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Life in pitch – Bacteria in a natural oil emitting lake help to understand anaerobic biodegradation of polycyclic aromatic hydrocarbons

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Für Jan

“So much as come before those battles lost and won

This life is shining more forever in the sun.”

Road Trippin’ - Red Hot Chili Peppers

Abstract

The research of anaerobic degradation of non-substituted polycyclic aromatic hydrocarbons is still in its infancy and most processes therein only poorly understood. Due to the poor bacterial degradation capabilities of PAHs, only few cultures exist that can be used to explore the underlying mechanisms. Their growth times are considerably longer than those with compounds with a smaller molecular weight and the production of biomass is substantially lower as shown by strain-specific FISH analyses and flow cytometry.

Elucidation of pathways started in enrichment cultures growing with naphthalene as sole carbon and electron source and is at a point where individual steps are fairly well characterized through years of research. The next logical step is to look at compounds with a higher molecular weight to find similarities or dissimilarities in the pathways. The compound of interest in this thesis was phenanthrene, a three-ringed PAH without known degradation steps apart from a carboxylation as initial reaction (Zhang and Young 1997; Davidova et al. 2007). Parallels to anaerobic naphthalene degradation are expected due to similarities in the aromatic ring structure. In this thesis, I wanted to find insights into the anaerobic degradation of phenanthrene as a bigger sized PAH. Therefore, we isolated bacteria able to degrade hydrocarbons under anoxic conditions from a naturally contaminated ecosystem, the pitch lake in Trinidad, Trinidad & Tobago. An enrichment culture growing anaerobically under sulfate-reducing conditions was set up from sediments from the pitch lake. This culture was able to degrade phenanthrene as sole carbon and energy source. It was used to elucidate the anaerobic phenanthrene degradation pathway. Beyond the carboxylation reaction in the C2 position, which could be shown indirectly within the framework of this thesis by metabolite analysis and biochemical enzyme assays, I was able to show the ligation reaction from 2-phenanthroic acid to 2-phenanthroyl-CoA within this culture. This step is therefore similar to the ligation in anaerobic naphthalene degradation from 2-napthoic acid to 2-naphthoyl-CoA. Further downstream reductions steps could not be shown with enzyme assays yet. Nevertheless, metabolite analysis was able to indicate a stepwise ring reduction,

which again would be in accordance with the naphthalene reduction steps (Eberlein, Estelmann, et al. 2013; Eberlein, Johannes, et al. 2013).

The main dominating bacterium within the culture belongs to the *Desulfobacteraceae* family and made up for 60% of the culture as confirmed by flow cytometry and genome-resolved metagenomics. It has a 93% similarity to the known naphthalene-degrading, sulfate-reducing strain NaphS2.

While looking for life in the naturally forming asphalt, minuscule water droplets have been discovered that contain living bacteria (Meckenstock et al. 2014). As an extreme and seemingly uninhabitable habitat within the naturally formed asphalt, important questions towards the origin of the bacteria within the bitumen, their way of coping with the lack of oxygen and their access to nutrients remain to be answered. In this thesis, sequencing of DNA extracted from both bitumen and water droplets gave further insights into the community composition in compartments and their possible interactions. The diversity in the bitumen was higher than in the water droplets, indicating a more specialized microbiota in the water droplets. Diversity within the droplets might change compared to the original diversity in the source water during upward movement due to a local selection and evolution on the “micro-scale”.

The diversity in both compartments was very low compared to other oil sources, with more than 30% of the contained bacteria without any known relatives. In other samples like marine oil sources, there are only 10% of the bacteria without known relatives. The dominant bacterium in all sequenced droplets was *Tepidiphilus sp*., it belongs to the family *Hydrogenophilaceae* within the β-Proteobacteria and is able to degrade organic acids as sole carbon source under nitrate-reducing conditions. It is therefore a plausible candidate to live in this extreme environment, but it still is not characterized well yet.

In summary this thesis advances our understanding of anaerobic phenanthrene degradation and I was able to discover the similarities between naphthalene and phenanthrene degradation on an enzymatic basis. This also allows us to assume similarities for PAHs of an even higher molecular weight than that of phenanthrene. The diversity of the degrader community just opened a small window into the life in oil and the background metagenome from the bitumen serves as a template for a deeper look into this extreme habitat.

Zusammenfassung

Die anaeroben Abbauwege polyzyklischer aromatischer Kohlenwasserstoffe (PAKs) sind bisher weitestgehend unerforscht geblieben. Aufgrund des langsamen Wachstums und geringer Biomassebildung existieren nur wenige Kulturen, anhand derer die Abbauwege aufgeklärt werden können. Des Weiteren sind deutliche Unterschiede der am Abbau beteiligten bekannten Gene zwischen verschiedenen Spezies aufgedeckt worden, was überdies die Aufklärung durch Sequenzierungsdaten verhindert.

Der einzig bislang untersuchte anaerobe Abbauweg eines PAKs ist der von Naphthalin, eines Aromaten mit zwei Ringen und einem Molekulargewicht von 128 g/mol. Für diesen Abbauweg wurden die initialisierende Carboxylierung, die Reduktion der aromatischen Strukturen sowie die letztendliche Ringöffnung bereits beschrieben.

Der naheliegend folgende Schritt betrifft die Untersuchung der Abbauwege für Schadstoffe mit höherem Molekulargewicht auf Gemeinsamkeiten und Unterschiede. Das Augenmerk dieser Arbeit liegt hierbei auf dem Schadstoff Phenanthren, einem aus drei aromatischen Ringen aufgebauter Kohlenwasserstoff mit einem Molekulargewicht von 178,23 g/mol. Aufgrund der Ähnlichkeiten in der aromatischen Ringstruktur ist diesbezüglich mit Parallelen zum anaeroben Naphthalinabbau zu rechnen.

Auf der Suche nach Spezialisten des Phenanthrenabbaus wurden Bakterien aus einem natürlichen Teersee in der Karibik isoliert und unter anoxischen Bedingungen angereichert. Diese Anreicherungskultur wächst auf Phenanthren als einziger Kohlenstoff- und Energiequelle und unter sulfatreduzierenden Bedingungen. Anhand dieser Kultur konnten weitere – über die initialisierende Carboxylierung hinausgehender Schritte – des anaeroben Kohlenwasserstoffabbaus für Phenanthren untersucht werden. Dabei wurde mittels einer Metabolitenanalyse die Carboxylierung in der C2-Position bestimmt. Die anschließende Ligase-Reaktion konnte in einem Enzym-Assay nachgewiesen werden, wobei nur 2-Phenanthroesäure zu 2-Phenanthroyl-CoA umgesetzt wurde, was die Bestimmung der C2-Position ebenfalls bestätigt. Bis

dahin verläuft der Abbau, parallel zum Naphthalin-Abbau, auch hier an der C2-Position. Die weitergehenden Schritte des Abbauprozesses konnten mit Enzym-Assays bislang nicht beschrieben werden. Metabolitenuntersuchungen ergaben jedoch erste Indizien für eine sukzessive Reduktion, ebenfalls vergleichbar mit dem Abbau des Naphthalins (Eberlein, Estelmann, et al. 2013; Eberlein, Johannes, et al. 2013).

Mittels Durchflusszytometrie nach einer FISH-Analyse konnte eine Bakterienspezies, in Übereinstimmung mit 60% der Sequenzdaten, als das in der untersuchten Kultur vorherrschenden Bakterium bestimmt werden. Diesem Bakterium wird auch kausal der Phenanthren-Abbau zugeschrieben. Das Bakterium gehört zur Familie der *Desulfobacteraceae* und zeigte einen mit 93% niedrigen Verwandtschaftsgrad zu dem bekannten sulfatreduzierendem Naphthalin-Abbauer NaphS2.

Auf der Suche nach Leben in natürlich entstehendem Asphalt fanden Meckenstock et al. (2014) kleine Wassertröpfchen im Teer, in denen wiederum lebende Bakterien entdeckt wurden. Durch weitere Isolierung und Sequenzierung, vor allem mittels Einzelzellanalyse, erwartete man neue Einsichten in eine gerichtete Evolution innerhalb der Tröpfchen, da diese als individuelle Ökosysteme fungieren. Zusammen mit den DNA-Sequenzen, die direkt aus dem Teer isoliert wurden, ergab sich ein Überblick über die Diversität innerhalb des Asphaltsees: Die Bakterien zeigten im Vergleich mit den Tröpfchen eine deutlich höhere Artenvielfalt. Dies lässt auf besser spezialisierte Bakterien in den Tröpfchen sowie auf eine breitere Verteilung von Bakterien im Teer schließen, die sich darin schneller und flexibler an sich verändernde Bedingungen anpassen können.

Im Vergleich zu anderen Öl-Proben ergab sich jedoch nur eine sehr geringe Diversität im Teer; etwa 30% der Bakterien konnten aber keiner Art zugewiesen werden. In Proben marinen Ursprungs liegt dieser Anteil bei etwa 10%. Mit über 50% Anteil war *Tepidiphilus sp.* das dominierende Bakterium in den Wassertröpfchen. Es gehört zur Familie der *Hydrogenophilaceae* innerhalb der β-Proteobakterien. *Tepidiphilus sp.* ist in der Lage, organische Säuren als einzige Kohlenstoffquelle unter denitrifizierenden Bedingungen abzubauen. Damit ist es ein plausibler Kandidat, um perfekt angepasst innerhalb des „Ökosystems

Wassertropfen“ zu existieren. Eine weitere Charakterisierung dieses Bakteriums ist bislang nicht erfolgt.

Mit dieser Arbeit konnten Fortschritte in der Aufklärung des anaeroben Phenanthrenabbaus beschrieben werden, die weitere Rückschlüsse auf generelle Abbauwege von hochmolekularen polyzyklischen aromatischen Kohlenwasserstoffen ermöglichen. Zudem wurden die Diversitäten von Bakterien, die in einem extremen Habitat wie dem Teersee überleben können, durch verschiedene Sequenzierungs- und Auswertungsmethoden differenzierter beleuchtet, um einen tieferen Einblick in das „Leben im Öl“ zu ermöglichen.

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List of Abbreviations

°C	Celsius
16S rRNA	Ribosomal RNA, small subunit
ADMA	4-Amino-N,N-dimethylaniline sulfate
BTEX	Benzene, Toluene, Ethylbenzene, Xylene
c	Concentration
cDNA	Complementary DNA
DAPI	4‘,6-Diamidin-2-phenylindol
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleoside triphosphate
DOM	Dissolved organic matter
FAM	6-carboxyfluorescein
FIG.	Figure
FISH	Fluorescence in situ hybridization
FT-ICR-MS	Fourier transform ion cyclotron resonance mass spectometry
GC-MS	Gas chromatography-mass spectrometry
gDNA	Genomic DNA
h	Hours
HMN	2,2,4,4,6,8,8-Heptamethylnonane
mV	Millivolts
min	Minutes
MTP	Microtiter plate
NCR	2-Naphthoyl-CoA reductase
OD	Optical Density
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffered saline
PCoA	Principal Component Analysis
PEG	Polyethylene glycol
PFA	Paraformaldehyde
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
s	Seconds
T	Temperature
t	time
T-RFLP	Terminal restriction fragment length polymorphism
Tab.	Table
TAE	Tris-acetate-EDTA
v/v	Volume per volume
w/v	Weight per volume

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1 Introduction

1.1 The structure and importance of polycyclic aromatic hydrocarbons in nature

As the world's human population is rising significantly, so is the problem of environmental pollution due to increases in demand for industrial commodities. Organic pollutants are a global issue, as contaminants can spread through air and water, even to countries far away from the producing states. A major concern is the pollution of water, shores, wetlands and beaches through oil spills, with a global annual release between 1.7 and 8.8 million metric tons (National Academy of Sciences, 1985). The most recent major oil spill happened in the year 2010 about 80 km away from the south coast of the USA next to the Mississippi-Delta when the Deepwater Horizon oil rig exploded after an unexpected blow-out (Paquette 2013). This was the largest anthropogenic release of hydrocarbons into the environment to date with a release of 795 million liters of oil (McNutt et al. 2012). Many wetlands in Louisiana, Mississippi and Alabama were affected.

Petroleum, which is the focus of this study, is a complex mixture of gaseous, liquid and solid hydrocarbons and the percentage of different fractions can vary widely. Physical properties, such as fluidity, color and density, can also vary significantly depending on the source of the petroleum (Zobell 1945). When testing a sample of crude oil with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) Marshall and Rodgers (2004) were able to identify more than 17.000 different chemical components in one sample, making it the most complex mixture of organic compounds on earth (Head, Jones, and Röling 2006).

Aromatic hydrocarbons are among the most hazardous contaminants in oil and pose a threat to all living organisms. They are highly toxic, mutagenic and potentially carcinogenic (Menzie, Potocki, and Santodonato 1992). Most abiotically produced hydrocarbons have their origin in oil and coal deposits. Additional introduction of these contaminants into the environment besides oil spills on sea and land are from gas production as well as all downstream

applications like automobile traffic and domestic heating (Johnsen, Wick, and Harms 2005).

The most persistent and environmental problematic hydrocarbons are polycyclic aromatic hydrocarbons (PAHs). The increasing release of PAHs into the environment started with the development of petroleum industries (Kiyohara and Nagao 1978). The major part of PAHs in the environment is of anthropogenic origin (Meckenstock, Safinowski, and Griebler 2004). The need for an enhanced biodegradation in order to remove organic pollutants including hydrocarbons is still rising with the industrialization of processes as well as anthropogenic impacts in daily life. Chemical manufacturers, airports and gas stations, power stations and harbors as well as offshore platforms all have a huge impact on the environment. Oil spills as large as the one following the explosion of the offshore platform Deepwater Horizon in the Gulf of Mexico in 2010 broaden the focus on bioremediation strategies which allow for faster degradation of contaminants in polluted environments as natural degradation processes. The biodegradation of those hydrocarbons is one of the primary mechanisms through which the pollutants are eliminated. However, the complete oxidation of aromatic substrates to CO_2 is a unique ability of microorganisms (Meckenstock, Safinowski, and Griebler 2004; Meckenstock and Mouttaki 2011).

Polycyclic aromatic hydrocarbons are ubiquitous recalcitrant substances that consist of two or more fused aromatic rings. Due to their known toxic, carcinogenic and mutagenic effects as well as their low volatility the microbial degradation is hindered. Their bioavailability is low due to low water solubility of the hydrophobic compounds within their structure (Annweiler, Richnow, et al. 2000; Wilkes and Schwarzbauer 2010). Sorption processes of the contaminant to natural organic matter are further decreasing the aqueous solubility (Coates et al. 1997; Grosser et al. 2000). For the same reasons they are considered a risk to human health (Habe and Omori 2003) and have been identified as priority pollutants by the U. S. Environmental Protection Agency (EPA) (Keith and Telliard 1979).

Aerobic biodegradation of PAHs is fairly well understood (Horvath 1972; Kiyohara and Nagao 1978; Cerniglia 1993; Juhasz and Naidu 2000; Habe and Omori 2003; Johnsen, Wick, and Harms 2005). During aerobic degradation, free oxygen is

used within an oxygenase attack, where the position of the attack is depending on the structure of the compound. During aromatic hydrocarbon degradation a dioxygenase oxidizes the compound to a dihydrodiol and further oxidation steps creating the key metabolite catechol (1,2-dihydroxybenzol) (Okoh 2006). Further degradation steps include the formation of succinate, acetyl-CoA and pyruvate before products are introduced into the citric acid cycle. The available oxygen is a key component to overcome the high resonance energy of the ring structure. Aerobic degradation is very fast compared to the anaerobic degradation, usually depleting all available oxygen and leaving hydrocarbons of a higher molecular weight behind. These then have to be degraded without oxygen via anaerobic degradation pathways. Up to now the anaerobic degradation pathways of aromatic hydrocarbons are only described for a few substances with only one aromatic ring like benzene and toluene as well as naphthalenes.

Naphthalene is a clear organic solid and belongs to the group of PAHs. Its structure consists of two fused benzene rings. As the smallest PAH, it has been in the focus of anaerobic degradation studies and is seen as a model compound for degradation pathways. It is a main ingredient in mothballs and a component of jet fuel, petroleum and tar coals (Tissot and Welte 1984). Naphthalene can undergo electrophilic aromatic substitution (Fujiwara et al. 1976). Naphthalene in the environment is mostly of anthropogenic origin, only a few magnolias, deer and some species of termites are known to be able to produce it; mostly to defend predators. Two fungi of the genus *Muscodor* are able to produce naphthalene as well (Daisy et al. 2002). It has been reclassified as a potential carcinogen due to evidence of carcinogenic activity in rats (Kavlock, Boekelheide, and Chapin 2002; McKee et al. 2004). Naphthalene is mainly used as a precursor to other chemicals for dispersants and tanning agents.

Besides naphthalene there are 23 other PAHs of a higher molecular weight consisting of three or more fused rings that are monitored by the EPA, the European Union (EU) and the EU Scientific Committee for Food (SCF). Out of this list, the three-ringed phenanthrene has been chosen as the main focus of this study as it has been shown to be degradable both aerobically and anaerobically (Zhang and Young 1997; Hayes, Nevin, and Lovley 1999; Habe and Omori 2003; Davidova et al. 2007; J.-L. Li and Chen 2009). Hence it is a

suitable contaminant to see if degradation pathways of PAHs with different molecular weights are comparable to each other and if it is possible to infer a common pathway for all PAHs of a higher molecular weight. Other PAHs as listed in Figure 1-1 have been used as substrates for enrichment cultures to test for the culture's degradation capacity of these contaminants. As the negative impacts of these hydrocarbons increase with their molecular weight, it is important to gain further insights into the microbial degradation processes of PAHs that are composed of a higher number of fused rings in order to remove them from our environment. This is especially true for contaminated groundwater aquifers, which are the basis of our drinking water, as well as for the protection of ecosystems that are negatively impacted by contamination with hydrocarbons.

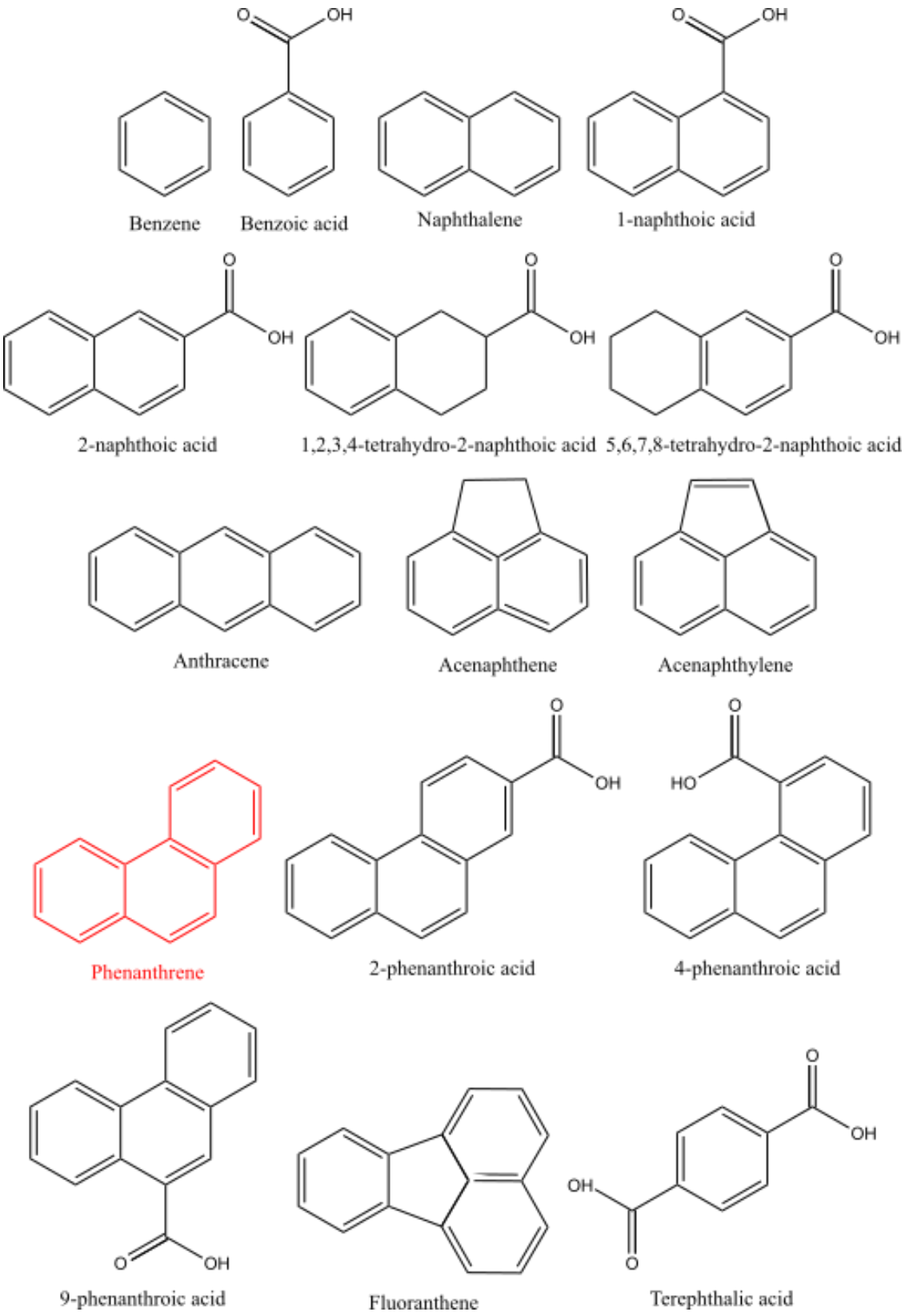


Figure 1-1: All substrates used in this thesis. Marked in red is the substrate of main interest, phenanthrene. Non-substituted PAHs are taken from the EPA-list of PAHs under observation. Substituted PAHs were chosen for likelihood of anaerobic degradation within the culture.

1.2 Anaerobic degradation of non-substituted PAHs

So far, anaerobic degradation processes of PAHs have been described for naphthalene and its derivatives under sulfate- and iron-reducing and methanogenic conditions (Meckenstock et al. 2016). The initial carboxylation reaction for phenanthrene has also been investigated (Zhang and Young 1997; Davidova et al. 2007), though further anaerobic degradation steps remain unknown. Anaerobic degradation of naphthalene and methylnaphthalene has mostly been studied using the two sulfate-reducing cultures NaphS2 and N47 (Meckenstock et al. 2000; Galushko et al. 1999). In Figure 1-2 the degradation pathway for methylnaphthalene and naphthalene are depicted. Methylnaphthalene is activated at the methyl group by fumarate addition through naphthylmethylsuccinate synthase (Meckenstock, Safinowski, and Griebler 2004) and, after activation with CoA, degraded to the central intermediate 2-naphthoyl-CoA through beta-oxidation like reactions (Annweiler, Materna, et al. 2000; Meckenstock et al. 2000; Annweiler, Michaelis, and Meckenstock 2002; Meckenstock et al. 2016). Naphthalene is activated through carboxylation (Zhang and Young 1997; Meckenstock and Mouttaki 2011). After addition of CoA by 2-naphthoate-CoA ligase, the ring system is reduced in successive two electron reduction steps by the new type III aryl-CoA-reductases 2-naphthoyl-CoA reductase and 5,6-dihydro-2-naphthoyl-CoA reductase yielding 5,6,7,8-tetrahydro2-naphthoyl-CoA (Eberlein, Estelmann, et al. 2013; Boll et al. 2014; Meckenstock et al. 2016). Then, the remaining aromatic ring I of the naphthalene skeleton is reduced, most likely to hexahydronaphthoyl-CoA, followed by beta-oxidation like reactions and ring cleavage. The downstream degradation pathway proceeds via cyclohexane derivatives, and the central metabolism is reached via pimeloyl-CoA after the second ring cleavage (Weyrauch et al. 2017).

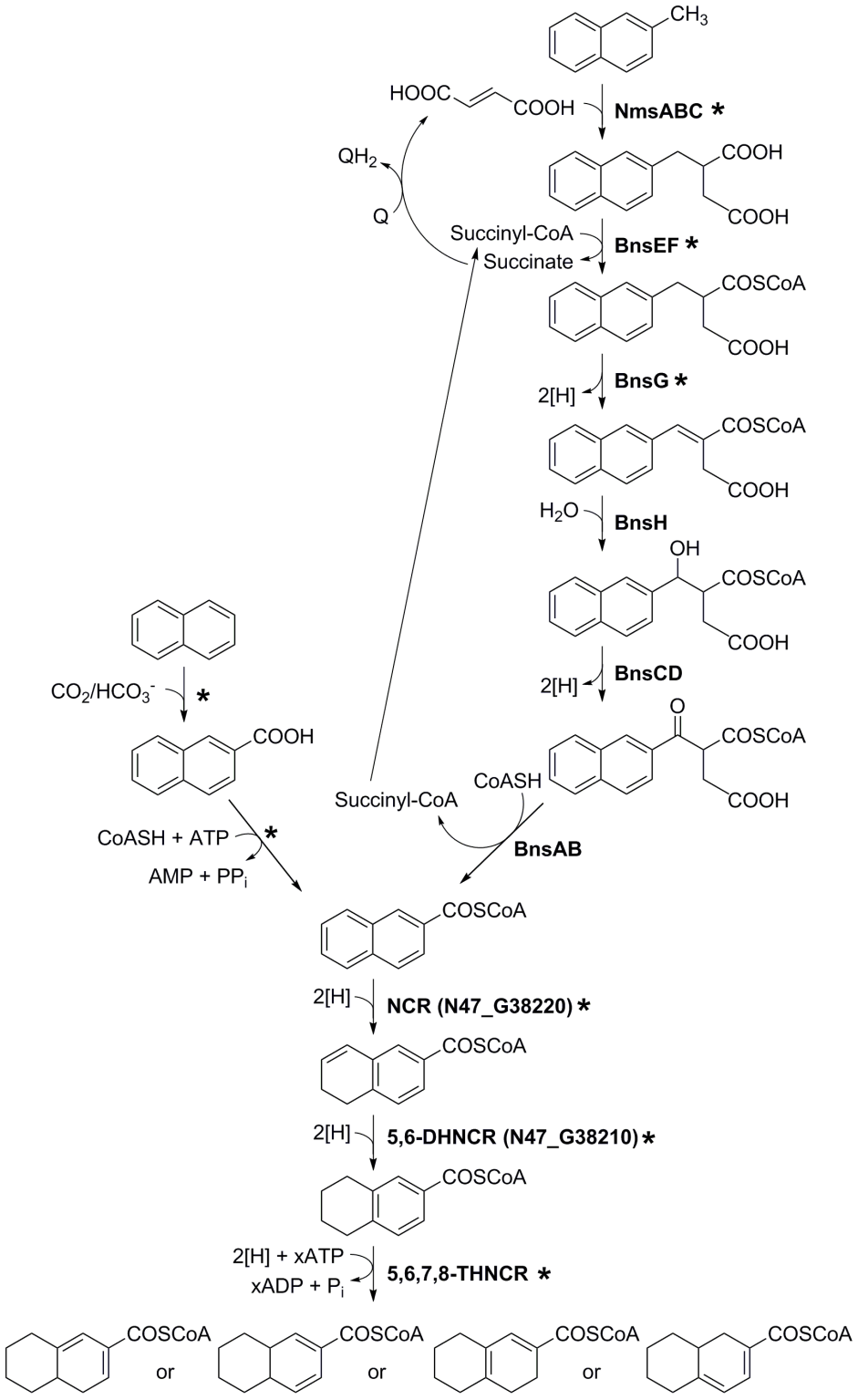


Figure 1-2: Proposed pathways for anaerobic naphthalene and 2-methylnaphthalene degradation in the enrichment culture N47. NmsABC = Naphthyl-2-methyl-succinate synthase; BnsEF = naphthyl-2-methyl-succinate-CoA transferase; BnsG = naphthyl-2-methyl-succinyl-CoA dehydrogenase; BnsH = naphthyl-2-methylene-succinyl-CoA hydratase; BnsCD = naphthyl-2-hydroxymethyl-succinyl-CoA dehydrogenase; BnsAB = naphthyl-2-oxomethyl-succinyl-CoA thiolase; NCR (N47_G38220) = 2-naphthoyl-CoA reductase; 5,6-DHNCR (N47_G38210) = 5,6-dihydro-2-naphthyl-CoA reductase; 5,6,7,8-THNCR = 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase. Reactions which have been identified and the activity measured in N47 cells grown with naphthalene or methylnaphthalene, respectively, are marked with an asterisk. Figure 1-2 was taken from Meckenstock et al. (2016).

So far, anaerobic phenanthrene degradation was reported for two sulfate-reducing cultures (Zhang and Young 1997; Davidova et al. 2007). Production of phenanthroic acid was documented in both cultures, which suggested that phenanthrene undergoes initial carboxylation similar to naphthalene.

For PAHs with more than two fused rings, only the initializing carboxylation reaction of phenanthrene is currently known (Zhang and Young 1997; Davidova et al. 2007). The position of the carboxylation reaction has been indicated to be in the C-2 position (Davidova *et al.* 2007).

The degradation of PAHs composed of more than three aromatic rings has so far only been shown under methanogenic conditions (Christensen et al. 2004; Trably, Patureau, and Delgenes 2003). However, these cultures do not grow with phenanthrene as sole carbon and energy source and hence differ from our novel freshwater culture. Substrates in the methanogenic cultures might rather be co-metabolized than being used as growth substrates (Meckenstock, Safinowski, and Griebler 2004), as opposed to the named naphthalene-degrading cultures that use naphthalene or phenanthrene as sole carbon and energy source.

In order to find more bacteria able to degrade PAHs under anoxic conditions we came up with the assumption, that in a hydrocarbon-rich environment bacteria specialized for this biodegradation should be detectable, which would help to further our understanding in anaerobic degradation of PAHs with a higher molecular weight.

1.3 Natural oil emitting sites

Besides anthropogenically introduced contaminations there are oil seeps on earth where a naturally occurring constant or intermittent stream of oil and its derivatives reaches the subsurface or surface and causes a natural overload of PAHs into the environment. There are many sources for upwelling oil within the oceans, but only a much smaller number on land. Especially for the latter, the oil quickly solidifies once it reaches the surface as the volatile parts evaporate and leave a tar-like mixture behind. One example for such “naturally contaminated” sites are tar lakes. Famous examples are the Rancho La Brea tar pits in the vicinity of Los Angeles, Lake Guanoco in Venezuela, the Carpinteria tar pits in

California, the pitch lake in Trinidad and Tobago, as well as smaller tar seeps in Turkey, Greece, Azerbaijan and Germany. Of all the above mentioned, the pitch lake in Trinidad and Tobago is the biggest natural asphalt deposit on earth. It is part of the UNESCO World Heritage tentative lists because of its outstanding universal value and great social and economic value for the Caribbean Island of Trinidad. For this thesis, all processed and analyzed samples derived from this location which is described in more detail in the following section.

1.3.1 The pitch lake in Trinidad & Tobago

The Trinidad pitch lake is not only the world's biggest natural tar lake situated in La Brea on the Caribbean Island of Trinidad (Trinidad and Tobago), but also the world's biggest source for natural asphalt. It is situated in the south-western peninsula of the Island of Trinidad. The pitch lake is self-refilling and its size from before 1893 decreased to its current size of about 0.36 km^2 by mining of the asphalt (Chaitan and Graterol 1991). Since the beginning of the 21st century the amount of bitumen is being mined sustainably, so the size of the pitch lake is stable at current (Personal communication to Lake Asphalt Trinidad and Tobago). The lake's depth was estimated at around 50 m in 1991 (Chaitan and Graterol 1991) but is expected to be at around 75 m today. Methods to measure the exact depth failed due to underground mass movement destroying the drilling material. It is assumed that the bitumen first reached the surface in Miocene times (Ponnamperuma and Pering 1967). Further, it has been suggested that the bitumen could be of abiotic origin (Dauvillier 1965) due to the uncommon chemical structure of its components, which resemble synthetic mixtures (Ponnamperuma and Pering 1967). The volcanic activities in this area served Dauvillier as a possible explanation for the origin of the bitumen (Dauvillier 1965). Tree trunks as old as 4000 – 5000 years have been found submerged in the bitumen (Ponnamperuma and Pering 1967). Until today the pitch lake is used as a recreational area, where families come to bath in water-filled foldings from the bitumen. Guided tours can be taken at what Trinidadians refer to as the 8^{th} Wonder of the World. The bitumen, which has been mined for export for more than half of a century, is of extraordinary quality and is therefore in great demand from the road surfacing industries. The construction of important airport runways

like JFK in New York and many highways in Germany have been covered with bitumen from the Trinidad pitch lake, at current the main import country is China (Personal communication with Lake Asphalt Company).

Today it is assumed that the bitumen is formed as a mixture of hydrocarbons that emerge under pressure from an underlying oil source rock through small upward leading dykes. This mixture picks up water and clay, mineral matter as well as gas and water and hence turns into bitumen (Figure 1-3). While gaseous components are volatilized, a viscous matter comparable to liquid bitumen forms. There are several spots on the lake where liquid bitumen directly surfaces. These are called “mother of the lake”. Numbers and locations for these active liquid spots are varying. The temperature of upwelling liquid bitumen has been measured at between 32°C and 56°C (Schulze-Makuch et al. 2011).

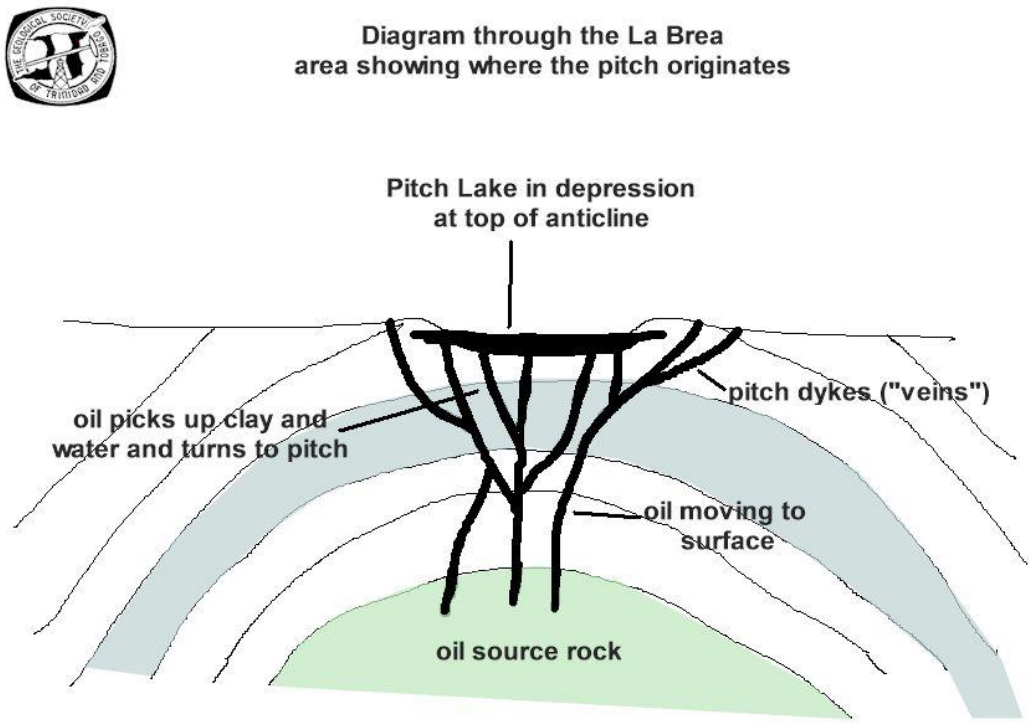


Figure 1-3: Origin of the bitumen. Picture originates from the ecological Society of Trinidad and Tobago (Chaitan and Graterol 1991).

