

Pathways for anaerobic hydrocarbon degradation in a nitrate reducing consortium

By

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“We learn only to ask more questions...”

- **Larry Niven**

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
A	Absorbance
a.a.	Amino Acids
ACE	Acetate
ATP	Adenosine triphosphate
ASS	Alkylsuccinate synthase
BLAST	Basic Local Alignment Search Tool
bp	Basepair
BSS	Benzylsuccinate synthase
CAS	(cycloalkyl) succinate synthase
Da	Dalton
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
dsDNA	Double stranded deoxyribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
EPA	Environmental Protection Agency
Fe(III)-reducing	Iron(III)-reducing
FID	Flame Ionization Detector
g	Gram
g/100 mL	Gram per 100 milliliter
g/L	Gram per liter
gDNA	Genomic DNA
GC	Gas Chromatography
h	Hour
HMN	2,2,4,4,6,8,8-Heptamethylnonane
HS	Head Space

kb	kilobasepare
kDa	kilodalton
LB	Luria-Bertani
LC	Liquid Chromatography
MAS	(1-methylalkyl) succinate synthase
µg	Microgram
µg/mL	Microgram per milliliter
µL	Microliter
µM	Micromolars
µmol	Micromole
M	Molar
mg/mL	Milligram per milliliter
MGD	Molybdopterin-guanine dinucleotide
MG-RAST	Metagenomic Rapid Annotation using Subsystem Technology
min	Minute
mL	Milliliter
mL /min	Milliliter per minute
mm	Millimeter
mM	Millimolar
Mr	Relative molecular mass
MS	Mass Spectrometry
NAPH	Naphthalene
ng/µl	Nanogram per microliter
nm	Nanometer
NRB	Nitrate respiring bacteria
OD	Optical density
ORF	Open reading frame
OTU	Operational Taxonomic Unit
PAH	Polycyclic aromatic hydrocarbon
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane

PTFE	Polytetrafluoroethylene
RDP	Ribosomal Database Project
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNR	type III ribonucleotide reductase
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SPME	Solid Phase Micro Extraction
TEA	Triethanolamine
TE-buffer	Tris-EDTA buffer
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
UST	Underground Storage Tank
UV	Ultraviolet
UV-Vis	Ultraviolet –visible
V	Volts
vol/vol	Volume per volume
w/v	Weight per volume
x g	Times gravity
x	Times

Chapter 1

Literature review

1. General introduction

Hydrocarbons are ubiquitous compounds originating from both natural and anthropogenic processes. Biosynthesis reactions present in bacteria, phytoplankton, plants and fungi (Ladygina *et al.*, 2006), as well as diagenesis and catagenesis (Horsfield and Rullkotter, 1994) all result in the natural occurrence of hydrocarbons. Hydrocarbons are environmentally distributed in deep subsurface oil reservoirs (Sephton and Hazen, 2013), microbial mats (Green and Jahnke, 2010), natural oil seeps (Kvenvolden and Cooper, 2003) and coalbeds (Strapoć *et al.*, 2011). Anthropogenic sources are mainly due to the increase in world liquid hydrocarbon consumption with an estimated 94 million barrels of oil used worldwide per day in 2015, a number which is projected to grow to 110.6 million by the year 2035 (U.S. Energy information administration, 2009). The increase in oil production can lead to higher incidences of accidental leaks and spills, during extraction, transport and consumption introducing crude oil and refined petroleum products into the environment. This is in addition to the input of oil through natural seeps. In the United States the Environmental Protection Agency (EPA) runs an Underground Storage Tank (UST) program with the purpose to regulate underground storage of petroleum and to provide broad based statistics for petroleum released into the environment. The UST program reports that there are 680 000 USTs and 9 000 new leaks of petroleum related products into the groundwater and soils are discovered annually clearly indicating that this is in no way a permanent solution to petroleum storage. These releases are significant since the contamination of natural resources, such as groundwater aquifers, by crude oil, petroleum products and additives can affect public water supply and natural environments, such as marshes, mudflats and sub-tidal areas, are extremely sensitive to contaminant impacts (Mills and Frankenberger, 1994).

The middle of the 20th century saw an increase of investigations into microbial hydrocarbon degradation capabilities mainly due to the fact that conventional methods of remediation, such as physical removal, can rarely complete the clean-up of oil spills. Bioremediation has moved to the forefront as a promising technology especially as a secondary treatment option for oil clean-up. Bioremediation by definition is: "The use of living organisms (e.g., bacteria) to clean up oil spills or remove other pollutants from soil, water, and wastewater." (National Safety Council, 2005), thus bioremediation aims to exploit the inherent capabilities of

microorganisms to degrade environmental contaminants. Bioremediation poses several advantages over conventional methods such as being significantly cheaper, less intrusive and more environmentally friendly in terms of the products formed.

Predominantly, the degraders of hydrocarbons are chemo-organotrophic species with the ability to utilize various natural and xenobiotic compounds as carbon sources and electron donors for the generation of growth (Fritsche and Hofrichter, 2001). A large number of microorganisms have shown the ability to utilize hydrocarbons as the sole energy source in their metabolism, but a single known bacterium does not currently exist that possess the enzymatic capabilities to degrade all or even most of the contaminants in a polluted environment. Studies have shown that mixed populations with overall broad enzymatic capabilities are needed for the degradation of complex mixtures of hydrocarbons (Das and Chandran, 2011).

Given the high carbon content available for biomass production, and the large energy content of such highly reduced compounds, it is hardly surprising that many microbes have evolved or acquired the ability to utilize hydrocarbons as sources of carbon and energy. However, accessing this energy requires the presence of novel enzymes. Claude Zobell (Zobell, 1946) was one of the first researchers to summarize the combined knowledge of microbial degradation of hydrocarbons in his article "Actions of microorganisms on hydrocarbons". He observed that nearly a hundred species of bacteria, fungi and yeasts have the ability to "eat" hydrocarbons and that they are widely distributed in nature. Microbial biodegradation can be considered the ultimate natural mechanism for the clean-up of hydrocarbon pollutants from the environment (Atlas and Bartha, 1992).

1.1 Microbial biodegradation of hydrocarbons

Biodegradation is the biologically catalysed reduction in complexity of chemical compounds. Hydrocarbons are compounds that consist exclusively of carbon and hydrogen and can be sorted into four different groups: the alkanes (saturated hydrocarbons), alkenes, alkynes and aromatic hydrocarbons. The non-aromatic (aliphatic) hydrocarbons can then be further divided into straight-chain, branched-chain and cyclic (alicyclic) compounds whereas the aromatic hydrocarbons may be either mono- or polycyclic and can contain aliphatic hydrogen chains (Widdel and Rabus, 2001). The biodegradation of hydrocarbons is a very complex process dependant on the nature and amount of hydrocarbons present in the system. Several bacteria have shown the ability to feed exclusively on hydrocarbons (Yakimov *et al.*, 2007) with biodegradation efficiency ranging anything from 0.13% (Jones *et*

al., 1970) to 50% (Pinholt *et al.*, 1979) for soil bacteria, determined by following degradation in a load of contaminated soil, and 0.003% (Hollaway *et al.*, 1980) to 100% (Mulkins-Phillips and Stewart, 1974) for marine bacteria, determined by taking water samples at different points at a depth of 5 meters.

A number of limiting factors pertaining to hydrocarbon degradation have been identified (Brusseu, 1998). Not all hydrocarbons are equally biodegradable and differ in their susceptibility to microbial attack, this susceptibility can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Barathi and Vasudevan, 2001; Ulrici, 2000). Temperature also plays a major role in biodegradation since it can both affect the chemical properties and solubility of the hydrocarbons, making them more susceptible to microbial attack, as well as the physiology and diversity of the microbial communities (Das and Chandran, 2011). The rate of hydrocarbon degradation will generally decrease with lower temperatures, but the environment also plays an important role regarding the optimum temperatures for degradation. The highest degradation rates in soil environments generally occur in the range 30-40°C whereas optimal rates for freshwater environments drop down to 20-30°C and marine environments display optimum rates at as low as 15-20°C (Bartha and Bossert, 1984; Cooney, 1984). Nutrients, such as nitrogen and phosphorus, are also very important since a hydrocarbon contaminated environment contains an abundance of carbon and the supply of nitrogen and phosphorus will generally become the limiting factor during biodegradation (Atlas, 1985). Despite all these factors, still the biggest obstacle for microorganisms to overcome is the chemical inertness at room temperature of the carbon-hydrogen bond due to the lack of functional groups. The following sections will cover the various mechanisms microorganisms have developed to overcome this restraint.

At present, even after years of intensive research, microorganisms and the factors involved in biodegradation of hydrocarbons are still not fully understood. The limitations of culture based techniques, which have traditionally been the primary tools utilized for studying hydrocarbon degradation ecologies, have contributed to this fact. Only a small percentage (1-10%) (Torsvik *et al.*, 1998) of the microbial diversity in nature can be cultured in the laboratory resulting in a lack of knowledge regarding the ecological functions of the majority of microorganisms in nature and their potential applications in biotechnology (Kellenberger, 2001).

1.1.1 Aerobic biodegradation of hydrocarbons

To illustrate the known differences in the mechanisms between anaerobic and aerobic biodegradation, aerobic biodegradation of hydrocarbons will only be briefly discussed in this chapter. Aerobic hydrocarbon degradation has been extensively studied and numerous research papers exist that summarize these mechanisms and reactions (Atlas and Bartha, 1992; Ronald M Atlas, 1981; Cerniglia, 1992; Das and Chandran, 2011; Leahy and Colwell, 1990; Smith, 1990; Van Hamme et al., 2003).

Aerobic conditions deliver the most rapid and complete biodegradation of hydrocarbons. From a microbe's metabolic perspective, the metabolism of hydrocarbons by oxidation with molecular oxygen delivers the largest thermodynamic benefit. Figure 1.1 illustrates the essential characteristics of microorganisms involved in aerobic degradation of hydrocarbons and can be summarized as follows (Fritsche and Hofrichter, 2001):

- Due to the water-insolubility of hydrocarbons the cell must have optimized metabolic processes for interaction with the contaminants, such as the production of biosurfactants. Biosurfactants increase the bioavailability of hydrocarbons by reducing the interfacial (liquid-liquid) tension and allowing the two phases to mix and interact more easily.
- An initial oxidative process is needed to activate the hydrocarbon for further catabolism. This activation and incorporation of oxygen forms the key enzymatic reaction and is catalyzed by well-defined oxygenases and peroxidases.
- In peripheral degradation pathways the hydrocarbons are degraded step by step into intermediates of the central intermediary metabolism, such as the tricarboxylic acid cycle.
- The central intermediary metabolites, e.g. acetyl-CoA, succinate, pyruvate, are converted to cell mass via biosynthesis. The sugars needed for the various biosynthesis and growth are obtained from gluconeogenesis.

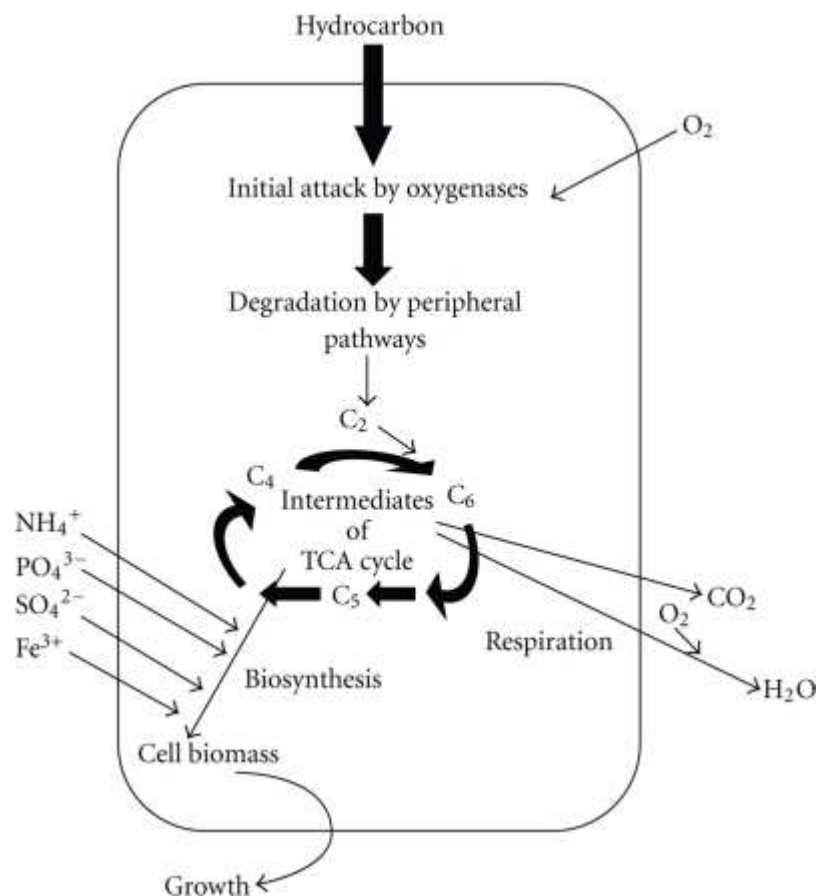


Figure 1.1: Main principle of aerobic degradation of hydrocarbons by microorganisms (taken from Das and Chandran, 2010).

The majority of bacteria present in hydrocarbon contaminated soils still cannot be cultured in a laboratory. The predominant bacteria of polluted aerobic soil environments, which are cultivable in nutrient rich media, belong to the genera and species listed in table 1.

Table 1.1: Predominant bacteria in soil samples polluted with aliphatic and aromatic hydrocarbons, polycyclic aromatic hydrocarbons and chlorinated compounds (taken from Fritsche and Hofrichter, 2000).

Gram-negative bacteria	Gram-positive bacteria
<i>Acinetobacter</i> spp.	<i>Arthrobacter</i> spp.
<i>Alcaligenes</i> sp.	<i>Bacillus</i> spp.
<i>Flavobacterium/Cytophaga</i> group	<i>Corynebacterium</i> spp.
<i>Pseudomonas</i> spp.	<i>Mycobacterium</i> spp.
<i>Xanthomonas</i> spp.	<i>Nocardia</i> spp.

Many of the hydrocarbon degrading bacteria are capable of versatile metabolism, and hydrocarbons are one of many other substrate classes that can serve as carbon sources (Harayama *et al.*, 2004; Margesin *et al.*, 2003). For most of these cases, hydrocarbons are not the preferred substrates and cells will tend to consume other, more easily accessible substrates before using hydrocarbons. To harness the free energy present in oxygen reactivity requires that an organism to not only possess the mechanisms of activation by oxygen but also to process the reactive intermediates in such a way to selectively oxygenate the desired substrates. Some bacterial species have been characterized that are highly specialized toward hydrocarbon degradation, called hydrocarbonoclastic bacteria (Harayama *et al.*, 2004; Head *et al.*, 2006; Wang *et al.*, 2010; Yakimov *et al.*, 2007). These organisms play a pivotal role in the removal of hydrocarbons from the environment. Hydrocarbonoclastic bacteria in the genera *Thalassolituus* (Yakimov *et al.*, 2004), *Oleiphilus* (Golyshin *et al.*, 2002), *Oleispira* (Yakimov *et al.*, 2003), *Marinobacter* (Duran, 2010), *Bacillus* and *Geobacillus* (Marchant *et al.*, 2006; Wang *et al.*, 2006) have all been identified as key organisms in the biodegradation of hydrocarbon spills in several environments. Of particular importance is the marine bacterium, *Alcanivorax*, capable of metabolising various linear or branched alkanes but unable to metabolize aromatic hydrocarbons, sugars, amino acids or other more commonly used carbon sources (Yakimov *et al.*, 2007).

Aerobic microorganisms usually initiate the metabolism of hydrocarbons through oxygenase and peroxidase reactions. Oxygenases utilise O_2 to incorporate oxygen into their substrates. Aerobic degraders need oxygen at two metabolic instances, firstly during the initial activation of the substrate and secondly at the end of the respiratory chain (Figure 1.1). The initial intracellular attack is an oxidative process which generates a highly reactive oxygen species. The incorporation of oxygen forms the key reaction in the activation of the hydrocarbon for degradation. The formed alcohol is then further oxidized and metabolized via the β -oxidation pathway (Rabus *et al.*, 2001). Figure 1.2 displays both types of enzymatic reactions involved in these processes. What kind of enzymatic reaction is realized will depend on the type of substrate and the nature of the enzymatic equipment possessed by the microorganisms.

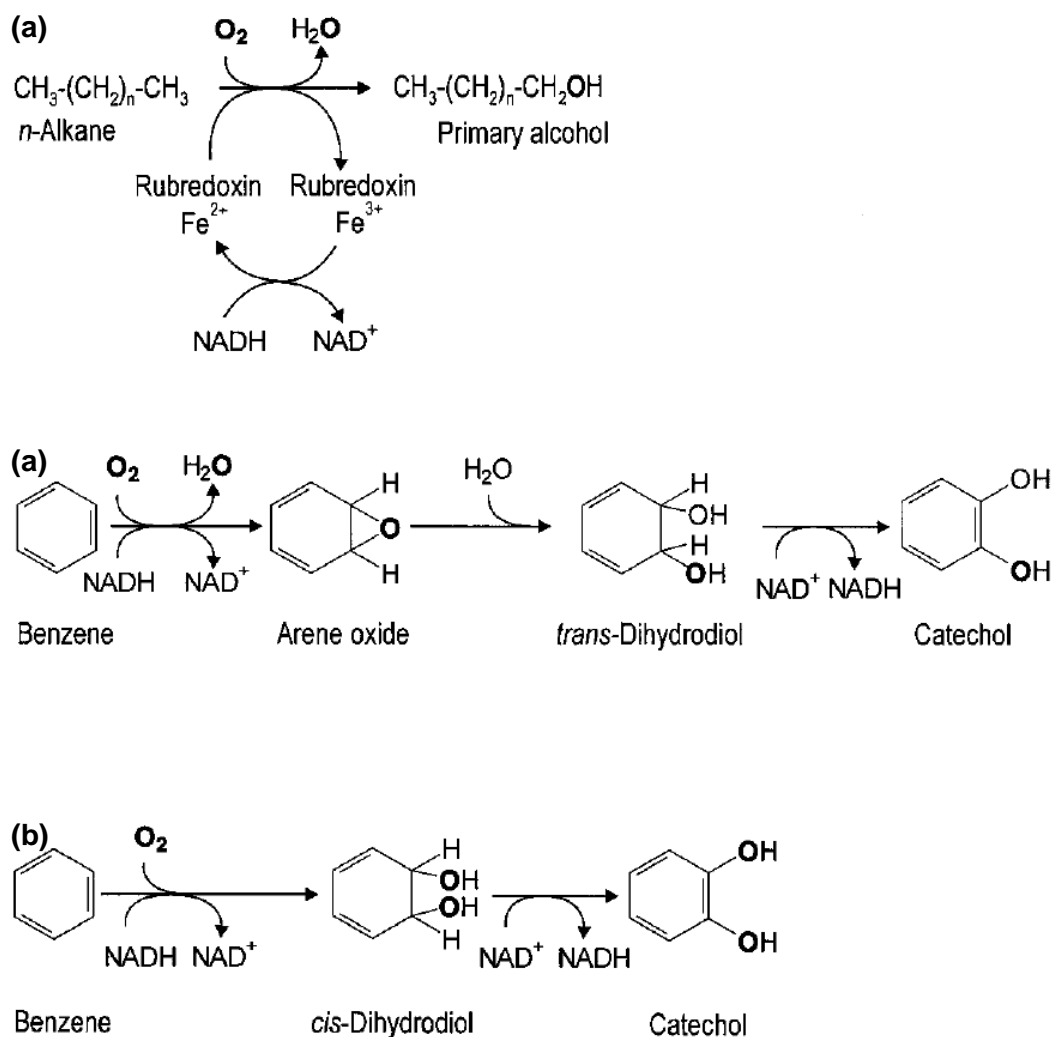


Figure 1.2: Initial attack on xenobiotics by oxygenases. (a) Monooxygenases incorporate one atom of O_2 into the substrate, the second atom is reduced to H_2O . (b) Dioxygenases incorporate both atoms into the substrate (Fritsche and Hofrichter, 2000).

1.1.2 Anaerobic biodegradation of hydrocarbons

The ability to utilize hydrocarbons in the presence of oxygen has been known since the beginning of the 20th century but molecular oxygen is not available in certain environments such as deep sediments or oil reserves. Since the oxygen dependant activation step is so pivotal in the degradation of hydrocarbons the question of whether or not hydrocarbons could be degraded under anoxic conditions was controversial. Hydrocarbons have been present in the biosphere all throughout the history of the world thus it was unimaginable that microorganisms could not have acquired pathways to utilize these compounds for growth in the absence of molecular oxygen (Widdel and Rabus, 2001).

In the late 80's studies started to implicate novel microbes capable of anaerobic hydrocarbon degradation (Figure 1.3) (Cerniglia, 1992). Later Vogel and Grbic-Galic (1986) conclusively demonstrated the biodegradation of hydrocarbons in the absence of molecular oxygen via methanogenesis of toluene and benzene, and Lovley and co-workers (1989) showed the complete oxidation of toluene to CO₂ by pure cultures of the Fe(III)-reducing bacterium *Geobacter metallireducens* strain GS-15. However, the first experimental demonstration alkane biodegradation was accomplished by Aeckersberg and co-workers (1991) in a study where they quantitatively measured the consumption of *n*-alkanes by sulphate-reducing bacteria, but since then the principle has been demonstrated with nitrate- as well as chlorate-reducing bacteria grown with saturated hydrocarbons as the sole carbon and energy source (Mbadinga *et al.*, 2011).

Since there is no biochemical agent under anoxic conditions with the same hydrocarbon activating properties as oxygen species when under anoxic conditions, these organisms seem to be able to activate hydrocarbons by reactions which are mechanistically unprecedented in biochemistry (Widdel and Rabus, 2001), completely different to those described in aerobic hydrocarbon metabolism. Rather than oxygen, these microorganisms have adapted to utilize sulphate, nitrate, oxidized metals – such as ferric iron and manganese(IV) - or CO₂ as electron acceptors for anaerobic respiration. They also grow in syntrophic co-cultures with other anaerobes or they can grow by anoxygenic photosynthesis. Table 1.2 clearly demonstrates that, thermodynamically speaking, the biodegradation of hydrocarbons under anaerobic conditions with these various electron acceptors is feasible (Mbadinga *et al.*, 2011).

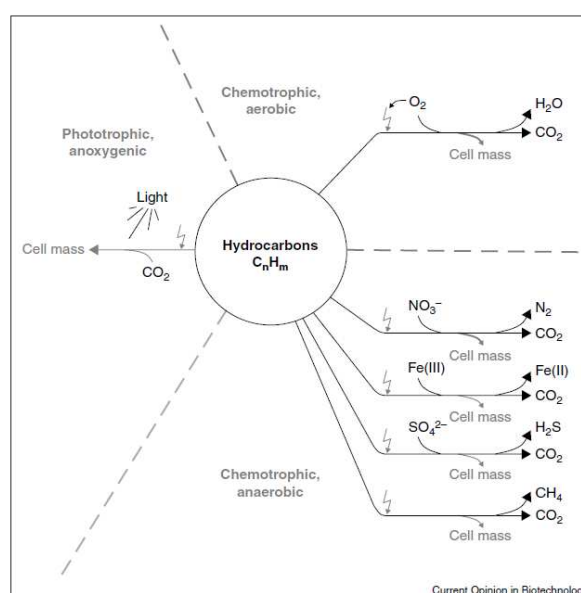


Figure 1.3: Experimentally verified possibilities for the microbial utilisation of hydrocarbons. Jagged arrows indicate hydrocarbon activation (taken from Widdel and Rabus, 2001).

