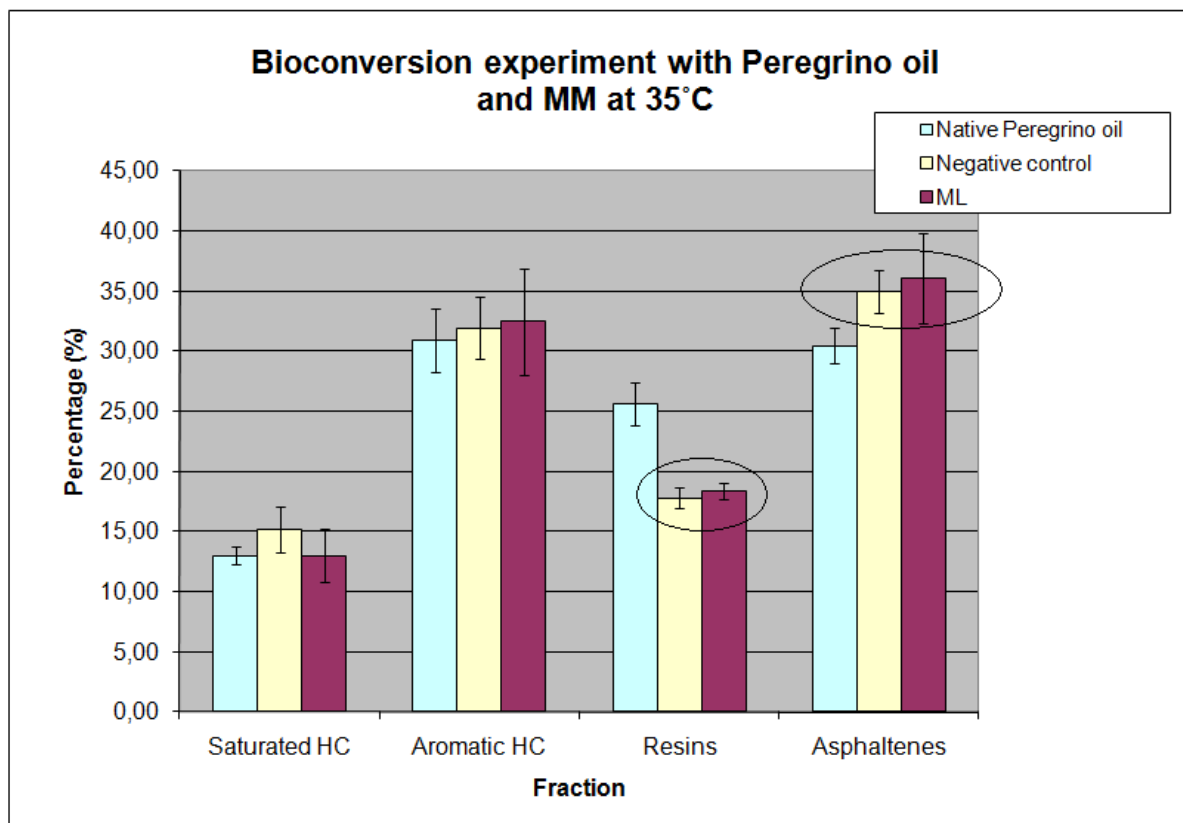
Figure 11. Bioconversion experiment with Peregrino oil and MM at 35˚C.
Comparison of native Peregrino, negative controls and the ancillary tests (inoculum ML). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relative differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relative to the total amount of fractions in the respective sample. Standard deviation is set as y and x error bars.

As figure 11 shows there is a significant reduction of the relative amount of resins in the negative control compared to native Peregrino oil, which results in an increased level of the relative amount of asphaltenes. No significant changes can be observed when comparing ML with the negative control, as the relative amounts of each fraction are equal. This indicates that there has been a change in oil composition due to physiochemical forces during the bioconversion experiment, which could have led to an elution of water-soluble resin fractions from the oil phase. In other words this indicates that the microorganisms did not have an impact on the conversion of the oil.

Bioconversion experiment with Peregrino oil and MMAc at 35˚C
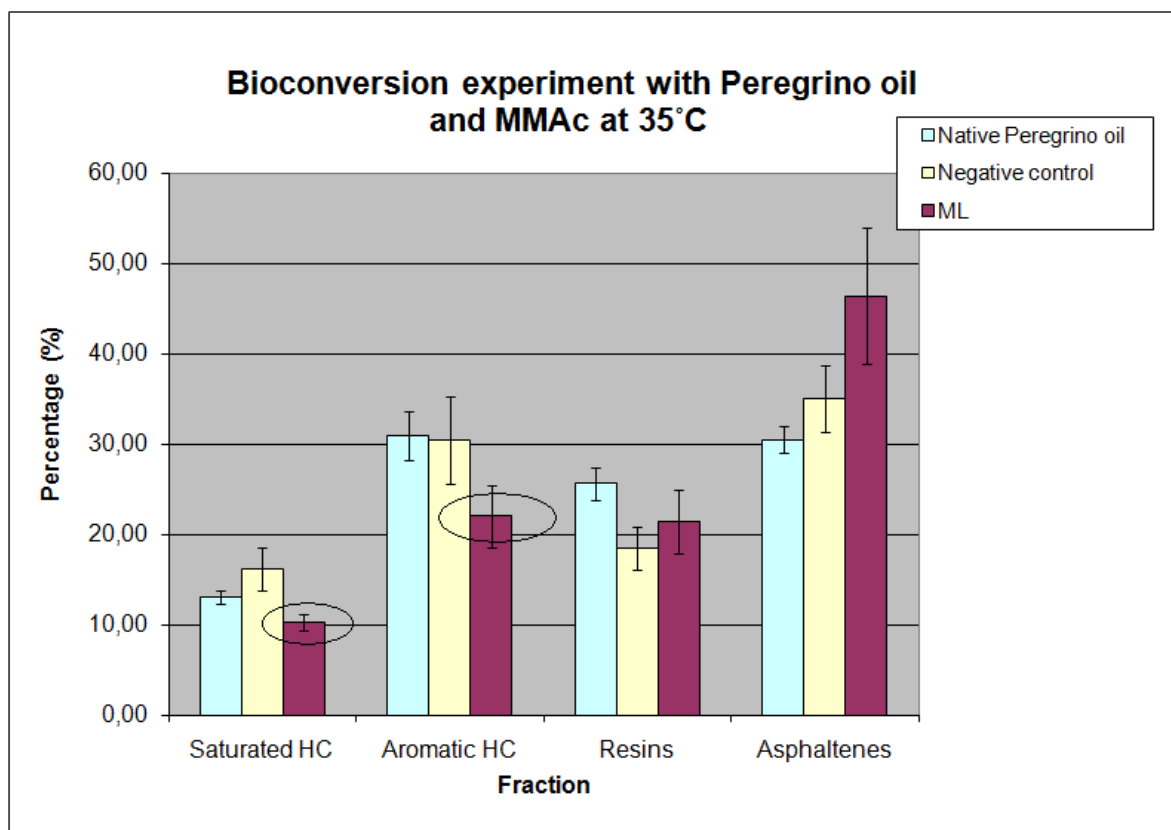
Figure 12. Bioconversion experiment with Peregrino oil and MMAc at 35˚C.
Comparison of native Peregrino, negative controls and the ancillary tests (inoculum ML). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

Results in figure 10 shows a significant decrease in the relative amount of saturated and aromatic HC for sample ML compared to the negative control, which in turn is relatively equal to the native Peregrino oil. This indicates that there has been a microbial influence on the composition of the oil.

Bioconversion experiment with Peregrino oil and MM at 60˚C
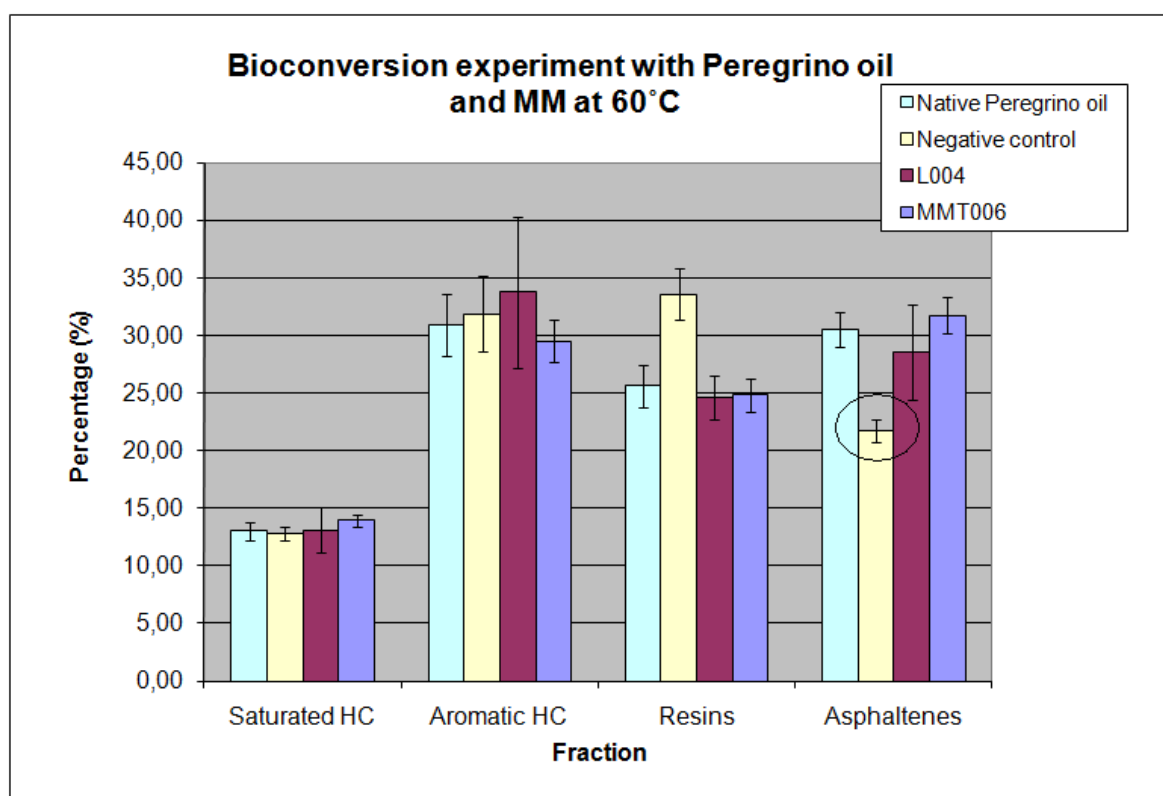
Figure 13. Bioconversion experiment with Peregrino oil and MM at 60˚C
Comparison of native Peregrino, negative controls and the ancillary tests (inocula L004 and
MMT006). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s).
SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively
differences between the native Peregrino oil and negative control, and the difference between the
negative control and the test. Y-axis shows the average percentage of each fraction relatively to the
total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

These results indicate that there has been a fractional change in the negative control due to
physiochemical forces, whereas the oil in the tests seem to have the same relatively
composition as the native oil. If the changes in the negative controls were caused by
physiochemical forces the results should also have indicated the same for the tests.

In conclusion it is difficult to say anything about the changes in the oil fractions of these
samples. These results should have been controlled by reanalyzing the samples if there were
more time.

Bioconversion experiment with Peregrino oil and MMAc at 60˚C
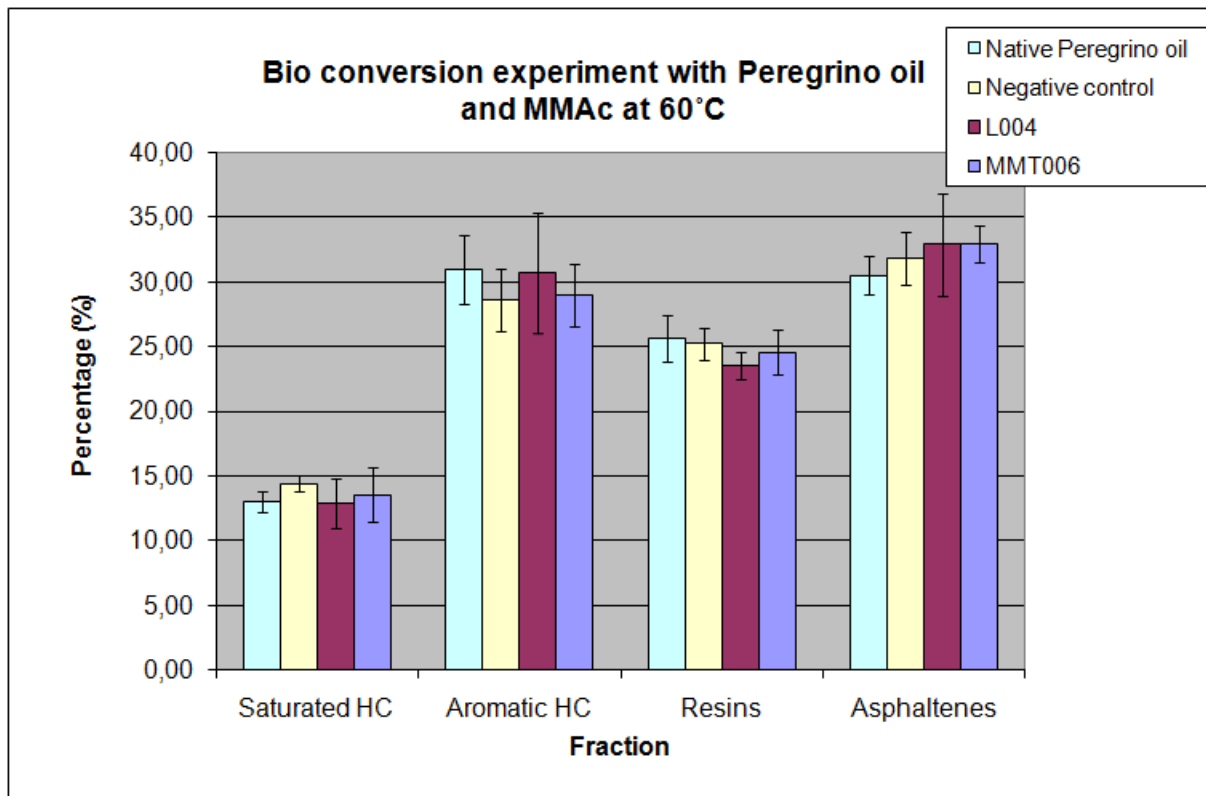
Figure 14. Bioconversion experiment with Peregrino oil and MMAc at 60˚C.
Comparison of native Peregrino, negative controls and the ancillary tests (inoculum ML). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

The results in figure 14 show no indication of fractional changes as both negative control and the tests have similar relative composition of the oil fractions.

Evaluation of standard deviation (SD)

The relative SD for several of the results was higher than the reference method (5 %). (Appendix G, page 108.) High SDs could result from several reasons like uneven sample application and uneven time of elution. It has also been shown that FID responses vary between different sets of Chromarods and from rod to rod within a set. (Shantha 1992) Suggestion has been made upon matching and selecting rods with similar response characteristics. Even though this could be a time – consuming and tedious method it could lower the variation between the rods. (Shantha 1992)

Considering that SD for several of the resulting peaks were high, one sample was analyzed using all the ten Chromarods to give an indication of the precision of the Chromarods (Table 14).

Table 14. Precision Chromarods used in the SARA method.
Sows the frequency between the relative amounts of each SARA fraction analyzed and computed by thin – layer chromatographic analysis. Sample analyzed is native Bressay Oil.

Chromarod No.	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	17,48	49,89	27,22	5,42
2	18,52	52,48	23,03	5,98
3	18,60	54,01	22,78	4,61
4	17,33	50,64	26,24	5,79
5	17,66	54,67	22,36	5,31
6	18,64	49,42	24,79	7,15
7	21,74	53,95	20,74	3,57
8	18,23	52,15	23,37	6,25
9	15,05	53,72	25,06	6,17
10	17,34	52,79	24,30	5,57
X	18,06	52,37	23,99	5,58
SD	1,668	1,836	1,926	0,974
CV (%)	9,235	3,507	8,029	17,441

The table shows that there is an internal variation between the Chromarods used in the TLC-FD analysis of oil in as three of the rods have a relative standard deviation over 5 %.

Had there been more time, TLC-FID results would have been conducted using several more parallels for each sample to lower the SD.

4.1.6 Liquid Chromatographic – Mass Spectrometry (LC-MS)

The LC/MS (QTOF) results did not arrive in time for processing because of a delay at Sintef Raw data are included but not interpreted. (Appendix K page 126)

4.1.7 DNA – extraction and measurement of DNA concentration

After extraction, DNA concentration was measured using NanoDrop® Spectrophotometer at two wavelengths; 260 and 280 nm (Appendix J, page 124). The ratio between these two wavelengths was calculated by the software program and gave an indication of the purity of the DNA. The resulting A260/A280 ratios for the purified the DNA was either over or below 1.8, which indicated the presence of co-purified proteins, phenol or other organic contaminants. (NanoDrop 2006; Qiagen 2006; Clark 2010).

4.1.8 Control of PCR amplified DNA with agarose gel electrophoresis

PCR for the detection of bacteria and archaea was conducted, and the products from each reaction were controlled with a standard of known molecular weight markers (DNA Mass Ladder, Invitrogen®) on agarose gel (2%).

Regular method of bacteria PCR did not produce any positive results; and because of this PCR was conducted with samples of diluted and upgraded DNA concentration. This produced sufficient positive results for both PCR bacteria and bacterial PCR bacteria to DGGE (Figure 15). The producing of positive results when upgrading or diluting the DNA concurs with the poor A260/A280 and A260/A230 ratios computed from measured DNA concentration. (Appendix J, page 124)
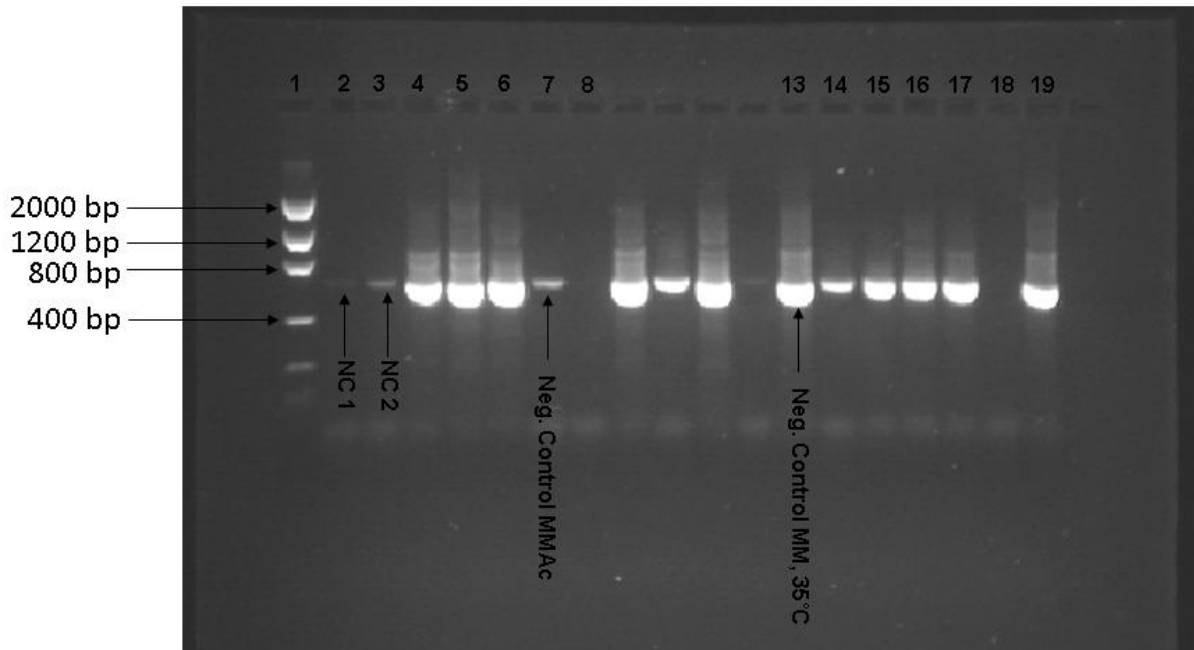
Figure 15. Bacteria PCR to DGGE.

The picture displays an agarose gel with results from PCR conducted with 16S-rRNA primer set "341 f Bac clamp" / "907 r Bac" DNA Mass Ladder (Invitrogen) in well 1 to the left with molecular weight markers of 400 and 2000 base pairs pointed out. DNA samples are listed in table 15.

Table 15. Bacteria PCR to DGGE.

Setup for agarose gel in figure 15. Negative controls from DNA purification (NC), pre bio- and post-bioconversion samples for each media serie (MM and MMAc). Negative control MM & MMAc, 35˚C including ML; corresponds to bioconversion experiment run at 35˚C. The others correspond to bioconversion experiment run at 35˚C.

Well	Sample id.	Well	Sample id.
1	DNA ladder	11	MMT006 MMAc (Post bio)
2	NC 1 (DNA purification)	12	MMT006 MM (Post bio)
3	NC 2 (DNA purification)	13	Neg.control MMAc, 35˚C
4	ML (Pre bio)	14	Neg.controlMM, 35˚C
5	L004 (Pre bio)	15	-
6	MMT006 (Pre bio)	16	-
7	Neg.control MMAc (Post bio)	17	-
8	Neg.control MM (Post bio)	18	ML MMAc (Post bio)
9	L004 MMAc (Post bio)	19	ML MM (Post bio)
10	L004 MM (Post bio)	20	-

Figure 15 shows several positive results with DNA fragments found between the molecular weight markers of the DNA ladder; 400 and 800 bp. This corresponds to the expected size of 606bp.There were also several larger DNA fragments, possibly due to non-specific primer hybridization. Some of the negative controls from the bioconversion experiment and DNA purification indicate positive results, which could mean that there has been a contamination during the bioconversion experiment or DNA purification.

Two samples which did not produce any positive results when upgrading DNA were; ML (MMAc) and negative control MMAc 35˚C. As a last check these samples were diluted (1:10) and PCR for Bacteria to DGGE conducted using the primer set "341 f Bac clamp" / "907r Bac". Because this did not result in any positive findings, the negative control was established to be negative (as it was expected to be). As for the sample ML (MMAc); measured DNA concentration was 446, 41 ng/µL, with an A260/A280 ratio of 1.16 and A260/A230 ratio of 0.57 (Appendix J, page 124). These ratios indicate the presence of RNA or co-purified contaminants respectively, and could mean that there was a high concentration of inhibitors blocking primer hybridization. Because of lack of time, the attempt to optimize ended here. Had there been more time the next step would have been to dilute ML MMAc even more before conducting PCR. Even so, both samples were conducted on DGGE confirm the results.

1st step of PCR for the detection of archaea was conducted using 16s rRNA primers "Arch 20 f mod" and "Arch 958 r mod", with expected product of 938 base pair. Results were poor, as there were several non-specific fragments. DNA was upgraded and PCR Archaea 1st step was conducted again. Neither this method produced any positive results, which could mean that there were no archaeal DNA in the original DNA samples or it could be co-purified contaminants blocking primer hybridization, and based on this, the DNA was diluted (1:10) before conducing PCR Archaea 1st step again. When this neither produced any positive results it was concluded to stop the search for archaeal DNA as the time limit was reached. If time had not been an issue, the next trial would have been to use BSA instead of dH_2O-water to check if these could help produce positive results from the PCR, as BSA could block inhibiting factors.

4.1.9 DGGE

To characterize the biodiversity in each DNA sample, PCR products were conducted on Denaturing Gradient Gel Electrophoresis (DGGE).

The analysis and interpretation of DGGE results are based on inspection of the gel images and comparison of the the diversity patterns of the ancillary samples; pre–and post bioconversion for both media series (MM and MMAc). Were samples pre-bio corresponds to DNA samples prior to bioconversion experiment, whereas post-bio corresponds to DNA sampled after the bioconversion experiment. Both clear and shadow –like fragments, which are a bit difficult to determine, are marked with arrows in the pictures (figure 16 – 20).

Bioconversion experiment with Peregrino oil inoculated with ML (MM) at 35°C
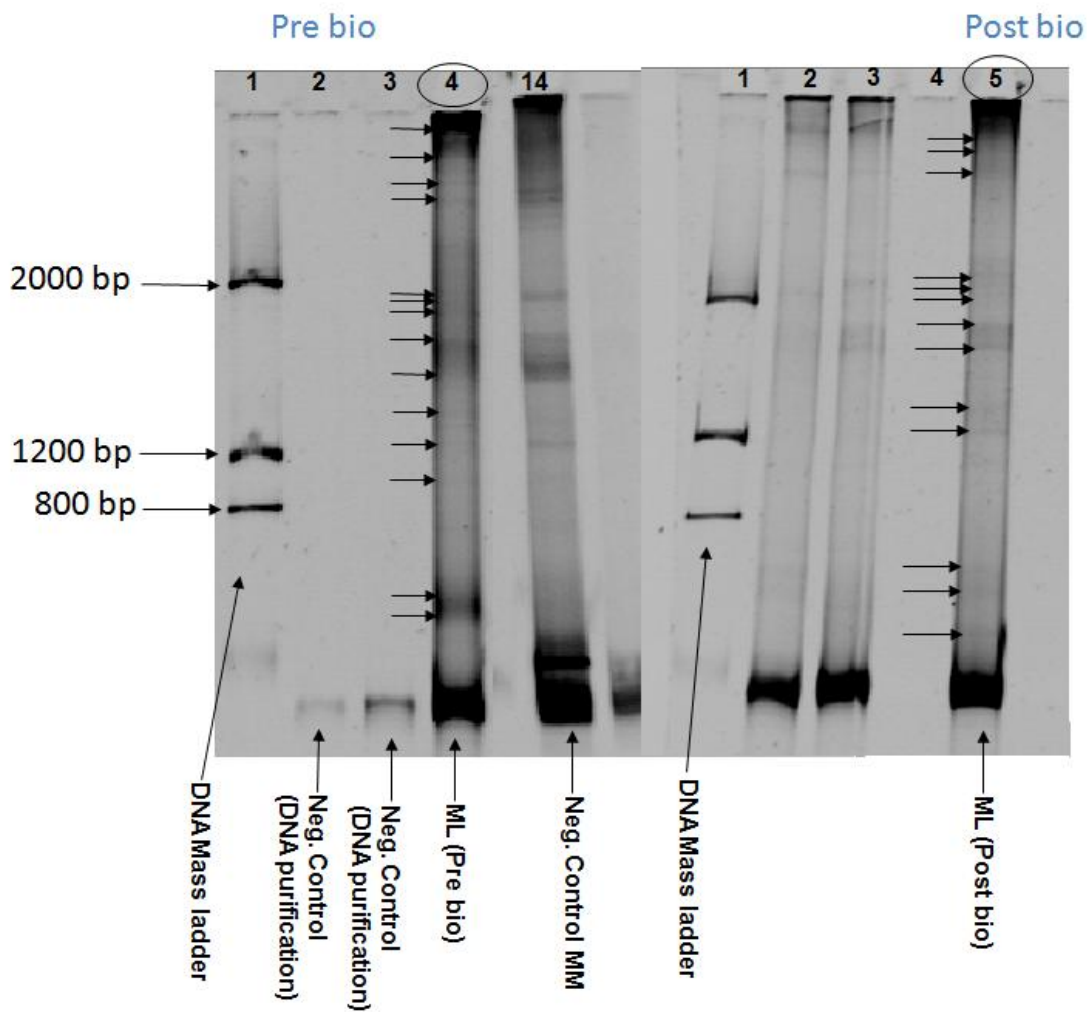
Figure 16. DGGE Bacteria for ML incubated with MM at 35°C.
The picture displays two polyacrylamide gels assembled for comparison. Samples; ML (Pre bio) and ML (Post bio) are marked with rings.

As figure 16 displays DNA was found in the negative control MM. This indicates contamination as stated previously in chapter 4.1.8, and the results were discarded. Had there been more time, the experiment would have been redone.