Once the gaseous fraction fumigates, the surface of the pitch lake solidifies and is hard enough to walk on it. The surface is interspersed with foldings from emerging bitumen, which are filled with water, depending on seasonal changes in rainfall. These water reservoirs are home to a variety of algae, small fish and

even caimans. Vulture-like birds called “Corbeaux” are feeding on the pitch lake. Gas bubbles can be observed directly in the bitumen as well as in the water filled foldings. The smell of sulphur is omnipresent on the whole pitch lake. The composition of the bitumen is depicted in Table 1-1 as shown by Attwool and Broome in 1954.

Table 1-1: Composition of the bitumen.

Bitumen	39,3%
Mineral matter	27,2%
Water, etc; volatile at 160°C	29,0%
Water of hydration	3,3%

The main components are identified as asphaltenes, which include molecular substances found in crude oil and along with resins like hydrocarbons and saturates (Schulze-Makuch et al. 2011). The composition of the bitumen is very uniform among the whole lake and especially the content of the bitumen is constant, only decreasing by 1.16% when samples were taken from the margins of the lake, 426 m away from the center.

1.3.1.1 Water droplets in the bitumen are a source of life in oil

Within the soft bitumen of the lake tiny water droplets about the size of only a few microliters have been found. Stable isotope analysis confirmed that these droplets stem from the water body underneath the underlying oil source rock (Meckenstock et al. 2014). If all droplets stem from the same water body, it has been assumed that they have been seeded with a comparable community that should be similar over all droplets according to the seeding hypothesis (for details to both seeding and endpoint hypothesis see section 1.5 below). Through the constant refilling processes of the bitumen, water droplets bud from their common origin and thus are separated from each other. In that way, they are forming tiny, individual habitats, yet remaining in an identical surrounding environment, namely the homogenously composed bitumen. Under these frame conditions, the individual droplets are forming ecological islands as explained in chapter 1.51.4 below. With the unique possibility to gain insights into community assembly over numerous replicates that are undisturbed from factors as dispersal and drift, only

influenced by diversification and selection opens up a window into a yet unknown world.

1.4 DNA-Extraction from difficult samples

In order to observe the diversity within a sample it is most useful to extract as many of the contained bacteria as possible. As only a small number of bacteria is culturable with state-of-the-art methods (Torsvik, Goksøyr, and Daae 1990), a direct DNA extraction is necessary. There is a wide variety of methods for total DNA extractions, most are suitable for a subset of different samples, like environmental samples from e.g. soil, sediments, oil, water and air, and human samples from e.g. blood, skin, feces and lungs. Commercially available kits have been developed for a variety of samples following a standard procedure, allowing for a fast and high throughput handling of standard samples like soils or blood samples. There are also quick and well-working options for environmental samples without the use of a commercial kit, like phenol-chloroform-isoamyl alcohol (PCI) and cetyltrimethylammonium bromide (CTAB) extractions.

One main concern with environmental samples is the amount of humic acids within the sample. Humic acids inhibit downstream applications like PCR and the digestions of the DNA with restriction enzymes (Tebbe and Vahjen 1993). They also cause false values when quantifying the extracted DNA by UV-Vis spectrophotometry. The results overestimate the concentration of nucleic acids in the eluate.

To extract DNA from environmental samples a direct lysis method can be used. This usually yields a higher amount of DNA, especially in soil/sediment samples, as not only bacterial DNA is extracted (Tebbe and Vahjen 1993). In order to separate bacteria from the sample matrix to gain a higher yield of bacterial DNA an extra step can be included in the protocol. For DNA samples within the bitumen this separation step is necessary, as bacteria adhering to the viscous bitumen or oil samples would hardly be affected by the procedure during lysis and purification steps (An et al. 2013). Gentle shaking incubations of a more degraded sample of bitumen did prove useful in a previous study to gain a higher

yield of DNA, but this DNA was inhibited from downstream enzymatic restriction steps.

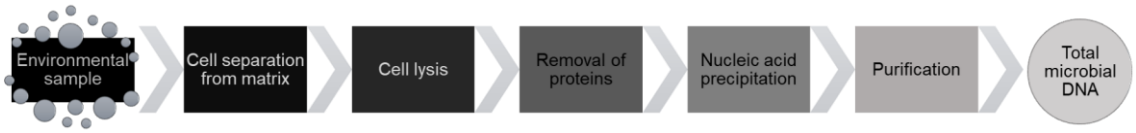


Figure 1-4: General workflow of DNA extractions, adapted from Roose-Amsaleg *et. al.* (2001)

The general workflow for DNA extractions is shown in Figure 1-4, as adapted from Roose-Amsaleg, Garnier-Sillam, and Harry (2001). Following the cell extraction, a cell lysis step is performed. This could be achieved both by mechanical as well as chemical methods. Heating up the viscous tar is preventing the chemicals from getting access to the bacterial cells as the bitumen melts to an almost solid mass, and therefore freeze-thaw-circles are not an option. Mechanical lysis, e.g. bead beating, can have negative effects on the size of the DNA fragments, as the DNA is also sheared by this method. Yet this is the only way to separate cells for further steps in order for the chemicals to get to them. The best amount of bead beating times and duration has to be determined in the process to find a compromise between DNA yield and fragment length. A crucial part is also the removal of protein contained in the sample, which can be done by the use of organic solvents or salting out. The viscosity of oil or tar samples make the whole extraction process far more complex that e.g. soil samples and require the development of a protocol that is suitable for these difficult samples. Within the framework of this thesis I developed a novel extraction method, which is a mixture of a PCI extraction as well as the use of a commercially available DNA extraction kit, which was the only way to get clean DNA that can be processed in the downstream applications up until sequencing.

To gain access to the bacteria within the water droplets, Meckenstock et al. (2014) used a direct PCR approach from each droplet, as the microliter amounts of water did not allow for a general extraction workflow as depicted above. The water droplets were directly used as the template in a nested PCR reaction and amplified with general 16S rRNA primers for a total of 25 cycles, which were then followed by a PCR with sequencing primers for an additional 6 cycles. This method has been adapted for the thesis at hand.

1.5 Island Ecology and Community Formation

Island ecology describes the ecology in habitats naturally or artificially distinct from its surroundings and thus building a separated ecosystem. It can be applied to macro-ecological questions concerning flora and fauna as well as micro-ecological ecosystem assemblies of microorganisms. Common examples for these kinds of habitats are holes within trees (Bell et al. 2005), or islands in a literal interpretation. In microbiology, island ecology is used to infer the development of a whole ecosystem that is with little influence from its surrounding environment, in order to be able to understand bacterial evolutionary processes on a small scale. However, these habitats are rarely found in nature, as hardly any ecosystem or habitat is separated completely from its surrounding environment. Despite this fact it is highly interesting to conduct research in these almost isolated habitats to gain insights into community assembly and evolutionary processes.

Community assembly as a de novo process itself is not yet understood in detail, but there are different theories considering possible options. Although continuous research, it is still not fully understood how complex communities are assembled and different theories considering possible explanations were developed. On the one hand are niche-based theories, where species with a specific function occupy a special niche. On the other hand there is the neutral theory of biodiversity as first stated by Hubbell in 2001. It is based solely on stochastic mechanisms like dispersal, drift and selection via abiotic factors and diversification (Nemergut et al. 2013). The specific function of a species is not considered in the neutral theory. The understanding of community assembly can possibly be derived from the observation and analysis of small ecological islands.

The pitch lake with its underlying water source and the formation of micro droplets is a special and rare embodiment of abundant separated yet ecologically similar natural micro islands.

With a variety of droplets from the same upcoming vein, the differences between droplets that stem most likely from the same water source can be used to look for differences in the community composition. Two theories can be distinguished here. On the one hand there is the endpoint hypothesis, were in all droplets a

very similar community composition can be found due to the droplets similar surrounding, and individual droplets are hard to tell apart. On the other hand, there is the seeding hypothesis, where the initial composition is identical as all droplets are stemming from the same water body, but the community shifts according to speciation processes such as adaptation to utilize high hydrocarbon concentrations within the individual droplets (Figure 1-5).

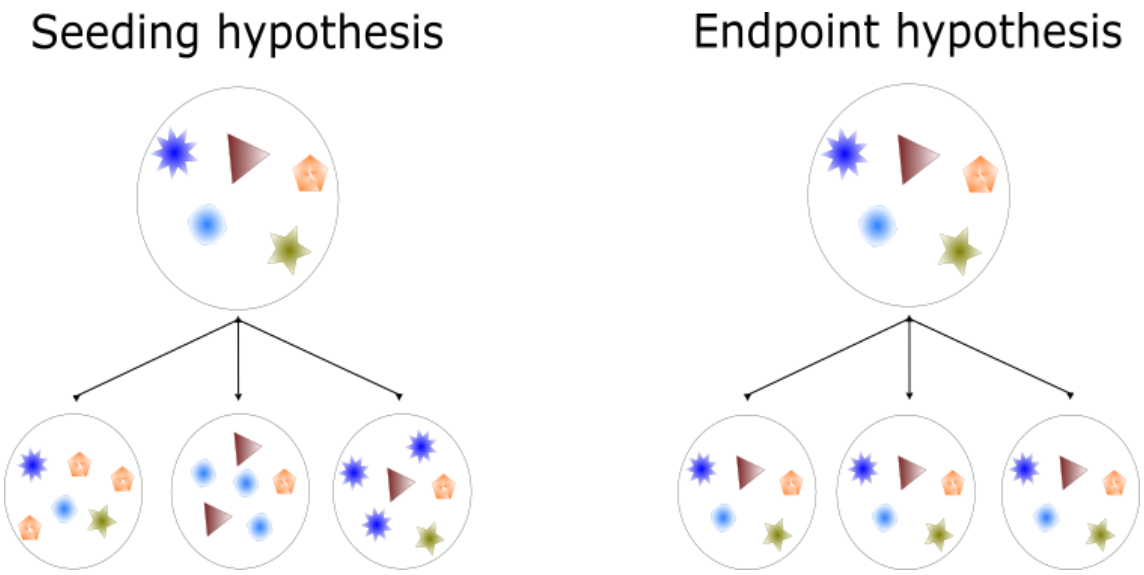


Figure 1-5: Seeding and endpoint hypothesis, schematic view. Symbols represent individual microorganisms. The seeding hypothesis follows neutral assembly, the endpoint hypothesis a deterministic assembly, where all communities end up in a similar composition

As neither strictly neutral nor strictly deterministic (no randomness involved) processes could be confirmed for all assembly processes, community assembly is most probably a mixture of both hypotheses, where the seeding community is identical based on the water body, and a core community of bacteria necessary. In this example for the degradation of the surrounding hydrocarbons, can be found in all droplets. The bacteria in the community with traits not necessary for the retrieval of nutrients can and might be obsolete and are possible candidates for either speciation or extinction. These generalists that exist alongside the core community can therefore differ between the droplets. (Figure 1-6).

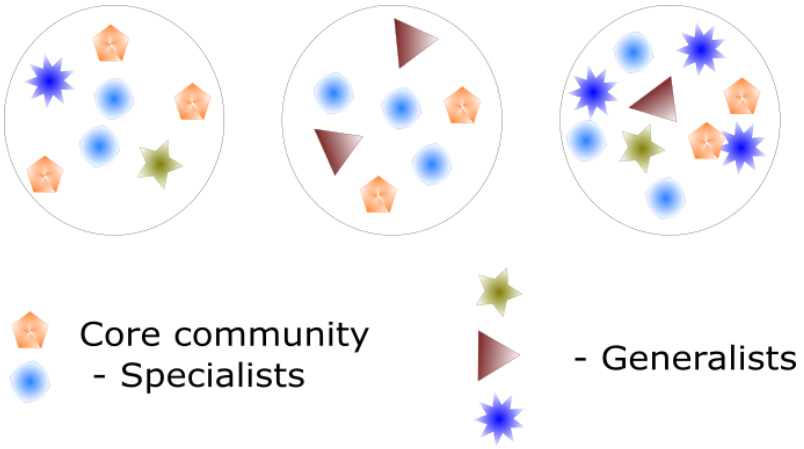


Figure 1-6: Schematic view of microbial communities assembled from a common seed bank. Core community (here probably bacteria able to degrade PAHs) can be found in all droplets, generalists are varying between the droplets.

The bacteria that are not involved for the degradation of PAHs are most likely metabolizing the products that the specialists produced during the degradation of PAHs.

1.6 Objectives

The main hypothesis of this dissertation was that bacteria living in a hydrocarbon rich environment like the pitch lake should be able to degrade high molecular weight PAHs under anoxic conditions. In order to gain knowledge about the community within the bitumen, total DNA had to be extracted. As the DNA extraction from these samples is not as straightforward as common soil samples and commercial kits cannot be used, a new protocol suitable for these special samples had to be developed. Furthermore, an enrichment culture from the pitch lake growing on the PAH of interest, phenanthrene, was set up and investigated for bacteria able to degrade this PAH under anoxic conditions.

The second hypothesis was that the water droplets from within the bitumen contain highly specialized bacteria from a subset of bacteria in the bitumen. The DNA sequences detected in water droplets were to be compared to those from the total bitumen DNA and also to the species within the enrichment culture.

The third hypothesis was that anaerobic degradation pathways of PAHs of a higher molecular weight are similar to the elucidated pathway of naphthalene. Therefore, the enrichment culture growing on phenanthrene was to be

characterized to elucidate degradation steps by various biochemical methods including enzyme assays and metabolite analyses.

The overarching goal was to advance our understanding anaerobic PAH degradation.

2 Material and Methods

2.1 Site description and sampling at the Trinidad pitch lake

The sampling site for the bitumen samples was the pitch lake in Trinidad, located near La Brea at the southern part of the Island of Trinidad, Trinidad & Tobago.

Figure 2-1: Map of Trinidad and the pitch lake (Trinidad & Tobago)

Samples have been taken from the south western part of the lake, close to the “Mother of the lake”, where mostly fresh bitumen comes up, which is still soft and almost impossible to walk on. The idea behind this sampling spot was that the water droplets are coming up with the fresh bitumen, which has not been exposed to the surface. Also, the composition of the bitumen has only been changed in moderately by evaporation of the volatile parts of the oil. All the sampling spots can be seen in Figure 2-2.

Figure 2-2: Pitch lake satellite picture (retrieved from Google Maps) with sampling spots 1 to 3.

Sampling spots were from 1) a 1 m deep hole created by an excavator as close as possible to the softest areas where the excavator was still able to work safely. The temperature of the bitumen in this area was measured at 33°C. Ten jam jars were filled with liquid bitumen that came up through thin fissures and gassed with nitrogen gas to keep the samples anoxic. Further on nine 205 mL Schott flasks have been filled with around 40 mL of this liquid bitumen. Two of these were also filled with 50 mL of n-Hexane to stop any biochemical reactions as blanks for methane measurements. The liquid bitumen was taken up with syringes which ends have been cut off to allow for a wider opening. While taking samples upcoming gas bubbles were visible.

In spot 2) the temperature of the bitumen was 27°C, this site was slightly higher than the level of the pitch lake itself. Liquid bitumen was coming out of small vents and is flowing down onto the lake. Four samples have been taken from that spot.

Spot 3) was a single soft spot of bitumen surrounded by hardened bitumen. The temperature here was measured at 36°C. Upcoming gas bubbles were also visible in that spot.

All samples were shipped to the Institute of Groundwater Ecology, Helmholtz Zentrum Munich, Germany for further extraction of droplets.

2.2 Chemicals, biochemical and gases

Chemicals used during this dissertation were purchased from AppliChem (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), Merck KGaA (Darmstadt, Germand), Carl-Roth (Karlsruhe, Germany), Sigma Aldrich (St. Louis, MO), and GE Healthcare Europe (Freiburg, Germany) in *p.a.* quality. Biochemicals were ordered from Bio-Rad Laboratories (Hercules, CA), Life Technologies (Carlsbad, CA), Promega (Fitchburg, WI), Qiagen (Hilden, Germany), Thermo Fisher Scientific (Waltham, MA), Biomers (Ulm, Germany), Roche (Basel, Switzerland) and 5Prime (Hamburg, Germany). Nitrogen gas (99.999%) and Biogon® (C20 E941/E29; carbon dioxide 20% ± 2%, rest nitrogen) were purchased from Linde AG (Pullach, Germany).

2.2.1 Media and Buffers

All solutions and media were prepared with MilliQ water (Merck KGaA, Darmstadt, Germany). Glass ware for growth media was washed with 1 M HCl, distilled water and MilliQ water prior to media preparation to remove traces of cleaning agents. All media and heat resistant solutions as well as autoclavable equipment were autoclaved prior to usage at 120°C for 45 minutes. Heat sensitive solutions were filtrated through a 22 µm filter. Equipment that cannot be autoclaved was sterilized under UV light for 15 minutes. Glass ware was heated to 180°C in dry heat for 2 hours. All anaerobic stock solutions were flushed with Biogon® (N_2/CO_2, 80:20 [v/v]) for at least 20 minutes after autoclaving.

2.2.1.1 Medium for the cultivation of TRIP1

The TRIP1 enrichment culture was enriched from soil of the pitch lake in Trinidad under anaerobic and sulfate-reducing conditions. The sole carbon and energy source was phenanthrene. Medium preparation and inoculation took place under strict anoxic conditions. The culture was stored at 30°C to mimic ambient temperatures of the pitch lake.

The TRIP1 enrichment is only the third enrichment culture growing on phenanthrene as sole carbon and energy source and the first culture enriched from a fresh water environment.

The medium was prepared from a 50X stock solution of the freshwater medium with sodium sulfate to a final concentration of 20 mM or 8 mM, respectively.

Table 2-1: Anaerobic freshwater medium without supplements.

	Amount
Stock Solution (50X)	14 mL
Na_2SO_4	1.98 g (20 mM) or 0.79 g (8 mM)
MilliQ	660 mL

The medium was incubated at 120°C for 90 minutes and subsequently flushed with Biogon® until cooled to RT. During the cool down process, resazurin, trace-elements (Table 2-3) and selenite-tungsten solution (Table 2-5) were added. The medium was aliquoted into serum bottles, sealed with butyl stoppers (Glasgerätebau Ochs, Göttingen, Germany) and aluminum crimp covers and autoclaved over night in a nitrogen atmosphere. Other supplements were added after autoclaving from sterile and anoxic stock solutions (Table 2-6).

Table 2-2: Stock solution (50X) of the anaerobic freshwater medium.

	Weighed-in quantity
NaCl	50 g/L
$MgCl_2 \cdot 6\ H_2O$	20 g/L
KH_2PO_4	10 g/L
NH_4Cl	12.5 g/L
KCl	25 g/L
$CaCl_2 \cdot 2\ H_2O$	7.5 g/L

Table 2-3: Trace elements SL10 (1000X; Widdel, Kohring, and Mayer (1983)).

	Weighed-in quantity
$FeCl_2 \cdot 4\ H_2O$	1500 mg/L
$ZnCl_2$	70 mg/L
$MnCl_2 \cdot 4\ H_2O$	100 mg/L
$CoCl_2 \cdot 6\ H_2O$	190 mg/L
$CuCl_2 \cdot 2\ H_2O$	2 mg/L
$NiCl_2 \cdot 6\ H_2O$	24 mg/L
$Na_2MoO \cdot 2\ H_2O$	36 mg/L
H_3BO_3	6 mg/L
HCl (25%)	10 mL/L

Table 2-4: Vitamin solution VL-7 (1000X; Pfennig (1978)).

	Weighed-in quantity
Cyanocobalamin (B12)	10 mg / 200 mL
p-Aminobenzoate	10 mg / 200 mL
$D(^+)$-Biotin	2 mg / 200 mL
Nicotinate	20 mg / 200 mL
Ca-$D(^+)$-Pantothenate	5 mg / 200 mL
Pyridoxamine dihydrochloride (B6)	50 mg / 200 mL
Thiamine dihydrochloride (B1)	10 mg / 200 mL

Table 2-5: Selenite-tungsten solution.

	Weighed-in quantity
NaOH	500 mg/L
$Na_2SeO_3 \cdot 5\ H_2O$	3 mg/L
$Na_2WO_4 \cdot 2\ H_2O$	4 mg/L

Table 2-6: Supplements added to the anoxic medium.

Stock solution	Weighed-in quantity	Final concentration in the medium
1 M $NaHCO_3$	12.6 g / 150 mL	30 mM
0.5 M $Na_2S \cdot 9\ H_2O$	6 g / 50 mL	0.5 mM
0.4% Resazurin	0.4 g / 100 mL	0.0004%
1,5% Phenanthrene in HMN	1.5 g / 100 mL	

Table 2-7: Supplemented medium for the anaerobic cultivation of TRIP1.

	Added amount of Stock solution
Carbonate buffer	30 mL/L
Reducing Agent (Na_2S, Table 2-6)	1 mL/L
Vitamin solution (Table 2-4)	1 mL/L
Trace elements (Table 2-3)	0.5 mL/L
Selenite-tungsten solution (Table 2-5)	0.5 mL/L
Resazurin (Table 2-6)	1 mL/L
Phenanthrene in HMN (Table 2-6)	20 mL/L

For substrate and terminal electron acceptor tests within this enrichment culture a variety of substrates were used as additives for the medium.

Table 2-8: Substrates used for substrate tests in TRIP1.

Substrate	Final concentration in the medium	Comment
2-Phenanthroic acid	100 µM	1 molecular equivalents NaOH
4-Phenanthroic acid	100 µM	1 molecular equivalents NaOH
Terephthalic acid	100 µM	2 molecular equivalents NaOH
1-Naphthoic acid	1 mM	5 mM stock solution
2-Naphthoic acid	1 mM	5 mM stock solution
1,2,3,4-Tetrahydronaphthoic acid	1 mM	5 mM stock solution
5,6,7,8-Tetrahydronaphthoic acid	1 mM	5 mM stock solution
Benzoic acid	1 mM	5 mM stock solution
Benzene	1 mM	5 mM stock solution
Naphthalene	20 mL / L	1,5 % in HMN
Acenaphthylene	100 µM	Solubilized in acetone
Acenaphthene	100 µM	Solubilized in acetone
Anthracene	100 µM	Solubilized in acetone
Fluoranthene	100 µM	Solubilized in acetone

To test the culture for growth on different terminal electron acceptors these were added to medium prepared without Na_2SO_4 as shown in Table 2-9.

Table 2-9: Terminal electron acceptors for growth tests in TRIP1.

Terminal electron acceptor	Final concentration in medium
Sulphur	In excess
Iron (III) Ferrihydrite	80 mM
Nitrate	5 mM
Without terminal electron acceptor	

Sulphur should not be heated above 110°C and was added after autoclaving from an anoxic stock solution. As sulphur can not be solubilized it was added to the medium in excess. The cultures with nitrate as terminal electron acceptor and the culture without terminal electron acceptors were not supplemented with Na_2S as reducing agent.

2.2.2 Preparation of cell-free extracts

2.2.2.1 Cell harvesting and preparation of cell-free extracts

Two 1.6 L cultures were separated from the HMN phase in a separation funnel in an anaerobic chamber under N_2-atmosphere. After centrifugation for 30 min at 10,000 × g and 4°C, cell pellets were resuspended with 0.5 mL, 100 mM MOPS buffer (3-(N-morpholino)propanesulfonic acid, pH 7.3. The cells were collected in an Eppendorf cup and centrifuged again for 15 minutes at 13,000 × g, 4°C, and the pellet resuspended in MOPS buffer. The cells were opened in a French press (Thermo Electron, Waltham, USA) and centrifuged for 15 min at 4°C, 19,000 × g. Enzyme assays were composed according to Table 2-26. The assay was started by adding the substrate and incubated at 30°C and 900 × g in a Thermomix Block (ThermoMixer® C, Eppendorf, Germany). Samples (40 µL) were mixed with double volume of methanol to stop the reaction, centrifuged again at 4°C, 13,000 × g for 15 minutes and transferred to LC-Vials. The supernatant after this centrifugation step is the cell-free extract (cfe). All transfer steps were carried out in the anaerobic chamber. The cfe can be stored at 4°C for only a few hours.

2.3 Molecular Methods

2.3.1 Polymerase chain reaction (PCR)

A PCR is used to amplify DNA in a cycling program that allows DNA denaturation, annealing of primers and elongation steps and is repeated according to its aim and needed copies of the original DNA segment. Different types of PCR have been used in this study.

Primers are short DNA strands, about 18 – 22 bp in length, constructed to anneal to the existing copy of DNA and targeting a specific region of this DNA. They are needed as a starting point for every PCR. A list of primers used in this thesis can be found in Table 2-10.

Table 2-10: PCR Primers used in this study.

Name	Sequence	Targeted region	Reference
B27f	AGAGTTTGATCMTGGCTCAG	Bacterial 16S rRNA	(Edwards et al. 1989)
B27f (-FAM)	FAM-AGAGTTTGATCMTGGCTCAG	Bacterial 16 S rRNA with FAM-label	(Edwards et al. 1989)
519r	TATTACCGCGGCKGCTG	Bacterial 16S rRNA reverse	(Lane 1991)
344f	AYGGGGYGCASCAGGSG	Archael 16S rRNA	(Stahl and Amann 1991)
915r	GTGCTCCCCCGCCAATTCCT	Archael 16S rRNA	(Stahl and Amann 1991)
MID13-MID17	CGTATCGCCTCCCTCGCGCCA	Bacterial 16S rRNA	

For each PCR reaction the following buffers and components have to be pipetted into a Master Mix for all reactions.

Table 2-11: Pipetting scheme for each PCR reaction (Master Mix).

Reagent	Concentration	Amount per DNA template
H_2O		Add to 50 µL
Taq-Buffer	10X	5.0 µL
$MgCl_2$	25 mM	3.0 µL
BSA	20 µg/µL	0.5 µL
dNTPs	10 mM	0.5 µL
Forward Primer	50 µM	0.3 µL
Reverse Primer	52 µM	0.3 µL
Taq-Polymerase	5 U/µL	0.25 µL

The 1 µL DNA-template is not included in the Master Mix but will be added into the 0.5 mL Eppendorf Tube individually. In Table 2-12 you can see the thermal profile for the PCR cycler:

Table 2-12: Thermal profile for PCR Cycler.

Temperature	Duration	Cycles
94°C	5 min	
94°C	30 sec	}
52°C	30 sec	} 25 – 30 cycles
70°C	60 sec	}
70°C	5 min	
8°C	Hold	

2.3.1.1 Droplet PCR

To amplify the minute amount of DNA available in the water droplets extracted from bitumen samples a nested PCR has been done. This means that a PCR with the common 16S rRNA primers is done for 25 cycles, and the in this way amplified DNA will be used as template for a 6 cycle DNA with adequate sequencing primers (MID-Primers, as shown in Table 2-10).

2.3.1.2 T-RFLP PCR

The PCR in preparation for a T-RFLP analysis is performed with FAM-labelled primers, as can be seen in Table 2-10. All conditions are similar to the PCR

described in 2.3.1. To avoid primer artifacts not more than 25- 30 cycles should be done.

2.3.2 Terminal restriction fragment length polymorphism (T-RFLP)

For a T-RFLP analysis the amplified DNA (2.3.1.2) is undergoing gel-electrophoresis (2.3.8) to analyze the functionality of the PCR with FAM-labels, a purification step (2.3.7), a restriction with MspI restriction enzyme (2.3.4) and desalting of the digested DNA products (2.3.5). The so prepared samples are loaded onto a 96-well PCR plate (Sigma-Aldrich, St. Louis, MO) with a HiDi-Formamide mixture with a 1/300 dilution of MapMarker-1000 ROX size standard. The samples are thereafter denatured in a thermal Cycler (Eppendorf, Hamburg, Germany) for 5 min at 95°C and placed on ice immediately after the denaturing step. Fragments were analyzed on an ABI 3730 Fragment Analyzer (Thermo Fisher, Waltham, MA). Analysis of the fragment data took place with the Gene Mapper software (Applied Biosystems).

2.3.3 Fluorescence in situ Hybridization (FISH)

Fluorescence in situ Hybridization is labelling microorganisms in situ with oligonucleotides, to which a fluorescent label is attached. This will emit light when activated at the correct wavelength. Choosing an oligonucleotide specific to a bacterial DNA sequence was done with the ARB Software Package (Ludwig et al. 2004). A probe designed to match only the dominating bacterium in the culture was created as well as a probe for a broader match for *Desulfobacteriaceae*, which should include all *Desulfobacteriaceae* within the culture. The probes were retrieved from Biomers (Ulm, Germany). To label microorganisms within the culture two different methods were used as described below (2.3.3.1; 2.3.3.2).

Table 2-13: Oligonucleotides used in this study.

Probe	Fluorescent Label	Sequence
TRIP-Desulfo_183	6-FAM	GACCAAAGUCUCUUGGAC
TRIP-Desulfo_1430	Cy3	GUUAGCCCAGCACCUUCU

2.3.3.1 FISH on microscopic slides

To add the oligonucleotides to microorganisms the latter have to be fixed in 4% formaldehyde at least over night after taking samples from the culture. Fixed cells can be stored at 4°C until further use. For staining 15 to 20 µL of the fixed cells were pipetted onto a microscopic slide and dried in an oven at 46°C for 1 hour. Slides are being dried after this incubation step in rising concentrations of ethanol (50%, 80% and 100%) for 3 minutes in each vessel. A subsequent short drying period in the oven followed. The slides are incubated with hybridization buffer (Table 2-14) plus 10 µL of the respective probe in a Falcon tube (Thermo Fisher) for at least 2 hours at 40°C for probe 183 and 46°C for probe 1430 (Pernthaler et al. 2001). During incubation a wet piece of paper is added to the Falcon tube to prevent drying out of the solution.

Table 2-14: Hybridization buffer for FISH staining.

Stock reagent	Volume	Final concentration in buffer
5 mM NaCl	18 µL	900 mM
1 M Tris/HCl	2 µL	20 mM
Formamide	55%	% depending on Probe
Distilled H_2O	Add to 100 µL	
1% SDS	2 µL	0,02%

After the hybridization excess label and reagents were washed off by incubation with wash buffer (Table 2-15). This also took place in 50 mL Falcon tubes. This time the slides were fully submerged in the buffer. Incubation was performed in a water bath at 35°C for 20 minutes. Slides are subsequently rinsed with MilliQ water and air-dried. To conserve the fluorescent label all steps after the hybridization should be carried out in the dark. Slides were stored in the freezer until use. Prior to microscopy all samples were stained with DAPI. Depending on the probe the labels were visible under specific wavelengths in the UV-range.

Table 2-15: Wash buffer for FISH staining.

Stock reagent	Volume	Final concentration in buffer
5 M NaCl	640 µL	Concentration depending on Formamide concentration
1 M Tris/HCl	1 mL	20 mM
0.5 M EDTA	500 µL	5 mM
MilliQ H_2O	Add to 50 mL	
10 % SDS	50 µL	0.01%

2.3.3.2 Liquid FISH

For a liquid FISH staining, where the cells are not fixed on a microscopic slide but left in liquid for downstream application of flow cytometry, there are a few differences in the protocol based on (Wallner, Erhart, and Amann 1995, modified by Thelen 2002). 10 µL of PFA fixed cells were added to a 1.5 mL microcentrifuge tube and dried at 46°C. After the addition of 80 µL ethanol (96%) the tubes were vortexed and centrifuged at 4000 g for 2 minutes. The supernatant was discarded and the cells dried again at 46°C. 50 µL of hybridization buffer (Table 2-14) and 5 µL of the oligonucleotide probe were added to the cells and incubated for 3 hours at 46°C. Centrifugation of the microcentrifuge tubes at 4000 × g for 2 minutes and discarding the supernatant made the cell pellet available for the addition of 100 µL of wash buffer (Table 2-15). Incubation took place for 30 minutes and 46°C. After a last centrifugation step under the above-mentioned conditions the cells were resuspended in 100 µL buffer PBS (Thermo Fisher Scientific).

2.3.4 Restriction of DNA

DNA amplicons were restricted for T-RFLP analysis following a purification step (2.3.7). Optimal amplicon quantities for the analysis should be in the range of 50 – 100 ng.

Table 2-16: Restriction of amplicons for T-RFLP analysis.

Reagent	Amount added per restriction
Restriction enzyme (MspI)	0.3 µL
10X Buffer incl. BSA	1 µL
H_20	Add to 10 µL

The reaction tubes were incubated for 2 h at 37°C in a PCR Cycler. Digests were stored frozen.

2.3.5 Desalting of DNA

The digested amplicons (2.3.4) need to be desalted for further downstream applications. This was carried out with the DyeEx Spin columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All centrifugation steps were carried out at 2800 × g and RT for 3 min. The resin in the spin columns was resuspended by vortexing, the bottom closure was snapped off and the cap loosened by a quarter turn to allow outflow of buffer during the first centrifugation step. The flow through was discarded and the digested amplicons added carefully to the resin. The spin column was placed in a new 1.5 mL microcentrifuge tube to collect the desalted restricted amplicons.

2.3.6 Isolation of DNA

2.3.6.1 Isolation of DNA from cultures

This protocol is based on Lueders, Manefield, and Friedrich (2003) and Gabor *et al.* (2003).