Table 1.2: Gibbs free energies for hexadecane (C₁₆H₃₄) degradation coupled to selected redox reactions at standard conditions (298.15 K) at pH = 7 (Taken from Mbadanga *et al.*, 2011).

Electron acceptor (ox/red)	ΔG° (kJ/mol of C ₁₆ H ₃₄) ^a	ΔG° (kJ/mol of C ₁₆ H ₃₄) ^b
O ₂ /H ₂ O ^c	-9677.07	-10316.27
NO ₂ ⁻ /	-12498.24	-11832.412
ClO ₃ ⁻ /Cl ⁻	-11764.86	-12404.06
NO ₃ ⁻ /N ₂	-9819.37	-9675.55
Fe ³⁺ /Fe ²⁺	-5335.67	-9891.78
S ₂ O ₃ ²⁻ /S ²⁻	-2472.845	-4091.46
SO ₄ ²⁻ /H ₂ S	-897.13	-557.55
HCO ₃ ⁻ /CH ₄	-204.15	-353.96

^a ΔG° : standard Gibbs free energy: reactants and products at 1 M concentration and gases at a partial pressure of 1 atm. Hexadecane (C₁₆H₃₄) was chosen as the model substrate for free energies calculations. Methane, hydrogen, nitrogen and oxygen are in the gaseous phase at partial pressures of 1 atm. All other compounds are in the aqueous phase.

^b $\Delta G^\circ = \Delta G^\circ + m \times 2.303RT \log 10^{-7}$ (m is the net number of protons formed in the equation).

^c The reaction with oxygen is shown for comparison.

1.2 Microbial communities involved in anaerobic degradation of alkanes

1.2.1 Nitrate-reducers

The anaerobic degradation of hydrocarbons coupled to nitrate reduction was first demonstrated with pure isolates (strain ToN1, mXyN1, EbN1 and PbN1) for the utilization of alkylbenzenes in crude oil (Rabus and Widdel, 1996). All four strains were isolated from a homogenized mixture of mud samples from ditches and the Weser river in Bremen, Germany. It has been regarded as a highly effective strategy due to the high water solubility and mobility of nitrate (92.1 g/100 mL H₂O at 25°C) and also since nitrate-reduction is a highly energetically favourable reaction. Interestingly, the ecological distribution of anaerobic hydrocarbon degrading denitrifiers is not restricted only to hydrocarbon-contaminated environments since they have actually been mainly isolated from non-contaminated habitats (Mbadanga *et al.*, 2011). Currently, known pure isolates are affiliated with the β - and γ -subclass of the Proteobacteria. So far at least six pure cultures of nitrate respiring bacteria (NRB) have been documented (Figure 1.4 and Table 1.3).

Table 1.3: Ecological distribution of nitrate and chlorate-reducing bacteria and consortia metabolizing alkanes under anoxic conditions (adapted from Mbadinga *et al.*, 2011).

Strains	Affiliation	Source	Temp (°C)	pH
Strain HxN1*	β-Proteobacteria	Ditch sediment	28	7.1
Strain HdN1	γ-Proteobacteria	Activated sludge	28	7.1
Strain OcN1*	β-Proteobacteria	Ditch sediment	28	7.1
<i>Marinobacter</i> sp. BC36	γ-Proteobacteria	Lagoon mats	nd	nd
<i>Marinobacter</i> sp. BC42	γ-Proteobacteria	Lagoon mats	nd	nd
<i>Pseudomonas balearica</i>	γ-Proteobacteria			
<i>Pseudomonas</i> strain BerOc6	γ-Proteobacteria			
Consortium	nd	Lake sediments	30	
	nd	Diesel fuel contaminated sediments	25	7.4
Consortium	δ-Proteobacteria	Lake sediments	nd	
Chlorate reducing <i>Pseudomonas chloritidismutans</i> AW-1	γ-Proteobacteria	Anaerobic chlorate-reducing sludge	30	7.3

nd: not documented

* *Rhodocyclaceae*

Strains OcN1 and HxN1 are both members of the family *Rhodocyclaceae* within the β-*Proteobacteria* and are both able to grow for complete oxidation C₆ to C₁₂ *n*-alkanes and co-metabolise short-chain (C₄ – C₅) and cyclic alkanes (Ehrenreich *et al.*, 2000). In contrast the anaerobic oxidation of long-chain *n*-alkanes (>C₁₂) coupled to the reduction of nitrate is apparently associated with the members of the γ-*Proteobacteria*. Pure cultures of strains HdN1, *Marinobacter* sp. BC36 and BP42 and *Pseudomonas balearica* strain BerOc6 are able to oxidize *n*-alkanes from C₁₄ to C₂₀ (Bonin *et al.*, 2004; Ehrenreich *et al.*, 2000; Grossi *et al.*, 2008). Aerobic oxidation of branched and cyclic alkanes under nitrate-reducing conditions has also been shown to occur, but only with enrichment cultures. The identity of the microorganisms capable of anaerobic cycloalkane degradation under nitrate-reducing conditions is still unknown, although some studies are pointing towards the *Geobacter* spp. (Mbadinga *et al.*, 2011).

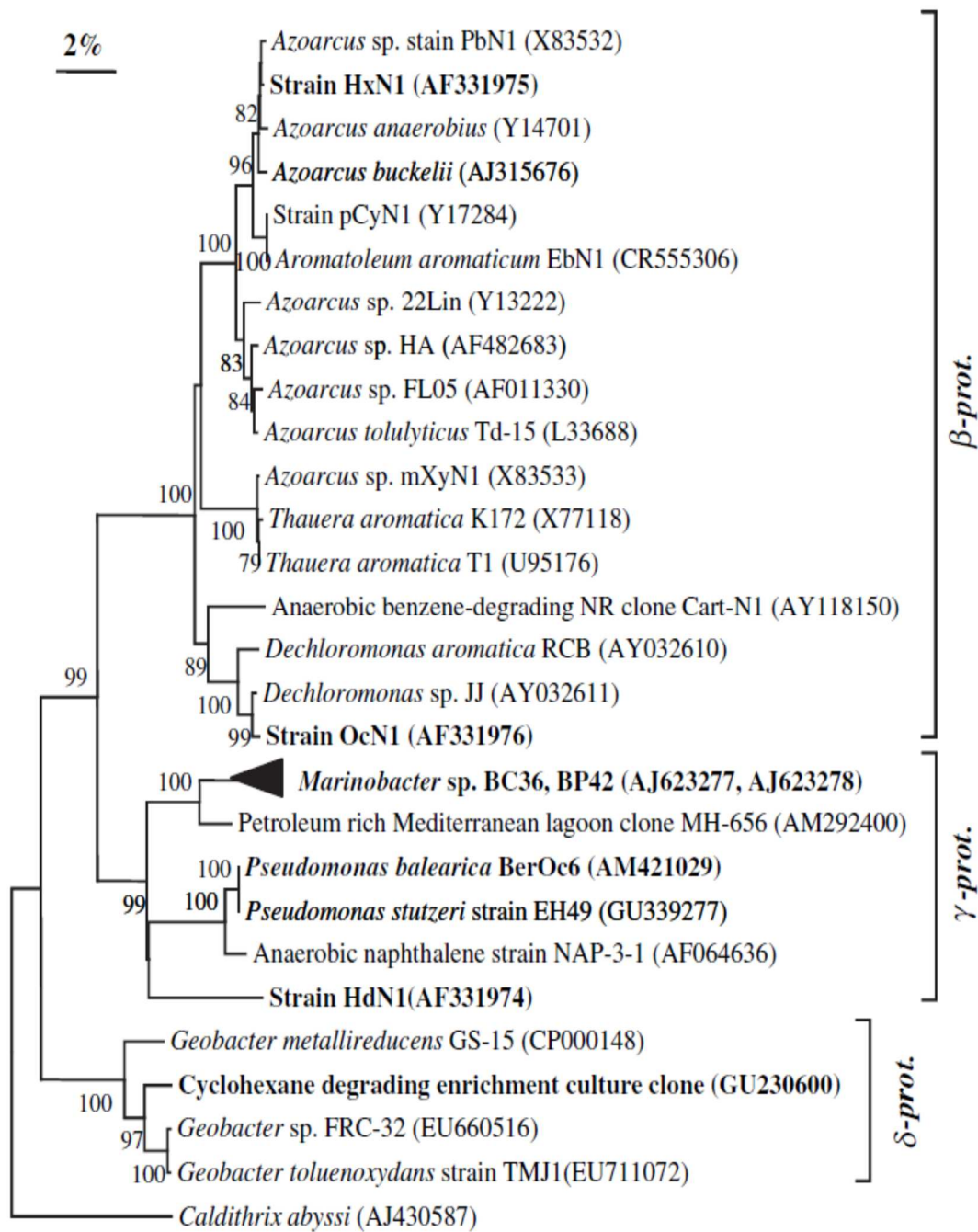


Figure 1.4: Phylogenetic tree of the 16S rRNA gene sequences of alkane-oxidizing, nitrate-reducing microorganisms (in bold) (taken from Mbadinga et al., 2010).

1.2.2 Sulfidogenic microorganisms

Sulphide formation during oil reservoir maturation resulted in a growing interest in the hydrocarbon utilization by sulfidogenic microorganisms. In contrast to nitrite reducers, sulfidogenic microorganisms capable of hydrocarbon utilization have always been identified or isolated from hydrocarbon-rich environments (Table 1.4) such as in petroleum deposits, hydrocarbon seeps, methane hydrates, petroleum deposits hydrothermal sediments, underground oil storage tanks and hydrocarbon-contaminated sediments (Magot et al., 2000; Watanabe et al., 2006). Phylogenetic analysis of the 16S rRNA genes and functional genes identify these sulphate reducing communities as part of the family *Desulfobacteraceae* which form part of the δ -Proteobacteria (Figure 1.5, Table 1.4), most of the members of this family are strict anaerobes.

So far eight mesophilic alkane degrading sulphate-reducers have been reported in literature (Mbadinga et al., 2011). Amongst them they are capable of oxidizing a wide range of alkanes. *Desulfatibacillum alkenivorans* AK-01 (So and Young, 1999) and *Desulfatibacillum aliphaticivorans* CV2803 (Cravo-Laureau et al., 2005) are able to grow by oxidizing long chain *n*-alkanes (C₁₂ to C₂₀). The *Desulfobacteraceae* strain PL2 can oxidize *n*-hexane and *n*-decane (Higashioka et al., 2009) whilst strain BuS5 will only grow on propane and butane (Kniemeyer et al., 2007). More recently, short chain (<C₁₂) degrading *Desulfobacteraceae*-affiliated propane and pentane oxidizing sulphate-reducing microorganisms have been found in non-marine sediments (Savage et al., 2010).

Desulfobacteraceae are not the only sulphide-reducing bacteria capable of anaerobic *n*-alkane degradation. Davidova and co-workers (2005) isolated two novel strains affiliated with the family *Synthrophobacteraceae*, also within the δ -Proteobacteria, these two strains, *Desulfoglaeba alkanexedens* ALDC and Lake, are able to grow by complete oxidation of C₆ to C₁₂. Thermophilic alkane-degrading sulphate reducers are extremely rare with only one isolate identified in literature. Rueter and co-workers (1994) were able to develop an anoxic enrichment with sulphate reduction in the presence of crude oil at 60°C, from this *Desulfothermus naphtha* TD3 was subsequently isolated, which was capable of *n*-alkane oxidation. Hyperthermophiles are more abundant and are represented by the hyperthermophilic archaeal members of the genus *Archaeoglobus* and the order *Thermococcales*. These microorganisms mostly live in hydrothermal environments and in subsurface thermophilic petroleum reservoirs (Gittel et al., 2009).

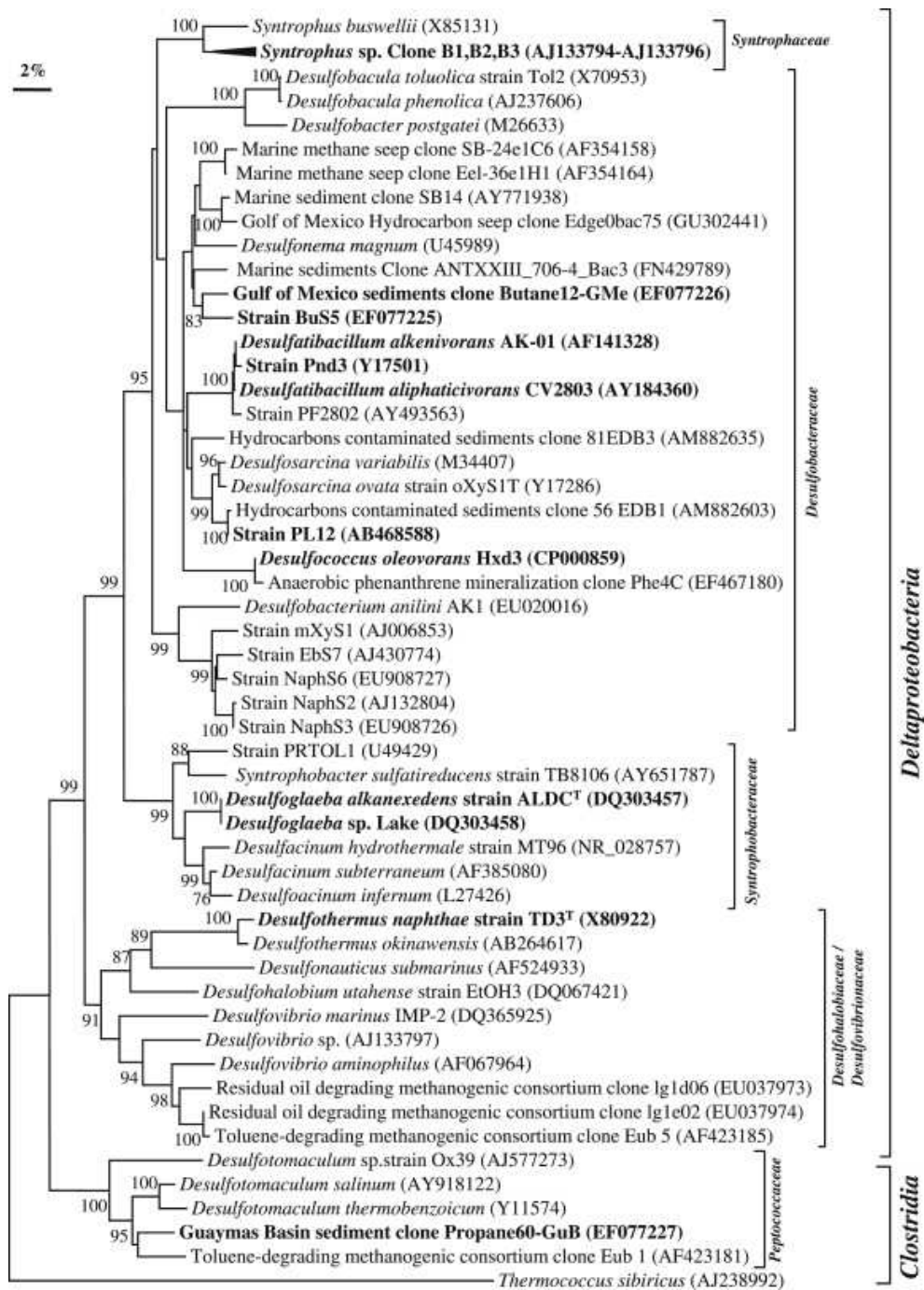


Figure 1.5: Phylogenetic tree of the 16S rRNA gene sequences of alkane-oxidizing, sulphate-reducing microorganisms (in bold) (taken from Mbadinda *et al.*, 2010).

Table 1.4: Ecological distribution of sulphate-reducing bacteria and consortia metabolizing alkanes under anoxic conditions (adapted from Mbadinga *et al.*, 2011).

Strains	Affiliation	Source	Temp (°C)	pH
Hxd3	γ-Proteobacteria	Oil-water separator	28-30	nd
Pnd3	γ-Proteobacteria	Marine sediments	28-30	nd
AK-01	γ-Proteobacteria	Petroleum-contaminated estuarine sediments	26-28	7.0-7.9
<i>Desulfoglaeba alkanexedens</i> ALDC	γ-Proteobacteria	Oily sludge	31-37	6.5-7.2
BuS5	γ-Proteobacteria	Marine hydrocarbon seeps	28	
PL12	γ-Proteobacteria	Petroleum-contaminated sediments	30-34	
<i>Desulfatibacillum aliphaticivorans</i> CV2803	γ-Proteobacteria	Hydrocarbon-polluted marine sediments	28-35	7.5
Clone Butane12-GMe	γ-Proteobacteria	Gulf of Mexico sediments	12	
Clone Propane60-GuB	γ-Proteobacteria	Guaymas Basin sediments	60	
Consortium	γ-Proteobacteria	Hydrocarbon seep	22	

nd: not documented

1.2.3 Methanogenesis

Methanogenesis is the biological formation of methane produced by strictly anaerobic organisms. The decomposition of complex organic matter under anaerobic conditions requires the concerted effort of a community of metabolically diverse microorganisms. In effect, this describes a syntrophic cooperation where formate, hydrogen and acetate are transferred from fermentative organisms to methanogens. This process is the least energetically favourable process in comparison with the other anaerobic respiration processes. The overall biodegradation process only becomes energetically favourable if the methanogenic substrates obtained from complex organic matter is used quickly in order to keep their concentrations at a low level (Schink, 1997).

Biodegradation of alkanes under methanogenic conditions with the ability to convert hexadecane to methane and CO₂ (Zengler *et al.*, 1999) has only been demonstrated in

highly enriched cultures obtained from ditch mud, but so far no isolates have been obtained and very few studies have been able to provide the phylogeny of the microorganisms involved in syntrophic association (Gieg *et al.*, 2008; Jones *et al.*, 2008; Zengler *et al.*, 1999). Assumptions made from a ditch mud methanogenic enrichment actively degrading hexadecane indicate that the community catalysed the following process (Zengler *et al.*, 1999):

Syntrophic proton-reducing acetogenic bacteria decompose alkane to acetate and H₂ → a group of archaea form methane and CO₂ from acetate → another group of archaea converts CO₂ and H₂ to methane.

1.2.4 Other electron donors

When compared to hydrocarbon oxidation with oxygen as the electron donor the anaerobic oxidation coupled to sulphate and nitrate reduction yields far less energy. Thus coupling other electron donors, such as perchlorate and nitrite, for anaerobic oxidation of alkanes might be possible, especially if taken into account that these alternative electron acceptors have a high redox potential (Table 1.5) and as such are ideal for microorganisms (Mbadanga *et al.*, 2010).

Table 1.5: Redox potentials of alternative electron acceptors (Zedelius *et al.*, 2011).

Electron acceptor (ox/red)	E ₀ (V)
ClO ₄ ⁻ /Cl ⁻	+1.287
ClO ₃ ⁻ /Cl ⁻	+1.03
2NO ₂ ⁻ /N ₂	+0.958
2NO/N ₂	+1.264
N ₂ O/N ₂	+1.3555

Mehboob and co-workers (2009) proposed a mechanism where aerobic oxygenases degrade alkanes under unusual anaerobic conditions. They reported that *Pseudomonas chloritidimutans* AW-1 utilizes chlorite not only as the electron acceptor but oxygen supplier for oxygenase activities. Chlorite disproportionates (ClO₂⁻ → Cl⁻ + O₂; ΔG⁰ = -148.4 kJ/mol) to produce molecular oxygen needed for the oxidation of the alkane substrate. Molecular oxygen can also be produced by dismutation of nitric oxide (NO) during nitrite reduction (NO₂⁻). During microbial nitrate reduction (NO₃⁻ to NO₂⁻ to NO to N₂O to N₂) nitric oxide is produced as an intermediate product, but in a methane-utilizing enrichment culture described by Ettwig and co-workers (2010) it occurs otherwise. It would appear that

'*Candidatus Methylomirabilis oxyfera*', which forms the dominant bacterium in the enrichment culture, nitrite (NO_2^-) is reduced to nitric oxide (2NO) which undergoes dismutation to nitrogen (N_2) and molecular oxygen (O_2) which is then utilized to oxidize the substrate.

1.3 Biochemical strategies for the anaerobic metabolism of alkanes

Even though hydrocarbons are considered to be chemically inert, they are still degraded by microorganisms. Figure 1.6 represents an overview of the main mechanisms and pathways utilized by microorganisms for the degradation of hydrocarbons under aerobic and anaerobic conditions. Since all known aerobic hydrocarbon degradation pathways start with the introduction of oxygen atom(s) into the substrates, usually through the action of oxygenase enzymes, this initial activation step in anaerobes must be initiated by other novel biochemical reactions. To date, several known pathways of anaerobic *n*-alkane activation have been identified and, surprisingly, a variety of reactions (Figure 1.7) seem to be employed by different microorganisms to overcome the activation barrier of different hydrocarbons (Heider, 2007).