Bioconversion experiment with Peregrino oil inoculated with (ML)MMAc at 35˚C
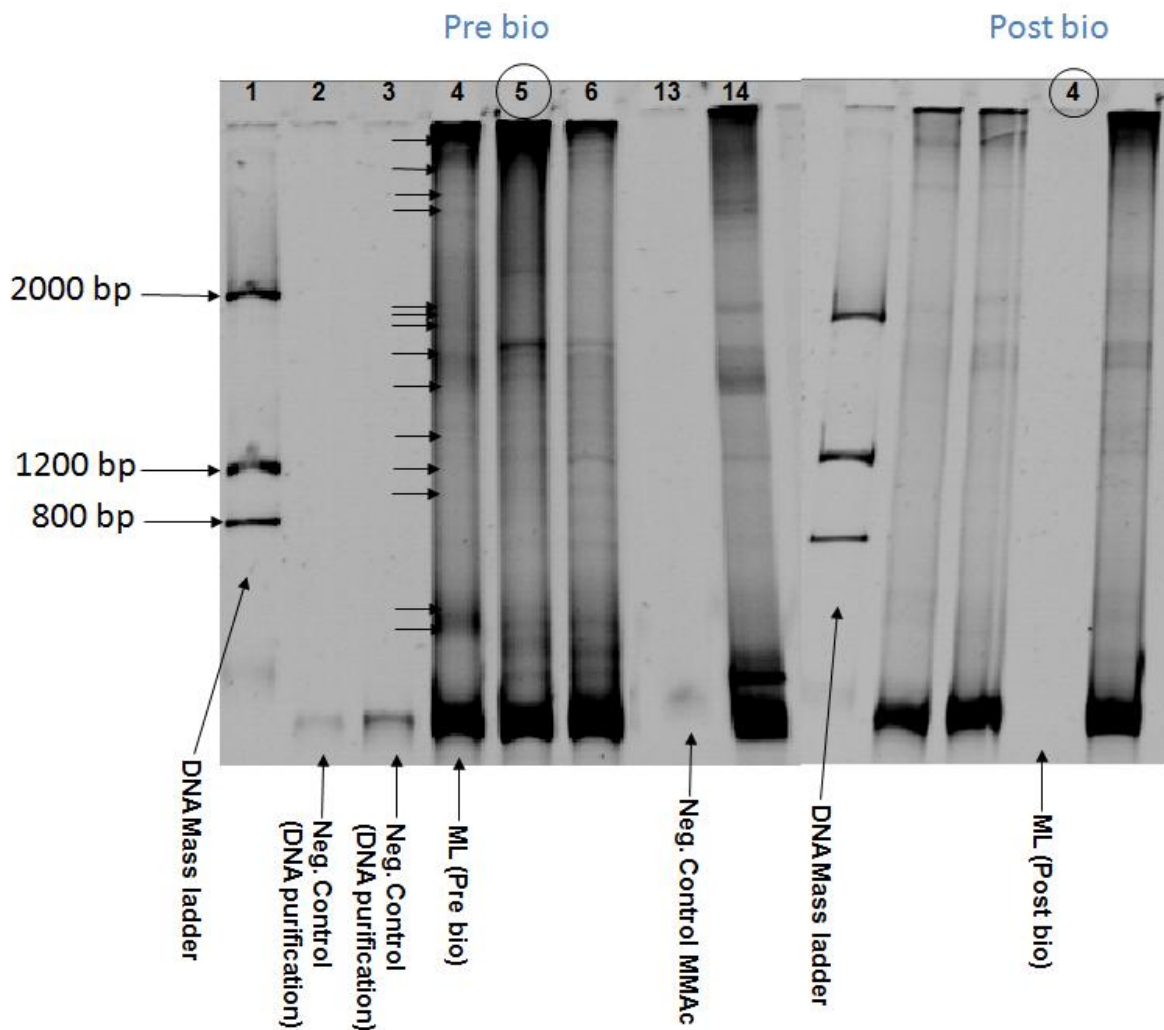
Figure 17. DGGE Bacteria for ML incubated with MMAc at 35˚C
The picture displays two polyacrylamide gels assembled for comparison. Samples; ML (Pre bio) and ML (Post bio) are marked with rings.

Results show that ML (Pre bio) was viable and functional, whereas ML (Post bio) was negative. As discussed in chapter 4.1.8, there might have been a high concentration of co-purified contaminants blocking primer hybridization.

Bioconversion experiment with Peregrino oil inoculated with L004 (MM) at 60˚C
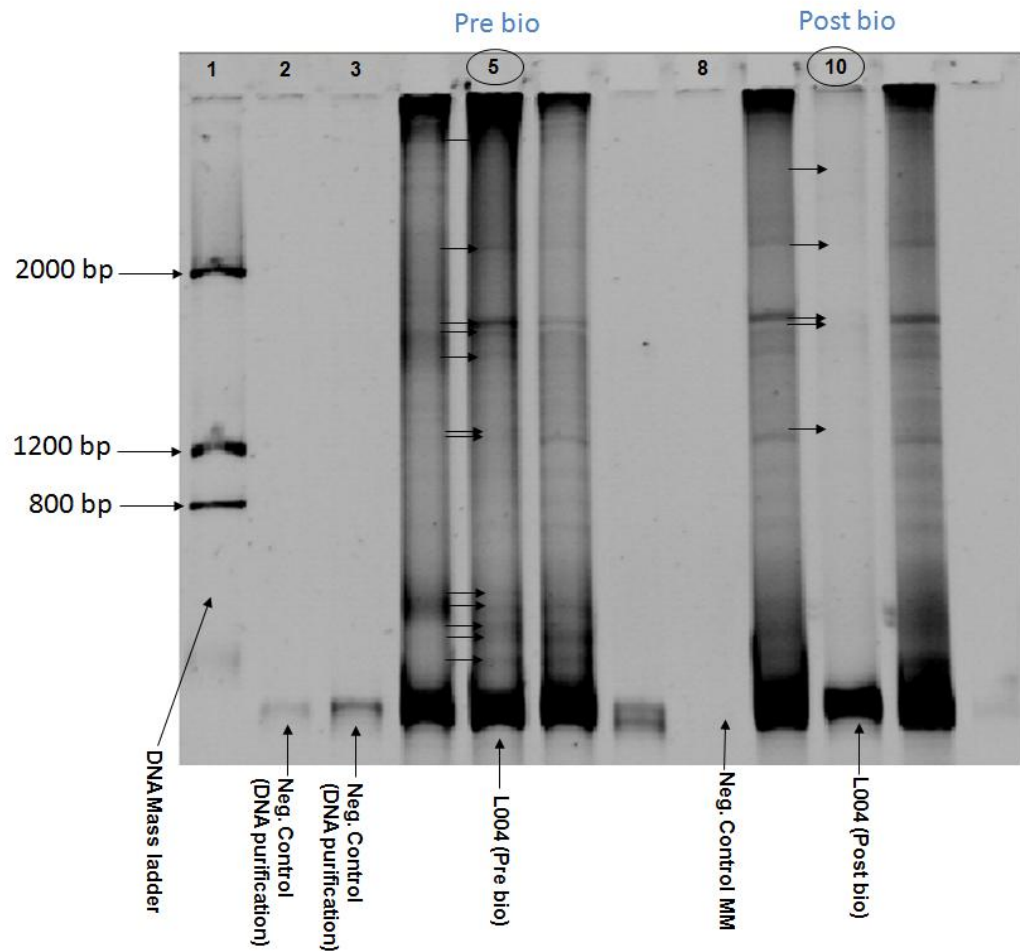
Figure 18. DGGE Bacteria for L004 incubated with MM at 60˚C.
The picture displays one polyacrylamide gel with samples; L004 (Pre bio) and L004 (Post bio) marked with rings.

Results indicates variation between the diversity patterns of samples L004 (pre-and post bio).

Bioconversion experiment with Peregrino oil inoculated with L004 (MMAc) at 60˚C
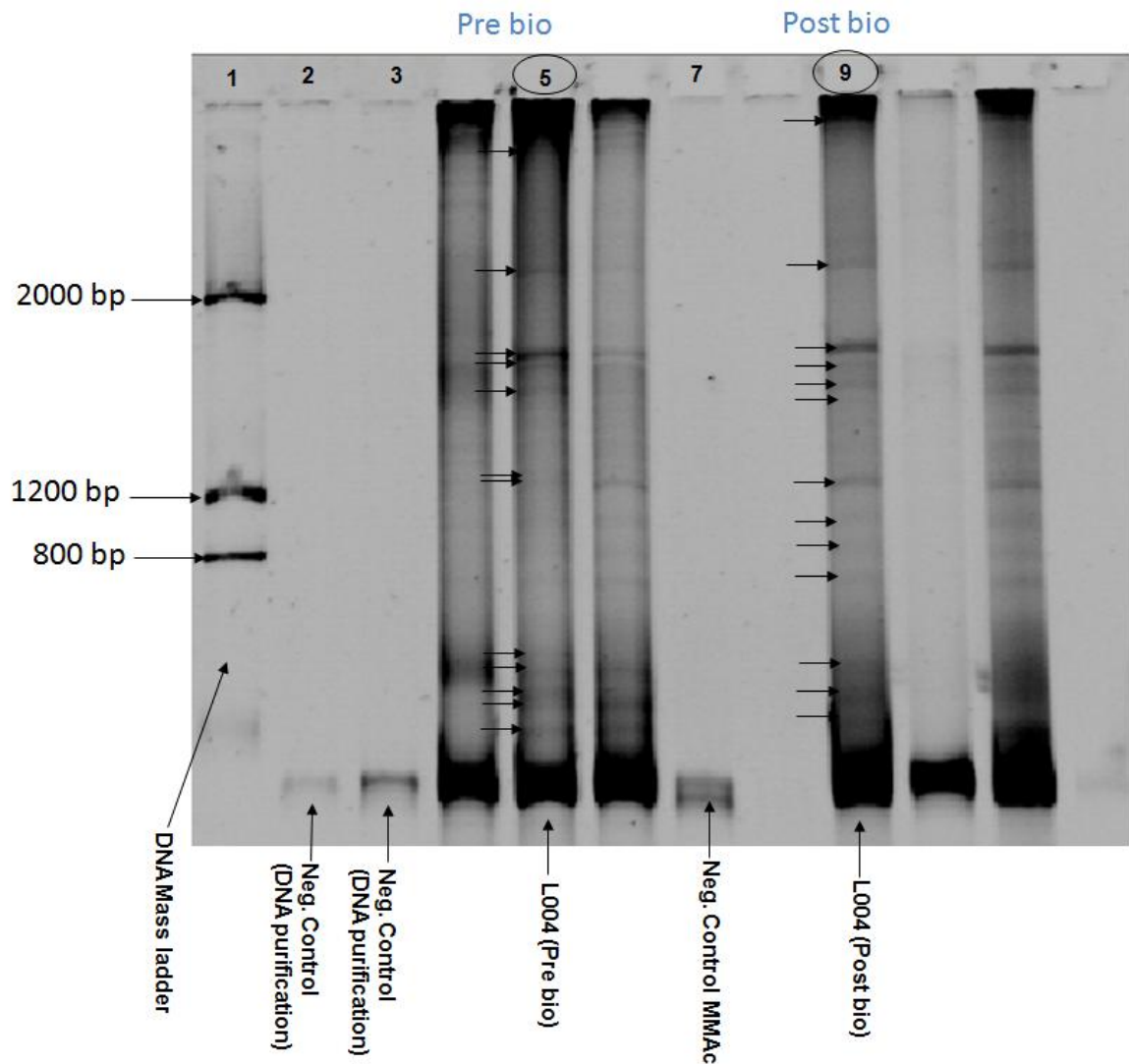
Figure 19. DGGE Bacteria for L004 incubated with MMAc at 60˚C.
The picture displays one polyacrylamide gel with samples; L004 (Pre bio) and L004 (Post bio) marked with rings.

Results indicates variation between the diversity patterns of samples L004 (pre-and post bio).

Bioconversion experiment with Peregrino oil inoculated with MMT006 (MM and MMAc) at 60°C
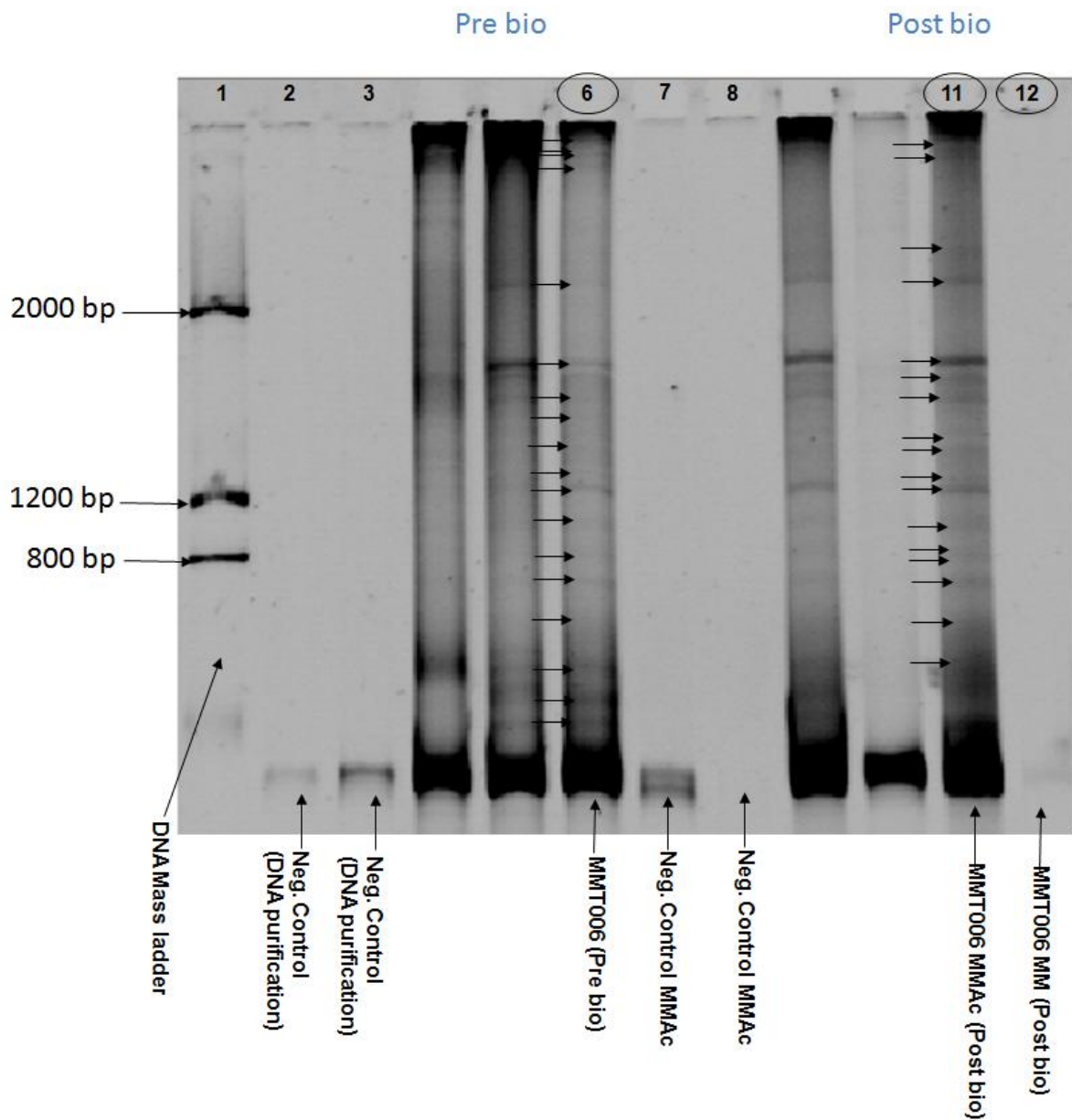
Figure 20. DGGE Bacteria for MMT006 incubated with MM and MMAc, respectively, at 60°C

The picture displays one polyacrylamide gel with samples; MMT006 (Pre bio), MMT006 (MM, Post bio) and MMT006 (MMAc, Post bio).

Positive results were found in MMT006 (Pre bio) and MMT006 (MMAc, Post bio) with an indication of different diversity patterns, whereas results for MMT006 (MM, Post bio) was negative. This indicates that the species in MMT006 had better growth opportunities with acetate as an additional energy source.

4.1.10 Account of the results from bioconversion experiment 1

Results from bioconversion experiment 1 were assembled in a table to make them more legible (Table 16), and discussed as a whole in chapter 5.

Table 16. Account of the results from bioconversion experiment 1
The results from each tools used to gather information about the oil and consortia from bioconversion experiment 1, in addition to a short comment. Indications of bioconversion are highlighted green.

	Sample id.	Visual inspection of the oil (grade)	TLC-FID (fractional change)	DGGE	Comment (in regard to bioconversion)
Pre bio	ML	-	-	Pos	Viable inoculum
	L004	-	-	Pos	Viable inoculum
	MMT006	-	-	Pos	Viable inoculum
Peregrino (35°C)	Neg.control MMAc	Neg	No	Neg	Negative as expected
	ML MMAc	1+	Yes	Neg	Indicating bio conversion and PCR inhibation, rerun of PCR necessary
	Neg.control MM	Neg	Yes	Pos	Indicating fractional change, discarded due to Pos DGGE
	ML MM	Neg	No	Pos	Discarded due to Pos DGGE for Neg. Control MM, rerun necessary
Peregrino (60°C)	Neg.control MMAc	Neg	No	Neg	Negative as expected
	L004 MMAc	1+	No	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	MMT006 MMAc	Neg/1+	No	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	Neg.control MM	Neg	No	Neg	Negative as expected
	L004 MM	Neg	No	Pos (weak)	Rerun necessary due to unsecure TLC-FID results
	MMT006 MM	Neg/1+	No	Neg	Rerun necessary due to unsecure TLC-FID results

4.2   Main experiment 2

The aim of main experiment 2 was to get a qualitative indication of the inocula ML, L004 and MMT006's ability to bioconvert Mariner Maureen-, Peregrino-, and Bressay oil, when being cultivated at the oils' respective reservoir temperatures.

4.2.1 Cultivation, standardization and normalization

Optic density (OD/0.2mL) was considered to be sufficient for ML and L004, based on that most of the readings were over 0.05 and the growth were in accordance to the master plates which they were re inoculated from. Measurement of optic density (OD/0.2mL) for MMT006 was considered to be insufficient, as the majority of the wells showed results under 0.05. Because of this, MMT006 was discarded and for practical reasons was not included in further experiments.

Standardization and normalization was successful, and the inoculum was ready for use in the bioconversion experiment. OD measurements are enclosed in appendix C, page 92.

4.2.2 Bioconversion experiment

Each shake flask of the bioconversion experiment was added 0. 1 g oil (corresponding to 0.2%) and later increased to total 2 % oil. This was done to create an even starting point for the shake flasks. Because of the high viscosity there were some challenges during transfer of the oil, which gave some of the shake flasks an uneven starting point compared to the rest. Even so, the total amount of oil was close to 2 % for all of the shake flasks after addition of the rest of the oil. Calculation of percentage oil added to each flask is enclosed in appendix E, page 103.

4.2.3 Separation of the oil- and water phase and preparation of oil samples

During experiment 2 the oil got stuck in the cork (Figure 21-23), probably due to too high rpm. This led to a lack of oil to be analyzed in some of the shake flasks. Because of this and the fact that there still was time to do a rerun, this experiment was terminated and experiment 3 was initiated.

Figure 21. Mariner Maureen oil after incubating for 7 days at 46˚C.
Negative controls to the left, and test flasks containing inoculum L004 and ML, in the middle and to the right, respectively. Showing the oil's appearance after seven days of incubation at 46˚C; most of the oil is stuck in the cork.

Figure 22. Peregrino oil after incubating for 7 days at 78˚C.
Negative controls to the left, and test flasks containing inoculum L004 and ML, in the middle and to the right, respectively. Showing the oil's appearance after seven days of incubation at 60˚C; the oil is stuck for two of the flasks.

Figure 23. Bressay oil after incubating for 7 days at 35˚C.

Negative controls to the left, and test flasks containing inoculum L004 and ML, in the middle and to the right, respectively. Showing the oil's appearance after seven days of incubation at 35˚C; a great amount of the oil is stuck in the cork.

4.3   Main experiment 3

The purpose of this experiment was to get a qualitative indication of the inocula ML and L004' ability to bioconvert Mariner Maureen-, Peregrino-, and Bressay oil, when being cultivated at the oils' respective reservoir temperatures.

4.3.1 Cultivation, standardization and normalization

Optic density (OD/0.2mL) was considered to be sufficient for both ML and L004, based on that most of the readings were over 0.05 and the growth were in accordance to the master plates which they were re inoculated from. MMT006 was not included in this experiment because of the time-consuming cultivation.

Inoculum was standardized and normalization was successful. OD measurements are enclosed in appendix C, page 92

4.3.2 Bioconversion experiment

Each shake flask of the bioconversion experiment was added 0.1 g oil (corresponding to 0.2%) and later increased to total 2 % oil. This was conducted to create an even starting point for the shake flasks Because of the high viscosity it was challenging to transfer the correct amount of the oil to the shake flasks, evens o, the variations was not that great. Calculation of percentage oil added to each flask is enclosed in appendix E, page 103.

4.3.3 Separation of the oil- and water phase and preparation of oil samples

After ending the experiment, the oil was visually inspected and graded based on its appearance in the water phase (Tables 17-19). Visual inspection criteria are shown in table 12. Figures 24-32 shows the shake flasks before prior to the bioconversion experiment; day three and after bioconversion experiment.

Bioconversion experiment of Mariner Maureen oil with MM and MMAc at 46°C

Figure 24. Mariner Maureen oil – before inoculation.
The oil's appearance in the water phase prior to bioconversion experiment.

Figure 25. Mariner Maureen – after incubating for three days at 46˚C.
Samples L004 MM and MMAc are enlarged.

Visual inspection show no observable changes.
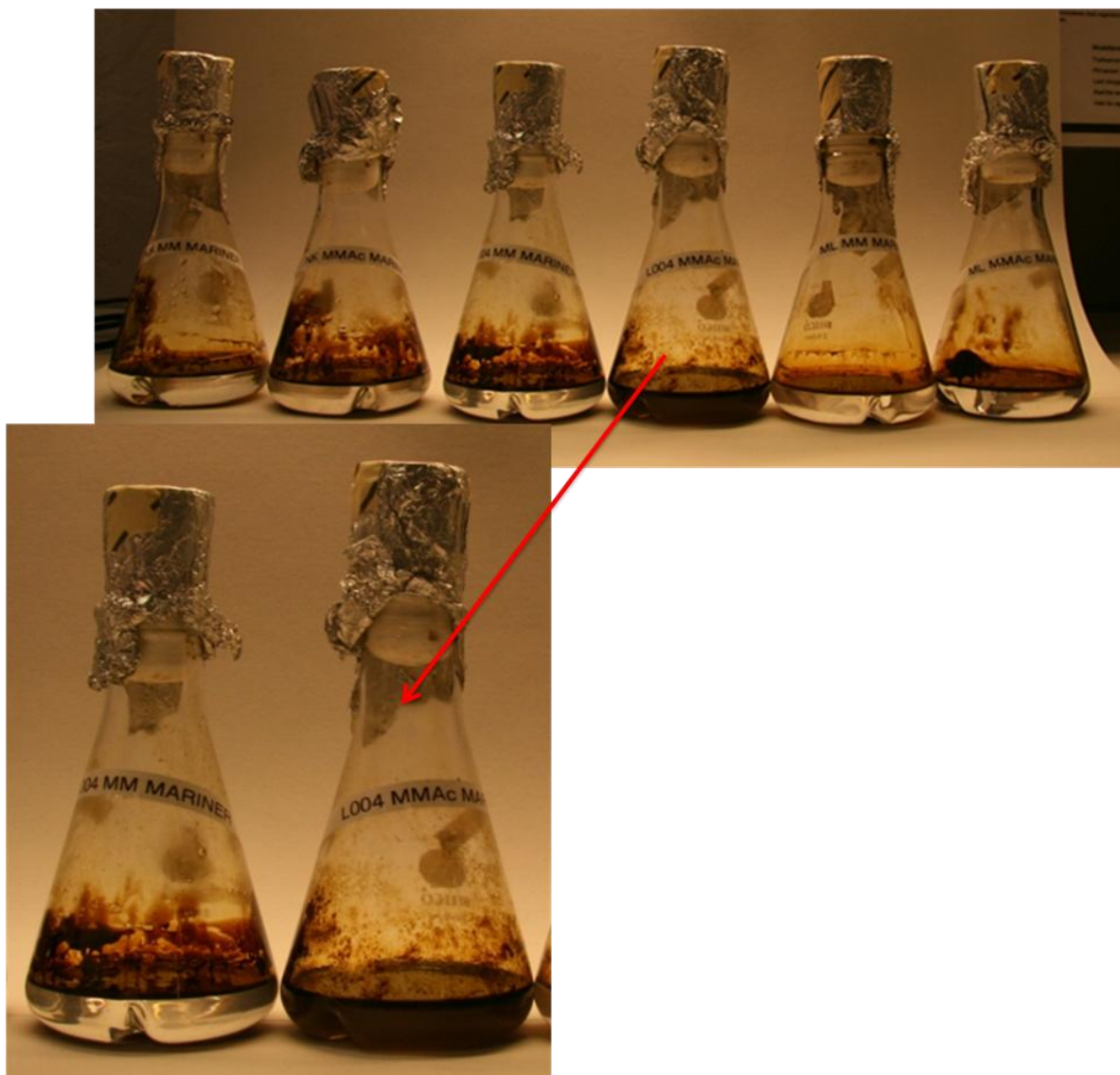
Figure 26. Mariner Maureen – after incubating for seven days at 46˚C
Sample L004 MMAc with promising results (enlarged).

Figure 26 shows the flasks day seven after ending the bioconversion experiment.
A positive visual indication of bioconversion is found in sample L004 MMAc as it seems like the oil has totally emulsified in the water.

Table 17. Visual inspection of Mariner Maureen oil after incubation for seven days at 46˚C
Oil in water is graded for both negative controls and tests with mediums (MM and MMAc).
Gradation is based on visual inspection criteria in table 12.