A bead beating tube was prepared by adding 0.2 mL of a 1:1 mix of 0.1 mm and 0.7 mm Zirconium/Silica beads to a 2 mL microcentrifuge tube with screw cap and autoclaved before further use. The cell pellet extracted from the enrichment culture was added to the bead beating tube together with 650 µL of buffer PTN (pH 8, Table 2-17) and this mix was vortexed for 2 to 3 minutes. 200 µL of 20% SDS (Table 2-19) were added as well as 100 µL of Phenol/Chloroform/Isoamyl alcohol (PCI, 25:24:1) at pH 8. A bead beating step was carried out for 45 sec at 6 m/s. Subsequent centrifugation at 7500 × g at RT for 5 minutes gave about

750 µL of supernatant which was transferred to a new 2 mL microcentrifuge tube placed on ice. One volume PCI was added and the DNA extracted by vigorous manual shaking. After a next centrifugation step at 4°C 800 µL supernatant were placed in a prepared “Phase Lock Gel Heavy” tube (Eppendorf), following a manual extraction with one volume Chloroform/Isoamyl alcohol (CI, 24:1) with a subsequent centrifugation step for 4 minutes at 4°C and 14.000 × g. About 650 µL of supernatant were transferred to a new 2 mL microcentrifuge tube and mixed with 2 volumes PEG (Table 2-20), which was incubated at 4°C for 2 hours. Precipitation took place while centrifugation for more than 30 minutes at 14.000 × g and RT, before PEG was gently removed with a pipette. Addition of 500 µL of cold (-20°C) 70% ethanol was used to wash the pellet before another centrifugation step for 5 minutes at 14.000 × g and 4°C was performed. The ethanol is removed carefully by pipetting and the DNA air dried briefly for not more than 10 minutes at RT. Elution was done with buffer EB (Table 2-21) in the desired amount for downstream applications and depending on yield by flipping before transferring the extracts to 0.5 mL microcentrifuge tubes and storing at -20°C until further use.

Table 2-17: Buffer PTN for DNA extraction, adjusted to pH8 with HCl.

Reagent	Final concentration in buffer
$NaPO_4$	120 mM
Tris	125 mM
NaCl, pH 8	0.25 mM
Na_2HPO_4	16.02 g/L
NaH_2PO_4	0.86 g/L
Tris-HCl	11.2 g/L
Tris-Base	6.6 g/L

Table 2-18: TE (pH 8) for DNA extraction.

Reagent	Final concentration in buffer
Tris-HCl	10 mM
EDTA	1 mM

Table 2-19: 20% SDS for DNA extraction.

Reagent	Weighed-in quantity
SDS	20 g
H_2O	100 mL

Table 2-20: 30% PEG for DNA extraction.

Reagent	Weighed-in quantity
Polyethylene Glycol 6000	150 g
NaCl	46,76 g
RNAse free water	Add to 500 mL

Table 2-21: Buffer EB for DNA extraction.

Reagent	Final concentration in buffer
Tris pH 8.5	10 mM
RNAse free water	

2.3.6.2 Isolation of genomic DNA from bitumen

To isolate DNA from bitumen a wide variety of methods have been tested to deal with the adhesion of humic acids from the bitumen directly to the DNA, which makes downstream processes impossible. The only protocol working for this kind of samples so far was a combination of the well-known Phenol-Chloroform extraction method and the use of the MoBio PowerSoil® DNA-Isolation Kit. All centrifugation steps were carried out at 10.000 × g, during the Phenol-Chloroform extraction cooled at 4°C, during Kit use at RT.

Bead beating tubes were prepared as in 2.3.6.1. Around 0.8 g of bitumen were put into prepared bead beating tubes. 300 µL of Miller phosphate buffer (Table 2-22) and Miller lysis buffer (Table 2-23) were added to the bead beating tube along with 600 µL of PCI (25:24:1). Contents were mixed by flicking and inverting. Cell lysis was done in the bead beater for 45 seconds at 5.5 m/s. Subsequent centrifuging of the tubes yielded around 560 µL of aqueous supernatant, which was transferred to a “Phase Lock Gel Heavy” tube (Eppendorf). One volume of CI (24:1) was added followed by mixing by inverting for 5 seconds followed. Another centrifugation step yielded two layers, whereas the upper layer was

transferred to a new 2 mL microcentrifuge tube. From this point on the PowerSoil® DNA Isolation Kit was used. To the top layer from the previous centrifugation step double the Volume of Solution C4 was added to the tube and mixed by inverting for 5 seconds. The spin filters from the kit were loaded with a 650 µL aliquot and centrifuged for 30 seconds. The flow through was discarded. This step was repeated until the remainder of the mixture was used up. 400 µL of Solution C5 was added to the spin filter and centrifuged again for 30 seconds. The flow through was discarded and the spin filter centrifuged for 1 minute to remove an excess of the Solution C5. The spin filter was transferred to a new 2 mL tube and 30 µL of Solution C6 were added for elution of DNA from the filter matrix. A 30 second centrifuge step was carried out; the elution steps were repeated if necessary. Eluted DNA was stored at -20°C.

Table 2-22: Miller phosphate buffer, pH 8.0.

Reagent	Final concentration in buffer
NaH_2PO_4	100 mM

Table 2-23: Miller SDS lysis buffer.

Reagent	Final concentration in buffer
NaCl	100 mM
Tris, pH 8.0	500 mM
SDS	10 %

2.3.7 Purification of DNA

Purification of DNA samples was done with a 5Prime PCRExtract Kit according to the manufacturer's instructions. All centrifugation steps took place at 13.000 × g for 1 min at RT. For that a PCRExtract column CB2 was placed into a 2 mL Collection Tube and 500 µL of buffer BL were added. After a centrifugation step the flow through was discarded. The amplified DNA was mixed with 5 volumes of buffer PD and added to the column. After a 2 min incubation step the columns were centrifuged and the flow through discarded. 700 µL buffer PW were added and centrifuged again after 2 to 5 min incubation. This step was repeated with 500 µL. Two additional centrifugation steps were added to remove residual

buffer PW. The column was allowed to air dry for several minutes before placing it into a 1.5 mL microcentrifuge tube. The desired amount of buffer EB (usually 25 µL) was added to the column to elute the DNA from the column matrix. The eluted DNA was transferred into a 0.5 mL tube for the following restriction step.

2.3.8 Separation of DNA by agarose gel electrophoresis

In order to check extracted or amplified DNA for correct length, integrity and quality and agarose gel electrophoresis was performed. DNA was mixed with 3X Loading Dye (Thermo Fisher Scientific) and added to a pocket of a 1.2% (w/v) agarose gel. The gel was placed in a gel chamber along with 1X TEA buffer (Sigma Aldrich). Separation took place at 80 to 120 V for 40 to 60 minutes. DNA size and concentration were estimated in comparison with GeneRuler™ 1kb DNA Ladder (Thermo Fisher Scientific). After the run the gel was stained for 10 minutes in GelRed™ (Biotium, Hayward, CA) and visualized under UV-light at a wavelength of 312 nm.

2.3.9 Gel-extraction of DNA

As a means to purify DNA the appropriate band can be cut out of the agarose gel and purified with the 5Prima PCR Extract kit as described under 2.3.7.

2.3.10 DNA Sequencing

DNAs (30 to 250 ng) were sonicated to a 100- to 800-bp size range using the E210 Covaris instrument (Covaris, Inc., USA). Fragments were end-repaired, then 3`-adenylated, and Illumina adapters were added by using NEBNext Sample Reagent Set (New England Biolabs, USA). Ligation products were purified by Ampure XP (Beckmann Coulter, USA) and DNA fragments (>200 bp) were PCR-amplified using Illumina adapter-specific primers and Platinum Pfx DNA polymerase (Invitrogen, USA). BIC AOSF Amplified library fragments were size selected on 3% agarose gel around 300 bp. After library profile analysis by Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and qPCR quantification (MxPro, Agilent Technologies, USA) BIC AOSF library was sequenced using 101 base-length read chemistry in a paired-end flow cell V3 on the Illumina

Hiseq2000 sequencer*//*(Illumina, USA) in order to obtain overlapping reads and generate long reads of 180 bp. version RTA 1.13.48

2.3.10.1 Nextera Mate Pair library preparation and Sequencing

The three mate pair libraries were prepared following Nextera protocols (Nextera Mate Pair sample preparation kit, Illumina). Briefly, genomic DNA was simultaneously enzymatically fragmented and tagged with a biotinylated adaptor. Fragments were size selected (3-5, 5-8 Kb and 8-11 Kb) through regular gel electrophoresis and circularized overnight with a ligase. Linear, non-circularized fragments were digested and circularized DNA was fragmented to 300-1000 bp size range using Covaris E210. Biotinylated DNA was immobilized on streptavidin beads, end-repaired, then 3`-adenylated, and Illumina adapters were added. DNA fragments were PCR-amplified using Illumina adapter-specific primers and then purified. Finally, libraries were quantified by qPCR and libraries profiles were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Each library was sequenced using 150 or 250 base-length read chemistry on a paired-end flow cell on the Illumina MiSeq (Illumina, USA).

BIC AOSN 8-11Kb library was sequenced using 151 base-length read chemistry in a paired-end flow cell on the Illumina MISEQ sequencer*//*(Illumina, USA) in order. Version RTA 1.18.54

2.3.11 Sequence analysis with SILVAngs

All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al. 2013). Each read was aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) (Pruesse, Peplies, and Glöckner 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al. 2013). Reads shorter than 250 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artefacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis. After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered (OTUs), on a per

sample basis, and the reference read of each OTU was classified. Dereplication and clustering was done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit) (W. Li and Godzik 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 128; http://www.arb-silva.de) using blastn (version 2.2.30+) with standard settings (Camacho et al. 2009). The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. This yields quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function (% sequence identity + % alignment coverage)/2 did not exceed the value of 93, remain unclassified. These reads were assigned to the meta group „No Relative" in the SILVAngs fingerprint and Krona charts (Ondov, Bergman, and Phillippy 2011). This method was first used in the publications of Klindworth *et al.*(2013) and Ionescu *et al.* (2012).

2.3.12 Metabolite extraction from cultures

150 mL were taken from a 1.5 L enrichment culture and the HMN phase was extracted in a separating funnel. The remaining water phase was brought to a pH of 12 with 10 M NaOH and after 15 minutes of stirring acidified with 6 M hydrochloric acid to pH 2. After mixing with 50 mL of ethyl acetate carboxylic acids and aromatic alcohols were extracted three times with ethyl acetate. The lower phase was collected for a subsequent extraction; the upper phase was dried through pulverized sodium sulfate. The combined ethyl acetate extracts were concentrated by vacuum evaporation until dry and resolubilized with 500 µL ethyl acetate. Resolubilized extracts were filled in 2 GC-Vials and derivatized once with BFTSA for 30 minutes at 65°C, and once with TMSH, which did not need any special treatment. Derivatized samples were measured with the GC-MS.

2.4 Analytical Methods

2.4.1 Sulfide measurement

To measure the sulfide concentration within the culture 0.5 mL of zinc acetate was added to 20 mL test tubes, followed by 1.5 mL of MilliQ water. The solutions have been mixed thoroughly and 100 µL of sample were introduced directly into the zinc acetate solution to prevent oxidation. 0.5 mL of ADMA solution was added carefully, followed by 0.5 mL of Fe(III) solution and another 2 mL of MilliQ water. After another mixing step the test tubes were set aside in a dark place at RT for 30 to 120 minutes maximum. The absorbance of the mixed solution was measured at 670 nm in an Ultrospec 2100 Pro UV-VIS spectrophotometer, along with standards prepared with 5 mM Na_2S x 9 H_2O in zinc acetate. Samples were measured in biological and technical triplicates.

ADMA: Dissolve 2 g of ADMA in 200 mL of MilliQ water. Add 200 mL of concentrated sulphuric acid (H_2SO_4) slowly while cooling. Dilute mixture to 1 L with MilliQ water. Solution can be stored at 4°C.

Fe(III) Solution: Dissolve 5 g of ferric ammonium sulfate [$FeNH_4(SO_4)_2$] in 40 mL MilliQ water. Add 1 mL sulphuric acid (H_2SO_4, 98%) and dilute solution to 1 L. Store at RT.

Zinc acetate solution: Add 2 g of zinc acetate [$Zn(CH_3COO)_2$] to 100 mL of MilliQ water and add 2 mL of acetic acid (99%) to dissolve if necessary. The solution must be clear. Store at 4°C.

2.4.2 Sulfate measurement

To measure sulfate in the enrichment culture the Barium-gelatin method was used, based on Tabatabai (1974). For the preparation of the Barium-gelatin reagent 0.75g of gelatin (from bovine skin, Sigma-Aldrich) was dissolved in 250 mL of boiling MilliQ water and cooled on ice. 10 g of barium-chloride ($BaCl_2 \cdot 2\ H_2O$) were added and the reagent stored in the fridge.

Samples were taken from the culture with a syringe flushed with Biogon® before inserting into the culture bottle via the butyl stopper. Samples were then

centrifuged in a table top centrifuge (Eppendorf) for 10 minutes at 14.000 × g. The supernatant was used for the measurement. Standards were prepared in a 0 – 10 mM range from a 0.5 mM stock solution and kept in microcentrifuge tubes in the fridge. To prepare the assay the barium-gelatin reagent was diluted 1:1 with 0.5 N HCl and 250 µL distributed in each needed well of a 96-well MTP. 30 µL of the sample's supernatant were added to the reagent. The plate was inserted into a Victor Spectrophotometer (PerkinElmer, Waltham, MA) and agitated continuously for ten minutes before measuring the turbidity at 450 nm.

2.4.3 Sulfate measurement on Ion Chromatograph (IC)

Samples were prepared by taking 150 µL of the culture into a 1.5 mL Eppendorf Cup. This was centrifuged at 13.000 × g for 10 minutes. 100 µL of the supernatant were transferred into a new Eppendorf Cup and subsequently diluted with 900 µL 0.1 M KOH to precipitate heavy metals. After vortexing of the sample, it was given 10 minutes to incubate, followed by a second centrifuging step under the same conditions. 20 µL of the sample were mixed with 980 µL of MilliQ water to get a total dilution of 1:500. The samples were measured on an Aquion IC System incl. Degaser (Thermo Fisher Scientific GmbH, USA) in biological triplicates under the following conditions: Isocratic Run, Suppressor current 7 mA, Flow rate 0.25 mL/min, Run time 26 min, Eluent: 0.954 g Na_2CO_3 plus 0.134 g $NaHCO_3$ filled up to 2 L with MilliQ water.

2.4.4 Gas chromatography (GC)

2.4.4.1 Methane measurement

Methane concentrations were measured in a Gas chromatograph (GC) from the headspace of n-Hexane-inactivated bitumen bottles, which were closed with a butyl stopper. In brief, 50 µL of the headspace of each culture were manually injected into the 8610C Gas Chromatograph from SRI Instruments Germany at constant helium (Linde AG) flow. The GC was equipped with a helium ionization detector and a thermal conductivity detector. The injected gas samples were separated on a 6 meter 1/8‘‘ S.S. HayeSep D column (SRI Instruments) at 50°C. Measuring time was 4 min, which allowed the recording of nitrogen, methane and

carbon dioxide. Measurement results from the helium ionization detector (HID) and the thermal conductivity detector (TCD) were used. For quantification, a 6-point standard curve (0.01%, 0.25%, 0.5%, 1%, 5% and 10% (v/v)) for methane and carbon dioxide was generated, using mixtures of nitrogen, methane and carbon dioxide (all gases from Linde AG). Injection volume for standards were 150 µL, they have been measured in technical triplicates.

Table 2-24: Methane concentration of standards for standard curve.

	mL	S1 10%	mL	S2 5%	mL	S3 1%	mL	S4 0,5%	mL	S5 0,25%	mL	S6 0,01%
N_2	120	0,8	120	0,902	120	0,980	120	0,990	120	0,995	120	0,998
CO_2	15	0,1	6,5	0,049	1,2	0,01	0,6	0,005	0,3	0,0025	0,12	0,001
CH_4	15	0,1	6,5	0,049	1,2	0,01	0,6	0,005	0,3	0,0025	0,12	0,001
Total	150		133		122,4		121,2		120,6		120,24	

2.4.4.2 Ion-ratio mass spectrometry (GC-IRMS)

40 g of liquid bitumen were sampled and filled into 250 mL glass bottles with screw cap and butyl stopper. These bottles were fumigated with nitrogen gas. After shipment, the bottles were stored at 30°C to mimic natural conditions until repeated measurements took place.

2.4.4.2.1 Hydrogen isotope measurement

The measurement of hydrogen isotope ratios was performed on a GC-IRMS in the Umweltisotopenchemie group of Dr. Martin Elsner, Institute of Groundwater Ecology, Helmholtz Zentrum München with the help of Dr. Armin Meyer. The column used for distinct separation was a RT-QPLOT column, 30 m×0.32 mm I.D. (Restek, Bellefonte, PA, USA). The system consisted of a Trace Ultra GC (Thermo Fisher Scientific, Mailand, Italien) linked to a Finnigan MAT 253 (Thermo Scientific, Bremen, Germany) IRMS via a Finnigan GC Combustion III Interface (Thermo Scientific, Bremen, Germany).

2.4.4.2.2 Carbon isotope measurement

Measurements of Isotope-Ratios were executed on a GC-IRMS. A RT-Qplot Column (Length 60 m, ID 32 mm; Restek GmbH, Bad Homburg, Germany) was used to achieve distinct separation of CH_4 and CO_2. The system consisted of a Trace Ultra GC (Thermo Fisher Scientific, Milano, Italy) linked to a Finnigan MAT

253 (Thermo Scientific, Bremen, Germany) IRMS via a Finnigan GC Combustion III Interface (Thermo Scientific, Bremen, Germany).

The temperature program was held at 50°C for 600 sec and rose with 30°C/min to 240°C, which were held for 240 sec. The injection volume was 0.3 mL of the headspace. The temperature of the injector was isotherm at 200°C, flow velocity in the column was at 1.2 mL/min and split velocity at 12 mL/min. The split was 1:10. Helium was used as carrier gas. Temperature of the pyrolysis reactor was at 940°C.

2.4.5 Liquid chromatography coupled to mass spectrometry (LC-MS/MS)

Samples were centrifuged for 45 minutes at 4°C and 4000 × g. The supernatant was analyzed on an Agilent 1200 HPLC system coupled to an Applied Biosystems Q-Trap mass spectrometer equipped with a TurboSpray ionization source. A Purospher Star C18 column was used in order to separate the analytes. The column oven was set to 35°C. A gradient of 25–40% acetonitrile in 0.1% acetic acid over 25 min at a flow rate of 0.3 ml/min was applied.

2.4.6 Microscopy

Culture samples were either added directly onto a microscopy slide and analyzed in an Axioskop 2 plus (Zeiss, Jena, Germany) under various magnifications. FISH-labelled cells were detected with fluorescence microscopy on the same microscope with light filters in the wavelengths depending on the FISH probe or DAPI staining (UV-light). For imaging, pictures were taken by the digital camera AxioCam HRm (Zeiss) and the software AxioVision (Version 4.8.2; Zeiss).

2.4.7 Flow Cytometry for absolute microbial cell counting

For a total cell count in the enrichment culture samples were prepared according to a protocol developed by Nina Weber (Group of Microbial Ecology, Institute of Groundwater Ecology, Helmholtz Zentrum München). A tube of reference beads (Trucount™ Absolute Counting Tubes, BD Bioscience, Franklin Lakes, NJ) was

sufficient for ten samples and prepared by dilution with 1 mL of RNAse free water, which has been filtered again (22 µm filter). It was mixed by vortexing for at least 10 seconds and incubated for 10 minutes in the dark at RT. 500 µL of culture sample (diluted if necessary) were mixed with 6 µL of a SYBR Green I working solution (SYBR Green I nucleic acid gel stain, Invitrogen/Molecular Probes #S7563, Table 2-25). Incubation of the mix took place at 37°C in a Thermomix at 900 rpm for 13 minutes in the dark. 100 µL of reference bead solution were added before measurement on the Cytomics FC 500 (Serial No. AM27235; Beckman Coulter, Krefeld, Germany). The machine was set to the following parameters:

FS:	398/1.0	SS:	972/5.0
G-FL (FL1):	569/1.0	R-FL (FL3):	640/1.0
Trigger/Threshold:	G-FL (FL1)/1	max. Events:	10.000.000
Flowrate:	medium	Measurement time:	60 seconds

Analysis of the cell count was done with the CXP software (Version 2.2; Beckman coulter). Each sample was measured in duplicates.

Calculation of Cells per mL:

$$\frac{Cells}{ml} = \frac{TCC\ x\ TrucountREF}{REF} x \frac{Samplevolume\ \mu L}{1000} x\ Dilution\ of\ Sample$$

Table 2-25: SYBR Green I working solution.

Reagent	Amount in Working Solution
10.000X SYBR Green I stock solution	10 µL
10 mM Tris-buffer pH 8.0	990 µL

2.5 Enzyme Assays

Two 1.6 L cultures were separated from the HMN phase in a separation funnel. After a two-step centrifugation of 400 mL each for 30 minutes at 10.000 g and 4°C the centrifuge flasks were washed with 0,5 mL MOPS buffer pH 7,3 each. The washed-out cells were collected in an Eppendorf cup and centrifuged again for 15 minutes at 4°C and 14.000 × g, the upper phase taken off and the pellet resuspended in the desired amount of MOPS buffer. The resuspended pellet was

filled into a French press cell and the cells opened. The crude extract was transported on ice, refilled into an Eppendorf screw cup and centrifuged for 15 minutes at 4°C and 14.000 × g. Assays were pipetted according to the scheme in Table 2-26. The cell-free extract (cfe) was added second to last, the substrate started the assay. Samples taken out at the desired time points were mixed with double Volume of 80% methanol to stop the reaction. Assays were incubated at 30°C and 900 × g in a Thermomix Block (ThermoMixer® C, Eppendorf, Germany). After all samples have been taken, they were centrifuged again at 4°C for 15 minutes and afterwards refilled into LC-Vials.

Table 2-26: Enzyme assay pipetting scheme.

	Carboxylase Assay	Ligase Assay	Reductase Assay
Cfe	80 µL	80 µL	100 µL
Phenanthrene	80 µL	80 µL	
2-PCA			40 µL
4-PCA			40 µL
50 mM $NaHCO_3$	20 µL	20 µL	
50 mM ATP	20 µL	20 µL	20 µL
10 mM CoA-SH		20 µL	20 µL
20 mM DTT			20 µL
20 mM $Na_2S_2O_4$			20 µL

2- & 4-PCA: 2- & 4-Phenanthroic acid; NaHCO3: Sodium bicarbonate; ATP: Adenosin triphosphate; CoA-SH: Coenzyme A; DTT: Dithiothreitol; $Na_2S_2O_4$: Sodium dithionite.

3 Results

3.1 Bacterial life in the bitumen

3.1.1 DNA-extraction from natural bitumen

DNA-extractions from bitumen samples were done in order to get an overview of bacteria present in the bitumen and for this community to be used as a “backbone” to the bacteria we found in the water droplets for community analyses. The extractions proofed to be difficult, as bitumen not only adheres tightly to the microbial cells but during the extraction process also to the DNA, which makes it difficult to use for downstream applications. The extracted DNA was not visible on agarose gels without a smear after electrophoresis. Even DNA extracted from gels would still be contaminated. Furthermore, the bacterial cell density in the bitumen was very low, so that the extraction method had to be very sensitive in order to get most of the cell mass out of the bitumen. The adhered bitumen also made optical quantification of DNA impossible, as humic acids have a similar absorbance and drive the results of a NanoDrop™ (Thermo Scientific) measurement up extremely high, with only minute amounts of DNA present in the sample. A variety of extraction protocols were tried and are explained in this section, until one combined method was successfully developed to produce DNA that could be sequenced (2.3.6.2).

In order to get bitumen samples into extraction tubes different methods were tested as bitumen is too viscous to fill desired amounts into microcentrifuge tubes. Heating up the bitumen to 40°C in a water bath made it more liquid and gave it a gooey consistency. On the other hand, it also made it almost impossible to fill into microcentrifuge tubes prepared with beads for downstream bead beating steps.

The next option was to cool down the bitumen with liquid nitrogen until it could be grinded to a powder-like consistency. The cooled powder could be quickly filled into microcentrifuge tubes to the desired amount of around 0.8 g before melting again at RT to a hardly distributable mass.

The following methods were tested for DNA-extraction directly from bitumen:

i) A Phenol-Chloroform-Isoamyl alcohol (PCI) extraction combined with bead beating resulted in one long smear on the gel. DNA concentrations as measured in a NanoDrop™ resulted in about 180 – 265 ng/µL, but the pellets were still black disturbing the NanoDrop™ results.

Figure 3-1: Agarose gel picture of extracted and pooled DNA from bitumen of the pitch lake. The lane marked with M contains the 1 kb DNA ladder, lane 1 contains pooled DNA samples from extraction method i).

ii) Phenol-Chloroform-Isoamyl alcohol (PCI) extraction with different shorter bead beating times to reduce shearing of DNA resulted in DNA-concentrations about 178 – 392 ng/µL. Pellets were still dark, and the NanoDrop™ curve did not show typical DNA shape but a high pollution between 221 and 230 nm. The gel showed long smears again and the highest concentration in a sample that is not the 392 ng/µL sample, showing that NanoDrop™ results should not be taken too seriously with this type of contamination. Samples were all taken from the same

jar. A PCR set up with these samples only yielded a product for one of the samples.

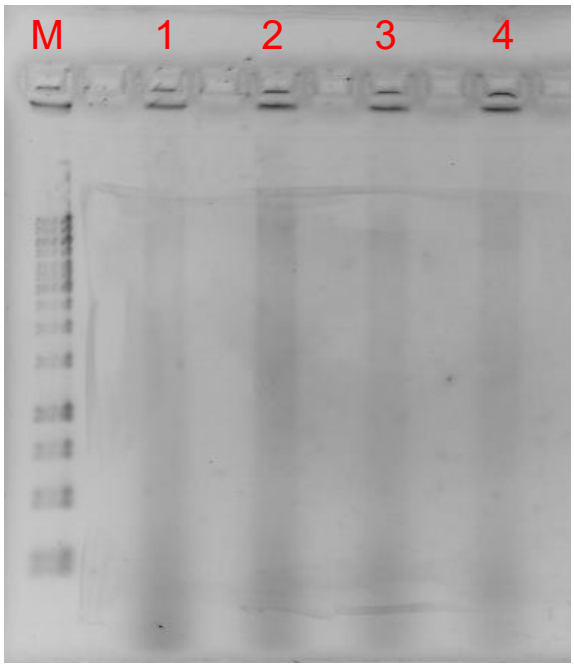


Figure 3-2: Agarose gel picture of DNA extracted from bitumen from the pitch lake with different bead-beating times. Lanes one to four contain DNA from samples from extraction method ii) with bead-beating times of 15, 20, 30 and 45 seconds, respectively.

iii) The next try to clean up the bitumen samples were different purification methods after the direct extraction. The first method included sephadex columns, which were equilibrated before use, but did not yield clean product after elution of the DNA from the matrix. The second method was to try another Phenol-Chloroform (PC) extraction in addition to the first one as stated in the protocol in 2.3.6.1. This cleaned up much of the dirt and yielded a clear liquid containing the DNA in the end. A concentration of the DNA could also be seen, as initially 300 µL were eluted in 10 µL to a concentration of 470 ng/µL from a starting concentration of 60 ng/µL.

iv) DNA extraction from bitumen was mixed with sterile water after pulverization, followed by extraction from the water phase. The water was filtered through paper filter and DNA was extracted from the filters directly with a PCI-Bead beating method. NanoDrop™ results for the pellet, which was still interspersed with fine filter particles, were given with 5350 – 5691 ng/µL, which is extremely high.

DNA was purified as described in 2.3.7. Gel results showed a clear cleaned up DNA. In lane 4 of Figure 3-3 a high molecular weight DNA can be seen. NanoDrop™ results changed to 23 - 71 ng/µL, but the shape of the DNA curve has its peak at 230 nm instead of at 260 nm, which still indicates for contamination of the sample and hardly any DNA.

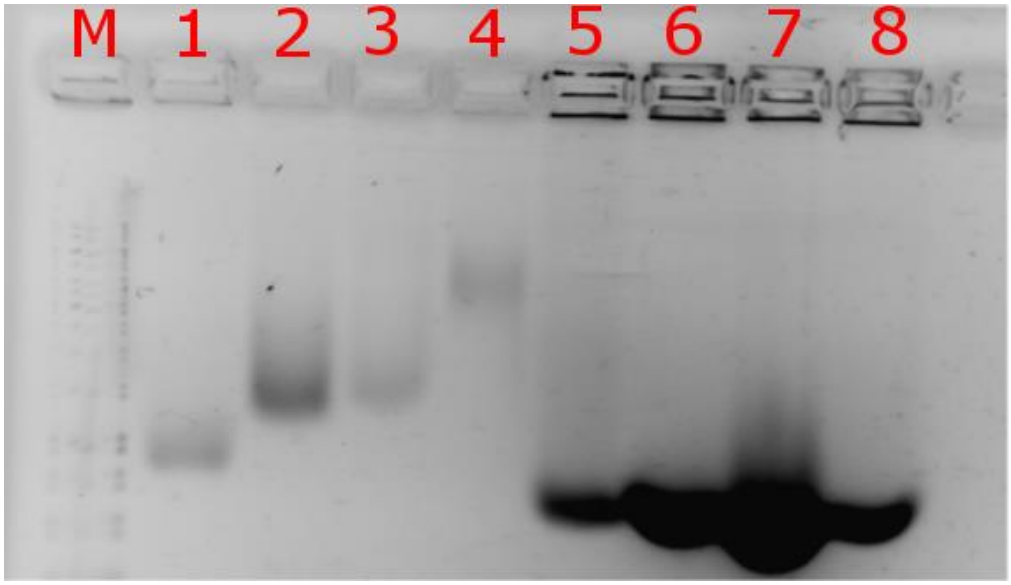


Figure 3-3: Agarose gel-electrophoresis of DNA-extraction from water phase of water mixed with the bitumen after the grinding step. Lane M contains the 1 kb DNA ladder, lanes 1 to 4 contain purified DNA from the water phase after the purification step described in iv) above. Lanes 5 to 8 contain DNA before the purification step from the same extractions. Remaining contaminations can be seen in the gel pockets.

v) The next try was to dissolve bitumen in three volumes of n-Hexane. The mixture was centrifuged for 30 minutes at 20.000 x g at 10°C. After the centrifugation there was a solid pellet at the bottom of the tube and a dark phase of n-Hexane on top. The pellet could not be re-dissolved by vortexing. In version 1 I added 0.5 mL of MilliQ to the n-Hexane phase and mixed both while vortexing. After a second centrifugation step the phases were separated and the n-Hexane phase on top could be taken away and stored separately.

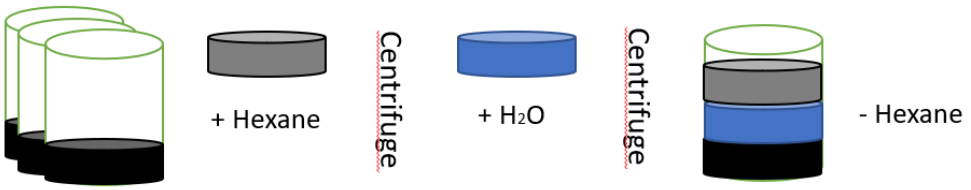


Figure 3-4: Schematic view of the n-Hexane DNA-extraction method development (version 1). n-Hexane (grey) was added to bitumen (black), followed by a centrifugation step. To separate all phases after the addition of water (blue) another centrifugation step was performed.

The water phase along with the pellet were frozen over night for a freeze-thaw-cell opening.

This method was repeated in triplicates with addition of MilliQ water in one tube before the first centrifugation step (Version 2, Figure 3-5), so more DNA could get into the water phase before the pellet settled at the bottom of the tube. The water was then transferred into a new tube after the first centrifugation step.

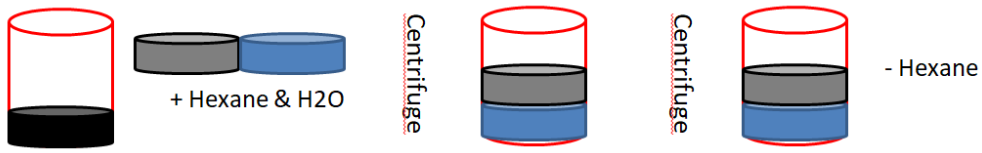


Figure 3-5: Schematic view of the n-Hexane DNA-extraction (Version 2). Water (blue) and n-Hexane (grey) were both added to the bitumen (black) during the first step, followed by a centrifugation step. Both layers were removed and centrifuged again for a good separation and the n-Hexane layer taken off.

Also, in this method the n-Hexane phase was transferred into a new tube before the second centrifugation step (Version 3, Figure 3-6) and the pellets were frozen for a direct extraction of DNA. After the second centrifugation step the phases were stored separately at -20°C until extraction.

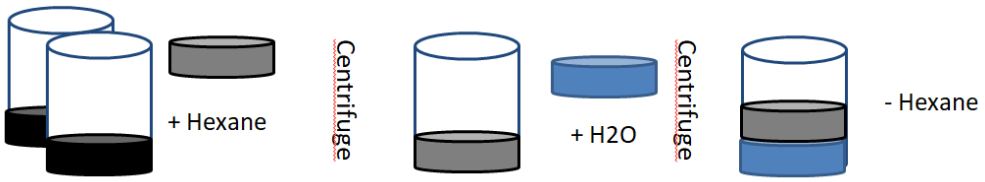


Figure 3-6: Schematic view of the n-Hexane DNA-extraction (Version 3). Water taken off after first centrifugation step, a complete separation was achieved after another centrifugation step, were the upper n-Hexane phase was removed and stored separately.