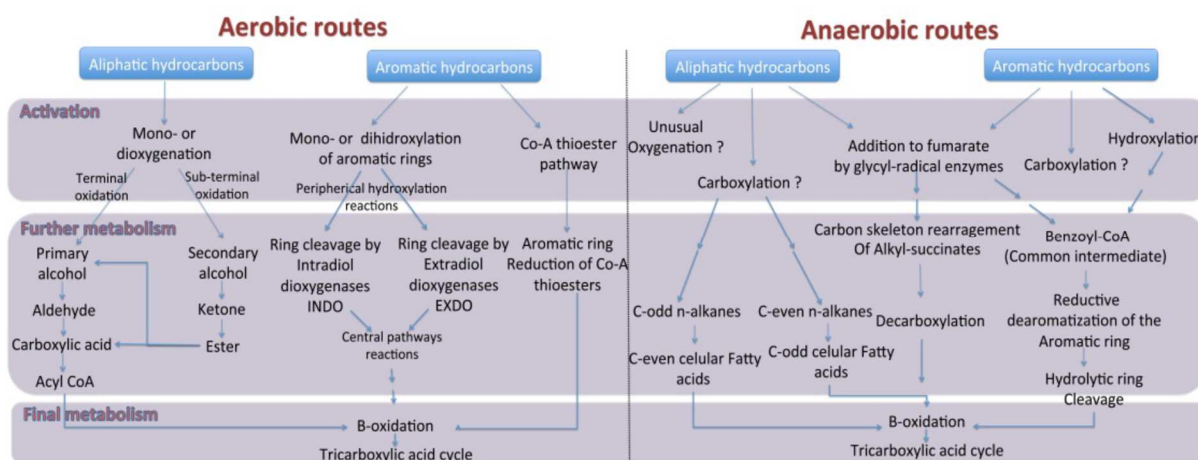


Figure 1.6: Pathways for aerobic and anaerobic bacterial degradation of hydrocarbon compounds (taken from Sierra-garcia and Oliveira, 2013)

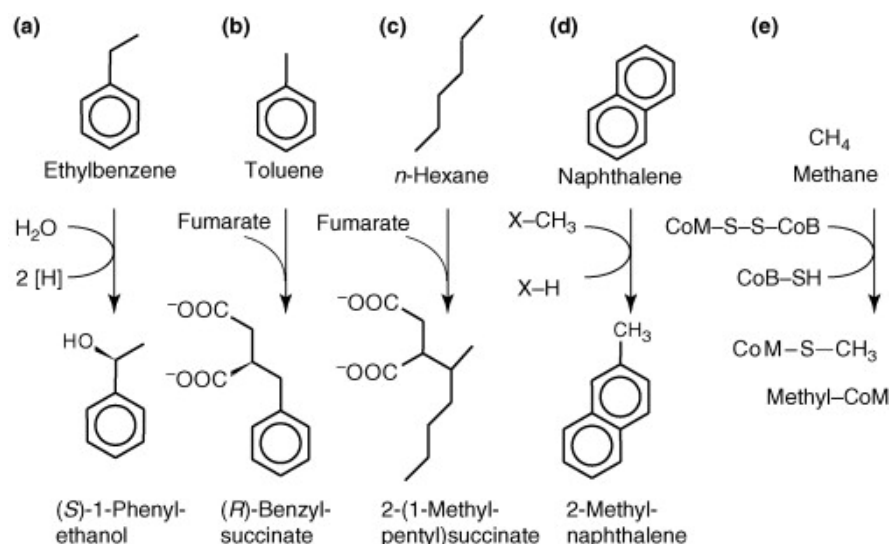


Figure 1.7: Examples of initial reactions involved in anaerobic degradation of hydrocarbons: (a) oxygen-independent hydroxylation, (b) fumarate addition reactions to methyl groups, (c) to methylene groups as observed in anaerobic n-hexane degradation; (d) methylation of the non-substituted aromatic hydrocarbon naphthalene, and (e) oxygen-independent methane oxidation by reverse methanogenesis (taken from Heider, 2007).

1.3.1 Alkane activation by oxygen independent hydroxylation

In denitrifying bacteria, the anaerobic metabolism of ethylbenzene and n-propylbenzene is initiated by hydroxylation of the C₁-methylene carbon atom of the sidechain (Figure 1.7). The best characterized enzyme for this reaction is ethylbenzene dehydrogenase from the β -proteobacterial strain EbN1 which is known to stereospecifically hydroxylate ethylbenzene to (S)-1-phenylethanol and was characterised as a soluble periplasmic molybdenum-cofactor-containing enzyme of the dimethyl sulfoxide (DMSO) reductase family (Hope A Johnson et al., 2001; Kniemeyer and Heider, 2001a). The enzyme consists of three subunits of which the α and β subunits share significant structural homology with those of nitrate reductases of *Escherichia coli* (Bertero et al., 2003; Jormakka et al., 2004) but the γ subunit appears to be unique. The catalytic centre is situated in the α -subunit, it contains a molybdenum atom which is coordinated by two molybdopterin-guanine dinucleotide (MGD) cofactors and an aspartate ligand from the protein. Also, both the α - and β -subunits contain unusually ligated $[\text{Fe}_4\text{S}_4]$ -clusters (one and three respectively) plus the β -subunit also contains $[\text{Fe}_3\text{S}_4]$ -clusters. The γ -subunit contains a heme b ligand which represents a type of heme protein with methionine and lysine as axial heme ligands (Kloer et al., 2006).

1.3.1.1 Proposed catalytic mechanism for hydroxylation

A catalytic mechanism (Figure 1.8) has been proposed based on the structure analysis and reactivity of a few substrate analogues and inhibitors. In the active site an oxo (or hydroxo) group should be bound to the molybdenum (in the Mo^{VI} oxidation state). After binding of the ethylbenzene substrate, the C-C bond of the ethylbenzene sidechain bond might be polarized enough by the aspartate ligand so that a hydride will be abstracted from C_1 by the molybdenum-oxo complex, resulting in the enzyme being reduced to Mo^{IV} state and the substrate being converted to a carbenium cation. The cation will then abstract the hydroxyl group from the molybdenum producing a Mo^{IV} -product complex. Previous observations concluded that external electron donors of high electron potential are needed to re-oxidize the reduced enzyme (Kniemeyer and Heider, 2001b), Mo^{IV} to Mo^{VI} , in two successive one-electron transfers (via Mo^{V}). These electrons are transferred via the five Fe-S clusters to the heme b (the expected exit site) from which it is transferred to a periplasmic cytochrome c (Kloer *et al.*, 2006). (S)-1-phenylethanol is then taken into the cytoplasm where it can be further oxidized to acetophenone by a stereospecific (S)-1-phenylethanol dehydrogenase (Kniemeyer and Heider, 2001a). Acetophenone is carboxylated to benzoylacetate by an ATP-dependant carboxylase, followed by the final step where the benzoylacetate is activated to benzoylactal-CoA, which is thiolitically cleaved to acetyl-CoA and benzoyl-CoA (Rabus *et al.*, 2005, 2002).

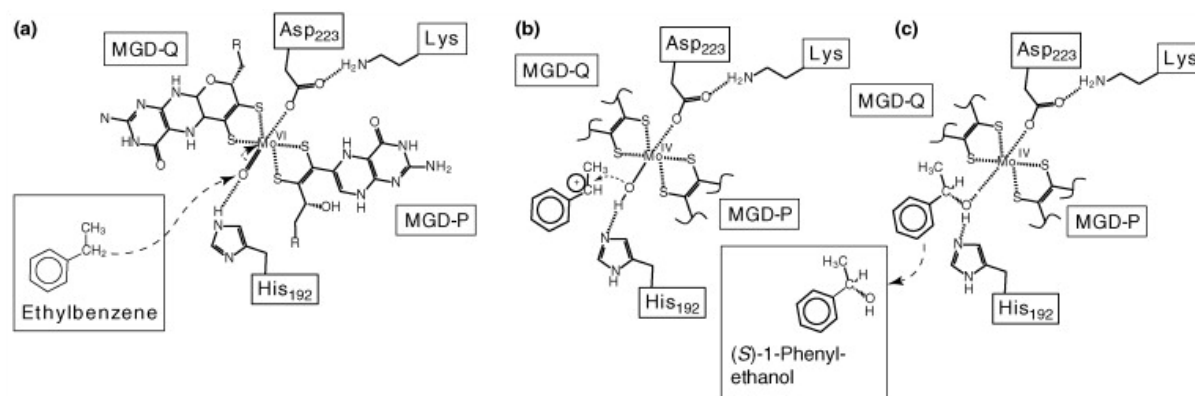


Figure 1.8: Putative reaction mechanism of ethylbenzene hydroxylation, as inferred from the structure of ethylbenzene dehydrogenase and its reactivity with substrate analogues. The active centre of the oxidized enzyme as shown in (a) was modelled from the known structure of the reduced enzyme by exchanging the position of a bound acetate ligand of the Mo with an oxo group. After binding of ethylbenzene to the active centre, a hydride transfer from the methylene carbon to the molybdenum cofactor is expected, leaving the substrate in a carbenium-ion transition state (b), which reacts with the coordinated hydroxyl group formed from the oxo ligand to generate the product still hydrogen-bonded to the Mo (c). The proposed steps of the reaction are indicated by dotted arrows (taken from Heider, 2007).

1.3.2 Alkane activation by addition to fumarate

The addition to fumarate (Figure 1.9) seems to be the dominant activation mechanism for anaerobic hydrocarbon degradation (Callaghan *et al.*, 2006). In brief, the reaction in which fumarate is added to the *n*-alkane is catalysed by a radical enzyme which yields (1-methylalkyl) succinate. This activated metabolite then undergoes a carbon skeleton rearrangement to form (2-ethylalkyl) malonyl-CoA that allows decarboxylation to 4-methylalkanoyl-CoA. This fatty acid can then undergo conventional β -oxidation to yield such intermediates as (2-methylalkyl)-CoA, a linear fatty acid containing two less carbon atoms than its parent, propionyl-CoA and acetyl-CoA. Acetyl-CoA can then be further oxidized to form CO₂. The fumarate can be regenerated from propionyl-CoA (via methylmalonyl-CoA and succinyl-CoA) or from acetyl-CoA (Callaghan *et al.*, 2006; Rabus *et al.*, 2002, 2001; Wilkes *et al.*, 2002). Alkane activation via fumarate addition is an exergonic reaction (thus $\Delta G^\circ < 0$) and as such does not require any exogenous energy input, such as ATP hydrolysis, for the reaction to be successful (Rabus *et al.*, 2001).

Apart from toluene, fumarate-addition reactions have been shown to be responsible for the activation of various other hydrocarbons or other chemically inert compounds. Fumarate addition to methyl groups have been reported in the anaerobic metabolism of *m*-xylene, 2-methylnaphthalene, *m*- and *p*-cresol (Chakraborty and Coates, 2004; Heider *et al.*, 1999; Widdel and Rabus, 2001) and also addition to the methylene groups of *n*-hexane (Rabus *et al.*, 2001) and ethylbenzene (Kniemeyer *et al.*, 2003).

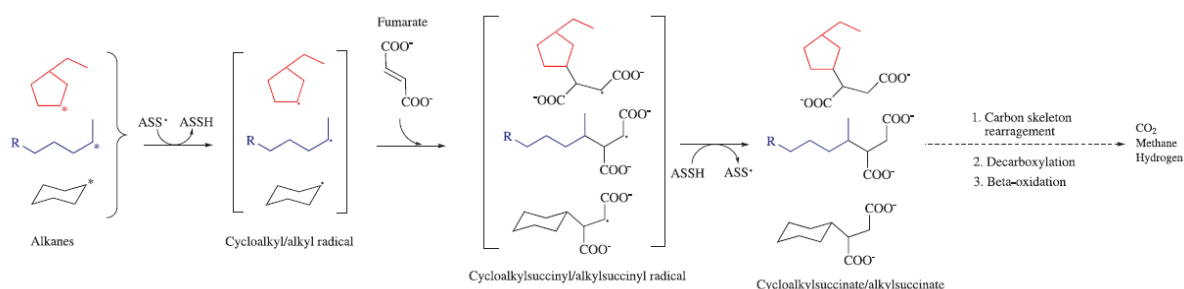


Figure 1.9: Alkane activation by fumarate addition (taken from Mbadinda *et al.*, 2011).

1.3.2.1 Enzyme mechanism for fumarate addition

The most well studied anaerobic enzyme mechanism involves the glycy radical enzyme-dependant stereospecific addition of the non-activated methyl group to a fumarate co-substrate to yield (R)-benzylsuccinate (Beller and Spormann, 1999; Leuthner *et al.*, 1998). The glycy radical enzyme in question, benzylsuccinate synthase (BSS) along with its glycy-

radical cofactor has been identified by Electron Paramagnetic Resonance (EPR) spectroscopic methods (Krieger *et al.*, 2001). BSS has been found to be the responsible biocatalyst for the addition of fumarate to aromatic hydrocarbons, such as toluene (Widdel *et al.*, 2006), in several hydrocarbon-degrading anaerobes (Heider, 2007; Rabus *et al.*, 2001). A conserved glycine motif (I/V-R-I/V-X-G-F/W/Y) located near the C-terminus and conserved cysteine residue(s) located in the middle of the polypeptide chain form the dominant features of the catalytic subunit of glycy radical enzymes such as BSS (Becker *et al.*, 1999; Logan *et al.*, 1999; O'Brien *et al.*, 2004). During activation of the hydrocarbon these conserved sites will transfer a radical from the glycine residue to a cysteine residue resulting in the thiyl radical initiating the abstraction of a hydrogen atom from the substrate. Thus, in the absence of molecular oxygen, the BSS glycy radical mechanism provides the means for anaerobic microorganisms to activate hydrocarbon substrates. The substrate specificity of benzylsuccinate synthases has been shown to be much broader than the bacterial strain itself can utilize as growth substrates. Also, the enzymes from different strains appear to differ in substrate specificity which increases the likelihood that multiple substrates are being activated via fumarate addition by the same benzylsuccinate synthase-like isoenzyme in the respective degradation pathways (Verfürth *et al.*, 2004).

Direct comparisons can be made between the formations of alkyl- or cyclic-branched succinates as catabolic intermediates and the well-established formation of benzylsuccinate, as the initial anaerobic activation step in anaerobic toluene metabolism, and as such the glycy radical enzymes have been termed alkylsuccinate synthase (ASS) or (1-methylalkyl) succinate synthase (MAS) for *n*-alkanes and (cycloalkyl) succinate synthase (CAS) for cycloalkanes. These enzymes form part of a novel class of glycy radical enzymes which forms a part of the superfamily pyruvate-formate lyase (PFL) and type III ribonucleotide reductase (RNR) (Widdel *et al.*, 2006). Recently, two ASS genes (*assA1* and *assA2*) which both display high similarity to the catalytic subunit gene of *bssA* were identified in the alkane degrader *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al.*, 2008). Similarly an *assA*-like gene, *masD*, was identified in the denitrifying, alkane degrading strain HxN1 (Grundmann *et al.*, 2008).

1.3.3 Alkane activation via carboxylation

Carboxylation reactions are based around the incorporation of $^{13}\text{C-CO}_2$ into intermediates. There is much debate surrounding this initial activation method since, energetically speaking, carboxylation of alkanes will not be feasible under standard conditions ($\Delta G^\circ = +28\text{kJ/mol}$), but the possibility of other reactions preceding carboxylation has been proposed

(So *et al.*, 2003). The carboxylation activation strategy was developed from the growth pattern of the sulphidogenic strain Hxd3 on the basis that C-even and C-odd fatty acids were formed from C-odd and C-even *n*-alkanes, respectively, thus one carbon shorter than the original alkane (Aeckersberg *et al.*, 1998; So *et al.*, 2003). It was concluded that strain Hxd3 would metabolize an alkane to fatty acids via carboxylation with inorganic carbon, most likely at C-3, resulting in the removal of two sub-terminal carbon atoms from the alkane chain. However, the hypothetical fatty acid intermediate has never been detected.

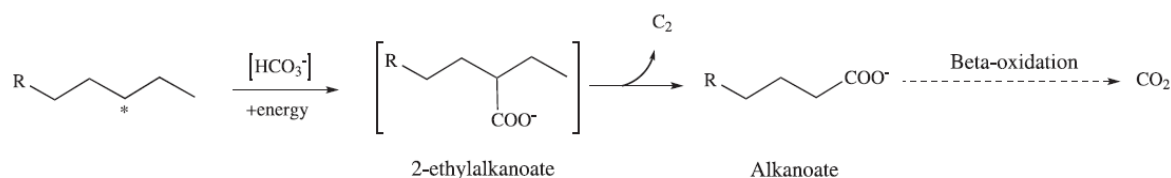


Figure 1.10: Alkane activation by carboxylation (taken from Mbadinga *et al.*, 2010).

1.3.4 Methylation of naphthalene

The degradation of naphthalene by sulphate-reducing enrichment cultures under anaerobic conditions apparently occurs by the conversion of naphthalene to 2-methylnaphthalene (Figure 1.6) which is then followed by the addition of fumarate and β -oxidation to succinyl-CoA and naphthoyl-CoA (Safinowski and Meckenstock, 2006a). Although the 2-methylnaphthalene-catabolic enzymes have been identified, naphthyl-2-methyl-succinate synthase, succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase and naphthyl-2-methyl-succinyl-CoA dehydrogenase, the enzyme responsible for the initial methyl transfer is still elusive, but it might possibly be generated from bicarbonate via a reverse CO-dehydrogenase pathway (Safinowski and Meckenstock, 2006a).

1.3.5 Anaerobic methane oxidation via “reverse methanogenesis”

The anaerobic oxidation of methane is apparently mediated by sulphate-reducing bacteria (Boetius *et al.*, 2000; Orphan *et al.*, 2001) or denitrifying bacteria (Raghoebarsing *et al.*, 2006) in a consortium with methane-oxidizing archaea closely related to the order *Methanosarcinales*. Cells from these consortia have been found to contain a modified form of the key methanogenesis enzyme, methyl-CoM reductase, which differs by containing a slightly larger modified form of the nickel-tetrapyrrol F430 cofactor (Krüger *et al.*, 2003). This modified cofactor has thus far been exclusively identified in mats of anaerobic methane oxidizers and as such is believed to be the active centre of a reversible methyl-CoM reductase isoenzyme which is capable of oxidizing methane to methyl-CoM (Figure 1.6). In conjunction, metagenomic analysis performed on anaerobic methane-oxidizing consortia

has revealed the presence of genes coding for a novel isoenzyme of methyl-coenzyme M reductase though to catalyse methane oxidation (Hallam *et al.*, 2004; Krüger *et al.*, 2003; Meyerdierks *et al.*, 2005).

1.3.6 Alternative mechanisms

Alternative mechanisms, not involving addition of fumarate or carboxylation, have been proposed depending on the availability of electron acceptors and the type of microorganisms present in an environment. In a process deemed as “unusual oxygenation”, *Pseudomonas chloritismutans* AW-1 has shown the ability to produce its own oxygen via chlorate respiration which can be utilized during alkane metabolism (Figure 1.11) (Mehboob *et al.*, 2009). This process would thus require oxygenases to incorporate the produced molecular oxygen into the alkanes. Similar activation strategies are also possible with electron acceptors such as nitrate or nitrite (Ettwig *et al.*, 2010).

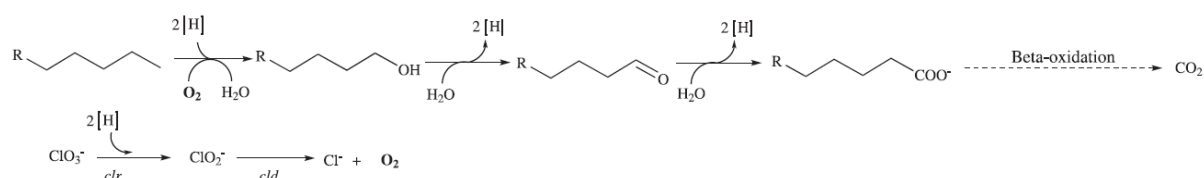


Figure 1.11: Alkane activation by “unusual oxygenation” (taken from Mbadinga *et al.*, 2010).

Another recently proposed strategy may involve the hydroxylation of oil alkanes and was identified in a methanogenic enrichment cultures degrading crude oil (Head *et al.*, 2010). Pyrosequencing of total genomic DNA revealed the presence of genes encoding the β -oxidation pathway and also alcohol and aldehyde dehydrogenase but no *assA*-like genes for fumarate addition or even carboxylation, thus indicating that the oil alkanes are probably activated via anaerobic hydroxylation (Head *et al.*, 2010).

1.4 Biomarkers for anaerobic hydrocarbon degradation

The assessment of the *in situ* anaerobic bioremediation at a hydrocarbon contaminated site can be achieved by studying various biomarkers or indicators connected to the biodegradation. These include signature metabolites of hydrocarbons (metabolic biomarkers) (Beller *et al.*, 1995; Young and Phelps, 2004), the functional genes involved in anaerobic biodegradation of target compounds (genetic/molecular biomarkers) (Callaghan *et al.*, 2009; Winderl, 2007) and isotope fractionation (compound specific isotope analysis) (Steinbach *et al.*, 2004). Recent studies (Beller *et al.*, 2008; Callaghan *et al.*, 2009; Yagi *et*

al., 2010) have started to indicate the importance of the analysis of multiple biomarkers to reliably prove that *in situ* biodegradation is occurring.

1.4.1 Glycyl radical genes as molecular biomarkers

Benzylsuccinate synthase (BSS) is a member of the glycyl radical family of proteins and has shown the ability to activate toluene under anaerobic conditions by addition of fumarate to the methyl group, thus producing benzylsuccinate (Selmer *et al.*, 2005). BSS is a heterohexamer with three different subunits, α (94 KDa), β (12 KDa) and γ (10 KDa), all, including the activating enzyme, which can be found on one single operon, the gene product is 860 amino acids long (Leuthner *et al.*, 1998). BSS catalyses toluene degradation in all known isolates (Heider *et al.*, 1999; Widdel and Rabus, 2001), but BSS-like enzymes have also been shown in activation, through fumarate addition, of several other aromatic substrates, such as *o*-, *m*- and *p*-xylenes; *o*-, *m*- and *p*-cresols and hexadecane (Beller and Spormann, 1999; Callaghan *et al.*, 2008a; Kniemeyer *et al.*, 2003; Verfürth *et al.*, 2004) and are believed to be active in 2-methylnaphthalene (Annweiler *et al.*, 2002; E. V. A. Annweiler *et al.*, 2000) and naphthalene degradation (Safinowski and Meckenstock, 2006b).

BSS-like enzymes have been shown to catalyse hydrocarbon degradation in a phylogenetically diverse group of microorganisms only under anaerobic conditions. The presence of the *bssA* gene, the gene coding for the α -subunit, in anoxic environments can therefore be used to indicate the presence of microorganisms capable of hydrocarbon degradation under anaerobic conditions. Beller and co-workers (2002) as well as Winderl (2007) have had great success in identifying *bssA* gene based communities in toluene contaminated sites with PCR based approaches.

For *n*-alkane activation Callaghan and co-workers (2009) devised a set of primers that target the recently discovered alkylsuccinate synthase genes (*assA1* and *assA2*), which were discovered in the alkane degrader *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al.*, 2008a). These primers have been proven to be very useful in providing a platform for further investigation of hydrocarbon contaminated environments. Analysis of 96 sequenced clones from sites that were negatively impacted by petroleum spills resulted in 21 OTUs which proved all similar to known *assA* genes. This same data set resulted in no hits observed with the primer set devised for the *bssA* gene by Winderl (2007) (Callaghan *et al.*, 2009). These results should in no way be interpreted in such a way to conclude that there are no *bssA* genes present, but rather to further highlight the importance of multiple biomarker screening.

1.4.2 Metabolic biomarkers for monitoring in situ hydrocarbon degradation

According to Young and Phelps (2004), an ideal metabolic marker should be a) formed during active biodegradation of the target compound, b) specific to the process being monitored, c) normally absent in unimpacted environments, d) water soluble for ease of sampling, and e) biodegradable. The benzy succinate synthase and similar pathways, as discussed above, have been found to be widely distributed throughout nitrate-, sulphur-, iron reducing, as well as, methanogenic consortia (Beller and Spormann, 1997; Rabus and Widdel, 1996; Zengler *et al.*, 1999). Since the pathway is well distributed in the environment, the intermediates become ideal candidates as biomarkers in a variety of conditions. As early as 1995, Beller and co-workers (1995) showed that the metabolites formed during the degradation of alkylbenzene could be useful as bioindicators during bioremediation. They observed that during the metabolism of toluene and all the xylenes, benzy succinate-like metabolites were being formed and could be used as indicators of *in situ* metabolism. An example of useful benzy succinate-like metabolites can be seen in figure 1.12 below.