Mariner Maureen (46˚C)	Gradation
Neg.control MM	Neg
Neg.control MMAc	Neg
L004 MM	Neg
L004 MMAc	4+
ML MM	Neg
ML MMAc	Neg

Bioconversion experiment of Peregrino oil with MM and MMAc at 60°C
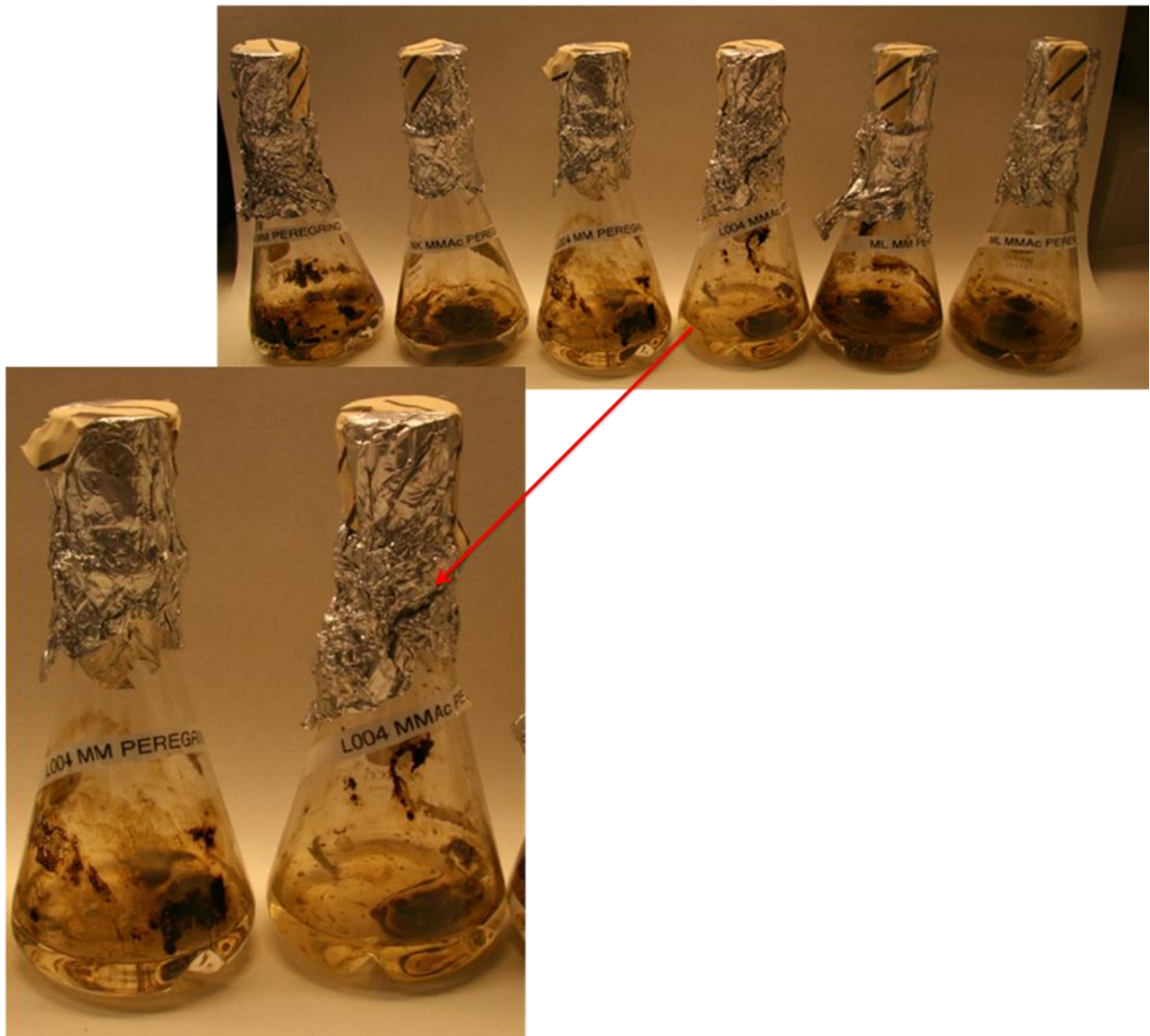
Figure 27. Peregrino – before inoculation.
The oil's appearance in the water phase prior to bioconversion experiment. Samples L004 MM and MMAc are enlarged
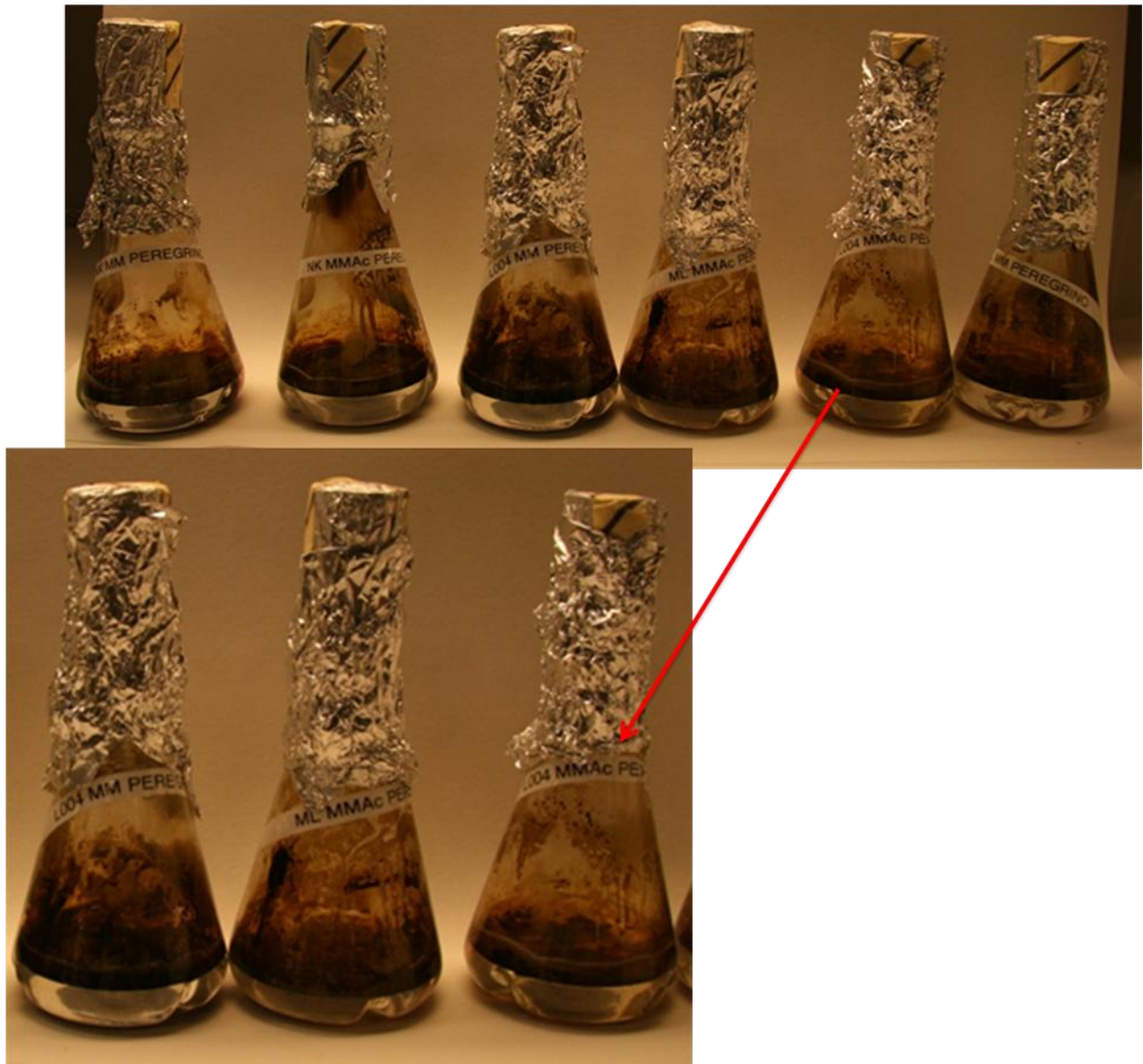
Figure 28. Peregrino – after incubating for three days at 60˚C
Samples L004 MM and MMAc, in addition to ML MMAc are enlarged

Visual inspection shows no indication of bioconversion.

Figure 29. Peregrino – after incubating for seven days at 60˚C
Visual growth is observed in sample L004 MM and L004 MMAc (enlarged).

Visual inspection show indication of bioconversion in tests; L004 (MM) and L004 (MMAc),

Table 18. Visual inspection of Peregrino oil after incubation for seven days at 60˚C
Oil in water is graded for both negative controls and tests with mediums (MM and MMAc).
Gradation is based on visual inspection criteria in table 12.

Peregrino (60˚C)	Gradation
Neg.control MM	Neg
Neg.control MMAc	Neg
L004 MM	1+
L004 MMAc	Neg / 1+
ML MM	Neg
ML MMAc	Neg

Bioconversion experiment of Bressay oil with MM and MMAc at 35°C
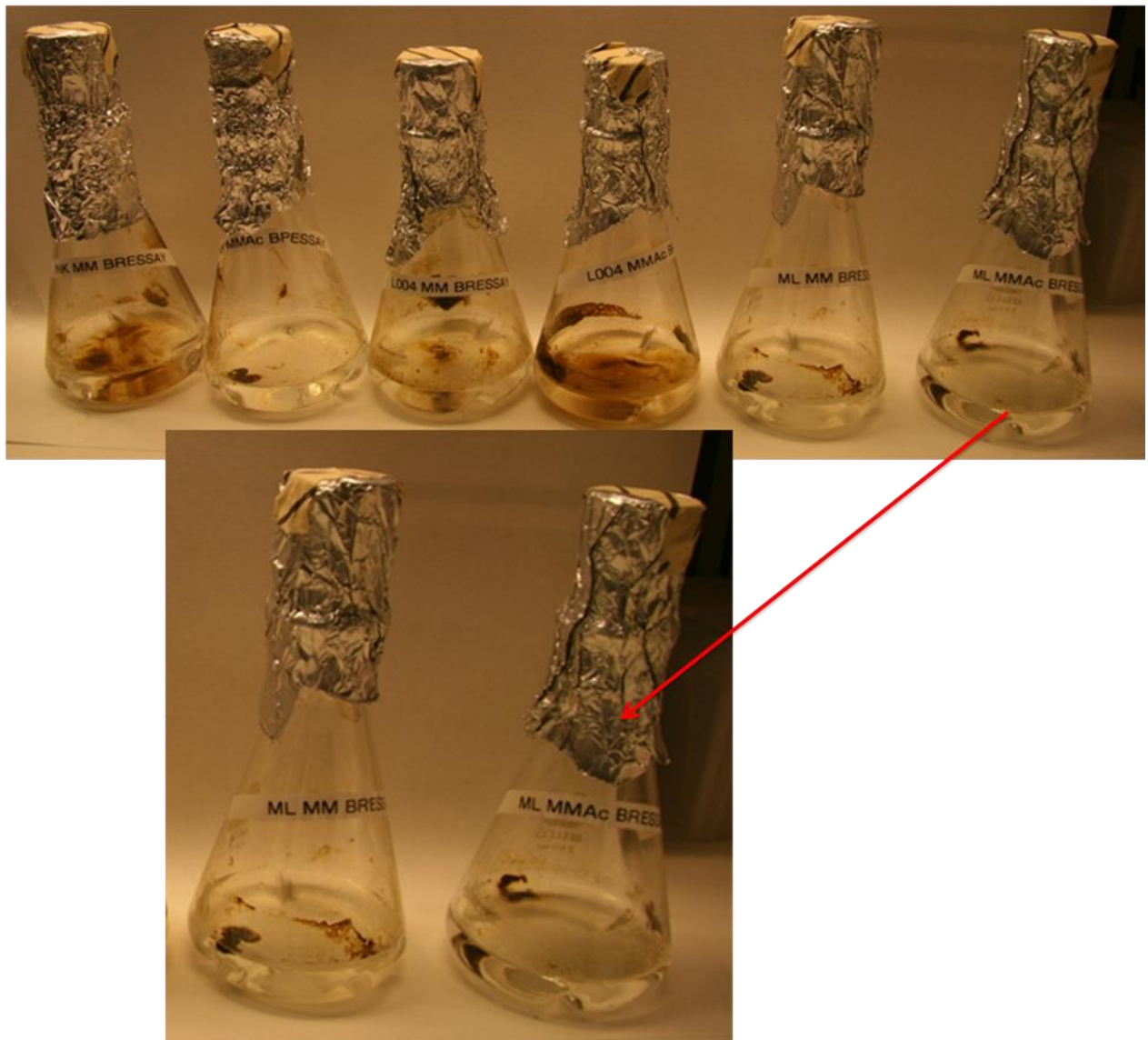
Figure 30. Bressay – before inoculation.
The oil's appearance in the water phase prior to bioconversion experiment; samples ML MM and ML MMAc are enlarged
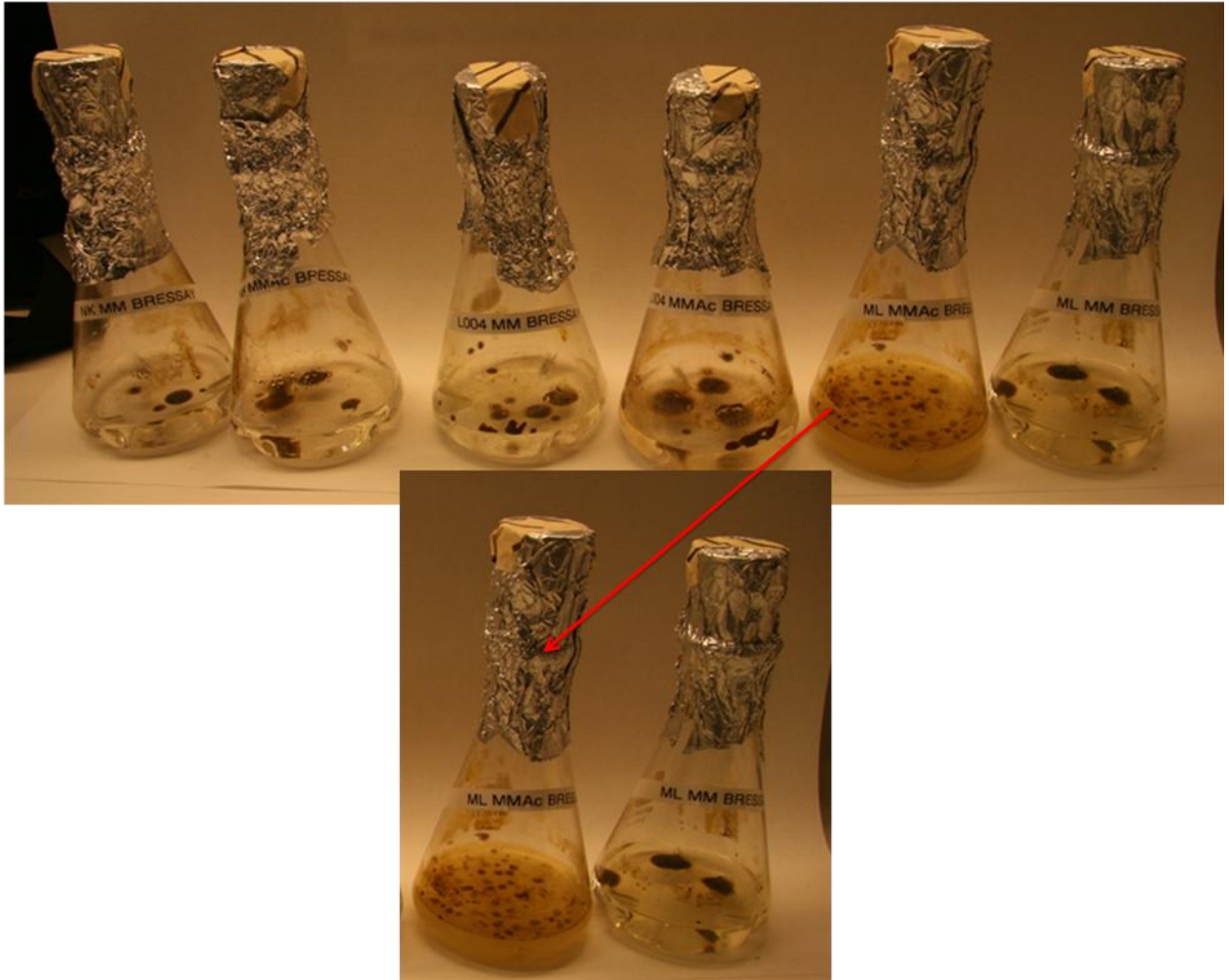
Figure 31. Bressay – after incubation for three days at 35˚C.
Samples ML MM and ML MMAc are enlarged

Visual inspection show indication of bioconversion and growth in ML
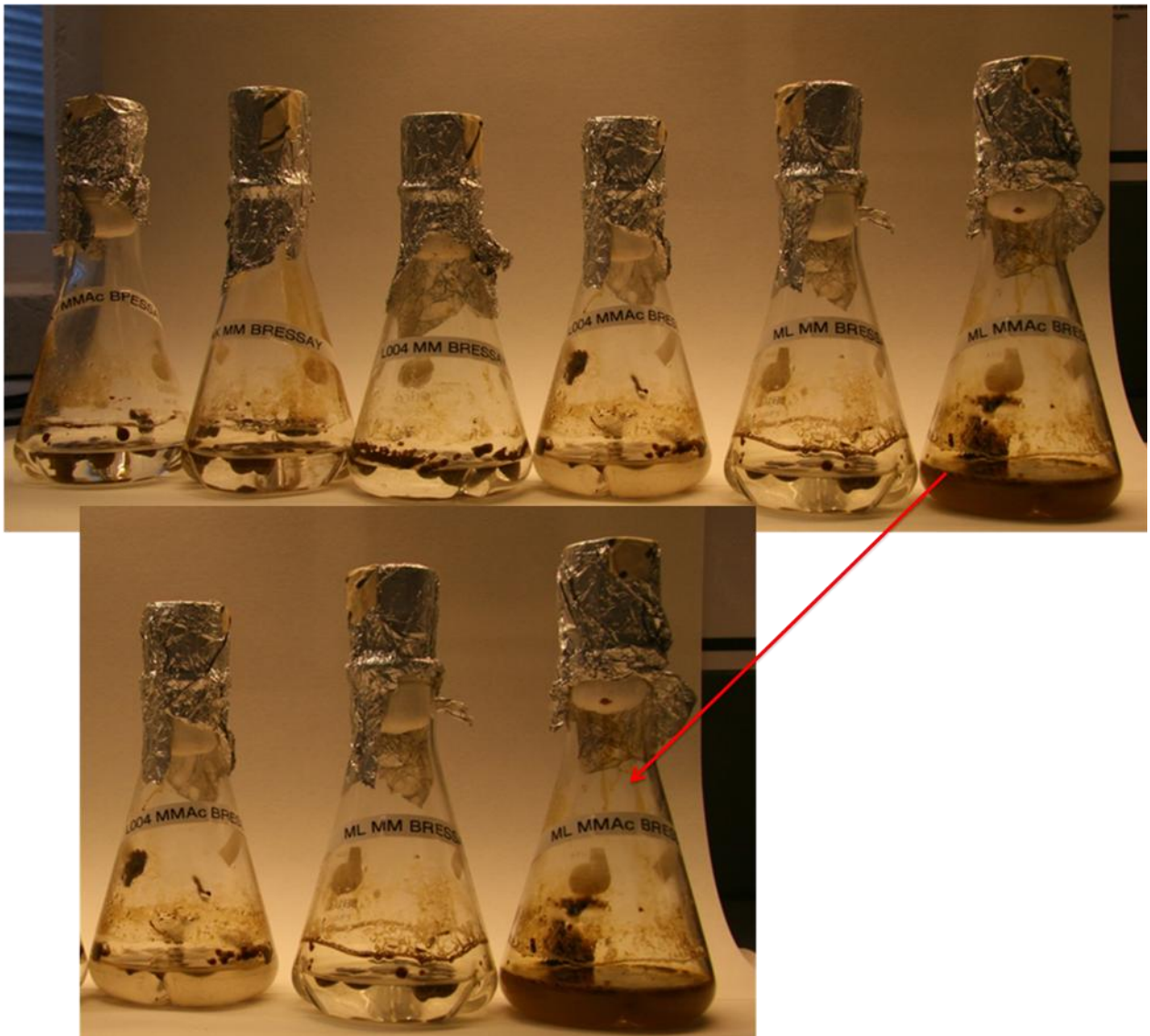
Figure 32. Bressay – after incubating for seven days at 35˚C
Visual growth is observed in sample L004 MMAc and ML MMAc (enlarged).

Visual inspection show indication of bioconversion in the tests; L004 (MM), L004 (MMAc) and especially ML (MMAc); were the oil seems to have totally emulsified in the water.

Table 19. Visual inspection of Bressay oil after incubation for seven days at 35˚C.
Oil in water is graded for both negative controls and tests with mediums (MM and MMAc). Gradation is based on visual inspection criteria in table 12.

Bressay (35˚C)	Gradation
Neg.control MM	Neg / 1+
Neg.control MMAc	Neg / 1+
L004 MM	1+
L004 MMAc	1+
ML MM	Neg
ML MMAc	1+/2+

4.3.4 Thin layer chromatography

The oil from bioconversion experiment three was analyzed by thin–layer chromatography with flame ionization detection to give an indication upon if there had been some compositional changes in the oil due to microbial of physiochemical forces.

The results, computed and processed, are illustrated in figure 33-38.Calculation of SD is enclosed in appendix G, page 108. Comparison was done between the results from native oil and negative control, whereas the tests were compared to the negative control.

Mariner Maureen oil
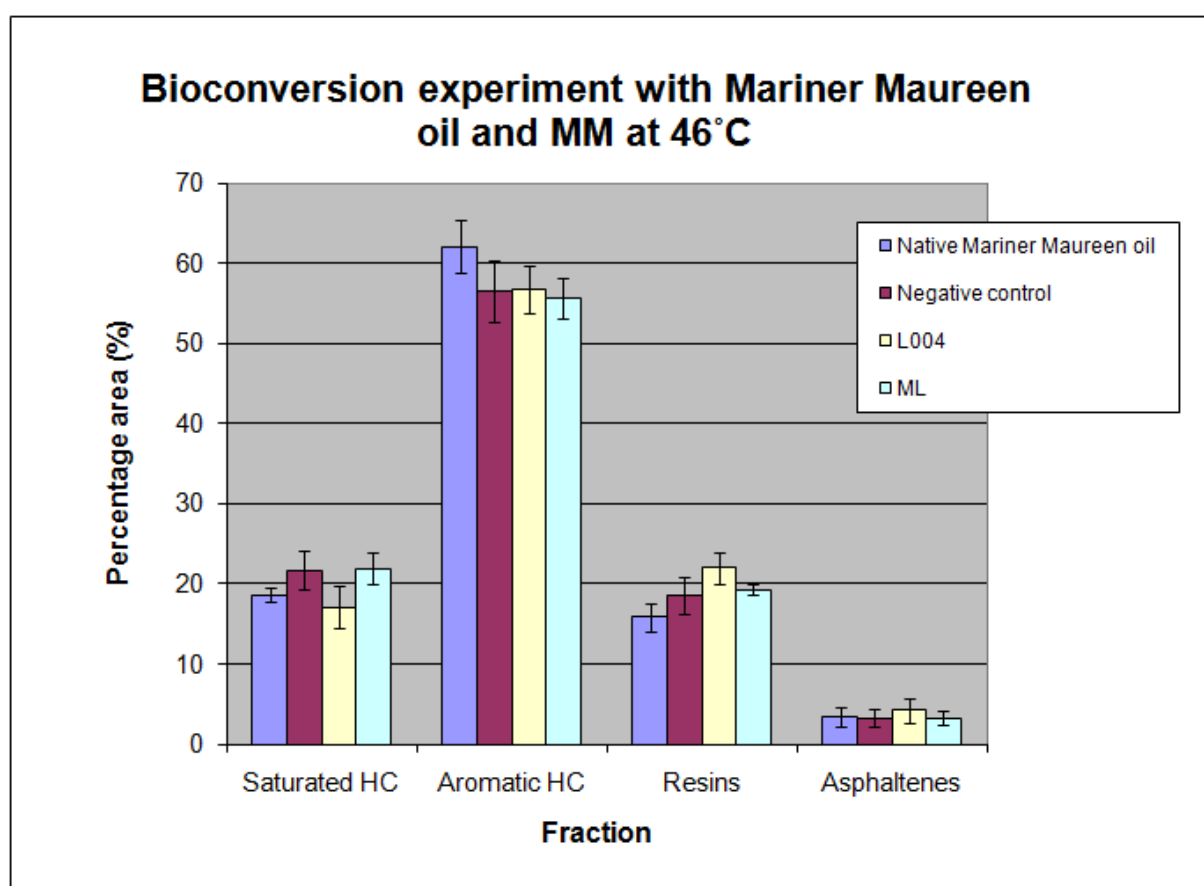
Figure 33. Bioconversion experiment with Mariner Maureen oil and MM at 46˚C
Comparison of native Mariner Maureen, negative controls and the ancillary tests (inoculum ML and L004). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Mariner Maureen oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

Results show no indication of fractional changes.
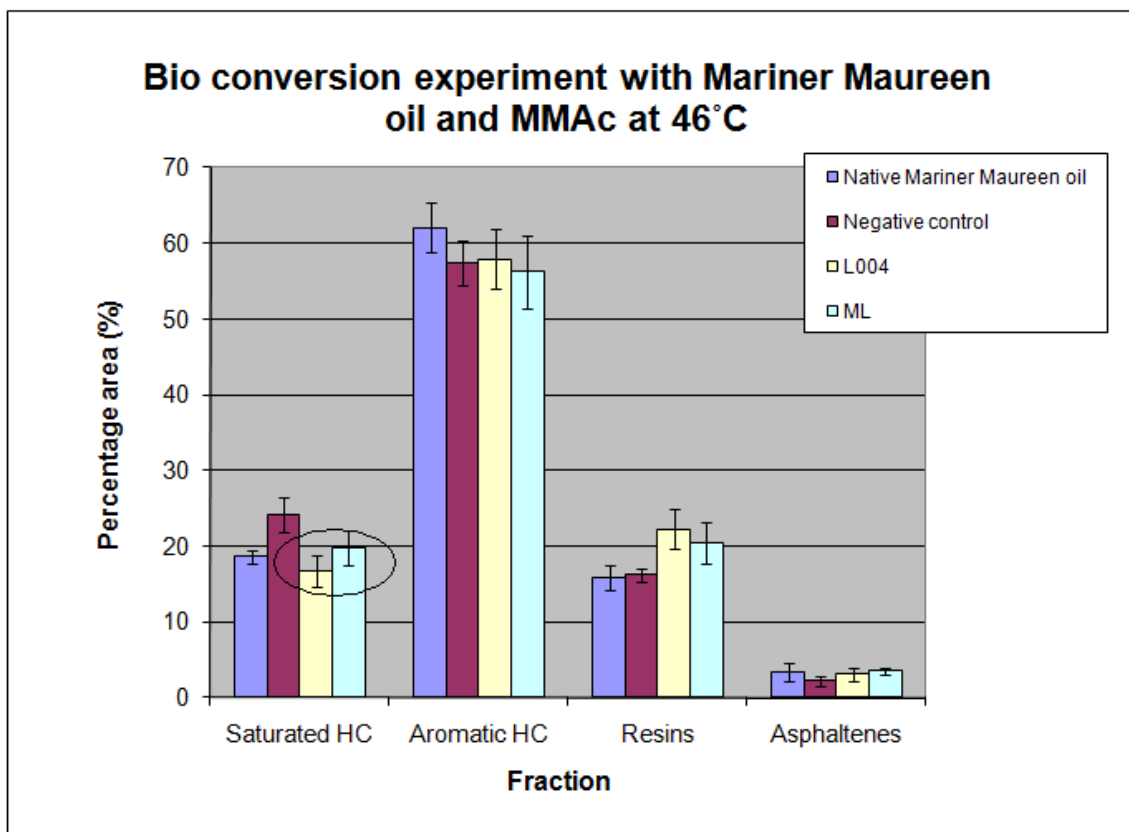
Figure 34. Bioconversion experiment with Mariner Maureen oil and MMAc at 46˚C
Comparison of native Mariner Maureen, negative controls and the ancillary tests (inoculum ML and L004). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Mariner Maureen oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

Results show an indication of fractional change in Mariner Maureen oil inoculated with L004; were there is a decrease in the relative amount of saturated hydrocarbons. Marginal changes can also be seen for Mariner Maureen oil inoculated with ML. As for the negative control, the relative amount of saturates has increased compared to the native oil, whereas the relative amount of reins is the same as in native oil.

