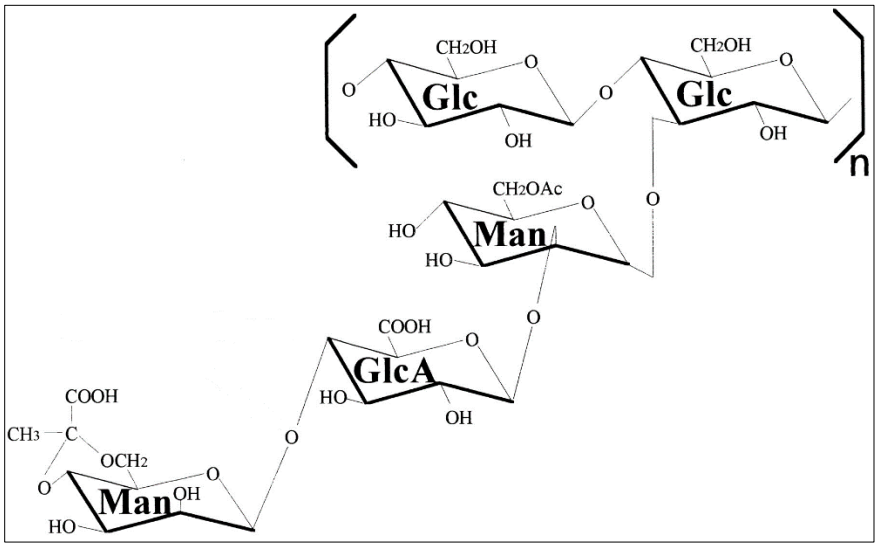
Assessment of the biodegradability of xanthan in offshore injection water



Master Thesis in Microbiology

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Abbreviations

APS: Ammonium Persulfate

ATP: Adinosine triphosphate

Bp: Base pairs

BSA: Bovine Serine Albumin

TAE: Tris-Acetate-EDTA

DGGE: Denaturing gradient gel electrophoresis

DNA: Deoxyribonucleic acid

LBA: Luria broth agar

PBS: Phosphate Buffered Seawater

PCR: Polymerase chain reaction

PWRI: Produced water reinjection

QPCR: Quantitative polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

SRP: Sulfate reduced seawater

Taq: Thermus aquaticus

TEMED: Tetramethylenediamide

w/v: Weight volume

Abstract

The application of biopolymers in EOR operations is considered environmental friendly compared to synthetic polymers. However, microbial degradation of the biopolymers may lead to a deterioration of effect in EOR applications. This thesis is part of an industrial project conducted by UNI Research CIPR for Statoil ASA, were the aim is to assess biodegradation of xanthan at specific oil field conditions.

Investigation of the biodegradation of xanthan was performed in two anoxic brines from a North Sea oil field; sulfate reduced sea water (SRP) and produced water for reinjection (PWRI). The biodegradation was assessed in a bioassay with xanthan as the sole added carbon source, at temperatures reflecting various locations in the oil reservoir. Reduction in viscosity, bacterial growth and shift in the bacterial community composition was used to verify xanthan degradation.

Biodegradation was not demonstrated in the bioassays. The SRP brine showed enrichment of bacteria affiliated to *Methylophaga* sp. and *Microbacterium* sp. after incubation at mesophilic conditions. Phylotypes affiliated to *Petrotoga halophila, Petrotoga mobilis, Thermosipho geolei* and *Kosmotoga olearia* were enriched in the PWRI brine at thermophilic conditions. A facultative anaerobic sugar degrading bacterium, with 100% sequence similarity to *Aeribacullus pallidus* was isolated from the PWRI brine at 60°C by plating on LBA. The strain preferred growing on solid medium. The strain was able to grow anaerobically on glucose (by fermentation), thereby differing from the type species classified as strict aerobic. Xanthan supported growth at aerobic conditions.

Biodegradation of xanthan was observed in a later experiment when the incubation time at mesophilic conditions was extended. A control experiment verified biodegradation, and a bacterium affiliated to *Prolixibacter bellariivorans* (98%sequence similarity) was identified as the putative xanthan degrader.

1 Introduction

1.1 Background for the study

Application of biopolymers is considered environmentally favorable compared to synthetic polymers, as biopolymers derive from renewable sources and are biodegradable. Biopolymers are considered a viable alternative to synthetic polymers in Enhanced oil recovery (EOR) application (Wei et al., 2014). Biopolymers like xanthan are mixed with the injected water to improve oil recovery. Application of synthetic polymers causes disposal problems after exploitation (Leja and Lewandowicz, 2009). The biodegradability of biopolymers is favorable when considering disposal and discharge, but is for the same reason a concern for oil companies due to the potential for deterioration of effect and performance during EOR application (McInerney et al., 2005). The objective of the current master project is to assess the potential for biodegradation of xanthan in injection waters from a North Sea oil field at relevant reservoir temperatures.

1.1.1 Biopolymers in EOR

Biopolymer is a general term for polymers produced by living organisms. Biopolymers are composed by monomeric units covalently linked to form larger structures. Polysaccharides are one form of biopolymers that are used in various commercial applications due to their special properties as viscosifying agents and stabilizers for water-based systems (Becker et al., 1998; García-Ochoa et al., 2000). The application range is broad, compromising the food, medicine, cosmetics and petroleum industry.

Flooding petroleum reservoirs with water soluble polymers may be the most economical tertiary chemical oil recovery method (Littmann, 1997). The polymers are utilized to increase the amount of crude oil extracted from oil reservoirs by increasing the viscosity of the injection water (Kreyenschulte et al., 2014). The high viscosity of the injected water improves oil recovery by reducing the water mobility and reducing fingering of the water (Nasr et al 2007; Wang and Dong, 2009). The exceptional rheological properties and stability at a wide range of temperature, salinities and pH has made biopolymers eligible candidates as viscosifying agent for enhanced recovery of heavy oil (Bryant, 1987; Van Hamme et al., 2003). High viscosity of solutions and water solubility makes biopolymers useful tools also in drilling fluids (García-Ochoa et al., 2000). Requirements from the Norwegian Environment Agency, demanding reduced use and emission of environmentally

harmful chemicals (classified as “black” and “red”) (Klima og forurensingsdirektoratet, 2010) has increased the focus within the oil industry to find environmentally friendly solutions for flow assurance, improved and enhanced oil production. These criteria’s can be covered by the use of biopolymers.

1.1.2 Biodegradation of biopolymers

Extensive research over the past decades has revealed large amounts of biopolymers produced by microorganisms (Kreyenschulte et al., 2014). Biopolymers offer a wide range of applications and can potentially replace less favorable materials. Industrial application of biologically produced polymers is considered environmental and economically beneficial, related to non-degradable synthetic alternatives.

The stability and hence performance of biopolymers are challenged by their biodegradability (McInerney et al., 2005). In sea water injected oil fields, sea water and reservoir microorganisms are potential degraders of biopolymers. In order for microorganisms to metabolize biopolymers, the macromolecule is broken down into smaller fragments outside the cell before uptake and further degradation inside the cell (Kreyenschulte et al., 2014). Biodegradation thus require secretion of specific enzymes that can depolymerize the biopolymer (Li et al., 2008). Enzymes acting on plant produced polysaccharides are well documented in the literature (Hashimoto et al., 1999). Reports on enzymes that depolymerize microbial produced biopolymers are more limited, but enzymes targeting bacterial exopolysaccharides like xanthan have been described (Ahlgren, 1991; Cadmus et al., 1988, Li et al., 2008).

1.1.3 Xanthan: Structure and application

Xanthan gum is a widely utilized biopolymer, applied in a numerous of different industries. Xanthan was the first biopolymer produced at a large scale (Kreyenschulte et al., 2014) exploited as a suspending and thickening agent in food since 1969 (Faria et al., 2011; García-Ochoa et al., 2000). Because of its many beneficial properties, xanthan has become a major commercially produced microbial polysaccharide, and is frequently used as a viscosifying agent in the petroleum industry (García-Ochoa et al., 2000; Katzbauer, 1997).

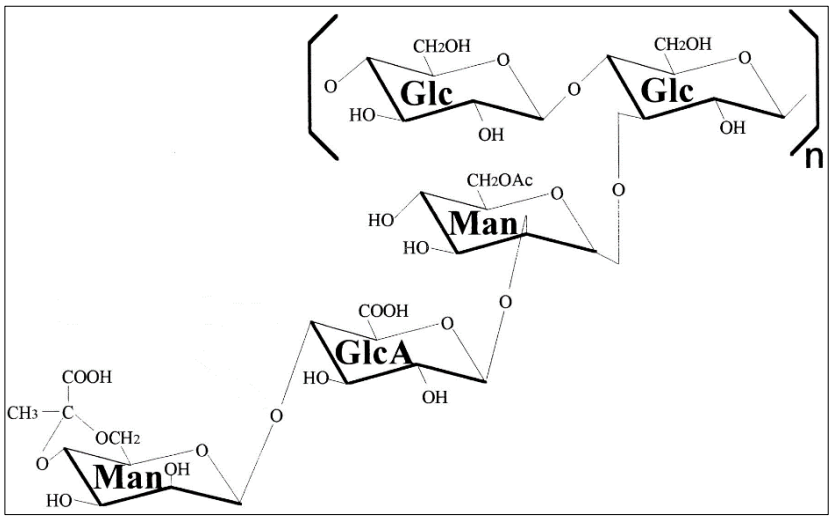

Figure 1: The chemical structure of xanthan. The biopolymer consists of a cellulosic backbone with trisaccharide side chains composed by a mannosyl- glucuronyl-mannose sequence linked to alternate glucosyl units (Qian et al., 2006).

Xanthan gum is produced by the plant-pathogenic bacterium *Xanthonomonas campestris* pv *campestris* (Li et al., 2008). The heteropolysaccharide is composed by repeated pentasaccharide units formed by two glucose units, two mannose units and one glucuronic units (Figure 1). The internal and terminal residues of the side chains can be acetylated or pyruvylated, determined by the growth conditions, substrates and bacterial strains (García-Ochoa et al., 2000; Li et al, 2008).

Xanthonomonas is a genus of the Pseudomonaceae family, comprising several plant pathogenic bacteria affecting a wide variety of different crops (García-Ochoa et al., 2000). *X. campestris* is an obligate aerobe, gram negative and rod shaped chemoorganotrophic bacteria, generating xanthan through fermentation of a broad range of substrates (Becker et al., 1998). The biopolymer is part of the bacterial capsule, and is an important virulence determinant (Li et al., 2008).

1.1.4 Biodegradation of xanthan

According to the literature available on the biodegradability of xanthan, there seems to be a disagreement in the scientific community regarding the resistance of the polymer against biodegradation. Some state that xanthan is a highly stable biopolymer, only known to be completely degraded by a few microorganisms (Cadmus et al., 1982; Hou et al., 1986;

Qian et al., 2006; Li et al., 2008). The fact that only a few enzyme systems hydrolyzing xanthan has been reported supports the assumption that xanthan is relatively resistant to biodegradation (Ahlgren, 1993; Cadmus et al., 1988; Ruijssenaars et al., 1999; Lui et al., 2005). However, reports from the oil industry state that application of xanthan may be problematic after experiencing degradation of xanthan in oil field applications (Bragg et al., 1983; McInerney et al., 2005).

The initial step in xanthan degradation is conducted by secreted enzymes, depolymerizing the polysaccharide. Several enzymes degrading the biopolymer have been reported in literature (Cadmus et al., 1988; Hou et al., 1986; Li et al., 2008). Degradation is mainly caused by xanthanase (endo-β-d-glucanase), catalyzing the hydrolysis of the cellulosic backbone (Hashimoto et al., 1998; Sutherland, 1987). Xanthanase is therefore the key enzyme reducing the viscosity of xanthan solutions (Chen et al., 2013). Another enzyme, xanthan lyase, eliminates the terminal mannose residue from the side chains of the biopolymer (Ahlgren, 1991; Hasimoto et al., 1998). Cultures growing on xanthan generally produced a mixture of xanthan degrading enzymes (Hashimoto et al., 1998; Ruijssenaars et al., 1999). Cellulases have also been shown to partially degrade the biopolymer, but only in unordered confirmation (Rinaldo and Milas, 1980).

Despite the supposed high stability of xanthan, microbial (enzymatic) degradation has been observed in enhanced oil recovery tests. Hou et al. (1986) investigated microorganisms that might have been responsible for loss of viscosity under EOR operation conditions. Xanthan degrading enzyme(s) excreted from an aerobe culture was not inhibited by anoxic conditions or different chemicals and biocides commonly used in enhanced oil recovery operations. Other authors have reported the isolation of salt tolerant and heat stable enzymes (Ahlgren, 1993; Cadmus et al., 1988), indicating potential for biodegradation of xanthan under reservoirs conditions. Cadmus at al. (1988) described a salt tolerant, heat stable xanthanase, functional in brines up to 65°C.

Cultures producing extracellular hydrolytic enzymes degrading xanthan have been reported by different authors. Cadmus et al. (1982) described the isolation of a salt tolerant *Bacillus* sp. K11, eliminating the side chains of xanthan. In later studies, several bacterial phyla producing xanthan degrading enzymes have been described. Xanthan lyase activity was first reported from a *Bacillus* sp., a *Corynobacterium* sp. and a mixed culture (Sutherland

et al., 1987). In later studies xanthan lyases have been purified from a mixed culture (Ahlgren, 1991), *Bacillus* sp. GL1 (Hashimoto et al., 1998) and from *Paenibacillus alginolyticus* XL-1 *(*Ruijssenaars et al., 1999). Liu et al. (2005) reported xanthan degradation by the newly isolated *Cellulomonas* sp. LX. In addition, Qian et al. (2007) isolated the xanthan degrading bacteria *Microbacterium* sp. strain XT11 from soil. More recently, *Enterobacter* sp. nov. LB37 was proven to degrade xanthan and thereby reducing the viscosity of xanthan solutions used in oil drilling processes (Chen et al., 2013). Figure 2 illustrates the extracellular depolymerization of xanthan by *Bacillus* sp. GL1, secreting xanthan lyase and xanthanase (Hashimoto et al., 1999).

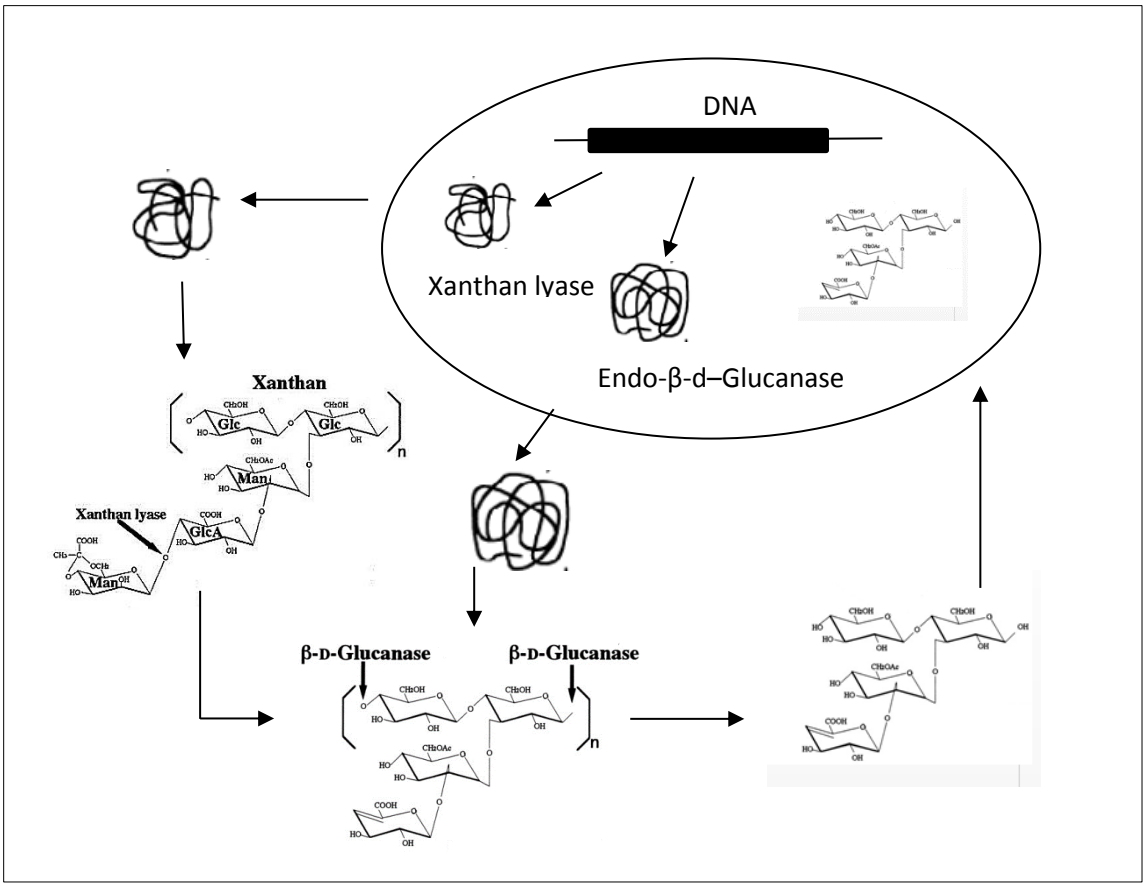

Figure 2: The enzymatic route for extracellular degradation of xanthan by *Bacillus* sp. GL1 (Hashimoto et al., 1999). Xanthan is depolymerized by the xanthanase (endo-β-d-Glucanase) after the mannose terminals are eliminated by xanthan lyase. The tetrasaccharide is assimilated into the cell, and internally degraded by intracellular enzymes.

1.1.5 Microbial growth in injection water

Sulfate reduces seawater (SRP) and produced water is considered as potential injection waters for biopolymer application in certain North Sea oil fields. Microbial growth in injection waters are influenced by the chemical and physical properties of the water. Generally, injection water is extensively processed in advance of injection, including de-oxygenation to prevent corrosion and biocide treatment to prevent microbial growth. In some oil fields, sulfate is removed from the injected sea water to prevent reservoir souring caused by sulfate reducing bacteria (SRB) (Myhr et al., 2001). SRB reduce sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S) when obtaining energy from organic and inorganic compounds.

Produced water is the aqueous liquid phase that is the byproduct during oil and/or gas recovery. This includes formation water, naturally occurring within the geological formation, as well as water injected into the reservoir. Produced water is anoxic, containing elevated concentration of metals, hydrocarbons and ammonium (Yeung et al., 2011). Reinjection of produced water (PWRI) as alternative to discharge into the sea is a governmental strategy to reduce the overall discharge of chemicals and petroleum components to the marine environment (Bjørnestad et al., 2005; Klima og forurensingdirektoratet, 2010).

Petroleum reservoirs are characterized by harsh physical conditions, with temperature and pressure as key parameters affecting microbial life. Temperature is the most limiting factor for microbial growth in these environments (Magot, 2005). In petroleum reservoirs, temperature increase with depth, greatly affecting the ability of organisms in the injected sea water to survive in the bulk part of the reservoir. pH, salinity and toxic oil components will also affect and potentially limit microbial growth in these habitats (Sunde and Torsvik, 2005). Application of cold sea water as injection water reduces the temperature near the injector, forming favorable growth temperatures for mesophilic sea water bacteria (Sunde and Torsvik, 2005).