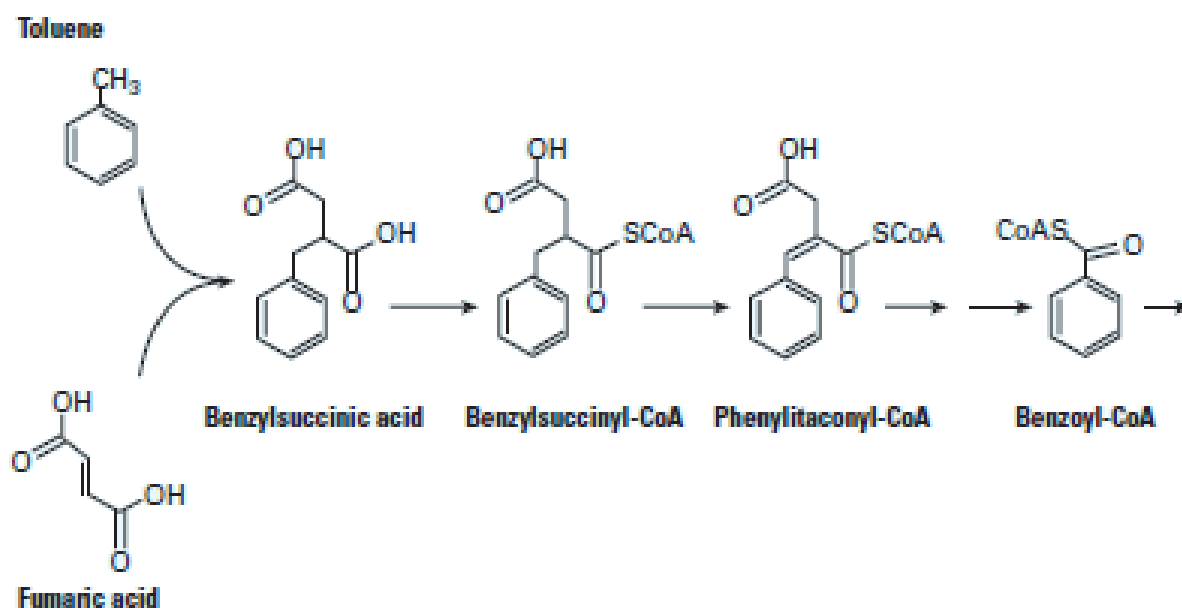


Figure 1.12: Toluene degradation pathway. An initial reaction involves the addition of fumarate to the methyl group resulting in benzy succinate which is activated with CoA (taken from (Young and Phelps, 2004)).

1.6 Conclusion

Recent studies have greatly contributed to our knowledge regarding the anaerobic catabolism of hydrocarbon compounds and have contributed significantly in our understanding of different aspects of the physiology, ecology biochemistry and mechanisms that allow anaerobic microorganisms to metabolise this highly abundant carbon source. These studies have started to show that the mechanisms for anaerobic catabolism of hydrocarbons is a very diverse process and that the capability for anaerobic degradation is widespread in natural environments with genus- and species-specific variations that can lead to substrate specificities. However, there are still many issues regarding anaerobic degradation that remain elusive or poorly studied. There is extensive generated knowledge regarding the regulation of genes responsible for aerobic degradation of hydrocarbons in many different bacteria yet in contrast the mechanisms that control gene expression in most anaerobes are unknown.

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Chapter 2

Introduction to the present study

2.1 Introduction

The aerobic degradation of hydrocarbon compounds is well known and various species of microorganisms from different genera are capable of breaking down hydrocarbons under these conditions. A key point in the degradation process is the activation of these chemically inert compounds by free molecular oxygen. As such it was long believed that this process could not occur in anaerobic conditions. However, as discussed in chapter 1, several studies concerning anaerobic hydrocarbon degradation have been performed and published but there are still large gaps in the knowledge about anaerobic hydrocarbon degradation. In particular the microorganisms involved and the initial activation mechanism has not been fully elucidated, especially in nitrate reducing organisms. That the degradability of these compounds by microorganisms under anaerobic conditions are not well understood has been the incentive for the present research programme.

In the present investigation, the microbial degradation of hydrocarbons will be studied under anaerobic conditions. Enrichment cultures were obtained from hydrocarbon contaminated environments since organisms capable of surviving under these conditions should inherently contain the mechanisms for survival as well as hydrocarbon metabolism. Techniques spanning different disciplines were applied to identify microorganisms actively involved in the degradation of hydrocarbons under anaerobic conditions and their preferred hydrocarbon substrates. In the following chapters, experiments are described in which the anaerobic degradation of naphthalene in the presence of nitrate is observed. Special attention was paid to the metagenomics potential for naphthalene degradation and metatranscriptomic analysis sheds light on the mechanisms employed by the enrichment culture.

These new insights into naphthalene degradation under anaerobic conditions coupled to nitrate reduction, as described in this thesis, adds to the greater scientific knowledge base regarding the anaerobic activation of polycyclic aromatic hydrocarbon compounds.

2.2 The broad aims of the study

The aim of the current study is to test the following hypotheses:

- Organisms present in hydrocarbon contaminated environments will inherently contain mechanisms for survival in these harsh environments as well as possess the pathways to metabolize these compounds.
- In the absence of oxygen, microorganisms will employ novel reactions for the activation of hydrocarbons for further degradation.
- Growth on a hydrocarbon under anaerobic conditions will result in the expression of gene transcripts relevant to the degradation.

2.3 Outline of the thesis

Chapter 3 describes the sampling of hydrocarbon contaminated environments and the enrichment of hydrocarbon degrading cultures using various mediums and conditions. The initial and enriched microbial composition of the different samples was assessed using molecular methods (PCR, DGGE, cloning and sequencing). The hydrocarbon degrading potential was investigated by screening various straight chain and cyclic hydrocarbons as well as known anaerobic degrading marker genes. Subsequently one of the enrichment cultures were selected for further investigation based on its novel diversity, presence of biomarker genes and anaerobic degradation potential.

Chapter 4 describes the further physiological and phylogenetic characterization of the hydrocarbon degrading nitrate reducing enrichment culture. Molecular methods showed that this enrichment culture was stable and was dominated mainly by *Citrobacter* sp. Hydrocarbon degradation studies indicated naphthalene as the hydrocarbon of choice for anaerobic degradation by the enrichment culture. Various methods were employed (total proteome analysis, growth studies on activated naphthalene derivatives) in an attempt to elucidate the activation mechanism for anaerobic naphthalene degradation coupled to nitrate reduction. However, these experiments only hinted at possible mechanisms for activation.

In chapter 5 the diversity, genetic potential and differential transcript analysis was studied using next generation sequencing technologies. Targeted 16S metagenomics presented the most complete view of the microbial population obtained in this study, while complete metagenomics sequencing highlighted the possible metabolic pathways potentially utilized by the enrichment culture for anaerobic naphthalene degradation. Comparing the transcriptomes of the enrichment culture grown on either acetate or naphthalene provided

differentially expressed gene data to draw final conclusions regarding the possibility of a methylation coupled to fumarate addition, but also a carboxylation reaction, for the activation of naphthalene for further degradation.

Chapter 3

Microbial diversity and hydrocarbon degradation potential of enrichment cultures obtained from contaminated environments

3.1 Introduction

Aliphatic and aromatic hydrocarbons are ubiquitous in the environment due to natural and anthropogenic processes. Under aerobic conditions these hydrocarbons can be rapidly biodegraded but oxygenated environments often quickly become anaerobic when microbial respiration is coupled to contaminant oxidation (Fritsche and Hofrichter, 2000). Anaerobic respiration is the process where microorganisms utilize alternative terminal electron acceptors in environments where molecular oxygen is limited or absent. Specific terminal electron acceptors utilized will vary depending on the redox conditions within an environment and also the adaptation of resident microorganisms. These alternative electron acceptors can include nitrate, sulphate, iron(III), carbon dioxide, among others (Canfield *et al.*, 2005). It is well known that these anaerobic, microbial respiratory processes can have a significant influence on the geochemical cycling of natural environments (Lovley and Phillips, 1994).

Even though it has been shown that hydrocarbons can be degraded anaerobically, very little is known about the microorganisms responsible for this activity with very few reports describing pure cultures with the ability to degrade hydrocarbons anaerobically (Austin and Callaghan, 2013) but degradation has been reported coupled to dissimilatory nitrate, iron(III) and sulphate reduction (E. Annweiler *et al.*, 2000; Callaghan *et al.*, 2009; Chakraborty and Coates, 2005; Coates *et al.*, 2001; Fukui *et al.*, 1999; So and Young, 1999). Various difficulties have been reported when attempting to isolate pure anaerobic cultures with this ability, anaerobic microorganism, these microorganisms have generally very low growth rates and conventional serial dilutions or plating techniques can result in the selection of bacteria growing on the nutrient content in the media rather than utilizing the hydrocarbon(s) (Rockne *et al.*, 2000). Another problem arises from the low water solubility of hydrocarbons making the effective addition to solid media problematic. Some isolation techniques have tried to counter this with some success, by either spraying ether dissolved hydrocarbons on a plate and letting the ether evaporate (Kiyohara *et al.*, 1982) or by adding ethanol dissolved hydrocarbons to the hot agar solution (Bogardt and Hemmingsen, 1992). These methods do however have their limitations including contamination and quantifying effective hydrocarbon accessibility, especially if one is to work under anaerobic conditions, these methods have to be adapted.

As such the isolation, cultivation and characterization of microorganisms from various environments, all capable of anaerobic hydrocarbon degradation, become extremely important. The extension of studies on pure cultures and mixed consortia can then lead to the elucidation of metabolic pathways, including their genes and gene products, responsible for hydrocarbon degrading processes. This information then paves the way for the prediction and modelling of these activities in novel pure culture isolates and within mixed communities (Callaghan *et al.*, 2012; Chakraborty and Coates, 2005).

The aims of this chapter were to:

- Characterize and document the sampling sites by proper sampling
- Elucidate the bacterial diversities of the collected samples
- Obtain sediment-free cultures enriched on hydrocarbons
- Assess hydrocarbon degradation potential of the enrichment cultures and final bacterial diversities of the enrichment cultures

3.2 Materials and methods

3.2.1 Chemicals

All chemicals used were of the highest available purity and purchased from Sigma-Aldrich® (Pty) Ltd.(Aston Manor, South Africa) and Merck (Pty) Ltd. (Modderfontein, South Africa), unless otherwise stated.

3.2.2 Sampling sites

Samples utilized in this study were obtained from three different sources. Two of them were from long term hydrocarbon contaminated soils due to exposure to petroleum hydrocarbons as a result of the areas being utilized as truck washing, refuelling and repairing areas. One was at the Free State Groundworks (Vrystaat Grondwerke) (-29.167878, 26.227841, Bloemfontein, S.A.) (Figure 3.1) where samples were taken from surface and 5 cm below the surface of the ramp entering the truck workshop. The other was at the surface of the truck washing area at the Star Diamonds mine (-28.319605, 26.794870, Theunissen district, S.A.) (Figure 3.2). The third was a microbial contaminated diesel sample obtained from Earthmoving Repair Services (-29.170033, 26.226544, Bloemfontein, S.A.) (Figure 3.3). This diesel sample was taken from one of the forklifts and microbial contamination was confirmed when Earthmoving Repair Services sent a sample to BP Petroleum industries before sample collection.



Figure 3.1: Free State Groundworks sampling site. Arrow indicates sampling point.



Figure 3.2: Star Diamonds sampling site. Arrow indicates sampling point.



Figure 3.3: Microbial contaminated diesel sample obtained from Earthmoving Repair Services (sample on the right). Sample on the left is fresh commercial diesel.

3.2.3 Initial sample diversity

3.2.3.1 Genomic DNA extraction

The Powersoil® DNA Isolation Kit was used to lyse cells and purify extracted DNA by following the manufacturer's instructions. Genomic DNA was extracted from samples by adding soil directly to the bead beating tube. For the diesel sample, autoclaved double distilled water was added to the diesel and stirred. After phase separation the aqueous

phase was added to the bead beating tube for extraction. DNA was eluted in 10 mM TE (Tris-EDTA) (pH8.0) elution buffer, concentrations were determined on the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific) and samples stored at -20°C until further use.

A 1% agarose gel (w/v) was prepared supplemented with 0.6 µl ethidium bromide (EtBr) (3 µl of a 10 mg/mL of stock in 50 mL gel) added for visualization of the DNA. Genomic DNA (10 µl) was added to 2 µl loading dye (Fermentas) before loading onto the gel. The MassRuler™ DNA ladder (Fermentas) was used to determine the molecular mass of bands visualized on the gel using the ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system.

3.2.3.2 16S rRNA gene amplification

The bacterial 16S rRNA gene was selectively amplified from the purified genomic DNA by PCR using the primers listed in Table 3.1 and consisted of the components indicated in Table 3.2 below.

Table 3.1: Primers used for 16S rRNA gene amplification.

Primer	Sequence	Reference
27F	5' - AGA GTT TGA TCM TGG CTC AG - 3'	(Lane, 1991)
1492R	5' - GGT TAC CTT GTT ACG ACT T - 3'	(Lane, 1991)

Table 3.2: Composition of 16S rRNA gene PCR.

Component	Amount (µL) for 50 µL reaction
Primer 27F (10 µM)	1
Primer 1492 (10 µM)	1
DreamTaq Green PCR Master Mix (2x)*	25
Template	Final concentration of 1 ng
Distilled H ₂ O	Up to 50 µL

*Thermo Scientific™ DreamTaq™ Green PCR Master Mix (2X) is a ready-to-use solution containing DreamTaq DNA polymerase, DreamTaq Green buffer, MgCl₂ and dNTPs.

Amplifications were run for 30 cycles in a thermal cycler PXE 0.2 (Thermo Electron corporation) after an initial denaturation at 95°C for 5 min. Each cycle was run at 95°C for 30 sec, 49°C for 45 sec and 72°C for 90 sec and final extension at 72°C for 10 min.

The DreamTaq™ Green PCR Master Mix is supplemented with two tracking dyes and a density gradient reagent that allows for direct loading of the PCR product on an agarose gel.

The dyes in the master mix do not interfere with PCR performance and are compatible with downstream applications. The agarose gel was prepared and visualization performed as described above in section 3.2.3.1.

3.2.3.3 Denaturing gradient gel electrophoresis (DGGE)

A +/-600 bp fragment of the 16S rRNA gene between position 341 and 907 of the 16S rRNA gene sequence was amplified as described by Heuer and co-workers (1997) using the primers listed in Table 3.3. Each reaction contained the components described in Table 3.4 below.

Table 3.3: Primers used for 16S rRNA gene fragment amplification.

Primer	Sequence	Reference
341F-GC Clamp	5'- CGC CCG CCG CGC GCG GCG GGC GGG -3'	(Muyzer <i>et al.</i> , 1993)
907R	5'- CCG TCA ATT CMT TTR AGT TT -3'	(Muyzer <i>et al.</i> , 1998)

Table 3.4: Composition of 16S rRNA gene fragment PCR.

Component	Amount (μL)
Primer 341F-GC (10 μM)	1
Primer 907R (10 μM)	1
Phusion Hot Start II DNA Polymerase (2 U/ μL)*	0.3
dNTPs	1
Buffer (5X)	10
Template	Final concentration of 1 ng
Distilled H ₂ O	Up to 50 μL

*Thermo Scientific

Amplifications were run for 30 cycles in a thermal cycler PXE 0.2 (Thermo Electron corporation) after an initial denaturation at 98°C for 1 min. Each cycle was run at 98°C for 10 sec, 55°C for 30 sec and 72°C for 20 sec and final extension at 72°C for 10 min. Amplicons were checked on a 1% agarose gel (w/v). The agarose gel was prepared and visualization performed as described above in section 3.2.3.1.

DGGE analysis was performed as described by Heuer and co-workers (1997), a linear denaturing gradient of 40 to 60% denaturant (100% denaturant consisted of 7M urea and 40% formamide) was used in a 7% (v/v) polyacrylamide gel. The DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) was used. Equal DNA amounts of the PCR products were loaded on the DGGE. Electrophoresis was performed in

1xTAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) at a constant voltage of 100V and a temperature of 60°C for 16h. After electrophoresis the gels were stained with ethidium bromide (10 µg/mL).

3.2.3.4 Cloning of plasmids containing the 16S rRNA gene fragments

The observed bands from the DGGE gel were excised and incubated in 50 µL distilled H₂O at 4°C overnight for elution of the DNA from the gel. Subsequently the eluted DNA was used as PCR template for re-amplification followed by sequence analysis. PCR amplification was performed as described in section 3.2.3.3, with the exception that the forward primer did not include the GC-clamp and distilled H₂O containing the eluted DNA was used to complete the reaction volume (50 µL) in addition to the volumes of the primers, dNTPs, buffer and polymerase.

After amplification, the PCR product was loaded onto a 0.8% (w/v) agarose gel for fractionation by electrophoresis, as described in section 3.2.3.1. Selected bands were excised from the gel using the Biospin Gel Extraction kit (Bioflux) according to the manufacturer's specifications. DNA concentrations were determined on the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific).

Purified amplicons were dried in the SpeedyVac Concentrator (Eppendorf). The phosphorylation reaction (Table 3.5) was added to the dried amplicons and incubated at 37°C for 30 minutes, followed by 1 hour at 85°C to inactivate the enzyme.

Table 3.5: Composition of phosphorylation reaction.

Component	Volume (µL)
T4 Polynucleotide Kinase Reaction Buffer*	2
ATP (10 mM)*	2
T4 Polynucleotide Kinase (10 U/ µL)*	1
ddH ₂ O	15
Total	20

*New England Biolabs, Inc.

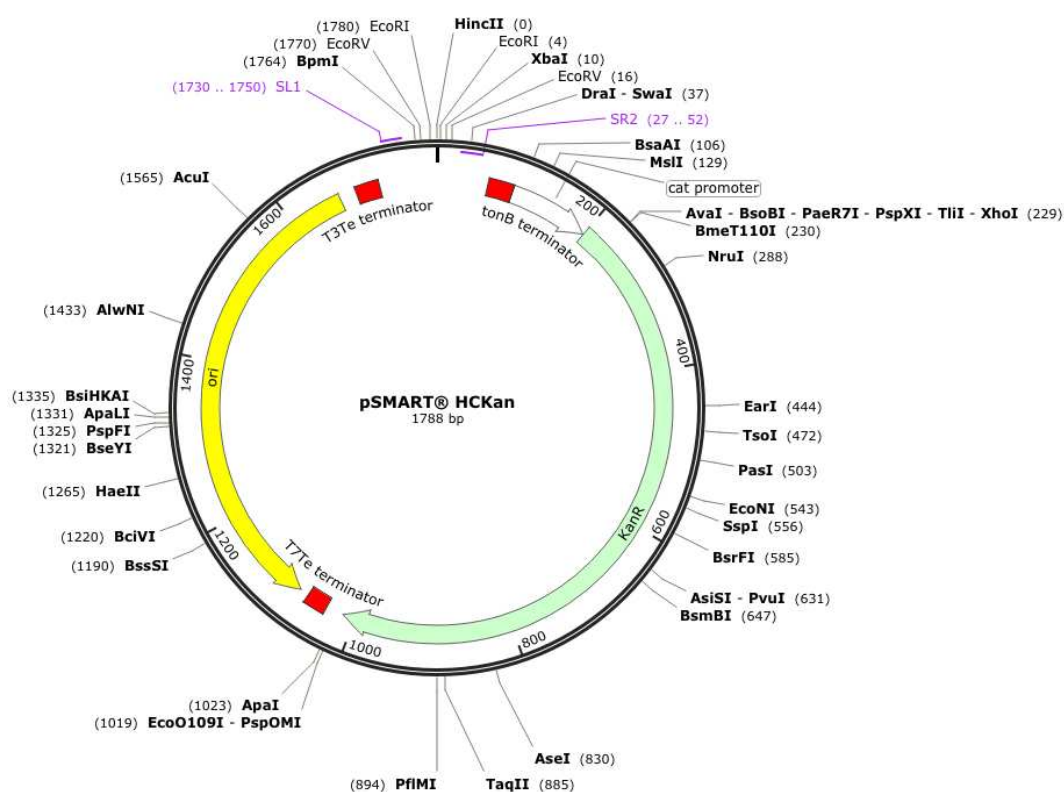


Figure 3.4: Schematic diagram of the pSMART® HCKan vector system. Ori, origin of replication; Kan, Kanamycin resistance gene.

The phosphorylated amplicons were ligated into the pSMART® HCKan vector system (Figure 3.4). The ligation reaction was assembled as described in table 3.6 below, incubated at 22°C for 1 h followed by 4°C for 8 h.

Table 3.6. Ligation mixture composition for the pSMART® HCKan vector system.

Component	Sample Volume (µL)
10X Rapid ligation buffer*	1
pSMART® HCKan**	1
Phosphorylated template*	7
T4 DNA ligase (10 U/ µL)	1
Total	10

*New England Biolabs, Inc.

**Lucigen

E. coli Top10 competent cells (50 µl), prepared using rubidium chloride, were thawed on ice and 5 µl of the ligation mixture added. This was incubated on ice for 30 min followed by a heat shock step at 42°C for 40 sec and immediate cooling on ice for 2 min, after which 700 µl of SOC medium [LB medium (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl)]

supplemented with 50 μ l 2 M magnesium and 100 μ l 1 M glucose solution] was added. The culture was incubated at 37°C for 1 h in a shaker rotating at 175 rpm. The transformation mix (50 μ l) was plated out on LB plates supplemented with 30 μ g/mL kanamycin (final concentration) to provide the adequate antibiotic pressure. The plates were incubated at 37°C for 16 h followed by selection of colonies. Colonies were inoculated into 5 mL LB medium supplemented with 30 μ g/mL kanamycin (final concentration) 37°C for 16 h while shaking at 175 rpm after which the cells were pelleted by centrifugation (6000 x g for 10 min, 4°C). Plasmid DNA was isolated with the Biospin Plasmid DNA Extraction Kit (Bioflux) according to the manufacturer's instructions. Plasmid DNA concentrations were determined on the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). Extracted plasmids were subjected to restriction fragment length polymorphism (RFLP) analysis as shown in table 3.7 with data verification using standard gel electrophoresis techniques, as described in section 3.2.3.1. Restriction digests were performed at 37°C for 3 hours.

Table 3.7. Restriction digests reaction composition.

Component	Volume (μL)
Plasmid DNA	5
Buffer (10X)	1
Distilled H ₂ O	2
<i>Eco</i> RI (5000 U/mL)	1
Total	10

3.2.3.5 Sequencing of 16S rRNA gene fragments

Inserts were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies, Inc.) according to the manufacturer's specification. The PCR reaction contained the components listed in table 3.8 below. Each reaction contained either a forward or a reverse primer, SL1 and SR2 respectively (Lucigen) on the pSMART® HCKan vector. Primer sequences are in table 3.9 below.

After completion of the PCR, primers and unincorporated dye-labelled nucleotides were removed following the EDTA/Ethanol precipitation protocol as recommended by the manufacturer.

Table 3.8. Sequencing PCR reaction composition.