Peregrino oil
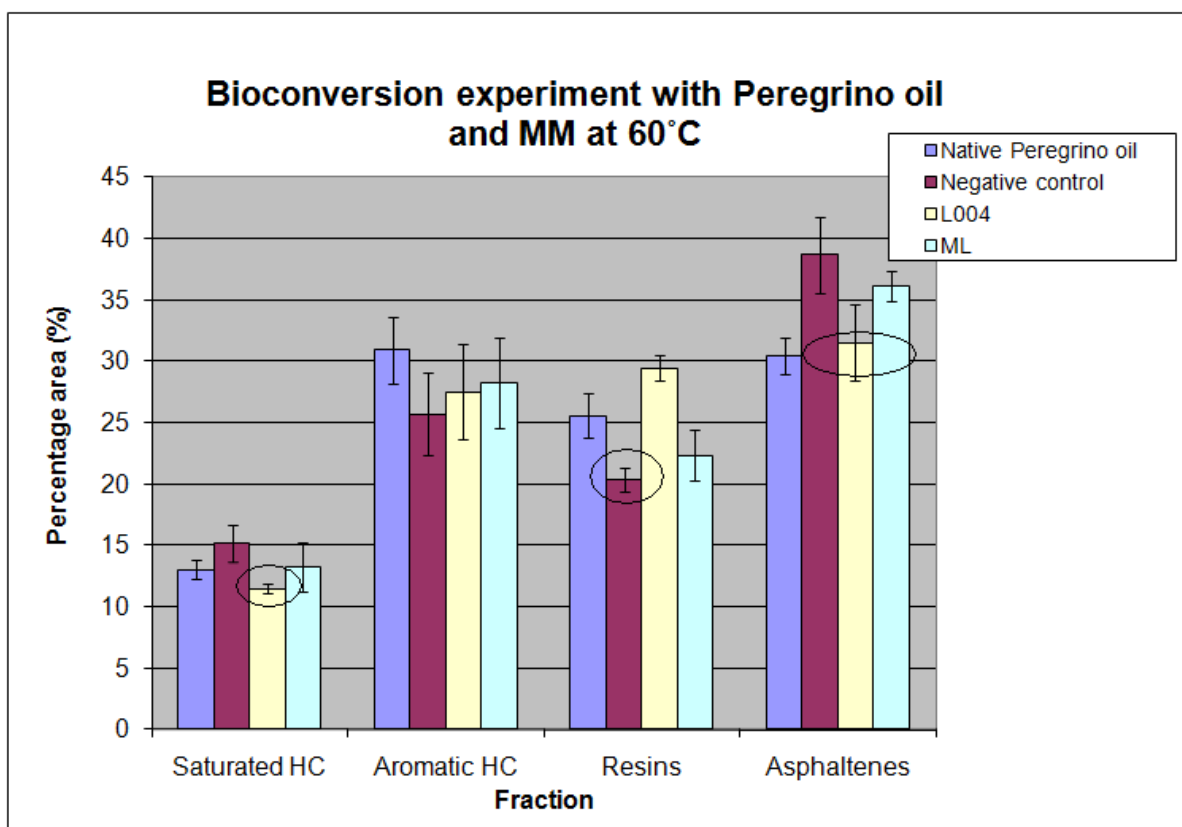
Figure 35. Bioconversion experiment with Peregrino oil and MM at 60˚C.
Comparison of native Peregrino, negative controls and the ancillary tests (inoculum ML and L004).
The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

The results indicate a fractional change in Peregrino oil inoculated with L004; there is a decrease in relative amount of saturated hydrocarbons and asphaltenes compared to the negative control. There are also indications of marginal changes in the negative control compared to the native Peregrino oil; a reduction in the relative amount of resins.
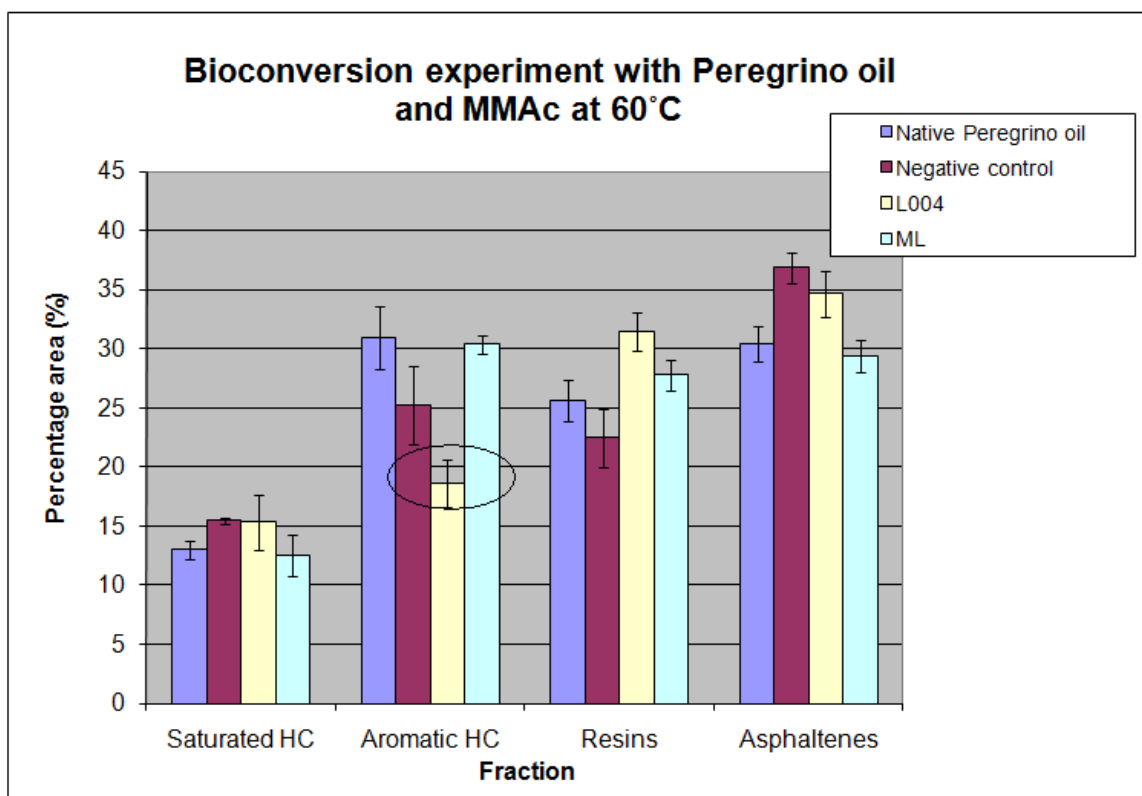
Figure 36. Bioconversion experiment with Peregrino oil and MMAc at 60˚C.
Comparison of native Peregrino, negative controls and the ancillary tests (inoculum ML and L004). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

The results indicate a decrease in the relative amount of aromatic hydrocarbons in Peregrino oil inoculated with L004.

Bressay oil
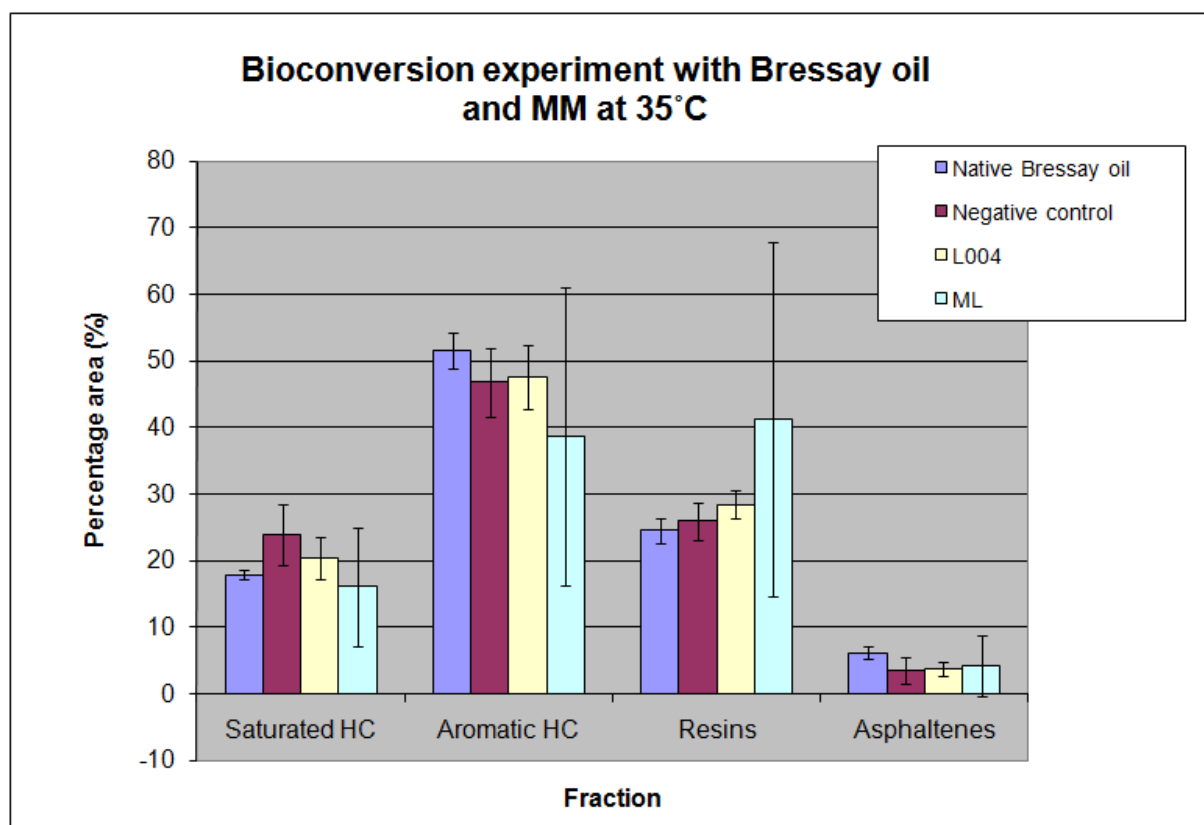
Figure 37. Bioconversion experiment with Bressay oil and MM at 35˚C.
Comparison of native Bressay, negative controls and the ancillary tests (inoculum ML and L004). The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA fractions are dispersed along the x-axis respectively to give an impression of the relatively differences between the native Peregrino oil and negative control, and the difference between the negative control and the test. Y-axis shows the average percentage of each fraction relatively to the total amount of fraction in the respective sample. Standard deviation is set as y and x error bars.

The results show no indication fractional changes. It was observed that the chromatograms of the test ML (MM) had very rare peaks (Appendix H, page 119), which indicates that a fault has occurred during analysis.
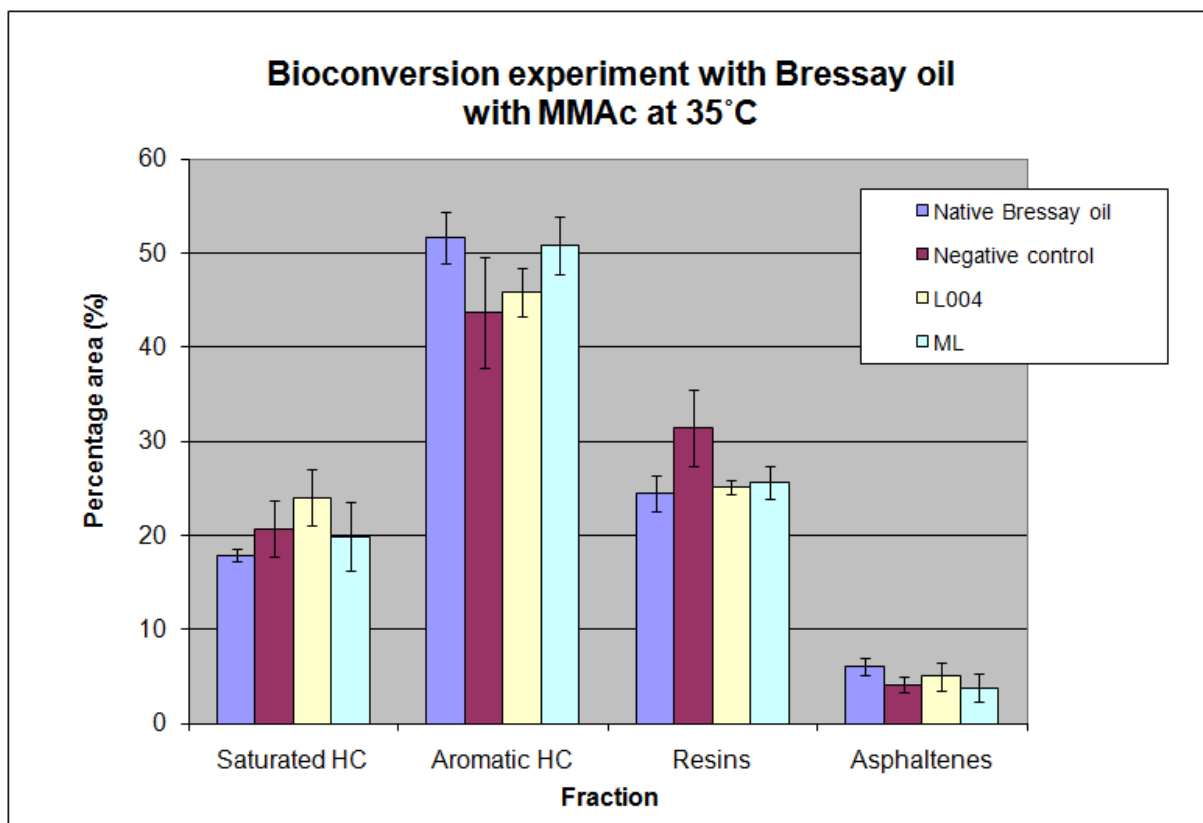
Figure 38. Bioconversion experiment with Bressay oil and MMAc at 35˚C.
Comparison of native Bressay, negative controls and the ancillary tests (inoculum ML and L004).
The fractions are relatively computed by the FID – instrument (Iatroscan™ MK-6/6s). SARA
fractions are dispersed along the x-axis respectively to give an impression of the relatively
differences between the native Peregrino oil and negative control, and the difference between
the negative control and the test. Y-axis shows the average percentage of each fraction
relatively to the total amount of fraction in the respective sample. Standard deviation is set as
y and x error bars.

Results show no indication of fractional changes.

4.3.5 DNA – extraction

After extraction of DNA, concentration was measured and the computed A260/A280 ratio showed either higher or less than 1.8, which indicates the presence of co-purified proteins, phenol, contaminants or RNA. (Appendix J, page 124).

4.3.1 Control of PCR amplified DNA with agarose gel electrophoresis

PCR for the detection of bacteria and archaea was conducted, and the products from each reaction controlled with a standard of known molecular weight markers on agarose gel (2%). As for experiment one, regular method, dilution and upgrading of the DNA concentration were done before producing sufficient positive results from bacterial DNA to DGGE. Figure 39-41 shows positive results produced from DNA sampled from shake flasks of both medium series (MM and MMAc) from bioconversion experiment 3, which were upgraded and conducted on PCR using 16S rRNA primer set "341 f Bac clamp"/ "907r Bac".
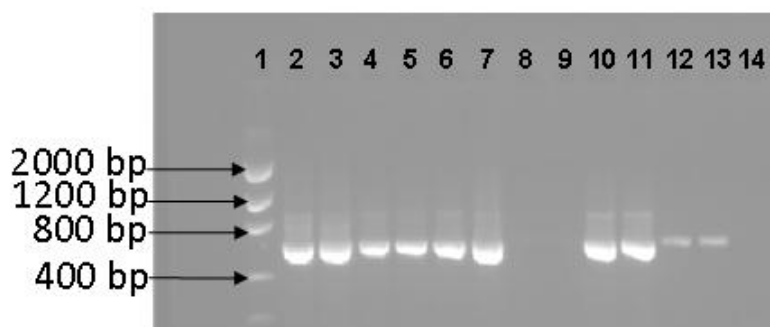
Figure 39. Bacteria PCR to DGGE
Displaying an agarose gel with results from PCR conducted with 16S rRNA primer set "341 f Bac clamp"/ "907 r Bac" on samples of upgraded DNA prior to- and day 3 of bioconversion experiment . DNA Mass Ladder (Invitrogen) in well 1 to the left with molecular weight markers of 400 and 2000 base pairs pointed out. DNA samples are listed in table 20.

Table 20. Bacteria PCR to DGGE.
Set up for agarose gel in figure 39. Samples of upgraded DNA prior to- and day 3 of bioconversion experiment.

Well	Sample id.	Well	Sample id.
1	DNA ladder	8	Neg. control Peregrino MM
2	ML (Pre Bio)	9	Neg. control Peregrino MMAc
3	L004 (Pre Bio)	10	L004 Peregrino MM
4	L004 Mariner MM	11	L004 PeregrinoMMAc
5	L004 Mariner MMAc	12	ML Peregrino MM
6	ML Mariner MM	13	ML Peregrino MMAc
7	ML Mariner MMAc	14	Neg. Control Bressay MM
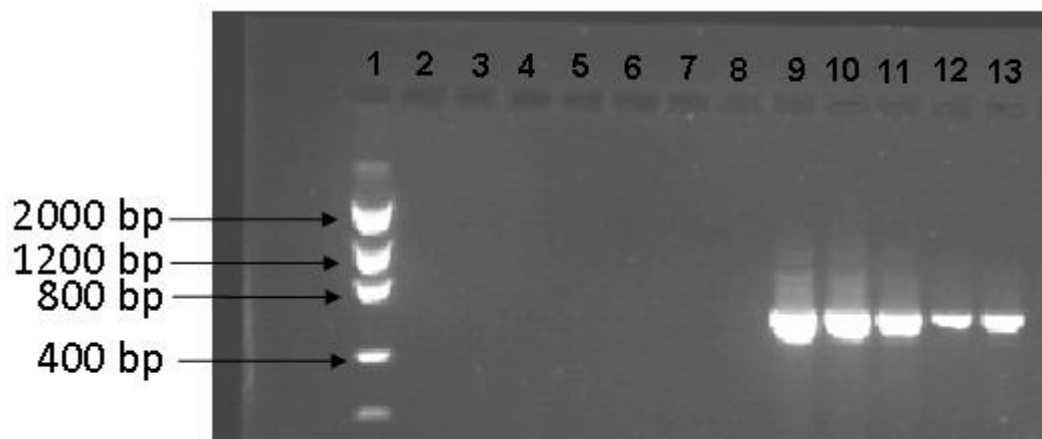
Figure 40. Bacteria PCR to DGGE

Picture of agarose gel displaying results from PCR conducted with 16S rRNA primer set "341 f Bac clamp"/ "907 r Bac" on the last samples of upgraded DNA from experiment three day three. Setup is shown in table 21. DNA Mass Ladder (Invitrogen) in well 1 to the left with molecular weight markers of 400 and 2000 base pairs pointed out. DNA samples are listed in table 21.

Table 21. Bacteria PCR to DGGE.

Setup for agarose gel in figure 40. The rest of the upgraded DNA samples from bioconversion experiment 3 day three. Negative controls from DNA purification ant tests for each media serie (MM and MMAc).

Well	Sample id.
1	DNA ladder
7	Neg. control (DNA purification)
8	Neg. control Bressay MMAc
9	L004 Bressay MM
10	L004 Bressay MMAc
11	L004 Bressay MMAc
12	ML Bressay MM
13	ML Bressay MMAc
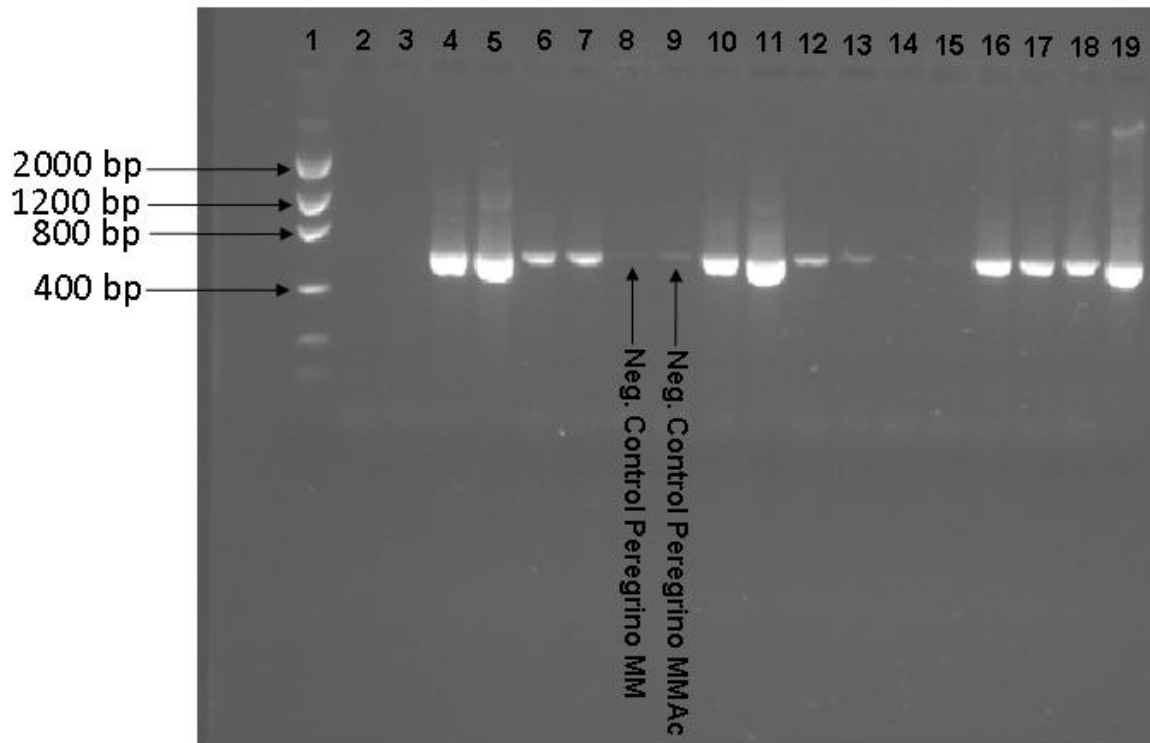
Figure 41. Bacteria PCR to DGGE.

Samples run with upgraded DNA. Picture of Agarose gel displaying results from PCR run with primer set 16S rRNA "341 f Bac clamp"/ "907 r Bac" on upgraded DNA samples from bioconversion experiment 3 day seven. DNA Mass Ladder (Invitrogen) in well 1 to the left with molecular weight markers of 400 and 2000 base pairs pointed out. DNA samples are listed in table 22.

Table 22. Bacteria PCR to DGGE

Setup agarose gel displayed in figure 41 with upgraded DNA samples from day 3 of the bioconversion experiment. Negative controls and tests for each media serie (MM and MMAc).

Post - bioconversion experiment 3			
Well	Sample id.	Well	Sample id.
1	DNA ladder	11	L004 Peregrino MMAc
2	Neg. control Mariner MM	12	ML Peregrino MM
3	Neg. control Mariner MMAc	13	ML Peregrino MMAc
4	L004 Mariner MM	14	Neg. control Bressay MM
5	L004 Mariner MMAc	15	Neg. control Bressay MMAc
6	ML Mariner MM	16	L004 Bressay MM
7	ML Mariner MMAc	17	L004 Bressay MMAc
8	Neg. control Peregrino MM	18	ML Bressay MM
9	Neg. control Peregrino MMAc	19	ML Bressay MMAc
10	L004 Peregrino MM	20	-

4.3.2 Denaturing Gradient Gel Electrophoresis (DGGE)

To characterize the biodiversity in each DNA sample PCR products were conducted on Denaturing Gradient Gel Electrophoresis (DGGE).

The analysis and interpretation of DGGE results is based on observation of the gel images and comparing the diversity patterns of the ancillary samples; pre–and post bioconversion experiment for both media series (MM and MMAc). Were samples pre-bio corresponds to DNA samples prior to bioconversion experiment, whereas post-bio corresponds to DNA sampled after the bioconversion experiment.Both clear and shadow –like fragments, which are a bit difficult to determine, are marked with arrows in the pictures (figure 42 – 47).

Bioconversion experiment with Mariner Maureen oil, inoculated with L004 at 46˚C
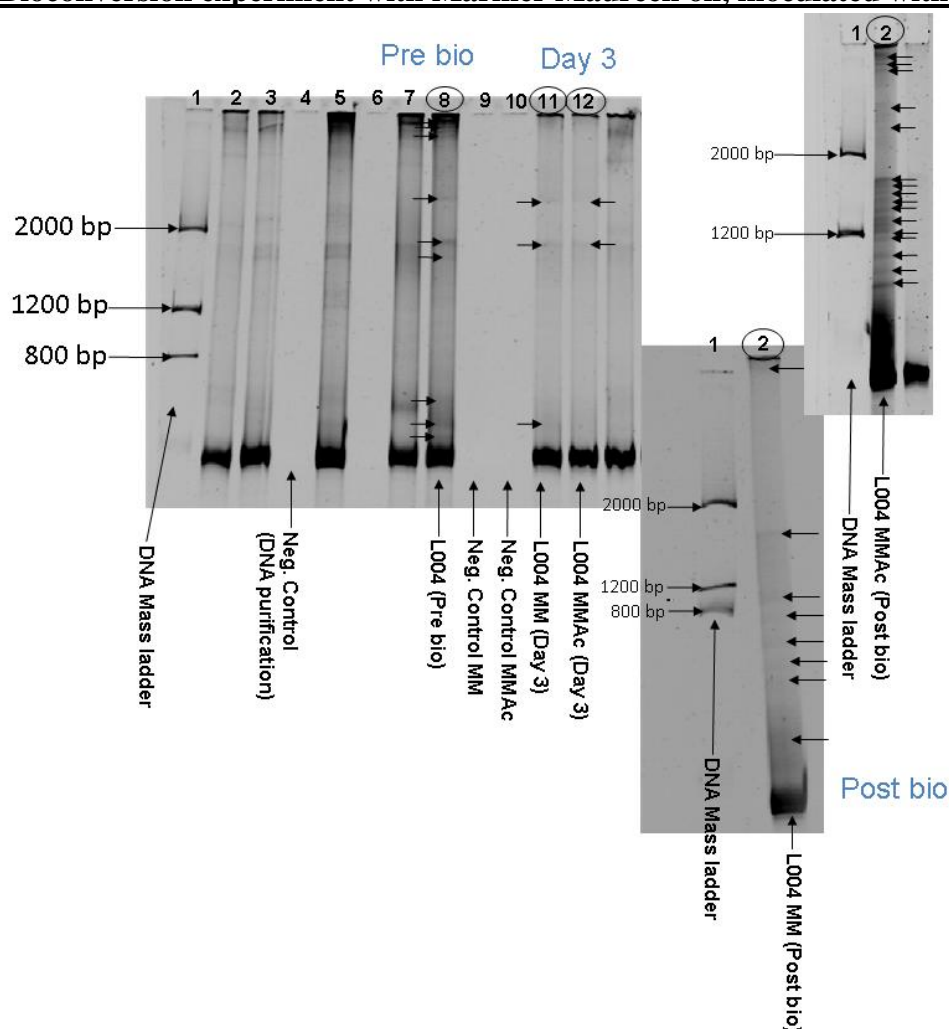
Figure 42. DGGE Bacteria for L004 incubated at 46˚C with Mariner Maureen oil.
The picture displays three polyacrylamide with samples; L004 (Pre bio), L004 (MM, day 3), L004 (MMAc, day 3), L004 (MM, Post bio) and L004 (MMAc, Post bio). Visible fragments are marked with arrows.

The results indicate a difference in diversity patterns of L004 (MMAc) than L004 (MM).

Bioconversion experiment with Mariner Maureen oil, inoculated with ML at 46˚C
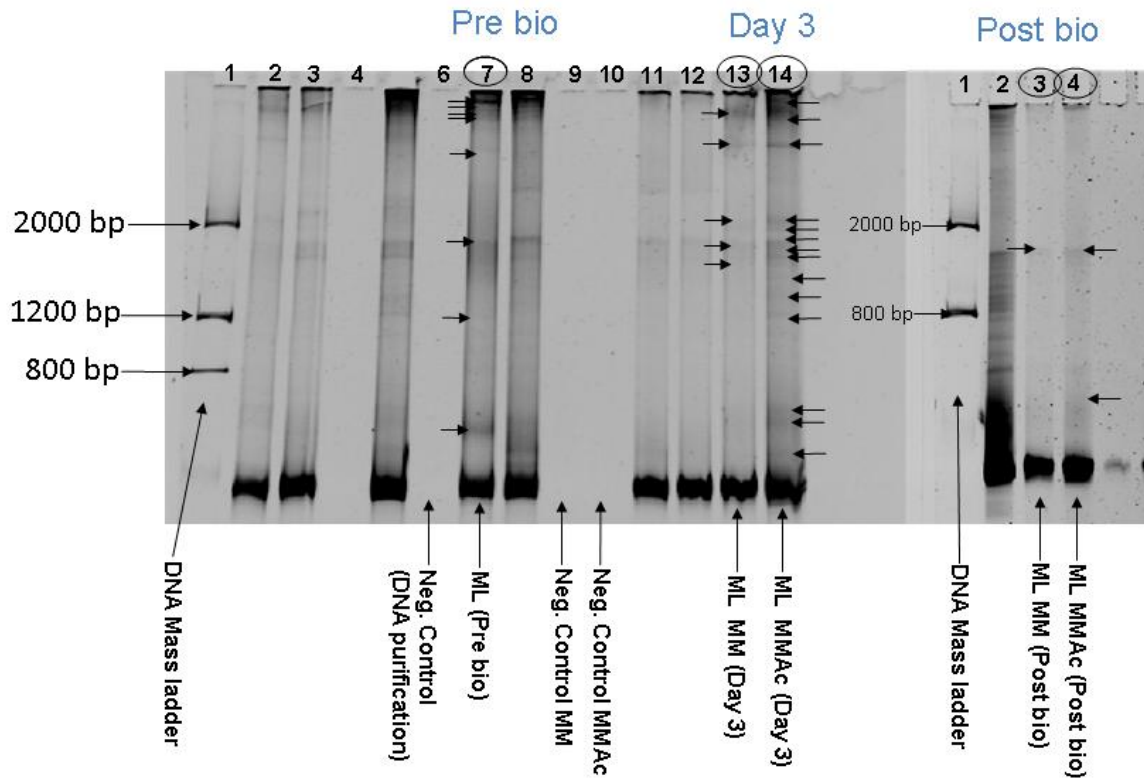
Figure 43. DGGE Bacteria for ML incubated at 46˚C with Mariner Maureen oil.
The picture displays two polyacrylamide gels aggregated for comparison. Samples; ML (pre bio), ML (MM, day 3), ML (MMAc, day 3), ML (MM, Post bio) and ML (MMAc, Post bio) are marked with rings. Visible fragments are marked with arrows.