The availability of electron donors and acceptors determines the bacterial metabolic processes within oil field environments (Magot et al., 2000). Because oil fields are subterranean environments isolated from the surface water, the redox potential is low. Important electron acceptors like oxygen, nitrate and ferric iron are generally absent (Magot et al., 2000). Multiple groups of physiological diverse microorganism have been recovered from petroleum reservoirs. Several studies have analyzed the microbial

communities in formation waters from petroleum reservoirs, indicating that presence of diverse populations of bacteria, including fermentative bacteria, sulfate and iron reducing bacteria, acetogenic bacteria and metanogenic archaea (Magot et al., 2000; Orphan et al., 2000; Pham et al., 2009). Stratal waters contain various concentrations of carbonate and sulfate, providing basis for these metabolic processes (Magot et al., 2000).

1.1.5 Fermentative bacteria

Under anoxic conditions, in shortage of external electron acceptors, organic compounds are catabolized by obligate anaerobic and facultative anaerobic bacteria by fermentation. In fermentation, the organic compounds sever as electron donor and electron acceptor, and ATP is produced by substrate level phosphorylation. The internal redox balance is maintained by producing and excretion of fermentation products like organic acids, alcohols, ammonium, CO_2 and hydrogen.

The diversity of bacterial fermentations is extensive, and they are commonly classified by the fermentation products. Fermentation of carbohydrates is a widespread process, utilized by a broad range of microorganisms. Fermentative bacteria are commonly isolated from oil reservoirs, where mesophilic to extreme thermophilic strains have been described (Grabowski et al., 2005, Magot et al., 2000). Thermophilic bacteria have been isolated with a greater frequency then mesophilic bacteria, a reflection of the high temperature in most oil reservoir (Magot et al., 2000).

1.1.7 Fermentation of glucose

Glucose is one of the main constituents of xanthan, constructing the backbone of the polymer. When xanthan is depolymerized, glucose is available for fermentation. A wide range of bacteria are capable of fermenting glucose and other hexoses. Glucose is commonly dissimilated by the Embden–Meyerhof–Parnas pathway (glycolysis), to yield pyruvate. Pyruvate is further fermented to a range of end products, related to fermentation process (table 1).

Table 1: Major pathways for fermentation of glucose (Müller, 2001). The figure includes fermentation products and examples of microorganism implementing the process.

Classification	End product	Microorganism
Ethanol fermentation	Enthanol + CO_2	*Saccharomyces, Zymomonas mobilis*
Homo lactic acid fermentation	Lactate	*Lactobacillus, Sporolactobacillus.*
Hetero lactic acid fermentation	Lactate, Ethanol + CO_2	*Leuconostoc, Bifidobacterium*
Propionic acid fermentation	Propionate + Acetate + CO_2	*Propionibacterium*
Mixed acid fermentation	Ethanol + Acetate + Lactate + Succinate + Formate + CO_2 + H_2	*Escherichia.*
2,3 butanedion fermentation	Ethanol + Butanediol + Lactate + Fomate + CO_2 + H_2	*Klebsiella, Enterobacter*
Butyrate fermentation	Butyrate + CO_2 + H_2	*Clostridium*
Acetone-butanol fermentation	Butanol + Acetone + CO_2 + H_2	*Clostridium*
Homoacetic acid fermentation	Acetate	*Acetobacterium*

Degradation of xanthan is expected to be conducted by bacteria implementing this process, yielding energy by fermentation of glucose (or potentially mannose). The diversity among these organisms is wide, distributed among a broad range of bacterial phyla and habitats.

1.2 Objectives and Hypothesis

The current master thesis is part of an industrial project conducted by UNI Research CIPR for Statoil ASA. The assessment of the potential for biodegradation of xanthan is fundamental before applying the biopolymer in EOR operation. Xanthan degradation due to microbial (enzymatic) activity reduces the water viscosity, and hence the effect of xanthan as mobility control in EOR. Monitoring the potential for microbial degradation of xanthan in a North Sea oil field is part of the quality assessment of the method when considering application to field. The experimental goal was to assess the potential for biodegradation in two potential injection brines, at mesophilic (30°C), thermophilic (60°C) and hyper thermophilic conditions (85°C). This was conducted by bioassays with xanthan as the sole added carbon source to the native brines. The following hypotheses were defined:

- ✓ Microbial degradation of xanthan will lead to cell growth.
- ✓ Growth on xanthan will cause shift in the community structure towards increased abundance of species capable of biopolymer degradation and glucose fermentation.
- ✓ The species degrading xanthan will be dominant, and can be isolated from the bioassay for further study.
- ✓ The viscosity of the brines will decrease if the biopolymer is degraded.

The following experimental approaches were conducted on the bioassays:

- ✓ Increase in bacterial cell number by QPCR.
- ✓ Fingerprinting by denaturation gradient gel electrophoresis (DGGE) was applied to assess shifts in the community structure.
- ✓ Isolation and partial characterization of dominant bacteria from a bioassay with positive growth.

Decrease in viscosity of xanthan brines was assessed by rheology measurements, conducted by UNI research CIPR staff. The results are not described in the current thesis. The results will be discussed in light of the results from the current microbial analysis.

2 Materials and Methods

2.1 Samples and sampling methods.

The biodegradability of xanthan was assessed in two types of injection water; sulfate reduced seawater (SRP) and produced water (for reinjection, PWRI), from a North Sea oil field. The brines were collected at the platform and transported to laboratory in jerry cans and shipped to Uni Research CIPRs laboratory in Bergen. The brines were transferred to Pyrex glass bottles under N_2-flushing for storage before use. The SRP bottles were stored in the refrigerator while the PWRI bottles were stored at room temperature to retain the potential for thermophilic microbial activity. The experimental and laboratory analysis were conducted at UNI Research CIPRs laboratories at Marineholmen.

2.2 The experimental design

The biodegradability of xanthan was assessed in a bioassay with native brines mixed with xanthan (figure 3). Before xanthan was added, the brines were degassed by water suction to reduce oxygen levels. Xanthan was added to the native brines, to a final concentration of 200 ppm and left to mix (magnetic stirrer) while flushing with N_2-gas for 1 hour. Thereafter, 45 ml of the biopolymer-brine were transferred to argon flushed 50 ml anaerobic culture bottles by use of a peristaltic filling machine (Flexicon, Ringsted, Denmark) while continuously flushing the xanthan-brine with argon-gas. Argon was chosen for this step because it is heavier than nitrogen and therefore serves as a better anoxic gas cap. The bottles were capped using butyl rubber stoppers and crimp seals. The pH of the xanthan-brine was measured before, during and at the end of the bottle-filling process.

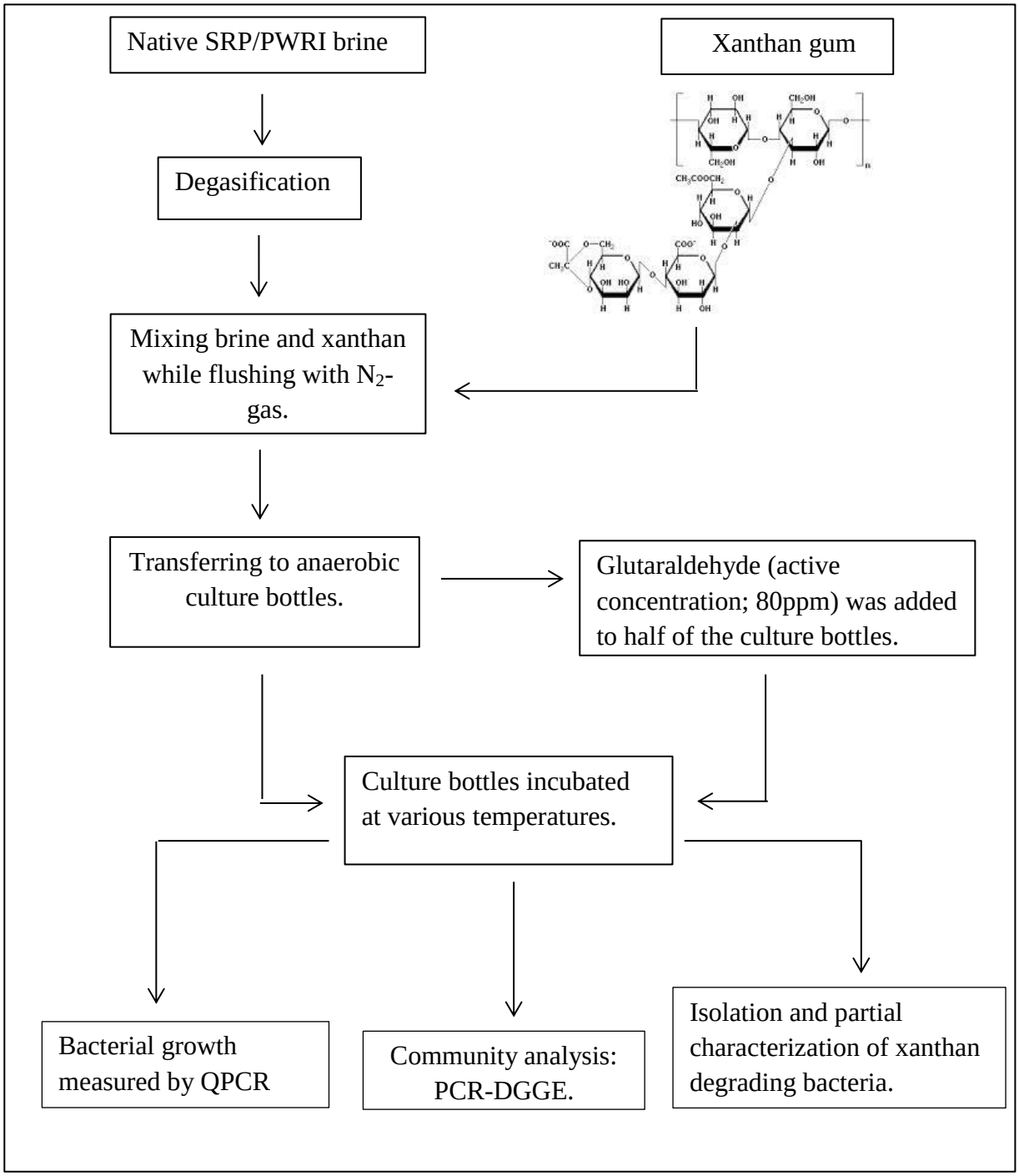

Figure 3: Schematic presentation of the procedure and processing of the injection waters. To remove oxygen, the brine was degassed before addition of xanthan. While mixing, the xanthan-brine was flushed with N_2-gas. The xanthan-brine was transferred to anaerobic culture bottles while flushing with argon. Glutaraldehyde (active concentration; 80ppm) was added to half of the bottles. The bottles were incubated at relevant reservoir temperatures, and analyzed for bacterial growth and shift in the bacterial community at permanent intervals. Bacteria was enriched and isolated from the PWRI brine.

A total of 54 bottles with xanthan-brine was made for each injection water. Half of the bottles were added glutaraldehyde (25% solution), to a final active concentration of 80 ppm. Three parallels with and without glutaraldehyde were analyzed at each sampling interval. Due to the potential bias of chemical oxidation in case of oxygen being introduced during sampling, new parallels were analyzed at regular intervals during sampling of the series incubated at high temperatures. Three parallels were incubated at 30°C, 6 parallels at 60°C and 18 parallels at 85°C.

Samples from the cultures were harvested at regular intervals, as described in table 2. A sample volume of 6 ml was harvested from the culture bottles using a sterile syringe. Two times 1.5 ml were harvested for QPCR and PCR-DGGE analysis. The samples were centrifuged at 16060 *g* for 20 minutes, before the supernatant was removed. This was follow by re-suspending the samples with 1 ml PBS (table A.2) before the centrifugation was repeated. The supernatant was carefully removed by pipetting. The cell pellet was stored at -20°C prior to molecular analysis. The residual volume of approximately 3 ml was used to assess change in viscosity during incubation.

Table 2: Overview of sampling. Description of the sampling intervals for the different temperatures. The highlighted numbers indicate incubation temperature. Molecular analysis (QPCR and PCR-DGGE) was conducted for all samples.

	Sample name	
Sampling time	Without biocide	With biocide
Day 0	SRP-X-**30**	SRP-XB-**30**
Day 3	SRP-X-**30**	SRP-XB-**30**
Day 7	SRP-X-**30**	SRP-XB-**30**
Day 0	SRP/PWRI-X-**60**	SRP/PWRI-XB-**60**
Day 7	SRP/PWRI-X-**60**	SRP/PWRI-XB-**60**
Day 14	SRP/PWRI-X-**60**	SRP/PWRI-XB-**60**
Day 21	SRP/PWRI-X-**60**	SRP/PWRI-XB-**60**
Day 28	SRP/PWRI-X-**60**	SRP/PWRI-XB-**60**
Day 0	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
1 Week	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
1 Month	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
2 Months	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
3 Months	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
4 Months	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
5 Months	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**
6 Months	SRP/PWRI-X-**85**	SRP/PWRI-XB-**85**

2.3 Enumeration by whole cell QPCR

Bacterial abundance in the batch cultures were monitored during the experiment by QPCR of the 16S rRNA gene. The primer set PRBA338f and PRUN518r were used, amplifying a product of approximately 180 bp in length. The PRBA338f primer complements a region conserved in the domain Bacteria, while the PRUN518r is a universal conserved region of the 16S rRNA gene (Øvreås et al., 1997). All primer sequences applied in this work are listed in the appendix (table A.7). According to the project protocol (Bødtker et al., 2015) DNA amplification was performed on whole cells without extraction of DNA. The samples were re-suspended in 100µl nuclease free water (Qiagen) prior to analysis. The samples were diluted if necessary. This was determined by preliminary analysis of dilution series (10-100 fold). Nuclease free water was included as a negative control to determine background of bacterial DNA in the QPCR mix. Extracted DNA from *E. coli* was applied as positive control. A series of DNA amplicons with known concentrations (copies/ml) were applied as standards for quantification.

A 20 µl QPCR reaction mix containing 10 µl SYBR® Green PCR kit, 0.06 µl primers (100uM), 8.88 µl nuclease free water and 1 µl template was made. The reaction was run by the following cycling conditions; denaturation of DNA at 95°C for 15 minutes, 36 cycles with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 1 minute at 72°C followed by a plate read. At the end, a melting curve from 55°C to 95°C was conducted. The reactions were carried out in an Opticon™ 2 monitor (MJ research, Switzerland). Quantification cycles and crossing values (C(t)) were calculated by the instrument software after manual determination of background in the samples. To account for multiple copies of the 16S rDNA among bacterial species, a correlation factor of 4.2 was applied to calculate the cell numbers (Větrovský and Baldrian, 2013).

2.4 Bacterial diversity in response to xanthan, biocide treatment and temperature based on PCR-DGGE fingerprint analysis

2.4.1 PCR

16S rDNA amplification for DGGE was performed with primers designated to amplify parts of the V3-region of bacteria. The primer combination pA8f and PRUN518r (Giovannoni, 1991; Muyzer et al., 1993) was used. The forward primer contained an additional 40-nucleotide GC rich sequence (GC clamp).

A 25 µl PCR reaction mix was made using 9.125 µl nuclease free water, 12.5 µl Hotstar Taq Mastermix (Qiagen), 0.125 µl of each primers (100 µM), 0.125 µl BSA (2%) and 3 µl template. PCR was performed in a Mini™ Gradient thermal cycler (Bio-Rad, USA). The following cycle conditions were used: enzymatic activation at 95°C for 15 minutes, before 35 cycles with denaturation of DNA at 94°C in 15 seconds, annealing for 30 seconds with 55°C, and extension for 1 minute at 72°C. After the 35 cycles a 10 minute final extension was conducted at 72°C.

2.4.2 Agarose gel electrophoresis

Positive PCR amplification was assessed by electrophoresis in 1.5 % Agarose (Seakem LE) gels strained with GelRed (Biotium, Hayward). The gel was cast by melting agarose in 1xTAE buffer (table A.1). 0.5 µl GelRed was then added to the cooled solution, before it was transferred to the gel camber. After solidification, the gel was covered with 1xTAE buffer in an electrophoresis chamber (HE33 mini submarine unit. Hoefer Inc, USA). 5 µl of the PCR products were mixed with 1 µl loading dye solution (table A.3) and loaded onto the gel. 1.5 µl DNA ladder (1 µg/µl) (pGEM ® DNA markers, Promega) were included as a reference. The electrophoresis on the gel was performed with a 200 volt current for 18 minutes, using Pharmacia Biotech Power Supply EPS 600 (GE Healthcare, Buckinghamshire). Gels were visualized using the molecular Imager system Chemidoc™ XRS+ with filter 1 and the Image Lab™ Software (Bio-Rad, Hercules).

2.4.3 Denaturation gradient gel electrophoresis

The DGGE analysis was prepared according to the principle in Øvreås et al., 1997. Glass plates were cleaned with detergent and alcohol and air dried to remove impurities. 150 µl silicon repel (Pharmacia Biotech) was wiped over the glass plates, before the plates were assembled on a stand, separated with rubber spacers. Beforehand, two different stock solutions containing 0% denaturant (DSSA) and 100% denaturant (DSSB) were prepared (table A.8). The stock solutions were mixed to 14.5 ml in two Erlenmeyer flasks, one containing a high level of denaturant (60%) and one a low level of denaturant (20%) (table A.9). 145 µl APS (Bio-Rad, Hercules) and 7.3 µl TEMED (Bio-Rad, Hercules) were added to each flask, before the solutions were transferred to a gradient maker. The gel was cast with a high denaturant concentration in the bottom and a decreasing, lower concentration towards the top of the gel.

The gel polymerized for 1 hour before the wells were washed with 0.5xTAE buffer. 15 µl of the PCR products was mixed with 3 µl loading dye and loaded onto the gel. A marker was included in each corner as a reference. The marker was provided by mixing PCR amplicons from five species with different GC-content. The amplification was performed using the same primer combination as for the samples. The gel was placed in a SE 600 Ruby Standard Dual Cooled Vertical Unit (GE Healthcare, Buckinghamshire) fillet with 0.5xTAE buffer preheated to 65°C. The electrophoresis was run at 10 minutes at 20 V, followed by 18 hours at 70 V, using a Pharmacia Biotech Electrophoresis Power Supply EPS 600 (GE Healthcare, Buckinghamshire).

After electrophoresis, the gel was stained with a mixture of 15 ml 1xTAE and 1.5 µl SYBR® Gold Nucleic Acid Strain (Invitrogen, Carlsbad, CA) for 45 minutes in the dark. The gel was visualized using a Chemidoc™ XRS+ with an XCitaBlue™ Conversion Screen and the Image Lab™ Software (Bio-Rad, Hercules). Bands selected for further analysis were picked from the gel using sterile pipette tips and transferred to Eppendorf tubes with 20 µl nuclease free water. These were stored for passive diffusion at 4°C overnight, and stored at -4°C until further analysis.

2.4.4 Reamplification of the DNA bands

The eluate was reamplified using the primer set described for PCR (section 2.4.1). A reaction mix was made by 11.25 µl nuclease free water, 12.5 µl Hotstar Taq Mastermix, 0.125 µl of the primers and 1 µl template. The cycling conditions are provided in section 2.4.1. Positive amplification was assessed by agarose gel electrophoresis (section 2.4.2).