Component	Sample	Control
Plasmid DNA Template	200-500 ng	-
Control Plasmid [pGEM-3Zf(+)]	-	2 µL
Premix	0.5	0.5
Primer (3.2 pmol/µL)	1 µL	-
Control Sequencing Primer	-	4 µL
Dilution buffer (2X)	2	2
Distilled H ₂ O	Up to 10 µL	1.5
Total	10	10

Table 3.9. Primers sequences used during sequencing PCR reaction.

Primer Name	Sequence
SL1	5'- CAG TCC AGT TAC GCT GGA GTC-3'
SR2	5'- GGT CAG GTA TGA TTT AAA TGG TCA GT-3'

Briefly, the sequencing reaction volume was adjusted to 20 µL and transferred to a 1.5 mL Eppendorf tube containing 5 µL 125 mM EDTA and 60 µL absolute ethanol and vortexed. The reaction was left to precipitate at room temperature for 15 min, centrifuged at 4°C (20 000 x *g*, 10 min) after which the supernatant was completely aspirated. To the tubes, 60 µL ethanol (70%) was added followed by centrifugation at 4°C (20 000 x *g*, 5 min). The supernatant was once again aspirated and the tubes dried in the SpeedyVac (Eppendorf) for 5 min. Samples were stored at 4°C until they were sequenced. Sanger sequencing was performed on a 3130x/ Genetic Analyser, HITACHI (Applied Biosystems).

3.2.3.6 Phylogenetic analysis

For each 16S rRNA gene sequence, the most closely related sequence was retrieved from the GenBank database by using BLAST and from the Ribosomal Database Project-II (RDP) by using the SEQUENCE MATCH tool. The sequences were aligned and the phylogenetic trees were calculated with the neighbour-joining method using tools on the RDP website (<http://rdp.cme.msu.edu/>) (Cole *et al.*, 2014). Bootstrap values were determined from 100 iterations.

3.2.4 Functional gene screening

A series of oligonucleotide primer pairs (Table 3.10) were utilized to screen for the presence of *assA* and *bssA* genes. These genes have been identified in known hydrocarbon degraders for the degradation of some aromatic and non-aromatic hydrocarbons (Callaghan *et al.*, 2010).

All samples were evaluated with each of the nine primer sets in 50 µL reactions containing 5-50 ng of DNA template. PCR conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 2 min followed by a final extension step at 72°C for 10 min.

Bands that presented with amplicons of the expected size were excised, as described in section 3.2.3.4, re-amplified and sequenced as described in section 3.2.3.5.

Table 3.10: Oligonucleotide primer set combinations and sequences targeting *assA* and *bssA* genes.

Primer set:	Forward and Reverse sequences	Amplicon size (bp)	Reference
1. (targets <i>assA</i> and <i>bssA</i>)	<i>ass/bssF</i> : 5'-TTTGAGTGCATCCGCCAYGGICT-3' <i>ass/bssR</i> : 5'-TCGTCRRTTGCCCCATTTIGGIGC-3'	<i>assA</i> : 661 <i>bssA</i> : 682	(Callaghan <i>et al.</i> , 2009)
2. (targets <i>bssA</i>)	7772F: 5'-GAC ATG ACC GAC GCS ATY CT-3' 8546R: 5'-TCG TCG TCR TTG CCC CAY TT-3'	793	(Winderl, 2007)
3. (targets <i>assA</i>)	1294F: 5'-TTSGARTGCATCCGNCACGGN-3' 1936R: 5'-TCRTCATTNCCCCAYTTNGG-3'	661	(Callaghan <i>et al.</i> , 2009)
4. (targets <i>assA</i>)	1294F: 5'-TTSGARTGCATCCGNCACGGN-3' 2457R: 5'-TTGTCCTGNGTYTTGCGG-3'	1180	(Callaghan <i>et al.</i> , 2009)
5. (targets <i>assA</i>)	1294dF: 5'-TTYGAGTGYATNCGCCASGGC-3' 1936R: 5'-TCRTCATTNCCCCAYTTNGG-3'	661	(Callaghan <i>et al.</i> , 2009)
6. (targets <i>assA</i>)	1294dF: 5'-TTYGAGTGYATNCGCCASGGC-3' 2457R: 5'-TTGTCCTGNGTYTTGCGG-3'	1180	(Callaghan <i>et al.</i> , 2009)
7. (targets <i>assA</i>)	1432F: 5'-CCNACCACNAAGCAYGG-3' 1936R: 5'-TCRTCATTNCCCCAYTTNGG-3'	523	(Callaghan <i>et al.</i> , 2009)
8. (targets <i>assA</i>)	1432F: 5'-CCNACCACNAAGCAYGG-3' 2457R: 5'-TTGTCCTGNGTYTTGCGG-3'	1042	(Callaghan <i>et al.</i> , 2009)
9. (targets <i>assA</i>)	1432F: 5'-CCNACCACNAAGCAYGG-3' <i>ass/bssR</i> : 5'-TCGTCRRTTGCCCCATTTIGGIGC-3'	523	(Callaghan <i>et al.</i> , 2009)

3.2.5 Enrichment media

The following media was used for initial enrichment of possible anaerobic hydrocarbon degrading microorganisms. All components of anaerobic versions of the medias were prepared in separate serum vials, sealed with a combination of Teflon-faced chlorobutyl stoppers and aluminium crimps (Bellco Glass, Vineland, N.J., U.S.) and purged with O₂-free N₂ (Air Liquide, Alrode, S.A.) to establish anaerobic conditions. Following purging, the indicated components were sterilized by autoclaving (120°C, 20 min) and allowed to be cooled to room temperature before being transferred into an anaerobic glove box (Coy Laboratories, Grass Lake, M.I., U.S.) with a 10% CO₂ / 10% H₂ / 80% N₂ headspace (Air Liquid, Alrode, S.A.) along with all other media components. Inside the anaerobic glove box the non-autoclaveable components were added by filter sterilization (0.22 µm, GVS Filter Technology, Indianapolis, I.N., U.S.). Filter sterilized diesel (1% v/v final concentration) was added as carbon source to all media (Palanisamy *et al.*, 2014).

3.2.5.1 Mineral salts-BTEX medium

The media was prepared according to the specifications of Taylor and Chen (1997). All components (Table 3.11) were prepared in separate serum vials. Following purging the serum vials containing only component one was autoclaved. Inside the anaerobic glove box components 2-8 were added by filter sterilization.

3.2.5.2 Bushnell Haas Broth

The media prepared according to the specifications of Bushnell and Haas (1941). All components (Table 3.12) were prepared in serum vials. Following purging the serum vials were autoclaved. Inside the anaerobic glove box hydrocarbons were added by filter sterilization (0.22 µm, GVS Filter Technology, Indianapolis, I.N., U.S.).

Bushnell Haas broth was also used for aerobic enrichments. The media was prepared exactly as described above with the exception that it was not purged with O₂-free N₂.

Table 3.11: Components of the mineral salts-BTEX medium (per litre) (Taylor and Chen, 1997).

Component	Concentration (per litre)	Component	Concentration (per litre)
1. Mineral salts medium		4. Vitamin solution (1.0 mL)	
Distilled water	993 mL	Distilled water	1000 mL
Na ₂ SO ₄	1.4 g	Biotin	0.01 g
NaCl	1.2 g	<i>p</i> -Aminobenzoic acid	0.05 g
KCl	0.3 g	Vitamin B12	0.05 g
NH ₄ Cl	0.3 g	Thiamine	0.1 g
MgCl ₂ .6H ₂ O	0.4 g	5. Resazurin solution (0.1%) (1.0 mL)	
KH ₂ PO ₄	0.2 g	6. BTEX compounds (1%)	
CaCl ₂ .6H ₂ O	0.15 g	7. Dithionite (Na ₂ S ₂ O ₄) solution (3%) (1.0 mL)	
NaNO ₃	0.35 g	8. Sulphide (Na ₂ S.9H ₂ O) solution (12%) (2.0 mL)	
(adjust pH to 7.5)		9. pH	7.0
2. Trace element solution (1.0 mL)			
Distilled water	993 mL		
HCl (25%)	6.5 mL		
FeCl ₂ .4H ₂ O	1.5 g		
H ₃ BO ₃	0.06 g		
MnCl ₂ .4H ₂ O	0.1 g		
CoCl ₂ .4H ₂ O	0.12 g		
ZnCl ₂	0.07 g		
NiCl ₂ .6H ₂ O	0.025 g		
CuCl ₂ .2H ₂ O	0.015 g		
Na ₂ MoO ₄ .2H ₂ O	0.025 g		
3. Selenite solution (1000 mL)			
Distilled water	1000 mL		
NaOH	0.5 g		
Na ₂ SeO ₃	0.3 g		

Table 3.12: Components of the Bushnell Haas Broth (per litre) (Bushnell and Haas, 1941).

Component	Concentration (per litre)
MgSO ₄	0.2
CaCl ₂	0.02
KH ₂ PO ₄	1.0
K ₂ HPO ₄	1.0
NH ₄ NO ₃	1.0
FeCl ₃	0.05
Resazurin	0.0001
pH	7.0

3.2.5.3 Mineral salts medium (MSM)

An adapted version of the mineral salts medium was prepared according to the specifications of Mittal and Rockne (2008). All components (Table 3.13) were prepared in separate serum vials. Following purging the serum vials containing all components, with the exception of component seven (Table 3.13) were combined and the medium autoclaved. Inside the anaerobic glove box component seven was added by filter sterilization.

3.2.5.4 Methanogenic medium

A medium designed to support methanogenic bacteria was prepared according to Edwards and Grbić-Galić (1994). All components (Table 3.14) were prepared in separate serum vials. The amorphous ferrous sulphide solution had been washed three times with deionized water to remove free sulphide. Following purging the serum vials containing all components, with the exception of components six, seven and eight (Table 3.14) were combined and the medium autoclaved (120°C, 20 min). Inside the anaerobic glove box components six, seven and eight were added by filter sterilization.

3.2.5.5 Sulphate reducing media

A medium designed to support sulphate reducing bacteria was prepared according to Widdel and Pfennig (1981). All components (Table 3.15) were prepared in separate serum vials. Following purging the serum vials containing all components, with the exception of component three, four and five (Table 3.15) were combined and the medium autoclaved. Inside the anaerobic glove box components three, four and five were added by filter sterilization.

3.2.5.6 Nitrate reducing media

A medium designed to support nitrate reducing bacteria was prepared according to Dou and co-workers (2009). All components (Table 3.16) were prepared in separate serum vials. Following purging the serum vials containing, vials containing component one (Table 3.16) of the medium was autoclaved. Inside the anaerobic glove box the other components were added by filter sterilization.

Table 3.13: Components of the mineral salts medium (per litre) (Mittal and Rockne, 2008).

Component	Concentration (per litre)	Component	Concentration (per litre)
1. Mineral salts medium		5. Reductant solution	
Distilled water	970 mL	(2.5 mL)	
Na ₂ SO ₄	3.97 g	Na ₂ S.9H ₂ O	4.0 g
NaCl	22.8 g	Cysteine-HCl	4.0 g
KCl	0.72 g	6. Resazurin solution	
NH ₄ Cl	0.25 g	(0.1%) (1.0 mL)	
NaHCO ₃	0.19 g	7. Wolfe's vitamin	
KBr	0.08 g	solution (0.01 mL)	
H ₃ BO ₃	0.026 g	Pyridoxine	
NaNO ₃	0.41 g	hydrochloride	0.01 g
NaF	0.01 g	Thiamine-HCl	0.005 g
CH ₃ COONa	0.082 g	Riboflavin	0.005 g
(adjust pH to 7.5)		Nicotinic acid	0.005 g
2. Trace metal solution		Calcium D-(+)-	
(0.5 mL)		pantothenate	0.005 g
Distilled water	1000 mL	<i>p</i> -Aminobenzoic acid	0.005 g
FeCl ₂ .4H ₂ O	0.2 g	Thioctic acid	0.005 g
H ₃ BO ₃	0.1 g	Biotin	0.002 g
MnCl ₂ .4H ₂ O	1.0 g	Folic Acid	0.002 g
CoCl ₂ .4H ₂ O	1.0 g	Vitamin B12	0.001 g
ZnCl ₂	0.1 g	8. pH	7.0
NiCl ₂ .6H ₂ O	2.0 g		
CuCl ₂ .2H ₂ O	0.06 g		
Na ₂ MoO ₄ .2H ₂ O	0.2 g		
Na ₂ SeO ₃ .5H ₂ O	0.1 g		
Na ₂ WO ₄ .2H ₂ O	0.1 g		
3. Phosphate solution			
(10.0 mL)			
Distilled water	1000 mL		
Na ₂ HPO ₄ .7H ₂ O	8.89g		
4. Divalent cation solution			
(20.0 mL)			
Distilled water	1000 mL		
MgCl ₂ .6H ₂ O	559.0 g		
CaCl ₂ .2H ₂ O	72.3 g		
SrCl ₂ .6H ₂ O	1.2 g		

Table 3.14: Components of the methanogenic medium (per litre) (Edwards and Grbić-Galić, 1994).

Component	Concentration (per Litre)	Component	Concentration (per Litre)
1. Phosphate buffer (10 mL)		7. Vitamin stock solution	
Distilled H ₂ O	1000 mL	(10mL)	
KH ₂ PO ₄	27.2 g	Distilled H ₂ O	1000 mL
K ₂ HPO ₄	34.8 g	Biotin	0.02 g
2. Salt solution (10 mL)		Folic Acid	0.02 g
Distilled H ₂ O	1000 mL	Pyridoxine hydrochlorid	0.1 g
NH ₄ Cl	53.5 g	Riboflavin	0.05 g
CaCl ₂ .6H ₂ O	7.0 g	Thiamine	0.05 g
FeCl ₂ .4H ₂ O	2.0 g	Nicotinic acid	0.05 g
3. Trace mineral solution (2.0 mL)		Pantothenic Acid	0.05 g
Distilled H ₂ O	1000 mL	<i>p</i> -aminobenzoic acid	0.05 g
H ₃ BO ₃	0.3 g	Cyanocobalamin	0.05 g
ZnCl ₂	0.1 g	Thioctic acid	0.05 g
NiCl ₂ .6H ₂ O	0.75 g	Mercaptothanesulfonic	
MnCl ₂ .4H ₂ O	1.0 g	acid	1 g
CuCl ₂ .2H ₂ O	0.1 g	8. Amorphous ferrous	
CoCl ₂ .6H ₂ O	1.5 g	sulphide solution	
Na ₂ SeO ₃	0.02 g	(10 mL)	
Al ₂ (SO ₄) ₃ .16H ₂ O	0.1 g	Distilled H ₂ O	1000 mL
H ₂ SO ₄	1 mL	(NH ₄) ₂ Fe(SO ₄) ₂ .6H ₂ O	39.2 g
4. Magnesium sulphate solution		Na ₂ S.9H ₂ O	24.0 g
(2 mL)		9. pH	6.8–7.0
Distilled H ₂ O	1000 mL		
MgSO ₄ .7H ₂ O	62.5 g		
5. Redox indicator solution (1 mL)			
Distilled H ₂ O	1000 mL		
Resazurin	1 g		
6. Saturated bicarbonate solution			
(10 mL)			
Distilled H ₂ O	1000 mL		
NaHCO ₃	260 g		

Table 3.15: Components of the sulphate reducing medium (per litre) (Widdel and Pfennig, 1981).

Component	Concentration (per Litre)	Component	Concentration (per Litre)
1. Basal medium component:		5. Vitamin solution (3 mL)	
Distilled H ₂ O	1000 mL	Distilled H ₂ O	1000 mL
Na ₂ SO ₄	3.0 g	Biotin	0.01 g
KH ₂ PO ₄	0.2 g	<i>p</i> -aminobenzoic acid	0.01 g
NH ₄ Cl	0.3 g	Pantothenate	0.01 g
MgCl ₂ .2H ₂ O	0.4 g	Pyridoxamine	0.02 g
NaCl	1.0 g	Nicotinic acid	0.02 g
KCl	0.5 g	Thiamine	0.02 g
CaCl ₂ .2H ₂ O	0.15 g	Vitamin B12 (50 mg/L)	5 mL
2. Trace element solution (1 mL)		6. Redox indicator solution (1 mL)	
Distilled H ₂ O	990 mL	Distilled H ₂ O	1000 mL
HCl (25%)	10 mL	Resazurin	1 g
FeCl ₂ .4H ₂ O	1.5 g	7. pH	7.1– 7.3
CoCl ₂ .6H ₂ O	0.19 g		
MnCl ₂ .4H ₂ O	0.1 g		
ZnCl ₂	0.07 g		
H ₃ BO ₃	0.062 g		
Na ₂ MoO ₄ .2H ₂ O	0.036 g		
NiCl ₂ .6H ₂ O	0.024 g		
CuCl ₂ .2H ₂ O	0.017 g		
3. Bicarbonate solution (1 mL)			
Distilled H ₂ O	1000 mL		
NaHCO ₃	84 g		
4. Sulphide solution (30 mL)			
Distilled H ₂ O	1000 mL		
Na ₂ S.9H ₂ O	30 g		

3.2.6 Enrichment procedure

Inside the anaerobic glove box, 50 mL of each of the liquid medias described in section 3.2.5 was dispensed in prepared 120 mL serum vials and inoculated with soil (5 g) from each of the different sampling sites. The flasks were once again sealed with a combination of Teflon-faced chlorobutyl stoppers and aluminium crimps (Bellco Glass, Vineland, N.J., U.S.). Controls for the enrichment of each medium and soil sample consisted of one flask that did not contain any diesel as carbon source, a sterile control where one flask was autoclaved (120°C, 20 min) twice to determine if the diesel would be absorbed on the sediment/sludge and an abiotic control where soil was omitted from the media (Palanisamy *et al.*, 2014).

Inoculated anaerobic serum vials as well as controls were statically incubated at 25°C, 37°C and 50°C in the dark. After three weeks of incubation the samples were re-inoculated (10% inoculum) into fresh anaerobic media containing 1% filter sterilized diesel. This process was repeated until soil and sediment free conditions were obtained within the vials.

Growth of sediment free cultures was monitored by measuring the optical density of each sample on a Spectronic® Genesys™ 5 at 600 nm.

Table 3.16: Components of the nitrate reducing medium (per litre) (Dou *et al.*, 2009).

Component	Concentration (per Litre)
1. Mineral salts	
Distilled H ₂ O	1000 mL
NaNO ₃	2.0 g
NH ₄ Cl	1.0 g
KH ₂ PO ₄	1.0 g
MgCl ₂	0.1 g
CaCl ₂ .2H ₂ O	0.05 mg
2. Sulphide solution (1.0 mL)	
Distilled H ₂ O	1000 mL
Na ₂ S.9H ₂ O	30 g
3. Vitamin solution (10 mL)	
Distilled H ₂ O	1000 mL
Biotin	0.02 g
Folic acid	0.02 g
Riboflavin	0.05 g
Thiamine	0.05 g
Nicotinic acid	0.05 g
Cyanocobalamin	0.001 g
<i>p</i> -aminobenzoic acid	0.05 g
Thiotic acid	0.05 g
4. Trace element solution (10 mL)	
Distilled H ₂ O	1000 mL
CoCl ₂ .6H ₂ O	0.03 g
CuCl ₂	0.15 mg
H ₃ BO ₃	0.0057 g
MnCl ₂ .4H ₂ O	0.02 g
Na ₂ MoO ₄ .2H ₂ O	0.0025 g
NiCl ₂ .2H ₂ O	0.0015 g
ZnCl ₂	0.0021 g
5. pH	6.8-7.2

3.2.6.1 Gram stain

Gram stains were performed on all samples using standard techniques which involved primary staining with crystal violet followed by iodine, subsequent washing of the slides with 95% ethanol, and finally, secondary staining with safranin (Gram, 1884).

3.2.6.2 Cell viability Stain

Cell viability was determined with the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies, Inc.) according to manufacturer's specifications.

3.2.6.3 Terminal electron acceptor reduction

Nitrate reduction was monitored as a production of nitrite with the Griess Reagent Kit for Nitrite Determination (Life Technologies, Inc.) according to the manufacturers' specification. Briefly, sulfanilic acid is quantitatively converted to diazonium salt by reaction with nitrite in an acid solution. The diazonium salt is then coupled to N-(1-naphthyl)ethylenediamine, forming an azo dye that can be spectrophotometrically quantified. Analysis was performed by mixing together equal volumes of N-(1-naphthyl)ethylenediamine and sulfanilic acid to form the Griess reagent. The Griess reagent (100 µL) was mixed with 300 µL of the nitrite-containing sample and 2.6 mL of deionized water. Samples were incubated at room temperature for 20 min and the absorbance was measured at 548 nm on a Spectronic® Genesys™ 5 (Thermo Scientific), against a reagent blank.

Sulphate concentrations were monitored according to the method described by Kolmert and co-workers (2000). The method is dependent on the precipitation of sulphate ions with barium chloride to form barium sulphate crystals of uniform size. Briefly, 1 mL of sample was mixed with 1 mL conditioning reagent (150 g NaCl, 100 mL glycerol, 60 mL concentrated HCl and 200 mL 95% ethanol per litre of distilled water). Approximately 60 mg barium chloride was added and the mixture vortexed (30 sec). Absorbance was measured at 420 nm against a reaction blank.

3.2.7 Enrichment culture hydrocarbon affinity

A modified version of the most-probable-number method for hydrocarbon degrading microbial populations used by Haines and co-workers (1996) was used to determine which hydrocarbons were utilized by the enrichment cultures.

Aerobic and anaerobic microbial enrichments were inoculated into Bushnell Haas broth (10 mL) (3.2.5.2) supplemented with 1 g/L of yeast extract and incubated at 25°C and 37°C for 24 hours for aerobic enrichments and 72 hours for anaerobic enrichments. After incubation cells were washed with Bushnell Haas broth and resuspended in 5 mL of Bushnell Haas broth. The experiment was carried out in 1.5 mL eppendorf tubes containing 300 µL of sterilized Bushnell Haas broth, 50 µL of harvested cells (OD₆₀₀ 0.5) and 7 µL of hydrocarbon (Boz *et al.*, 2015). Hydrocarbons tested include benzene (0.23 µM), toluene (0.19 µM), xylene (0.16 µM), naphthalene (0.18 µM), phenanthrene (0.13 µM), hexane (0.15 µM) and octane (0.12 µM). Naphthalene and phenanthrene was dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN). HMN was shown to not be a microbial growth substrate (Law and Aitken, 2003). Chemical controls did not contain any cells. Samples were incubated for 21 days at 25°C and 37°C respectively. At the end of the incubation period 50 µL *p*-iodonitrotetrazolium violet (INT) was added to each sample. Samples were incubated for 24 hours after addition of the indicator. A red precipitate indicated a positive result and growth was observed as increase in optical density measured at 600 nm on a Spectronic® Genesys™ 5 (Thermo Scientific).

3.2.8 Enriched consortium diversities

After obtaining sediment free cultures, enrichments were grown aerobically and anaerobically in Bushnell Haas broth liquid and solid media after which it was subjected to diversity studies once again. Diversity assessment was performed as described in section 3.2.3. Briefly, genomic DNA was extracted followed by 16S rRNA gene fragment amplification, DGGE, ligation into plasmids and finally sequencing of inserts.