The results indicate a variation in biodiversity patterns between ML incubated with MM and MMAc, in addition to indicating a lower biodiversity post bio than pre bio, for both ML MM and MMAc.

Bioconversion experiment with Peregrino oil, inoculated with L004 at 60˚C
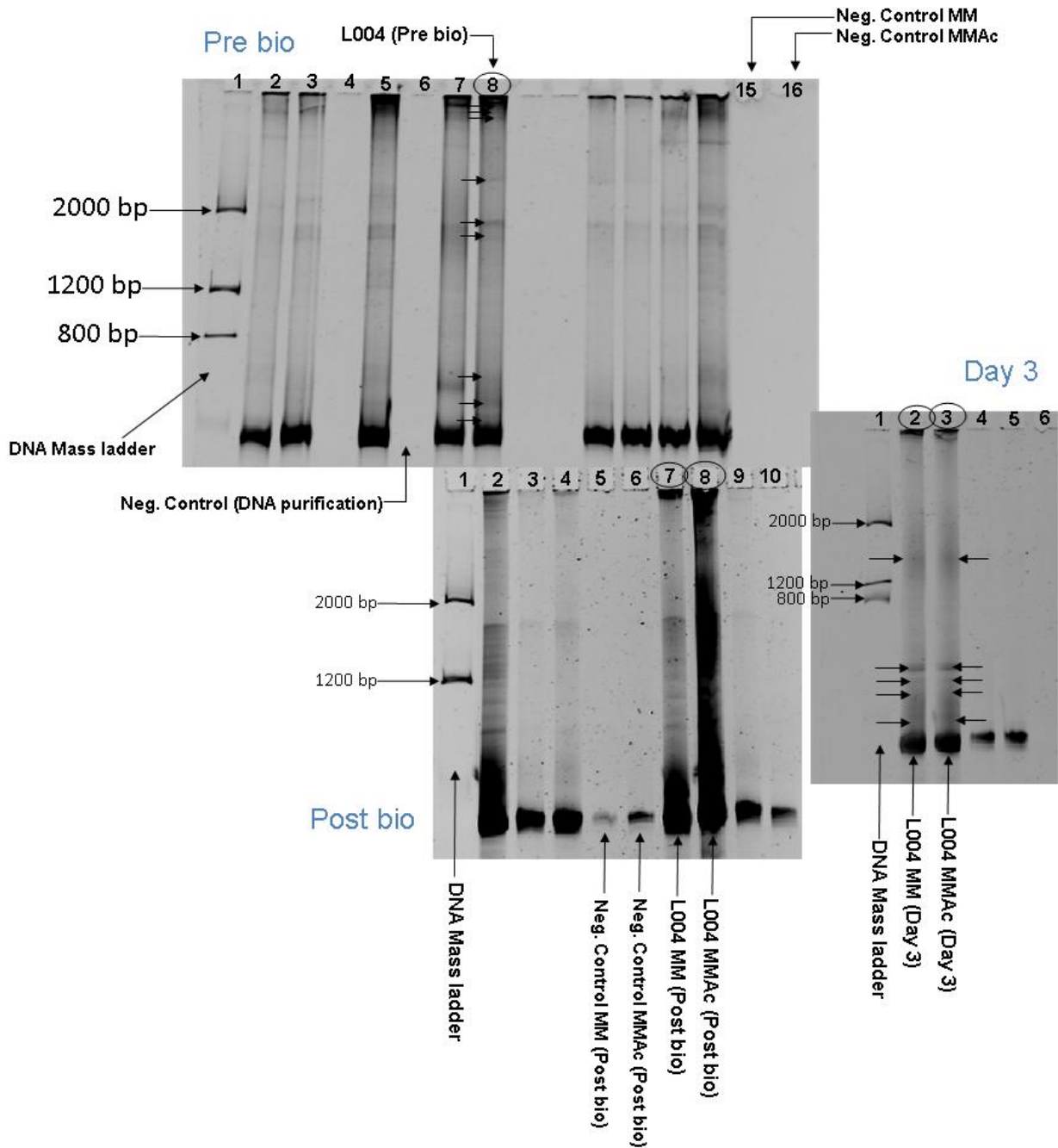
Figure 44. DGGE Bacteria for L004 incubated at 60˚C with Peregrino oil.
The picture displays three polyacrylamide comprising of samples; L004 (Pre bio), L004 (MM, day 3), L004 (MMAc, day 3), L004 (MM, Post bio) and L004 (MMAc, Post bio). Samples of interest are marked with circles.

The results indicate a variation between diversity patterns of L004 (MM) and L004 (MMAc).

Bioconversion experiment with Peregrino oil, inoculated with ML at 60˚C
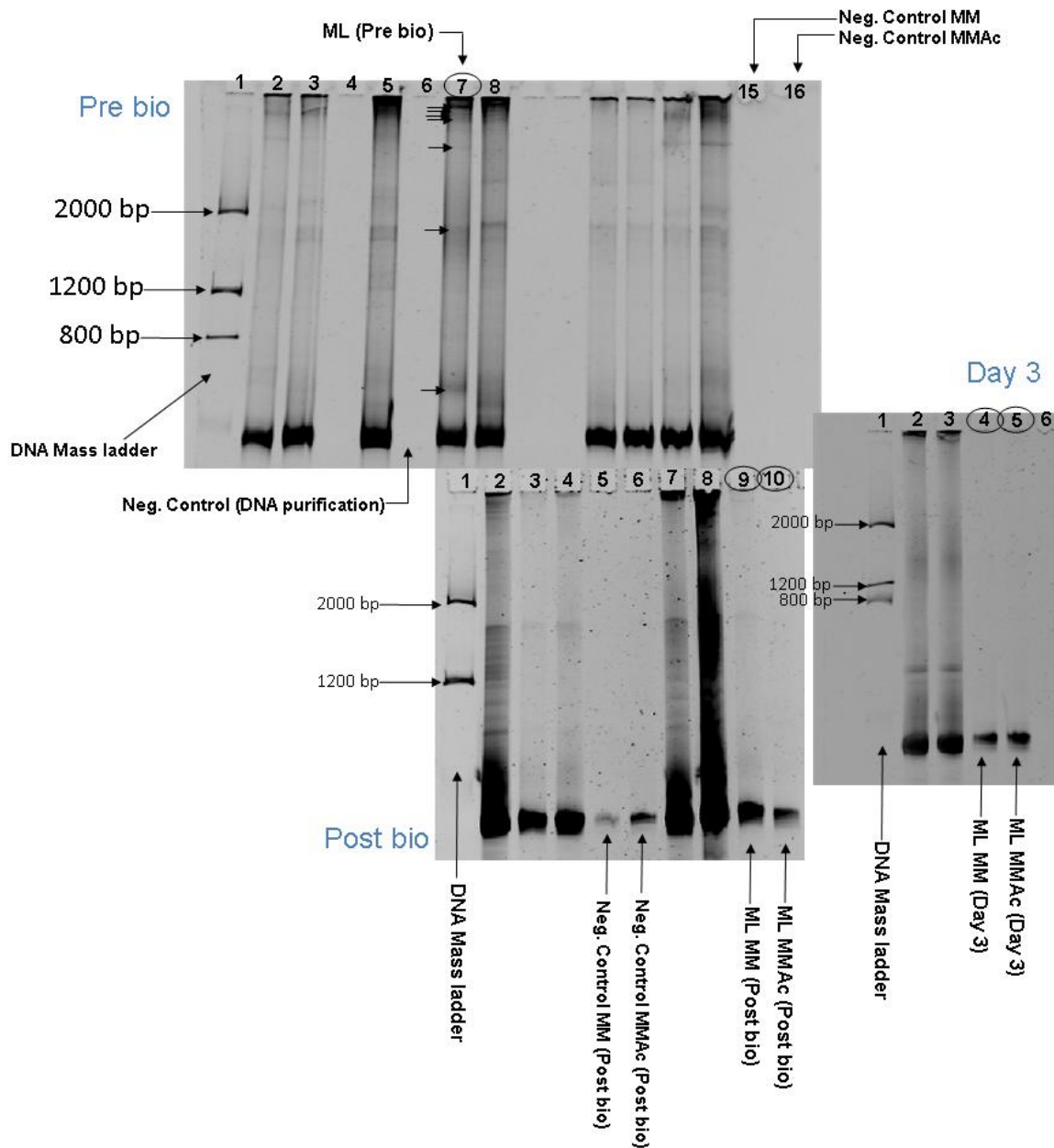
Figure 45. DGGE Bacteria for ML incubated at 60˚C with Peregrino oil.
The picture displays three polyacrylamide comprising of samples; ML (Pre bio), ML (MM, day 3), ML (MMAc, day 3), ML (MM, Post bio) and ML (MMAc, Post bio). Samples of interest are marked with circles.

The results indicate low biodiversity of both tests ML (MM) and ML (MMAc).

Bioconversion experiment with Bressay oil, inoculated with L004 at 35°C
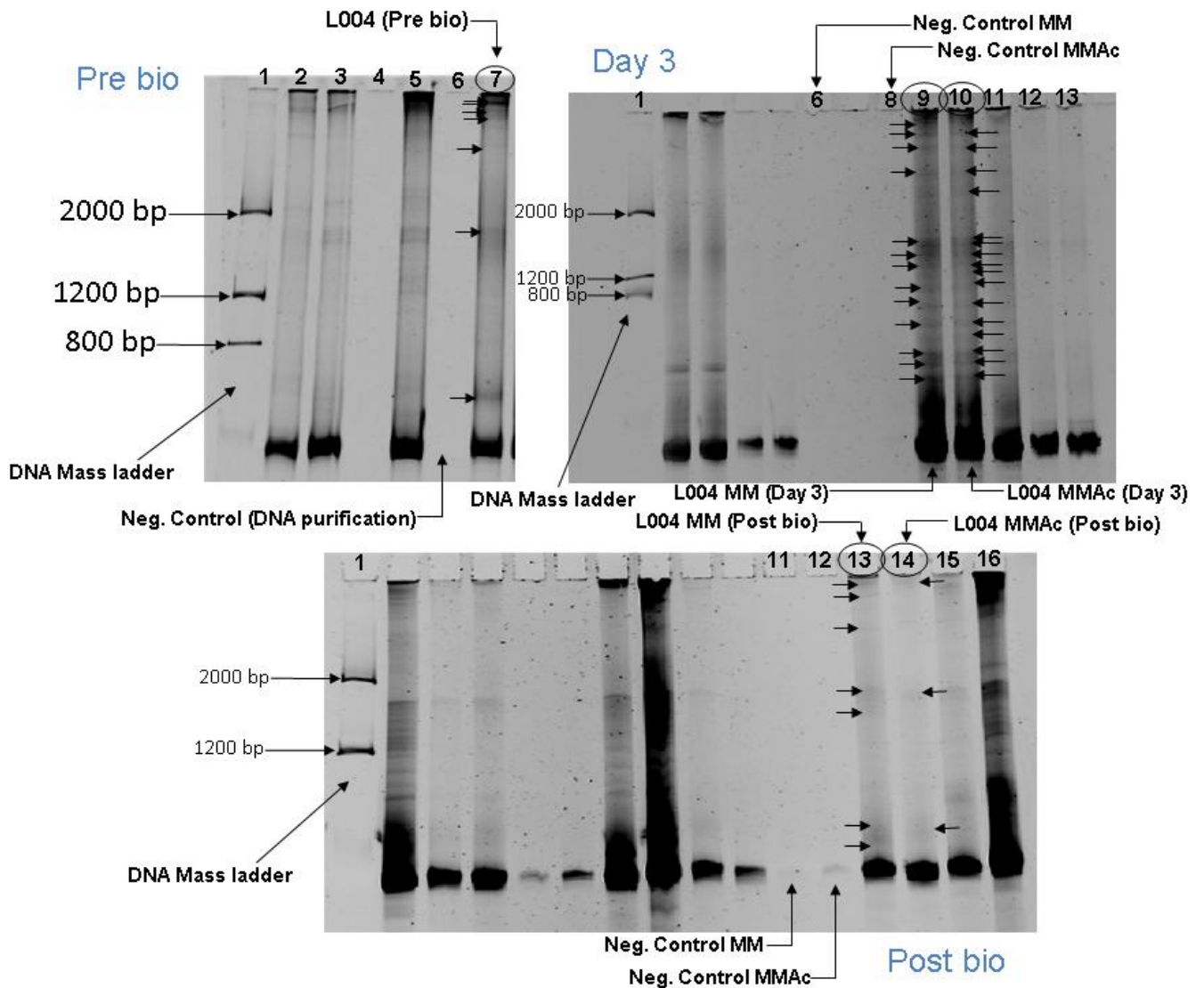
Figure 46. DGGE Bacteria for L004 incubated at 35°C with Bressay oil.
The picture displays three polyacrylamide comprising of samples; L004 (Pre bio), L004 (MM, day 3), L004 (MMAc, day 3), L004 (MM, Post bio) and L004 (MMAc, Post bio). Samples are marked with circles.

The results indicate a lower biodiversity in both media series of L004 (Post bio) compared to L004 (Day 3).

Bioconversion experiment with Bressay oil, inoculated with ML at 35˚C
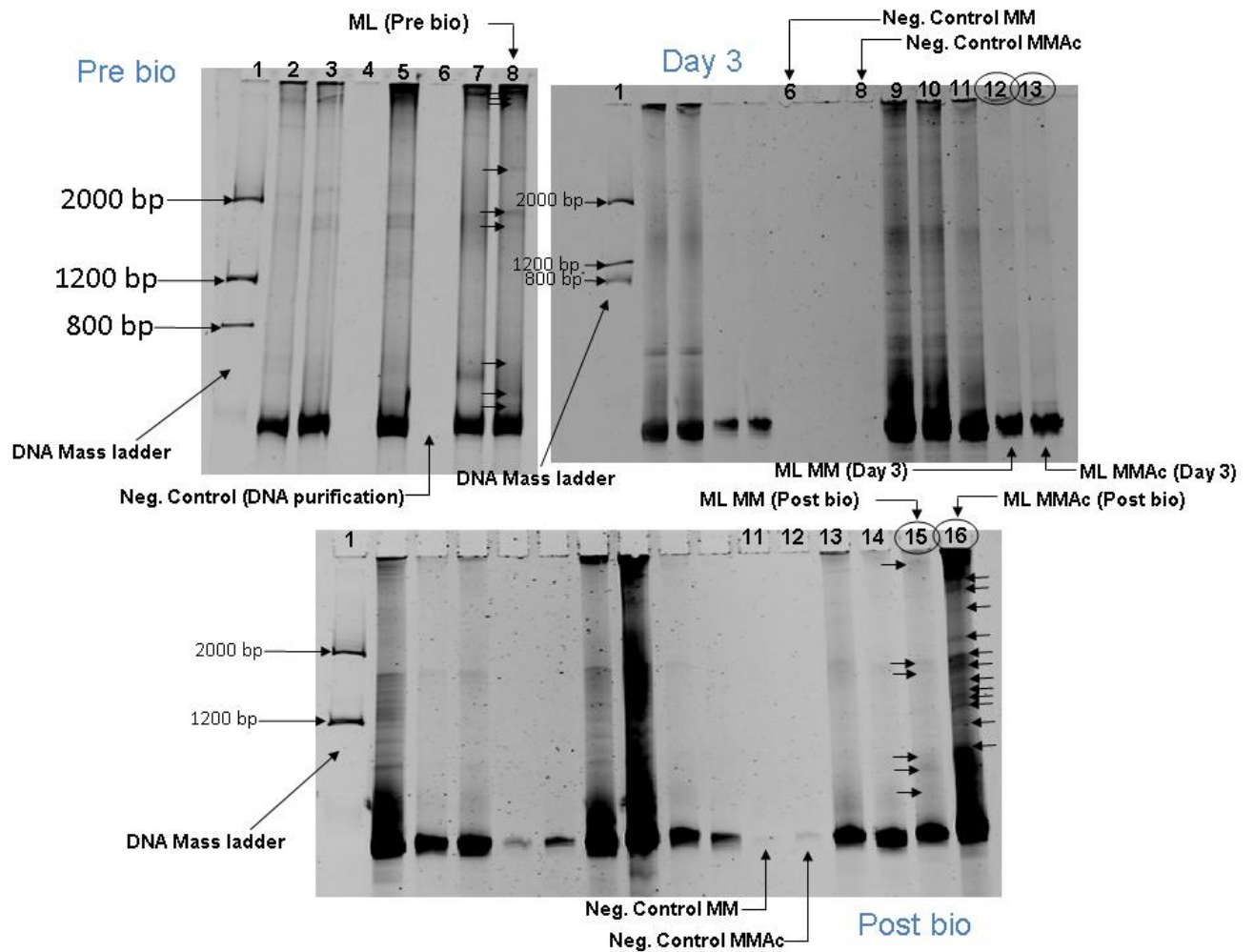
Figure 47. DGGE Bacteria for ML incubated at 35˚C with Bressay oil.
The picture displays three polyacrylamide comprising of samples; ML (Pre bio), ML (MM , day 3),
ML (MMAc, day 3), ML (MM, Post bio) and ML (MMAc, Post bio). Samples of interest are marked
with circles.

Results indicate a difference in biodiversity pattern between ML (MMAc) and ML (MM)
This could mean that ML incubated with MMAc had better growth conditions than ML
incubated with MM.

4.3.3 Account of the results from bioconversion experiment 3

Results from bioconversion experiment 3 more were assembled in a table to make them more legible (Table 23), and discussed as a whole in chapter 5.

Table 23. Account of the results from bioconversion experiment 3
The results from each tools used to gather information about the oil and consortia from bioconversion experiment 3, in addition to a short comment. Indications of bioconversion are highlighted green.

	Sample id.	Visual inspection of the oil	TLC-FID (fractional change?)	DGGE	Comment
Pre bio	ML	-	-	Pos	Viable inoculum
	L004	-	-	Pos	Viable inoculum
	MMT006	-	-	Pos	Viable inoculum
Mariner Maureen (46°C)	Neg.control MMAc	Neg	Yes (weak)	Neg	Indicating fractional changes, rerun of TLC-FID necessary
	L004 MMAc	4+	Yes (weak)	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	ML MMAc	Neg	Yes (weak)	Pos (weak)	Indicating fractional changes, rerun of TLC-FID necessary
	Neg.control MM	Neg	No	Neg	Negative as expected
	L004 MM	Neg	No	Pos (weak)	No indication of fractional change, rerun of TLC-FID necessary
	ML MM	Neg	No	Pos (weak)	No indication of fractional change, rerun of TLC-FID necessary
Peregrino (60°C)	Neg.control MMAc	Neg	Yes (weak)	Neg	Indicating fractional changes, rerun of TLC-FID necessary
	L004 MMAc	Neg/1+	Yes	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	ML MMAc	Neg	No	Neg	No indication of fractional changes as expected (Opt.g.t ML 35°C)*
	Neg.control MM	Neg	Yes (weak)	Neg	Indicating fractional changes, rerun of TLC-FID necessary
	L004 MM	1+	Yes (weak)	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	ML MM	Neg	No	Neg	No indication of fractional changes as expected (Opt.g.t ML 35°C)*
Bressay (35°C)	Neg.control MMAc	Neg/1+	No	Neg	No indication of fractional change, rerun of TLC-FID necessary
	L004 MMAc	1+	No	Pos (weak)	Indicating bio conversion, rerun of TLC-FID necessary
	ML MMAc	1+/2+	No	Pos	Indicating bio conversion, rerun of TLC-FID necessary
	Neg.control MM	Neg/1+	No	Neg	No indication of fractional change, rerun of TLC-FID necessary
	L004 MM	1+	No	Pos (weak)	Indicating bio conversion, rerun of TLC-FID necessary
	ML MM	Neg	No	Pos (weak)	No indication of fractional change, rerun of TLC-FID necessary

* Opt.g.t ML 35°C; optimal growth temperature for ML is 35°C.

5.   Discussion

Results achieved from analysis of main experiment 1 and 3 indicate bioconversion in several cases. Regarding experimental design one (main experiment 1), which was based on cultivating the inocula; MMT006, L004 and ML at their respective optimal growth temperature with Peregrino oil, the results indicated that inocula cultivated at their respective optimal growth temperature with nutritious media (MMAc) might have led to bioconversion of Peregrino oil. This also indicates a possible co-metabolism of acetate by the microorganisms.

Regarding experimental design two (main experiment 3), which was based on cultivating the inocula; L004 and ML at the respective reservoir temperatures of Mariner Maureen-, Peregrino- and Bressay oil, the results suggested a much stronger indication of bioconversion for oils inoculated with L004 than ML. This was based on positive indications of bioconversion in five out of six tests inoculated with L004. As the optimal growth temperature of ML is 35°C (Kjellsen 2010), it is not surprising that the results were poor when cultivating with Mariner Maureen- and Peregrino oil at 60°C and 46°C, respectively. Optimal growth temperature of L004 is 60°C (Kotlar 2011), and this could possibly have given L004 a better chance of bioconverting the oils.

Visual inspection of the oils showed cases were the oil had totally emulsified in the water. This made the oil- and water separation difficult and might have led to a high transfer of organic compounds which later was co-purified during DNA extraction. Measured concentration of extracted DNA gave calculated A260/A230 –ratios which also indicated co-purified contaminants in bioconversion experiment 1 and 3. It is possible that by choosing another method of extraction it might have helped in increasing the purity of the DNA from the most challenging samples. FastDNA® SPIN Kit for soil has been established as a useful method in extraction of DNA from oil contaminated soil. (Evans 2004) This method might also work for extracting DNA from oil samples.

DGGE results from PCR amplified DNA was difficult to interpret as the ancillary samples (pre-and post-bioconversion experiment) were conducted on separate gels. Because of this, characterizing was based on comparison between diversity patterns between the ancillary samples. This gave a crude and qualitative indication of changes between pre- and post-bioconversion. To confirm the results, advanced image analysis software could have been used to detect a more precise position of the bands in the gel (Rf –value) and their intensity. In combination with a program for interpolation of the data provided, and a multivariate analysis might have given a more precise basis for comparison of the fragments within a series of ancillary samples. (Tourlomousis 2010)

Results from both visual inspection of the oil samples in the shake flasks after ending the bioconversion experiments and TLC-FID results from the extracted oil, suggested fractional changes due to bioconversion experiment. TLC-FID results suggest a significant reduction of saturated and aromatic hydrocarbons in some of the conducted oil samples, which also has

been observed in earlier bioconversion experiments executed at Statoil's Research Centre. (Kotlar 2011) Some inconsistency was found between the results achieved for visual inspection and TLC-FID results and might be because both methods are crude and thus, only give an indication of fractional changes and bioconversion. TLC–FID only gives a qualitative impression of the relative distribution of heavy oil fractions; SARA. Consequently, the method does not detect the smaller changes on molecular level. The LC/MS (QTOF)-results (unfortunately not ready at time of submission of this report) might have given more detailed information of compositional changes in the bio converted oil.

In bioconversion experiment 1 and 3, there were some cases of oil inoculated with microorganism that showed negative results (Table 16 and 23). Possible factors like non-optimal growth conditions regarding temperature and the supply of nutrients could possibly serve as reasons for this. It is also a well-known fact that biological experiments are not easily conducted and since much of the biochemistry of microorganisms is still unknown, we have no way of predicting the outcome of a biological experiment. (Sen 2008)

There are several reports which suggest that microorganisms might have an impact on heavy oil fractions (Lazar 2007; Sen 2008; Kotlar 2011). Overall the microbial consortia used in these bioconversion experiments show indications of bioconversion potential of heavy oil fractions in Mariner Maureen, - Peregrino- and Bressay oil, both observed visually and supported by TLC-FID results indicating fractional changes in the oil. More research must be done to identify the specific changes and prove microbial impact by repeating these experiments, and alternately using more advanced analytical methodology for analysis of the oil.

Thoughts about experience achieved during the experiments of this Master's project and further work

The experimental designs of this Master's project were based on gathering as much qualitative information as possible without bringing to many variables into consideration which could make the results difficult to interpret. Because of limited time, no parallel series were carried out. To confirm the results produced in these bioconversion experiments regarding the promising inoculum L004, several parallels could have been carried out to obtain a quantitative impression of the bioconversion potential of the consortium.

Concerning the analysis of the inoculum, it could also be interesting to sample DNA directly from the oil conducted with the bioconversion experiment in addition to the water phase. Then do a more thorough characterization of the biodiversity in the consortium. DNA samples could be analyzed with DGGE and advanced software analysis to give a more precise identification of the resulting fragments in the DGGE gel. (Tourlomousis 2010)

A hybridization technique like southern blotting could have been used to transfer the fragments of interest to a membrane and use specific labelled probes to identify species. In addition, the fragments could be excised from the DGGE gel, re-amplified, sequenced and identified.

In conclusion, the results from the bioconversion experiments of this Master's project indicate that all three of the selected inocula have the potential of bioconverting oil, with inoculum L004 being the most promising one; suggesting that this consortia might have an impact on the heavy oil fractions of Mariner Maureen,- Peregrino- and Bressay oil. Additional research in the laboratory with specific, simulating reservoir conditions must be done, and finally (after producing sufficient results at laboratory scale) research and experiments must be conducted in the actual oil fields by introducing the inocula to the reservoir of current interest. Research and analysis of the production rate of the reservoir must be done to see whether the injection of the inocula enhances oil recovery and eventually leads to improved oil production of the reservoir. In other words, there is still much research to do before one can establish the microbial effect of the inocula on these oils.

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Appendix A. Medium recipes and procedures

Each of the mediums (RMMAcYE, MMAc and MM) was made fresh before each experiment and completed with TMS, vitamins and phosphate, as described table 1.

Medium volume: 1 Litre

Table 1. Chemicals used in each of the mediums and the amount per Litre.

Chemical	RMMAcYE (solid) / RMMAcYE Contents (per Litre)	MMAc (solid) / MMAc Contents (per Litre)	MM Contents (per Litre)
Ammonium nitrate (NH_4NO_3)	0.9 g	0.9 g	0.9 g
Calcium chloride ($CaCl_2$ 2* H_2O)	0.05 g	0.05 g	0.05 g
Magnesium sulphate ($MgSO_4$ 7* H_2O)	0.2 g	0.2 g	0.2 g
Sodium acetate (NaAcetate)	5 g	5 g	-
Yeast (YE)	0.5 g	-	-
Tryptone (T)	0.5 g	-	-
Peptone (P)	0.5 g	-	-
MQ - water / RO - water	900 g	900 g	900 g

NOTE: The chemical content marked with "-"is not used in the specific medium.