2.4.5 Dideoxy thermal cycle sequencing

Reamplified DGGE bands were nucleotide sequenced using dideoxy sequencing. The sequences were purified for excess primers and nucleotides using ExoSAP-IT® (USB®), as described in the procedure by the manufacturer (appendix 1). A reaction mix containing the following reagents was made: 5 µl nuclease free water, 1 µl Bigdye® 3.1, 1 µl BigDye® sequencing buffer, 3.2 pmol Prun518r primer and 1 µl purified PCR product, to a total volume of 10 µl. The cycling conditions were as follows: 25 cycles of 15 second at 96°C, 10 seconds at 55°C followed by 2 minutes at 60°C. The products were diluted in 10 µl nuclease free water, and delivered to the University of Bergen Sequencing Facility for reading of the sequences.

2.4.6 Bioinformatics

The quality of the DNA sequences was controlled in chromatograms visualized in Chromas lite 2.1.1 from Technelysium Pty Ltd. Sequences with isolated, restricted peaks without disturbing background were assessed as good quality sequences.

Nucleotide sequences were aligned in the Basis Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information's (NCBI). This allows rapid screening in a large database maintained by NCBI, comparing the similarity between sequences and calculating the statistical significance of the results. Hits from the database (max identity) were used to affiliate the phylotypes to bacterial taxa by genetic similarity in the 16S rDNA. Sequences with low homology to known genera where analyzed by Ribosomal database project (RDP) classifier for reference and taxonomic classification.

2.5 Verification of xanthan degradation by culturing

A control experiment was conducted to verify biodegradation of xanthan by culturing in synthetic brine. The experiment was performed at 30°C in anoxic synthetic SRP brine (described in section 2.6.1) with xanthan as sole carbon source (300ppm). The synthetic brine (45 ml) was inoculated with 0.5 ml from selected bioassay bottles and incubated at 30°C for 4 weeks. Samples from the cultures were harvested and analyzed at permanent intervals, comprising the same analysis as in the bioassay (table 3). Sampling was conducted as defined previously (section 2.2). The pH was measured using an Inolab 720 pH meter (Germany). Viscosity measurements were conducted by UNI Research CIPR staff, and are included as part of the result section.

Table 3: Overview of sampling time and analysis included in the control experiment.

Sampling time	Viscosity	pH	QPCR/PCR-DGGE
Day 0	+	+	+
Day 3	-	-	+
Day 7	-	-	+
Day 14	-	-	+
Day 21	-	-	+[a]
Day 28	+	+	+

[a] Only QPCR, + = analysis included, - = analysis not included

2.6 Isolation and characterization of dominant Bacteria

2.6.1 Anaerobic enrichments:

Enrichments of dominant bacteria from the bioassay (SRP/PWRI brines without biocide at 60°C) were performed in synthetic brine based on the ionic composition in the SRP (table A.11) and PWRI brines (table A.10). The salts were dissolved in 1 liter water before autoclaving. The medium was flushed with N_2-gas while cooling, before nitrogen and phosphorus were added to the solution (final concentrations; 116 µM NH_4^+ and 10 µM PO_4^{3-}). In addition, 1 ml trace element solution (table A.4) (Widdel et al., 1983) and 0.5 ml of a 10x vitamin solution (table A.5) were added per liter (Bødtker et al., 2009). The pH was measured and adjusted to approximately 7. The pH was monitored during dispensing of 15 ml brine to 30 ml anaerobic culture tubes and 45 ml to 50 ml anaerobic culture bottles.

Enrichment of bacteria from the bioassays was performed by transferring 1.5 ml of the original cultures to anoxic synthetic SRP and PWRI brine (15 ml) with xanthan as the sole carbon source (figure 4). The culture bottles were incubated at 60°C. Visual assessment of growth was performed by microscopy weekly (Leitz Laborlux K, Wetzlar, Germany). When growth was detected, 1 ml of the cultures was transferred to fresh brine containing glucose (20mM) or a mixture of organic acids (butyric and caproic acid to a final concentration of 10mM). Assessment of the bacterial community composition in the enrichment cultures was performed by PCR-DGGE. Samples from the cultures were harvested after 4 weeks of incubation as described in section 2.2. The PCR-DGGE analyses were performed as described in section 2.4. An additional transfer to fresh brine was performed for cultures with glucose as substrate.

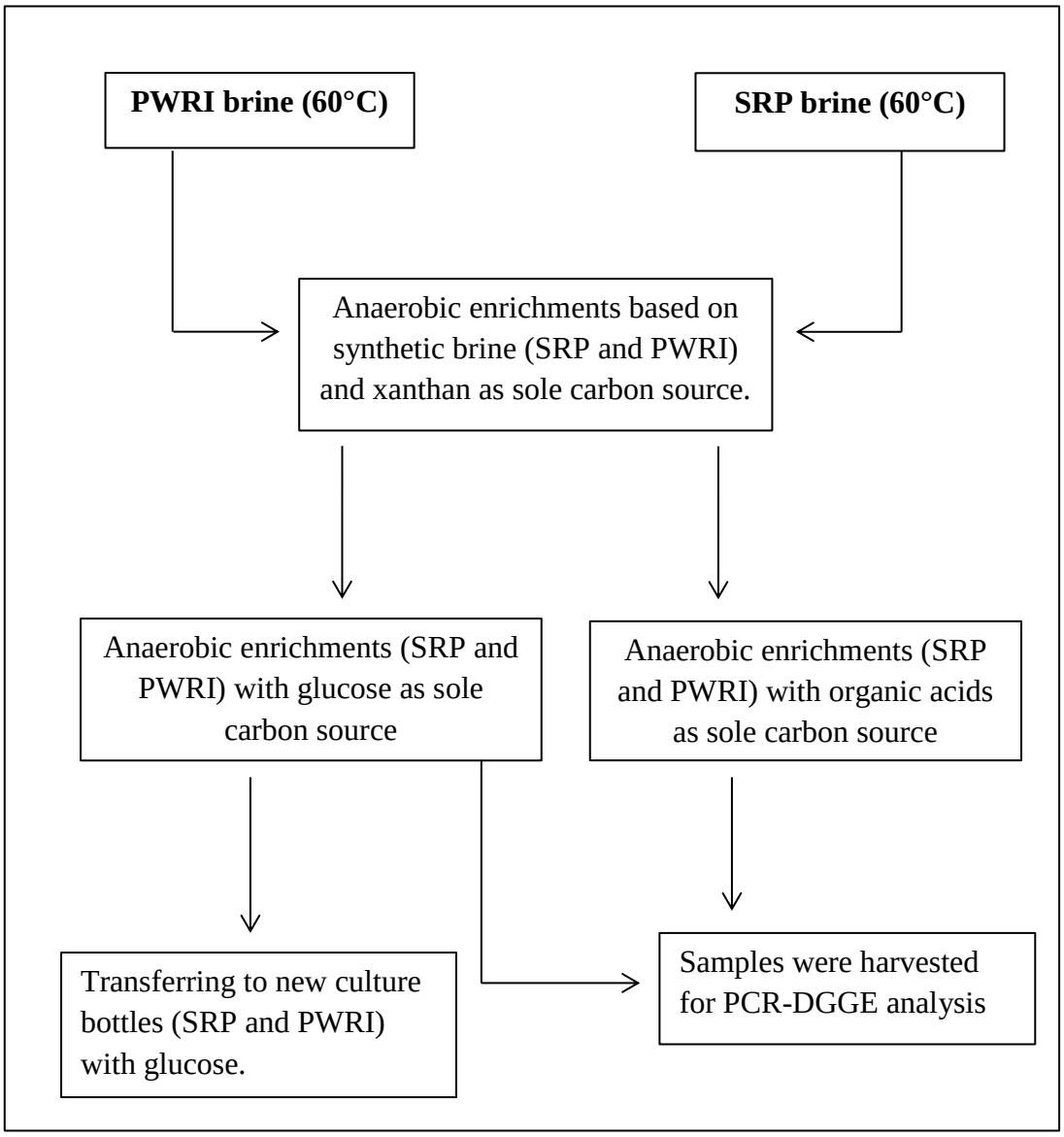

Figure 4. Culturing in anoxic synthetic brines. Culture bottles from the bioassay (60°C) were used as inoculum. Growth was initial examined using xanthan as sole carbon sources. Inoculums from the initial cultures were transferred to new bottles containing glucose or organic acids (C_4+C_6). To profile the bacterial community in the enrichments, a PCR-DGGE analysis was performed. An additional transfer was performed with glucose as substrate.

2.6.2 Aerobic enrichments:

Aerobic isolation of facultative anaerobic sugar degraders was performed by plating technique on LBA medium (table A.6) Cultures from the original bioassay (SRP and PWRI brines without biocide, 60°C) were used as inoculum. Approximately 0.1 ml of each culture was transferred to LBA plates before the volume was spread evenly using a sterile glass rod. The plates were incubated at 60°C. When growth was detected, single colonies were transferred to new plates using streaking technique. The process was repeated three

times to provide pure colonies. The colonies were examined in the microscope to assess the purity and morphology of the isolates.

When pure colonies were obtained, colonies from the LBA plates were transferred to aerobic and anaerobic synthetic brine (15 ml) (provided in section 2.6.1) containing glucose (20 mM). The enrichments were incubated at 60°C, and frequently visualized by microscopy to assess growth. When growth was identified, an isolate deriving from the PWRI brine was selected for a partial characterization.

The purity and morphology of the culture was controlled by microscopy. For genotypic characterization, 1 ml of the culture was harvested for PCR analysis and centrifuged at 16060 *g* for 20 minutes. The supernatant was discharged and the pellet was stored at -4°C until further analysis.

2.6.3. Characterization of isolates

Genotypic characterization

The 16S rDNA was amplified by whole cell PCR using the primer combination pA8f and Hr (table A.7), which amplifies a fragment corresponding to the position 8-1541 of the *Escerichia coli* 16 RNA gene (Giovannoni, 1991). A PCR reaction was conducted using the following reagents: 0.25 µl of each primer (100 µM), 25 µl HotStarTaq Master Mix, 2 µl cell suspension and nuclease free water to a final volume of 50 µl. The cycling conditions are provided in section 2.4.1. Positive amplification was determined by agarose gel electrophoresis as described in section 2.4.2.

The PCR product were purified for excess primers and nucleotides by ExoSaP-IT® and sequenced using BigDye® 3.1 (section 2.4.5). The sequencing was performed using various primers corresponding to different positions in the 16S rDNA. The forward primers pA8f, PRBA338f, PRE927f and reverse primers PRUN518r, PRE944r and HR were used in a parallel sequencing reaction (table A.7). The sequences were assessed in Chromas lite V.2.1.1, to ensure the purity of the isolate. The partial DNA sequences obtained from sequencing was assembled using the CAP3 DNA assembly program and manually controlled for gaps and designated bases (Huang and Madan,1999) The DNA sequence was align in the BLAST database (section 2.4.6), for identification of the closest validated relative.

Phenotypic characterization

Optimal growth temperature

Optical density (OD) was applied as measure for growth when assessing the physiological properties of the isolate. Optimal growth temperature was determined by aerobic growth in liquid culture added glucose (20mM) as carbon and energy source. To stimulate growth, one drop of yeast extract (10%) was added. 3 parallel cultures were incubated at 5 different temperatures; 30°C, 54°C, 60°C, 65°C and 70°C. OD was measured in a Spectronic 21 (Milton Roy Company, USA) at 600nm for 9 days until the maximal value had passed. A blank control was included as a reference at each incubation temperature.

Substrate diversity

Aerobic utilization of different substrates was analyzed in liquid PWRI brine with 1% substrate. The following substrates were tested (concentration of solution): fructose (10% w/v), cellobiose (10% w/v), maltose (10% w/v), starch (30% w/v), cellulose (10% w/v), sucrose (2M), galactose (10% w/v), organic acids (C_4+C_6, 1M) and crude oil. In addition, aerobic growth on xanthan (final concentration: 300ppm) was assessed. A drop of yeast extract (10% initially, 1% after 2 transfers) was added in all parallels. A parallel containing only yeast extract was included to assess growth on this substrate alone. Fermentation of glucose was determined in anoxic brine. In addition, growth on glucose was assessed with nitrate (6.7 mM) and thiosulfate (6.7 mM) as alternate electron acceptors. Growth was determined by OD measurements (600nm) in addition to microscopic examinations of the cultures.

3 Results

3.1 Enumeration by QPCR of numbers of bacteria in the bioassays.

3.1.1 Mesophilic conditions

The initial cell number in the SRP water was estimated to be approximately $1x10^6$ cells per ml. Two of the parallels without biocide showed an increase in cell number during the 7 days. The last parallel showed an increased abundance after 3 days, before the cell number had dropped below the original value at the end of the incubation (Figure 5). One of the parallels with biocide showed an increased cell number after the incubation session, while the two remaining parallels had a declining cell number after 7 days.

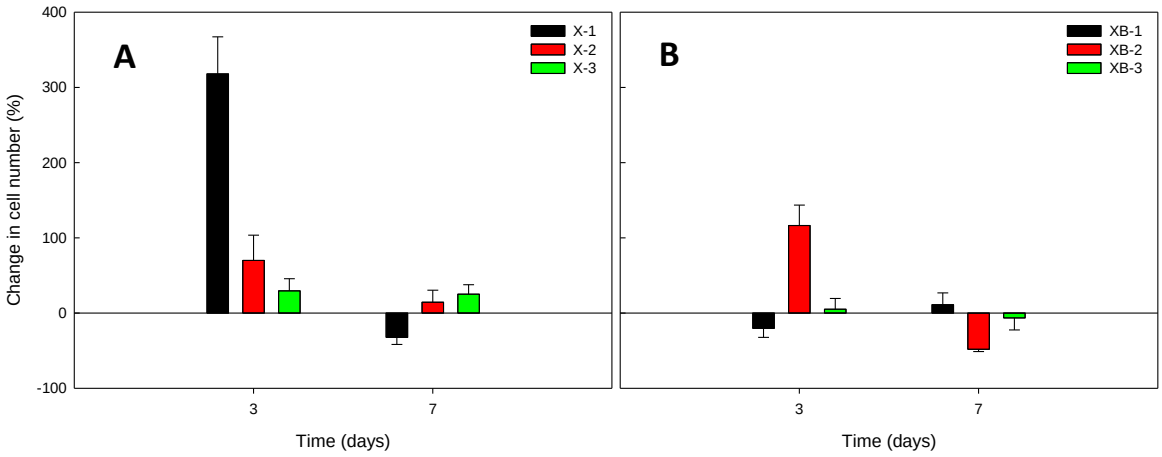

Figure 5: Changes in cell number (%) in the xanthan-brine incubated at 30 °C for 7 days. Parallels without biocide are presented to the left (A) while parallels with biocide are presented to the right (B). Error bars show standard error (SE) of average (n = 3).

3.1.2 Thermophilic conditions

The SRP brine incubated at 60°C showed a declining cell number in 4 of the 6 parallels without biocide during the incubation (figure 6). Growth was detected in one of the parallels, showing a 40% increase in cell number after 2 weeks. One parallel showed an unaltered cell number after 2 weeks. The remaining parallels showed a reduction in cell number, ranging from 20 to 60%. The parallels with biocide showed a greater reduction, corresponding to a 60-80% decline related to the original abundance.

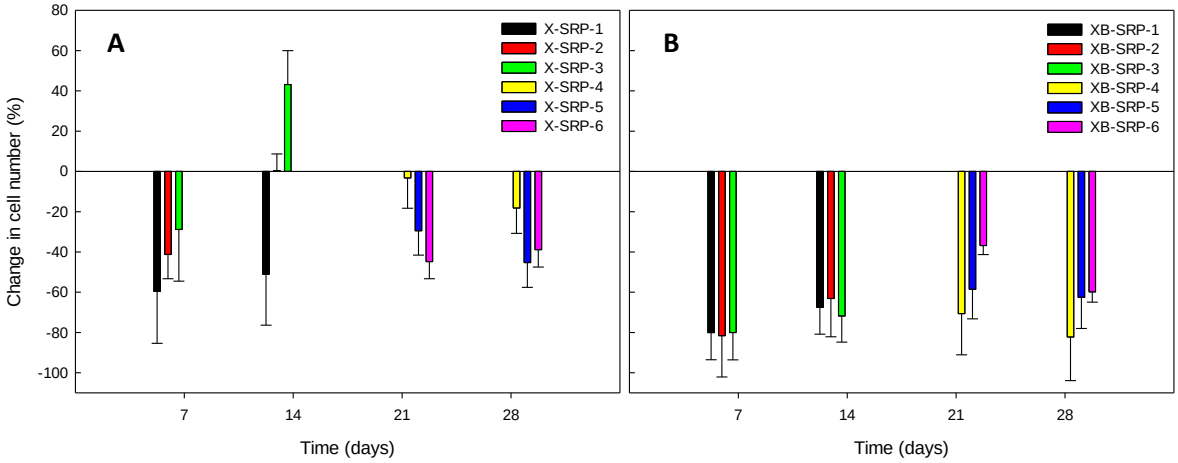

Figure 6: Percentage change in cell numbers measured in the SRP brine incubated at 60°C. Results from the parallels without biocide are presented to the left (A), while parallels with biocide are presented to the right (B).Error bars show standard error (SE) of average (n = 3).

The PWRI brine had a higher initial cell numbers then the SRP brine, ranging from $5x10^7$ to $1x10^8$cells per ml. All parallels showed a declining cell number during the incubation (figure 7). The parallels without biocide showed a reduction in cell number equivalent to 70-80% after 4 weeks. Parallels with biocide had a similar development, with a reduction in cell abundance equivalent to 70-80% in two of three parallels. The remaining parallel had a reduction corresponding to 30% of the original cell number.

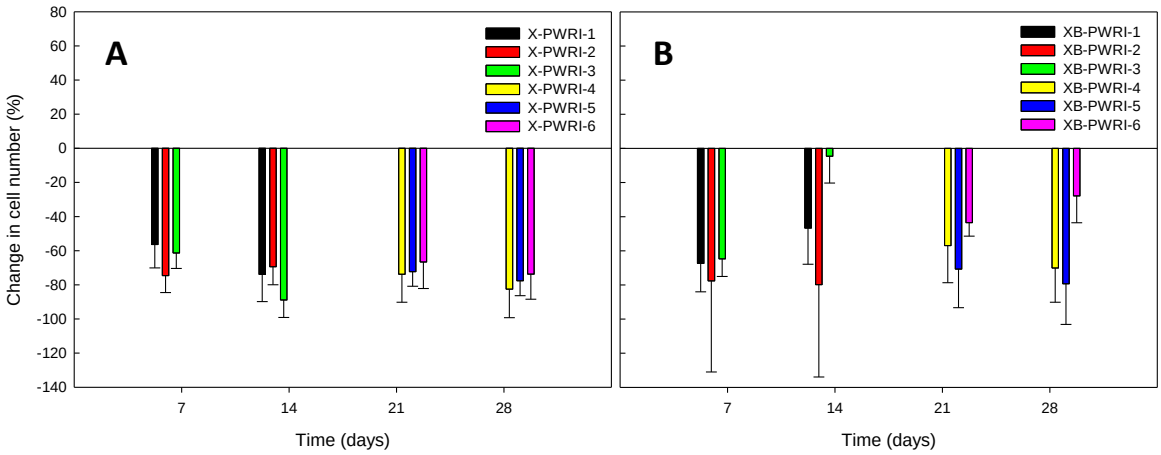

Figure 7: Change in cell number (%) measured in the PWRI brine incubated at 60°C. Results from the parallels without biocide are presented to the left (A), while parallels with biocide are shown to the right (B). Error bars show standard error (SE) of average (n = 3).