Phylogenetic analysis was also performed using the RDP website as described in section 3.2.3.6.

3.3 Results and discussions

3.3.1 Site description and sampling

Samples were collected between July 2011 and January 2012. Soil samples were collected with sterilized spatulas in Whirl-Pak® Sampling Bags (Sigma-Aldrich) and the contaminated diesel sample in a sterile glass bottle. Samples were stored in the dark at 4°C until used. Samples were taken from the surface and 5 cm below surface since the top soil is generally subjected to additional conditions with regard to temperature fluctuations, water exposure, solar irradiation and continued hydrocarbon contamination possibly resulting in a greater impact on biota present (Martínez-Alonso *et al.*, 2010).

The Star Diamonds mine is situated in the Theunissen district, 40 km south of Welkom in the Free State province and has been in operation for more than 60 years. According to local sources the area where samples were taken has been used as a truck repair and wash bay area ever since the inception of the mine resulting in multiple years of hydrocarbon contamination.

Free State Groundworks (Vrystaat Grondwerke) was established in 1965 and specializes in the rental of vehicles and equipment for digging, earth moving, etc. As such they have many vehicles that need to be serviced and repaired, again resulting in multiple years of soil hydrocarbon contamination.

Earthmoving Repair Services specializes in the repair of earthmoving equipment and trucks. The origin of the contaminated diesel samples was a forklift, which had a clogged system. Prior to contacting our lab, a sample of the diesel was sent to the labs of BP Petroleum Inc. that confirmed the presence of microbial contamination in the diesel to the owners.

3.3.2 Initial sample diversity

Various investigation strategies exist for the determination of biodiversity in soil that involve environmental sampling and extraction of target molecules. To date the most commonly used methods for determining microbial classification is the sequencing of the 16S rRNA gene since it is a highly conserved marker, with a slow and constant mutation rate. As such the 16S rRNA gene can be used to measure taxonomic distance between species based on differences in the DNA sequence (Ding *et al.*, 2013).

The metabolic diversity of microorganisms in natural environments is an important factor in the biodegradation of hydrocarbons. As more complete degradation of hydrocarbon pollutants is generally accomplished by mixed microbial species, one of the objectives of this study was to isolate a novel hydrocarbon degrading consortium to determine the hydrocarbon biodegradation potential in standardized culture conditions.

Genomic DNA was extracted from sediment samples (Figure 3.5a, b). For the extraction of genomic DNA from the contaminated diesel sample, two strategies were followed. One was to attempt to extract genomic DNA directly from the contaminated diesel sample using the Powersoil® DNA Isolation Kit, this approach did not yield any results (Figure 3.5c). The second approach was to create an artificial water bottom in the diesel (an aqueous phase at the bottom of the diesel), the diesel sample was shaken vigorously and the phases were left to separate. The resulting aqueous phase was then used for genomic DNA extraction using the Powersoil® DNA Isolation Kit, this approach was more successful (Figure 3.5c).

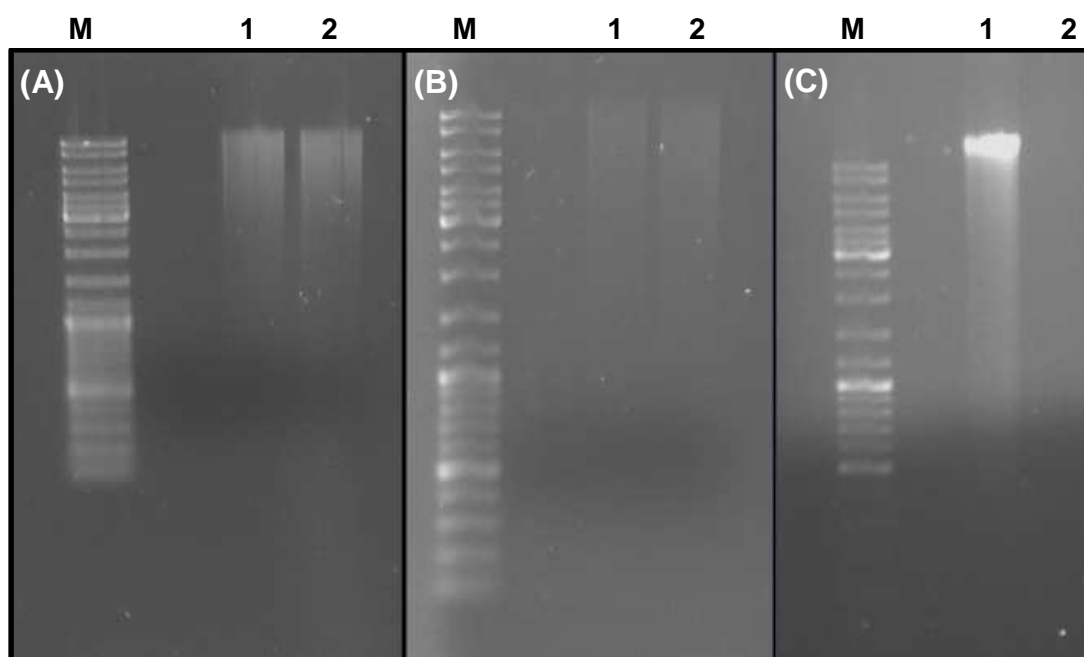


Figure 3.5: Genomic DNA extracted from collected samples. Lane M, MassRuler™ DNA ladder (Fermentas). (A) Free State Groundworks soil; Lane 1, surface sample; Lane 2, 5cm below surface. (B) Star Diamonds soil; Lane 1, surface samples; Lane 2, 5cm below surface. (C) Earthmoving Repair services diesel sample; Lane 1, false water bottom sample; Lane 2, diesel sample.

The presence of bacteria in the hydrocarbon contaminated soils, as well as in the contaminated diesel sample, were confirmed by PCR using universal primers to amplify the 16S rRNA genes (Figure 3.6a) as described in section 3.2.3.2. The presence of bacteria in

these samples is very encouraging as the contamination has been occurring for years, increasing the probability of the presence of bacteria capable of hydrocarbon degradation.

The utilization of molecular biological techniques to identify microorganisms in environmental samples have aided in overcoming some of the limitations of traditional cultivation techniques. However, the extensive 16S rRNA library and sequencing to diversity saturation approach exclusively can become very time consuming and labour intensive. As such, the bacterial community profile was studied by a fingerprinting techniques based on the 16S rRNA gene, in this case DGGE, to assess the diversity of the whole soil community at a molecular level. This technique is based on the separation of dsDNA during electrophoresis in an acrylamide gel containing a denaturing gradient. During electrophoresis the DNA will encounter an appropriate denaturant concentration resulting in a sequence-dependant partial separation of the double strands which leads to a reduced migration rate and a DNA band pattern representative of the sampled microbial community (Ding *et al.*, 2013).

Amplicons, a +/- 600 bp fragments of the 16S rRNA gene (V3/4 region), with the expected size (Figure 3.6b) were separated by DGGE and the results showed a clear difference in the bacterial populations within the three studied samples (Figure 3.7) indicating that, although the site conditions were similar, all hydrocarbon contaminated, that different populations were present.

The bands indicated in figure 3.7 were excised, DNA eluted and subjected to PCR amplification and sequencing. The generated DNA sequences were assembled by aligning the forward and reverse sequences in Geneious® Pro v 7.1.7 (Biomatters, Ltd, <http://www.geneious.com>). Well-known databases of 16S rRNA gene sequences were consulted for sequence similarities. The closest BLAST hits from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were tabulated (Table 3.17) and the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/html/>) was used to construct dendrograms (Figure 3.8, 3.9 and 3.10).

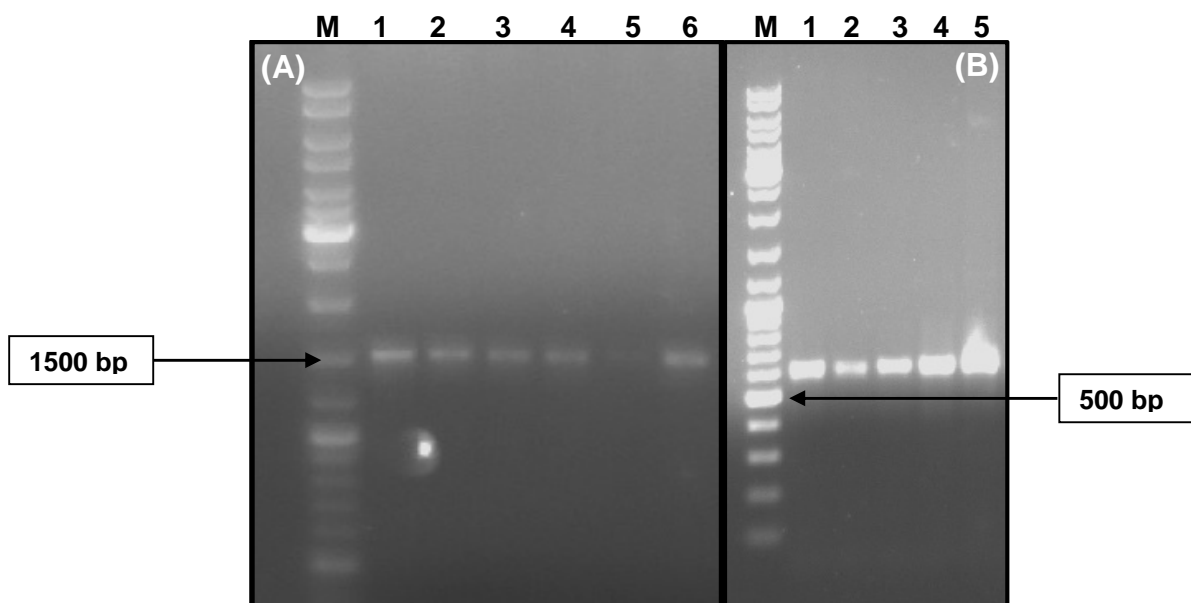


Figure 3.6: Amplification of 16S rRNA fragments using genomic DNA. Lane M, MassRuler™ DNA ladder (Fermentas). (A) Lane 1, Free State Groundworks soil surface sample; Lane 2, Free State Groundworks soil 5 cm below surface; Lane 3, Star Diamonds soil surface sample; Lane 4, Star Diamonds soil 5 cm below surface; Lane 5, Earthmoving Repair Services Diesel; Lane 6, Earthmoving Repair Services false water bottom sample. (B) Lane 1, Free State Groundworks soil surface sample; Lane 2, Free State Groundworks soil 5 cm below surface; Lane 3, Star Diamonds soil surface sample; Lane 4, Star Diamonds soil 5 cm below surface; Lane 5, Earthmoving Repair Services false water bottom sample.

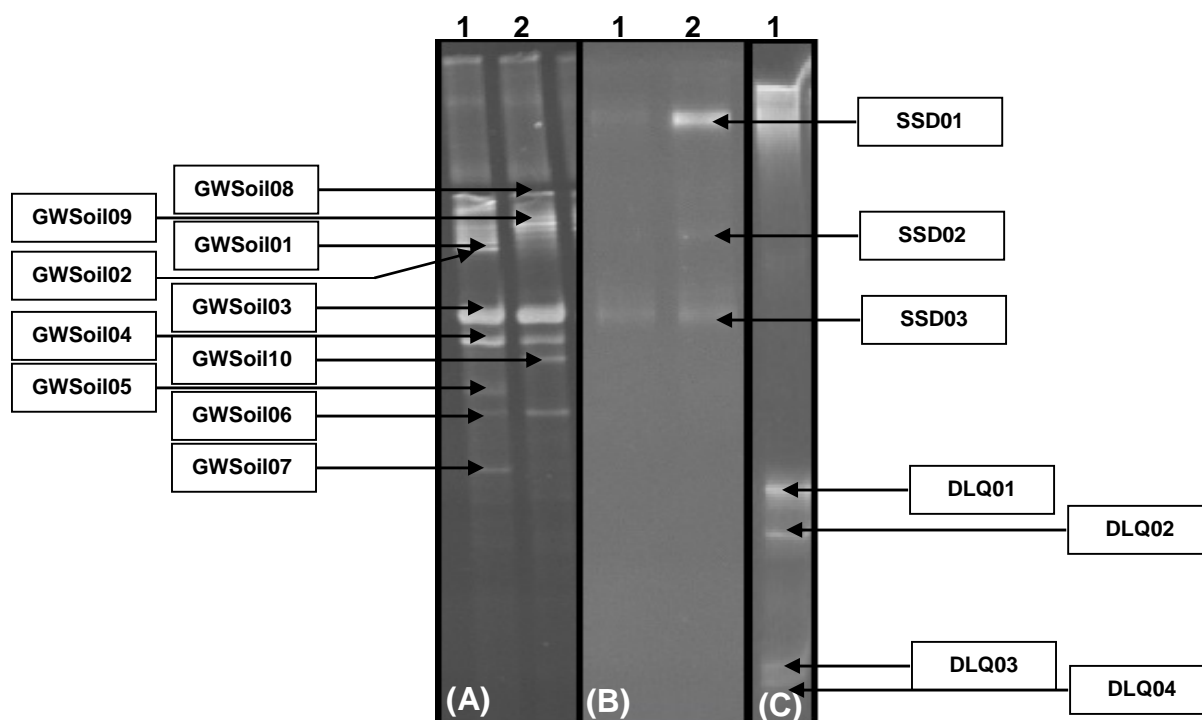


Figure 3.7: Initial sample diversity assessment by DGGE. (A) Free State Groundworks Soil; Lane 1, Surface soil; Lane 2, 5cm below surface. (B) Star Diamonds Soil; Lane 1, Surface soil; Lane 2, 5cm below surface. (C) Earthmoving Repair Service Diesel; Lane 1, False water bottom.

Phylogenetic studies of hydrocarbon contaminated environments can be a useful tool to speculate as to which species can possibly be involved in degradation activities and also for further development of strategies for selecting the best degrading consortium or even isolating the putative degraders (Chang *et al.*, 2005). As discussed in chapter 1, various bacterial species are known to degrade hydrocarbons and most of them are isolated from contaminated soil or sediments. Some of the most commonly studied bacteria found in contaminated soils and sediments include *Agrobacterium*, *Bacillus*, *Burkholderia* and *Sphingomonas*, *Rhodococcus*, *Mycobacterium*, *Pseudomonas* and *Flavobacterium* species (Ding *et al.*, 2013). Thus the presence of *Pseudomonas*, *Flavobacterium* and other γ -Proteobacteria in the sampled environments correlate to the potential biodegradation of hydrocarbons. When diversity fingerprints are established with DGGE profiling, the number of DGGE bands can be taken as a rough indication of the species present in each sample (Fromin *et al.*, 2002). As can be seen in figure 3.7, the Free State Groundworks soil sample displayed the richest microbial diversity when compared to the other two samples, dominated by the genera *Citrobacter* and *Pseudomonas*. The Star Diamonds soil and Earthmoving Repair Services diesel samples contained lower diversities consisting mainly of γ -Proteobacteria. Many of the species present in the different samples are capable of anaerobic and facultative anaerobic respiration and some have been previously reported as capable of anaerobic hydrocarbon degradation (Table 3.18).

Table 3.17: Closest GenBank reference obtained for DGGE band sequences from different sampling sites.

Consortium	Phylotype	Phylogenetic Affiliation	Closest Relative	Identity
Star Diamonds	SSD01	Deinococci	Uncultured bacterium clone SEV1DH051	97%
	SSD02	γ -Proteobacteria	Uncultured bacterium clone TFAgeg168	98%
	SSD03	γ -Proteobacteria	Uncultured <i>Syntrophorhabdus</i> sp., clone P39F	75%
Free State Groundworks	GW Soil01	Flavobacteria	<i>Epilithonimonas tenax</i> strain M0417	100%
	GW Soil02		Uncultured low G+C Gram-positive bacterium clone ML623J-26	79%
	GW Soil03		<i>Citrobacter</i> sp. enrichment culture clone M3C15	100%
	GW Soil04	γ -Proteobacteria	<i>Pseudomonas putida</i> strain MB10	89%
	GW Soil05	γ -Proteobacteria	<i>Pseudomonas</i> sp. C2SS10	87%
	GW Soil06	γ -Proteobacteria	<i>Pseudoxanthomonas</i> sp. BZ60	99%
	GW Soil07	γ -Proteobacteria	Uncultured <i>Chromatiaceae</i> bacterium	84%
	GW Soil08		Uncultured epsilon proteobacterium clone S1-65	96%
	GW Soil09		Uncultured bacterium isolate DGGE gel band TS07	94%
	GW Soil10		Uncultured bacterium isolate DGGE gel band OTB26	88%
Earthmoving Repair Diesel	DLQ01		Uncultured bacterium isolate DGGE gel band B15	96%
	DLQ02	β -Proteobacteria	Uncultured <i>Delftia</i> sp. clone 6TA04	99%
	DLQ03	γ -Proteobacteria	<i>Stenotrophomonas</i> sp. DB-17	99%
	DLQ04	γ -Proteobacteria	<i>Stenotrophomonas</i> sp. I28-J6NNFC6A2	100%

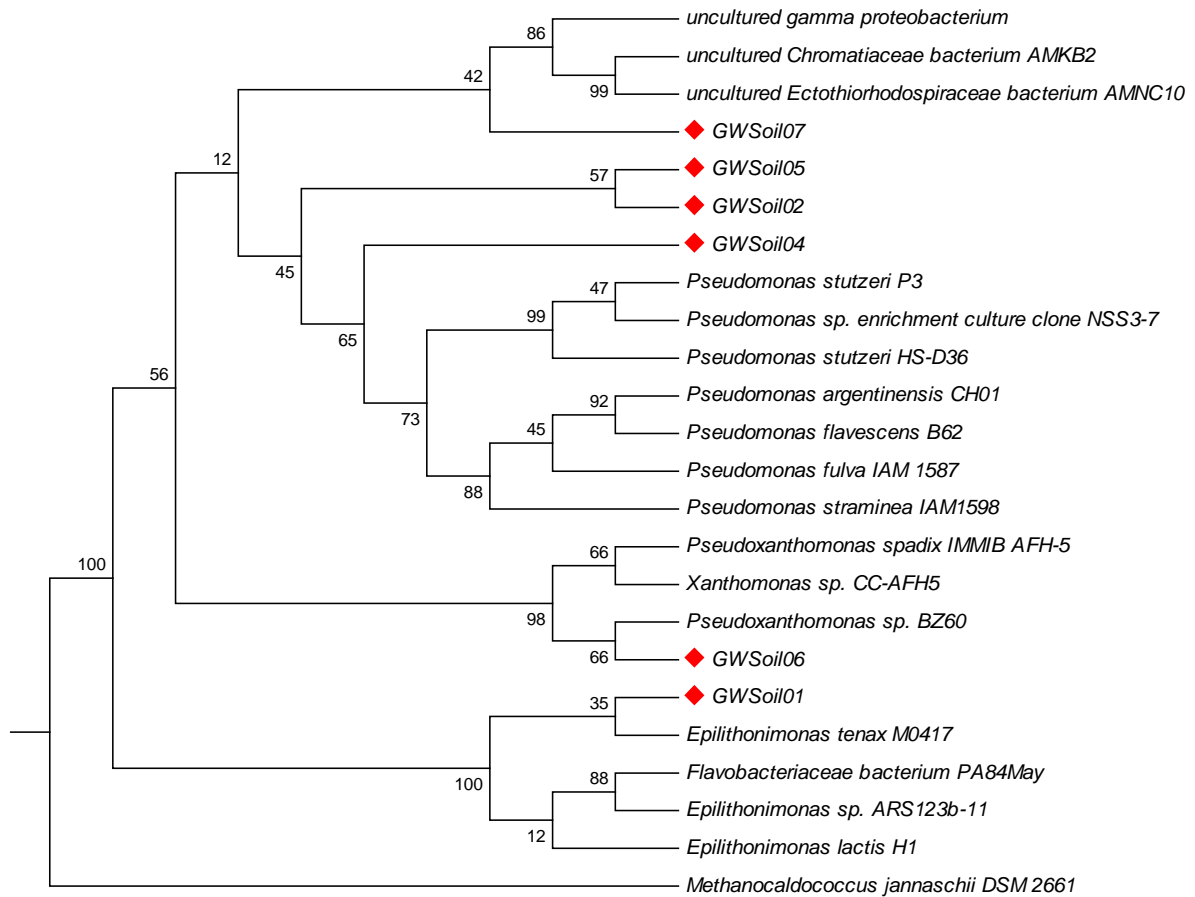


Figure 3.8: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Free State Groundworks Soil sample and reference sequences from the Ribosomal Database Project (RDP).

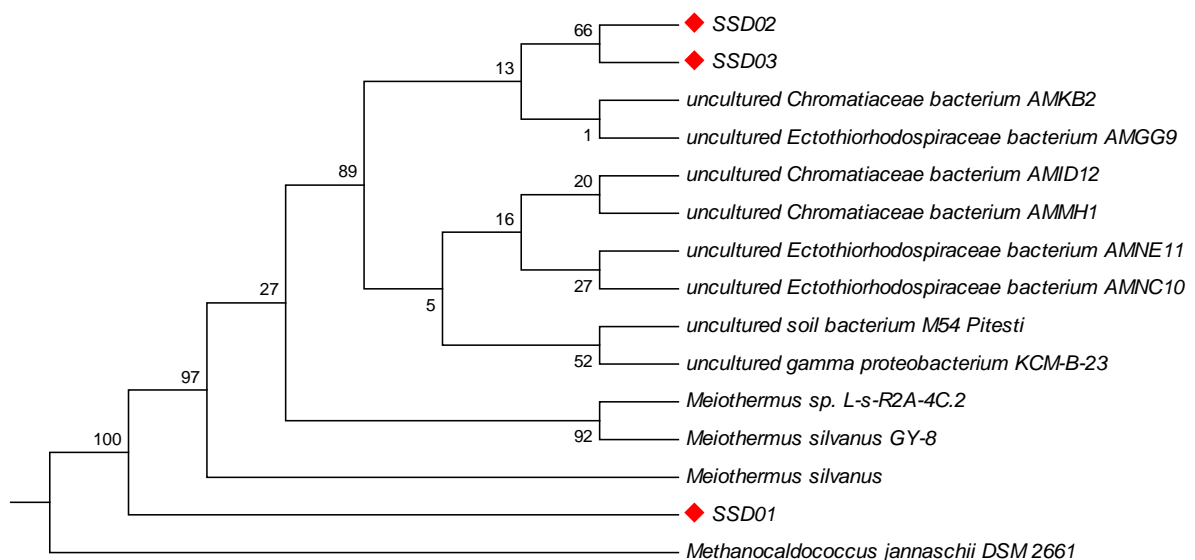


Figure 3.9: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Star Diamonds Soil sample and reference sequences from the Ribosomal Database Project (RDP).