All tubes containing bitumen pellets were extracted following the bead beating protocol, the water-only tubes did not go through bead beating

to avoid unnecessary shearing of DNA. The agarose gel with all extracted DNA can be seen in Figure 3-7:

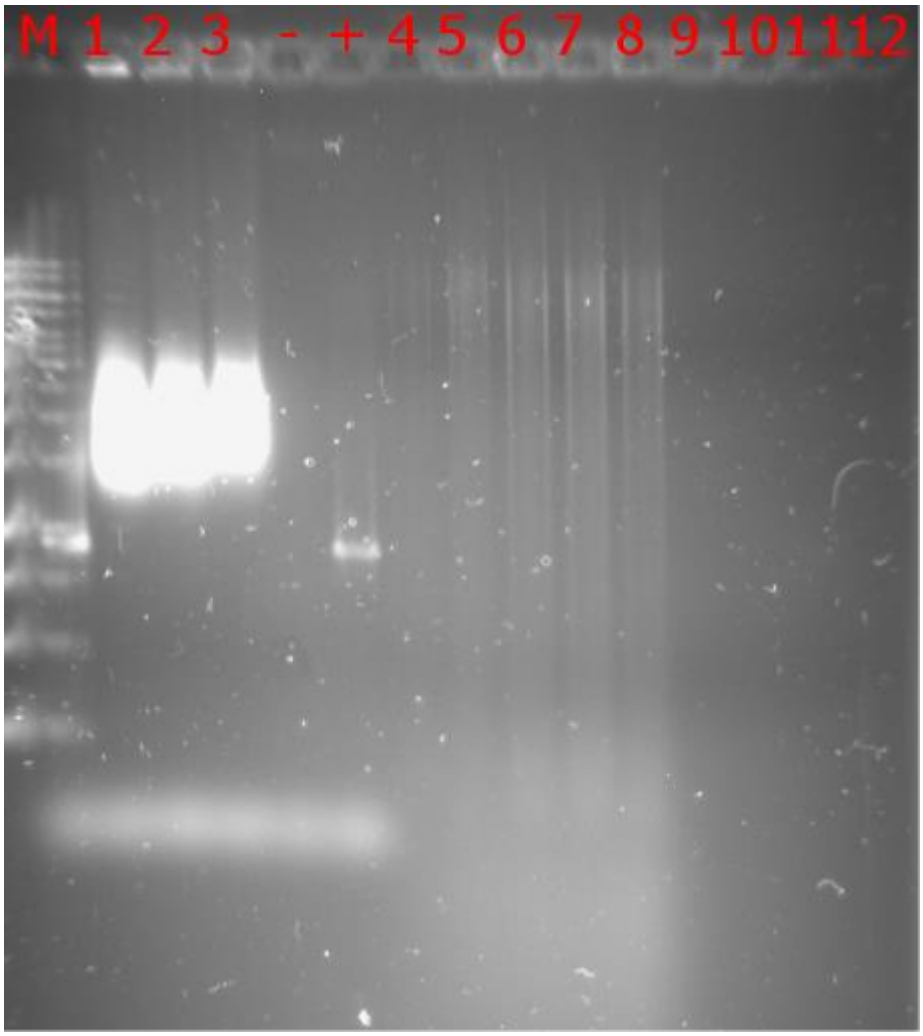


Figure 3-7: Agarose gel picture of DNA extracted with the n-Hexane method versions 1, 2 and 3. Lane M contains 1 kb DNA ladder, lane 1-3 contain n-hexane phase from version 1, lane - as negative control, lane + as positive control, lanes 4 - 8 contain DNA from extractions with pellets from version 1, 2, and 2 from version 3; lanes 9 - 12 contain extractions from the water phase from version 1, 2, and 2 from version 3.

The DNA from this extraction was not usable, neither from the pellets, pellets and water, nor just the water phase alone. The DNA did not seem to separate into the water phase at all during dilution with n-Hexane and water or addition of the water in a later step of the protocol.

vi) As another purification protocol I tried a kit with magnetic beads. The extracted DNA was incubated for a short period of time with a double volume of PEG. After washing and elution steps as in the original protocol, the magnetic bead kit (Thermo Fisher) was used according to the manufacturer's instructions. The eluate was clearer than with

previous clean up steps, but still not as clear as water. That is why the eluted DNA was not measured with the NanoDrop™ but added directly to an agarose gel (Figure 3-8).

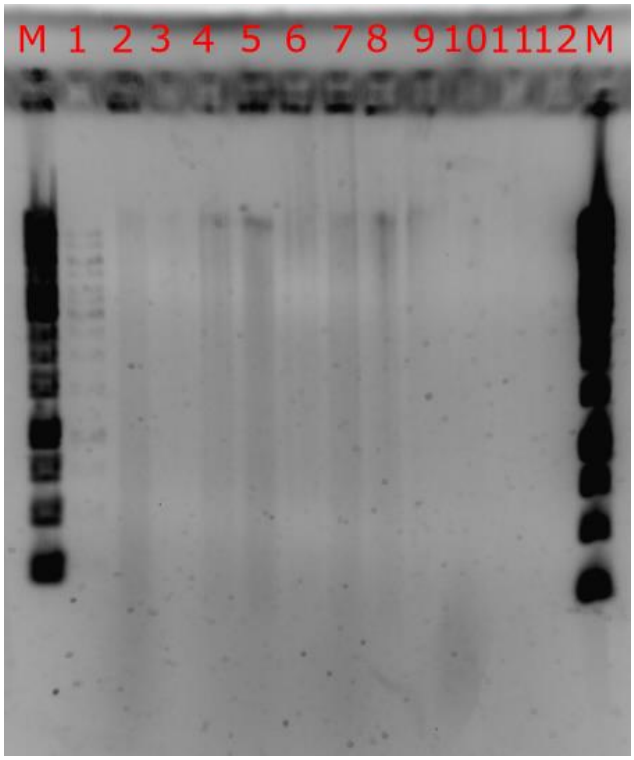


Figure 3-8: Picture of agarose gel loaded with DNA from an extraction of bitumen samples after triple clean up with magnetic beads. Lanes 2 throughout to 12 contain samples of parallel DNA extractions with a commercially available kit that works with magnetic beads (Dynabeads DNA DIRECT Universal Kit, ThermoFisher Scientific).

The DNA again only showed a big smear over the whole length of the gel but started from a higher molecular weight than previous extractions. Hence, the DNA was tested on a Bioanalyzer and showed that the quality was not good enough for downstream applications.

vii) The DNA-extraction method tested last was a combination of Phenol-Chloroform-Isoamyl alcohol extraction and the use of the MoBio PowerSoil DNA-Isolation kit as described in (2.3.6.2). It was on one hand the fastest protocol and on the other one the protocol that yielded the clearest eluate and the highest quality of DNA. The size of the DNA was at about 200 kb, which is a good size for sequencing applications as used in the laboratory of Genoscope (France). Three samples were extracted with the Kit only, and three with the combined methods. There is a clear difference in DNA yield for these methods, as shown in Figure 3-9.

Figure 3-9: Picture of an agarose gel loaded with DNA from an extraction from bitumen with combined methods from PCI-extraction and commercially available kit as described above. Lane M contains 1 kb DNA ladder, lane 1 - 3 contain extracted DNA from combined methods, lane 4 to 6 contain DNA from three parallel extractions with the commercially available Kit (MoBio PowerSoil® DNA-Isolation Kit).

The DNA looked better in the combined method; the concentration is much higher compared to the DNA extracted with the commercial kit alone. The DNA was still degraded from the process, which can be seen by the long smear. To optimize the extraction method, several different approaches were used. The bitumen was again prepared for extraction with either liquid nitrogen or preheated and filled in tubes carefully. From both methods, three samples each were preheated before adding the Miller phosphate buffer (2.3.6.2), and three were not preheated. From these three, each tube was subjected to different bead beating times from 20 over 30 to 45 seconds. As bead beating was only mixing both beads and bitumen completely in the preheated samples all samples were heated before the bead beating step to 65°C. This made the bitumen soft enough to allow for the samples to be completely mixed in the tube. After the centrifugation step at 4°C and 10.000 x g I continued the extraction as stated in the protocol in 2.3.6.2. Following the extraction was a purification step as stated in 2.3.7. The agarose gel of the so extracted and cleaned up DNA showed its high molecular weight and a distinct band above the 1 kb ladders highest band.

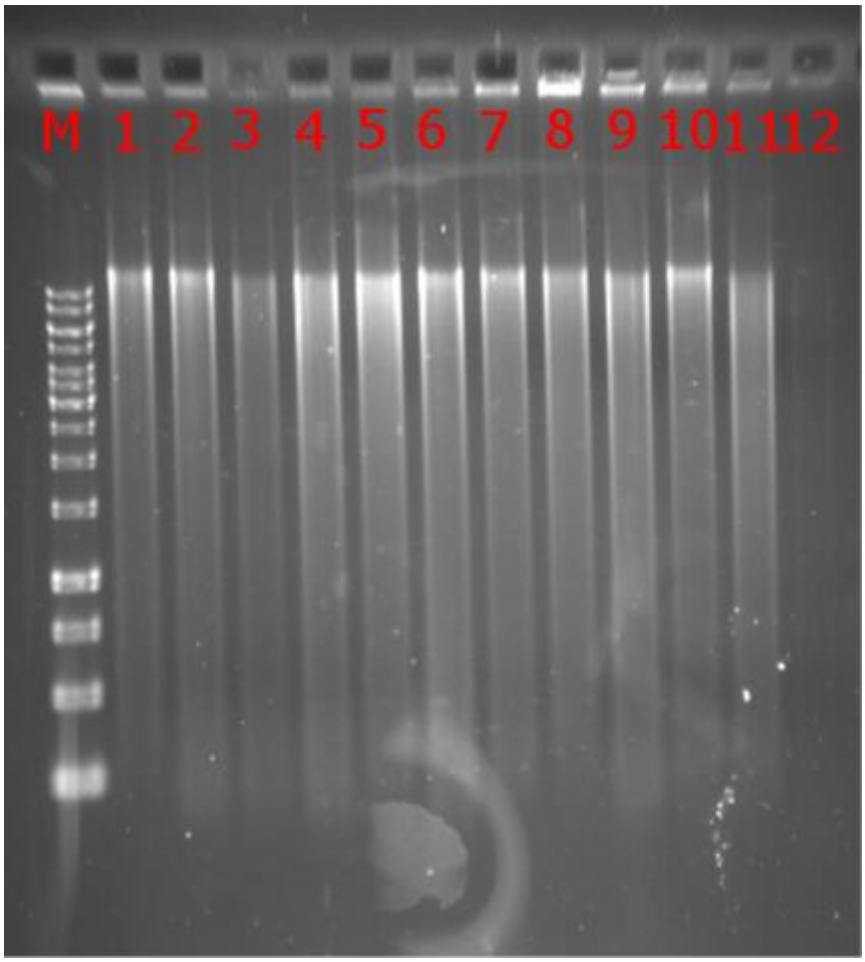


Figure 3-10: Agarose gel picture loaded with DNA extractions performed with combined methods of PCI-extraction and commercially available kit. Lane M contains the 1 kb DNA ladder, lanes 1 through 12 show extracted DNA that is hardly distinguishable from the different preparation steps. Samples in lane 1 to 6 were grinded with liquid nitrogen, lanes were preheated to be filled into Eppendorf cups. Samples in lanes 1 to 3 and 7 to 9 were preheated before the addition of Miller phosphate buffer. Bead-beating times for lanes 1, 4, 7 and 10 were 20 seconds, for lanes 2, 5, 8 and 11 30 seconds, for lanes 3, 6, 9 and 12 bead-beating times were 45 seconds.

The different preparation methods of the bitumen (e.g. preheated or grinded with liquid nitrogen; preheating steps before the addition of Miller phosphate buffer; different bead-beating times), especially the bead beating times, did not seem to have a high influence on the quality and amount of DNA extracted. Nevertheless, the preheating of the bitumen before the addition of the lysis buffer as well as another preheating step before the bead beating seem to make a difference in cell lysis as compared to the previously extracted DNA with the same method (Figure 3-9).

This DNA was measured in the NanoDrop™ and yielded concentrations between 10 ng/µL (Lane 10) and 306 ng/µL (Lane 9).

These samples were finally sent to Genoscope (France) for metagenomic sequencing.

3.1.1.1 16S rRNA gene sequencing

The above extracted DNA was sequenced by our collaboration partner GENOSCOPE in France, the sequencing technique is described in 2.3.10. A metagenomic library was build and the results were analyzed with the SILVAngs online tool (2.3.11).

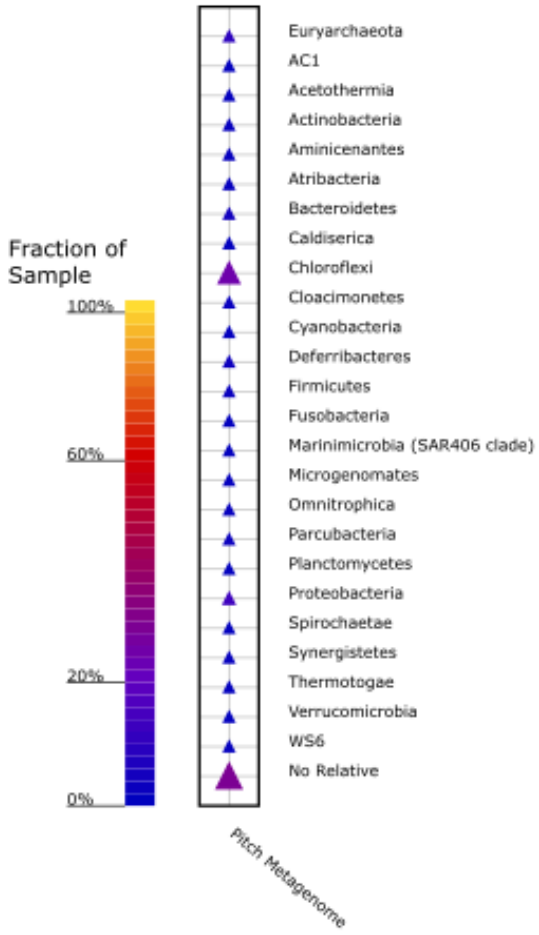


Figure 3-11: Taxonomic fingerprint based on 16S rRNA genes of the sequenced bitumen metagenome at phylum level, calculations were performed with the SILVAngs tool as described in 2.3.11. Triangles indicate a total sequence number of less than 100 sequences. The size of the shapes is accounting for the number of OTUs per phylum. Color indicates the fraction of the sequences in this sample belonging to the corresponding phylum.

It can be seen from picture Figure 3-11 that a high fraction of bacteria of about 30% had no known relative, and the phylum with the greatest amount of species were the Chloroflexi. For all of them less than 100 sequences were found within the sample after the quality trimmings within the SILVAngs work flow.

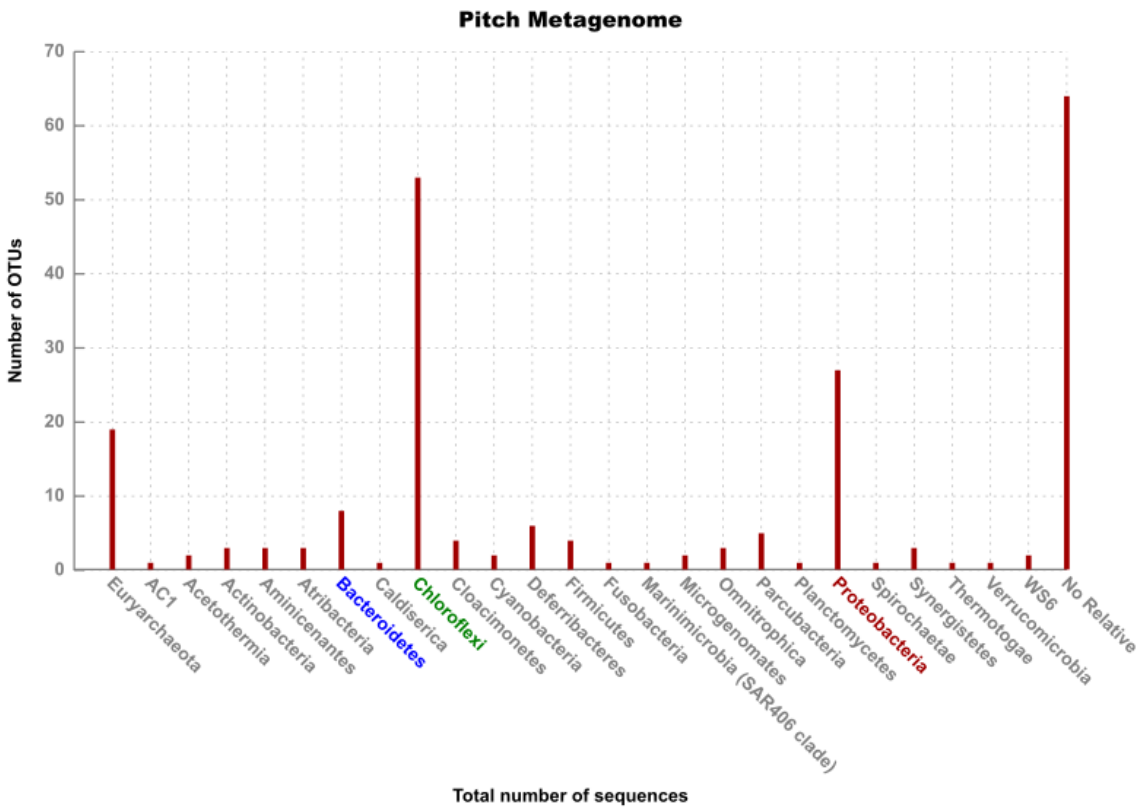


Figure 3-12: Number of OTUs based on unique reads per phylum in the bitumen metagenome. Colored phyla names indicate Proteobacteria, Bacteroidetes and Chloroflexi, the three main groups besides the biggest group without known relatives.

The more detailed graph in Figure 3-12 shows the total number of OTUs within the metagenome of the bitumen. Besides the already accounted for groups of the bacteria without a known relative at the phylum level and the Chloroflexi there are Proteobacteria and Bacteroidetes that seem to play an important role within the bitumen metagenome compared to all other phyla.

Below are some Krona plots that give more details of the bacterial sequences found in the bitumen metagenome.

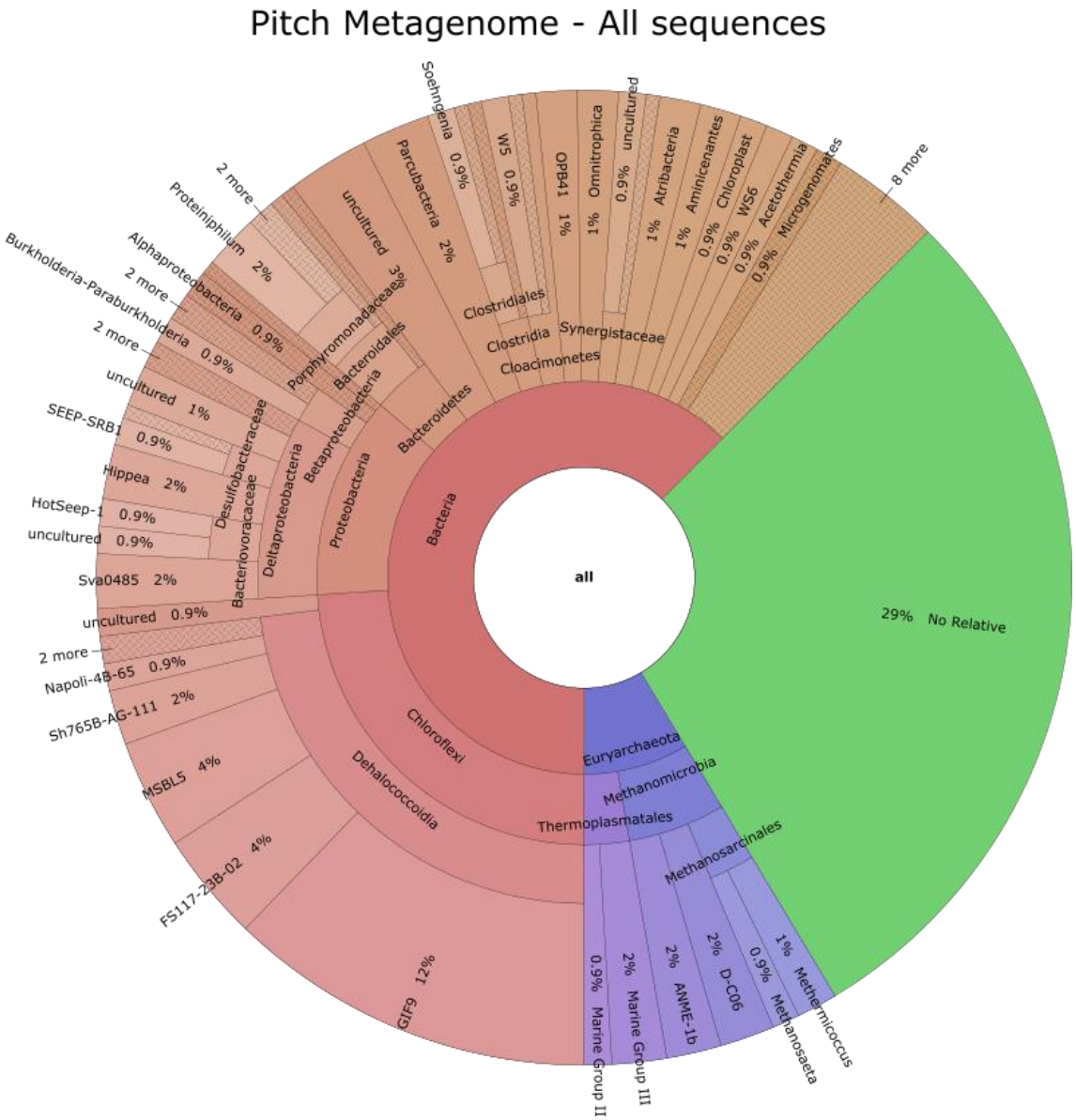


Figure 3-13: Krona Plot of the microbial community composition based on the bitumen metagenome calculated with the SILVAngs taxonomy of 16S rRNA genes.DNA was extracted from bitumen of the pitch lake. All sequences after the quality trimmings as described in 2.3.11 are included.

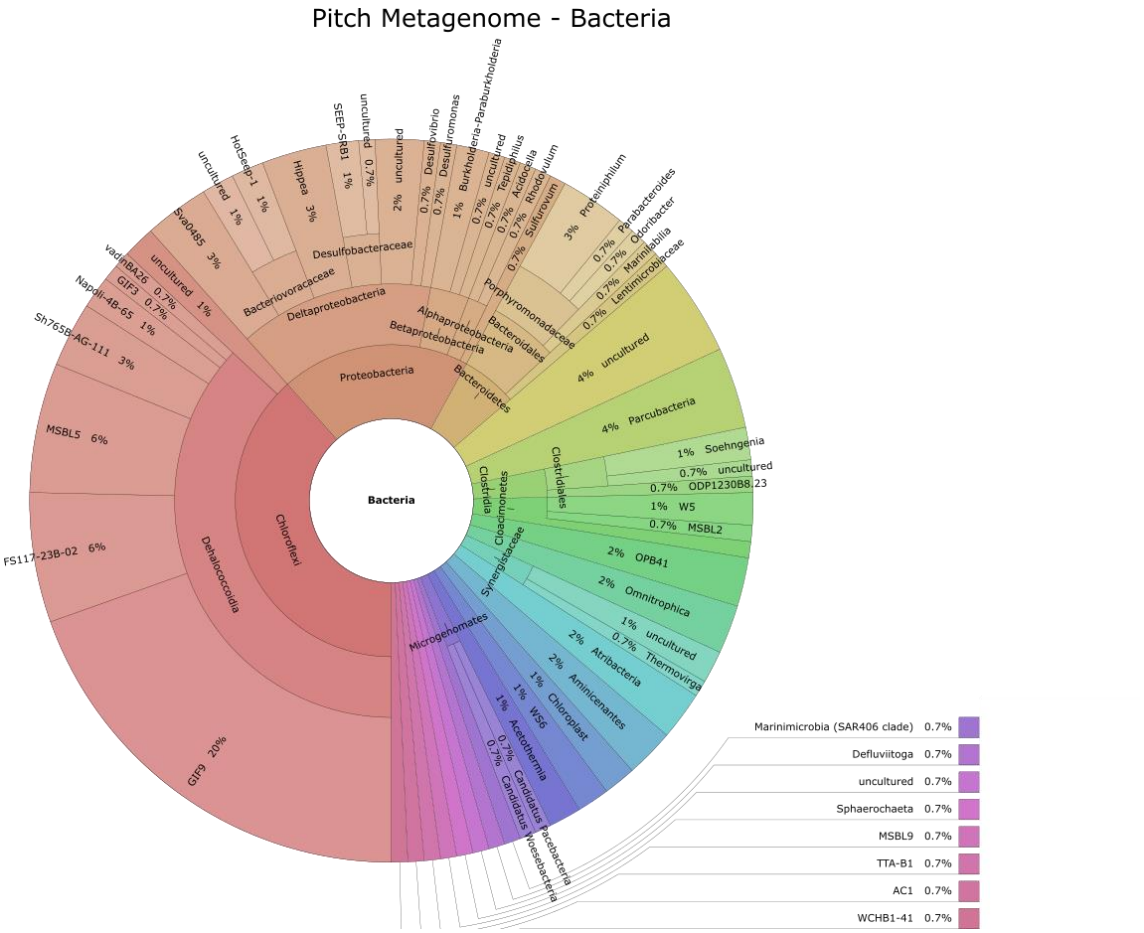


Figure 3-14: Krona Plot of the bitumen metagenome based on the SILVAngs calculated taxonomy of 16S rRNA genes within the extracted bitumen DNA, zoomed to the level of all Bacteria.

In Figure 3-14 we can see an overview of all bacterial rRNA sequences contained within the DNA extracted directly from the bitumen. More than one third can be assigned to the phylum of Chloroflexi, which will be shown in more detail below. The second biggest group, the Proteobacteria, are also depicted in more detail below. There are no bacterial rRNA sequences within this plot that seem unreasonable for such a habitat. A possible bias introduced by the applied extraction method can not be accounted for at this stage.

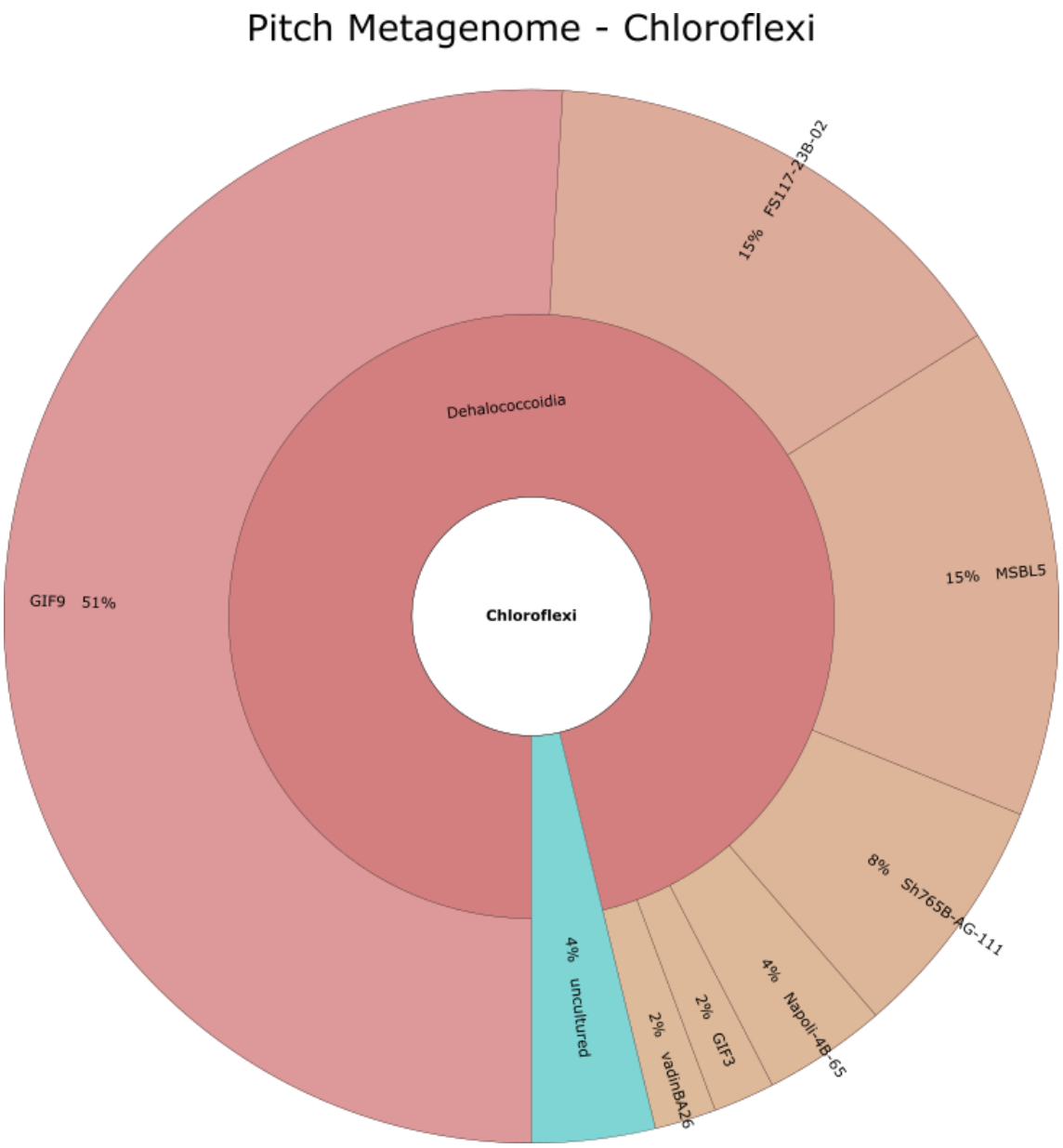


Figure 3-15: Krona Plot of the bitumen metagenome based on the SILVAngs calculated taxonomy of rRNA genes within the extracted bitumen DNA, zoomed to the level of all Chloroflexi as the most abundant group.

All Chloroflexi in the bitumen metagenome that could be assigned fall into the class of Dehalococcoidiales, and 51% of those belong to the group of GIF9 bacteria. These make up for about 25% of all bacteria within the bitumen and are therewith the biggest group in absolute numbers.

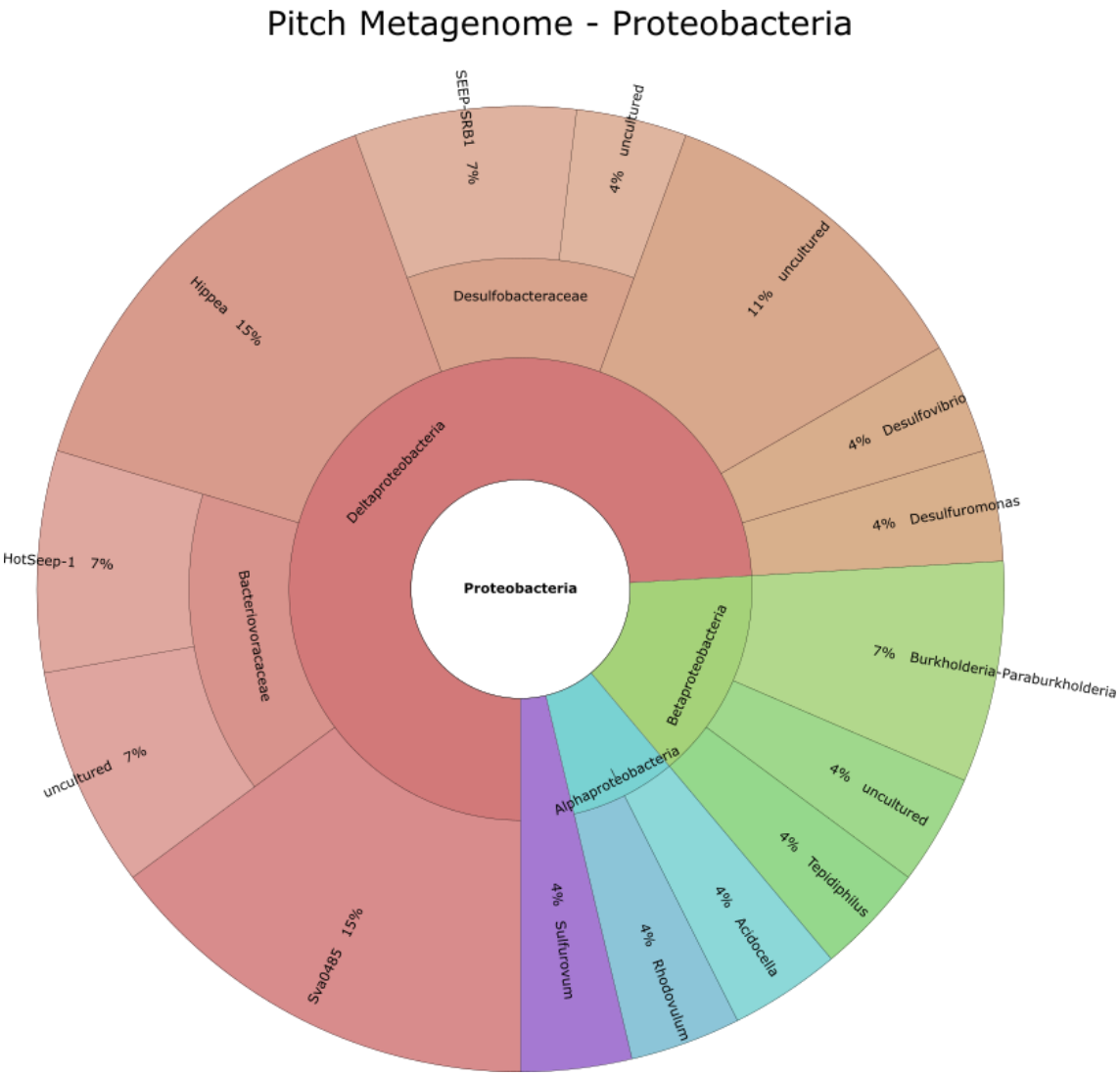


Figure 3-16: Krona Plot of the bitumen metagenome based on the SILVAngs calculated taxonomy of rRNA genes within the extracted bitumen DNA, zoomed to the level of all Proteobacteria, as most abundant group in water droplets (3.2.2).

The Deltaproteobacteria accounted for the biggest proportion of Proteobacteria within the bitumen metagenome. Among them were well known degraders of contaminants like aromatic hydrocarbons under sulfate and sulfur-reducing conditions and therewith fit well in the expected classes of bacteria within this metagenome.

3.1.2 Methane measurements in a time series of bitumen incubations

Methane contained in long time incubations of bitumen samples stored at 30°C was measured on a GC in order to distinguish if the contained methane is of biological origin. The measurement of the initial concentration of methane in the headspace of bitumen filled bottles (~40 mL) took place after stopping all

biological reactions with 50 mL of n-Hexane as a control. The 6-point standard curve was measured with concentrations as described in the methods part 2.4.4. Samples were measured in biological duplicates and technical triplicates with an injection volume of 150 µL each.

Table 3-1: Results of methane measurements of two bottles of long-time bitumen incubations in triplicates.

	14.04.2014	15.05.2014
Mean R1 Area CH_4	8228,4977	3245,650
Mean R1 Height CH_4	1354,381333	771,8983333
Mean R2 Area CH_4	7378,916567	3203,480733
Mean R2 Height CH_4	1729,741333	768,233

The samples were measured again after the sample taking for isotope measurements, giving consistently only about 40% of the previous measured data values.

Compared to the standard measurements these results give a total methane concentration of about 1% in the headspace of the bottles. This is important to know for upcoming measurements of isotope ratios on a GC-IRMS.