3.1.3 Hyper thermophilic conditions

The SRP brine incubated at 85°C showed a significant reduction in cell number during the first 4 weeks (figure 8). The SRP brine without biocide showed a residual bacterial number of approximately $1x10^4$ cells per ml, while no cells were detected in the SRP brine with biocide. After 2 months, no cells were detected in parallels without biocide. After 5, growth was identified in parallels with biocide, showing approximately $1x10^4$ cells per ml. After 6 months, growth was detected in all parallels, regardless of biocide treatment. The cell number was approximately $1x10^4$ cells per ml.

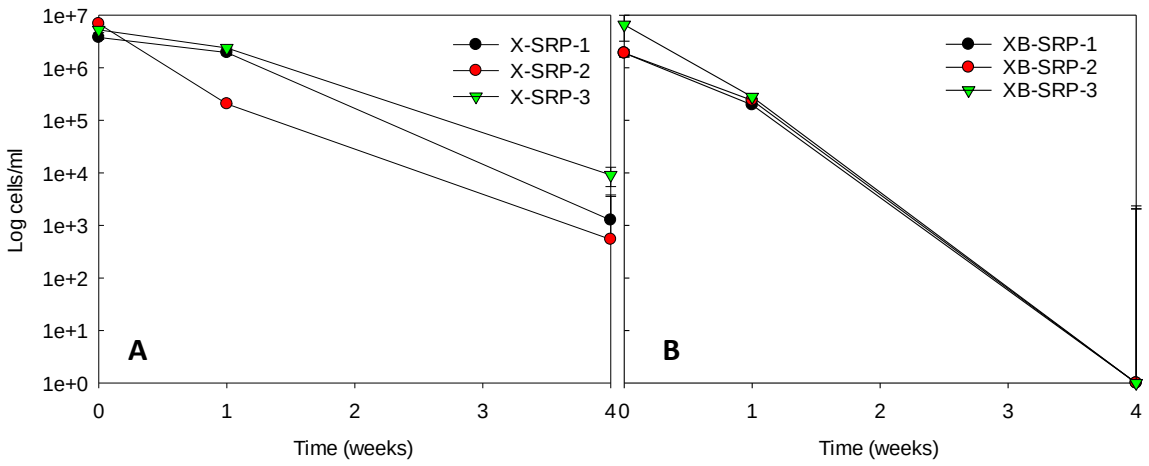

Figure 8: The cell numbers in the SRP brine without (A) and with (B) biocide incubated at 85°C for 4 weeks. A rapid decrease in cells was observed during the first 4 weeks. Error bars shows standard error (SE) of average (n=3).

The PWRI brine showed a similar development as the SRP brine (figure 9). However, no increase in cell number was observed at the end of incubation.

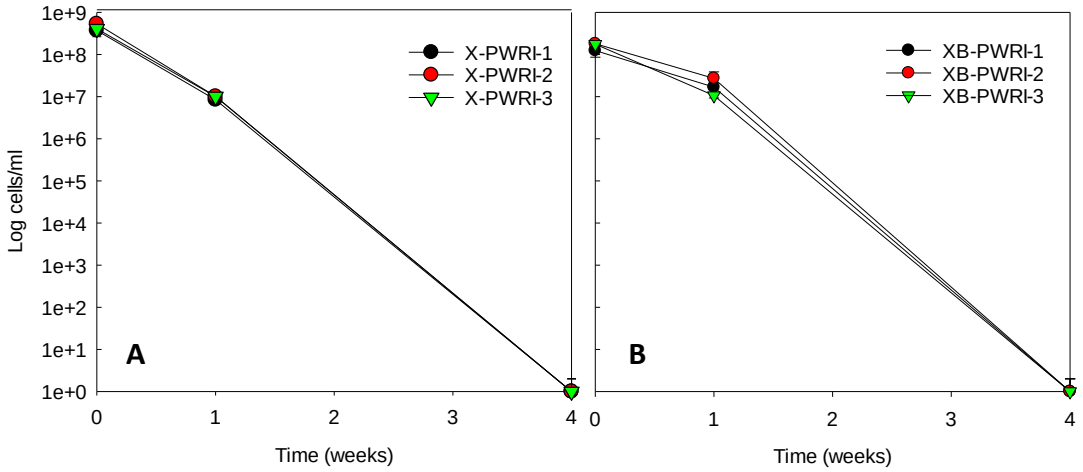

Figure 9: The cell numbers in the PWRI brine during the first 4 weeks incubation at 85°C. Parallels without biocide (A) and parallels with biocide (B) showed a similar rapid reduction in cells. Error bars shows standard error (SE) on average (n=3).

3.2 Bacterial diversity in response to xanthan, biocide treatment and temperature based on PCR-DGGE fingerprint analysis

3.2.1 Mesophilic conditions

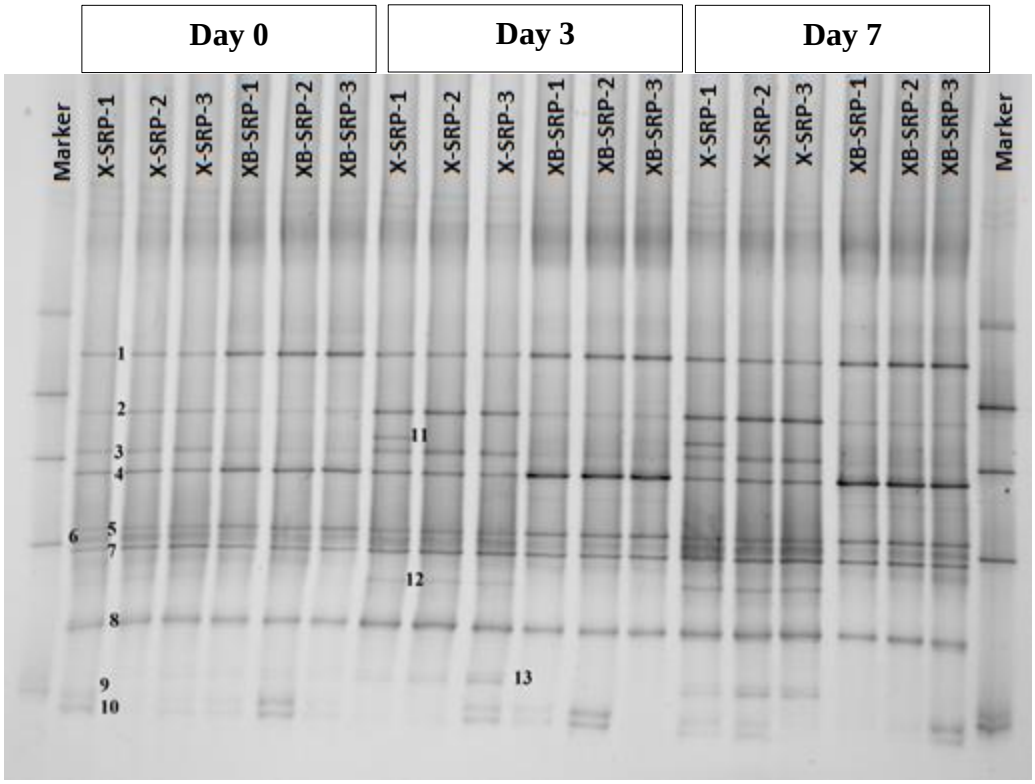

Figure 10: The DGGE profile of the bacterial community in the SRP brine incubated at 30°C. Marker: A mixture of PCR product from species with different GC-content. Sample names and sampling time are described at the top of the profile.

All samples from the SRP water incubated at 30°C yielded positive PCR amplification. The PCR amplicons were separated in a total of 13 different bands (figure 10). Nine of the bands were observed in the initial samples. The same pattern was observed in all parallels, with the exception that some bands were less pronounced in the parallels with biocide (band 2 and 3). Band 8 and 9 were unevenly distributed among different parallels, both with and without biocide.

Three new bands occurred in the parallels without biocide after day 3. These bands were also present in the samples after day 7. Band 2, which was present originally, showed an increased abundance during incubation in the parallels without biocide. The band-pattern was unaltered in parallels with biocide, but variations over time were observed related to

the strength of the band. Band 1 and 4 showed an increased abundance at day 7, while band 2 and 3 were reduced in strength.

The bands marked with a number were excised and reamplified, followed by nucleotide sequencing. BLAST alignments of the sequenced fragments in the NCBI nucleotide collection (nr/nt) database including accession number and identity are listed in the appendix (table A.12). A summary of the identified phylotypes are presented in table 4.

Two bands are not included in the tables or in the further discussions in this study. Band 5, affiliated to *Sphingomonas* sp. was proven to derive from the xanthan solution. Band 4 showed 100% sequence similarity to several species in the genus *Xanthanomonas*, including *Xanthanomonas campestris* (JX852722.1). Even though that this band was not identified from the xanthan solution, the presence of this species suggest that it has been added to the brine with xanthan.

Table 4: A summary of the community analysis of SRP brine incubated at 30°C. The table includes the phylotypes identified in the brine. A color code is applied to introduce the presence and strength of the bands, related to time of incubation. Strong bands are presented with a dark color. Band numbers refer to the DGGE profile (figure 10).

		Without Biocide			With Biocide		
Bands	**Phylotypes**	**Day 0**	**Day 3**	**Day 7**	**Day 0**	**Day 3**	**Day 7**
1	*Colwellia* sp.						
2	*Hyphomonas* sp.						
3	*Cupriavidus basilensis*						
7	*Cupriavidus basilensis*						
6	*Thioclava* sp.						
8	*Ralstonia* sp.						
9,10	*Rhodococcus* sp						
11	*Methylophaga* sp.		a	a			
12,13	*Microbacterium* sp.						

a) *Methylophaga* sp. was only identified in one of the three parallels.

Band 10 was only detected in one of the parallels without biocide. The band showed 95% sequence similarity to the genus *Methylophaga* (AJ244762.1). The two additional bands identified after day 3 (band 11 and 12) showed 98% and 99% sequence similarity to related

species in the genus *Microbacterium* (AF474330.1, GQ250443.1). The most dominant band in these parallels was band 2, showing 94% similarity to a *Hyphomonas* sp. (AY690712.1).

The microbial diversity was reduced during incubation in parallels with biocide. At day 7, band 2 and 3 were lost. Band 3 was affiliated to *Cupriavidus basilensis* (CP010537.1), showing 97% sequence similarity. Band 1 displayed an increasing abundance in this environment. The band showed 96% sequence similarity to *Colwellia* sp. (HQ836463.1).

3.2.2 Thermophilic conditions

Positive PCR amplification was obtained from all samples incubated at 60°C. Four separate DGGE analyses were conducted to separate the PCR amplicons obtained from the different brines and biocide treatment.

The SRP brine

A total of nine bands were identified from the SRP brine without biocide (appendix 2, figure A.1). A cluster of bands were located in the middle of the gel, indicating a high similarity in GC content of these species. An attempt for improved separation was made by casting a gel with a higher denaturation gradient (40/60) without success. All the nine bands were detected in the initial SRP community. One of the bands (band 2) disappeared at day 7. Besides this, the community structure was constant during the incubation. The BLAST alignments, including accession number and similarity of the phylotypes are presented in the appendix (table A.13).

The PCR amplicons obtained from the SRP brine with biocide was separated in nine bands (appendix 2, figure A.2). The bands had a broader distribution along the gradient compared with the amplicons from the brine without biocide. One of the bands (band 4) disappeared after day 7. No new bands were detected during the incubation, but variation in strength was identified. Band 12 and 15 showed an increased abundance after day 28. The affiliations of the bands are presented in the appendix (table A.14). The bands that were lost from both profiles were affiliated to the genus *Xanthanomonas*, and are not further discussed. A summary of the phylotypes in the SRP brines are provided in table 5.

Table 5: A summary of the community analysis of SRP brine incubated at 60°C. The table includes the phylotypes of the sequenced bands. The band numbers represents numbering on the DGGE profiles, presented in the appendix (figure A.1 and figure A.2). A color code is applied to introduce the presence and strength of the bands, related to time of incubation. Strong bands are presented with a dark color.

Bands	Phylotypes	Without Biocide		With Biocide		
		Day 0	Day 7-28	Day 0	Day 7	Day 14-28
1, 5, 12	*Cupriavidus basielsis*					
3, 4, 15	*Thioclava* sp.					
6, 16	*Phyllobacterium myrsinacearum*					
7, 17	*Ralstonia* sp.					
8, 9, 18,19	*Rhodococcus* sp.					
10	*Colwellia* sp.					
11	*Hyphomonas* sp.					

The community structure was stable in the SRP brine without biocide, without any modifications. The most dominant band in the brine without biocide was affiliated to the genus *Ralstonia* (DQ374437.1), showing 100 % similarity in gene sequence.

The parallels supplied with biocide showed a higher microbial diversity then the parallels without biocide. Some of the bands observed under mesophilic conditions were preserved in these parallels. Band 10 and 11, affiliated to *Colwellia* sp. (HQ836463.1) and *Hyphomonas* sp. (AY690712.1) and were only identified in the brine with biocide. The dominating band in these parallels showed highest sequence similarity to *Phyllobacterium myrsinacearum* (98%, KJ147062.1). In addition, band 12 and 15, affiliated to *Cupriavidus basilensis* (97%, CP010537.1) and *Thioclava* sp. (99%, KM255690.1) were stronger after day 28.

The PWRI brine

PCR amplicons from the PWRI brine without biocide yielded a total of 15 bands along the gradient (appendix 2, figure A.3). Seven of the bands were identified in the initial community. The bands were distributed along the center and lower parts of the gradient. After day 7, 8 new bands were identified on the gradient, located in upper and lower parts of the gel. All bands showed a similar strength during the incubation. No further changes were identified during the next 3 weeks.

The PCR products from the brine with biocide were separated in 5 bands after DGGE analysis (appendix 2, figure A.4). In contrast to the brine without biocide, no new bands were detected during the incubation. Similarity, habitat and accession number for the sequenced bands are presented in the appendix (table A.15 and table A.16). A summary of the phylotypes in the PWRI brine are provided in table 6.

Table 6: A summary of the community fingerprinting from the PWRI brine incubated at 60°C. The table includes the phylotypes of the sequenced bands. The band number represents numbering on the DGGE profiles, presented in the appendix (figure A.3 and figure A.4). A color code is applied to introduce the presence and strength of the bands, related to time of incubation. Strong bands are presented with a dark color.

		Without Biocide		With Biocide	
Band	**Phylotypes**	**1 Hour**	**1-4 Weeks**	**1 Hour**	**1-4 Weeks**
1, 16	*Geotoga Petraea*				
2, 5, 17	*Marinobacterium* sp.				
3	*Curiavidus basilensis*				
4,6,7,18,19	*Pelobacter carbinolicus*				
8, 10	*Petrotoga halophila*				
9,11	*Petrotoga mobilis*				
12	*Phyllobacterium myrsinacearum*				
13	*Thermosipho geolei*				
14,15	*Kosmotoga olearia*				

The initial community structure identified several species which previously has been isolated from brines collected from oil fields (Davey et al., 1993; Lenchi et al., 2013; Van Der Kraan et al., 2009). The new bands identified after day 7 belonged to additional species isolated from the same habitat (Dipippo et al., 2009; Miranda-Tello et al., 2007; L'haridon et al., 2001). Two of the new bands were classified as *Petrotoga halophila* (NR_043201.1), showing 98 and 99% sequence similarity. Two bands were classified as *Petrotoga mobilis* (NR_074401.1), both showing 99% sequence similarity. Additional bands showed highest sequence similarity to *Phyllobacterium myrsinacearum* (98%, KJ147062.1), *Thermosipho geolei* (92%, NR_025389.1) and *Kosmotoga olearia* (99%, NR_044583.1). The dominating band in the brine with biocide was classified as *Geotoga petraea* showing 99% sequence similarity (NR_104910.1).

3.2.3 Hyper thermophilic conditions

PCR amplification for the SRP brine incubated at 85°C was not successful, despite several attempts with different primer sets. A community analysis was therefore not possible. Consistent with the reduction in cell numbers in the PWRI brine, a PCR-DGGE was not performed.

3.3 Verification of xanthan degradation by culturing

Due to lack of biodegradation in the current experiment, the incubation time was extended in later bioassays. Positive biodegradation was observed at 30°C after 4 months incubation in a new experiment not included in the current thesis. In order to verify xanthan degradation in the later bioassay, a control experiment was performed in synthetic SRP brine. The series of SRP brine incubated for 4 months was used as inoculum to synthetic brine with xanthan as sole carbon source.

3.3.1 Estimation of bacterial growth by QPCR

The QPCR analysis revealed extensive bacterial growth in one of the parallels (figure 11). SRP-2, the parallel that was inoculated with the sample that originally showed xanthan degradation (results not shown), showed a significant bacterial growth during the first 7 days. The cell number showed a decreased the following week, before the highest cell number was reached after day 21. Growth was also observed in the two other parallels (SRP-1 and SRP-3), but less pronounced than in SRP-2.

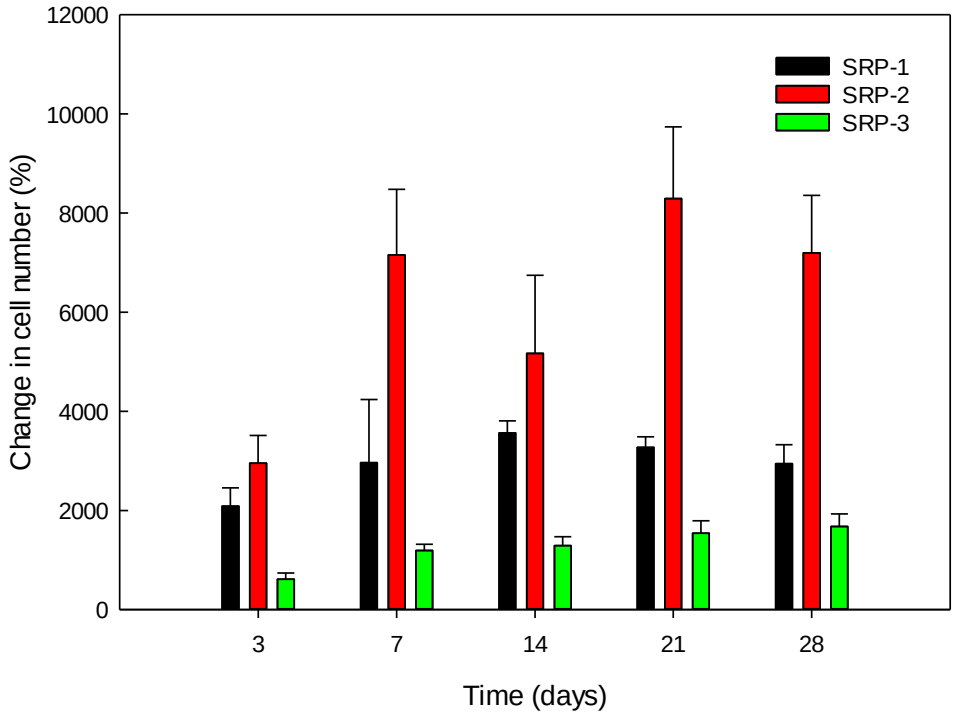

Figure 11: Change in cell number (%) measured in the synthetic SRP brine incubated at 30°C for 4 weeks. Error bars show standard error (SE) of average (n = 3).