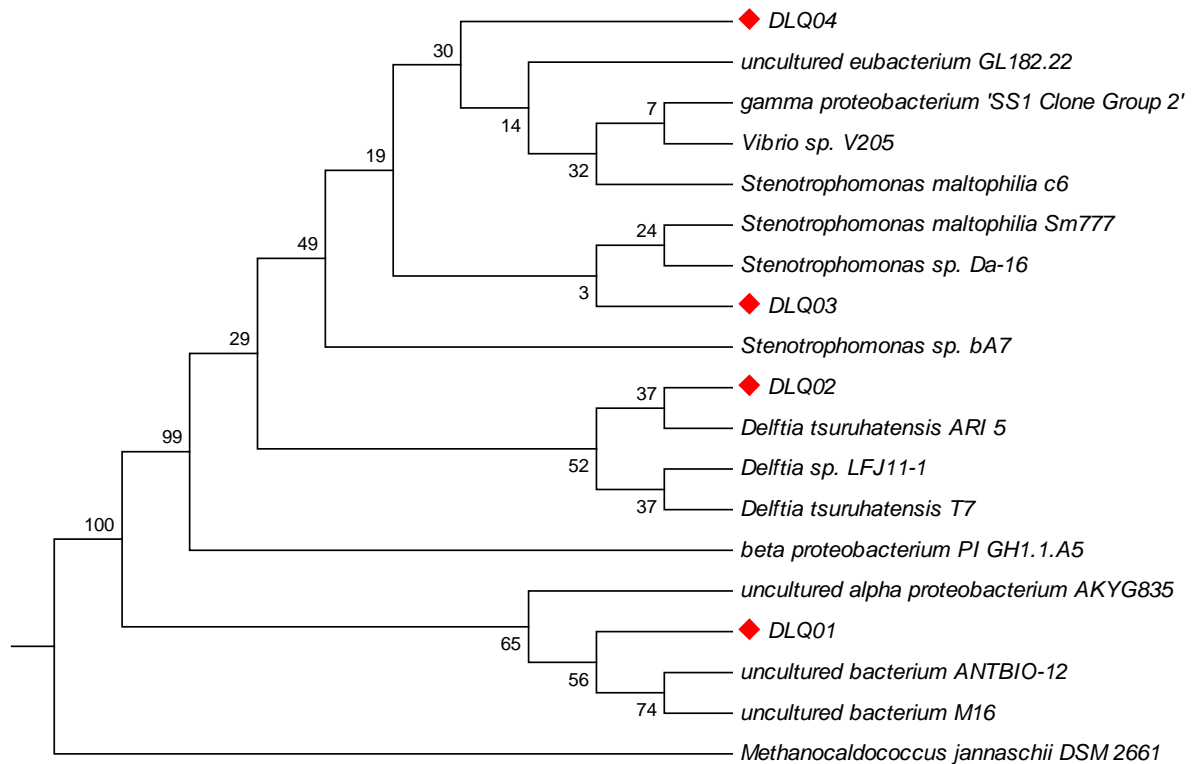


Figure 3.10: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Earthmoving Repair Service false water bottom sample and reference sequences from the Ribosomal Database Project (RDP).

Table 3.18: Species present in environmental samples previously associated with anaerobic growth coupled to hydrocarbon degradation.

Specie	Identified anaerobic species	Anaerobic Hydrocarbon degraded
<i>Syntrophorhabdus</i>	Yes	Phenol (Shakéd <i>et al.</i> , 2010)
<i>Citrobacter</i>	Facultative	Biphenyl (Grishchenkov <i>et al.</i> , 2002) Phenanthrene (Zhang <i>et al.</i> , 2012)
<i>Pseudomonas</i>	Facultative	Benzo[a]pyrene, fluoranthene, and phenanthrene (Liang <i>et al.</i> , 2014)
<i>Pseudoxanthomonas</i>	Facultative	Dinitrotoluene (Cheng <i>et al.</i> , 2007)
<i>Stenotrophomonas</i>	Facultative	Isolated from hydrocarbon contaminated sludge (Assih <i>et al.</i> , 2002)

3.3.3 Functional gene screening

Petroleum hydrocarbon degradation pathways are distinct and it is believed that n-alkane-utilizing strains will not grow on aromatic hydrocarbons, and vice-versa (Rabus *et al.*, 2011). Currently there are two elucidated mechanisms for anaerobic degradation of alkanes. One involves the addition of fumarate to the subterminal carbon of the alkane to produce an alkylsuccinate compound. The enzymes involved in this process are alkylsuccinate synthases, AssA1 and AssA2, encoded by *assA1* and *assA2* genes respectively (Callaghan *et al.*, 2008; Callaghan *et al.*, 2010). In the other process the alkane is carboxylated (Grossi *et al.*, 2008). Aromatic hydrocarbons on the other hand can be converted into a few central intermediates before being further metabolized, benzoyl-CoA being the most common central intermediate of anaerobic aromatic hydrocarbon degradation (Fuchs, 2008). A good target for the detection of anaerobic hydrocarbon degrading microorganisms is the enzyme benzylsuccinate synthase (Bss). This enzyme is involved in the anaerobic degradation of toluene and xylene and also involves the addition of fumarate. Benzylsuccinate synthase will add fumarate to the methyl group of the aromatic compound transforming it into benzylsuccinates. To date Bss has been identified in all anaerobic toluene degrading microorganisms and the highly conserved α subunit, encoded by the *bssA* gene, has been employed as a molecular marker for the characterization of environmental samples (Beller *et al.*, 2002; Winderl, 2007).

To verify the potential for anaerobic petroleum hydrocarbon degradation within the microbial populations of the sampled environments, PCR analysis was performed targeting the *assA* and *bssA* genes (Table 3.10). Primer set 1 was designed to target both *assA* and *bssA* genes (Callaghan *et al.*, 2010). Primer set 2 was designed to specifically target only the *bssA* gene and was shown to be very successful on generating PCR products in a wide array of toluene degraders (Winderl, 2007). Primer sets 3-10 were designed by considering a full-length *assA* gene sequence and has been shown to successfully amplify *assA* in *Desulfoglaeba alkanexedens* (Callaghan *et al.*, 2010).

Only genomic DNA from the Free State Groundworks soil sample gave rise to PCR products using these primers (Data not shown for Star Diamonds soil and Earthmoving Repair Services diesel), despite the fact that all three samples had a history of petroleum contamination. The gel band indicated in red displayed the correct size (~793 bp) for part of the *bssA* gene (Figure 3.11).

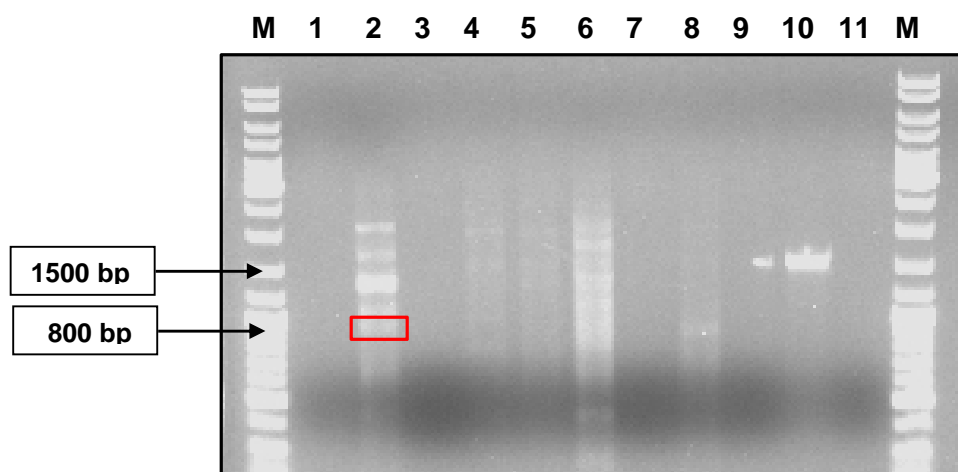


Figure 3.11: PCR amplification of *bssA* and *assA* genes from Free State Groundworks soil sample genomic DNA. Lanes in Table 3.19 below.

Table 3.19: Legend for figure 3.11.

Lane	Primer set target:	Amplicon size (bp)
M	MassRuler™ DNA ladder (Fermentas)	
1	targets <i>assA</i> and <i>bssA</i>	<i>assA</i> : 661 <i>bssA</i> : 682
2	targets <i>bssA</i>	793
3	targets <i>assA</i>	661
4	targets <i>assA</i>	1180
5	targets <i>assA</i>	661
6	targets <i>assA</i>	1180
7	targets <i>assA</i>	523
8	targets <i>assA</i>	1042
9	targets <i>assA</i>	523
10	16S rRNA gene (positive control)	1500
11	Negative control	n.a

The indicated band (Figure 3.11) was excised, re-amplified and sequenced. Obtained sequences were compared to sequences present in the GenBank database and resulted in positive identification of the sequences band with other *bssA* gene sequences. Specifically a 94% identity match was obtained with the *bssA* gene sequence from *Thaura aromatica* a known toluene degrader (Leuthner and Heider, 2000).

ClustalΩ (<http://www.ebi.ac.uk/Tools/msa/Clustalo/>) alignment of the obtained sequence with the sequence from *T. aromatica* shows high sequence similarities (Table 3.20). All the gene sequences in the GenBank database for *bssA* genes from *Thaura* spp. were aligned with the obtained sequence and a dendrogram constructed using MEGA6 (Tamura *et al.*, 2013). As can be seen in figure 3.12 and table 3.20, although there are definite similarities between the obtained sequence and the *bssA* gene sequence from *T. aromatica*, the obtained sequence still differs enough to be grouped apart from the reference sequences. Furthermore, no *Thauera* sp. sequence was obtained in the soil sample (Table 3.17). These results already earmarked the Free State Groundworks soil sample for further investigation since, although *T. aromatica* is an anaerobic toluene degrader, Bss like enzymes have been found to catalyse anaerobic biodegradation of a variety of aromatic substrates, including *o*-, *m*-, and *p*-xylene, ethylbenzene, 2-methylnaphthalene and may also be involved in the anaerobic degradation of naphthalene (Beller and Spormann, 1999; Heider *et al.*, 1998; Musat *et al.*, 2009; Widdel and Rabus, 2001). Oka and co-workers (2011) even identified *bss* gene analogues capable of anaerobic alkane degradation.

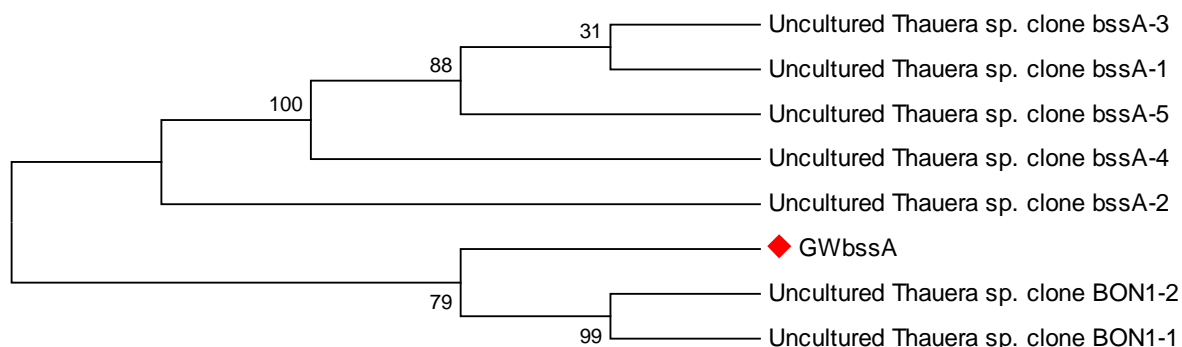


Figure 3.12: Phylogenetic tree of *bssA* gene sequences in the Free State Groundworks Soil sample and reference sequences from GenBank.

Table 3.20: ClustalΩ alignment of the sequences band and the *bssA* gene from *Thaura aromatica*.

gi GWbssA	TCCGTTGCCCACTTCGGCGCCCGCTTGAAGTCGACGCGCA-TTCCTCGAAGCCTTCCCA 59
gi Thauera	--CGTTGCCCACTTCGGCGCGCGCTTGAAGTCGACGCGCATTTCTCGAAACCTTCCCA 58 *****
gi GWbssA	GTTGCGCTTCAGCGCCTGGCTGAGCTGCTCGAGCGTGTACTTCTTGTCTCGAACACCAG 119
gi Thauera	GTTGCGCTTCAGTCGCTGGCTGAGTTGTTGAGGGGTACTTCTTCTCGAACACCAG 118 ***** ** ***** *
gi GWbssA	CTTCTTGAGCGCCACGAGGGAGTTCGCCGCTACGATCGTCTGTGATCGGGTTGTGCCAGCC 179
gi Thauera	TTTCTTGATGGCCACGAGGGAGTTCGCCGCGACGATCGTCTGTGATCGGGTTGTGCCAGCC 178 *****
gi GWbssA	GTTGGGCTGCTCGGACAGGGCGCACGCGTCCATGCCGAGCTCCATGCAGCCGTCGTCGAT 239
gi Thauera	GTTGGGTTGCTCGGACAGGGCACAAAGCGTCCATGCCGAGTGCCATGCAGCCGTCGTCGAT 238 ***** ** *****
gi GWbssA	TGCGGACACGAAGGGCATCTGCAGGAAGCGCTGTTTGAAGTAGCGCGACACGTCCTTGGT 299
gi Thauera	TGCGGACACGAAGGGCATCTGTAGAAAGCGTTGCTCGAAGTAGCGCGACACGTCCTTGGT 298 ***** * *****
gi GWbssA	GCTGATGCACAAGTTGATCGCGTACTGATACTGCTCGGGAAGGCCCTCCCAAACGTCCTC 359
gi Thauera	GCTGATACAGAGTTGATCGCGTATTGATACTGTTTGCAGGAAGGCCCTCCCAAACGTCCTC 358 ***** * *****
gi GWbssA	GAAGGTCTTCAAGGACGAGAGATCACCGTCTTCGGTCCGAGCTGCATGTCCGGCTACGA 419
gi Thauera	GAAGGTCTTCAAGGACGAGAGATCTCCGGTCTTCGGGCCCAACTGCATGTCCGGCTACGA 418 ***** **** ***** ** *
gi GWbssA	CCAGTCATAGCCGTCATTGAGCGTGATTTCCGAGCAGCTTGGCCGGGAAGATTGAGCCGCC 479
gi Thauera	CCAGTCGATAGCCGTCGTTAAGCGTGATTTCCAGTAGCTTGGCCGGGAAGATTGAGCCACC 478 ***** ***** ** ***** ***** **
gi GWbssA	GCCTTCCGAACGGGTCTTTTGCCTTTCGCGCGCCGCTGGATGCCGGGCGACATGCACAG 539
gi Thauera	GCCTTCCGAGCGGGTCTTTTGCCTTTCGCGCGCCGCTGGATGCCGGGCGACATGCACAG 538 ***** *****
gi GWbssA	CACGTTGACCCAGTTGTGGGCTTCCTCGTCGGTGGCGCCRTTGCCGTTGAGGCTGAACTT 599
gi Thauera	CACGTTGACCCAGTTGTGGGCTTCCTCGTCGGTGGCGCCGTTGCCGTTGAGGCTGAACTT 598 ***** *****
gi GWbssA	GGCGTATTCCTTCATCTGTTCCGTCGCGATCTCGTCRTGCTTGATGCAGCGATAGCCGAG 659
gi Thauera	TGCGTATTCCTTCATCTGCTCCGTCGCGATCTCGTCGCTTGATGGACGATAGCCGAG 658 ***** *****
gi GWbssA	ACCGTCGCGGATGCACTCGAAAACCCAGTGCAGCGTCTTTTACGGTTCTTCTTGAATA 719
gi Thauera	ACCGTCGGAATGCACTCGAAAACCCAGCGCAGCGTCTTCTCACGGTTCTTCTTGAATA 718 ***** ***** *****
gi GWbssA	GCGGAAGACGATGGAGGGCTCGGCCGTGCGGATCCGCTTGGCCGCTC----- 767
gi Thauera	GCGGAAGACGATGGAGGGCTCGGCTGTGCGAATCCGCTTGGCCGCTTCGAGGATCGCGTC 778 ***** ***** **
gi GWbssA	----- 767
gi Thauera	GGTCA 783

3.3.4 Enrichment cultures

Culture conditions were manipulated to select only for microorganisms capable of growth on hydrocarbons as sole carbon source, in this case added as filter sterilized diesel. Various minimal enrichment medias were utilized with slight differences as described in section 3.2.5, for example, nitrate or sulphate or both added as terminal electron acceptors, to further facilitate enrichment. Enrichment cultures were also incubated at increasing temperatures (25°C, 37°C and 50°C) in an attempt to select with physical conditions, as the solubility of hydrocarbons increases at elevated temperatures, thus increasing the bioavailability of these sparingly soluble compounds (Feitkenhauer and Märkl, 2003).

Initially, while sediment was still visible in media, no tests were performed on the enrichment cultures. Cultures were only incubated at their respective temperatures and passaged to fresh media every three weeks until sediment free cultures were obtained (Figure 3.13) as the effective concentration of the site contaminated hydrocarbons is difficult to determine and also might interfere with downstream analysis. The diesel sample did not contain any sediment, but was re-inoculated at the same time intervals as the sediment samples, for uniformity.

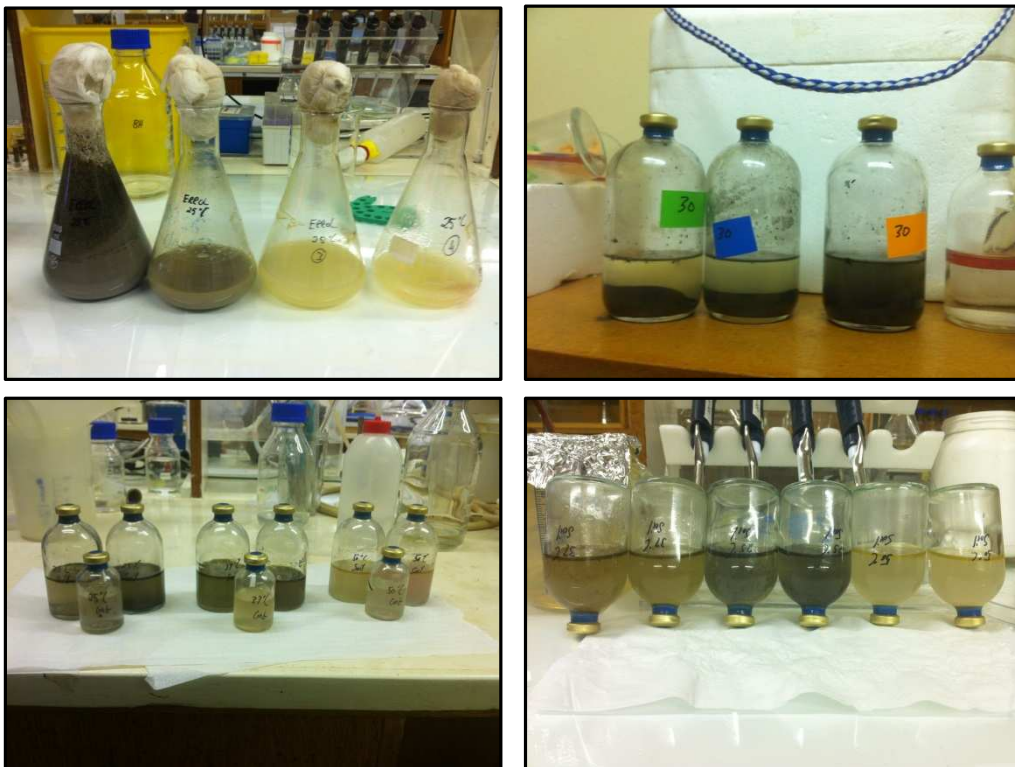


Figure 3.13: Pictures of random enrichment vials showing sediment containing cultures at the beginning and sediment free cultures towards the end of enrichment.

After sediment free enrichments were obtained, enrichment cultures were re-inoculated into fresh media and growth was determined by measuring the optical density at 600 nm (OD_{600}). Active growing cultures were then screened for electron acceptor reduction (nitrate and sulphate) as well as Gram staining and Live/Dead staining performed. All results are recorded in table 3.21. All the bacteria present in the enrichment cultures are gram negative rods (Figure 3.14) with growth observed in most of the medias at 25°C under aerobic and anaerobic conditions. A methanogenic medium was the exception, as no increase in optical density at 600 nm was observed. Some live cells were identified during Live/Dead staining in the Star Diamonds enrichment. The Star Diamonds enrichment also displayed growth at 37°C under anaerobic conditions. In growing culture s nitrate reduction was observed in all medias, but no sulphate reduction could be observed. Nitrate is not thought to be a significant electron sink and, consequently, nitrate reducers are generally not considered important anaerobic microorganisms for significant removal of hydrocarbon contaminants. As such sulphate reducers are more commonly described in literature regarding anaerobic hydrocarbon degradation (Rockne et al., 2000). Growth was observed in the sulphate reducing media, however significant levels of sulphate reduction was not obtained. Selected results can be seen below in figure 3.15 for example purposes.

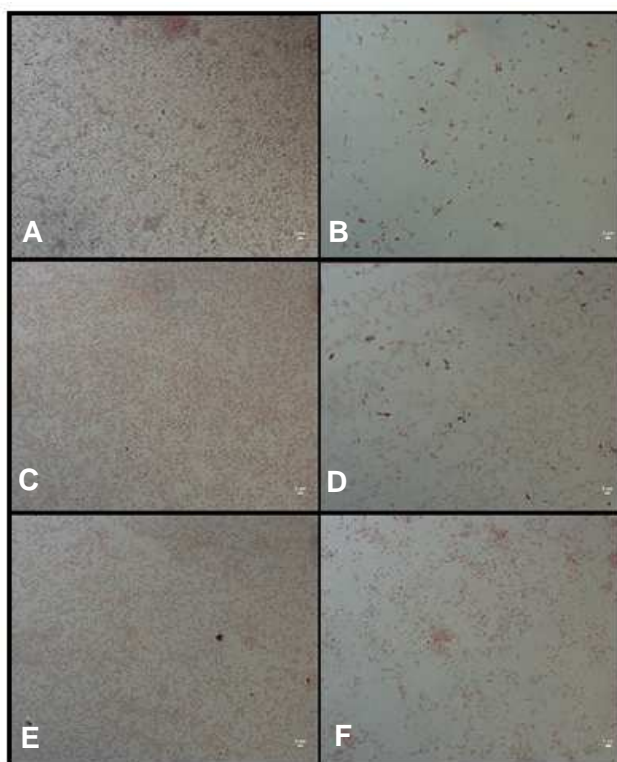


Figure 3.14: Gram stains performed on enrichment cultures grown in Mineral Salts Medium at 25°C under aerobic and anaerobic conditions. Free State Groundworks Soil sample (A) Aerobic and (B) Anaerobic. Star Diamonds Soil sample (C) Aerobic and (D) Anaerobic. Earthmoving Repair Services Diesel (E) Aerobic and (F) Anaerobic. Scale bar represents 2 μ m.

Table 3.21: Observations for the different environmental samples grown in various medias and results pertaining to growth and terminal electron acceptor reduction.