3.1.2.1 Carbon and hydrogen isotope measurements as indicators of methane production in the bitumen

Isotope measurements were obtained as described in 2.4.4.2. Both hydrogen and carbon isotopes were measured to get an idea if there is a shift not just in concentrations of methane over the incubation period, but also in the ratios between these two isotopes in order to be able to detect a biological production of methane as a result of biochemical processes in the incubation.

The measurements for the initial sample point in April 2014 are depicted in

Table 3-2.

Table 3-2: Results of initial Isotope ratio measurements of methane from two bitumen incubations from April 2014.

Carbon Isotope Ratio				Mean	SD
Sample 1	-46,62	-46,879	-46,814	-46,77	0,13
Sample 2	-46,513	-46,583	-46,298	-46,46	0,15
Hydrogen Isotope Ratio					
Sample 1	-166,42	-174,52	-176,65	-172,53	5,40
Sample 2	-164,07	-166,71	-168,05	-166,28	2,03

Three other bottles were measured 30 months later, as the expected change in methane isotope ratios will take a lot of time due to the little biomass in the samples. The results can be found in Table 3-3.

Table 3-3: Results of isotope ratio measurements of methane from three undisturbed bitumen incubations from August 2016, 28 months after initial measurements of samples 1 and 2, compare

Table 3-2.

Carbon Isotope Ratio				Mean	
Sample 4	-37,226	-37,277		-37,25	
Sample 5	-46,059	-45,857		-45,96	
Sample 6	-45,945	-44,116		-45,03	

Hydrogen Isotope Ratio				Mean	SD
Sample 4	-284,827	-289,458	-293,241	-289,18	4,21
Sample 5	-281,102	-279,44	-287,919	-282,82	4,49
Sample 6	-273,545	-274,743	-274,897	-274,40	0,74
Standard before measurement	-120,426	-123,221	-119,136	-120,93	2,09
Standard after measurement	-120,051	-120,527	-125,587	-122,06	3,07

Since the standard measurement was varying significantly from the original composition, the values of the hydrogen measurements were corrected for the values of the standard difference and lead to the results depicted in Table 3-4. Corrected values caused a shift of the mean values from between -274 and -289 to values between -152 to -167. This is a significant difference as can be seen in Figure 3-17, a schematic diagram of the origin of methane in isotope ratios.

Table 3-4: Corrected values for hydrogen isotope ratios of methane from bitumen incubations 4, 5 and 6 from August 2016. Corrections were necessary because of varying values for the measured standard.

Hydrogen Isotope Ratio	Mean	SD
R4	-167,12	4,21
R5	-161,33	4,49
R6	-152,90	0,74

The shift in isotope ratios within the bitumen incubations over a period of 28 did not differ much. To evaluate the ratios a paper from Whiticar (1999) used a diagram to compare isotope ratios with the origin of methane. This was used to determine the origin of the methane in the headspace.

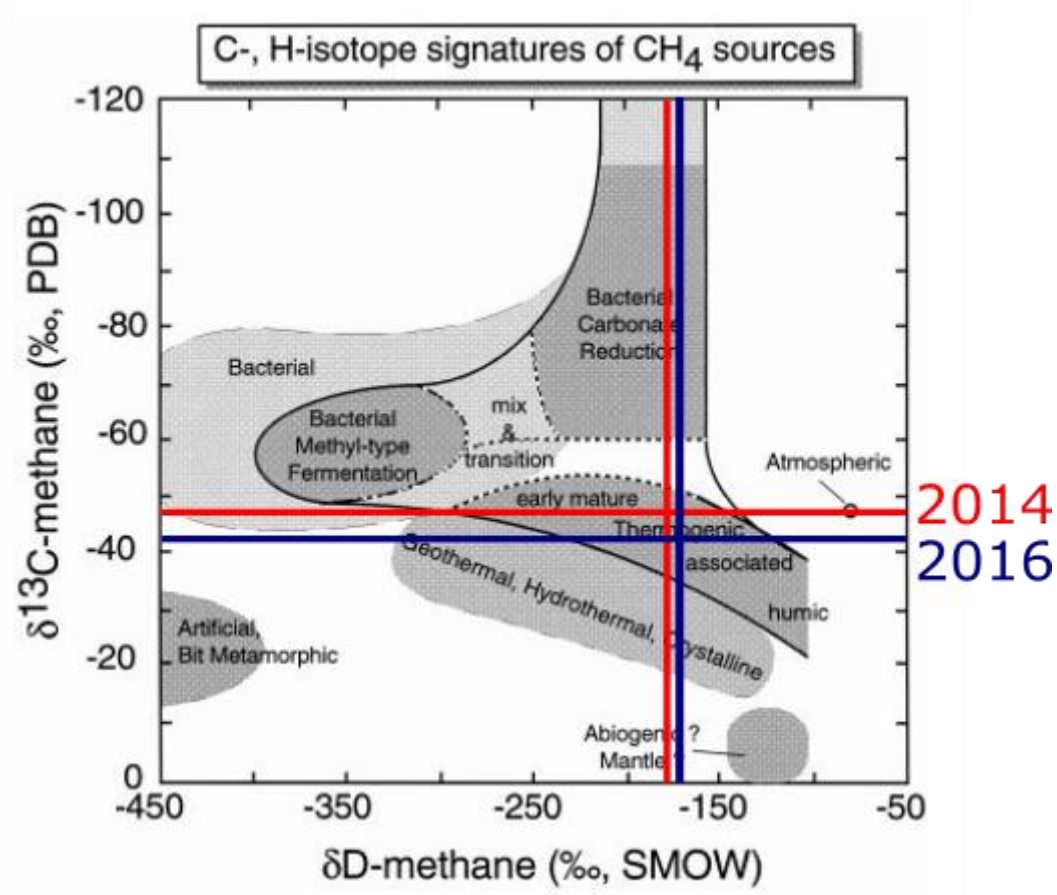


Figure 3-17: Dual Isotope Plot with characteristic signatures for different methane sources. Crosshairs depict the measured isotope ratios of methane in the years 2014 and 2016. Figure adapted from Whiticar (1999).

It can be seen that the two signatures of April 2014 and August 2016 still fall into the area of thermogenic methane production. The shift of the deuterium would have been extreme to at least 300 ‰ for an indication of a biological origin, the ^{13}C isotope should have become more negative.

From this point of view, we cannot say that bacterial methanogenesis is an important process within the bitumen lake, even though methanogenic archaea within the Methanosarcinales and Methanomicrobiales have been found according to Meckenstock et al. (2014).

3.2 Water droplets as a source of PAH-degrading specialists in the bitumen?

Tiny water droplets were isolated from bitumen that has been refilled into small glass jars. Bubbles were visible on the top of the bitumen and sometimes filled with minute amounts of water. These were collected in PCR centrifuge cups and

the size of each water droplet was estimated by pipetting. Droplets were filled up with MilliQ water to a volume of 3 µL to have enough material for three PCRs. It was important to keep the droplets separate at all times to be able to see differences in the community structure within each droplet. Other individual droplets were sent to Genoscope for single cell sequencing.

3.2.1 DNA Amplification from bacterial DNA within the water droplets

DNA amplification took place from individual droplets in a nested PCR as described in 2.3.1.1. A 6-cycle PCR with 16s rRNA primers was performed and the so amplified DNA was used as template for a 30-cycle PCR with sequencing primers (MID-Primer, Table 2-10). The amplification worked out well for bacterial DNA, but archaeal DNA could not be amplified with this method, as can be seen in the following gel picture (Figure 3-18):

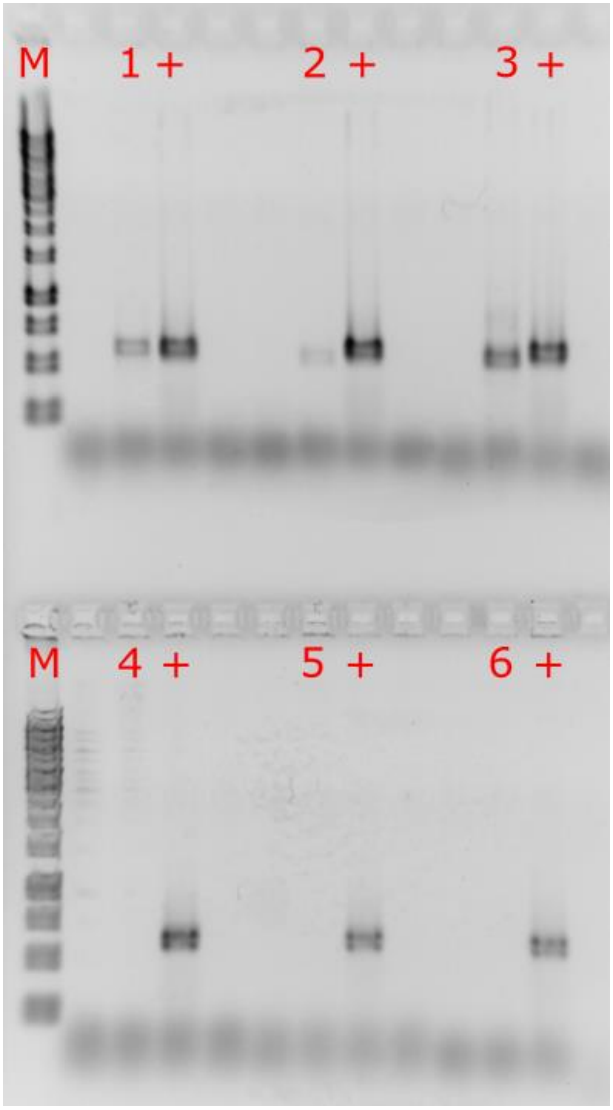


Figure 3-18: Agarose gel picture of DNA amplified by a direct droplet PCR after nested PCR with MID-Primers in second PCR. Lane M contains 1 kb DNA ladder, all lanes marked with + contain a positive control, lanes 1 – 3 contain bacterial DNA amplified with bacterial 16S rRNA gene primers, lanes 4, 5 and 6 contain no DNA, DNA was tried to be amplified with archaeal 16S rRNA primers.

Several runs of nested PCR have been performed to get clean and HMW DNA for sequencing of the droplets, and a set of four droplets has been sent out for sequencing to see if the method is able to produce DNA that is good enough for sequence evaluation. The primers used for these four droplets were MID 14 to MID 17 primers as shown in Table 2-10. After several clean up steps of the amplified DNA and control of the integrity of the DNA on a Bioanalyzer the four samples have been loaded on a pyrotag sequencing plate. The evaluation of the four droplets took place with the SILVAngs online tool as described in 2.3.11 in order to be comparable with the extracted and sequenced bitumen DNA. Only three out of the four droplets could be evaluated with this method, as one sample did not meet the quality standards for SILVAngs evaluation.

Another approach to evaluate the droplet sequences was the use of MOTHUR (Schloss et al. 2009) and R (CoreTeam 2011) for analysis.

Both methods differ in their evaluation, as different databases for species classification are used. In themselves they are consistent, so evaluations and discussions of these results will be done for both methods and major differences will be pointed out.

As previous results have shown (Meckenstock et al. 2014) archaeal DNA can be found in at least a few droplets. A novel DNA extraction method for the water droplets should be developed to have access to more DNA than that can be amplified with bacterial and/or archaeal 16S rRNA primers, including the harder to amplify gram-positive bacteria.

3.2.2 Single Cell Sequencing of bacteria within the water droplets

One droplet was also subjected to single cell sequencing, performed by Genoscope, France. This led to a list of bacterial classes found within that droplet.

Table 3-5: List of bacterial classes found within a single droplet via single cell sequencing. The phylogenetic anchoring was based on 16S rRNA SSU tags identified in the assembly.

	Family level	**Genus level**	**Oxygen use**	**Earlier extraction**
	Burkholderiaceae	Chitinimonas	aerobic	freshwater flagellate
2 x	Clostridiales_Incertae Sedis XI	Soehngenia	anaerobic	anaerobic sludge bed
	Enterobacteriaceae	Kosakonia	facultative anaerobe	Gut microbiota
3 x	*Enterobacteriaceae*	Salmonella	facultative anaerobe	Gut microbiota, pathogenic
	Enterobacteriaceae	Mangrovibacter	facultative anaerobe	Gut microbiota
	Enterobacteriaceae	Sodalis	facultative anaerobe	hemolymph tse tse fly
3 x	*Fusobacteriaceae*	Fusobacterium	anaerobic	human pathogen
4 x	*Hydrogenophilaceae*	Petrobacter	aerobic	oil well
2 x	*Hydrogenophilaceae*	Tepidiphilus	anaerobic	hot spring (Poddar, Lepcha, and Das 2014)
	Nanohaloarchaea	Candidatus Haloredivivus	aerobic	Hypersaline pond (Ventosa et al. 2014)
	Porphyromonadaceae	Dysgonomonas	facultative anaerobe	Various human sources, pathogenic (Vaughan and Forbes 2014)
	Porphyromonadaceae	Proteiniphilum	anaerobic	reactor brewery wastewater
3 x	*Porphyromonadaceae*	Petrimonas	anaerobic	oil reservoir

	Thermodesulfobacteriaceae	Caldimicrobium	strictly anaerobic	hot springs

Annotation of all available genes within this droplet was performed automated and a list of interesting monooxygenase genes has been found, including those for phenol, toluene, xylene, pyrimidine and a putative quinol monooxygenase, as well as enoyl-CoA-hydratase, which are essential for metabolizing fatty acids to produce acetyl-CoA. Also, the key-enzyme for anaerobic toluene activation, a benzylsuccinate synthase (bssA) has been found.

3.2.3 Ecological analysis of bacteria living in the water droplets

Out of the four droplets sequenced by amplicon sequencing a biom-file was generated using MOTHUR. Within MOTHUR Greengenes (Version 13_8_99) was used as a taxonomy database (DeSantis et al. 2006). The MOTHUR biom-file was imported into R and merged with a mapping file to identify droplets with their respective sequences in order to be able to evaluate them individually.

Rarefaction curves were computed with MOTHUR in order to see the species richness depending on the amount of sequences (Figure 3-19). The flatter the curve is at the top, the rarer species have been found in a sample. If the curve does not appear to flatten when reaching the top, only the more abundant species have been found or sequenced so far, as is the case for all the droplets shown below. Diversity plots, which follow later in the evaluation, can give further insights into the species richness and diversity within each droplet and also between droplets.

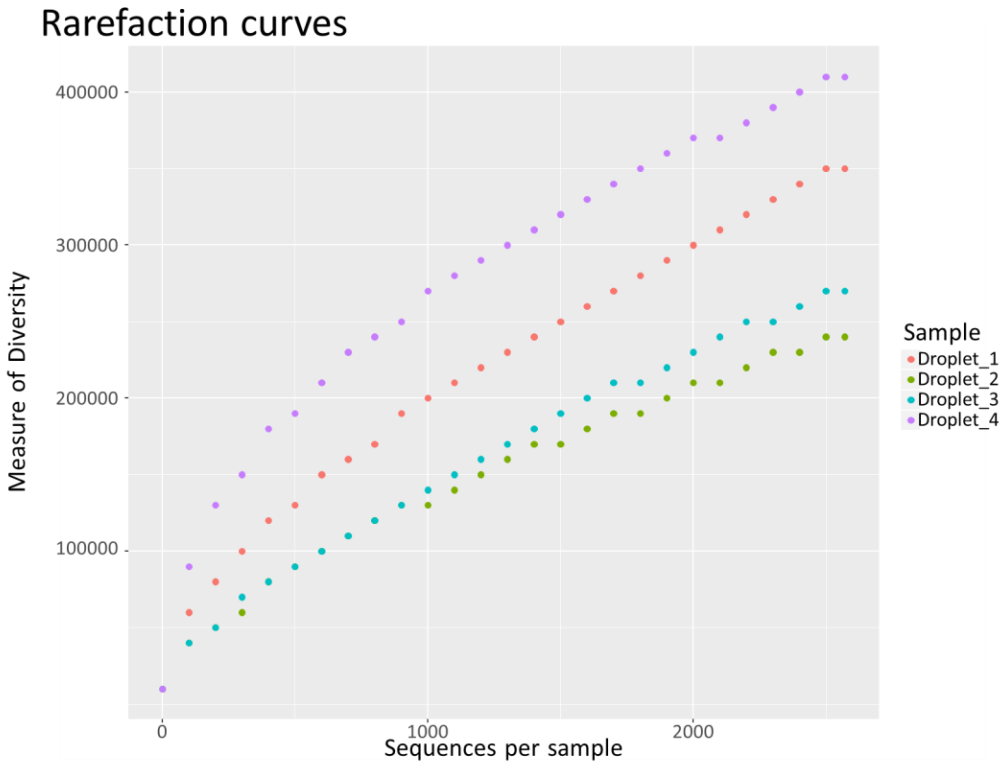


Figure 3-19: Rarefaction curve for water droplets one to four as calculated within the MOTHUR algorithm.

The first step after the rarefaction curves was the transformation of absolute OTU values into relative values. OTUs have then been collapsed into family groups to get a better overview at the family level. This was done in a way that sequences that did not identify to a family level have not been removed. The same has been done at the genus level for all sequences where this was possible. A biplot was created showing both the samples and the matrix points of the phylum level evaluation of the droplets (see Figure 3-20). In this plot the relative abundances of bacterial sequences at the phylum level can be seen. The ecological distances based on the relative abundances can be seen as well. Bacteroidetes and Proteobacteria can be identified as the main phyla in this plot.

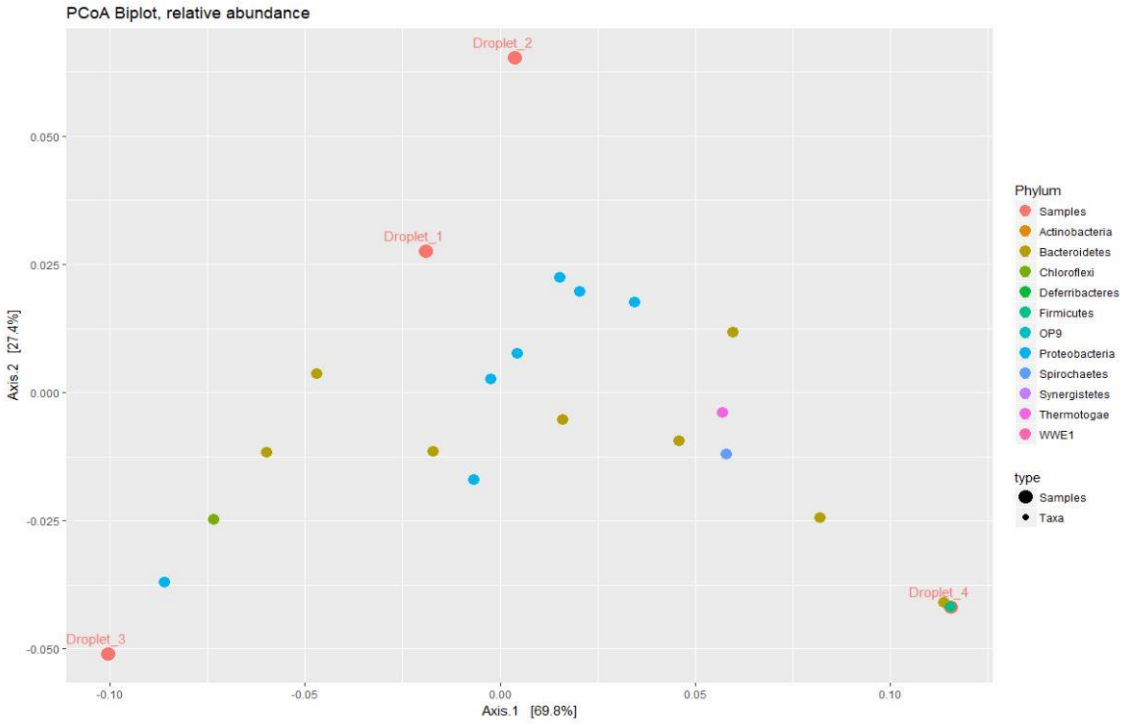


Figure 3-20: Biplot of relative abundances at the phylum level calculated by Bray-Curtis dissimilarity, shown together with the Droplets.

Another version of this plot, this time at the Order level, is shown in Figure 3-21 in a Principle Component Analysis. Burkholderiales and GIF9 are the most abundant 16S rRNA genes found within the droplet communities. Rhodocyclales and Sphaerochaetales as well as Clostridiales are abundant enough to make up for a noteworthy amount of bacterial sequences. It is important to point out that the communities in the droplets are centered between the droplets, which points to the high similarities between the droplets in their dominating bacterial community, and leads to the assumption that the rare species make up for the overall differences between the droplets. There is no clear clustering visible within the plot

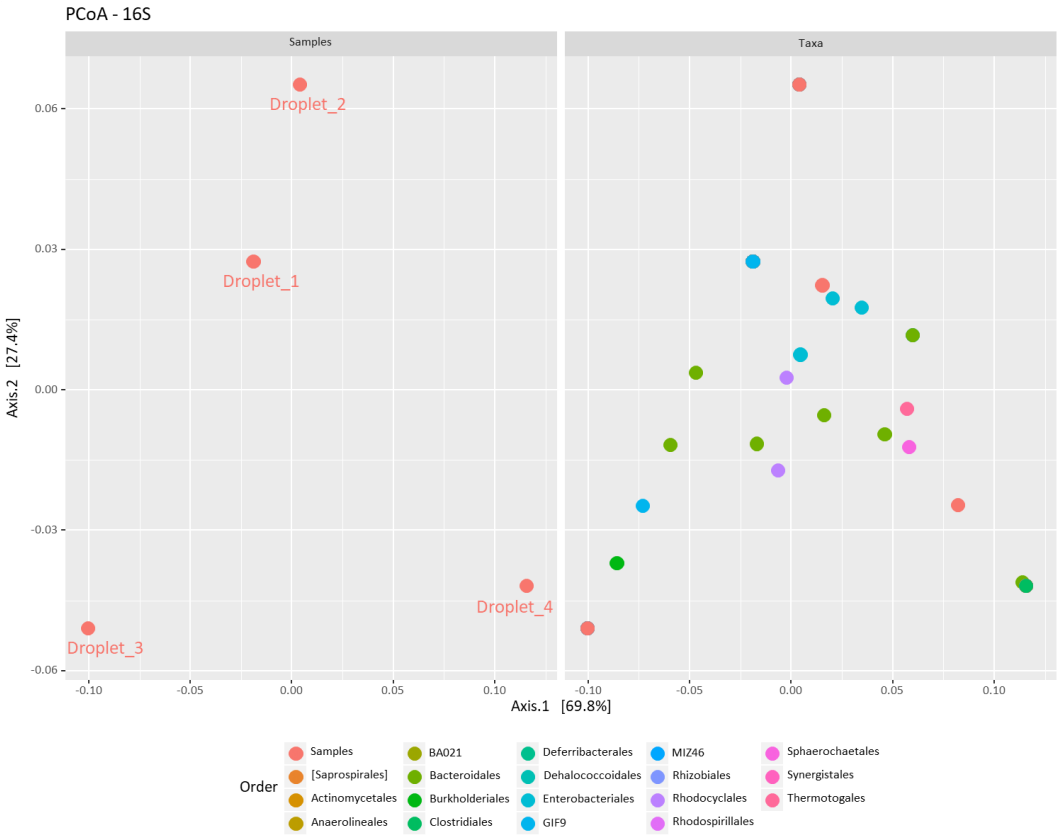


Figure 3-21: Principal Component Analysis of the microbial community composition, based on 16S rRNA genes for the four individual droplets.

As a next step, different diversity indices were calculated for the four sequenced individual droplets as shown in Figure 3-22. A comparison of the different options to calculate species richness and diversity within the droplets (Alpha-Diversity) showed clearly that the diversity is highest in Droplet 4 and lowest for most calculations in Droplet 2. This is in agreement with the rarefaction curves as shown in Figure 3-19, where the curve for Droplet 4 shows the steepest slope and the curve for Droplet 2 the one with the least steep slope. Only in the Chao1 and ACE diversity indices this order is changed. The diversity of Droplet 1 is higher than the diversity of Droplet 3 in five out of the 7 calculations, with this difference being the most prominent in the ACE index.

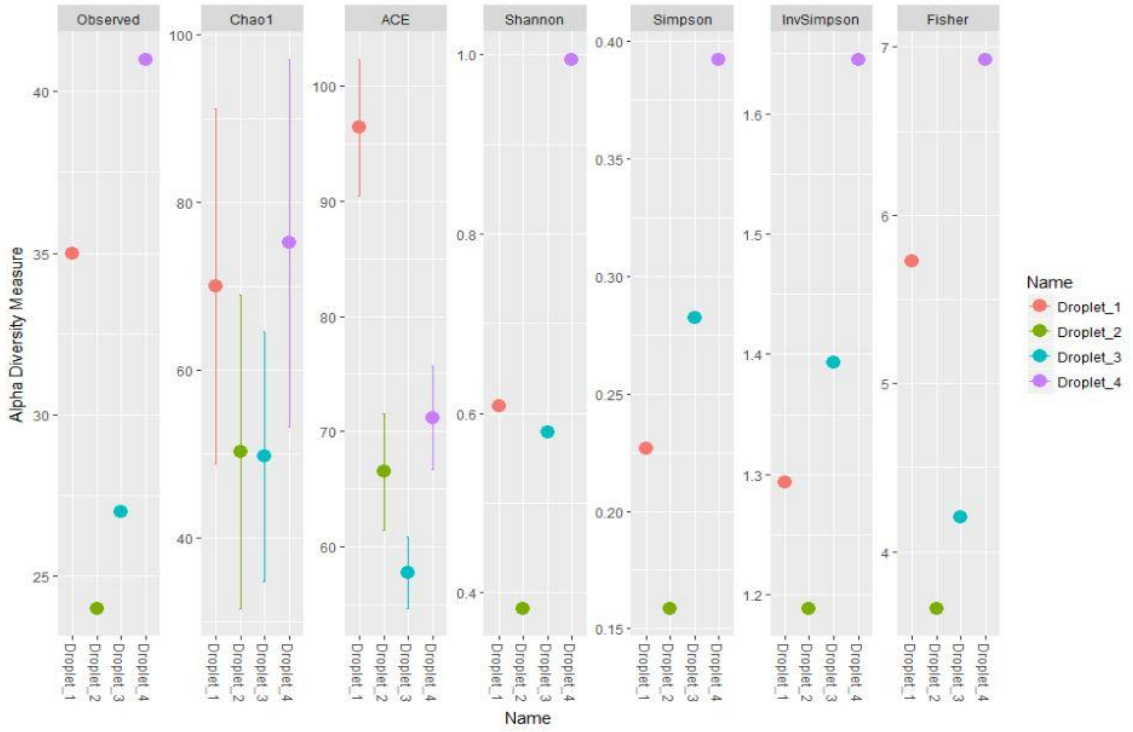


Figure 3-22: Comparison of computed diversity indices for the four sequenced droplets. The vertical distances are a measure of alpha-diversity between the four droplets in the depicted diversity indices. Error bars depict the standard error for the respective models for estimating richness.

Also, in the PCoA plots (Figure 3-21 and Figure 3-22) in can be observed that Droplet 2 and 4 are furthest apart on two axes calculated with a Bray-Curtis-dissimilarity, so the pattern continues.

To get an overview a diversity plot has been calculated for all Proteobacteria within the droplets, as the majority of bacteria belong to this phylum.

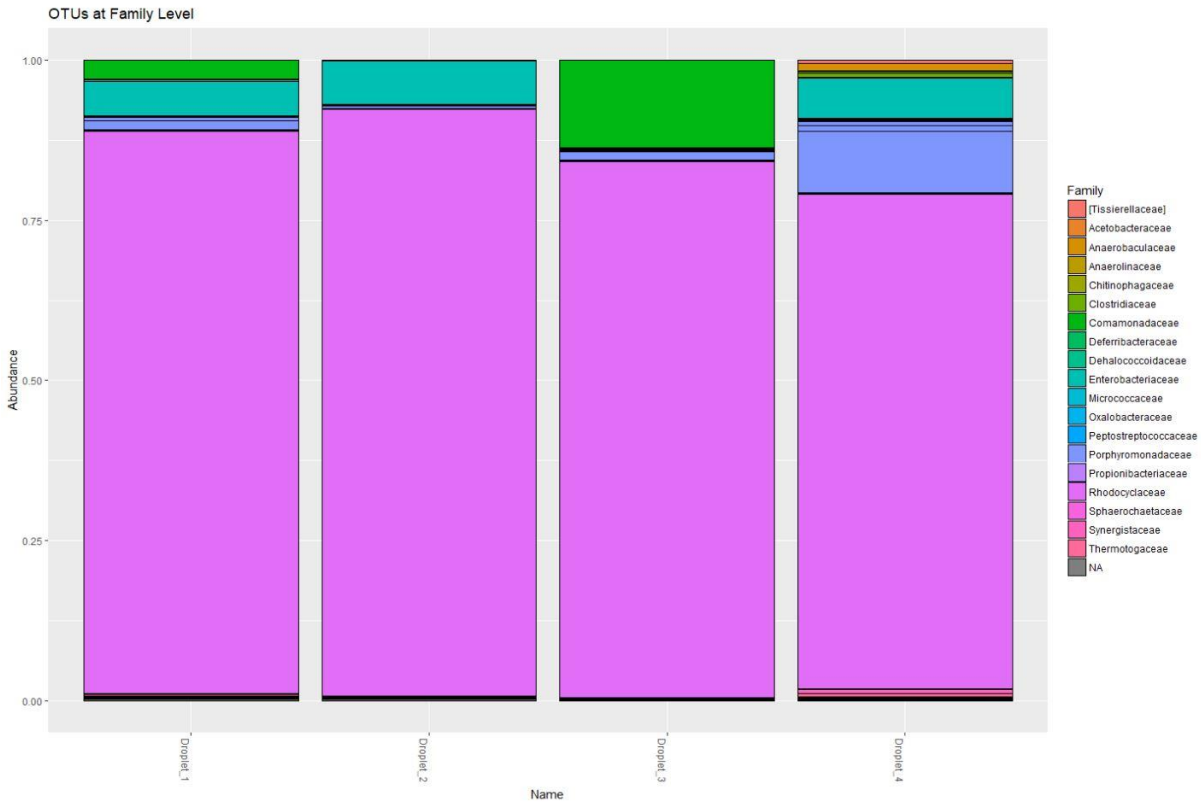


Figure 3-23: Relative composition of OTUs at the family level of all Proteobacteria within the droplets.

According to this plot the most abundant bacteria classified within the family of *Rhodocyclaceae*. The diversity shown in this plot is also in accordance with the calculated diversities in Figure 3-22 and the rarefaction curves in Figure 3-19 with Droplet 4 being the most and Droplet 2 being the least diverse.

The evaluation of the sequences from all four droplets also took place with the use of the SILVAngs platform online. Rarefaction curves have been calculated as well and are very similar in their order and the steep increase in the curves as compared to the rarefaction curves calculated with R (Figure 3-19).

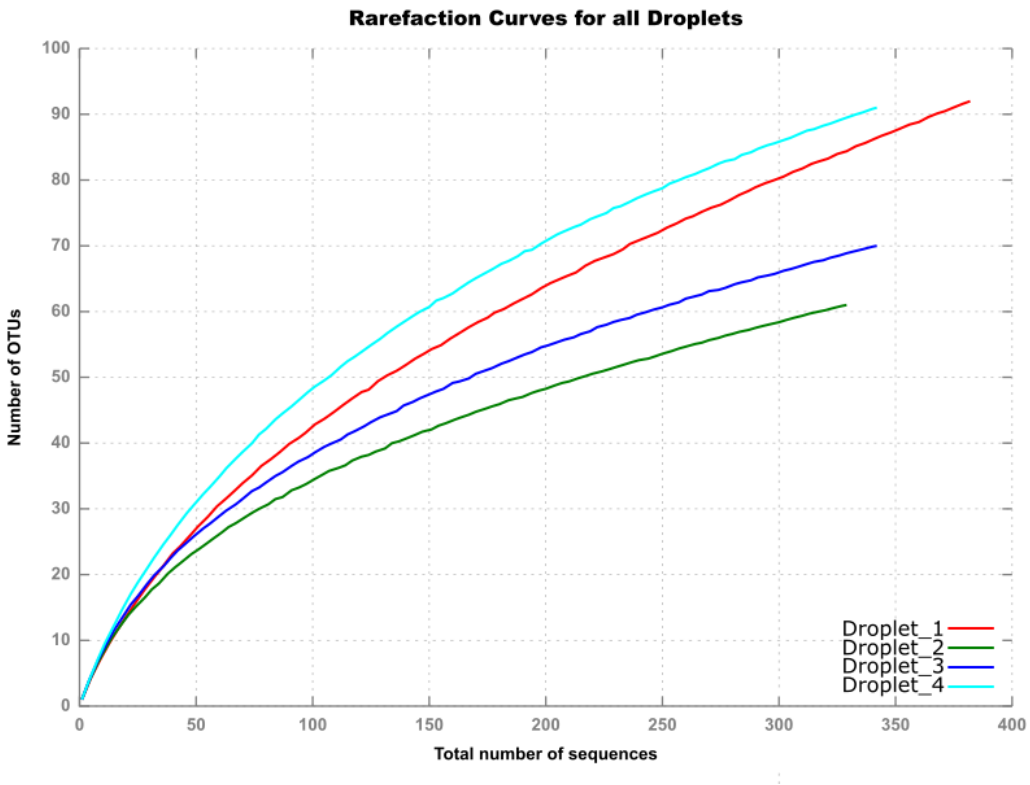


Figure 3-24: Rarefaction curves for the four sequenced droplets as calculated in the SILVAngs online tool. Both samples of rarefaction curves (Figure 3-19Figure 3-24) show comparable results.

An overview of the containing Phyla can be seen in Figure 3-25.

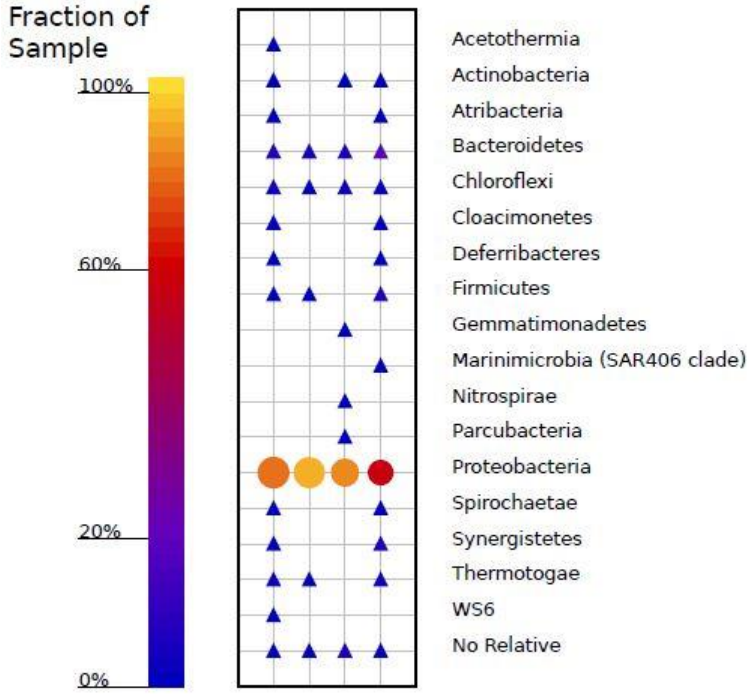


Figure 3-25: Taxonomic fingerprint at the phylum level of all four droplets as calculated by SILVAngs online tool. Triangles indicate a total sequence number of less than 100 sequences, circles indicate a range between 100 and 1000 sequences. The size of the shapes is accounting for the number of OTUs per phylum. Color indicates the fraction of the sequences in this sample belonging to the corresponding phylum.