3.4.2 Community fingerprinting by PCR-DGGE

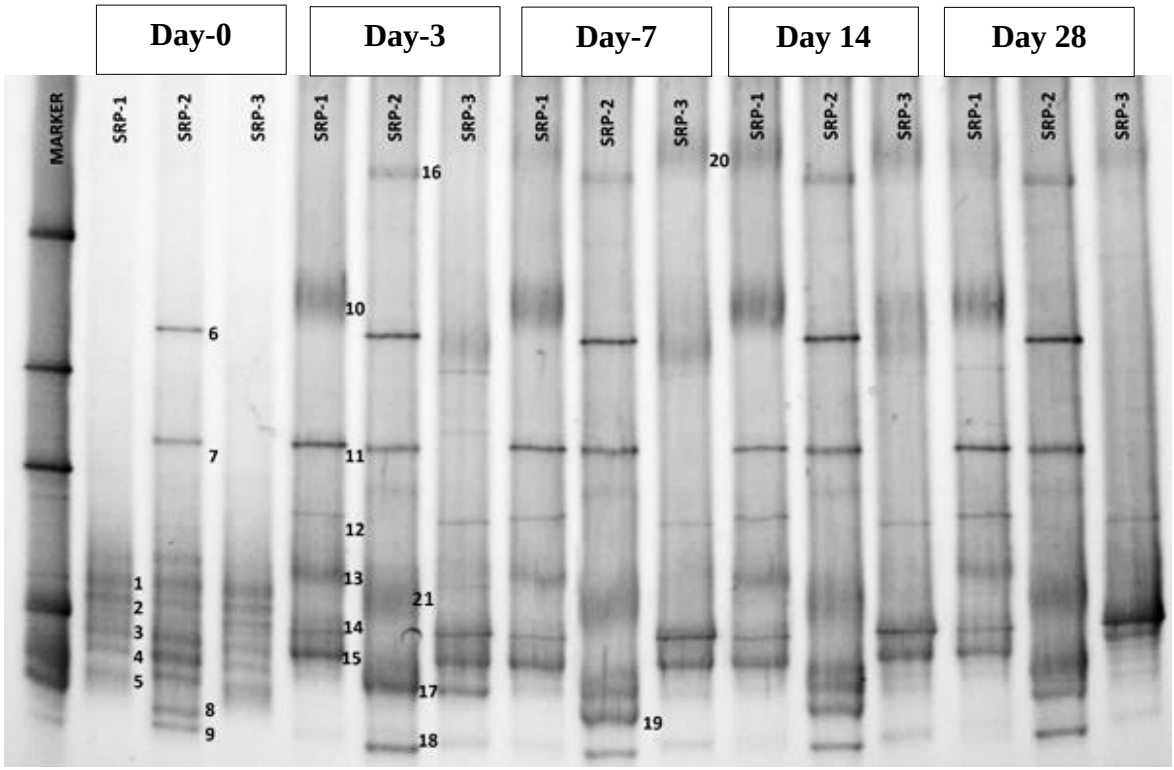

Figure 12: The DGGE profile of the bacterial community in the synthetic SRP medium. Five different sampling times were analyzed. Sample names are provided in the top. Marker: A mixture of PCR product from species with different GC-content. Numbers represent bands that were excised, re-amplified and sequenced.

The DGGE gel in figure 12 shows the samples from the control experiment. A total of 21 bands were identified during the incubation. The initial community structure identified 5 bands present in all parallels. Four additional bands were found in SRP-2. Two of these bands (band 6 and 7) were dominating throughout the incubation.

After day 3, the 5 bands originally present in all parallels were lost. The parallels showed differences in the development of the number and distribution of the new identified bands. Six new bands were detected in SRP-1 (band 10-15), 4 new bands were identified in SRP-2 (band 16-18, 21), while 4 new bands were detected in SRP-3 (band 12, 14, 15 and 17). After day 7, one additional band was detected in SRP-2 (band 19) and SRP-3 (band 20). The remaining incubation did not showed any changes in the community structure. The BLAST alignments, accession numbers and habitats of the sequenced bands are presented in appendix 2 (table A.17). A summary of the phylotypes identified in the control

experiment are provided in table 7. Two of the initial bands are not included in the table or in the further discussion of the results. Band 2 and 3, showing highest sequence similarity to *Sphinhomonas* sp. (97%, AF385529.1) and *Burkholderia* sp. (95%, KC160738.1) were proven to derive from the xanthan solution.

Table 7: The table shows the community development in control experiment, during incubation at 30°C for 28 days. The band number represents the numbering on the DGGE profile (figure 12). A color code is applied to introduce the presence and strength of the bands, related to time of incubation. Strong bands are presented with a dark color. Code 1, 2 and 3 refers to the parallels; SRP-1, SRP-2 and SRP-3.

		Day 0			Day 3			Day 7-28		
Bands	**Phylotypes**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1	*Celeribacter/Roseobacter* sp.									
4,5	*P. myrsinacearum*									
6,7	*Prolixibacter bellariivorans*									
8,9	*Labrenzia* sp.									
10	*Sunxiuqinia faeciviva*									
11	*Spongiibacter tropicus*									
12,13	*Roseobacter denitrificans*				a					a
14	*Thalassospira xiamenensis*									
15	*Muricauda beolgyonensis*									
16	*Prolixibacter bellariivorans*									
17	Sequence of low quality									
18	*Labrenzia* sp.									
19	*Pelobacter seleniigenes*									
20	*Lutibacter aestuarii*									
21	*Phycisphaerae bacterium*									

a) Only band 12 was present in SRP-3.

After day 3, 6 new bands were identified in SRP-1. Band nr 10 showed 99% sequence similarity to *Sunxiuqinia faeciviva* (NR_108114.1). The strongest band (band nr 11) in this parallel was classified as *Spongiibacter tropicus* (NR_118017.1), showing 99% similarity in 16S rDNA. Additional bands showed highest sequence similarity to *Roseobacter*

denitrificans (90-96%, NR_102909.1), *Thalassospira xiamenensis* (99%, EU603449.1) and *Muricauda beolgyonensis* (99% NR_117844.1).

The two dominating bands from SRP-2 were classified as *Prolixibacter bellariivorans* (NR_113041.1), with 97% and 98% similarity in the partial 16S rDNA sequence. Band 16, observed after day 3, showed highest sequence similarity to the same species (96%). Three additional bands were observed after day 3. Band 17 did not provide an alignment, due to low quality of the sequence. Band 18 was identified as *Labrenzia* sp. (KC878323), showing 98% sequence similarity. This genus was also identified in the initial sample (band 8 and 9). Band 21 was affiliated to the class *Phycisphaerae*, without any closer affiliation. An additional band observed after day 7 (band 19) was classified as *Pelobacter seleniigenes* (NR_044032), showing 98% sequence similarity.

Bands affiliated to *Roseobacter denitrificans*, *Thalassospira xiamenensis* and *Muricauda beolgyonensis* were also observed in SRP-3 after day 3. In addition, a new band (nr 20) was identified after day 7. The band was identified in the upper part of the gel, showing 99% sequence similarity to *Lutibacter aestuarii* (NR_108995.1). Simultaneously, band 17 disappeared from this parallel.

3.4.3 Physical and chemical parameters: Viscosity and pH

The viscosity profiles from the three parallels are presented in figure 13. SRP-1 and SRP-3 did not show any significant alterations in the viscosity during the incubation. In contrast, the final viscosity profile from SRP-2 was approximately 1 centipoise, corresponding to the viscosity of water.

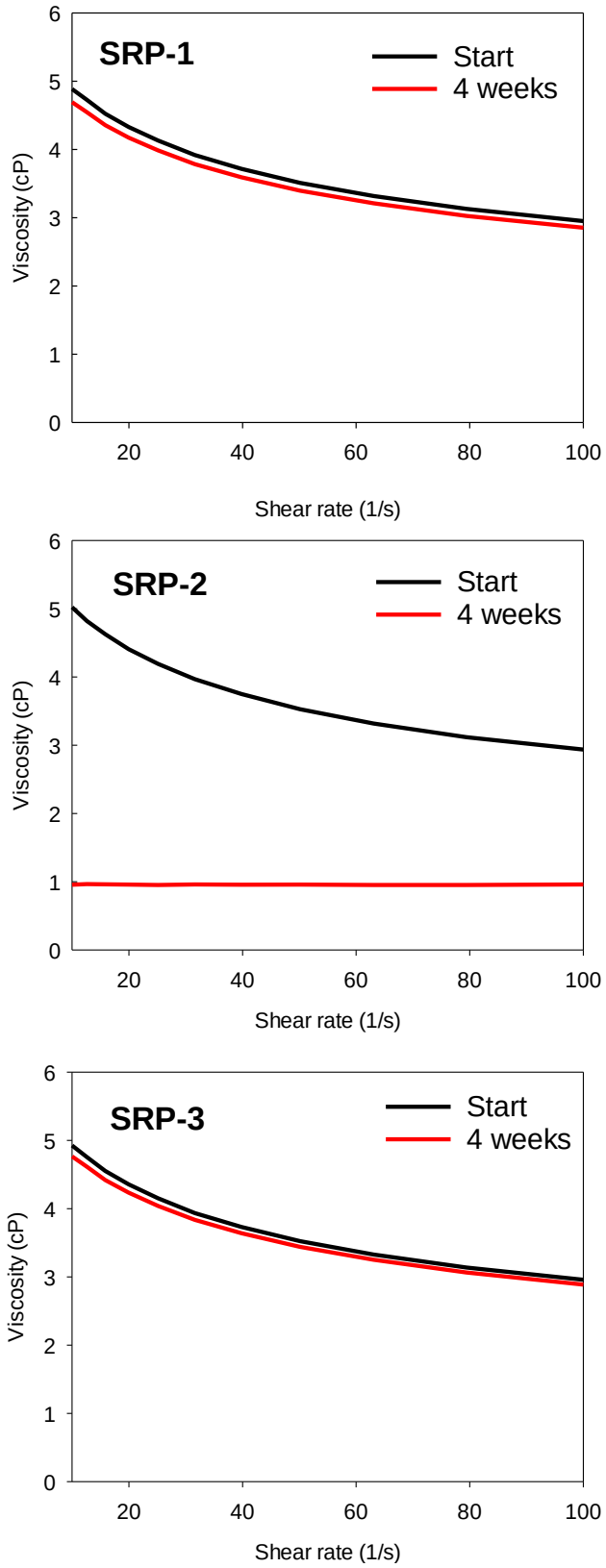

Figure 13: The viscosity profile of the parallels in the control experiment. Sample name are provided in the left corner. Viscosity was measured in the start of the experiment and after 4 weeks of incubation.

The pH in the enrichments was approximately constant during the experiment. The pH measurements are listed in table 8. The initial pH in SRP-2 was lower than the other parallels, probably due to a low pH in the inoculum.

Table 8: The pH in the different parallels, measured at the start and at the end of the experiment.

Sample names	Start	Day 28
SRP-1	7.5	7.5
SRP-2	6.9	6.9
SRP-3	7.3	7.4

3.4 Isolation of dominant bacteria

3.4.1 Anaerobic enrichments

Inoculation in synthetic brine containing xanthan gum as the sole carbon source was performed in the first step to enrich bacteria from selected cultures bottles. The original SRP and PWRI brines incubated at 60°C were chosen as inoculum, as cells were detected by microscopy. After 3 weeks, microscopy analysis revealed cocci and rod-shaped cells in all the enrichments. The cell density was very low; reflecting the small alteration in bacterial numbers observed in the original culture bottles. This observation emphasizes the lack of ability to degrade xanthan, thereby not providing sufficient energy for growth.

One ml of the initial enrichment cultures were transferred to new culture bottles containing organic acids (C_4+C_6) or glucose. After 3 weeks of incubation, cocci and rod shaped cells were detected in microscope. A DGGE analysis was performed to monitor the enrichment cultures and to comparing the bacterial community composition in the different brines (figure 14).

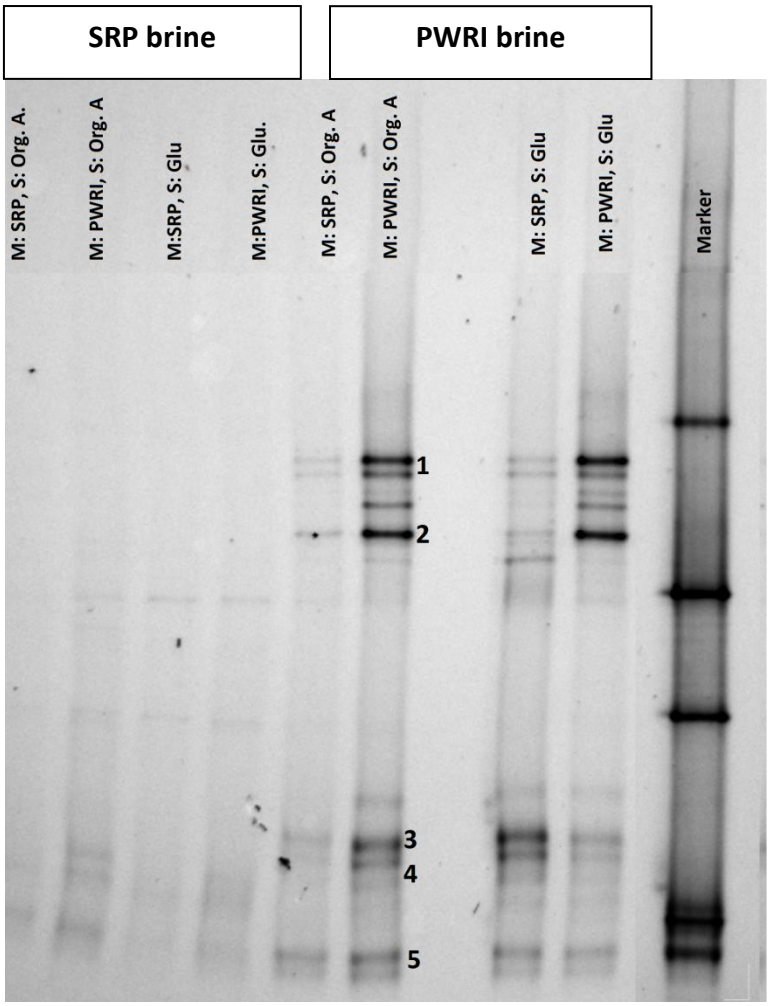

Figure 14: The DGGE of the bacterial community in the anaerobic enrichments. Samples in well 1-4 were inoculated with the original SRP brine, while samples in well 5, 6, 8 and 9 were inoculated with the PWRI brine. Numbers 1-5 indicate excised and sequenced bands. Marker: A mixture of PCR product from five species with different GC-content. Sample codes: M: Medium (synthetic PWRI or SRP brine), S: Substrate (Org. A: Organic acids, Glu: Glucose)

The enrichments inoculated with the original SRP brine (well 1-4) revealed a few, very weak bands, regardless of carbon source and synthetic brine. This indicates the lack of bacteria able to grow in the synthetic brine on the available carbon source. Enrichments inoculated with the PWRI brine revealed several strong bands, mainly present in the synthetic PWRI brine. Two of the bands (band 3 and 4) were also present in the synthetic SRP brine with glucose as substrate. The bands marked with a number were excised, re-amplified and sequenced (table 9).

Table 9: BLAST alignments for bands identified from the DGGE profile from the anaerobic enrichments (figure 14). The table include similarity, habitat and accession number for the sequences.

Bands	Phylotypes	Similarity	Habitat	Accession nr
1	*Petrotoga halophila*	100%	Petroleum reservoirs.	NR_043201.1
2	*Petrotoga mobilis*	99%	Petroleum reservoirs.	NR_074401.1
3, 4	*Thermosipho geolei*	98%	Petroleum reservoirs.	NR_025389.1
5	*Phyllobacterium myrsinacearum*	100%	Associated with Plants	KJ147062.1

The identified phylotypes showed similarity to the phylotypes observed in the original PWRI brine. Band 1 and 2, classified as *Petrotoga halophila* (NR_043201.1) and *Petrotoga mobilis* (NR_074401.1) were the most dominant in the PWRI brine regardless of substrate, indicating the ability to utilize both carbon sources. They were not identified in the SRP brine, suggesting that the species are adapted to the specific ionic composition in the PWRI brine. Band 3 and 4 showed 98% sequence similarity to *Thermosipho geolei (*NR_025389.1) also identified in the original bioassay. The sequence obtained from the original bioassay was of low quality, and showed only 92% similarity. Identification of the same phylotype with a higher sequence quality supports the classification performed in the original brine. Band 5 was affiliated to *Phyllobacterium myrsinacearum* (KJ147062.1), showing 100% sequence similarity.

Assessment by microscopy indicated that growth was more pronounced in the cultures containing glucose, and they were therefore selected for further culturing. An additional transfer was performed in an attempt to progress the growth rate in the enrichments. Microscopy analysis detected only a few cocci shaped cells after 2 weeks at 60°C. The incubation continued for two additional weeks, without a significant increase in visible cells. Because of the time limit for the current thesis, the anaerobic enrichment approach could not be used for isolation of a dominant bacterium.

3.4.2 Aerobic enrichments

Due to the low growth rate observed in anaerobic cultures, attempts to isolate aerobic, facultative anaerobic sugar degraders were performed by plating technique on LB medium. Culture bottles from both brines were inoculated on LBA, to assess the potential for aerobic growth. All plates' revealed growth after 3 days incubation at 60°C. The colonies had a similar irregular shape and a brown-yellow color when growing on the surface (figure 15).

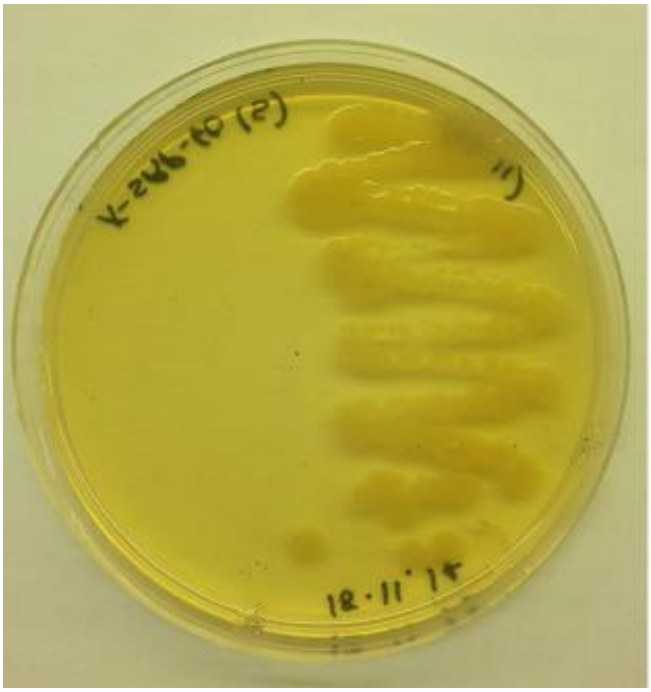

Figure 15: Positive growth on LBA.

After isolation of single colonies, the isolates were transferred to aerobic and anaerobic synthetic brines containing glucose and incubated at 60°C. After approximately one week, growth was detected in all the enrichments. One of the isolates originating from the PWRI brine showed the most pronounced growth, and was therefore selected for a partial characterization.

The isolate had a rod-shaped morphology, with a wide variety in lengths (figure 16). The cells were motile. Microscopic examinations revealed several assemblies of cells indicating that the cells prefer growing in aggregates.