Mix and dissolve each component in MG - / RO – water in a bottle (1L) with a cork that can endure high pressure and temperature. Adjust pH to 7.5 with 1 M NaOH

Measured pH: _____   Adjusted pH:_____

If an agar solution is to be made; add bacteriological agar (15 g/L)
Autoclave for 20 minutes at 120 ℃.

After autoclaving, complete the medium (see table 2):

Table 2. Completion of the medium

Solution	Amount (ml/L)
Vitamins 1:100	1
TMS 1:1:1	3
Phosphate solution	100

Add phosphate solution (autoclaved); 100 ml/ l medium. See table 3.
Add autoclaved Trace Mineral solution (1:1:1); 3 ml/l. See table 4
Sterile filtrate vitamins (1ml, 1:100) and add to medium; 1ml/l. See table 5
If agar solution; make agar plates. Medium: ready for use.

Table 3.Recipe phosphate solution

Chemical	Manufacturer	Amount (g/L)
Di - sodium hydrogen phosphate ($Na_2HPO_4 * 2H_2O$)	ROTH	30,6
Potassium di -hydrogen phosphate (KH_2PO_4)	Merck	15,2
MQ - water / RO – water		1000

Table 4. TMS 1:1:1

TMS 1:1:1	Contents
Trace Mineral Solution 1 (TMS 1)	$FeSO_4* 7H_2O$ (5g/L), $CuSO_4* 5H_2O$ (0,39g/L), $ZnSO_4*7H_2O$ (0,44g/L), $MnSO_4*H_2O$ (0,15 g/L), $Na_2MoO_4*2H_2O$ (0,010 g/L), $CoCl_2*6H_2O$ (0,020 g/L), Ion free water (950 mL), Conc. HCl (50 mL)
Trace Mineral Solution 3 (TMS 3)	$Na_2SiO_3*9H_2O$ (2042,2 mg/L), NaF (445,5 mg/L), $K_2B_4O_7*4H_2O$ (5651,7 mg/L), $NaIO_3$ (47,9 mg/L), $KAl(SO_4)_2*12H_2O$ (180,7 mg/L), $SnCl_2*2H_2O$ (10,9 mg/L), Ion free water (950mL), Conc. HCl (50 mL).
Trace Mineral Solution 4 (TMS 4)	$NiCl_2*6H_2O$ (346,8 mg/l), $Na_2SeO_3*5H_2O$ (101,4 mg/l), V_2O_5 (18,0 mg/l), $K_2Cr_2O_7$ (14,0 mg/L), $Na_2WO_4*2H_2O$ (3,6 mg/L), Ion free water (950 mL), Conc. HCl (50 mL)

Mix solutions of TMS 1, 3 and 4 (made by lab workers at Statoil), in the proportions 1:1:1, autoclave and added to medium (3 ml/l).

Table 5. Vitamin Stock Solution

Chemical	Amount (g/L) / volume (mL/L)
Biotin	2,00 g/L
Folic acid	2,00 g/L
Pyridoxine-HCl	10,00 g/L
Thiamine-HCl*$2H_2O$	5,00 g/L
Riboflavin	5,00 g/L
Nicotinic acid	5,00 g/L
p-aminobenzoic acid	0,1 g/L
Vitamin B12 or cyanocobalamin	5,00 g/L
Lipoic acid or DL-Thioctic Acid	5,00 g/L
Ion free water	1000 mL

Dilute vitamin Stock Solution into proportion of 1:100, and transfer to ampoules (1 ml). This was previously made by lab workers at both Sintef and Statoil. Vitamin solution of 1 ml is further sterile filtrated before use.

Appendix B. Chemicals, kits and equipment used in the project

Table B1. Chemicals

Aim	Chemical / reagents	Contents / manufacturer
Freezing down inoculum	86-88 % glycerol, 85 % glycerol	Merck KGaA, Germany, Sigma-Aldrich Norway AS; kat.nr.33224
Preparation of oil samples	Dichloromethane (HPLC qual.)	Lab-Scan, Analytical Sciences, Poland
Thin - Layer Chromatography w/ flame ionization detection (TLC-FID)	n-Hexane (100%), HPLC quality	Lab-Scan, Analytical Sciences, Poland / Merck KGaA, Germany
	Toluene (100 %), HPLC quality	Merck KGaA, Germany / VWR, US
	Dichloromethane: Methanol (95:5),	Lab-Scan, Analytical Sciences, Poland
Electrophoresis of DNA (Agarosegel)	0,5 X TBE	(0,045M Tris, 0,045M borsyre, 1mM EDTA). PRIME, Germany
	Agarose	Carl ROTH GmbH, Denmark
	Blue/Orange 6 X Gel loading Dye	Promega corporation, U.S
	SYBR®Safe DNA gel Stain, 10 000	Invitrogen, Life Technologies, U.S
	Low DNA Mass Ladder	Invitrogen, Life Technologies, U.S
Denaturating Gradient Gel Electrophoresis (DGGE)	50 X TAE	(2M Tris-Acetate, 0,05 M EDTA), Eppendorf
	40 % Acrylamide/bis	Carl ROTH GmbH, Denmark
	Formamide	Fisher Bioreagents, Thermo Fisher Scientific Inc. US
	Urea	Invitrogen, Life Technologies, U.S
	Ammonium persulfate (APS, 10%)	Bio-Rad Laboratories.Inc, U.S
	TEMED	Bio-Rad Laboratories.Inc, U.S
	SYBR®Gold nucleic acid stain	Invitrogen, Life Technologies, U.S
	Low DNA Mass Ladder	Invitrogen, Life Technologies, U.S
	DNA gel loading buffer, 10 X	PRIME, Germany
	Denaturing solution (20 %)	40% acrylamide/bis (15 mL, Carl ROTH), 50x TAE – buffer (2 mL, Eppendorf), formamide (8 mL, Fisher Reagents), urea (8,4 g, Invitrogen)
	Denaturing solution (70 %)	40% acrylamide/bis (15 mL, Carl ROTH), 50x TAE – buffer (2 mL, Eppendorf), 28 mL, Fisher Bioreagents), Urea (29,4 g, Invitrogen)

Table B2. Kits

Method	Kits, reagents and enzyme	Manufacturer
DNA extraction	Dneasy® Blood & Tissue kit (250)	Qiagen, Germany
	50 med mer Tris-HCL, pH 8	Sigma-Aldrich Norway
	Lysozyme, 50000 u/mg cryst.	Merck KGaA, Germany
	0,1 M EDTA, pH 8	FLUKA BioChemica, Sigma Aldrich, Norway
	Triton - X 100	Kemetyl, Sweden
	Ethanol (96 %)	Carl ROTH GmbH, Denmark
PCR	PCR buffer, 10x	Qiagen, Germany
	dNTP -mix, 10 mM	Qiagen, Germany
	HotStarTaq® Plus DNA Plymerase	Qiagen, Germany

Table B3. Equipment

Equipment / instrument	Producer
Microwell	F96 MicroWell™ Plate, Polysterene clear — Nunc™ Thermo Fisher Scientific Inc, U.S
	24 Microwell Nunc polysterene plate — Nunc™ Thermo Fisher Scientific Inc, U.S
	V96 deep well, MASTERBLOCK storage plate, poly propylene — Greiner - Bio - One International GmbH, Germany
Incubation cabinet	BINDER incubator — BINDER, GmBH, U.S
	Incubation shaker; Multitron — Infors AG, Bottmingen, Switzerland
	Incubation shaker; Minitron — Infors AG, Bottmingen, Switzerland
Hybridization oven	ProBlot 125 — Labnet International.inc, US.
Reciprocal table	Heigar reciprocal table — Heigar, HT, Norway
Heating block	Grant QBT2 — Grant Instruments (Cambridge) Ltd
Centrifuge	Centrifuge 5424 — Eppendorf, Germany
	Centrifuge 5430 R — Eppendorf, Germany
	Centrifuge 5804 — Eppendorf, Germany
Measurement of DNA concentration	NanoDrop® Spectrophotometer, ND - 1000 — Thermo Fisher Scientific Inc, U.S
PCR - instrument	Mastercycler® ep — Eppendorf, Germany
Electroporator	Consort E132, PowerPac™Basic — Bio-Rad Laboratories.Inc, U.S
Electrophoresis cell	Sub-Cell® GT System — Bio-Rad Laboratories.Inc, U.S
DGGE instrument	DCODE™System — DCODE™, Bio-Rad Laboratories.Inc, U.S
	Gradient Delivery System, Model 475 — Bio-Rad Laboratories Inc, U.S
Photo system	Bio imaging system (Gel-Doc) — Syngene, Synoptics Ltd, England
Thin-Layer Chromatografi w/ flame ionization detection (TLC-FID)	Iatroscan™ MK-6/6s — Mitsubishi Kagaku Iatron, Inc. Tokyo, Japan
OD measurement	Sample spotter Mod. SES 3202/IS03 — SES Analysesysteme GmbH, Germany
	Spektrophotometer FLUOstar Omega — BMG LABTECH GmbH, Offenburg, Germany
Re-inoculation and standardization of inoculum	Robotic workstation 200 — Tecan group Ltd., Switzerland

Appendix C. OD measurements of 96-microwell plates

OD measurements of ML master plates made from picking colonies. Wells which has been added colonies and has an OD measurement over OD 0.05 (660nm), are marked yellow.

	ID1: Masterplate_ML_1											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,417	0,445	0,631	0,054	0,032	0,03	0,029	0,028	0,029	0,032	0,035	0,029
B	0,381	0,293	0,61	0,355	0,063	0,034	0,03	0,03	0,027	0,029	0,034	0,043
C	0,628	0,312	0,456	0,575	0,035	0,038	0,037	0,031	0,029	0,028	0,028	0,031
D	0,462	0,347	0,402	0,356	0,034	0,042	0,036	0,029	0,033	0,039	0,027	0,03
E	0,663	0,375	0,391	0,353	0,034	0,041	0,03	0,036	0,044	0,029	0,028	0,028
F	0,345	0,276	0,459	0,031	0,029	0,03	0,033	0,025	0,033	0,035	0,033	0,046
G	0,51	0,412	0,24	0,336	0,033	0,034	0,052	0,027	0,033	0,029	0,029	0,026
H	0,501	0,225	0,454	0,61	0,031	0,032	0,031	0,031	0,033	0,033	0,03	0,031

Figure C1 ML master plate 1. OD/ 0.2 mL

	ID1: Masterplate_ML_2											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	1,529	0,365	0,344	0,319	0,054	0,036	0,029	0,029	0,029	0,03	0,028	0,029
B	0,364	0,52	0,405	0,549	0,04	0,037	0,029	0,028	0,029	0,028	0,029	0,031
C	0,562	0,507	0,025	1,449	0,032	0,033	0,028	0,025	0,028	0,027	0,027	0,027
D	0,504	0,492	0,026	1,406	0,041	0,027	0,023	0,028	0,029	0,029	0,03	0,026
E	0,509	0,513	1,478	0,024	0,028	0,028	0,036	0,026	0,024	0,026	0,029	0,023
F	0,651	0,427	0,382	0,036	0,032	0,032	0,025	0,03	0,025	0,026	0,027	0,027
G	0,522	0,55	0,603	0,029	0,047	0,028	0,027	0,026	0,027	0,027	0,028	0,028
H	0,028	0,485	1,478	0,032	0,039	0,028	0,024	0,04	0,03	0,029	0,029	0,028

Figure C2 ML master plate 2. OD/0.2 mL

	ID1: Masterplate_ML_3											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,714	0,638	1,66	0,522	0,589	0,621	0,026	0,023	0,026	0,027	0,029	0,028
B	0,818	1,494	0,557	0,622	0,562	0,596	0,025	0,028	0,021	0,028	0,028	0,029
C	1,625	0,671	0,604	0,576	0,024	0,6	0,026	0,027	0,028	0,027	0,027	0,026
D	1,803	0,636	0,585	1,48	0,024	0,696	0,029	0,027	0,027	0,026	0,028	0,026
E	0,795	0,599	0,612	0,627	0,6	0,02	0,029	0,027	0,027	0,028	0,027	0,028
F	0,025	0,588	0,626	0,632	1,534	0,023	0,023	0,022	0,027	0,027	0,036	0,027
G	0,729	1,711	0,02	0,603	0,572	0,024	0,028	0,028	0,025	0,027	0,028	0,025
H	1,767	0,652	1,265	0,601	0,603	0,024	0,028	0,027	0,025	0,026	0,026	0,028

Figure C3 ML master plate 3. OD/0.2 mL

Backup plates

Backup plates were made of each master plate. Figures C4- C6 shows the backup plates used for making the fusions plate. An overview of the transferred colonies is found in figure C7.

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: ML_1A_Statoil											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
A	0,049	0,753	0,626	0,027	0,026	0,028	0,024	0,028	0,024	0,026	0,03	0,028
B	0,046	0,046	0,066	0,74	0,033	0,026	0,026	0,027	0,022	0,024	0,031	0,028
C	0,073	0,824	0,677	0,79	0,026	0,022	0,023	0,03	0,026	0,027	0,027	0,028
D	0,052	0,054	0,687	0,739	0,028	0,022	0,03	0,028	0,027	0,031	0,027	0,027
E	0,071	0,053	0,652	0,046	0,026	0,028	0,025	0,028	0,035	0,025	0,029	0,028
F	0,047	0,046	1,903	0,024	0,025	0,025	0,027	0,029	0,031	0,028	0,026	0,026
G	0,073	0,092	0,04	0,047	0,03	0,025	0,026	0,033	0,029	0,043	0,028	0,03
H	0,7	0,037	0,059	0,059	0,029	0,027	0,027	0,029	0,026	0,026	0,028	0,03

Figure C4. Backup plate 1A, from master plate ML 1. OD/0.2 mL

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: ML_2A_Statoil											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
A	2,119	0,824	0,787	0,611	0,043	0,025	0,034	0,036	0,031	0,034	0,037	0,032
B	0,869	0,903	0,715	0,91	0,044	0,033	0,034	0,03	0,033	0,029	0,032	0,031
C	1,465	0,953	0,033	2,13	0,04	0,034	0,029	0,029	0,03	0,031	0,029	0,032
D	0,943	0,956	0,032	2,206	0,035	0,033	0,031	0,032	0,032	0,033	0,044	0,041
E	0,943	0,947	2,248	0,032	0,032	0,029	0,034	0,029	0,03	0,031	0,032	0,044
F	1,125	0,842	0,803	0,032	0,029	0,028	0,03	0,028	0,032	0,04	0,032	0,034
G	0,982	0,905	0,933	0,032	0,036	0,029	0,029	0,031	0,03	0,034	0,045	0,039
H	0,033	0,937	1,661	0,027	0,028	0,034	0,038	0,033	0,031	0,03	0,033	0,032

Figure C5. Backup plate 2A, from master plate ML 2. OD/0.2 mL

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: ML_3A_Sintef											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
A	0,758	0,84	2,262	0,727	0,79	0,653	0,048	0,037	0,027	0,027	0,028	0,028
B	0,939	1,956	0,653	0,639	0,688	0,714	0,078	0,03	0,027	0,028	0,026	0,024
C	2,057	0,931	0,904	0,935	0,026	0,988	0,066	0,028	0,023	0,025	0,027	0,028
D	2,128	0,611	0,925	1,742	0,024	0,98	0,045	0,029	0,024	0,023	0,029	0,027
E	0,924	0,864	0,942	0,888	0,868	0,024	0,034	0,028	0,026	0,022	0,025	0,028
F	0,03	0,883	0,857	0,869	1,849	0,025	0,027	0,026	0,026	0,025	0,031	0,028
G	0,898	2,168	0,037	0,913	0,616	0,026	0,031	0,026	0,026	0,035	0,027	0,03
H	2,192	0,933	1,979	0,895	0,918	0,029	0,03	0,033	0,031	0,025	0,031	0,027

Figure C6. Backup plate 3A, from master plate ML 3. OD/0.2 mL

	1	2	3	4	5	6	7	8	9	10	11	12
				ID1: ML fusion plate								
A	1A	2B	3D	4E	1F	2E	-	1B	2C	3C	4C	5E
B	1C	2C	3E	4G	1G	2F	3H	1C	2D	3D	4D	5F
C	1C	2E	3F	4H	2A	2G	3G	1D	2E	3E	4E	5G
D	1D	2G	3H	1A	2B	2H	4A	1E	2F	-	4F	5H
E	1E	3A	4B	1B	-	3A	4B	1G	2G	3H	4G	6A
F	1G	3B	4C	1C	-	3B	4C	1H	2H	3F	4H	6B
G	1H	3C	4C	1D	2C	3E	4D	2A	3A	4A	5A	6C
H	2A	3C	4D	1E	2D	3F	1A	2B	3B	4B	5B	6D

Figure C7. Overview of the colonies from each of the three back up plates (figure C4-C6) transferred into this fusion plate. Cells coloured orange, green and blue represents wells filled with colonies from ML back up plate 1A, 2A and 3A, respectively.

This fusion plate of ML was used for making of backup plates, which in turn was used for inoculum making in each experiment.

Re inoculum used for experiment 1

As described in chapter 3.3.2, master plates were thawed, re inoculated and cultivated for some days. Then OD was measured to be sufficient and the inoculum was standardized for the shake flask experiment. Figure D8-D10 shows the OD measurements of the re inoculated plates.

	1	2	3	4	5	6	7	8	9	10	11	12
	Absorbance			ID1: ML	Absorbance values are displayed as OD							
	Raw Data (660)											
A	0,157	1,154	0,064	0,965	0,917	0,265	1,878	1,197	0,711	0,308	0,155	0,145
B	0,106	0,059	0,126	1,954	1,423	0,435	1,796	0,569	0,928	0,126	0,295	0,19
C	0,05	0,04	0,809	0,574	1,365	2,015	0,207	1,299	0,158	0,196	0,024	0,574
D	0,092	1,275	0,887	1,009	1,291	0,58	0,537	0,619	0,589	1,3	0,18	0,024
E	0,868	1,279	0,772	0,798	1,266	1,569	0,927	0,096	0,167	0,333	1,482	0,025
F	1,135	1,353	0,064	0,991	0,365	0,782	0,844	0,163	0,597	0,376	0,551	0,02
G	1,152	1,396	0,047	0,627	1,317	0,446	0,61	0,164	0,156	0,194	0,187	0,023
H	0,057	1,007	0,914	0,896	1,101	1,361	0,231	0,931	0,978	0,194	0,108	0,024

Figure C8. Re inoculum of backup plates made from fusion of ML – master plates

ID1: L004
Absorbance — Absorbance values are displayed as OD
Raw Data (660)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,892	0,615	0,932	0,035	1,032	1,062	0,921	0,946	1,042	1,368	1,021	1,112
B	0,663	0,651	1,175	0,264	0,229	0,992	1,07	0,916	0,89	0,877	1,971	0,159
C	0,707	0,576	1,05	0,275	0,95	1,559	0,455	0,303	1,088	1,084	0,489	1,138
D	0,384	0,38	0,905	2,051	1,082	1,078	1,129	0,44	1,141	1,083	0,999	0,865
E	0,842	0,393	0,857	0,393	0,674	1	0,612	1,089	1,112	1,215	0,988	1,22
F	1,156	0,808	0,854	1,023	0,438	0,434	0,792	0,423	1,212	1,27	1,922	0,419
G	0,449	0,446	0,45	0,471	2,355	1,161	0,448	1,141	1,133	1,16	0,45	0,45
H	1,334	1,929	1,124	0,445	0,441	0,447	0,446	0,836	0,591	1,585	1,938	0,97

Figure C9. Re inoculum of L004

ID1: MMT006
Absorbance — Absorbance values are displayed as OD
Raw Data (660)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,667	0,631	0,58	0,776	0,751	0,845	0,699	0,683	0,51	1,277	0,469	0,455
B	0,591	0,586	0,692	0,654	0,725	0,817	0,634	0,748	0,392	0,444	0,356	0,794
C	0,586	0,453	0,607	0,682	0,529	0,589	1,439	0,633	1,024	1,524	0,61	0,283
D	0,475	0,775	0,602	0,682	0,896	0,56	0,635	0,631	0,698	0,69	0,589	0,547
E	0,607	0,659	0,405	0,465	0,652	0,666	0,779	0,63	0,362	1,619	0,596	0,539
F	0,585	0,794	0,441	0,478	0,533	0,315	0,631	0,668	0,539	0,568	1,14	1,821
G	2,394	0,469	0,315	0,31	0,697	0,698	0,715	0,747	2,004	1,725	0,57	0,512
H	0,583	0,544	0,36	0,42	0,76	0,751	0,701	0,784	1,958	1,984	0,79	0,541

Figure C10. Re inoculum of MMT006

Re inoculum used for experiment 2

ID1: ML
Absorbance — Absorbance values are displayed as OD
Raw Data (660)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,03	0,03	1,05	1,057	1,723	1,193	0,994	1,12	1,876	1,104	1,236	1,079
B	0,027	1,039	1,043	1,101	1,013	1,142	1,159	2,147	1,124	1,163	2,444	2,351
C	0,027	0,029	1,115	1,04	1,162	1,025	0,978	2,374	1,042	1,488	1,245	0,947
D	0,028	0,943	1,21	2,245	1,032	1,041	1,008	1,13	1,049	1,227	1,187	1,226
E	0,032	1,01	0,996	1,382	1,137	1,226	0,965	1,209	1,891	1,933	1,197	1,245
F	1,087	0,028	1,014	1,783	1,019	1,337	2,184	2,134	1,796	1,16	1,25	1,098
G	1,03	1,006	1,004	1,235	1,081	2,184	1,997	0,976	1,969	0,871	1,102	1,108
H	1,029	1,009	1,024	1,005	1,025	1,138	1,123	2,41	1,074	1,125	0,928	0,969

Figure C11. Re inoculum of backup plates made from fusion of ML – master plates

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: L004											
Absorbance				Absorbance values are displayed as OD								
Raw Data (660)												
A	0,532	0,503	0,583	0,019	0,386	1,065	0,86	0,846	0,815	0,879	0,23	0,187
B	0,651	0,761	0,8	0,75	0,062	0,752	0,731	0,715	0,752	0,757	0,937	0,031
C	0,707	0,163	0,516	0,773	0,686	0,281	0,573	0,037	0,747	0,731	0,031	0,818
D	0,628	0,11	0,083	0,25	0,765	0,677	0,717	0,582	0,707	0,755	0,9	0,959
E	0,817	0,576	0,735	0,017	0,763	0,586	0,148	0,666	0,715	0,717	0,036	0,812
F	0,627	0,046	0,614	0,686	0,747	0,767	0,788	0,031	0,037	0,636	0,377	0,039
G	0,829	0,187	0,744	0,879	0,931	0,497	0,765	0,593	0,593	0,434	0,429	0,031
H	0,916	0,726	0,923	0,775	0,96	0,216	0,024	0,272	1,117	0,227	1,158	0,747

Figure C12. Re inoculum of L004

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: MMT006											
Absorbance				Absorbance values are displayed as OD								
Raw Data (660)												
A	0,036	0,042	0,038	0,04	0,035	0,028	0,029	0,049	0,037	0,031	0,046	0,027
B	0,067	0,032	0,027	0,03	0,034	0,027	0,028	0,042	0,037	0,033	0,047	0,032
C	0,032	0,036	0,027	0,027	0,055	0,027	0,032	0,047	0,029	0,029	0,057	0,033
D	0,039	0,038	0,03	0,027	0,054	0,029	0,029	0,059	0,051	0,026	0,038	0,042
E	0,039	0,059	0,027	0,029	0,045	0,025	0,025	0,038	0,06	0,03	0,059	0,037
F	0,038	0,068	0,027	0,033	0,086	0,025	0,025	0,043	0,044	0,026	0,061	0,047
G	0,037	0,141	0,027	0,025	0,029	0,027	0,031	0,031	0,037	0,032	0,032	0,027
H	0,034	0,027	0,026	0,031	0,049	0,026	0,026	0,045	0,055	0,026	0,034	0,042

Figure C13. Re inoculum of MMT006

Re inoculum used for experiment 3

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: ML											
Absorbance				Absorbance values are displayed as OD								
Raw Data (660)												
A	0,011	0,037	1,691	1,795	2,081	1,771	1,811	1,829	1,861	1,991	2,247	1,997
B	0,089	1,859	1,899	1,763	1,907	1,449	1,271	2,341	1,743	1,367	1,787	2,561
C	0,009	0,011	1,865	1,839	1,517	1,937	2,013	2,567	1,153	1,839	1,413	1,967
D	1,365	0,301	1,879	3,003	1,755	1,799	1,101	1,827	1,573	1,593	1,341	1,407
E	0,561	1,989	1,863	1,649	1,899	1,553	1,815	1,301	2,631	2,485	1,513	2,435
F	1,943	0,245	1,957	2,355	1,931	1,545	2,507	2,473	1,819	1,323	1,449	1,703
G	1,681	1,909	2,001	1,797	1,931	2,635	2,923	1,967	2,705	0,837	1,209	1,465
H	2,113	2,113	2,097	2,043	2,105	1,449	1,689	2,403	1,035	0,851	0,961	1,897

Figure C14. Re inoculum of backup plates made from fusion of ML – master plates

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: L004											
	Absorbance values are displayed as OD											
	Raw Data (660)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1,113	1,297	0,751	0,037	0,323	0,063	0,195	0,719	0,709	0,827	0,635	0,833
B	0,667	0,623	0,069	0,899	0,465	1,119	0,839	0,789	0,627	0,799	0,733	0,655
C	1,447	0,069	0,451	0,507	0,789	0,813	0,015	0,047	0,641	0,711	0,059	0,589
D	0,585	0,045	0,697	0,813	0,683	0,829	0,751	0,637	0,671	0,405	0,955	0,559
E	1,133	0,069	0,703	0,035	0,485	0,671	0,065	0,521	0,597	0,555	0,041	0,433
F	0,621	0,325	0,403	0,703	0,015	0,573	0,627	0,043	0,145	0,597	0,443	0,243
G	1,129	0,213	1,777	0,051	1,203	1,175	0,633	0,509	0,621	0,475	0,019	0,021
H	0,609	1,719	0,835	2,591	1,301	0,169	0,169	0,367	0,759	0,687	0,305	0,341

Figure C15. Re inoculum of L004

Appendix D. Normalization of inocula

Normalization of inoculum experiment 1

Figure D1- D8 show OD measurements (Optic density (OD/0.2mL) 96-microwell plate) before and after normalization of inoculum. Calculations for normalization of OD are under each figure.

Normalization calculations are based on the formulas;

1. Calculation of OD in the total volume of standardized inoculum

$$\frac{\text{Measured OD}}{0,2 \text{ mL}} \text{ X volume inoculum (mL)} = \text{OD}$$

And 2; dividing the calculated total OD by the same amount in volume gives the volume MM which the inoculum is diluted in.

Ex. Calculated OD is 0.1. This makes up the calculation;

$$\frac{\text{OD } 0,1}{0,1 \text{ mL}} = 1 \text{ (OD/mL)}$$

Note; For several of the standardized inoculums the measured OD was very high and resulted in high dilution volumes and time-consuming work. It was later realized that a smarter way to normalize the inoculum could be to calculate the volume of inoculum needed for bioconversion experiment, and using this as total volume and basis for dilution calculation.