From Figure 3-25 it is evident that all droplets contained a majority of Proteobacteria, which is as calculated with MOTHUR (Schloss et al. 2009). An overview over the number of OTUs that can be found in each droplet belonging to individual phyla can be seen in Figure 3-26 to Figure 3-29.

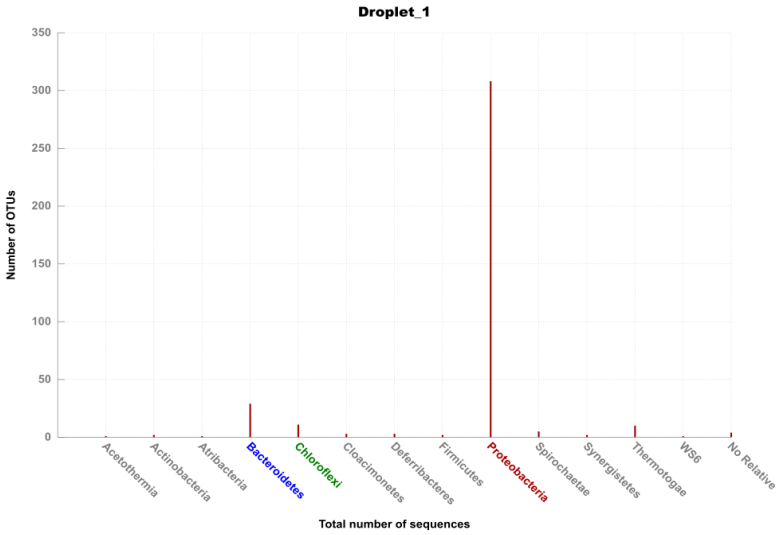


Figure 3-26: Community composition based on the absolute number of OTUs based on unique reads per phylum found in Droplets 1. Special colored phyla names indicate Proteobacteria, Bacteroidetes and Chloroflexi as the most abundant amplicon sequences in each droplet.

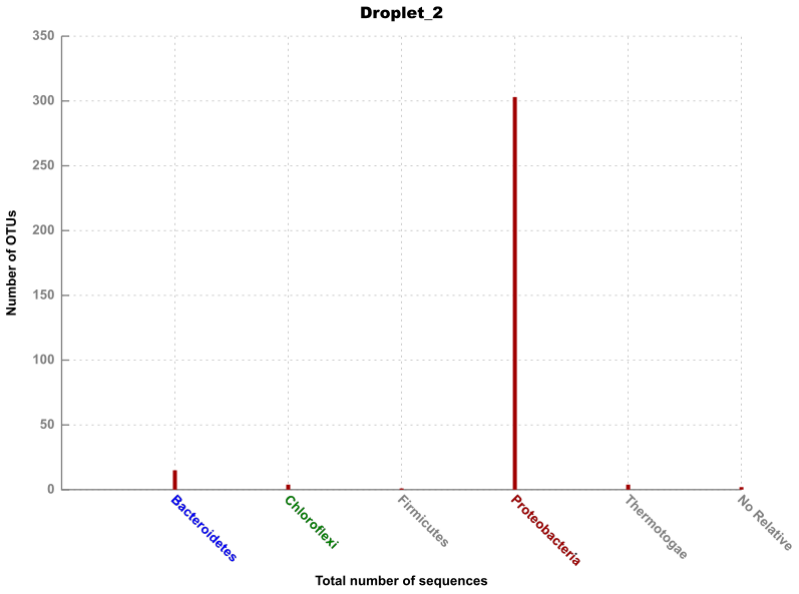


Figure 3-27: Community composition based on the absolute number of OTUs based on unique reads per phylum found in Droplets 2. Special colored phyla names indicate Proteobacteria, Bacteroidetes and Chloroflexi as the most abundant amplicon sequences in each droplet.

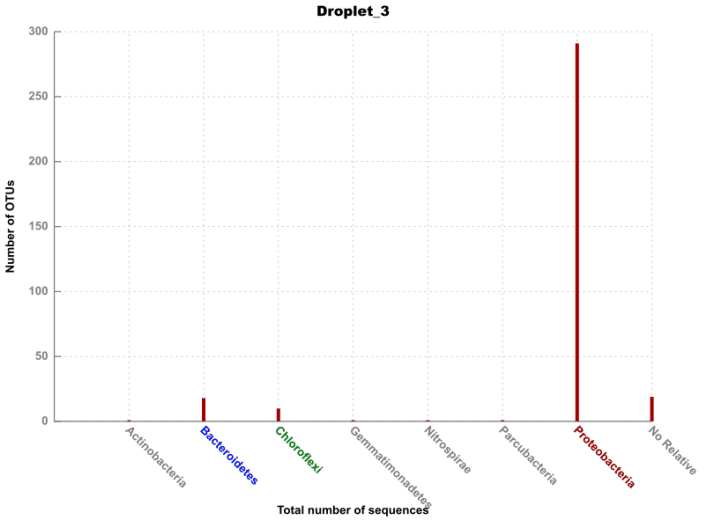


Figure 3-28: Community composition based on the absolute number of OTUs based on unique reads per phylum found in Droplets 3. Special colored phyla names indicate Proteobacteria, Bacteroidetes and Chloroflexi as the most abundant amplicon sequences in each droplet.

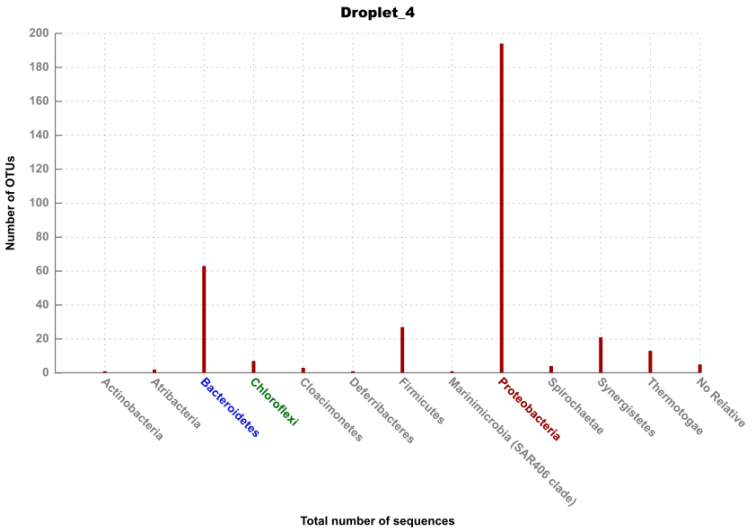


Figure 3-29: Community composition based on the absolute number of OTUs based on unique reads per phylum found in Droplets 1. Special colored phyla names indicate Proteobacteria, Bacteroidetes and Chloroflexi as the most abundant amplicon sequences in each droplet.

With SILVAngs it is easier to browse through all organisms in one droplet as its being depicted in a Krona plot. On the genus level *Tepidiphilus sp*. accounted for 62% of the bacteria in the droplet and 90% of the Betaproteobacteria within the droplet. It belongs to the family of *Hydrogenophilaceae*, which is not even mentioned in Figure 3-23, where all OTUs belonging to the *Proteobacteriaceae* are depicted. This is a main difference between the evaluation using Greengenes as taxonomy database versus the Silva database.

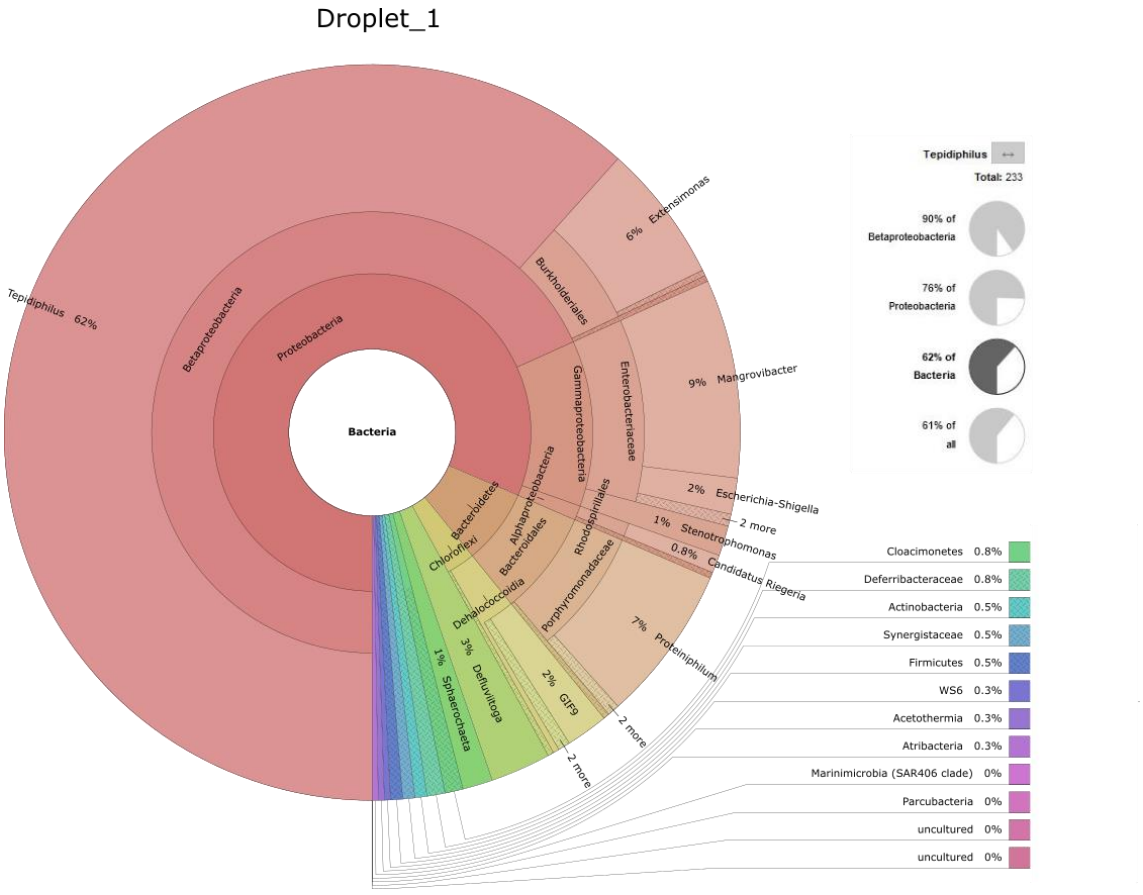


Figure 3-30: Krona plot of the taxonomic composition of Droplet_1, zoomed in to a level where the most dominating bacteria *Tepidiphilus sp*. can be identified at genus level. Charts in the upper right depict the portion of *Tepidiphilus sp*. of all Betaproteobacteria, Proteobacteria, Bacteria and all sequences, respectively.

In the second droplet with the lowest diversity of all four droplets *Tepidiphilus sp*. as the dominating bacterial genus was even more present and accounted for 77% of all bacteria (Figure 3-31).

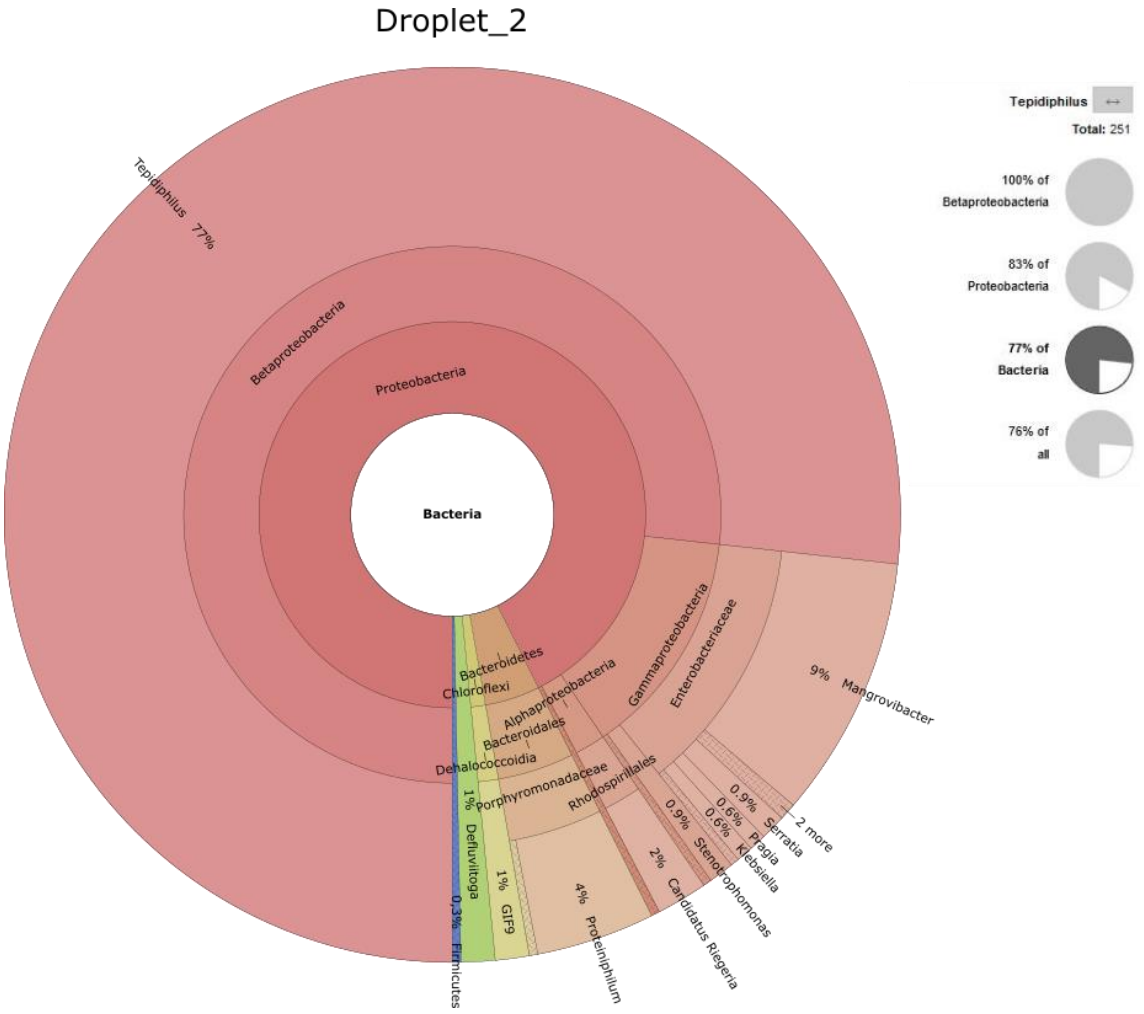


Figure 3-31: Krona plot of the taxonomic composition of Droplet_2, zoomed in to a level where the most dominating bacteria *Tepidiphilus sp*. can be identified at genus level. Charts in the upper right depict the portion of *Tepidiphilus sp*. of all Betaproteobacteria, Proteobacteria, Bacteria and all sequences, respectively.

Droplet_3 still got *Tepidiphilus sp*. as most abundant bacterium, with a total of 73% of the contained bacteria. The remaining bacteria did not differ vastly from Droplets 1 and 2.

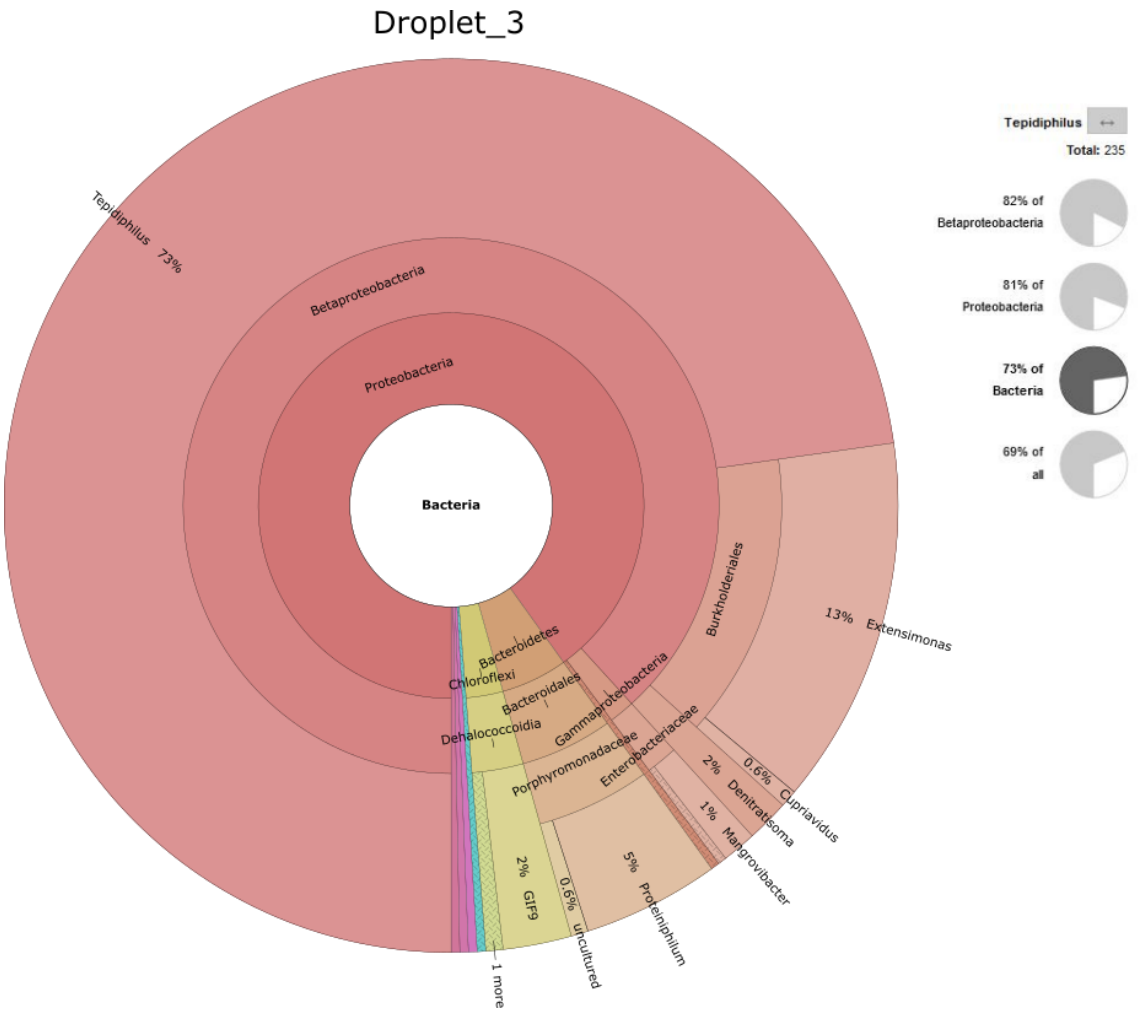


Figure 3-32: Krona plot of the taxonomic composition of Droplet_3, zoomed in to a level where the most dominating bacteria *Tepidiphilus sp*. can be identified at genus level. Charts in the upper right depict the portion of *Tepidiphilus sp*. of all Betaproteobacteria, Proteobacteria, Bacteria and all sequences, respectively.

In Droplet_4 (Figure 3-33) the proportion of *Tepidiphilus sp*. accounted for 50% of all bacteria. This was also in accordance with the rarefaction curves (Figure 3-24) where the curve for Droplet_4 is highest and the least steep, indicating that the sequencing depth for this droplet is not high enough to find most bacteria.

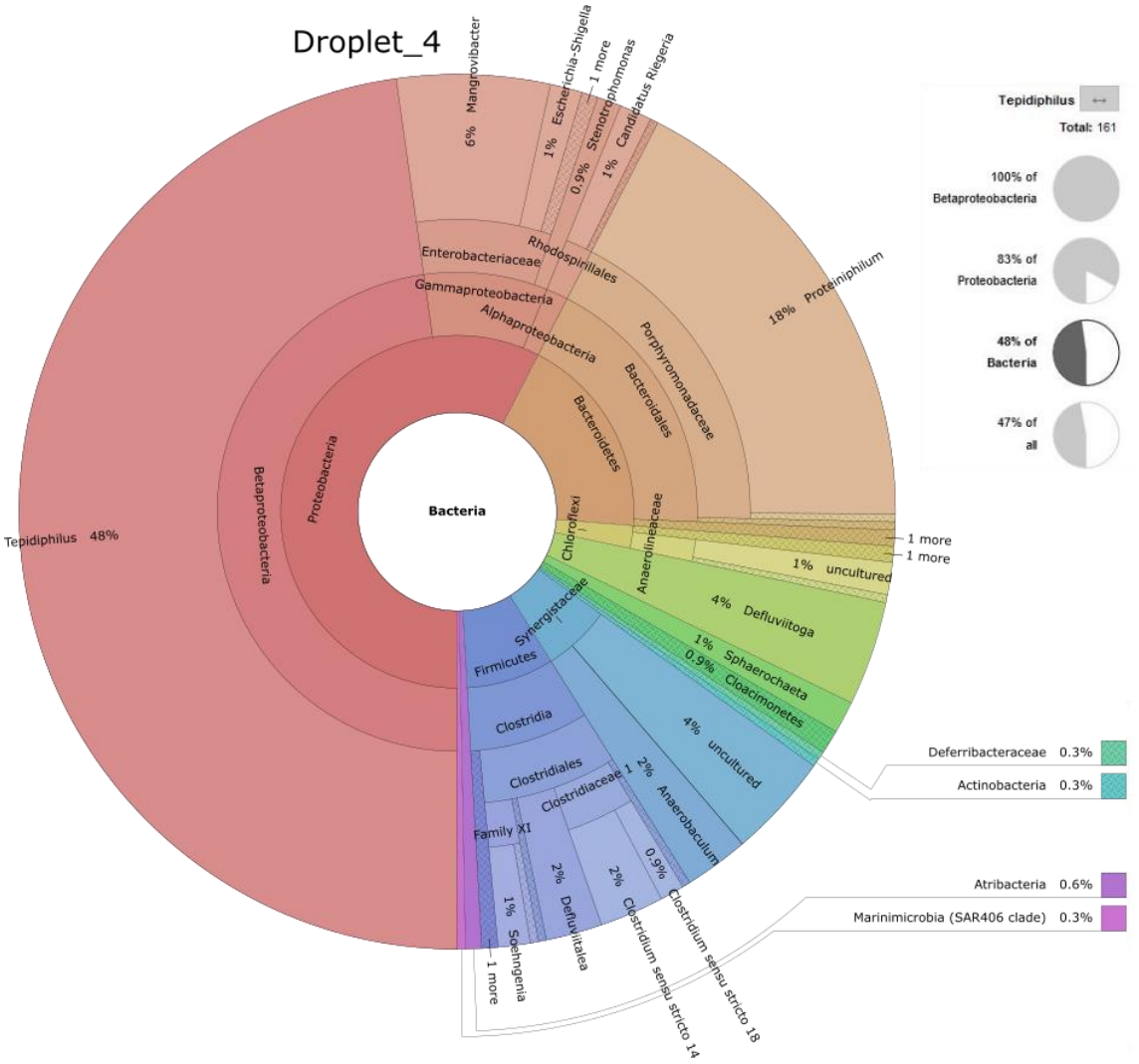


Figure 3-33: Krona plot of the taxonomic composition of Droplet_4, zoomed in to a level where the most dominating bacteria *Tepidiphilus sp*. can be identified at genus level. Charts in the upper right depict the portion of *Tepidiphilus sp*. of all Betaproteobacteria, Proteobacteria, Bacteria and all sequences, respectively.

All droplets were compared to the bacterial DNA extracted directly from the bitumen samples as shown in Figure 3-14. The dominating bacteria in all four droplets were *Tepidiphilus sp*., which accounted for total of 0.7% in the bitumen metagenome.

About 15% of bacteria in the bitumen DNA were δ-Proteobacteria. As many known hydrocarbon degraders are from that class, an anaerobic enrichment culture has been set up from samples directly adjacent to the bitumen in order to find bacteria capable of degrading PAHs anaerobically. The enrichment proved to be successful and is described in detail in the following section.

3.3 TRIP1 Enrichment culture

To gain first insights into the phenanthrene degradation capacity of bacteria growing in an extreme habitat as a “naturally contaminated” hydrocarbon lake a sulfate-reducing enrichment culture was established by Prof. Dr. Rainer Meckenstock and Gabriele Barthel (Anaerobic Degradation Group, Institute of Groundwater Ecology, Helmholtz Zentrum München). The culture was transferred three times with sediment before it was transferred sediment free. A variety of Terminal Electron Acceptors (TEAs) were tested, the sulfate-reducing culture was chosen to be cultured further. The interest in anaerobic degradation pathways of phenanthrene as a PAH of a higher molecular weight than the model PAH naphthalene was the main reason to establish this culture. Pathways are expected to be similar to naphthalene degradation pathways in their step wise manner and enzymatic reactions. As carboxylation (Zhang and Young 1997; Meckenstock et al. 2000; Musat et al. 2009), ligation with CoA (Meckenstock et al. 2016) and ring reduction steps (Eberlein, Johannes, et al. 2013) have been characterized well by now for the anaerobic naphthalene degradation; these will be the enzymatic reactions we are going to test for in this culture.

3.3.1 Culture description

The “Trinidad Phenanthrene” (TRIP1) enrichment culture was grown on sediment from the vicinity of the pitch lake. It is growing under sulfate-reducing conditions with a sulfate concentration of 20 mM, but can also grow with as little as 8 mM, as calculated in Meckenstock *et al.* (2016):

$$C_{14}H_{10} + 8.25\ SO_4^{2-} + 9\ H_2O \rightarrow 14\ HCO_3^- + 8.25\ HS^- + 5.75\ H^+$$

3.3.1.1 Sulfide / Sulfate Measurements as a means to determine culture growth

Sulfide and sulfate were measured in the culture according to 2.4.1 and 2.4.2. Sulfide measurements were done in weekly turns; the sulfide production is expected to rise over time as sulfate is reduced to sulfide. The sulfide production is depicted in the following picture:

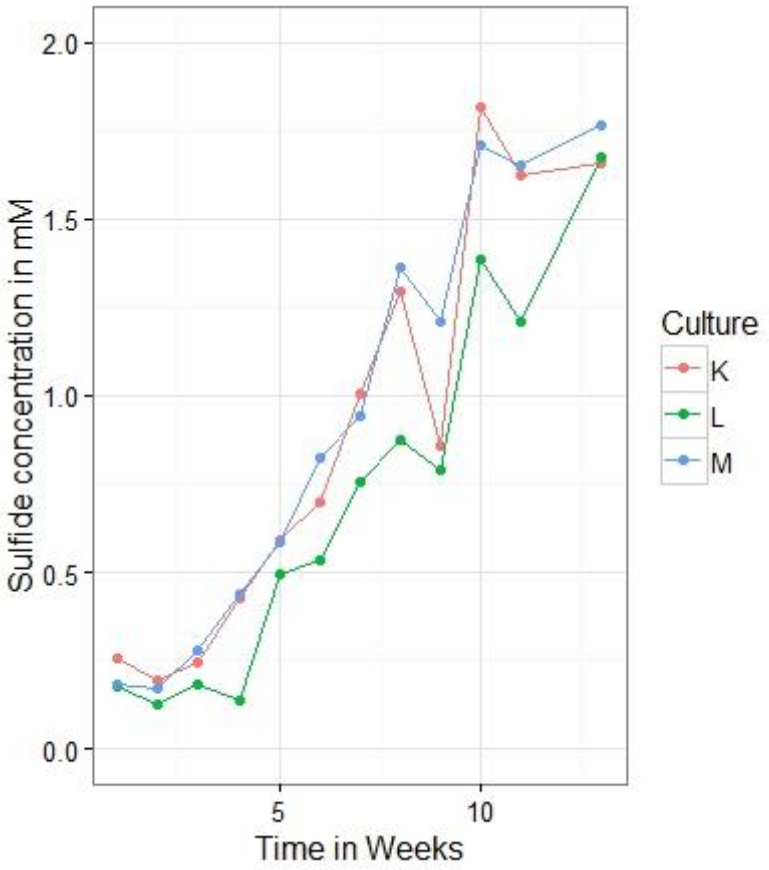


Figure 3-34: Sulfide production of the enrichment culture TRIP 1 with phenanthrene as electron donor and sulfate as electron acceptor over 14 weeks; K, L and M depict biological triplicates.

The increase of sulfide was from 0.25 mM over 14 weeks to 1.75 mM to a total of 1.5 mM.

Expected sulfate concentration curves would start at 20 mM of sulfate at measuring point t_0 at the point of inoculation and sulfate concentrations would decrease over time, as sulfate is being reduced to sulfide in the culture. The following sulfate concentration curves could be obtained:

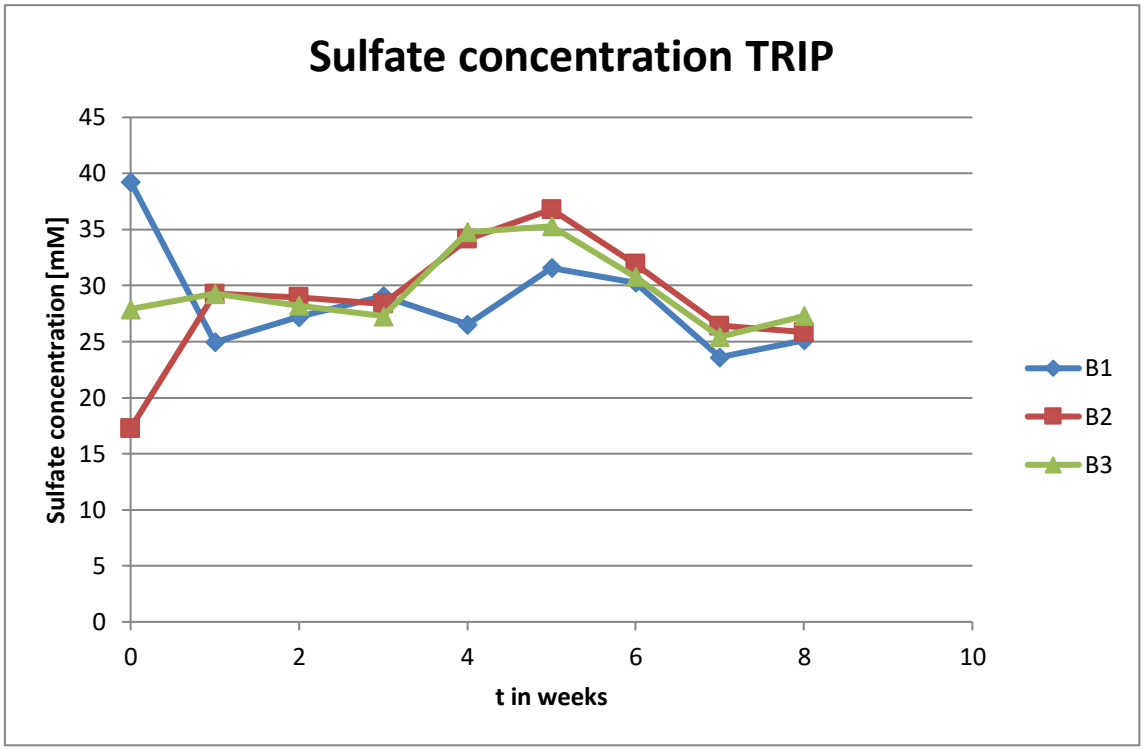


Figure 3-35: First sulfate concentration curve of enrichment culture TRIP1 over a time span of eight weeks. B1, B2 and B3 depict biological triplicates

In contrast to the expectation of a decrease in sulfate concentrations in connection to the rise in sulfide concentrations as depicted in Figure 3-34, no decrease in sulfate concentrations was visible in the sulfate measurements that could account for the sulfide increase.

Basically, there is no decrease in sulfate concentration over the time of eight weeks, even though the cultures have been growing visibly. Sulfate growth curves were therefore repeated (Figure 3-36). That curve shows variations in sulfate concentrations of about 10 mM, but in the end the sulfate concentrations were measured within ±3mM of the original concentrations.

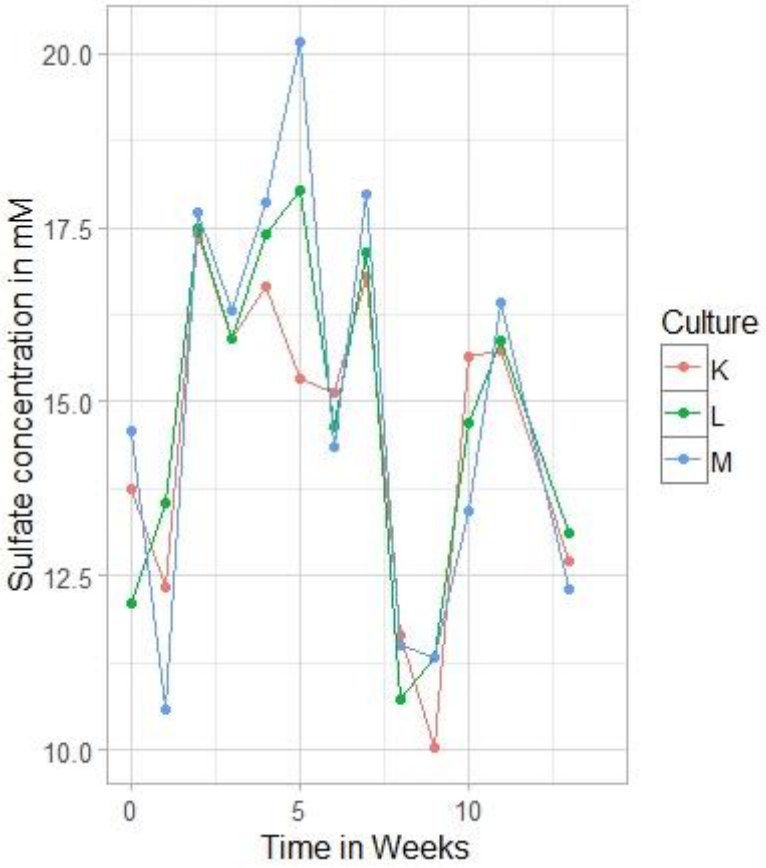


Figure 3-36: Final sulfate concentration curve from sulfate measurements over 16 weeks. K, L and M depict biological triplicates.