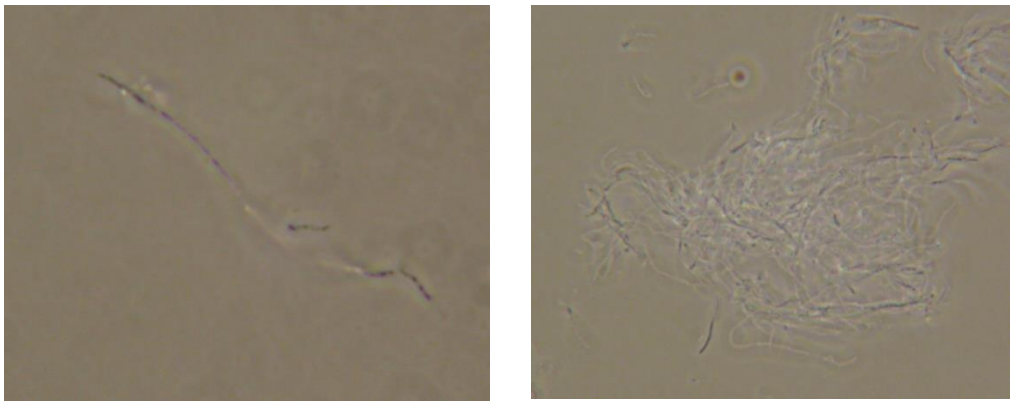

Figure 16: Microscopic examination of the isolate revealed rod-shaped cells, showing a wide variety in lengths. The picture to the right shows cell aggregates of the isolates strain.

3.3.3 Partial Characterization of the isolate

Genotypic characterization

Genotypic characterization was performed by amplification of the whole 16S rRNA gene. The obtained contig was approximately 1500 bp. Comparison of the sequence with available sequences in the Genbank identified the isolate as *Aeribacillus pallidus* (KJ722452.1), showing 100% sequence similarity. The sequence also provided a control for the purity of the isolate, indicated by single peaks with low disturbance seen in the chromatograms (example shown in appendix 2, figure A.5). The isolate is further referred to as strain BH-1.

Phenotypic characterization

The optimal growth temperature of the isolate was determined by turbidity analysis at a variety of temperatures. Growth occurred at 54, 60 and 65°C, with an optimal growth temperature between 60-65°C. Growth was not observed at 30°C and 70°C.

Utilization of substrates differed significantly between strain BH-1 and other described *A. pallidus* strains (table 10). Strain BH-1 demonstrated growth on starch, xanthan, cellobiose galactose, maltose, sucrose and organic acids. Growth was observed in minimum one of three parallels after three transfers. Cellulose, fructose and crude oil did not support growth. Yeast extract was needed to stimulate growth.

Table 10: Physiological properties of strain BH-1, *A. pallidus* DSM 3670^T (type species, Scholz et al., 1987) and *A. pallidus* TD1 (Yasawong et al., 2011).

Utilization of	**Strain BH-1**	***A. pallidus* DSM 3670^T**	***A. pallidus* TD1**
Starch	+	+^{w}	-
Cellulose	-	ND	ND
Xanthan	+	ND	ND
Fructose	-	+	ND
Cellobiose	+^{d}	-	+
Galactose	+^{d}	-	ND
Maltose	+^{d}	+^{d}	+
Sucrose	+^{d}	ND	+
Organic acids (C_4+C_6)	+^{d}	ND	ND
Crude Oil	-	ND	ND
Anaerobic growth*	**+**	-	ND

+ = positive, W = weakly positive, - = negative, d = reactions differ, ND = not determined.

* = On glucose

Strain BH-1 showed similarity to *A. pallidus* DSM 3670^T (type species, Scholz et al., 1987), by the ability to grow on starch and maltose. Similarities was also observed between strain BH-1 and *A. pallidus* TD1 (Yasawong et al., 2011) both growing on cellobiose, maltose and sucrose. The ability to grow on xanthan, galactose and organic acids has not been demonstrated by strain TD1 and DSM 3670^T. In contrast to strain TD1 and DSM 3670^T, strain BH-1 grew anaerobically by fermentation of glucose. The strain did not utilize nitrate or thiosulfate as alternate electron acceptors.

4 Discussion

4.1 The Bioassay: Xanthan degradation

Biodegradation of xanthan was assessed by reduction in viscosity, increase in cell number and shifts in the bacterial community composition in oil field brines added xanthan. Reduction in pH, due to accumulation of fermentation products, was applied as an additional indication for biodegradation. Results from the viscosity analysis, as assessed by UNI research CIPR, did not show a decrease in viscosity in any of the bioassays. This was consistent with an unaltered pH after incubation. Evaluation of bacterial growth and shifts in the community structure was required to assess the development in the microbial communities, and the possibility for biodegradation with an extended incubation time.

4.1.1 Degradation of xanthan at mesophilic conditions

Only limited bacterial growth was observed in the SRP brine incubated at 30°C. Unaltered viscosity confirmed the absence of biodegradation within 7 days. Only minor shifts in the bacterial community composition were observed, which is in agreement with the limited bacterial growth. However, some samples showed variations from the initial community structure. Development of new DNA bands may indicate an onset of enrichment of bacteria able to utilize xanthan (or other organic material deriving from xanthan), and it is possible that a prolonged incubation may have resulted in biodegradation of xanthan.

The new phylotypes enriched from the SRP-brine was affiliated to *Methylophaga* and *Microbacterium*. Members of the genus *Methylophaga* are strict aerobic, moderate halophilic bacteria that utilize substrates like methanol and methylamine (Boden, 2012; Janvier et al., 1985). The fact that a strain was enriched under anaerobic conditions contradicts this statement. However, the band showed low homology (95%), and might belong to another genus. The genus *Microbacterium* is known to include a xanthan degrading species. The xanthan-degrading *Microbacterium* sp. strain XT11 was isolated from soil by Qian et al. (2007). In later studies, the endoxanthanase and xanthan lyase produced by this strain have been purified and characterized (Li et al., 2008; Yang et al., 2014), which suggests that strain XT11 completely degrade the biopolymer. The presence of this genus after three days of incubation suggests that further incubation may have resulted in degradation of xanthan. The series was therefore incubated further for a total of 32 days (Bødtker et al., 2015), but no reduction in viscosity was observed.

Members of the genus *Microbacterium* are generally described at strict aerobic, but poor anaerobic growth are known for some species (Goodfellow et al., 2012). The poor ability to grow at anaerobic conditions suggests that xanthan degradation in the anoxic brines would be insignificant. The bands observed at day 3 had not increased in strength at day 7, indicating that the abundance of the species was persistent. The unaltered viscosity after the extended incubation indicates that phylotype affiliated to *Microbacterium* sp. was not able to degrade xanthan at the current conditions.

Positive biodegradation of xanthan was observed in SRP brine in a later experiment (Bødtker et al., 2015), not included in the current thesis. The experiment was performed with the same xanthan quality, but the SRP brine had been sampled at a different time from the platform. After sampling at day 7, the bottles were re-incubated for another 106 days. Within 4 months, xanthan degrading species had grown sufficient to alter the viscosity of the xanthan-brine in one of three parallel culture bottles. This parallel also demonstrated a reduction in pH, indicating fermentation of glucose followed by an accumulation of fermentation products (e.g. H_2, organic acids, CO_2). The long incubation time required for xanthan degrading species to be enriched suggest that the number of bacteria able to degrade xanthan are low in oil field brines not previously subjected to xanthan. Due to the low initial abundance of these species, the demonstrated effects were not obtained within the incubation time included in the first bioassay.

The stability of xanthan has previously been discussed in several studies stating that the biopolymer is completely degraded by only a few microorganisms (Cadmus et al., 1982; Li et al., 2008; Qian et al., 2007). This is supported by the few xanthan degrading species described in literature. The main habitat for xanthan degrading species is assumed to be soil, as the producer of the biopolymer, *Xanthanomonas campestris* inhabits this habitat (Garcia-Ochoa et al., 2000). The reported isolation of numerous xanthan degraders from soil supports this assumption. These species, affiliated to *Bacillus, Paenibacillus, Corynobacterium, Cellulomonas* and *Microbacterium*, were mainly isolated from soil and enriched aerobically at mesophilic conditions (Cadmus et al., 1982; Hashimoto et al., 1998; Liu et al., 2005; Ruijssenaars et al., 1999; Qian et al., 2007). In several of the studies, xanthan degrading species was purified from mixed cultures growing on xanthan. However, the incubation time spent before xanthan degradation was demonstrated in the initial cultures is not provided.

The studies show that when the xanthan degrading species was enriched, xanthan degradation occurred fast, extending from hours to a couple of days (Hashimoto et al., 1998; Liu et al., 2005; Ruijssennars et al., 1999). Degradation of the biopolymer was induced when xanthan was the only carbon source, and repressed by other substrates (Liu et al., 2005; Ruijssennars et al., 1999; Yang et al., 2014). Despite the few studies reporting xanthan degrading species, the degradation rate demonstrated from these studies was fast, suggesting a rapid depolymerization of xanthan when the degrading enzymes are produced. In addition, the quality of the xanthan molecule might affect the modification caused by xanthan degrading enzymes (Hashimoto et al., 1998; Ruijssennars et al., 1999).

Xanthan degradation in synthetic brine

To verify the xanthan degradation observed at the extended incubation, samples from the bioassay was transferred to synthetic brine added xanthan as sole carbon source. The parallel that was inoculated with the originally xanthan degrading culture showed pronounced growth during the incubation. The same parallel showed a complete reduction in viscosity after 28 days, confirming the utilization of xanthan as growth substrate. The cells showed a significant growth after day 7 and the highest abundance after day 21.

Bacterial growth was also demonstrated in the two other parallels, but was less pronounced and not associated with significant reduction in viscosity. Growth without xanthan degradation was probably supported by cellular debris and glucose-residues from the xanthan production, utilized as a carbon source by species not able to degrade xanthan. In addition, nitrogen, phosphorus and trace elements are added to the synthetic brine, providing essential nutrients for growth. These nutrients were not included in the original bioassay, which was constructed to assess xanthan degradation without addition of other nutrients. This might have stimulated growth in the synthetic brine, despite the lack of xanthan degradation.

The bacterial community composition revealed great difference between the parallels. The culture which demonstrated xanthan degradation showed several strong bands, not present in the other parallels. The bands were observed from the start of the experiment, indicating that the phylotype was dominating in the original culture bottle. The bands showed highest sequence similarity to *Prolixibacter bellariivorans* (98% sequence similarity), characterized as a psychrotolerant, facultative anaerobe bacterium, which can ferment sugars by mixed acid fermentation (Holmes et al., 2007). The type species can ferment a

wide range of substrates, but xanthan was not included in the study (Holmes et al., 2007). No available literature discusses xanthan degradation relative to this species, but the physiological properties of the species propose it as an eligible candidate for degrading the biopolymer at the current conditions. The type species can grow at temperatures from 4°C to 42°C, degrade starch and ferment a range of substrates, including glucose and mannose. Identification of this species in the parallel that demonstrated xanthan degradation suggests that the phylotype affiliated to *Proloxibacter bellariivorans* is responsible for the degradation. The observed reduction in viscosity indicates a complete degradation of the xanthan molecule.

Prolixibacter bellariivorans implements a mixed acid fermentation when grown on glucose, producing acetate, propionate and succinate as end products (Holmes et al., 2007). When considering the metabolic traits of other bacteria detected in the xanthan degrading culture, it is possible that the fermentation products were further degraded by other species. A phylotype affiliated to *Pelobacter seleniigenes* (98% sequence similarity) were identified after day 7. This strain implements fermentation of short organic acids, suggesting that the fermentation products from the xanthan degrader supported growth of this species (Narasingarao and Häggblom., 2007). In the original bioassay, biodegradation of xanthan was accompanied by a reduced pH at the end of incubation, indicating accumulation of fermentation products. This was not observed in the control assay, despite a complete degradation of xanthan. The unaltered pH was presumable due to the buffering capacity in the synthetic brine. Addition of $NaHCO_3$ to adjust the pH in the brine has formed a buffer effect that probably has neutralized the CO_2/acids produced by fermentation.

In conclusions, no significant bacterial growth was observed within 7 days of mesophilic incubation in the original bioassay. Viscosity measurements confirmed that no degradation of xanthan had occurred and prolonged incubation for another 25 days did not change the viscosity (results not shown). Increasing the incubation time to 4 months in a later bioassay gave a positive indication of xanthan degradation by reduction in viscosity in one of three parallel bottles. Overall, the results suggest that the number of xanthan degrading bacteria in the SRP brine is low. However, when the species are enriched, they are able to grow readily on xanthan as sole carbon and energy source. The significant loss in viscosity suggests that the putative degrader *Prolixibacter bellariivorans* completely depolymerize the biopolymer.

4.1.2 Degradation of xanthan at thermophilic conditions

Limited growth was demonstrated in only 1 of the 12 parallels with SRP brine incubated at 60°C. The remaining parallels showed a declining bacterial number, indicating that xanthan was not utilized as a growth substrate. Unaltered viscosity in the brines supported the results.

The small alterations demonstrated in the community composition also correspond with these results. The brine with biocide showed a greater diversity in bands than the brine without biocide. Several of these bands were also identified in the brine incubated at 30°C. These bands were lost in the brine without biocide, demonstrating the preservation effect of glutaraldehyde. These bands belonged to mesophilic species, which presumably had lysed in the brine without biocide.

The PWRI brine showed a reduction in cell numbers in all parallels. The viscosity of the brine was unaltered, indicating that the biopolymer was preserved. However, the bacterial community structure from the brine without biocide showed numerous new bands after day 7. Several bands affiliated to members of the order *Thermotogales* were identified. This group includes strict anaerobic, mostly thermophilic and hyper thermophilic prokaryote, often obtained from oil reservoir and oil production fluids (Magot et al., 2000; Miranda-Tello et al., 2004). Environmental analysis suggests that members of *Thermotogales* play important roles in these habitats (Magot et al., 2000). The identified bands belonged to thermophilic, fermentative organisms, previously isolated from oil reservoirs (Dipippo et al., 2009; L'haridon et al., 2001: Lien et al., 1998; Miranda-Tello et al., 2007).

Two bands affiliated to *Petrotoga* were identified after 7 days of incubation. Species in this genus has previously only been isolated from oil reservoirs (Ollivier & Cayol, 2005). The identified bands were affiliated to *Petrotoga halophila* (99% sequence similarity) and *Petrotoga mobilis* (99% sequence similarity). The type species can degrade biopolymers such as starch and xylan, and are able to ferment a wide range of carbohydrates, including glucose (Lien et al., 1998; Miranda-Tello et al., 2007). An additional band showed highest sequence similarity to *Thermosipho geolei* (92%), but the sequence was of low quality with a lot of disturbance. The type species is not able to degrade sugar-based biopolymers, but grow by fermenting glucose (L'haridon et al., 2001). Bands affiliated to *Kosmotoga olearia* (99% sequence similarity) were also identified after day 7. The type species does

not utilize glucose, but growth is supported by fermentation of mannose (Dipippo et al., 2009).

The physiological and metabolic properties of these strains make them suitable candidates to degrade xanthan in an oil reservoir. The species are adapted to the high temperatures and anoxic conditions in these habitats. Fermentation is a common catabolic process in oil reservoirs, where the access to external electron acceptors is generally low (Grabowski et al., 2005; Magot et al., 2000). Despite the lack of xanthan degradation, enrichment of species possessing these properties is significant, as they have the metabolism necessary to ferment glucose or mannose. *Petrotoga* also has the ability to degrade other biopolymers, suggesting that these species produce and secrete other extracellular enzymes (Lien et al., 1998; Miranda-Tello et al., 2007).

A final band affiliated to *Phyllobacterium myrsinacearum* was also identified, a species associated with plant roots (Mergart et al., 2002). This phylotype was also identified in the SRP brine, present in the initial community structure. Due to the species natural habitat, it was first assumed that the bacteria derived from the xanthan solution. However, the appearance of the band after day 7 suggests that the bacteria are active growing. The genus *Phyllobacterium* is classified as mesophilic and aerobic, arguing against growth at the current conditions (Mantelin et al., 2006). The absence of the band in the initial community may be due to the sensitivity in the PCR reaction. The high cell abundance in initial samples might have obstructed the reaction, leading to amplification only of the dominant sequences. However, this is difficult to state with certainty.

4.1.3 Degradation of xanthan at hyper thermophilic conditions

All parallels showed an extensive decrease in bacterial cells during the first month. After 6 months, an increase in bacterial numbers was demonstrated in the SRP brine, regardless of biocide treatment. However, as the sample did not yield a PCR product for DGGE analysis, it is impossible to determine whether the enhanced cell numbers were linked to hyper thermophilic growth or residual intact DNA present in the sample. The low cell numbers also question the validity of the measurements, as small amount of target DNA leads to an elevated risk for contamination DNA affecting the results. Impurities linked to the QPCR mix and the laboratory environment might lead to calculation of an enhanced cell number. Such impurities were detected in the negative control, but small variations between the negative control and the samples reduce the reliability of the results.

Amplification of the 16S rDNA is very sensitive to contamination by bacterial DNA, as the primers anneal with the conserved regions of the bacterial genes (Wintzingerode et al., 1997).

4.2 Enrichments and isolation of a dominant bacteria

The lack of xanthan degradation observed in the original bioassays was confirmed by missing growth on xanthan in the anaerobic enrichments. Cultures inoculated with PWRI brine demonstrated growth on alternate carbon sources. The DNA fingerprinting analysis from the enrichments identified several strong bands originating from the PWRI brine. The bands were affiliated to *Petrotoga halophila* (100% sequence similarity), *Petrotoga mobilis* (99% sequence similarity) and *Thermosipho geolei* (98% sequence similarity), also identified in the original bioassay. Finding of these species confirms the results from the bioassay, and demonstrates their ability to ferment glucose. The presence of the phylotype affiliated to *Thermosipho geolei* supports the classification of the low quality sequence in the original PWRI brine.

None of the these strains are assessed for growth on organic acids (L'haridon et al., 2001; Lien et al., 1998; Miranda-Tello et at., 2007,) but the presence of strong bands in the DGGE profile indicates the ability to utilize the substrate. *Kosmotoga oleari*, present in the original bioassay was not found in the enrichments. This strain is not able to degrade glucose, and has therefore not grown after additional transfers.

An additional band was identified as *Phyllobacterium myrsinacearum*. As already mentioned, the presence of this species is difficult to explain. However, the identification of the same sequence in the SRP brine, PWRI brine and the anaerobic enrichments supports the expectations that *P. myrsinacearum* derive from the xanthan solution.

The low growth rates observed in the anaerobic enrichments suggest that the bacteria require additional nutrients to stimulate growth. Addition of yeast extract could have enhanced the growth rate in the cultures. Yeast extract was added as a growth factor when the physiological properties of the type species was analyzed, suggesting that yeast extract is required to improve the growth rate (Lien et al., 1998; L'haridon et al., 2001; Miranda-Tello et al., 2007).