Experiment 1

OD measurements before normalization;

				ID1: 1A -ML, 3A-L004,5A-MMT006								
	Absorbance			Absorbance values are displayed as OD								
	Raw Data (660)											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,181	0,027	0,462	0,028	0,668	0,041	0,026	0,027	0,026	0,029	0,026	0,026
B	0,026	0,026	0,032	0,038	0,028	0,026	0,026	0,026	0,026	0,027	0,027	0,025
C	0,026	0,026	0,029	0,027	0,029	0,026	0,029	0,024	0,025	0,027	0,026	0,026
D	0,027	0,025	0,023	0,025	0,025	0,029	0,023	0,024	0,026	0,026	0,027	0,025
E	0,027	0,027	0,02	0,023	0,025	0,024	0,028	0,022	0,021	0,024	0,027	0,027
F	0,03	0,027	0,026	0,021	0,019	0,027	0,026	0,023	0,03	0,026	0,027	0,028
G	0,026	0,027	0,024	0,026	0,027	0,026	0,024	0,021	0,02	0,028	0,026	0,025
H	0,022	0,027	0,028	0,026	0,024	0,025	0,021	0,026	0,028	0,026	0,028	0,03

Figure D1.Measurement of OD/0.2 mL, before normalization. Inoculum ML, L004 and MMT006 is found in well 1A, 3A and 5A, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: ML_12_A											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
A	0,187	0,026	0,455	0,026	0,665	0,042	0,025	0,027	0,024	0,024	0,024	0,177
B	0,025	0,026	0,025	0,026	0,025	0,024	0,026	0,024	0,024	0,023	0,026	0,025
C	0,025	0,028	0,019	0,023	0,025	0,026	0,028	0,023	0,024	0,023	0,025	0,024
D	0,024	0,022	0,023	0,024	0,024	0,026	0,02	0,018	0,026	0,025	0,024	0,025
E	0,024	0,025	0,021	0,029	0,023	0,022	0,031	0,02	0,02	0,024	0,02	0,023
F	0,029	0,026	0,019	0,022	0,018	0,022	0,021	0,028	0,024	0,021	0,031	0,023
G	0,024	0,024	0,024	0,025	0,028	0,023	0,018	0,02	0,018	0,024	0,023	0,025
H	0,021	0,025	0,028	0,024	0,029	0,023	0,017	0,025	0,025	0,023	0,027	0,028

Figure D2. Measurement of OD/0.2 mL after the second round of standardization (chapter 4.1.2), before normalization. Inoculum ML is found in well 12A. Normalization calculations;

ML; because measured OD was low, OD/mL was decreased to 0.9

$$\frac{OD\ 0,177 \times 4,6\ mL}{0,2\ mL} = \underline{4,071}$$

$$OD\ 0,9 = \frac{0,471}{OD\ 0,9} = 4,5\ mL$$

→ ML was diluted with 4.5 mL

MMT006;

$$\frac{OD\ 0,668 \times 0,6\ mL}{0,2\ mL} = OD\ 2,004$$

→ MMT006 was diluted with 2.004 mL MM

L004;

$$\frac{OD\ 0,462 \times 2,0\ mL}{0,2\ mL} = OD\ 4,62$$

→ L004 was diluted with 4.62 mL

OD measurements after normalization;

	1	2	3	4	5	6	7	8	9	10	11	12
	ID1: Inoculum; 8H_MM006-10H_L004-12H_ML											
Absorbance	Absorbance values are displayed as OD											
Raw Data (660)												
A	0,256	0,027	0,463	0,026	0,685	0,026	0,03	0,027	0,024	0,026	0,025	0,264
B	0,025	0,026	0,024	0,025	0,027	0,023	0,02	0,021	0,027	0,025	0,029	0,026
C	0,025	0,022	0,029	0,024	0,023	0,023	0,02	0,024	0,024	0,027	0,024	0,026
D	0,025	0,024	0,024	0,023	0,02	0,024	0,02	0,024	0,021	0,026	0,026	0,026
E	0,026	0,024	0,023	0,022	0,021	0,022	0,02	0,023	0,019	0,023	0,02	0,029
F	0,031	0,026	0,022	0,022	0,019	0,025	0,02	0,021	0,023	0,021	0,024	0,025
G	0,025	0,026	0,037	0,019	0,027	0,023	0,02	0,029	0,024	0,026	0,025	0,025
H	0,025	0,027	0,059	0,025	0,05	0,023	0,02	0,164	0,024	0,162	0,028	0,062

FigureD3. Measurements of OD/0.2 mL for the three inoculums after normalization to OD/ml =1. MMT006, L004 and ML are found in wells; 8H, 10H and 12 H, respectively.

Multiplying OD/0.2 ml with a factor of 5, should give OD/ml = 1. This was almost correct for MMT006 and L004, with the OD/ml being 0.82 and 0.81, respectively. OD/ml for ML was only 0.31, and there is a great possibility that there has been a personal misreading and calculation fail when diluting the inoculum.

Normalization of inoculum experiment 2

OD measurements before normalization;

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,2	0,025	0,026	0,289	0,026	0,025	0,026	0,026	0,027	0,027	0,025	0,025
B	0,025	0,025	0,025	0,025	0,027	0,027	0,025	0,025	0,024	0,025	0,026	0,025
C	0,025	0,033	0,026	0,024	0,025	0,027	0,025	0,026	0,024	0,026	0,025	0,024
D	0,375	0,025	0,025	0,219	0,025	0,024	0,026	0,028	0,021	0,025	0,025	0,025
E	0,026	0,023	0,02	0,025	0,025	0,027	0,024	0,024	0,025	0,026	0,023	0,026
F	0,025	0,028	0,024	0,025	0,025	0,025	0,029	0,025	0,025	0,025	0,025	0,025
G	0,025	0,025	0,025	0,025	0,027	0,024	0,025	0,023	0,026	0,021	0,024	0,026
H	0,026	0,026	0,026	0,025	0,023	0,021	0,026	0,028	0,028	0,025	0,026	0,024

ID1: L004_1D, ML_4D before normalization to OD/ml=1
Absorbance values are displayed as OD
Raw Data (660)

Figure D4. Measurement of OD/0.2 mL before normalization. Inoculums L004 and ML are found in well 1D and 4D respectively

Normalization calculations;

L004;

$$\frac{OD\ 0,375}{0,2\ mL} \times 16,8\ mL = OD\ 31,5$$

→ L004 was diluted with 31.5 mL MM

ML;

$$\frac{OD\ 0,219}{0,2\ mL} \times 16,8\ mL = OD\ 18,396$$

→ ML was diluted with 18.396 mL MM

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,201	0,026	0,027	0,301	0,026	0,025	0,027	0,027	0,028	0,027	0,027	0,023
B	0,027	0,026	0,026	0,026	0,028	0,027	0,027	0,027	0,025	0,028	0,024	0,026
C	0,024	0,031	0,026	0,026	0,028	0,027	0,027	0,029	0,026	0,027	0,027	0,025
D	0,389	0,026	0,026	0,218	0,025	0,026	0,026	0,028	0,025	0,026	0,026	0,027
E	0,026	0,023	0,021	0,024	0,026	0,028	0,024	0,025	0,027	0,028	0,023	0,028
F	0,026	0,028	0,024	0,026	0,026	0,025	0,025	0,025	0,021	0,024	0,026	0,026
G	0,028	0,024	0,027	0,024	0,022	0,023	0,026	0,025	0,026	0,027	0,026	0,025
H	0,213	0,028	0,026	0,201	0,024	0,025	0,027	0,029	0,029	0,027	0,027	0,026

ID1: L004_1H, ML_4H after norm.
Absorbance values are displayed as OD
Raw Data (660)

Figure D5. Measurement of OD/0.2 mL after normalization. Inoculums L004 and ML are found in well 1H and 4H respectively

Multiplying OD/0.2 ml with a factor of 5 results in OD/ml = 1.065 for L004 and 1.005

Normalization of inoculum experiment 3

				ID1: 1EL004_12EML_before norm									
	Absorbance			Absorbance values are displayed as OD									
	Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,029	0,026	0,025	0,027	0,025	0,025	0,028	0,024	0,025	0,025	0,023	0,023
B	0,028	0,026	0,033	0,023	0,025	0,025	0,024	0,025	0,024	0,025	0,027	0,024
C	0,024	0,025	0,024	0,025	0,025	0,026	0,022	0,025	0,021	0,025	0,025	0,024
D	0,025	0,025	0,026	0,023	0,022	0,025	0,024	0,028	0,024	0,024	0,024	0,027
E	0,435	0,025	0,024	0,024	0,023	0,021	0,025	0,022	0,023	0,022	0,024	1,667
F	0,025	0,024	0,025	0,026	0,026	0,026	0,024	0,026	0,023	0,025	0,022	0,026
G	0,027	0,023	0,025	0,025	0,024	0,024	0,023	0,026	0,023	0,025	0,024	0,025
H	0,432	0,025	0,025	0,025	0,023	0,024	0,024	0,026	0,021	0,025	0,021	1,584

Figure D6. Measurement of OD/0.2 mL, before normalization. Inoculums L004 and ML are found in wells 1E and 12E, respectively.

Normalization calculations;

L004;

$$\frac{OD\ 0,435}{0,2\ mL} \times 20\ mL = OD\ 43,5$$

→ L004 was diluted with 43.5 mL MM

ML;

$$\frac{OD\ 1,667}{0,2\ mL} \times 20\ mL = OD\ 166,7$$

→ ML was diluted with 166.7 mL MM

				ID1: 5HL004_8HML_after norm1									
	Absorbance			Absorbance values are displayed as OD									
	Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,455	0,026	0,025	0,025	0,026	0,025	0,025	0,026	0,022	0,025	0,025	0,022
B	0,026	0,026	0,027	0,022	0,025	0,024	0,024	0,024	0,027	0,026	0,025	0,024
C	0,024	0,026	0,025	0,025	0,025	0,025	0,023	0,025	0,022	0,023	0,025	0,022
D	0,025	0,027	0,021	0,021	0,021	0,025	0,021	0,027	0,025	0,025	0,024	0,029
E	0,42	0,025	0,025	0,025	0,021	0,023	0,021	0,023	0,019	0,022	0,025	1,617
F	0,027	0,025	0,026	0,024	0,024	0,025	0,026	0,026	0,021	0,024	0,021	0,023
G	0,026	0,024	0,025	0,025	0,019	0,022	0,023	0,022	0,023	0,025	0,024	0,024
H	0,437	0,024	0,027	0,025	0,205	0,023	0,023	0,277	0,02	0,026	0,021	1,593

Figure D7. Measurement of OD/0.2 mL after normalization. Inoculums L004 and ML are found in well 5H and 8H respectively

Multiplying OD/0.2 ml with a factor of 5 results in OD/ml = 1.025 for L004 and 1.385 for ML. It was discovered that it was accidently added 10 mL less than the dilution volume, therefore it was tried to dilute once more, by adding the rest of the 10 mL.

				ID1: ML									
	Absorbance			Absorbance values are displayed as OD									
	Raw Data (660)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0,35	0,027	0,026	0,024	0,026	0,025	0,265	0,025	0,024	0,025	0,025	0,022
B	0,028	0,024	0,023	0,021	0,025	0,026	0,024	0,022	0,027	0,025	0,026	0,022
C	0,023	0,026	0,025	0,025	0,024	0,026	0,023	0,026	0,021	0,023	0,023	0,021
D	0,026	0,026	0,023	0,022	0,019	0,024	0,022	0,027	0,025	0,024	0,023	0,024
E	0,42	0,023	0,024	0,024	0,023	0,02	0,021	0,021	0,022	0,023	0,026	1,579
F	0,02	0,023	0,029	0,023	0,025	0,023	0,021	0,274	0,02	0,021	0,02	0,021
G	0,027	0,021	0,024	0,023	0,02	0,02	0,022	0,023	0,02	0,023	0,024	0,022
H	0,438	0,025	0,023	0,022	0,214	0,023	0,022	0,272	0,022	0,025	0,021	1,551

Figure D8. Measurement of OD/0.2 mL after normalization of ML. ML is found in well 7A.

When multiplying the measured OD – value with a factor of 5; OD/mL = 1.325. This was accepted, and the inoculum used in the bio conversion experiment.

Appendix E. Percentage oil added to the shake flasks

Calculations;
The shale flasks were induced with 0.2 % oil day 1 and up to 2 % day 3. Oil (g) added was calculated using these formulas;

$$\underline{Day\ 1} \qquad \frac{50\ mL}{100\%} \times 0,2\% = 0,1\ g$$

$$\underline{Day\ 3} \qquad \frac{50\ mL}{100\%} \times 2\% = 1\ g$$

Percentage oil (w/v) was calculated by dividing the weight of added oil (g) by the initial total volume in the shake flasks day 1 (media, inoculum, and oil). Note that the total volume of negative shake flasks day 3 was 49 mL, whereas it was 48 mL for the tests. The inoculum added to each shake flask constitute the last millilitre (mL).

- Induction percentage (w/v);

$$\frac{Oil\ (g)}{Total\ volume} \times 100\%$$

Percentage oil total (w/v) was calculated by dividing the total weigh of added oil (g) with the total volume in the shake flask.
Note that, the total volume was compensated for DNA sampling day 3 for experiment 3, by subtraction of 2 mL. This means that amount of oil (g) added day 3 was calculated using this formula;

$$\frac{Oil\ (g)}{Initial\ volume\ day\ 1 - (2mL)} - (oil\ (g)\ added\ day\ 1) = oil\ (g)\ added\ day\ 3$$

Table E1. Amount of oil (g) added; day 1 and day 3 of bio conversion experiment 1. (Calculated percentage of oil is included).

	ID	Day 1				Day 3		
		Volume medium	oil (g)	Total volume	% - oil (w/v)	oil (g) added	Total volume	% - oil total (w/v)
Peregrino (35°C)	Ktr. P MMAc	49,00	0,10	49,10	0,20	0,95	50,05	2,10
	Ktr. P MM	49,00	0,13	49,13	0,26	0,89	50,02	2,04
	ML P MMAc	48,00	0,14	48,14	0,29	0,85	48,99	2,02
	ML P MM	48,00	0,09	48,09	0,19	0,96	49,05	2,14
Peregrino (60°C)	Ktr. P MMAc	49,00	0,12	49,12	0,24	0,91	50,03	2,06
	Ktr. P MM	49,00	0,12	49,12	0,24	0,90	50,02	2,04
	L004 MMAc	48,00	0,14	48,14	0,29	0,88	49,02	2,08
	L004 MM	48,00	0,12	48,12	0,25	0,90	49,02	2,08
	MMT006 MMAc	48,00	0,13	48,13	0,27	0,88	49,01	2,06
	MMT006 MM	48,00	0,11	48,11	0,23	0,89	49,00	2,04

Table E2. Amount of oil (g) added; day 1 and day 3 of bioconversion experiment 2. (Calculated percentage of oil is included).

ID	Day 1				Day 3			
	Volume medium (mL)	Oil added	Total volume (mL)	Oil (%)	Volume medium after DNA sampling	Oil added (g)	Total volume (mL)	Total oil (%)
Mariner Maureen 46°C								
Neg.control, MM	49,00	0,13	49,13	0,26	47,00	0,95	47,95	2,25
Neg.control, MMAc	49,00	0,18	49,18	0,37	47,00	0,86	47,86	2,17
L004, MM	48,00	0,12	48,12	0,25	46,00	0,89	46,89	2,15
L004, MMAc	48,00	0,16	48,16	0,33	46,00	0,87	46,87	2,20
ML, MM	48,00	0,11	48,11	0,23	46,00	0,89	46,89	2,13
ML, MMAc	48,00	0,12	48,12	0,25	46,00	0,89	46,89	2,15
Peregrino 78°C								
Neg.control, MM	49,00	0,12	49,12	0,24	47,00	0,88	47,88	2,09
Neg.control, MMAc	49,00	0,13	49,13	0,26	47,00	0,87	47,87	2,09
L004, MM	48,00	0,10	48,1	0,21	46,00	0,90	46,90	2,13
L004, MMAc	48,00	0,12	48,12	0,25	46,00	0,88	46,88	2,13
ML, MM	48,00	0,11	48,11	0,23	46,00	0,89	46,89	2,13
ML, MMAc	48,00	0,11	48,11	0,23	46,00	0,89	46,89	2,13
Bressay 35°C								
Neg.control, MM	49,00	0,13	49,13	0,26	47,00	0,87	47,87	2,09
Neg.control, MMAc	49,00	0,16	49,16	0,33	47,00	0,84	47,84	2,09
L004, MM	48,00	0,14	48,14	0,29	46,00	0,86	46,86	2,13
L004, MMAc	48,00	0,13	48,13	0,27	46,00	0,87	46,87	2,13
ML, MM	48,00	0,19	48,19	0,39	46,00	0,81	46,81	2,14
ML, MMAc	48,00	0,20	48,20	0,41	46,00	0,80	46,80	2,14

Table E3. Amount of oil added day; 1 and day 3 of bioconversion experiment 3. (Calculated percentage of oil is included).

ID	Day 1				Day 3			
	Volume medium (mL)	Oil added	Total volume (mL)	Oil (%)	Volume medium after DNA sampling of 2 mL	Oil added (g)	Total volume (mL)	Total oil (%)
Mariner Maureen 46°C								
Neg.control, MM	49,00	0,10	49,10	0,20	47,00	1,02	48,02	2,33
Neg.control, MMAc	49,00	0,10	49,10	0,20	47,00	0,95	47,95	2,19
L004, MM	48,00	0,11	48,11	0,23	46,00	0,92	46,92	2,20
L004, MMAc	48,00	0,12	48,12	0,25	46,00	1,07	47,07	2,53
ML, MM	48,00	0,13	48,13	0,27	46,00	0,87	46,87	2,13
ML, MMAc	48,00	0,10	48,10	0,21	46,00	0,86	46,86	2,05
Peregrino 60°C								
Neg.control, MM	49,00	0,25	49,25	0,51	47,00	0,97	47,97	2,54
Neg.control, MMAc	49,00	0,20	49,20	0,41	47,00	0,79	47,79	2,07
L004, MM	48,00	0,22	48,22	0,46	46,00	0,88	46,88	2,35
L004, MMAc	48,00	0,14	48,14	0,29	46,00	0,85	46,85	2,11
ML, MM	48,00	0,17	48,17	0,35	46,00	0,81	46,81	2,09
ML, MMAc	48,00	0,20	48,20	0,41	46,00	0,79	46,79	2,12
Bressay 35°C								
Neg.control, MM	49,00	0,12	49,12	0,24	47,00	1,07	48,07	2,48
Neg.control, MMAc	49,00	0,10	49,10	0,20	47,00	0,92	47,92	2,13
L004, MM	48,00	0,12	48,12	0,25	46,00	0,88	46,88	2,13
L004, MMAc	48,00	0,22	48,22	0,46	46,00	0,99	46,99	2,58
ML, MM	48,00	0,21	48,21	0,44	46,00	0,86	46,86	2,28
ML, MMAc	48,00	0,20	48,20	0,41	46,00	0,80	46,80	2,14

Appendix F. Pre-treatment and dilution of oil samples for analysis by TLC- FID and LC/MS (QTOF).

Oils samples prepared for TLC-FID was diluted to a concentration of 10 mg/mL. Volume of the oil samples needed for dilution was calculated using the formula:

$$C1 \times V1 = C2 \times V2 \rightarrow V1 = \frac{(C2 \times V2)}{V2}, \text{ with tot. volume } V2: 10 \text{ mL and } C2: 10 \text{ mg/mL}$$

Volume (**V1**) was then diluted with DCM (10 mL).

For analysis on LC/MS (QTOF), the oil samples were diluted to 1 mg/mL by addition of oil (0.150 mL) to DCM (1.350 mL). This was calculated using the same formula;

$$V1 = \frac{(1 \text{ mg/mL} \times 1.5 \text{ mL})}{10 \text{ mg/mL}} = 0.150 \text{ mL}$$

Table F1. Experiment 1

ID	Weight beaker (g)	Weight beaker with oil (g)	Diff =weight oil (g)	Weight oil in mg	Added DCM (mL)	Conc. Oil sample (mg/mL)	Dilution volume (V1) in mL
Ktr. P MM 35°C	67,24	68,43	1,190	1190	50,00	23,80	4,20
Ktr. P MMAc 35°C	73,05	74,56	1,510	1510	50,00	30,20	3,31
Anrikn. P MM 35°C	68,32	69,35	1,030	1030	40,00	25,75	3,88
Anrikn. P MMAc 35°C	68,17	69,21	1,040	1040	40,00	26,00	3,85
Ktr. P MM 60°C	70,26	71,22	0,9600	960,0	50,00	19,20	5,21
Ktr. P MMAc 60°C	68,59	69,56	0,9700	970,0	50,00	19,40	5,15
L004, MM	68,03	69,04	1,010	1010	100,0	10,10	No dilution needed
L004 MMAc,	67,55	68,52	0,9700	970,0	50,00	19,40	5,15
MMT006 MM	67,52	68,39	0,8700	870,0	50,00	17,40	5,75
MMT006 MMAc	67,22	68,29	1,070	1070	50,00	21,40	4,67

Table F2. Experiment 3

ID	Weight beaker (g)	Weight beaker with oil (g)	Diff=weight oil (g)	Weight oil in mg	Added DCM (mL)	Conc. Oil sample (mg/mL)	Dilution volume (V1) in mL
Mariner Maureen							
Neg.control, MM	67,25	68,18	0,9300	930,0	50	18,60	0,806
Neg.control, MMAc	70,29	71,12	0,8300	830,0	50	16,60	0,904
L004, MM	72,59	73,4	0,8100	810,0	50	16,20	0,926
L004, MMAc	67,74	68,77	1,030	1030	50	20,60	0,728
ML, MM	70,26	70,94	0,6800	680,0	50	13,60	1,103
ML, MMAc	57,01	57,6	0,5900	590,0	25	23,60	0,636
Peregrino							
Neg.control, MM	71,08	72,47	1,390	1390	50	27,80	0,540
Neg.control, MMAc	68,17	69,05	0,8800	880,0	50	17,60	0,852
L004, MM	69,67	70,59	0,9200	920,0	50	18,40	0,815
L004, MMAc	57,85	58,68	0,8300	830,0	50	16,60	0,904
ML, MM	70,11	71,01	0,9000	900,0	50	18,00	0,833
ML, MMAc	69,68	70,43	0,7500	750,0	50	15,00	1,000
Bressay							
Neg.control, MM	68,08	68,94	0,8600	860,0	50	17,20	0,872
Neg.control, MMAc	67,18	67,94	0,7600	760,0	50	15,20	0,987
L004, MM	68,33	69,12	0,7900	790,0	50	15,80	0,949
L004, MMAc	68,41	69,43	1,020	1020	25	40,80	0,368
ML, MM	57,23	58,07	0,8400	840,0	25	33,60	0,446
ML, MMAc	58,33	59,06	0,7300	730,0	50	14,60	1,027
Native oil							
Mariner Maureen	67,60	68,42	0,8200	820,0	50	16,40	0,915
Peregrino	73,03	73,85	0,8200	820,0	50	16,40	0,915
Bressay	68,42	69,44	1,020	1020	50	20,40	0,735

Appendix G. Standard deviation and %CV for TLC-FID results

The following tables contain percentage area for each fraction in the samples, including standard deviation and variation between the parallels of each sample.
% CV should be <5 % for the method.

Experiment 1. Tables show calculated standard deviation and %CV between the parallels of each sample.

Experiment at 35°C

Chromarod No.	Native Peregrino			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	12,67	31,74	23,81	31,78
2	12,49	33,07	25,60	28,85
3	13,91	27,91	27,40	30,77
X	13,02	30,91	25,60	30,47
SD	0,776	2,677	1,796	1,493
CV (%)	5,956	8,661	7,015	4,899

Chromarod No.	Negative control MM 35°C			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	17,88	29,33	18,88	33,92
2	15,21	30,32	16,77	37,70
3	13,40	34,74	17,58	34,27
4	14,38	33,46	18,02	34,13
X	15,22	31,96	17,81	35,00
SD	1,920	2,557	0,880	1,802
CV (%)	12,615	8,000	4,938	5,149

Chromarod No.	Negative control MMAc 35°C			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	15,83	30,56	21,23	32,38
2	13,86	35,10	17,50	33,55
3	18,62	25,47	16,67	39,24
X	16,10	30,37	18,47	35,06
SD	2,395	4,818	2,425	3,667
CV (%)	14,873	15,861	13,132	10,460

	ML MM			
	Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,37	37,19	17,79	33,65
2	12,11	28,31	19,11	40,46
3	15,54	31,94	18,37	34,15
X	13,01	32,48	18,43	36,09
SD	2,222	4,463	0,660	3,798
CV (%)	17,085	13,741	3,580	10,525

	ML MMAc			
	Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,42	26,44	24,78	37,36
2	10,17	19,34	17,23	53,26
3	10,13	23,12	23,80	42,95
4	9,26	19,24	19,58	51,92
Average	10,24	22,04	21,35	46,37
SD	0,889	3,447	3,553	7,553
CV (%)	8,676	15,641	16,645	16,289

Experiment at 60°C

Chromarod No.	Negative control MM 60°C Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	12,42	31,00	33,85	22,72
2	13,53	29,10	35,63	21,74
3	12,56	35,50	31,19	20,74
X	12,84	31,87	33,56	21,74
SD	0,602	3,289	2,234	0,989
CV (%)	4,688	10,321	6,659	4,549

Chromarod No.	Negative control MMAc 60°C Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	15,08	29,54	26,03	29,36
2	14,80	26,84	25,20	33,15
3	14,08	31,60	23,48	30,84
4	13,70	26,39	26,18	33,73
X	14,42	28,59	25,22	31,77
SD	0,635	2,437	1,238	2,035
CV (%)	4,402	8,523	4,910	6,407

Chromarod No.	L004 MM Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,92	35,30	25,57	27,21
2	11,06	36,34	24,94	27,66
3	15,21	24,25	26,05	34,48
4	14,19	39,15	21,81	24,85
X	13,09	33,76	24,59	28,55
SD	1,931	6,543	1,909	4,141
CV (%)	14,749	19,382	7,762	14,504

| Chromarod No. | L004 MMAc | | | |
| | Area (%) | | | |
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	12,48	25,60	24,64	37,28
2	11,28	34,69	22,46	31,57
3	14,96	31,75	23,50	29,79
X	12,91	30,68	23,53	32,88
SD	1,875	4,636	1,089	3,914
CV (%)	14,528	15,112	4,627	11,904

| Chromarod No. | MMT006 MM | | | |
| | Area (%) | | | |
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	13,43	29,82	23,70	33,05
2	14,49	31,15	24,36	30,00
3	13,83	27,55	26,44	32,18
X	13,92	29,51	24,83	31,74
SD	0,534	1,822	1,434	1,570
CV (%)	3,837	6,173	5,776	4,945

| Chromarod No. | MMT006 MMAc | | | |
| | Area (%) | | | |
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,10	29,42	25,16	34,32
2	14,76	26,41	25,91	32,92
3	14,84	31,14	22,57	31,45
X	13,57	28,99	24,54	32,90
SD	2,139	2,393	1,753	1,435
CV (%)	15,765	8,253	7,141	4,361

Experiment 3. Tables show calculated standard deviation and %CV between the parallels of each sample.