In the end it can be said that for the several different cultures there is neither decrease nor increase in sulfate values albeit the visible increase in biomass and the sulfide increase as depicted in Figure 3-34.

A growth curve prepared by Zahra Farmani within the framework of her Master Thesis in the laboratory of Prof. Rainer Meckenstock in Essen on TRIP1 cultures inoculated from the above shown cultures and measured after the same protocol gave following sulfate depletion curve Figure 3-37:

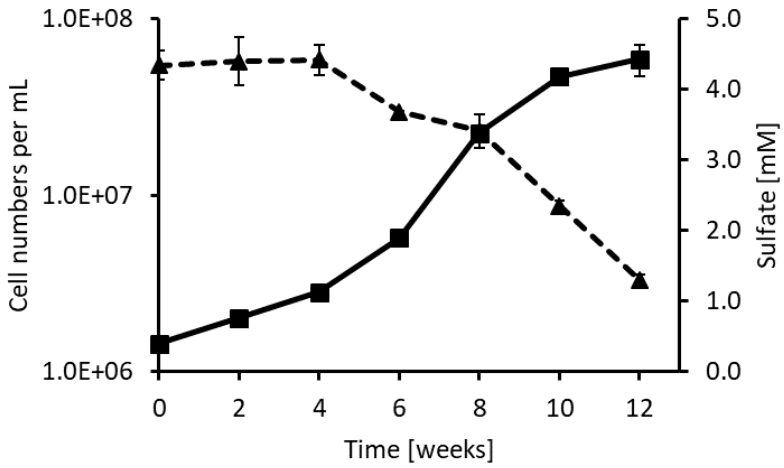


Figure 3-37: Sulfate depletion curve coupled to cell counts as measured by Zahra Farmani, University of Essen, on TRIP1 cultures. The solid squares indicate the cell numbers per mL of culture. The solid triangles depict the sulfate concentrations, determined by barium chloride method (Tabatabai 1974). The error bars depict standard deviations of triplicate incubations.

In culture TRIP1, sulfate-depletion of 2.42 mM was coupled to phenanthrene depletion of 0.08 mM (theoretical concentration in the aqueous phase, as calculated from the phenanthrene loss in the HMN carrier phase). The resulting electron recovery of 341% can be explained with the loss of phenanthrene by absorption into the butyl stopper. Nevertheless, we propose that culture TRIP1 fully oxidizes phenanthrene to CO2, as members of the *Desulfobacteraceae* are commonly documented as complete oxidizers. The culture is growing under sulfate-reducing conditions and it took a growth time of around eight weeks until the culture was transferable. This has been determined by cell counting of DAPI stained cells from the culture in a biweekly mode (Figure 3-37).

The growth curve showed a lag phase of about four weeks when growing the culture at 30°C. After the fourth week, the culture entered the exponential growth phase, and the time point of eight weeks to transfer the culture to fresh medium falls well within this phase. Even with a fully-grown culture the medium never turned turbid, only very little biomass was obtained and the turbidity was hardly visible to the naked eye.

3.3.1.2 Microbial community composition

Genome-centric metagenomic analyses (Gkanogiannis et al. 2016) of the community genome from the culture TRIP1 led to the reconstruction of a handful of nearly complete genomes, including the genome of the most prominent organism. Table S1 (Supplemental Material) displays the ten organisms from

NCBI's Refseq database (https://www.ncbi.nlm.nih.gov/refseq) that are more closely related to the dominant *Desulfobacteraceae* organism in terms of genome-level syntenic relationships (D. Vallenet et al. 2006; David Vallenet et al. 2013).

Table 3-6: Top ten Refseq genomes closest related to the dominant PITCH1 *Desulfobacterium* of culture TRIP1 in terms of syntenic relationships. The different columns provide the number (Nb) and percentage (%) of genes which are involved in BBH (Bidirectional Best Hit) relationships between our key player's genome and the genome. The key players genome is compared to (named in the last column), along with the number of synteny groups (Synton Nb, defined as in Boyer *et al.*, 2005), along with the syntons minimum, maximum and average size (expressed in number of genes). The genome of the naphthalene degrading bacterium NaphS2 clearly tops this list.

Nb	%	Nb	%	Synton Nb	Min	Avg	Max	Replicon Name
1499	30.79	2203	45.25	661	1	3.12	23	Deltaproteobacterium NaphS2 NZ_ADZZ
1191	24.46	1913	39.29	449	1	3.45	39	*Syntrophobacter fumaroxidans* MPOB NC_008554
1046	21.48	1893	28.88	398	1	3.09	39	*Desulfobacterium autotrophicum* HRM2 NC_012108
1040	21.36	1965	40.36	419	1	3.03	39	*Desulfatibacillum alkenivorans* AK-01 NC_011768
1018	20.91	1708	35.08	346	1	3.39	38	*Desulfarculus baarsii* DSM 2075 NC_014365
950	19.51	1722	35.37	323	1	3.13	39	*Desulfococcus oleovorans* Hxd3 NC_009943
909	18.67	1788	36.72	350	1	2.86	37	*Geobacter uraniireducens* Rf4 NC_009483
902	18.53	1701	34.94	338	1	3.04	36	*Desulfobulbus propionicus* DSM 2032 NC_014972
872	17.91	1651	33.91	347	1	2.86	38	*Geobacter metallireducens* GS-15 NC_007517

The 16S rRNA gene sequence of this bacterium was most closely related to *Desulfobacterium anilini* strain Ani1 (= DSM 4660(T)) and *Desulfobacterium anilini* strain AK1 (94% identity). The latter were recently reclassified as *Desulfatiglans anilini* species (Suzuki et al. 2014). Another closely related bacterium was *Desulfobacterium* spec. strain NaphS2 (93% similarity, Galushko *et al.*, 1999), with only weaker (87%) similarity to the Phe4A strain from the phenanthrene-degrading culture Phe4 (Davidova et al. 2007). Other organisms with nearly complete reconstructed genomes showed similarities to *Desulfatiglans anilini* strain AK1 (98% similarity), *Paludibacter propionicigenes* (91% similarity) as well as to an unknown *Spirochaetales*. As the similarity to the *Spirochaetales* is deduced from genome-level syntenies but no 16S rRNA

sequence could be recovered from the corresponding organism in the TRIP1 culture, it is not included in the tree depicted in Figure 3-38.

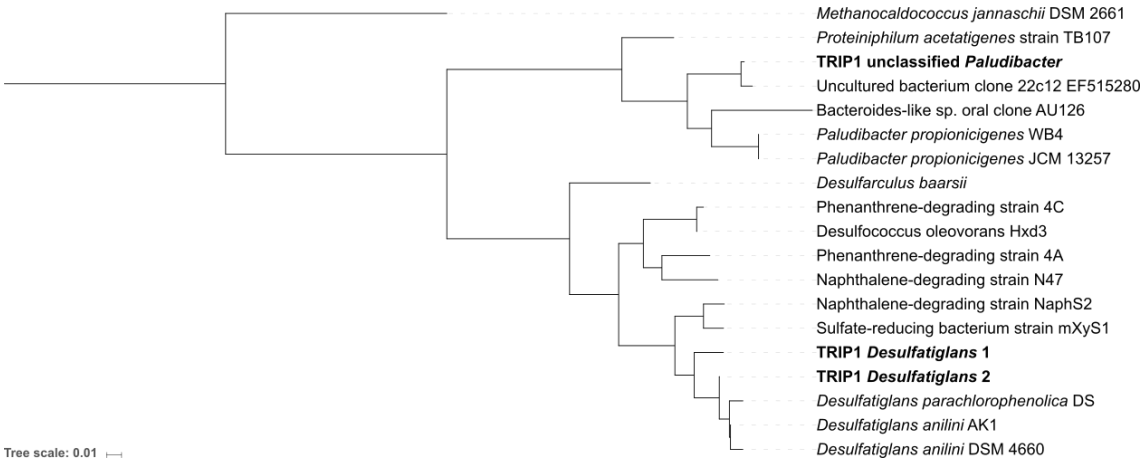


Figure 3-38: Phylogenetic tree of selected members of the enrichment culture TRIP1, including the dominant Desulfatiglans organism (Desulfatiglans-1), and closely related species together with other known PAH-degrading bacteria, based on neighbor-joining analysis of full length 16S rRNA gene sequences obtained from the metagenome. Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2011) and are expressed in units of base substitutions per site. Members of the TRIP1 enrichment culture are marked in bold. Geobacter and Paludibacter like species were present at less than 2% relative abundance, and were not included in this analysis.

3.3.1.3 FISH analysis for the determination of the main dominating bacterium

The unknown *Desulfatiglans*, determined as the most dominant bacterium in the culture, was calculated to account for 60.36% of all reads based on raw read mapping of five almost closed genomes within the culture. The other genomes make up for 2.1%, 1.17%, 0.51% and 0.5%, respectively. The negative control for probe TRIP_Desulfo183-Fam was a sample of the naphthalene degrader N47. The estimate for the abundance of the dominant *Desulfatiglans* could be independently confirmed by flow cytometric counting of samples from the TRIP1 cultures stained with fluorescence in situ hybridization (FISH) probes specific for this organism. The cell count in a flow cytometer of cell stained with FISH probes showed about 50% of the cells stained with the FISH-Probe designed for the most dominating bacteria with 3.4×10^6 counts (TRIP_Desulfo183-Fam) with a total cell count of 6.8×10^6 cells (sd = 4.8×10^6).

3.3.1.4 Metabolite extraction as a first indication of the degradation pathway

Metabolites were extracted in weekly modus from duplicates of cultures set up in larger volumes to account for the 50 mL of culture necessary for the extraction. Sulfate was measured simultaneously in both enrichments to monitor growth. The

results were similar to the sulfate extraction in the 150 µL culture bottles; no trend of sulfate reduction could be measured. After extraction of the metabolites the compounds were derivatized and stored until they could be measured with GC-MS. From this measurement a possible metabolite identified could be Terephthalic acid, with a structure that resembles a possible reduced metabolite:

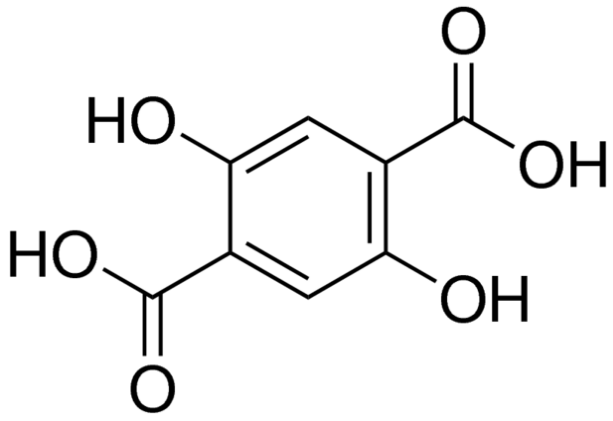


Figure 3-39: Chemical structure of a metabolite within the culture as measured on GC-MS, possibly an oxidized derivate of terephthalic acid.

Further metabolites could not be detected; especially 2- or 4-phenanthroic acid could not be identified, which led to a preparation of samples to be measured on the LC-MS/MS, because of its higher detection limit.

A time series showed a gradual increase in metabolites, starting from week four at an intensity of around 500 to its peak in week nine with an intensity of 12.000, before declining again. In Figure 3-40 we see the relative abundances of the extracted metabolites from the cultures in black compared to the standards of 2-phenanthroic acid and 4-phenanthroic acid in blue and red, respectively. A clear overlap in retention times from the extracted metabolites appeared with 2-phenanthroic acid, whereas the retention time for 4-Phenanthroic acid was much shorter and could be excluded as possible metabolite for the carboxylation reaction. 1- and 3- phenanthroic acid could up until now not be ruled out due to unavailability of standards (Figure 3-40).

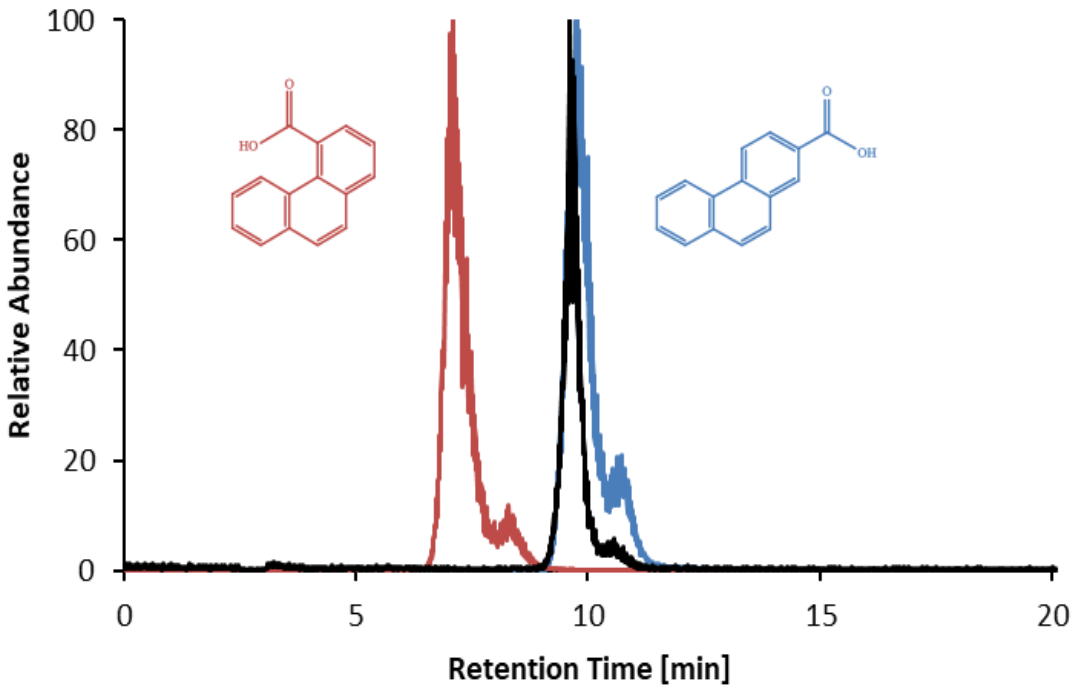


Figure 3-40: Diagram of retention times as measured by LC-MS/MS of 2- and 4-phenanthroic acid (blue and red lines, respectively) compared to extracted metabolites from TRIP1 culture (black line).

3.3.1.5 Culture characterization with substrate and TEA tests

Further on we conducted substrate tests. Substrates have been chosen by their likelihood to be degraded (benzene, benzoic acid, 2- and 4-phenanthroic acid, naphthalene, 1- and 2-naphthoic acid) or the higher molecular weight (HMW) PAHs of interest, e.g. acenaphthylene, acenaphthene, anthracene and fluoranthene. Growth could not be detected on the HMW PAHs, but on 2- and 4-phenanthroic acid, terephthalic acid as possible intermediate during degradation processes, 1, 2, 3, 4-tetrahydro-naphthoic acid; 5, 6, 7, 8-tetrahydronaphthoic acid; 1- and 2-naphthoic acid, benzoic acid and on a phenanthrene control. All cultures with visible growth have been suspect to T-RFLP analysis and a total cell count on the flow cytometer to evaluate whether the dominant *Desulfatiglans* bacterium remained the same. Benzene showed growth in different stages but that could not be confirmed after a second transfer four months after initial inoculation.

Table 3-7: Substrate utilization by culture TRIP1. a) Growth was evaluated as positive (+) if the cell count in the second transfer increased more than ten-fold compared to the control without electron acceptor (9.8 x 104), as measured by flow cytometry, (-) bacterial growth lower than ten-fold compared to non-substrate control or no detectable growth, (*) PCR amplification not possible b) Fragment length in bold describes the dominant peaks in the TRFLP analysis.

Substrate	**Total cell count** [a]	**T-RFLP fragment length bp** [b]	**Assignment to organism**

Phenanthrene (positive control)	6.8 x 10^6 (+)	**53**; 74	*Desulfatiglans* 1
Naphthalene	*(*)*	_	
Acenaphthene	*(*)*	_	
Anthracene	*(*)*	_	
Fluoranthene	*(*)*	_	
Benzene	7.0 x 10^6 (+)	53; 74; **515**	*Desulfatiglans* 2
Benzoic acid	5.1 x 10^7 (+)	53; 74; **515**	*Desulfatiglans* 2
1-Naphthoic acid	2.3 x 10^6 (+)	*(*)*	
2-Naphthoic acid	2.7 x 10^6 (+)	**53**; 74; 97	*Desulfatiglans* 1, unclassified bacterium
1,2,3,4-Tetrahydro-2-naphthoic acid	5.3 x 10^4 (-)		
5,6,7,8-Tetrahydro-2-naphthoic acid	5.2 x 10^4 (-)		
2-Phenanthroic acid	1.3 x 10^7 (+)	53; **404**	unknown
4-Phenanthroic acid	7.1 x 10^5 (+)	**53**; 74	*Desulfatiglans* 1
Terephthalic acid	8.6 x 10^6 (+)	**156**	unknown

We conducted growth tests on terminal electron acceptors different from sulfate and determined growth by total cell count (TCC) on a Flow cytometer of the cultures growing on nitrate, elemental sulphur, iron-(III)-ferrihydrite as well as in the culture growing without a terminal electron acceptor. A T-RFLP analysis was done after the second transfer in order to evaluate if the diversity in the culture changed due to different terminal electron acceptors. We found that the most dominant bacterium within the culture was the same in the cultures growing on nitrate and iron-(III)-ferrihydrite. A slight shift in the dominant bacterium took place in the well growing cultures on sulphur and without terminal electron acceptor. The latter cultures also looked more turbid after the eight weeks period than that of the phenanthrene control.

Growth on elemental sulphur yielded both a rise in sulfate and sulfide as determined by ion chromatography, of 3,48 mM (sd = 0,22 in biological triplicates) and 7,00 mM (sd = 0,39 in biological triplicates), respectively. In the phenanthrene control the decrease in sulfate was about 5,67 mM (sd = 0,3 in biological triplicates), the increase in sulfide was 6,23 mM (no sd, just one control measured). Nitrate and ammonium measurements in the culture using nitrate as terminal electron acceptor showed an increase in the following amounts: 6.58 mM (sd = 0.59 in biological triplicates) for nitrate and 0.25 mM (sd = 0.003 in biological triplicates and technical duplicates) for ammonium.

3.3.1.6 Flow Cytometer cell counts

Growth was regarded as positive when the counts where tenfold higher than in the control of the culture without terminal electron acceptor.

Table 3-8: Cell counts on different tested substrates. Indicated replicates are biological replicates.

Substrate	Number of replicates	Average TCC
Phenanthrene control	3	6,8*10^6
1-Tetrahydro-2-naphthoic acid	3	5,3*10^4
5-Tetrahydro-2-naphthoic acid	3	5,2*10^4
2-Phenanthroic acid	3	1,3*10^7
4-Phenanthroic acid	2	7,1*10^5
Benzene	1	7,0*10^6
1-Naphthoic acid	3	2,3*10^6
2-Naphthoic acid	3	2,7*10^6
Terephthalic acid	3	8,6*10^6
Benzoic acid	3	5,1*10^7

The total cell counts were higher than in the phenanthrene control on 2-phenanthroic acid, benzene, terephthalic acid and benzoic acid. These substrates seem to be more degradable, as they are of a lower molecular weight. When these results are combined with the results from the T-RFLP assignment to the available bacs it can be seen that 2-phenanthroic acid was most likely degraded by the main dominating *Desulfatiglans* in the culture, as this substrate was already activated by carboxylation. However, the T-RFLP-peaks for the other substrates showed a shift. These substrates were therefore presumably degraded by other bacteria than the dominant phenanthrene degrader.

Table 3-9: Total cell counts and counts of cells stained with FISH probes.

Terminal electron acceptor	FISH stained cell counts	Total cell counts
Sulfate Control (TRIP1 Culture)	1,0*10^5	3,4*10^6
Nitrate	9,5*10^4	7,4*10^4
Sulphur	9,0*10^4	6,1*10^4
No TEA	9,8*10^4	5,8*10^4

It is evident, that in both the FISH stained cells counts and the total cell counts sulfate was the TEA with the highest growth yield.

3.3.2 Enzyme assays

3.3.2.1 Carboxylase Assay

We set up an enzyme assay for the carboxylase reaction both with crude cell suspension and cell-free extract with phenanthrene as substrate and sodium bicarbonate ($NaHCO_3$) both with and without ATP as described in the methods section 2.5, Table 2-26. Cell-free extracts as well as crude cell extracts were used to start the assay respectively, as previous assays also had been performed with crude cell extracts. The first assay was set up with cultures being 18 weeks old, with biomass being visible in amounts that were deemed to be useful to set up assays. No carboxylase enzyme activity could be measured in these cells, even though they were from the same cell batch that yielded the ligase enzyme activity displayed below in 3.3.2.2.

The assay was repeated with cultures being 10 weeks old, to assure a higher enzymatic activity, but it seems that the biomass in these cultures was not sufficient to provide results measurable in the HPLC, no carboxylated metabolites could be detected. However, it can not be inferred that a direct carboxylation reaction is not happening.

3.3.2.2 Phenanthroate-CoA-ligase Assay

The ligase assay with 2-phenanthroic acid and coenzyme A as substrates was performed on an 18-week-old 1.6 L culture, which was beyond the usual transfer time, but yielded enough biomass to perform the assays. We were able to show the ligation reaction with cell free extract taking place with coenzyme A and 2-phenanthroic acid as substrate (Figure 3-41). Other tested substrates were 4- and 9-phenanthroic acid, none of which yielded a conversion of the substrate. A clear shift from 2-phenanthroic acid to 2-phenanthroyl-CoA could be detected via HPLC measurement within a 90-minute incubation period, during which the substrates were completely used up (Figure 3-41). We tested different reducing agents in order to activate downstream reactions, but they did not yield a peak detectable with the HPLC, e.g. the initial reduction steps as expected from the anaerobic naphthalene degradation pathway.

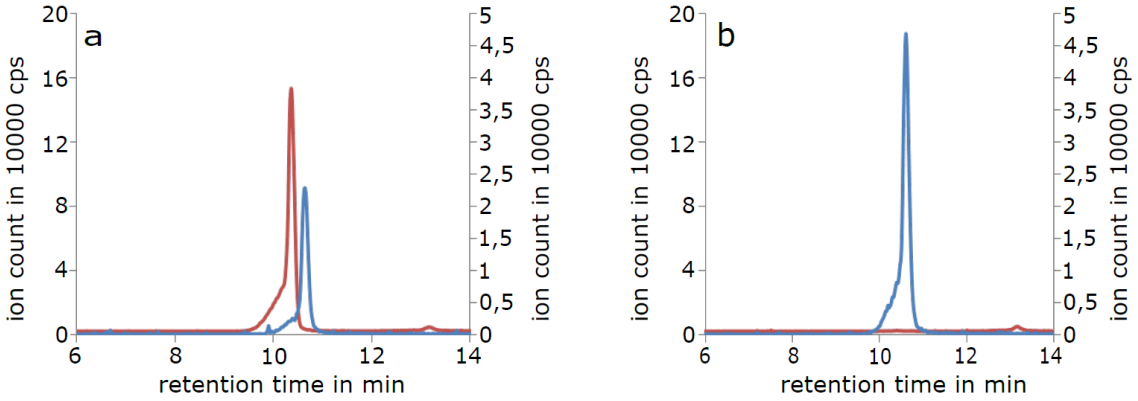


Figure 3-41: LC/MS chromatogram of the ligation reaction of 2-phenanthroic acid (red line) to 2-phenanthroate-CoA (blue line). Time Points during ligase assay: Panel a) t = 0 min, Panel b) t = 10 min

The complete vanishing of the 2-phenanthroic acid as seen after only 10 minutes is a strong indication of the complete use up of this substrate for this reaction.

3.3.2.3 Reductase Assay

The reductase assay was performed in two different ways. The first option was to add different reducing agents to the ligase reaction, like NADH, NAD^+, NADPH or 2-α-ketoglutarate to achieve a seamless downstream conversion of the produced 2-phenanthroyl-CoA. As described above no reduction steps could be confirmed in the HPLC spectrogram that indicated a stepwise ring reduction up until the ring-opening reduction. However, it is interesting that during metabolite extraction from the culture metabolites varying states of reduced ring structures have been found.

The second way to test for the reductase in an assay was to add chemically synthesized 2-phenanthroyl-CoA as a direct substrate for the reductase, and the above-mentioned reducing agents. During this round of assays no peaks could be detected, most likely due to too little biomass in the assay. The cultures were grown in 1.6 L batches, for only 10 weeks, which is most likely too short to build up enough biomass in such a big batch.

3.3.2.4 Metabolite Analysis / Downstream pathway

After the full growth time of 12 weeks, the culture was subject to metabolite analysis on a High-Resolution GC-MS. The following masses were detected (Figure 3-42):

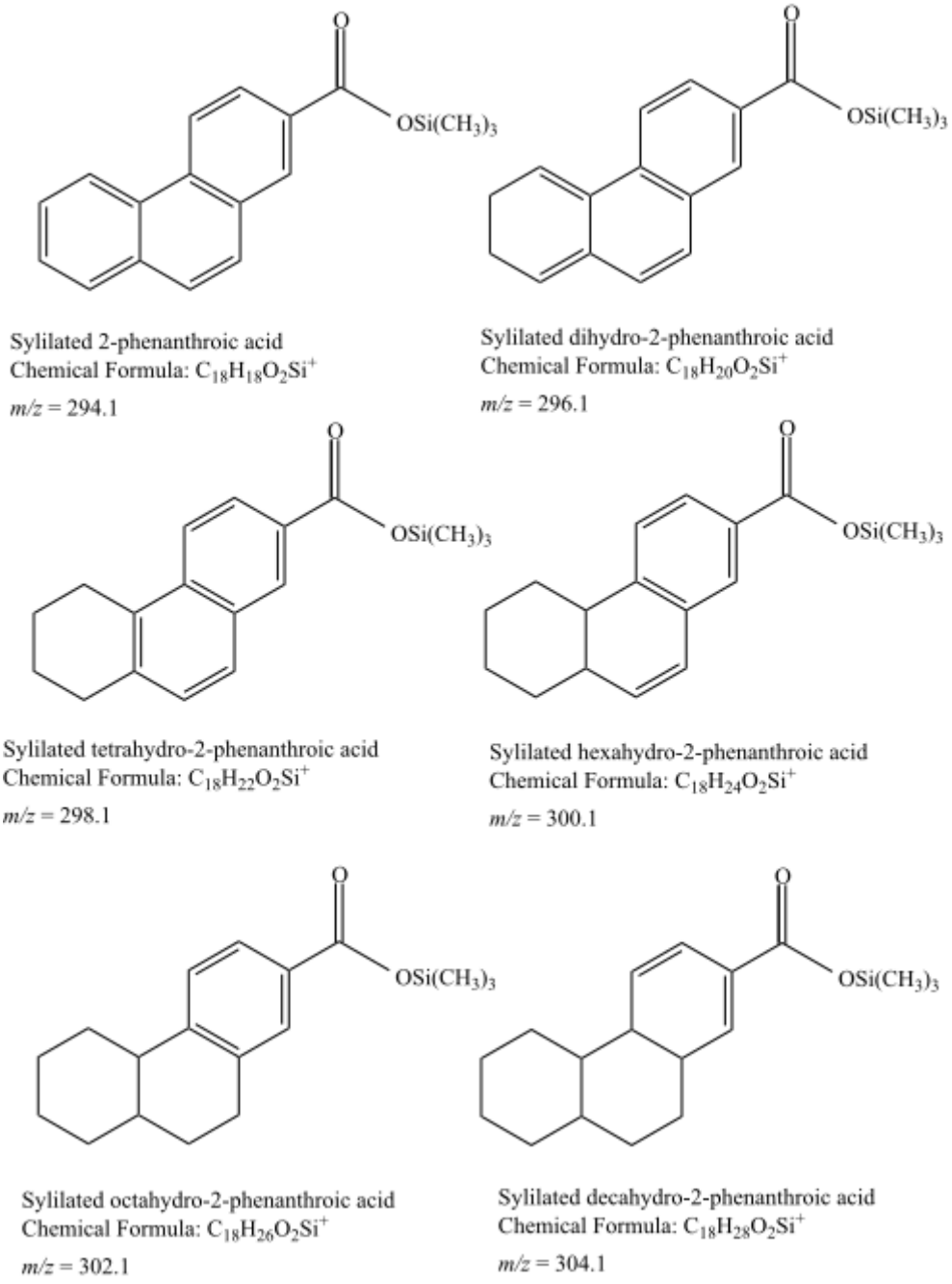
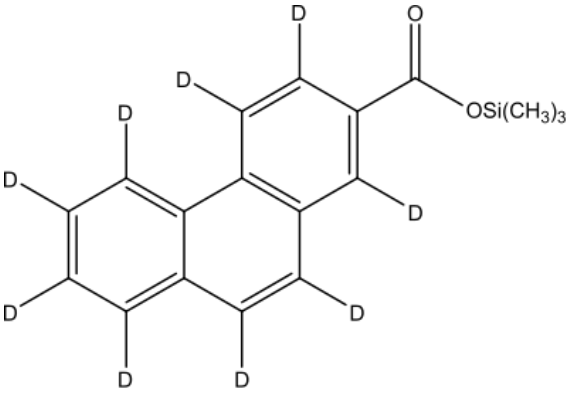


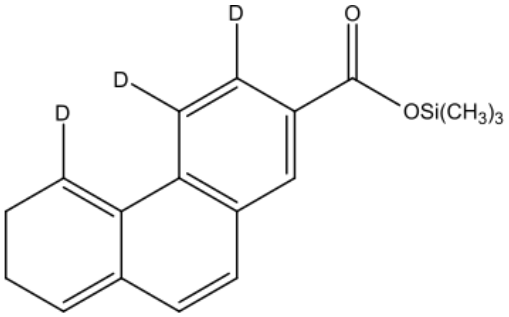
Figure 3-42: Molecular masses and possible structures of metabolites characterized in culture TRIP1 by GC-MS; metabolites are depicted as ions of sylilated acids.

The detected metabolites indicate a stepwise ring reduction of 2-phenanthroyl-CoA. Thus, we assume a ring reduction starting from ring III of phenanthroic acid, opposite to the carboxyl group, similar to the anaerobic reduction of naphthalene (Eberlein, Johannes, et al. 2013; Estelmann et al. 2015). For further elucidation of the degradation pathway, culture TRIP1 was grown with fully deuterated phenanthrene ($C_{14}D_{10}$) as substrate. Metabolites were extracted and analyzed with high-resolution GC-MS. A number of putative metabolites of phenanthrene degradation containing deuterium were detected, indicating that they originated

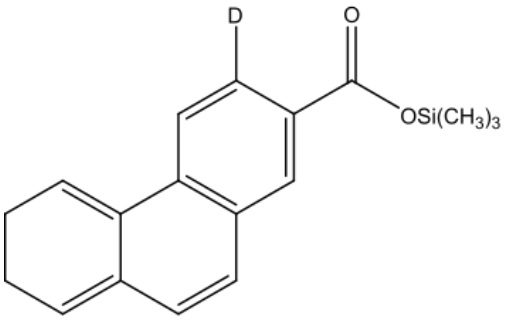
from the deuterated phenanthrene. The measured molecular masses supported the metabolite analysis with non-labeled phenanthrene indicating a stepwise reduction of phenanthroyl-CoA. However, the calculated number of saturated and unsaturated bonds did not agree with the number of deuterium atoms in the molecules, indicating that isotope exchange with protons from water occurred during the ring reduction process.



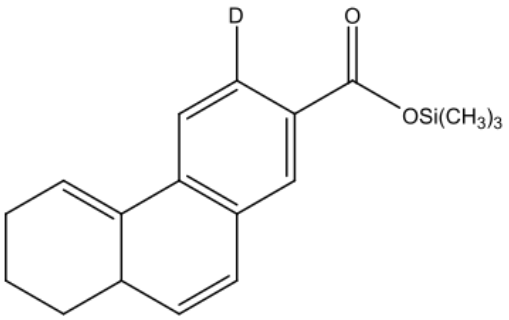
Silylated 2-phenanthroic acid (D_{10})
m/z = 303.16



Silylated 6,7-dihydro-2-phenanthroic acid (D_3)
m/z = 299.14



Silylated 6,7-dihydro-2-phenanthroic acid (D_1)
m/z = 297.13



Silylated 6,7,8,9-tetrahydro-2-phenanthroic acid (D_1)
m/z = 299.14

Figure 3-43: Metabolites characterized from incubations of culture TRIP1 with fully deuterated phenanthrene, measured by high-resolution GC-MS (metabolites are depicted as ions of silylated acids).

4 Discussion

This thesis is structured in two main parts. On one hand, the direct extraction of DNA from bitumen samples and water droplets was developed to better characterize microbial communities within the bitumen ecosystem, to understand their community assembly and strategies of coping with live in such an extreme habitat. On the other hand, a major focus was given to the phenanthrene degradation potential of an enrichment culture established from bitumen samples growing with phenanthrene as sole carbon and energy source and yielding insights into the biochemistry of anaerobic PAH degradation. Despite the tedious nature of bitumen samples and extremely slow anaerobic microbial growth, both objectives were central to allow for a better understanding of anaerobic PAH degradation in the environment.

4.1 Are bacteria in bitumen able to degrade high molecular weight PAHs under anoxic conditions?

4.1.1 DNA-extraction from soils with high humic acid contents

Microorganisms have previously been shown to be growing within the pitch lake (Schulze-Makuch et al. 2011; Meckenstock et al. 2014). In order to successfully extract intact and high molecular weight (HMW) DNA directly from bitumen samples, it proved to be necessary to grind the bitumen to particles as small as possible to get access to the microorganisms trapped between the particles. Trying to remove contained humic acids was also important for downstream applications, as they adhere tightly to the DNA and prevent further steps. This was not easily doable with commercially available kits specialized for soil extractions optimized to remove as many humic acids as possible, as the viscosity of the bitumen during cell opening steps made it adhere directly to the DNA. The biggest hurdle here was to remove these compounds without shearing of the DNA into pieces too small for sequencing steps. Most protocols were not able to generate clean DNA pellets in the process, even when the pellets were cleaned up through sephadex filters or agarose gels.