Due to the slow growth in the anaerobic enrichment, isolation could not be conducted within the timeframe of the current thesis. It was therefore decided to isolate facultative

anaerobic sugar degrading bacteria by aerobic growth on solid media. By streak-plate technique, a bacterium was isolated from the PWRI brine at 60°C. The strain was affiliated to *Aeribacillus pallidus*, showing 100% similarity in 16S rDNA. The genus *Aeribacillus* are described as strict aerobic, thermophilic, motile, gram positive rods (Miñana-Galbis et al., 2010; Scholz et al., 1987). At this point, the genus only possesses one species, *A. pallidus.*

Strain BH-1 had an optimal growth temperature between 60-65°C, corresponding to properties of the type species (Scholz et al., 1987). Strain BH-1 was able to grow anaerobically by fermentation of glucose, showing a significant difference from the other described *Aeribacillus* strains. In the available literature, anaerobic growth from *A. pallidus* is assessed in two studies (Radchenkova et al., 2013; Scholz et al., 1987), reporting that growth was not demonstrated at anoxic conditions. Other strains have also been characterized and classified as strict aerobic (Miñana-Galbis et al., 2010; Yasawong et al., 2011). To achieve anaerobic growth, the isolate needed a long incubation time. Anaerobic growth was demonstrated after several weeks of incubation, while aerobic growth was detected within days. A limited growth period may be the reason why anaerobic growth has not been observed in previous studies. The ability to grow under anaerobic conditions clarifies the enrichment of *A. pallidus* from the anoxic PWRI brine.

The characterization of strain BH-1 was conducted in liquid medium. The process was challenging, as the isolate grew poor at low cell densities. However, the strain was fast growing on a solid surface, giving visible colonies within 2-3 days. The growth rate was less extensive in the liquid media, indicating that the cells preferred growing on a solid surface. This suggests that growth might be improved in a pipe system or in reservoir rocks, with high access to solid surfaces.

As the isolate required a high cell abundance to grow in liquid media, it was challenging to assess the substrate range of strain BH-1. Pronounced growth was only obtained when the original cultures were very turbid, suggesting that the cells required a high inoculum to grow. For several substrates, growth was only demonstrated in one or two of the parallels after three transfers. This might be due to a low initial cell number in some of the cultures, thereby obstructing further growth. The tendency of cells to form aggregates in liquid cultures supports the affinity to grow in biofilms.

Strain BH-1 showed some significant difference in substrate utilization related to other *Aeribacillus pallidus* strains. Growth was supported by glucose, starch, xanthan, cellobiose, galactose, sucrose, organic acids and maltose. Growth on xanthan, galactose and organic acids has not been reported for strain DSM 3670^T and TD1 (Scholz et al., 1987; Yasawong et al., 2011). Growth was not supported by cellulose, fructose and crude oil. In addition, yeast extract was required for growth. The requirement of yeast extract and the low growth rate in anaerobic brine might explain why this strain was not identified in the original bioassay.

Growth on xanthan demonstrates the ability to produce and secrete xanthan degrading enzyme. However, the low growth rate at anaerobic conditions suggests that degradation of xanthan would be low in anoxic brine. The preference for a solid surface might provide enhanced growth conditions in an oil reservoir with growth in biofilms and on pipe walls. Biodegradation could therefore be significant in field conditions. Additional experiment should be performed to assess anaerobic growth on solid surfaces and the potential for xanthan degradation in anoxic brine.

5 Methodological approaches

Sampling and preservation of the brines

The injection waters were collected at the platform on jerry cans before being shipped to UNI Research CIPRs laboratories. After being transferred to pyrex bottles, PWRI brine was stored in room temperature, to maintain the potential for thermophilic bacterial grow. This preservation would provide growth conditions for mesophilic bacteria, thereby possibly enhancing the initial cell numbers in the samples. The PWRI brine has a high presence of organic compounds, which may serve as carbon for a variety of bacteria (Yeung et al., 2011). The initial cell numbers were high ($1x10^7$-$1x10^8$) suggesting that growth had occurred prior to incubation. However, this was not significant for the experiment, as the cell numbers were monitored to detect growth on xanthan. The results were coupled to parallel viscosity measurements, which was the major indicator for degradation of the biopolymer.

Whole cell PCR-amplification

The QPCR and PCR for DGGE analysis were performed on whole cells sampled by centrifugation of a small volume. This approach was chosen due to the extensive number of sample bottles and samplings in the experimental matrix, and the need to perform the analysis on a small sample volume. To detect biodegradation of xanthan, cell numbers were monitored to detect alterations. As xanthan was the only significant carbon source in the samples, growth would be an indicator for xanthan degradation. The analyses were therefore conducted to detect potentially increases in cell numbers to indicate growth, and not to calculate the absolute bacterial numbers.

The PCR amplification for DGGE analysis was performed with the same approach. The DGGE analysis was conducted to evaluate potential shifts in the bacterial community structure, and possibly identify the xanthan degraders. Based on these criteria's, and the extensive amount of samples, whole cell QPCR and PCR was considered as suitable for the enumeration and fingerprinting analysis. Domain specific primers were applied to amplify a partial sequence of the 16S rDNA from Bacteria. Because fermentative bacteria were expected to be the major contributors to biodegradation of xanthan under the experimental conditions, the 16S rDNA from Archeaea was not included in the analysis.

The exclusion of DNA extraction may have affected the final results obtained by the PCR analysis. DNA extraction protocols are known to include mechanical or enzymatic steps to ensure lysis of the cells and exposure of the DNA (Frostegård et al., 1999). The procedures often include proteinase, degrading proteins that may be contaminating or destructive to the DNA. These steps are applied to extract DNA from all bacteria in a sample, including gram-positive bacteria which can be extremely resistant to cell lysis because of the thick cell walls surrounding the cells. Insufficient disruption of the cells can bias the view of the bacterial community structure, as DNA that is maintained within the cells is excluded from the further analysis (Wintzingerode et al., 1997). However, the considerable temperature gradient the cells were exposed to after sampling and during the PCR reaction might have been sufficient to lyse the major parts cells in the sample. The finding of gram positive bacteria on the DGGE profile suggests that the thermal lysis was sufficient to degrade the robust peptidoglycan layer of these cells. Assessment by QPCR showed that whole cells and extracted DNA from native brines gave a similar cell number, indicating that the majority of the cells had lysed during the reaction. However, it should be accounted for that parts of the microbial community might have been excluded due to the missing DNA extraction.

PCR amplification for DGGE analysis

Application of PCR to amplify specific regions of the DNA molecule is a well-established method in molecular microbiology. Amplification of the 16S rDNA provides phylogenetic information about the microbial diversity in environmental studies, providing basis for several techniques for investigating the microbial community (Takahashi et al., 2014). The accuracy and quality of the PCR are essential for further analysis. However, the PCR are prone to bias by PCR selection, PCR artefacts and contamination among others (Wintzingerode et al., 1997).

PCR selection favor amplification of certain templates related to others, producing an overrepresentation in some of the amplicons. Different primer affinities in the sequences, due to the accessibility of the target DNA, denaturation properties and binding efficiency, may changing the template-to-product ratio obtained from the original community (Wagner et al., 1994). PCR selection may also occur due to different gene copy numbers within the genome. The number of *rrn* operons among bacterial species differs greatly, and is an important source for overrepresentation (Boon et al., 2002).

PCR drift is a result of random events in first cycles of the reaction, leading to a skewing abundance of various organisms in the PCR amplicons (Wagner et al., 1994). To overcome this challenge, multiple PCR reactions should be used to analyze the microbial community. This work was based on multiple QPCR analysis to ensure the consistency in the measurements. Single PCR-DGGE analysis was performed from the various samples. However, the samples had the same origin, and showed a comparative banding pattern and the similarities in identified phylotypes. In addition, some of the profiles were repeated several times to provide the reproducibility of the results.

Formation of PCR artefacts can greatly influence the interpretation of diversity studies. Chimeras are hybrid products formed between DNA fragments with high sequence (Lahr and Katz, 2009). The chimeras can be falsely interpreted as novel organism, thus bias the apparent diversity (Haas et al., 2011). This study did not include any algorithm for detecting chimeras, which may be a potential error in the diversity analysis.

Fingerprinting analysis of bacterial communities

A fingerprinting analysis was included as a methodological approach to assess alterations in the microbial community structure, and potentially identify new species linked to xanthan degradation. The PCR amplicons was obtained using bacterial specific primers, to include all bacteria in the samples. When using such primers, the banding pattern on the profile is generally view as a reflection of the composition of the dominant bacterial community (Brunvold et al., 2007). The profile only represents the major constituents of the community, as less abundant sequences are amplified insufficient to provide a visible band on the gel (Boon et al., 2002; Heuer et al., 1997). Identical position of the bands might be due to identical sequences, but also as a result of the same melting behavior of different sequences (Muyzer et al., 1993).

Sequence heterogeneity between different copies of the rRNA gene operons within a single species can produce multiple bands deriving from the same species (Wintzingerode et al., 1997). This was observed by the affiliation of several bands at different positions to the same phylotype. Bands can also represent more than organisms, as partial 16S rDNA sequences can be identical in close related species (Boon et al., 2002). BLAST alignments for some of the sequences showed similar affiliations to several species within the same genus. Community richness estimations based on DGGE-fragments should therefore be

interpreted as an indicator of the microbial diversity, and not an absolute measurement (Jousset et al., 2010).

Sequence analysis of the excised bands

Sequencing of partial 16S rDNA was conducted to affiliate the DGGE bands to bacterial taxa. The 16S rDNA is a suitable phylogenetic marker in ecological surveys, due to its essential function, its universal presence and the conserved sequence of the gene (Pace, 1997). Comparative sequence analysis of the gene is a common way to identify bacteria, and the majority of bacterial phyla are only known from 16S rDNA analysis (Rappe and Giovannoni, 2003). The guideline for the similarity level for bacterial specification is a controversy topic, and there are no set universal "threshold value" where there is general agreement for ranking of bacterial species (Janda and Abbott, 2007; Woo et al., 2008). However, the similarity value for specification is always above 97%. In addition, different species may have identical 16S rDNA sequences, while single species may different heterogeneous copies of the gene (Fox et al., 1992).

As previously mentioned, some bands showed identical sequence similarity to several species, indicating the limitations of using partial 16S rDNA sequences to distinguish closely related species. The similarity index obtained by the GenBank when comparing sequences gives an indicator of how close the sequences are related. Sequences can be categorized in the same species if the similarity is >97%, and within the same genus if the similarity is >95%. Sequences with a similarity higher than 80% are defined as within the same phylum (Schloss and Handelsman., 2005). Some of the obtained sequences showed a low quality with a lot of disturbance. Some of these sequences had alignments with sequence similarities lower than the limit for species and genus assignment. The phylogeny of the sequences must not be interpreted as defined properties, but as an indicator for the bacterial diversity within the different brines.

Cultivation approach for enrichment and isolation

Isolation and cultivation of microorganism tend to discriminate many species, not adapted to the given environment. The viable-but-non culturable (VBNC) hypothesis was developed by Colwell et al. (1982), explaining how bacteria may be underestimated if culture methods is used (Xu et al., 1982). Cultivation depended techniques are known to be selective, are not considered representative for the microbial community (Ranjard et al.,

2000). The cultivability of bacteria in natural environments is proven to be very low, estimated to be between 0.1% and 10% of the population. Cultivation media are often designated to facilitate growth of microorganisms with certain traits, thus excluding the occurrence of many species present in the native samples.

The enrichments performed in this study were based on synthetic brines assembled from the ionic composition in original brines. This approach was chosen in an attempt to isolate a potential xanthan degrading species in a media based on the original brine. The anaerobic isolation approach was not feasible, because of the timeframe available for the current work. However, if a greater number of substrates, incubation temperatures, salinities and pH were applied, the enrichments may have grown faster. Because the major focus of the current master thesis was the bioassay, further work was not conducted.

6 Conclusions

- ✓ Biodegradation of xanthan did not occur in the original bioassay. This was verified by lack of bacterial growth and unaltered viscosity.
- ✓ Some of the bacterial communities showed alterations related to the initial community. At mesophilic conditions, a phylotype affiliated to *Microbacterium* sp. was identified after day 3. However, the poor anaerobic growth of this species suggests that anaerobic biodegradation would be limited. Extended incubation did not lead to biodegradation. The PWRI brine incubated at 60°C identified phylotypes affiliated to *Petrotoga halophila, Petrotoga mobilis, Thermosipho geolei* and *Kosmotoga olearia*. The enrichment was not linked to biodegradation of xanthan.
- ✓ Due to the missing xanthan degradation, an attempt to isolate sugar degrading facultative anaerobic bacteria was conducted. The isolated strain was affiliated to *Aeribacillus pallidus,* originating from the PWRI brine at thermophilic conditions. The strain was characterized and described as facultative anaerobic, as it grew anaerobically by glucose fermentation. This property distinguish the strain from other *Aeribacillus* strains described in literature, characterized as being strict aerobic. The isolate was fast growing on a solid medium and with high cell densities. This may indicate that the isolate would thrive in a pipe system or in reservoir rocks, where it can grow on the surfaces and in assemblies with other cells. Xanthan degradation was observed at aerobic conditions, suggesting the excretion of xanthan degrading enzymes.
- ✓ Biodegradation was demonstrated at 30°C in a later experiment, after a total incubation period of 4 months. A control experiment verified the demonstrated xanthan degradation. Pronounced bacterial growth was detected in the culture bottle inoculated with the xanthan degrading culture. Consistent with the bacterial growth, a reduction in viscosity was demonstrated. Dominant bands classified as *Prolixibacter bellariivorans* was only observed in the xanthan degrading culture, indicating that the degradation was conducted by this species. This species has not previously been linked to degradation of xanthan.

Further work

This study was part of an experiment conducted to assess the biodegradation of xanthan in a bottle experiment. Based on the results of the current thesis, the following additional experiments should be performed/the following topics should be studied further

- ✓ A repeated bioassay in SRP brine at 30°C with an extended incubation, to further assess xanthan degradation in the injection water.
- ✓ Isolation and characterization of the xanthan degrading species in the control experiment.
- ✓ Further characterization of *Aeribacillus pallidus.* Assessment of viscosity reduction by aerobic growth on xanthan.
- ✓ Isolation and characterization of dominant bacteria from the anaerobic enrichments.

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8 APPENDIX

Appendix 1: Materials and protocols

Solutions

Table A.1: 50xTAE

Concentration	Component
20mM	Tris-acetate
10mM	Sodium.acetate
0,5 mM	Sodium EDTA

Add distilled water to a total volume of 1 liter. Sterilize in autoclave for 20 min, 121°C.

Table A.2: PBS - Phosphate buffered seawater

Quantity	Component
8.5 gram	NaCl
1.44 gram	Na_2HPO_4 x $2H_2O$
0.25 gram	KH_2PO_4

Dissolve in 1 liter distilled water. Sterile by autoclaving for 20 min, 121°C.

Table A.3: Loading dye – Agarose gels and DGGE

Quantity	Component
0.025 gram	Bromophenol blue
4 gram	Saccharose

Mix in 10 ml distilled H_2O.

Table A.4: Trace elements

Quantity	Component
10 ml	HCL (32%)
1.5 g	FeCL2 x $4H_20$
70 mg	$ZnCl_2$
100 mg	$MnCl_2$ x $4H_2O$
6 mg	H_3BO_3
190 mg	$CoCl_2$ x $6H_2O$
2 mg	$CuCl_2$ x $2H_2O$
24 mg	$NiCl_2$ x $6H_2O$
36 mg	Na_2MoO_4 x $2H_2O$

Dissolve $FeCl_2$ x $4H_2O$ in HCl. Add dissolved water and the remaining salt to a total volume of 1 liter.

Table A.5: Vitamin solution

Quantity	Component
8.0 mg	4 – Aminobenzoic acid
2.0 mg	D-(+) Biotin
20.0 mg	Nicotinic acid
10.0 mg	Ca-D (+) Pantothenate
30.0 mg	Pyridoxamine x 1HCl
20.0 mg	Thiamine dichloride
10.0 mg	Cyanocobalamine

Dissolve in 1 liter distilled water. 4 – Aminobenzoic acid and D-(+) Biotin dissolved in boiling water.

Table A.6: LBA

Quantity	Component
10 g	Tryptone
10 g	NaCl
5 g	yeast extract
15 gram	Agar

Mix with distilled water to a total volume of 1 liter. Sterilize in autoclave, 20min at 121°C.

Table A.7: Primers

Primer	Sequence	Reference
pA8f	5'-AGA GTT TGA TCC TGG CTC AG-3' with 40bp GC clamp	Giovannoni, 1991
PRBA338f	5'-ACT CCT ACG GGA GGC AGC AG-3'	Lane, 1991
PRE927f	5'GGG CCC GCA CAA GCG GTG-'3	Steinsbu, 2003
PRUN518r	5'-ATT ACC GCG GCT GCT GG-3'	Muyzer et al., 1993
PRE944r	5'-CAC CGC TTG TGC GGG CCC-3'	Steinsbu, 2003
Hr	5'-TGC GGC TGG ATC ACC TCC TT-3'	Giovannoni, 1991

Table A.8: Stock solutions for DGGE

DSSA, 0% denaturant		**DSSB, 100% denaturant**	
Quantity	Component	Quantity	Component
100 ml	40% acrylamide stock solution (BioRadLa Inc, Ca, USA)	50 ml	40% acrylamide stock solution
		2.5 ml	50xTAE
5 ml	50x TAE	105 g	UREA
Sterile distilled water to a total volume of 500ml.		100ml	Deionized formamide (Pharmacia biotech)
		Sterile distilled water to a total volume of 250ml.	

Table A.9: Composition of the DGGE gradient

Component	Level of denaturant	
	20 %	**60%**
DSSA	11.6 ml	5.8 ml
DSSB	2.9 ml	8.7ml
APS (Ammonium Persulfate Solution)	145µl	145µl
TEMEB	7.3µl	7.3µl

Table A.10: Synthetic PWRI brine

Synthetic PWRI brine	
Salts	**Quantity (mg/L)**
NaCl	37469
KCl	679
$CaCl_2 \times 2H_2O$	2340
$MgCl_2 \times 6H_2O$	1882
$SrCl_2 \times 6H_2O$	207
$BaCl_2 \times 2H_2O$	162
Na_2SO_4	154

Dissolve in distilled water in the specified direction. Autoclave at 121°C for 20 minutes.

Table A.11: Synthetic SRP brine

Synthetic SRP brine	
Salts	**Quantity (mg/L)**
NaCl	26945
KCl	688
$CaCl_2 \times 2H_2O$	1705
$MgCl_2 \times 6H_2O$	2358
$SrCl_2 \times 6H_2O$	9
$Na_2 \times SO_4$	59

Dissolve in distilled water in the specified direction. Autoclave at 121°C for 20 minutes.

ExoSAP-IT® protocol

Mix 5µl PCR product with 2µl ExoSAP-IT. Incubate the mixture in a thermocycler at 37°C for 15 minutes, followed by an inactivation of the reagent at 80°C for 15 minutes.

Appendix 2: Additional results

The DGGE profiles and tables with the BLAST alignments not included in the results section is shown in the figures and table in the following pages.

Table A.12: The table shows the phylotypes obtained from the SRP brine incubated at 30°C (figure 10). BLAST alignments for each band, including similarity, habitat and accession nr for the sequence are included.