Mariner Maureen

Chromarod No.	Native Mariner Maureen			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	17,98	64,64	15,28	2,09
2	19,64	58,47	17,81	4,08
3	18,26	63,19	14,51	4,04
Average	18,63	62,10	15,87	3,40
SD	0,889	3,226	1,723	1,136
CV (%)	4,771	5,194	10,860	33,371

Chromarod No.	Negative control MM			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	24,52	51,62	19,26	4,59
2	22,80	58,15	16,86	2,19
3	20,24	60,69	16,53	2,54
4	19,27	55,75	21,43	3,55
Average	21,71	56,55	18,52	3,22
SD	2,395	3,855	2,289	1,080
CV (%)	11,035	6,816	12,359	33,582

Chromarod No.	Negative control MMAc			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	23,70	56,21	17,22	2,87
2	22,22	60,79	15,35	1,64
3	26,56	55,12	16,07	2,25
Average	24,16	57,37	16,22	2,25
SD	2,207	3,011	0,944	0,616
CV (%)	9,135	5,248	5,824	27,367

ML MM			
Area (%)			
Chromarod No.	**Peak 1 Saturated HC**	**Peak 2 Aromatic HC**	**Peak 3 Resins**	**Peak 4 Asphaltenes**
1	22,71	56,27	18,47	2,55
2	23,22	52,84	19,74	4,20
3	19,58	57,82	19,48	3,12
Average	21,84	55,64	19,23	3,29
SD	1,974	2,547	0,669	0,837
CV (%)	9,039	4,578	3,480	25,429

ML MMAc			
Area (%)			
Chromarod No.	**Peak 1 Saturated HC**	**Peak 2 Aromatic HC**	**Peak 3 Resins**	**Peak 4 Asphaltenes**
1	20,04	58,11	18,90	2,95
2	20,68	51,74	23,67	3,91
3	21,70	53,08	21,82	3,40
4	16,49	62,04	17,66	3,81
Average	19,73	56,24	20,51	3,52
SD	2,267	4,740	2,732	0,438
CV (%)	11,490	8,428	13,316	12,449

L004 MM			
Area (%)			
Chromarod No.	**Peak 1 Saturated HC**	**Peak 2 Aromatic HC**	**Peak 3 Resins**	**Peak 4 Asphaltenes**
1	15,20	55,73	23,35	5,73
2	20,04	54,46	22,77	2,73
3	15,89	60,14	19,72	4,25
Average	17,04	56,77	21,95	4,23
SD	2,621	2,985	1,950	1,499
CV (%)	15,377	5,258	8,883	35,408

L004 MMAc			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	19,15	55,06	22,45	3,33
2	16,00	62,40	19,58	2,03
3	15,12	56,23	24,75	3,90
Average	16,76	57,90	22,26	3,09
SD	2,120	3,941	2,593	0,959
CV (%)	12,652	6,806	11,649	31,055

Peregrino oil

Native Peregrino			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	12,67	31,74	23,81	31,78
2	12,49	33,07	25,60	28,85
3	13,91	27,91	27,40	30,77
Average	13,02	30,91	25,60	30,47
SD	0,776	2,677	1,796	1,493
CV (%)	5,956	8,661	7,015	4,899

Negative control MM			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	14,57	28,09	21,08	36,26
2	17,06	23,29	20,12	39,53
3	15,60	29,04	19,15	36,22
4	13,58	22,47	21,19	42,76
Average	15,20	25,72	20,38	38,69
SD	1,488	3,321	0,955	3,124
CV (%)	9,787	12,909	4,687	8,073

Chromarod No.	Negative control MMAc			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	15,25	23,19	24,46	37,10
2	15,38	23,43	23,16	38,02
3	15,79	29,02	19,76	35,43
Average	15,47	25,21	22,46	36,85
SD	0,281	3,298	2,429	1,311
CV (%)	1,819	13,081	10,813	3,558

Chromarod No.	ML MM			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	12,56	25,51	24,68	37,25
2	11,72	32,42	20,99	34,87
3	15,55	26,72	21,43	36,30
Average	13,28	28,21	22,37	36,14
SD	2,015	3,690	2,012	1,196
CV (%)	15,179	13,078	8,996	3,310

Chromarod No.	ML MMAc			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,74	30,87	28,60	28,79
2	14,16	30,23	27,49	28,12
3	13,75	31,06	26,05	29,14
4	10,39	29,25	29,01	31,35
Average	12,51	30,35	27,79	29,35
SD	1,764	0,818	1,325	1,397
CV (%)	14,098	2,696	4,770	4,760

	L004 MM			
	Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	11,93	27,16	28,50	32,41
2	11,47	23,87	30,59	34,06
3	11,07	31,57	29,32	28,03
Average	11,49	27,53	29,47	31,50
SD	0,428	3,864	1,054	3,117
CV (%)	3,728	14,034	3,576	9,895

	L004 MMAc			
	Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	17,37	20,25	29,86	32,51
2	17,28	17,28	31,42	34,01
3	12,77	16,44	33,60	37,19
4	13,95	20,40	30,83	34,82
Average	15,34	18,59	31,43	34,63
SD	2,343	2,030	1,581	1,956
CV (%)	15,270	10,915	5,032	5,647

Bressay oil

	Native Bressay			
	Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	17,33	50,64	26,24	5,79
2	17,66	54,67	22,36	5,31
3	18,64	49,42	24,79	7,15
Average	17,87	51,58	24,46	6,09
SD	0,679	2,745	1,959	0,951
CV (%)	3,799	5,321	8,008	15,631

Chromarod No.	Negative control MM			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	19,40	48,05	26,86	5,68
2	23,93	51,18	22,72	2,16
3	28,47	41,01	28,09	2,43
Average	23,94	46,75	25,89	3,42
SD	4,535	5,209	2,811	1,960
CV (%)	18,947	11,142	10,858	57,268

Chromarod No.	Negative control MMAc			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	21,36	39,90	35,51	3,23
2	23,29	40,72	31,47	4,52
3	17,51	50,53	27,30	4,66
Average	20,72	43,72	31,43	4,14
SD	2,945	5,913	4,104	0,791
CV (%)	14,212	13,525	13,058	19,111

Chromarod No.	L004 MM			
	Area (%)			
	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	18,86	49,80	27,19	4,15
2	19,62	44,35	31,04	4,98
3	24,95	42,73	28,99	3,32
4	17,95	53,12	26,51	2,42
Average	20,34	47,50	28,43	3,72
SD	3,146	4,815	2,030	1,098
CV (%)	15,464	10,136	7,140	29,524

L004 MMAc			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	20,55	48,28	24,98	6,19
2	26,07	46,07	24,53	3,32
3	25,47	43,05	25,92	5,56
Average	24,03	45,80	25,14	5,03
SD	3,032	2,625	0,707	1,508
CV (%)	12,618	5,731	2,814	29,999

ML MM			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	20,85	51,12	26,58	1,45
2	5,83	12,89	71,94	9,34
3	21,38	52,04	25,03	1,55
Average	16,02	38,68	41,18	4,12
SD	8,830	22,341	26,647	4,528
CV (%)	55,123	57,755	64,703	110,004

ML MMAc			
Area (%)			
Chromarod No.	Peak 1 Saturated HC	Peak 2 Aromatic HC	Peak 3 Resins	Peak 4 Asphaltenes
1	23,85	49,19	24,58	2,38
2	18,51	54,82	24,00	2,67
3	21,66	47,90	25,83	4,60
4	15,47	51,20	27,94	5,40
Average	19,87	50,78	25,59	3,76
SD	3,664	3,017	1,743	1,469
CV (%)	18,436	5,942	6,811	39,068

Appendix H. Chromatograms from TLC-FID results

Chromatograms for sample ML (MMAc) are included to demonstrate the deviating SD as described in chapter 4.1.5.

Experiment 3, Bressay oil ; ML MMAc – Parallel no.

```
Data File C:\CHEM32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA000001.D
Sample Name: 17

=================================================================
Acq. Operator   : Sandra                      Seq. Line :   1
Acq. Instrument : IatroScan                   Location :   -
Injection Date  : 16.02.2011 13:45:00                   Inj :   1
Acq. Method     : C:\Chem32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA1.M
Last changed    : 22.06.2009 13:18:33
Analysis Method : C:\CHEM32\1\METHODS\SARA1.M
Last changed    : 04.05.2011 10:22:25   by Hege
```
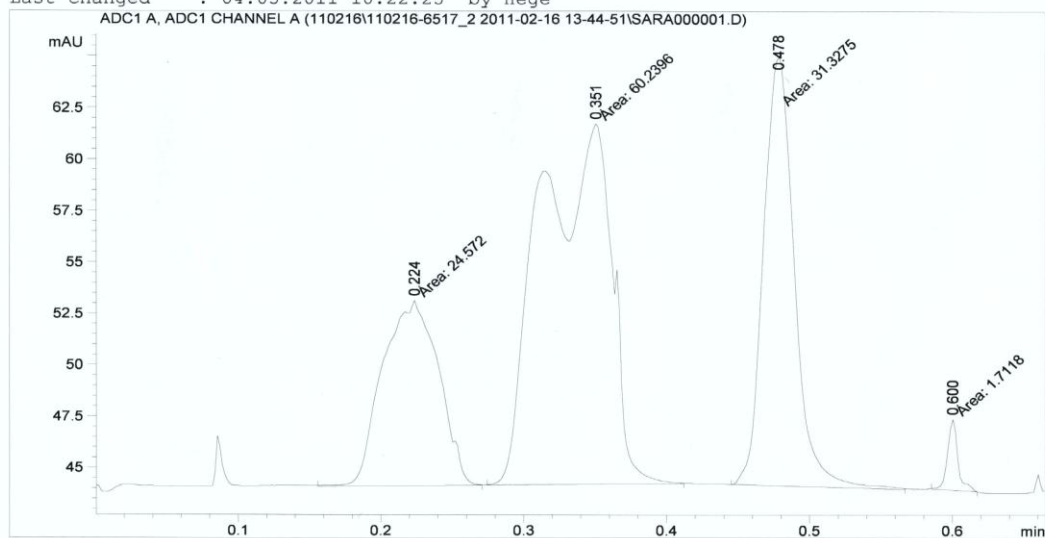


```
=================================================================
                      Area Percent Report
=================================================================

Sorted By             :       Signal
Multiplier            :       1.0000
Dilution              :       1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: ADC1 A, ADC1 CHANNEL A

Peak RetTime Type  Width      Area        Height       Area
  #   [min]        [min]   [mAU*s]       [mAU]          %
----|-------|----|-------|----------|----------|--------|
   1   0.224 MM   0.0453   24.57198    9.03970   20.8500
   2   0.351 MM   0.0572   60.23964   17.53900   51.1151
   3   0.478 MM   0.0250   31.32753   20.87338   26.5823
   4   0.600 MM   8.22e-3   1.71180    3.47185    1.4525

Totals :                  117.85095   50.92393


=================================================================
                    *** End of Report ***
```

IatroScan 06.05.2011 08:44:32 Hege   Page   1 of 1

1

```
Data File C:\CHEM32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA000002.D
Sample Name: 17
```

```
========================================================================
Acq. Operator   : Sandra                        Seq. Line :   2
Acq. Instrument : IatroScan                      Location  :   -
Injection Date  : 16.02.2011 13:46:10              Inj :     1
Acq. Method     : C:\Chem32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA1.M
Last changed    : 22.06.2009 13:18:33
Analysis Method : C:\CHEM32\1\METHODS\SARA1.M
Last changed    : 04.05.2011 10:22:25   by Hege
```


ADC1 A, ADC1 CHANNEL A (110216\110216-6517_2 2011-02-16 13-44-51\SARA000002.D)

```
========================================================================
                       Area Percent Report
========================================================================

Sorted By             :       Signal
Multiplier            :       1.0000
Dilution              :       1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: ADC1 A, ADC1 CHANNEL A

Peak RetTime Type  Width     Area        Height     Area
 #   [min]        [min]    [mAU*s]       [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  0.180 MM   0.0430    25.75285     9.98358   5.8274
   2  0.318 MM   0.0519    56.96803    18.28667  12.8907
   3  0.382 MM  8.46e-3   317.91730   626.18591  71.9384
   4  0.463 MM   0.0255    41.29176    26.95103   9.3435

Totals :                  441.92994   681.40719


========================================================================
                      *** End of Report ***
```
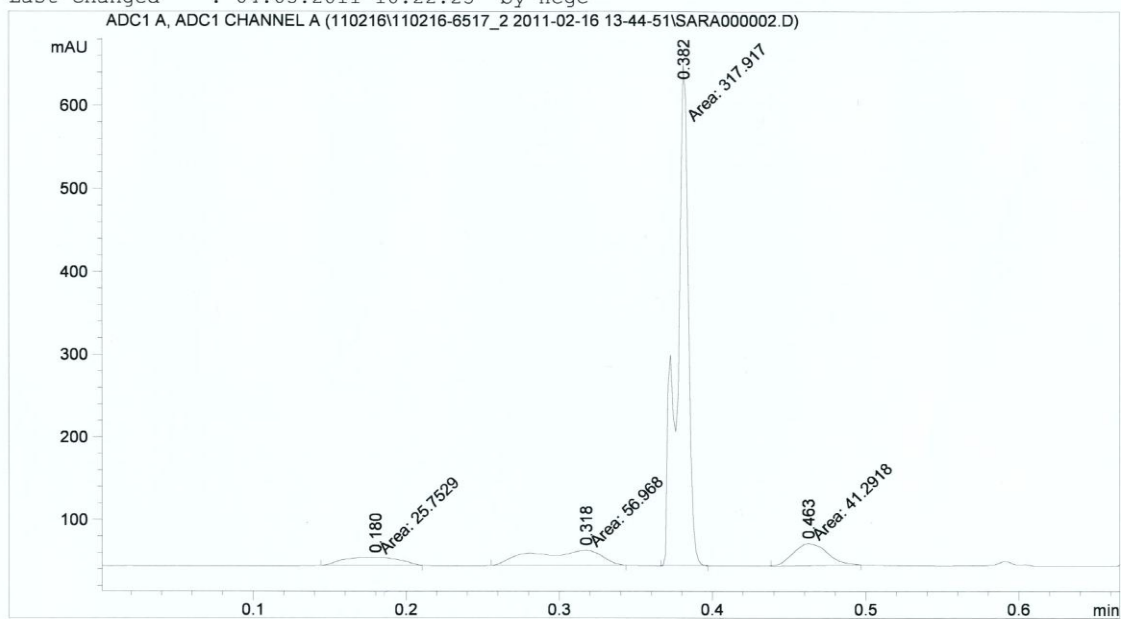
Experiment 3, Bressay oil ; ML MMAc – Parallel no. 3

```
Data File C:\CHEM32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA000003.D
Sample Name: 17
```

```
=================================================================
Acq. Operator   : Sandra                       Seq. Line :   3
Acq. Instrument : IatroScan                     Location :   -
Injection Date  : 16.02.2011 13:47:20               Inj :   1
Acq. Method     : C:\Chem32\1\DATA\110216\110216-6517_2 2011-02-16 13-44-51\SARA1.M
Last changed    : 22.06.2009 13:18:33
Analysis Method : C:\CHEM32\1\METHODS\SARA1.M
Last changed    : 04.05.2011 10:22:25  by Hege
```



```
=================================================================
                     Area Percent Report
=================================================================
```

```
Sorted By            :      Signal
Multiplier           :      1.0000
Dilution             :      1.0000
Use Multiplier & Dilution Factor with ISTDs
```
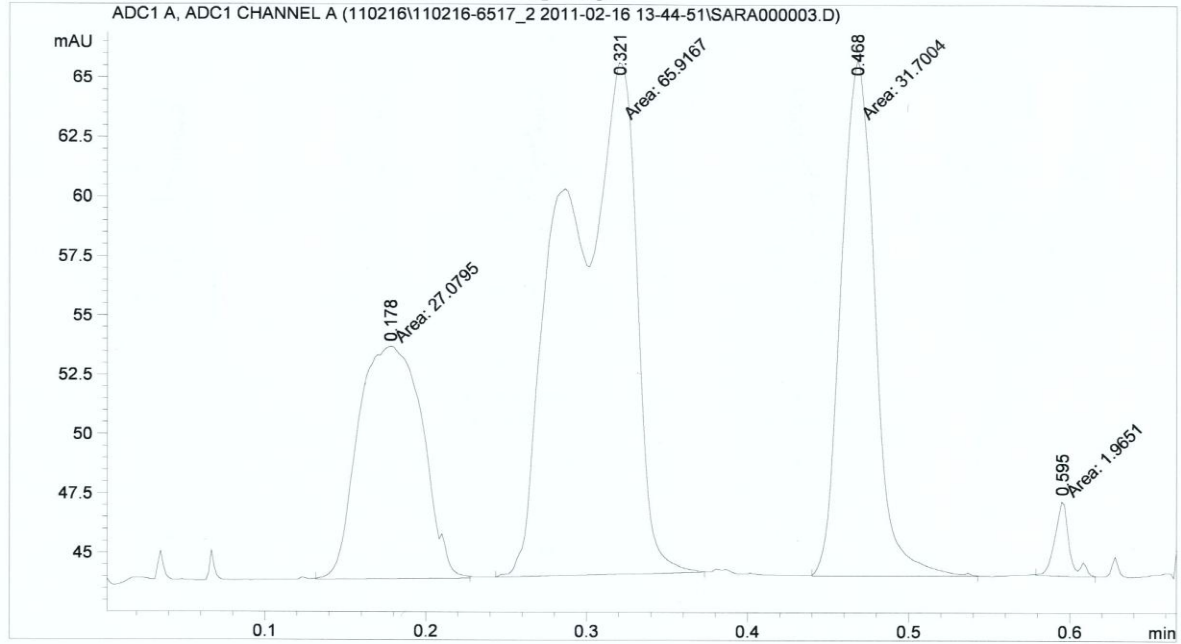
```
Signal 1: ADC1 A, ADC1 CHANNEL A

Peak RetTime Type  Width     Area       Height     Area
  #   [min]        [min]    [mAU*s]     [mAU]        %
----|-------|----|-------|----------|----------|--------|
   1  0.178 MM   0.0461   27.07952    9.78457   21.3794
   2  0.321 MM   0.0509   65.91666   21.58392   52.0415
   3  0.468 MM   0.0243   31.70038   21.74994   25.0276
   4  0.595 MM   0.0103    1.96510    3.18137    1.5515

Totals :                 126.66165   56.29980
```

```
=================================================================
                     *** End of Report ***
```

Apendix I. DNeasy procedure for Pre-treatment and DNA purification of Gram positive bacteria

Protocol: Pretreatment for Gram-Positive Bacteria

This protocol is designed for purification of total DNA from Gram-positive bacteria, such as *Corynebacterium* spp. and *B. subtilis*. The protocol describes the preliminary harvesting of bacteria and incubation with lysozyme to lyse their cell walls before DNA purification.

Important points before starting

■ See "Quantification of starting material", page 17, for details of how to collect and store samples, and how to determine the number of cells in a bacterial culture.

■ Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).

■ This pretreatment protocol has not been thoroughly tested and optimized for high-throughput DNA purification using the DNeasy 96 Blood & Tissue Kit. As a general guideline, we recommend to decrease the amount of starting material when using this protocol with the DNeasy 96 Blood & Tissue Kit.

Things to do before starting

■ Prepare enzymatic lysis buffer as described in "Equipment and Reagents to Be Supplied by User", page 14.

■ Preheat a heating block or water bath to 37°C for use in step 3.

Procedure

1. Harvest cells (maximum 2 x 10⁹ cells) in a microcentrifuge tube by centrifuging for 10 min at 5000 x g (7500 rpm). Discard supernatant.

2. Resuspend bacterial pellet in 180 µl enzymatic lysis buffer.

3. Incubate for at least 30 min at 37°C.

   After incubation, heat the heating block or water bath to 56°C if it is to be used for the incubation in step 5.

4. Add 25 µl proteinase K and 200 µl Buffer AL (without ethanol). Mix by vortexing.

   Note: Do not add proteinase K directly to Buffer AL.

   Ensure that ethanol has not been added to Buffer AL (see "Buffer AL", page 18). Buffer AL can be purchased separately (see page 56 for ordering information).

5. Incubate at 56°C for 30 min.

   Optional: If required, incubate at 95°C for 15 min to inactivate pathogens. Note that incubation at 95°C can lead to some DNA degradation.

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.*

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x *g* (14,000 rpm).

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.

Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. Recommended: For maximum DNA yield, repeat elution once as described in step 7.

This step leads to increased overall DNA yield.

A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

---

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

Appendix J. Mesurement of DNA concentration

Experiment 1

Table J1. Measurements of DNA concentrations in samples purified with DNeasy Blood and Tissue kit. Measurement is done with spectrophotometer (NanoDrop® Spectrophotometer, ND - 1000) at 260- and 280 nm. Table shows the computed concentration and the 260/280 – ratio, calculated for the evaluation of DNA purity in each sample. The extinction coefficient used for computing DNA concentration is 50 ng-cm/ml. Blue cells marks the ratios being over 1.8, and cells marked green represents ratios being below 1.8.

Sample ID	DNA conc. (ng/µL)	A260 nm (AU)	A280 nm (AU)	A260/ A280	A260/ A230
BLANK	-0,06	-0,001	-0,021	0,05	0,28
NC 1 (DNA purification)	6,87	0,137	0,106	1,3	0,18
NC 2 (DNA purification)	3,18	0,064	0,02	3,25	0,11
ML (Pre Bio)	31,1	0,622	0,282	2,2	0,86
L004 (Pre Bio)	20,63	0,413	0,273	1,51	0,62
MMT006 (Pre Bio)	17,61	0,352	0,201	1,75	0,48
Neg.control, Peregrino, MMAc	10,85	0,217	0,162	1,34	0,29
Neg.control, Peregrino, MineralM	4,41	0,088	0,081	1,08	0,19
L004, Peregrino, MMAc	7,75	0,155	0,068	2,27	0,37
L004, Peregrino, MineralM	11,07	0,221	0,186	1,19	0,41
MMT006, Peregrino, MMAc	49,43	0,989	0,554	1,79	1,13
BLANK	0,13	0,003	-0,007	-0,37	1,81
MMT006, Peregrino, MinearlM	6,44	0,129	0,1	1,29	0,2
Neg.control, Peregrino, MMAc, 35°C	62,46	1,249	0,992	1,26	0,66
Neg.control, Peregrino, MineralM, 35°C	25,03	0,501	0,246	2,03	0,63
ML, Peregrino, MMAc, 35°C	446,41	8,928	7,673	1,16	0,57
ML, Peregrino, MinearlM, 35°C	96,97	1,939	1,024	1,89	1,68
BLANK	-0,16	-0,003	-0,023	0,14	0,45

Experiment 3

Table J2. Measurements of DNA concentrations in samples purified with DNeasy Blood and Tissue kit. Measurement is done with spectrophotometer (NanoDrop® Spectrophotometer, ND - 1000) at 260- and 280 nm. Table shows the computed concentration and the 260/280 – ratio, calculated for the evaluation of DNA purity in each sample. "NC" is negative control for the DNA purification procedure, the rest of the samples are named. The extinction coefficient used for computing DNA concentration is 50 ng-cm/ml. Blue cells marks the ratios being over 1.8, and cells marked green represents ratios being below 1.8.

Sample ID	DNA conc. (ng/ µL)	A260 nm (AU)	A280 nm (AU)	A260/ A280	A260/ A230
BLANK	-0,16	-0,003	-0,023	0,14	0,45
Exp. 3, day 3 - NC DNA purification	2,39	0,048	0,02	2,34	0,09
Exp. 3, day 3 - ML (Pre bio)	24,58	0,492	0,263	1,87	0,81
Exp. 3, day 3 - L004 (Pre bio)	12,59	0,252	0,182	1,38	0,36
Exp. 3, day 3 - NC MM Mariner	4,68	0,094	0,062	1,52	0,18
Exp. 3, day 3 - NC MMAc Mariner	2,58	0,052	0,034	1,5	0,11
Exp. 3, day 3 - L004 MM Mariner	3,82	0,076	0,071	1,07	0,12
Exp. 3, day 3 - L004 MMAc Mariner	2,2	0,044	0,006	7,51	0,13
Exp. 3, day 3 - ML MM Mariner	3,78	0,076	0,04	1,88	0,14
Exp. 3, day 3 - ML MMAc Mariner	22,4	0,448	0,263	1,7	0,55
Exp. 3, day 3 - NC MM Peregrino	5,08	0,102	0,073	1,4	0,16
BLANK	0,03	0,001	-0,004	-0,15	-0,16
Exp. 3, day 3 - NC MMAc Peregrino	5,72	0,114	0,089	1,29	0,22
Exp. 3, day 3 - L004 MM Peregrino	5,07	0,101	0,082	1,24	0,23
Exp. 3, day 3 - L004 MMAc Peregrino	6,71	0,134	0,082	1,64	0,31
Exp. 3, day 3 - ML MM Peregrino	5,43	0,109	0,085	1,28	0,23
Exp. 3, day 3- ML MMAc Peregrino	5,45	0,109	0,083	1,32	0,18
Exp. 3, day 3 - NC MM Bressay	3,91	0,078	0,06	1,31	0,13
BLANK	1,63	0,033	0,044	0,74	2,54
BLANK	1,25	0,025	0,038	0,66	1,26
BLANK	1,07	0,021	0,023	0,95	1,7
BLANK	-0,73	-0,015	-0,023	0,64	2,03
Exp. 3, day 3 - NC MMAc Peregrino - again	4,88	0,098	0,051	1,92	0,2
Exp. 3, day 3 - L004 MM Peregrino - again	2,46	0,049	0,032	1,56	0,17
Exp. 3, day 3 - ML MM Peregrino -again	0,6	0,012	0,01	1,16	0,04
Exp. 3, day 3 - ML MMAc Peregrino- again	3,17	0,063	0,012	5,49	0,11
Exp. 3, day 3 - NC MM Pergrino - again	1,82	0,036	-0,007	-4,92	0,06
Exp. 3, day 3 - NC DNA purification	3,58	0,072	0,038	1,89	0,15
Exp. 3, day 3 - NC MMAc Bressay	6,27	0,125	0,075	1,66	0,2
Exp. 3, day 3 - L004 MM Bressay	2,09	0,042	0,017	2,49	0,12
Exp. 3, day 3 - L004 MMAc Bressay (old)	3,16	0,063	0,034	1,87	0,11
Exp. 3, day 3 - L004 MM Bressay (fresh)	3,46	0,069	0,037	1,88	0,11
BLANK	-0,38	-0,008	-0,011	0,7	-5,1
Exp. 3, day 3 - ML MM Bressay	0,95	0,019	-0,003	-6,06	0,06
Exp. 3, day 3 - ML MMAc Bressay	3,71	0,074	0,023	3,17	0,2
Exp. 3, day 7 - NC MM Mariner	1,7	0,034	0,018	1,86	0,1
Exp. 3, day 7 - NC MMAc Mariner	1,77	0,035	0,018	1,95	0,12
Exp. 3, day 7 - L004 MM Mariner	1,23	0,025	0,008	3,24	0,08
Exp. 3, day 7- L004 MMAc Mariner	9,21	0,184	0,076	2,42	0,53
Exp. 3, day 7 - ML MM Mariner	1,61	0,032	0,006	5,84	0,12
Exp. 3, day 7 - ML MMAc Mariner	4,71	0,094	0,058	1,62	0,25
Exp. 3, day 7 - NC MM Peregrino	1,86	0,037	0,016	2,31	0,16
Exp. 3, day 7 - NC MMAc Peregrino	3,4	0,068	0,017	3,89	0,1
BLANK	-0,14	-0,003	-0,004	0,65	-0,16
Exp. 3, day 7 - L004 MM Peregrino	2,15	0,043	0,024	1,76	0,11
Exp. 3, day 7 - L004 MMAc Peregrino	3,09	0,062	0,026	2,37	0,22
Exp. 3, day 7 - ML MM Peregrino	3,54	0,071	0,032	2,18	0,11
Exp. 3, day 7 - ML MMAc Peregrino	3,04	0,061	0,04	1,54	0,18
Exp. 3, day 7 - NC MM Bressay	4,88	0,098	0,064	1,52	0,22
Exp. 3, day 7 - NC MMAc Bressay	2,32	0,046	0,042	1,1	0,13
Exp. 3, day 7 - L004 MM Bressay	2,29	0,046	0,009	4,91	0,13
Exp. 3, day 7 - L004 MMAc Bressay	1,79	0,036	0,022	1,62	0,09
Exp. 3, day 7 - ML MM Bressay	3,89	0,078	0,047	1,67	0,2
Exp. 3, day 7 - ML MMAc Bressay	28,81	0,576	0,307	1,88	0,77
BLANK	-0,41	-0,008	0,001	-5,76	-1,36

Appendix K Rawdata LC/MS (QTOF)

+ESI Scan (0.225-0.370 min, 10 scans) Frag=50.0V SAMPLE19-r001.d Subtract

294.2204

744.5773

+ESI Scan (0.242-0.387 min, 10 scans) Frag=50.0V SAMPLE20-r001.d Subtract

312.2641

706.5508

+ESI Scan (0.243-0.355 min, 8 scans) Frag=50.0V SAMPLE21-r001.d Subtract

320.2382

740.5638

+ESI Scan (0.229-0.373 min, 10 scans) Frag=50.0V SAMPLE41-r001.d Subtract

229.1627

+ESI Scan (0.241-0.386 min, 10 scans) Frag=50.0V SAMPLE42-r001.d Subtract

229.1622

+ESI Scan (0.234-0.379 min, 10 scans) Frag=50.0V SAMPLE43-r001.d Subtract

259.2100

746.5818

Counts (%) vs. Mass-to-Charge (m/z)