A newly developed protocol combining a basic phenol-chloroform-isoamyl alcohol-approach with the humic acid removal used in commercially available kits generated DNA clean enough for sequencing. However, the bias introduced to the full community by this extraction method could not be evaluated in the course of this thesis.

The sequenced DNA was used as a backbone to compare the droplet communities. We assumed a higher variety of bacteria in the bitumen as opposed to the communities being more specialized within the droplets.

4.1.2 Sequencing results of 16S rRNA gene amplicons from bitumen

Although the whole metagenome of the bitumen was sequenced, only the 16S rRNA sequences were evaluated in the thesis at hand. A detailed look into the metagenome will follow at a later point in time. The overview of bacterial communities within the bitumen as depicted in Figure 3-12 showed that about a third of the bacterial sequences did not indicate known relatives, which is quite a large part of the community when compared to soil samples with only about 10% of bacteria with no known relatives (Lauber et al. 2009). In the DNA extracted from the bitumen we expected a majority of anaerobic bacteria with the ability to degrade hydrocarbons. What we found were three main groups of bacteria within the bitumen samples that are belonging to the phyla of Chloroflexi (32% of all sequences, 46% of bacterial sequences), Proteobacteria (11% and 16%, respectively) and Bacteroidetes (5% and 4%, respectively).

The Chloroflexi are a highly diverse group of bacteria consisting of aerobic thermophiles, anoxygenic phototrophs, anaerobic dehalorespirers and cellulose degraders. The main group of bacteria belong to the class of *Dehalococcoidiales* (43%) and within those to a group of GIF9 bacteria (22%). None of these could be grown in culture until now (Biderre-Petit et al. 2016). They are commonly found abundant in anoxic methane rich layers of lakes at around 58 to 65 meters depth. This would be consistent to the estimates of the origin of the water body underneath the pitch lake. Two almost complete genomes of this group stem from an aquifer and marine sediment (Biderre-Petit et al. 2016), which shows its ability to grow under varying conditions and could well live in the brackish water identified from below the pitch lake. Within these two genomes

there was no gene for organohalide respiration, as it is common within the *Dehalococcoidiales*, which leads to the assumption that they have other means to conserve energy (Biderre-Petit et al. 2016). This stands in stark contrast to the droplet community, where the Chloroflexi do not play an important role with a total abundance of 1-3% depending on the droplet.

The second group of Proteobacteria on the other hand is far more important in the droplet community and consists of mainly δ-Proteobacteria within the bitumen (Figure 3-16). δ-Proteobacteria made up 12% of the bacterial sequences in the bitumen and 16% within the water droplets. Along with them are β- and α-Proteobacteria. Belonging to the δ-Proteobacteria is a main group of *Desulfobacteraceae* (2% in the bitumen, and 0.8% in the water droplets), known as strict anaerobic sulfate-reducing bacteria (Kuever 2014; Kuever et al. 2015). Also similarly abundant within the δ-Proteobacteria is the group of *Bacteriovoracaceae*, a Gram negative family of bacteria that predate on other Gram negative bacteria (Davidov and Jurkevitch 2004).

Belonging to the Bacteriodetes, similarly abundant both in bitumen and water droplets with 5% of all bacterial sequences, are both aerobic and anaerobic bacteria and they are common in a wide variety of habitats, for example fresh and salt water, waste water, soil, and they are a major group in the guts of humans as well as fish, insects, termites and molluscs (van Kessel et al. 2011; Kadnikov et al. 2012; Chen and LaPara 2006). In an environment like this it is not surprising to find bacteria that are able to live under such varying conditions.

The higher abundance of δ-Proteobacteria within the droplets compared to the bitumen is a sign of higher specialization and adaptation to the surrounding within the droplets and indicates a high ability to degrade available PAHs, as smaller hydrocarbons are degraded first. Bacteroidetes, being equally abundant, do not seem to play a major role for the degradation process and can be counted as “background”, ready to thrive under the right circumstances.

4.1.3 Water droplets within the bitumen as a small insight into degrader ecology

The chemistry of the small water droplets found within the bitumen have been characterized previously in detail (Meckenstock et al. 2014). Water stable isotopes as well as enrichment of $\delta^{18}O$ values indicate a deep origin from ancient sea water and brines, further supported by the salinity of the water. The droplets pH is at 7.2. These data combined indicate the possibility for microorganisms to be able to live within this water, as also all necessary nutrients are available within each droplet and the basic needs for life are fulfilled. Also, within that publication it has been shown that motile bacteria were found in droplets extracted from undisturbed bitumen samples. The bacterial community composition has been studied by pyrotag sequencing and a typical community as often found in oil samples could be described (Schulze-Makuch et al. 2011; Meckenstock et al. 2014).

Active degradation could be detected within the droplets by FTICR, which made it possible to identify metabolites of PAH degradation within the water of the droplets (Meckenstock et al. 2014). A key question of this thesis was to identify a degrader community within the droplets, as the assumption was, that a core community of active degraders is surrounded by a community of variable bacteria that live of the degraders, either by consuming their products or living by predation.

Already during the evaluation of DNA sequences extracted from the droplets with bioinformatical methods (e.g. MOTHUR) the assumption that those sequences stem from an extreme habitat came up, as the diversity was extremely low and very atypical for oil samples. They were also very different from the droplets previously sequenced by Meckenstock et al. (2014), indicating either a high between-sample-diversity or a change in the seeding community. The main bacterium in all droplets sequenced within the framework of this thesis was the β-Proteobacterium *Tepidiphilus sp*. The β-Proteobacteria are a highly diverse group of bacteria consisting of photoheterothrophs, autotrophs and chemolithothrophs. They are common in many niches and play an important role in environmental processes, like nitrification and wastewater treatments. There are also pathogenic species among them. Well known orders of the

β-Proteobacteria are *Burkholderiales*, which where the dominating species in the previous characterizations by Schulze-Makuch et al. (2011) and Meckenstock et al. (2014); *Rhodocyclales, Nitrosomonadales* and *Neisseriales*.

Tepidiphilus sp. belongs to the family of *Hydrogenophilaceae*, in the order *Hydrogenophilales* and hence are thermophilic oxidizers of hydrogen or organic molecules. *Tepidiphilus sp*. is closely related to bacteria of the genus *Petrobacter sp*. (98,9% similarity, Poddar, Lepcha, and Das 2017). One member of the genus *Petrobacter* has previously been extracted from a non-water-flooded oil well in Australia (Salinas et al. 2004) and has since been transferred to the genus of *Tepidiphilus*. It has been observed to grow both aerobically and anaerobically in the presence of nitrate and prefers organic acids as sole carbon source (Poddar, Lepcha, and Das 2014). When comparing the concentrations of sulfate and nitrate within the water samples, it becomes clear that the sulfate concentrations are three orders of magnitude higher than nitrate concentrations. Naturally occurring amounts of nitrate in the water phase as measured by Meckenstock et. al. (2014, values not published) were between 32 µM to 258 µM, whereas concentrations of sulfate were in the order of 160 mM to 285 mM. Nevertheless, the measured nitrate concentration was sufficient for naphthalene degradation under nitrate reducing conditions as shown by Rockne et al. (2000). One possible explanation for the very low concentration of nitrate in the water samples is that the nitrate within the droplets was already used up for degradation processes by *Tepidiphilus sp*., whereas no major processes involving sulfate reduction took place yet. Also, their ability to use fermentation products of hydrocarbon degradation gives them a broad range of substrates to live on.

The arising question when sequencing the tiny water droplets is the sequence depth for each individual droplet. In the rarefaction curves in Figure 3-19 it can be seen that the curves remain steep, which is due to the fact that rare sequences have less coverage and sequencing depth, and thus are more prone to sequencing errors. This causes quality steps during the sequence evaluation to reject these sequences, keeping mostly those that are more abundant with higher sequencing depth. At least the rarefaction curve for Droplet 2 seems to have more species within the sequenced droplet.

Overall it can only be said that all four droplets are very similar to each other due to the fact that the majority of bacteria in each of them belong to the same species, *Tepidiphilus sp*., according to the SILVAngs platform evaluation. During the evaluation with MOTHUR based on the Greengenes Database the dominating bacteria in all the droplets belong to the family of *Rhodocyclaceae*.

In Figure 3-22 seven different calculations of the alpha diversity of each droplet are shown and compared. These are the observed diversities, Chao1 and ACE index, Shannon, Simpson and inverted Simpson diversities as well as the Fisher diversity.

The Chao1 index describes the species richness estimated from the total numbers of species present in a community. It is commonly used and based upon the number of rare classes (i.e. OTUs) found in a sample (Chao 1984). The calculations showed that Droplets 1 and 4 were more diverse than Droplets 2 and 3, which were almost equal in that regard, with Droplet 4 showing the highest diversity. The picture was very different when calculated for the ACE index. This is only incorporating data from species with less than ten individuals and therefore a better representation of the rare species within the sample (Hughes et al. 2001). Using this method, the highest diversity was found in Droplet 1, whereas Droplets 2 and 4 were very similar and Droplet 3 was the least diverse. In all other calculations the highest diversity by far was found in Droplet 4, with Droplet 2 being the least diverse and Droplets 1 and 3 interchangeably in the middle section. This was in agreement with the rarefaction curves as shown in Figure 3-19, where the curve for Droplet 4 showed the steepest slope, showing that the sequencing depth is not enough for all the included species and the curve for Droplet 2 the one with the least steep slope. The Shannon index accounts for both abundance and evenness of species present, e.g. the amount of different species and the absolute number of individuals per species. This is different to the Simpson index, which focuses more on the species present and its relative abundances (Hunter and Gaston 1988). There is no correction for small sample sizes within the Simpson index. Droplet 4 being of the highest diversity in both calculations and therewith indicated that not just relative species numbers but also absolute numbers were highest in this droplet.

The picture of the same sequences being evaluated with the Greengenes platform gave similar results concerning the species diversity, but the actual composition changed dramatically based on the database used to identify the sequences, Silva and Greengenes, respectively. Proteobacteria were still the main group within each of the droplets, but at the family level the *Hydrogenophilaceae* are not identified, instead the majority of the bacteria belonged to the family of *Rhodocyclaceae*. This family consists of mostly aerobic or denitrifying bacteria. Many of them also appear in waste water treatments. Comparing both families, the *Hydrogenophilaceae* seem to be more fitting to the extreme habitat of the pitch lake considering the degradation capabilities of the species. It is important to mention that Greengenes is an outdated database, whereas SILVA is well curated, thus these results are of higher accuracy.

To make a reasonable discussion of the ecological inferences of the diversity in the droplets a much bigger sample size must be considered. Differences between within-sample and between-sample diversities must be researched in more detail and from different sites on the pitch lake in order to better understand the possible origin of the droplets and to test the varying hypotheses of seeding or endpoint hypothesis or a mixture thereof. Practical constraints in sequencing and gaining enough sample material were reasons for the small sample volume being sequenced within the framework of this thesis, a final conclusion to the droplet communities cannot be given at this point. However, the aforementioned results of my thesis give a more detailed look into the life in extreme habitats of the aromatic hydrocarbon rich environment these bacteria are able to grow in despite its hostile conditions.

Concluding the diversity analyses of bitumen and droplets I was able to confirm our hypothesis that the diversity in the bitumen itself is much higher than in the droplets. This gives the inhabitant bacteria a wider range of possibilities to adopt to changes in the composition of the bitumen and enclosed nutrients. The high specialization within the droplets was expected due to the conserved conditions in these “ecological islands” within the bitumen, and the low level of adaptation necessary to survive under the harsh conditions for life within the bitumen. It did not work as expected to use the DNA sequences derived from bitumen extractions as blueprint or background sequences for those from the water

droplets, as the main sequenced bacterium *Tepidiphilus sp*. within the *Hydrogenophilaceae* in the droplets did not show up at the family level of the bitumen sequences. Both bitumen DNA and water droplet DNA were extracted from the same sampling site, the bitumen was used after the water droplets had been removed and stored separately in the freezer. The differences in the species composition might be caused by the different extraction methods (direct PCR for the water droplets and the above introduced protocol for bitumen extractions) and the differences in abundance of the species.

4.2 TRIP1 Enrichment

4.2.1 Culture description

From the pitch lake an anaerobic enrichment culture has been set up with phenanthrene as sole carbon and energy source. Under sulfate-reducing conditions the culture is able to grow, but produced only very little biomass, which makes analyses very complicated. By now, it is only the third described enrichment culture growing under these conditions and the first being of freshwater origin. The other two cultures were of marine origin and were described by Zhang and Young in 1997 and Davidova *et al.* in 2007. They have been able to elucidate the initial activation reaction for anaerobic phenanthrene degradation, which has been shown to be a carboxylation and Davidova *et al*. (2007) discovered the carboxylation reaction in the C2 position based on experiments with deuterated phenanthrene.

However, more comprehensive information on the anaerobic degradation of phenanthrene is still missing. In order to investigate anaerobic phenanthrene degradation in more detail, we enriched an anaerobic phenanthrene-degrading culture with sulfate as electron acceptor with an inoculum from the pitch lake in Trinidad and Tobago. The culture degrades phenanthrene with rates similar to anaerobic naphthalene-degrading cultures (6-8 days doubling times for NaphS2, and more than two weeks for N47, Galushko *et al.* 1999; Meckenstock *et al.* 2000) which is remarkable given the much lower aqueous solubility of phenanthrene

(7.3 µM at 30°C for phenanthrene versus 242 µM at 25°C for naphthalene). This relatively fast growth allowed studying the culture in more detail.

The dominant organism, a *Desulfatiglans* species, accounted for more than 60 % of all microbial cells in the culture as calculated from mapping the original shotgun reads using the BWA software (H. Li and Durbin 2009), leading to a 60.4% figure for the first unclassified *Desulfobacteraceae* (*Desulfatiglans*-1), 2.1% for the second *Desulfobacteraceae* (*Desulfatiglans*-2), 1.2% for the unclassified *Bacteroidetes* and 0.51% for the unknown *Spirochaete* bacterium. The full length 16S rRNA gene sequence shared 93% identity to the naphthalene-degrading strain NaphS2. Assuming that this organism is responsible for phenanthrene degradation, it appears only remotely related to the previously reported phenanthrene-degrading microorganism Phe4A (Davidova et al. 2007). Its 16S rRNA gene sequence affiliates it to the family *Desulfobacteraceae*, which already includes several sulfate-reducing members that are known to degrade aromatic hydrocarbons, such as the aniline-degrading *Desulfatiglans anilini*, or the naphthalene-degrading strains NaphS2 and N47 (Schnell, Bak, and Pfennig 1989; Meckenstock et al. 2000; Galushko et al. 1999).

The reconstructed genome sequences are currently being analyzed and will be reported elsewhere, but as the enzymes for the anaerobic degradation of phenanthrene (and to some extent naphthalene as well) are currently unknown, genomic sequences by themselves do not point to obvious candidate genes for the degradative enzymes. For example, genes from TRIP1's dominant *Desulfatiglans* that are closest to the NaphS2 genes encoding its naphthoate-CoA ligase share only 35% identity to the latter. On the other hand, some genes encoding flavoenzymes of the "old yellow enzyme" (OYE) family, a large family that includes 2-naphthoyl-CoA reductases (Eberlein, Estelmann, et al. 2013), showed stronger similarities (60% identity) to NaphS2 OYEs, and are plausible candidates for encoding similar activities on phenanthrene-derived substrates.

As sequence information could not help to gain more insights into degradation pathways, we were prompted to gain information from metabolite analysis of culture supernatants. The dominant metabolite was 2-phenanthroic acid, consistent with observations from the other two known phenanthrene-degrading cultures (Zhang and Young 1997; Davidova et al. 2007)

High resolution mass spectrometric analysis of the TRIP1 culture revealed further derivatives of phenanthroic acid showing molecular masses indicative of a stepwise reduction of phenanthroyl-CoA. This would again be analogous to the biochemical strategy in anaerobic naphthalene degradation, where the ring system is reduced in two-electron steps to overcome the resonance energy and prepare the compound for further degradation (Annweiler, Michaelis, and Meckenstock 2002; Eberlein, Estelmann, et al. 2013; Weyrauch et al. 2017). This scenario was further supported by analyzing metabolites arising from the degradation of deuterated phenanthrene, among which reduced compounds were identified. However, the seemingly erratic conservation of deuterium atoms in the metabolites of deuterated phenanthrene degradation indicated that some detected metabolites might undergo isotope exchange reactions with protons, either in the culture or later during the chemical analysis. Therefore, it cannot be excluded that some of the detected reduced phenanthroic acid derivatives are not derived from the degradation pathway, even though a stepwise reduction of the aromatic ring system is highly probable.

Our study sheds light on the first steps of the anaerobic degradation pathway of phenanthrene, and indicates that the biochemical strategies identified in the anaerobic degradation of naphthalene and benzene (Meckenstock et al. 2016) most likely also hold for larger non-substituted three-ringed PAHs, namely a carboxylation as the initial activation reaction and a stepwise reduction of the aromatic ring system to overcome the resonance energy. The biochemical proofs provided here open the way to the identification of further degradation reactions and the corresponding genes.

4.2.2 Enzyme assays

Enzyme assays are performed to elucidate degradation pathways. Their aim is to test different substrates for their conversion to products that themselves are used as substrates for subsequent reactions. If the conditions are mimicking the natural conditions in the culture well, more than one reaction can be observed by the reaction to products, which are further downstream in the suggested pathway. This also can pose problems, as small steps can be overlooked and intermittent metabolites are transformed faster than the samples can be taken. This case has

happened during the uncovering of the anaerobic naphthalene degradation, where two 2-electron reduction steps have been observed to be one 4-electron step initially (Eberlein, Estelmann, et al. 2013; Eberlein, Johannes, et al. 2013). The upcoming of products has therefore to be handled with great care and the time points for taking samples during an assay have to be thoroughly thought through. A problem faced with enzyme assays during this thesis was the small amount of biomass available even from bigger volumes of culture grown for a longer time, which was not always enough to show the expected reactions. Another problem was faced with the growth time of the culture, as some enzymes might not be active after an elongated growth period, which was thought necessary to gain enough biomass for the assay. The optimization of both amount and time to grow for large scale cultures is a necessary step in order to gain better understanding of the degradation pathways within the TRIP1 culture.

4.2.2.1 Carboxylase

Carboxylation is the initial activation reaction during anaerobic naphthalene degradation (Mouttaki, Johannes, and Meckenstock 2012) and for smaller substituted aromatic compounds like phenol, catechol, hydroquinone and resorcinol (Gorny and Schink 1994; He and Wiegel 1996; Zhang and Wiegel 1994). We therefore assumed a similar reaction for the activation of phenanthrene during the anaerobic degradation pathway, as has been assumed by Zhang and Young (1997) and Davidova *et al*. (2007). An experimental approach to test this hypothesis was performed by Zhang and Young in 1997 in a way that they added ^{13}C-labeled bicarbonate directly into the medium and detected the incorporated labels during metabolite analysis. As retention times of the eluated phenanthrene carboxylic acid during HPLC analysis differed from the only available standard of 9-phenanthrene carboxylic acid they excluded the C9 position as place of the initial carboxylation reaction.

Davidova *et al.* were able to show that a carboxylation reaction took place in the C2 position by incubating their phenanthrene degrading culture with labeled ^{13}C-bicarbonate for 28 weeks and synthesizing the standards for 2-, 4- and 9-phenanthrene carboxylic acid. Retention times during GC-MS analysis performed after metabolite extractions were similar to the retention times of the 2-phenanthrene carboxylic acid standard and differed from the other two tested

standards. This is also backed by the net atom charge of the carbon atoms in the C2 and C3 position, that are most negative within the phenanthrene structure and therefore most reactive (Hites and Jr 1987). As the heavy isotope label was incorporated in the carboxyl group, a direct carboxylation was assumed as the most likely reaction.

Carboxylase assays have been performed under varying conditions of growth times. Substrates with unlabeled and labeled ^{13}C bicarbonate for a more exact determination of the position of the carboxylation reactions have been executed but did not result in the formation of a peak when evaluated with HPLC. We assume that the detection limit of the produced peak was too small due to little biomass to be detected with the HPLC. A higher detection limit could have been reached by analysis on an LC-MS/MS, but the machine was not available at the time when the assays were performed. An earlier experiment with a culture growing for a longer period did not result in a peak for phenanthrene carboxylic acid either, where we suppose an overgrowing of the culture relating to growth times and a following inactivity of the necessary enzymes.

4.2.2.2 Ligase

Following the carboxylation reaction during anaerobic naphthalene degradation a ligase reaction with coenzyme A has been shown, forming a naphthoyl-CoA ester (Eberlein, Estelmann, et al. 2013; Eberlein, Johannes, et al. 2013; Estelmann et al. 2015). Similar ligation reactions with coenzyme A for smaller compounds like benzoate and succinate have been shown earlier (Hutber and Ribbons 1983). The ligase has been shown to be ATP-dependent in the anaerobic naphthalene degradation pathway (Weyrauch 2016). With our ligase assay we could give clear evidence of the ligation reaction happening as the substrate 2-phenanthroic acid was completely used up and a peak with the exact mass of the 2-phenanthroyl-CoA came up. This reaction happened within 10 minutes of the start of the assay. This gives more evidence of the degradation pathways of naphthalene and phenanthrene being similar and comparable.

Phenanthroic acid was proven as a real metabolite of the degradation pathway in enzyme assays with crude cell extracts measuring phenanthroate:CoA ligase. Among the three tested isomers (2-, 4-, and 9-phenanthroic acid), only 2-phenanthroic acid was converted, indicating that the measured ligase reaction is

specific and not due to a non-specific side reaction. The measurement of this enzymatic activity in anaerobic phenanthrene degradation also indicated that the initial carboxylation reaction most likely produces 2-phenanthroic acid. This is similar to anaerobic naphthalene degradation where 2-naphthoic acid is the product of the naphthalene carboxylase reaction (Mouttaki, Johannes, and Meckenstock 2012). However, due to the lack of the 1- and 3-phenanthroic acid isomers as reference compounds or substrates, it cannot be excluded that these isomers can also be converted by the phenanthroate:CoA ligase.

4.2.2.3 Reductase

The reduction of the very stable ring structure of aromatic compounds is in high demand of energy to break the double bonds. The reduction of naphthalene is happening in 2-electron steps beginning with the ring opposite the position of the carboxylation (Eberlein, Johannes, et al. 2013; Meckenstock et al. 2016; Weyrauch et al. 2017). Again, assuming a similar pathway for the phenanthrene degradation we set up assays starting from the ligase reaction and adding reducing agents to keep the reaction going. Reducing agents of choice where sodium dithionite, NADH, NAD+, NADPH and 2-alpha-keto-glutarate. Dithionite as was able to serve as an artificial electron donor both in benzoyl-CoA reduction (Boll and Fuchs 1995) and in assays set up with NaphS2-cfe. Nevertheless, it also seemed to inhibit a reaction of the NADH-dependent reduction of hexahydronaphthoyl-CoA. As no reaction could be detected with any of the reducing agents, it could either mean the reaction was inhibited by the used electron donors or the enzyme itself was not active in that state of the culture. A peak of the expected mass could not be detected by HPLC analysis. On the other hand, it might be that the detection limit of the HPLC was too low to record the amount of substrate being turned into reduced structures.

A second approach was the synthesis of 2-phenanthroyl-CoA to directly use as substrate for the reduction reaction. The substrates were synthesized by Philip Weyrauch and Ivana Kraiselburd in the laboratory of Rainer U. Meckenstock in the Biofilm Centre at the University of Duisburg/Essen, Germany. Up until now no reduction reaction could be shown for the TRIP1 culture. This is an urgent next step in order to be able to identify the downstream pathway.

4.2.2.4 Metabolite analysis / possible downstream pathway

A possible downstream pathway starting from the described ligase reaction within the culture would be similar to the pathway shown and proposed for anaerobic naphthalene degradation (Eberlein, Johannes, et al. 2013; Meckenstock et al. 2016; Weyrauch et al. 2017). At the moment there is no indication of a deviating mechanism, as the carboxylation reaction and the subsequent ligation reaction could be shown for this culture within this thesis. The metabolite analysis on the LC-MS/MS gives distinct hints for the position of the carboxylation reaction at the C2 position of the molecule, further supported by the sole conversion of 2-phenanthrene carboxylic acid to 2-phenanthroyl-CoA as opposed to the utilization of substrates with the carboxyl group at the C4 or C9 position. This is a strong indication of the similarity of the anaerobic degradation pathways between naphthalene and phenanthrene. Further downstream reactions have to be evaluated individually and keeping close to the steps from anaerobic naphthalene degradation might proof very useful. For this to work out, I strongly recommend to optimize growth conditions of the culture in bigger batches, as the optimum between growth time and enzyme activity has yet to be determined. Metabolite analysis gives great opportunities to elucidate the downstream pathway further, and machines with a higher detection limit for low metabolite concentrations in biochemical enzyme assays would be of great help.

5 Conclusion

Microbial community specialization in extreme habitats like the naturally occurring bitumen is high, as I was able to show for the tiny water droplets dwelling up from below the pitch lake. Even though droplets from one seep were fully separated, their diversity was extremely even when comparing the low diversities and the majority of bacteria in all four droplets were associated with one genus, *Tepidiphilus sp*. The role of these known degraders of fermentation products in hydrocarbon breakdown in the droplets remains to be further elucidated. Still their high abundance in the investigated droplets suggests an important role in natural bitumen.

Bacteria isolated from this extreme habitat in an enrichment culture were used to successfully elucidate anaerobic degradation pathways. Slow and very limited biomass growth under sulfate-reducing conditions in these cultures were a major obstacle in investigating the biochemistry of individual steps. Nonetheless, I was able to provide new insights into the first steps of the anaerobic degradation of phenanthrene, the diversity of bacteria living in the bitumen and also in the tiny water droplets emerging from the bitumen. Their discussion as ecological islands provides an important perspective for future research into the system. Also, the protocol for DNA extractions from difficult bitumen samples developed in this thesis will be a major facilitator of future related work. More than one third of the bacteria detected in the bitumen metagenome were without known relatives, a much higher number compared to i.e. anoxic sediment samples. This emphasizes the great prospects of a continued functional characterization of these intriguing microbiomes, not only in a bioremediation context, but also in a biotechnological perspective.

6 Literature

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Appendix

Batch File for MOTHUR including taxonomical matching and creation of .biom-file:

```
#extract .sff file

sffinfo(sff=I41HLDC04.sff, flow=T)

#check seqs

summary.seqs(fasta=current)

#separate flowgrams according to the barcode and primer, minimum
length

trim.flows(flow=I41HLDC04.flow, oligos=16S_bac.oligos, pdiffs=2,
bdiffs=1, minflows=360, maxflows=720)

#denoise seq data

shhh.flows(file=I41HLDC04.flow.files, processors=8)

summary.seqs(fasta=current)

#remove barcodes and primers

trim.seqs(fasta=I41HLDC04.shhh.fasta, name=I41HLDC04.shhh.names,
oligos=16S_bac.oligos, pdiffs=2, bdiffs=1, maxhomop=8, minlength=200,
flip=T)

summary.seqs(fasta=I41HLDC04.shhh.trim.fasta,
name=I41HLDC04.shhh.trim.names)

#trim without quality score

trim.seqs(fasta=I41HLDC04.fasta, oligos=16S_bac.oligos,
qfile=I41HLDC04.qual, maxambig=0, maxhomop=8, flip=T, bdiffs=1,
pdiffs=2, qwindowaverage=35, qwindowsize=50)

summary.seqs(fasta=I41HLDC04.trim.fasta)

#simplify the dataset by working with the unique sequences (nothing is
removed)

unique.seqs(fasta=I41HLDC04.shhh.trim.fasta,
name=I41HLDC04.shhh.trim.names)
```

```
summary.seqs(fasta=I41HLDC04.shhh.trim.unique.fasta,
name=I41HLDC04.shhh.trim.unique.names)

#go on with forward files only

#align dataset (flip=t allows reverse reads to be aligned)

align.seqs(fasta=I41HLDC04.shhh.trim.unique.fasta,
reference=silva.bacteria.fasta, flip=t, processors=8)

#check alignment

summary.seqs(fasta=I41HLDC04.shhh.trim.unique.align,
name=I41HLDC04.shhh.trim.unique.names)

#test for alignment in the correct region

screen.seqs(fasta=I41HLDC04.shhh.trim.unique.align,
name=I41HLDC04.shhh.trim.unique.names, group=I41HLDC04.shhh.groups,
start=1044, optimize=end, criteria=95, processors=8)

summary.seqs(fasta=I41HLDC04.shhh.trim.unique.good.align,
name=I41HLDC04.shhh.trim.unique.good.names)

#keep sequences only overlapping in the same region (result from
screen.seqs) and remove any columns in the alignment that don't
contain data

filter.seqs(fasta=I41HLDC04.shhh.trim.unique.good.align, vertical=T,
trump=.)

summary.seqs(fasta=I41HLDC04.shhh.trim.uniue.good.filter.fasta)

#further simplify Dataset

unique.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.fasta,
name=I41HLDC04.shhh.trim.unique.good.names)

summary.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.fasta
)

list.seqs(name=I41HLDC04.shhh.trim.unique.good.filter.names)

get.seqs(group=I41HLDC04.shhh.good.groups, accnos=current)

#merge rare sequences with common sequences with differences of less
than x bp
```

```
pre.cluster(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.names,
group=I41HLDC04.shhh.good.groups, diffs=2, processors=8)

summary.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.precl
uster.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.names)

#find chimera, there are several chimera tools on mothur
http://www.mothur.org/wiki/Chimera.seqs

chimera.uchime(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.pre
cluster.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.names,
group=I41HLDC04.shhh.good.groups)

#remove chimeras

remove.seqs(accnos=I41HLDC04.shhh.trim.unique.good.filter.unique.precl
uster.uchime.accnos,
fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.names,
group=I41HLDC04.shhh.good.groups, dups=T)

summary.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.precl
uster.pick.fasta)

#classify your sequences

classify.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.prec
luster.pick.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.pick.nam
es, group=I41HLDC04.shhh.good.pick.groups, template=gg_13_8_99.fasta,
taxonomy=gg_13_8_99.gg.tax, cutoff=80, processors=8)

#remove "Chloroplasts" and "Mitochondria"

remove.lineage(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.pre
cluster.pick.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.pick.nam
es, group=I41HLDC04.shhh.good.pick.groups,
taxonomy=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.pick
.gg.wang.taxonomy, taxon=Mitochondria-Chloroplast-Archaea-Eukaryota-
unknown)
```

```
summary.seqs(fasta=I41HLDC04.shhh.trim.unique.good.filter.unique.precl
uster.pick.pick.fasta,
name=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.pick.pic
k.names)

#go on with analysis

#build OTUs

dist.seqs(fasta=current, cutoff=0.15, processors=8)

#cluster

cluster(column=current, name=current)

#create OTU Table

make.shared(list=current, group=current, label=0.03)

count.groups()

#subsampling to compare groups (e.g. compare sampling effort within
different plots)

#option: size='minimum size'

sub.sample(shared=current)

classify.otu(list=current, name=current, taxonomy=current, label=0.03)

#create biom table

make.biom(shared=current, label=0.03,
constaxonomy=I41HLDC04.shhh.trim.unique.good.filter.unique.precluster.
pick.pick.an.0.03.cons.taxonomy, reftaxonomy=gg_13_8_99.gg.tax)

#phylogenetic tree

dist.seqs(fasta=current, output=phylip, processors=8)

clearcut(phylip=current)
```

Publications and Authorship Declaration

6.1 Published

1. Meckenstock, Rainer U., Frederick von Netzer, Christine Stumpp, Tillmann Lueders, Anne M. Himmelberg, Norbert Hertkorn, Philipp Schmitt-Kopplin, Mourad Harir, Riad Hosein, Shirin Haque, Dirk Schulze-Makuch. "Water droplets in oil are microhabitats for microbial life." Science 345, no. 6197 (2014): 673-676.

2. Meckenstock, Rainer U., Matthias Boll, Housna Mouttaki, Janina S. Koelschbach, Paola Cunha Tarouco, Philip Weyrauch, Xiyang Dong, and Anne M. Himmelberg. "Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons." Journal of Molecular Microbiology and Biotechnology 26, no. 1-3 (2016): 92-118.

3. Himmelberg, Anne M., Thomas Brüls, Zahra Farmani, Philip Weyrauch, Gabriele Barthel, Wolfgang Schrader, Rainer U. Meckenstock. "Anaerobic degradation of phenanthrene by a sulfate-reducing enrichment culture." Environmental Microbiology 20, no. 10 (2018): 3589 - 3600.

6.2 Authorship clarification

1. The outline of the Science publication was developed by Rainer U. Meckenstock. The PhD candidate Anne Himmelberg executed experiments concerning the stable isotope measurements of the methane and designed supplementary figure S2 during revisions.

2. The review article is part of a special issue of the journal JMMB. All invited authors of the special issue were members of the DFG priority program “Biologische Umsetzungen von Kohlenwasserstoffen in Abwesenheit von Sauerstoff: Von molekularer zu globaler Ebene” (SPP-1319). The outline of the review article was developed by Rainer U. Meckenstock. The PhD candidate Anne Himmelberg wrote parts of the paragraph ‘Detecting Biodegradation of Benzene and PAHs in the Environment’, which is also

part of the introduction and the discussion of the current PhD thesis. The manuscript draft was revised and edited by Rainer U. Meckenstock.

3. The experiments were conducted by the PhD candidate as long as not indicated differently. The manuscript was written by the PhD candidate, along with the design of all used figures. Data from metabolite analysis were provided by Zahra Farmani in the laboratories of Wolfgang Schrader and Rainer U. Meckenstock. She also measured the sulfate depletion and the cell counts within the culture as shown in Figure 3-37 in the framework of her Masters thesis. Philip Weyrauch performed the HPLC measurements of the enzyme assays. Thomas Brüls at GENOSCOPE sequenced the metagenome of the enrichment culture and performed the sequence analysis. Parts of the manuscript are also part of the introduction and the discussion of the thesis in hand. The manuscript draft was revised and edited by Thomas Brüls, Philip Weyrauch, Wolfgang Schrader and Rainer U. Meckenstock.

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Lebenslauf

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Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich diese Dissertation selbstständig ohne Hilfe Dritter und ohne Benutzung anderer als der angegebenen Quellen und Hilfsmittel verfasst habe. Alle den benutzten Quellen wörtlich oder sinngemäß entnommenen Stellen sind als solche einzeln kenntlich gemacht.

Diese Arbeit ist bislang keiner anderen Prüfungsbehörde vorgelegt worden und auch nicht veröffentlicht worden.

Ich bin mir bewusst, dass eine falsche Erklärung rechtliche Folgen haben wird.

_____

Ort, Datum, Unterschrift