Band	Phylotypes	Similarity	Habitat	Accession nr
1	*Colwellia* sp.	96 %	Marine habitat	HQ836463.1
2	*Hyphomonas* sp.	94 %	Marine habitat	AY690712.1
3, 7	*Cupriavidus basilensis*	97 %	Soil.	CP010537.1
4	*Xanthanomonas* sp.	100 %	Xanthan producer.	JX852722.1
5	*Sphingomonas* sp.	94 %	*Xanthan solution*	AF385529.1
6	*Thioclava* sp.	96 %	Marine habitat	KM255690.1
8	*Ralstonia* sp.	99 %	Soil, rivers.	DQ374437.1
9,10	*Rhodococcus* sp.	99-100 %	Soil, water.	GU726528.1
11	*Methylophaga* sp.	95 %	Marine habitat	AJ244762.1
12	*Microbacterium* sp.	98 %	Several different habitats	AF474330.1
13	*Microbacterium* sp.	99 %	Several different habitats	GQ250443.1

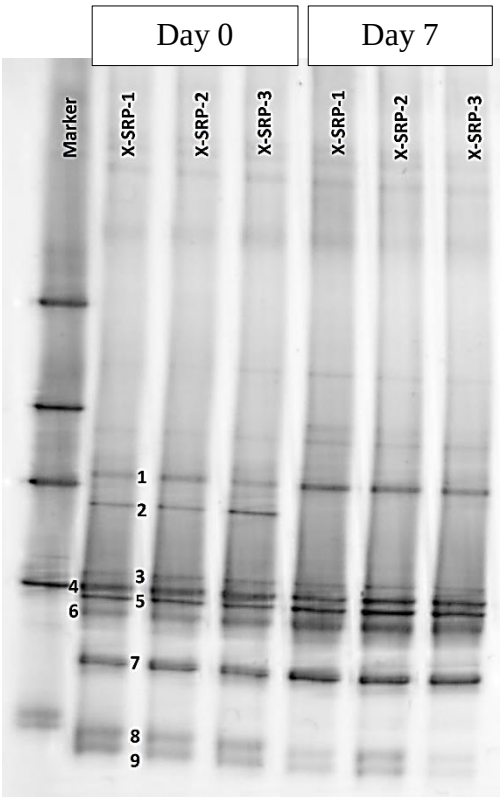

Figure A.1: DGGE profile of the bacterial community in the SRP brine without biocide, incubated at 60°C. The profile displays the initial community compositions and the alterations detected after 7 days of incubation. No further changes were detected after the first week (results not shown). Marker: A mixture of PCR product from species with different GC-content. Sample names and sampling times are provided in the top of the profile. Numbers indicates excised bands that were further sequences. BLAST alignments are provided in table A.13.

Table A.13: Phylotypes in the SRP brine without biocide (figure A.1). The table includes similarity, habitat and accession nr for the identified strain.

Band	Phylotypes	Similarity	Habitat	Accession nr
1,5	*Cupriavidus basilensis*	99 %	Soil	CP010537.1
2	*Xanthanomonas* sp.	96 %	Xanthan producer.	JX852722.1
3,4	*Thioclava* sp.	98-99 %	Marine environment.	KM255690.1
6	*Phyllobacterium myrsinacearum*	99 %	Assosiated with plants	KJ147062.1
7	*Ralstonia* sp.	100 %	Soil, rivers.	DQ374437.1
8, 9	*Rhodococcus* sp.	100 %	Several different habitats	GU726528.1

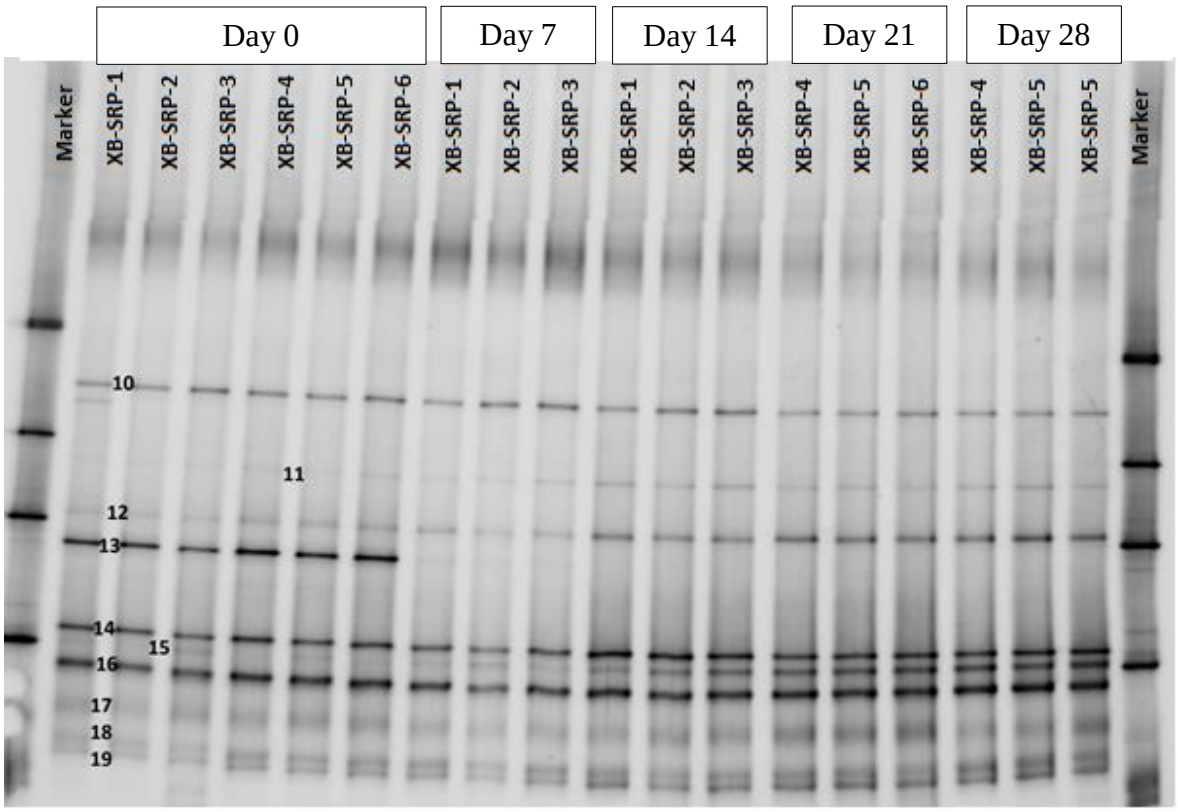

Figure A.2: The DGGE of the bacterial community in the SRP water with biocide, incubated at 60°C for 4 weeks. Marker: A mixture of PCR product from species with different GC-content. Sample names and sampling times are provided in the top of the gel. BLAST alignments are provided in table A.14.

Table A.14: The affiliation of the bands attained from the SRP brine with biocide (figure A.2). The table includes the phylotypes, similarity, habitat and accession nr.

Band	Phylotypes	Similarity	Habitat	Accession nr
10	*Colwellia* sp.	96 %	Surfacewater	HQ836463.1
11	*Hyphomonas* sp.	94 %	Marine habitat	AY690712.1
12	*Cupriavidus basilensis*	99 %	Soil.	CP010537.1
13	*Xanthanomonas* sp.	96 %	Xanthan producer.	JX852722.1
14	*Sphinhomonas* sp.	94 %	*Xanthan solution.*	AF385529.1
15	*Thioclava* sp.	96 %	Marine habitat	KM255690.1
16	*Phyllobacterium myrsinacearum*	98 %	Associated to plants.	KF479624.1
17	*Ralstonia* sp.	99 %	Soil, rivers	DQ374437.1
18, 19	*Rhodococcus* sp.	99-100 %	Several different habitats	GU726528.1

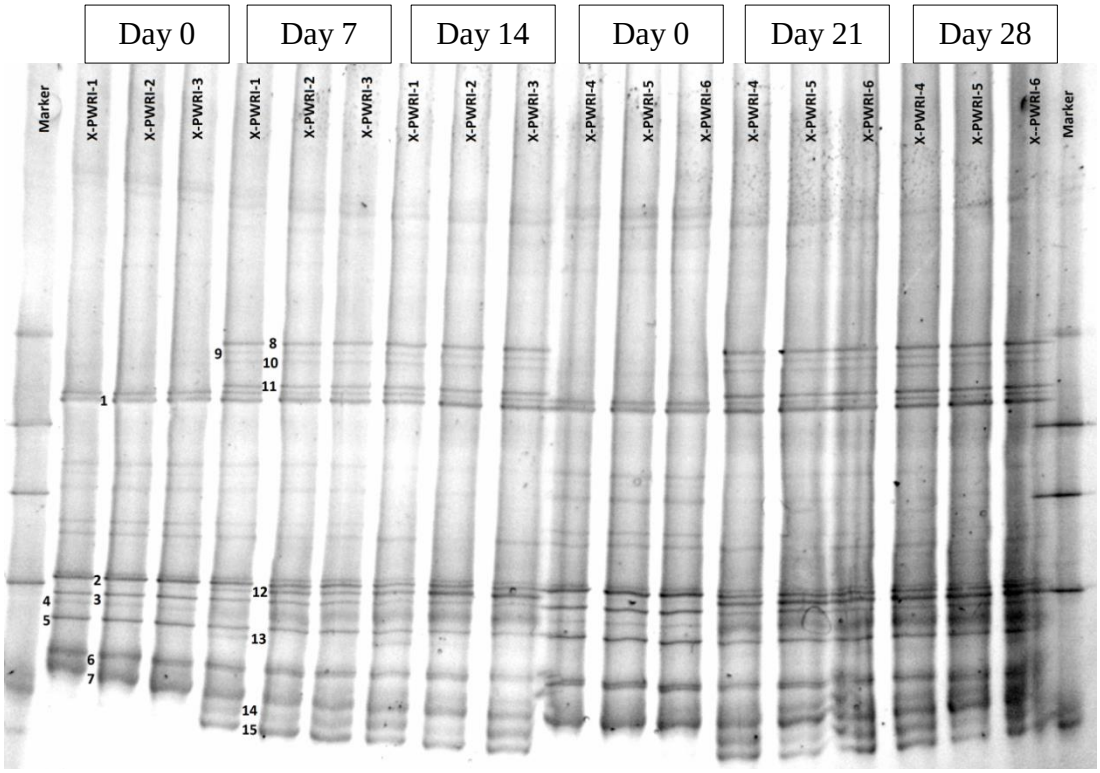

Figure A.3: DGGE profile from PWRI brine without biocide, incubated at 60°C for 4 weeks. Marker: A mixture of PCR product from species with different GC-content. Sample names and sampling times are provided in the top of the profile. Bands marked with a number were excised, re-amplified and sequenced. The BLAST hits are presented in table A.15.

Table A.15: The table presents the phylotypes obtained from the PWRI brine incubated at 60°C for 4 weeks (figure A.3). Similarity, habitat and Accession nr are included.

Band	Phylotypes	Similarity	Habitat	Accession nr
1	*Geotoga petraea*	99 %	Anaerobe, termofile.	NR_104910.1
2, 5	*Marinobacterium* sp.	96-98 %	Marine, oil reservoir.	AB196257.1
3	*Cupriavidus basilensis*	99 %	Soil.	CP010537.1
4, 6, 7	*Pelobacter carbinolicus*	89-97 %	Aquatic, anaerobic.	NR_075013.1
8, 10	*Petrotoga halophila*	98-99 %	Oil reservoir.	NR_043201.1
9, 11	*Petrotoga mobilis*	99 %	Oil reservoir.	NR_074401.1
12	*Phyllobacterium myrsinacearum*	98 %	Associated to plants.	KJ147062.1
13	*Thermosipho geolei*	92 %	Oil reservoir.	NR_025389.1
14, 15	*Kosmotoga olearia strain*	99 %	Oil reservoir.	NR_044583.1

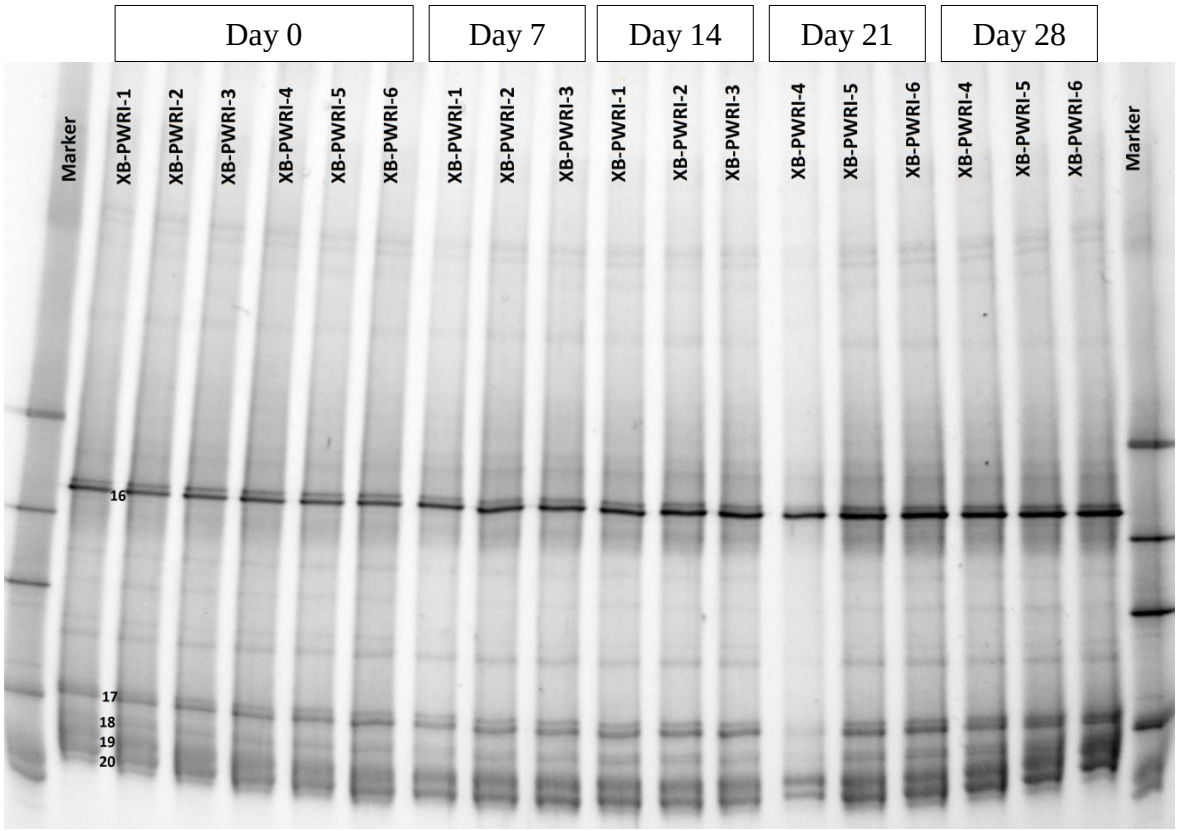

Figure A.4: The DGGE of the bacterial community in the PWRI brine with biocide, incubated for 4 weeks at 60°C. Marker: A mixture of PCR product from species with different GC-content. Sample names and sampling times are provided in the top of the profile. Numbering represents bands that were further analyzed.

Table A.16: Presentation of the phylotypes, similarity, habitat and accession nr for the dominating bands in the PWRI brine with biocide, shown in figure A.4.

Band	Phylotypes	Similarity	Habitat	Accession nr
16	*Geotoga Petraea*	99 %	Oil reservoir	NR_104910.1
17	*Marinobacterium* sp.	98 %	Marine, oil reservoir.	AB196257.1
18	*Sphingomonas* sp.	99 %	*Xanthan solution.*	AF385529.1
19, 20	*Pelobacter carbinolicus*	89-97 %	Aquatic, anaerobic.	NR_075013.1

Table A.17: BLAST alignments and similarity index for the sequences obtained from the DGGE analysis from the control experiment. The table includes habitat and accession nr for the sequences. The numbering represents bands on the profile (figure 12).

Band	Phylotypes	Similarity	Habitat	Accession nr.
1	*Celeribacter* sp./ *Roseobacter* sp.	99 %	Marine environments	JX844513.1/ GU584168.1
2	*Sphingomonas* sp.	97%	Xanthan solution	AF385529.1
3	*Burkholderia* sp.	95%	Xanthan solution	KC160738.1
4, 5	*Phyllobacterium myrsinacearum*	99%	Associated with plants	KJ147062.1
6, 7, 16	*Prolixibacter bellariivorans*	96 - 98 %	Marine sediments	NR_113041.1
8, 9, 18	*Labrenzia* sp.	94- 98 %	Marine environments	JQ342690.1
10	*Sunxiuqinia faeciviva*	99 %	Marine sediments	NR_108114.1
11	*Spongiibacter tropicus*	99 %	Marine environments	NR_118017.1
12, 13	*Roseobacter denitrificans*	90-96 %	Several different habitats	NR_102909.1
14	*Thalassospira xiamenensis*	99 %	Marine environments	EU603449.1
15	*Muricauda beolgyonensis*	99 %	Marine environments	NR_117844.1
17	Sequence of to low quality.	-	-	-
19	*Pelobacter seleniigenes*	98 %	Sediments.	NR_044032
20	*Lutibacter aestuarii*	98 %	Marine environments	NR_108995.1
21	*Phycisphaerae* bacterium	90 %	Aquatic environments	KC491303

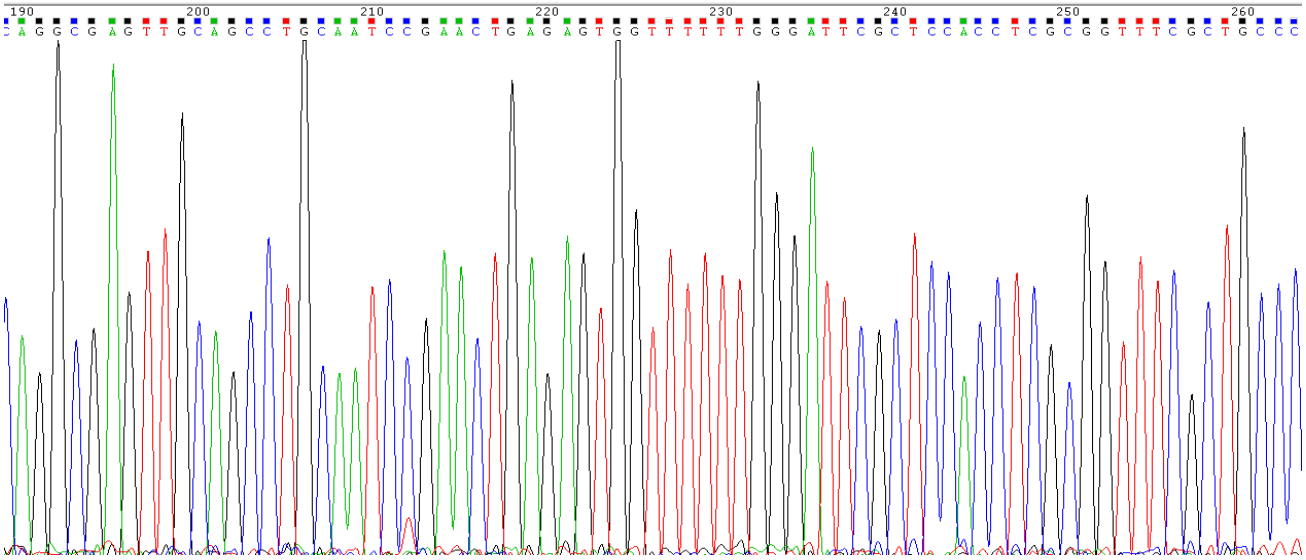

Figure A.5: A short excerpt from the chromatogram, presenting a part of the sequence of isolate BH-1.