Sample	Enrichment media	Aerobic Growth Observed			Anaerobic Growth Observed			Gram stain Results	Live/Dead Stain results	Reduction of electron acceptor
		25°C	37°C	50°C	25°C	37°C	50°C			
Free State Groundworks	Mineral salts-BTEX medium	Yes	Yes	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Bushnell Haas Broth	Yes	Yes	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Mineral salts medium	Yes	Yes	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Methanogenic medium	No	No	No	No	No	No	Not Tested	Not Tested	Not tested
	Sulphate reducing media	No	No	No	No	No	No	Gram negative rods	Small amount of live cells observed	No sulphate reduction
	Nitrate reducing media	Yes	Yes	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction
Star Diamonds	Mineral salts-BTEX medium	Yes	Yes	Yes	Yes	Yes	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Bushnell Haas Broth	Yes	Yes	Yes	Yes	Yes	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Mineral salts medium	Yes	Yes	Yes	Yes	Yes	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Methanogenic medium	No	No	No	No	No	No		Very few live cells observed	Not tested
	Sulphate reducing media	Yes	Yes	Yes	Yes	Yes	No	Gram negative rods	Most cells were alive	Low sulphate reduction
	Nitrate reducing media	Yes	Yes	Yes	Yes	Yes	No	Gram negative rods	Most cells were alive	Nitrate reduction
Earthmoving Repair Diesel	Mineral salts-BTEX medium	Yes	No	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Bushnell Haas Broth	Yes	No	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Mineral salts medium	Yes	No	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction; no sulphate reduction
	Methanogenic medium	No	No	No	No	No	No	Not tested	No cells observed	Not tested
	Sulphate reducing media	No	No	No	No	No	No	Not tested	Small amount of live cells observed	No sulphate reduction
	Nitrate reducing media	Yes	No	No	Yes	No	No	Gram negative rods	Most cells were alive	Nitrate reduction

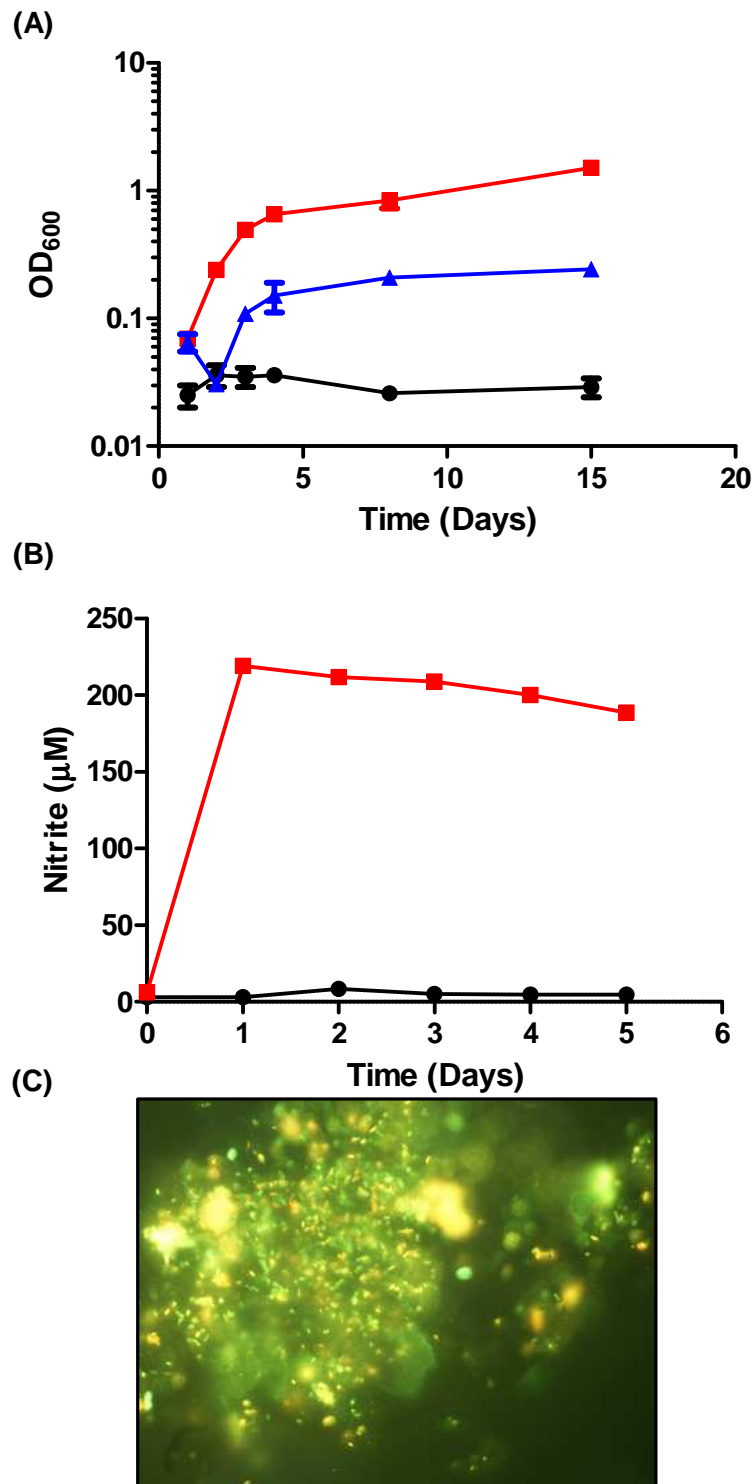


Figure 3.15: Example of results obtained by monitoring growth, terminal electron acceptor reduction and Live/Dead staining. (A) Growth of Free State Groundworks enrichment in Bushnell Haas Broth at 25°C, with diesel as sole carbon source, under aerobic (■) and anaerobic conditions (▲) with a cell free control (●). (B) Nitrate reduction by the Free State Groundworks enrichment in Bushnell Haas Broth at 25°C, with diesel as sole carbon source, under anaerobic conditions. Nitrate reduction as indicated by nitrite formation (■) with cell free control (●). (C) Live/Dead stain on Free State Groundworks enrichment in Bushnell Haas Broth at 25°C, with diesel as sole carbon source, under anaerobic conditions.

3.3.4.1 Enrichment culture diversity

No real difference in growth or electron acceptor reduction could be observed in the different medium containing nitrate (data not shown), as such, further enrichment was performed exclusively in Bushnell Haas Broth with diesel as sole carbon source. Enrichment of hydrocarbon degrading microorganisms was ongoing for nearly six months, at temperatures and conditions determined from table 3.21. Briefly, the Free State Groundworks soil enrichments were continued at 25°C and 37°C under aerobic conditions and at 25°C under anaerobic conditions, the Star Diamonds soil enrichments were continued at 25°C, 37°C and 50°C under aerobic conditions and at 25°C and 37°C under anaerobic conditions and the Earthmoving Repair Services diesel enrichment only at 25°C under aerobic and anaerobic conditions.

Diversity assessment was once again performed using PCR-DGGE and sequencing and the bands obtained. The generated DNA sequences were assembled by aligning the forward and reverse sequences in Geneious® Pro v 7.1.7 (Biomatters, Ltd, <http://www.geneious.com>). Well-known databases of 16S rRNA gene sequences were consulted for sequence similarities. The closest BLAST hits from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was tabulated (Table 3.22) and the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/html/>) was used to construct dendrograms (Figure 3.16 to 3.23).

When the initial sample diversity, table 3.17, is compared to the enriched diversity, table 3.22, a significant increase in hydrocarbon degraders is observed. This is mostly due to the fact that some community members were present in very low numbers, resulting in poor identification using PCR methods. Selection due to enrichment conditions has now drastically increased the numbers of the diesel degradation members over the soil members due to more favourable culture conditions and the removal of other inhibitory factors that might have been present in the soil. Most of the members now present have been identified as capable of growing on various hydrocarbons, with the exception of the Free State Groundwork soil enrichment. The Earthmoving Repair Diesel enrichment are *Stenotrophomonas* sp., *Microbacterium* sp., *Bacillus* sp., *Paenibacillus* sp and *Acinetobacter* sp., all whom have either shown to be able to grow on hydrocarbons as sole carbon source or have been identified in hydrocarbon contaminated environments (Alisi *et al.*, 2009; Arulazhagan *et al.*, 2010; Bayoumi, 2009; Boonchan *et al.*, 2000; Daane *et al.*, 2001; Ganesh and Lin, 2009; Guzik *et al.*, 2009; Heitkamp and Cerniglia, 1989; Juhasz and Naidu, 2000; Kayode-Isola *et al.*, 2008; Kebria *et al.*, 2009; Lease *et al.*, 2011; Lee *et al.*, 2003; Li *et*

al., 2008; Liu *et al.*, 2012; Xie *et al.*, 2010). The same applies for the Star Diamonds soil enrichment which contained *Achromobacter* sp., *Ochrobactrum* sp., *Pseudomonas* and *Enterobacter* sp. (Abdel-el-haleem, 2003; Al-thani *et al.*, 2009; Mazzeo *et al.*, 2010; Mishra *et al.*, 2004; Nielsen *et al.*, 2006; Owsianiak *et al.*, 2009; Zhang *et al.*, 2005). On the other hand, members of the Free State Groundworks soil enrichment included *Edwardsiella* sp. and *Citrobacter* sp., both with very little literature pertaining to their usage of hydrocarbons as carbon sources, but which have been found on some occasions in hydrocarbon contaminated soil. Under anaerobic conditions *Citrobacter freundii* has been shown to be capable of biphenyl degradation (Grishchenkov *et al.*, 2002), but no other anaerobic degradation of other hydrocarbons have been found in literature.

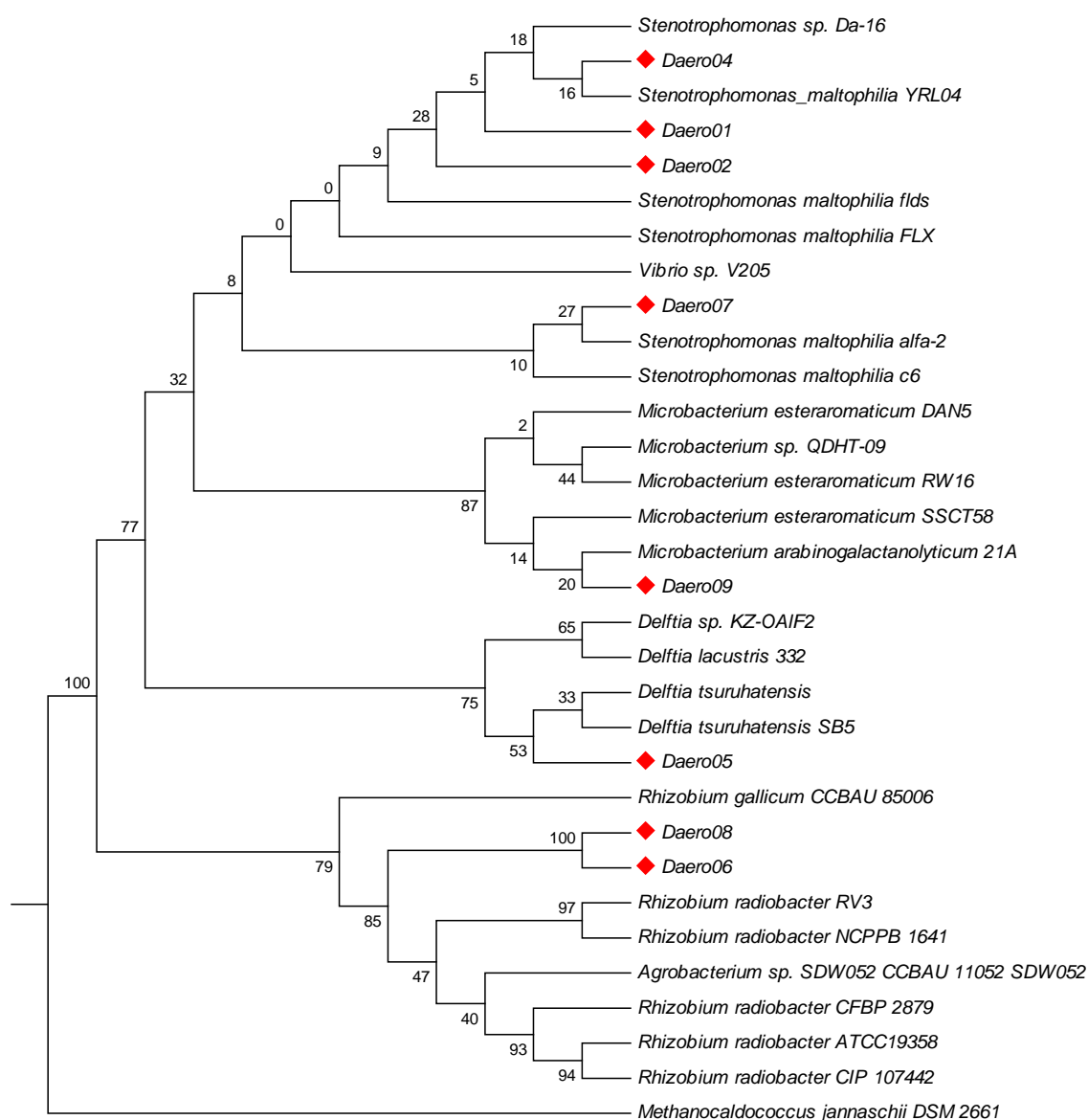


Figure 3.16: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Earthmoving Repair Service 25°C aerobic enrichment and reference sequences from the Ribosomal Database Project (RDP).

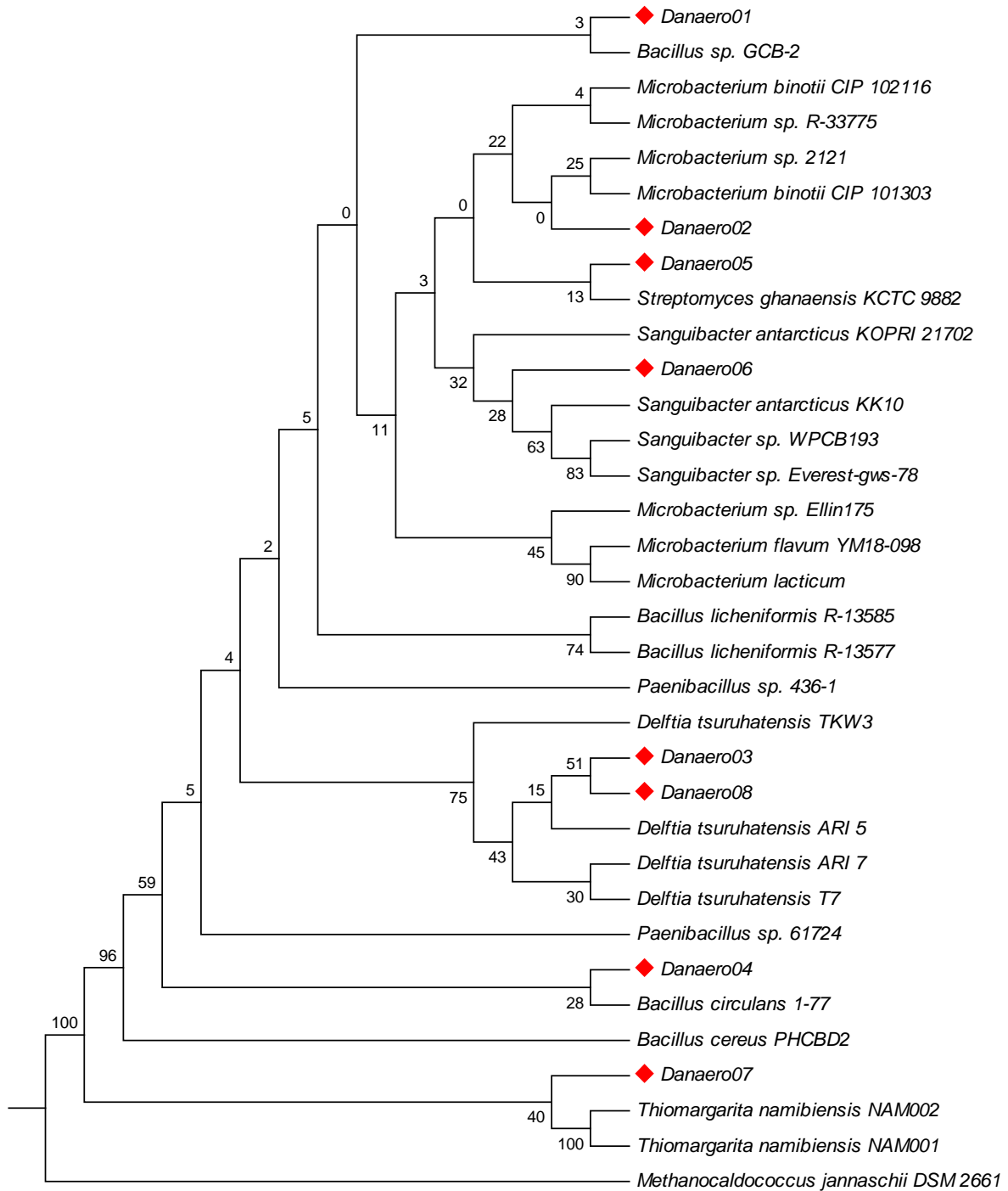


Figure 3.17: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Earthmoving Repair Service 25°C anaerobic enrichment and reference sequences from the Ribosomal Database Project (RDP).

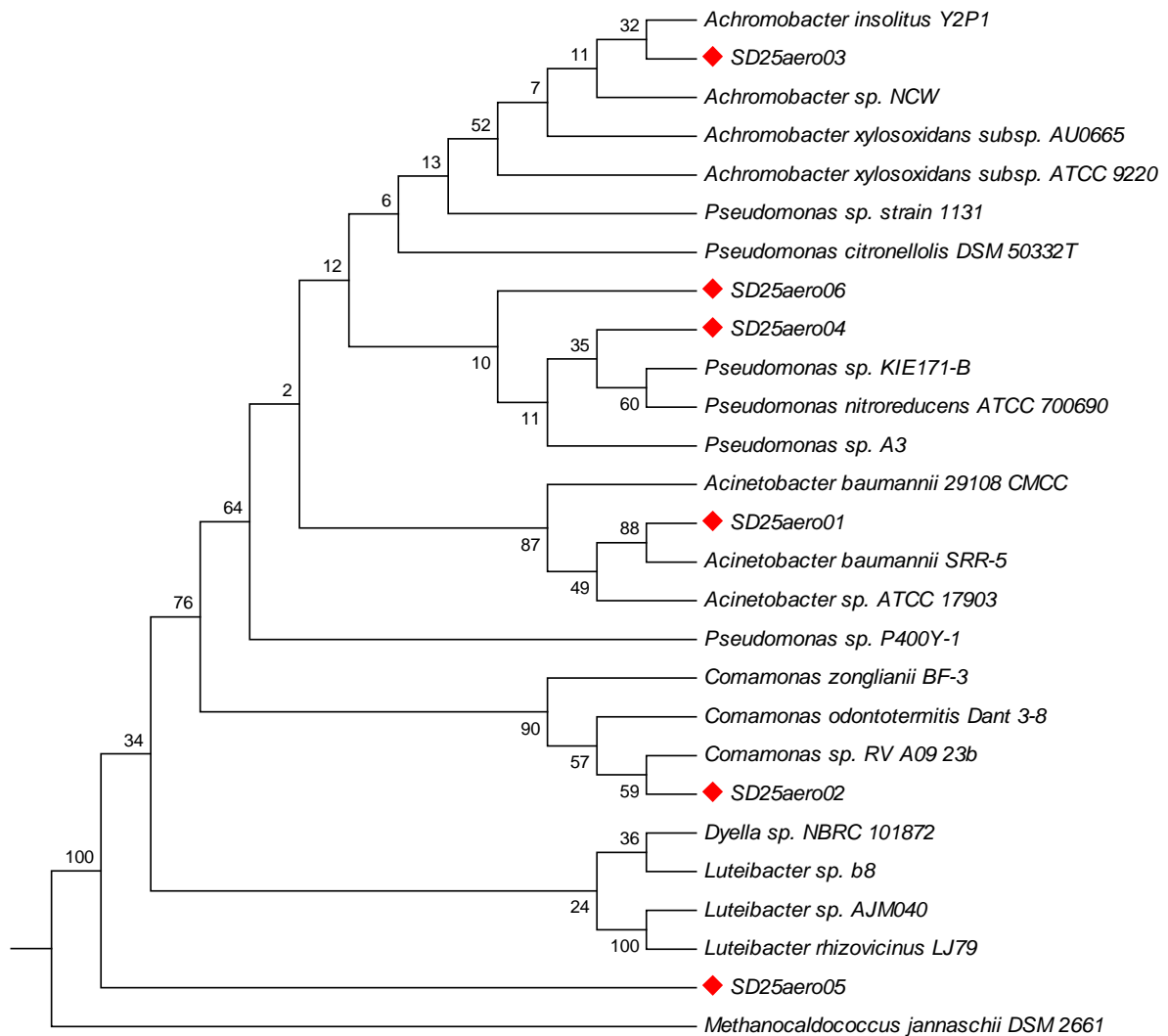


Figure 3.18: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Star Diamond soil 25°C aerobic enrichment and reference sequences from the Ribosomal Database Project (RDP).

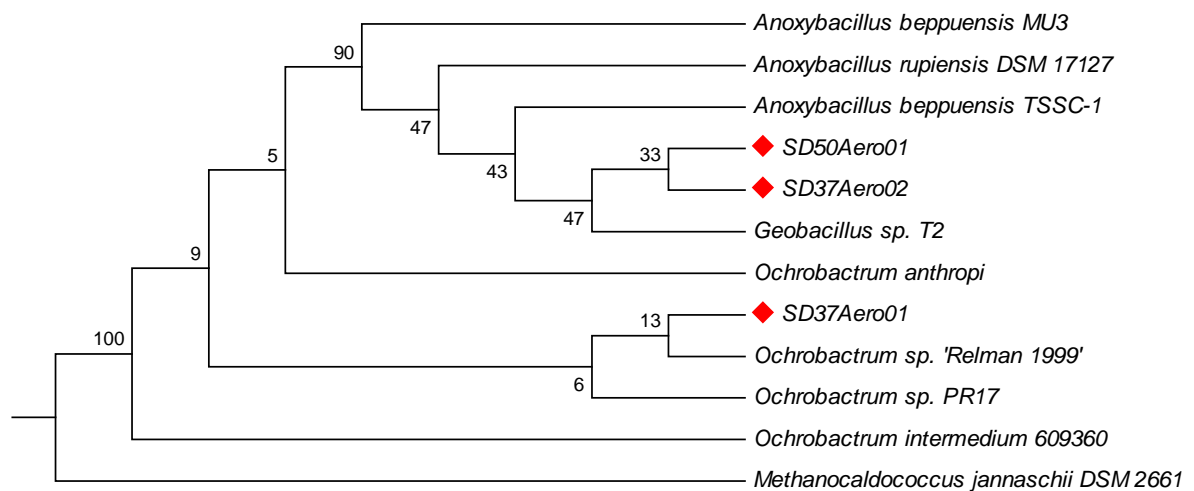


Figure 3.19: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Star Diamond soil 37°C and 50°C aerobic enrichments and reference sequences from the Ribosomal Database Project (RDP).

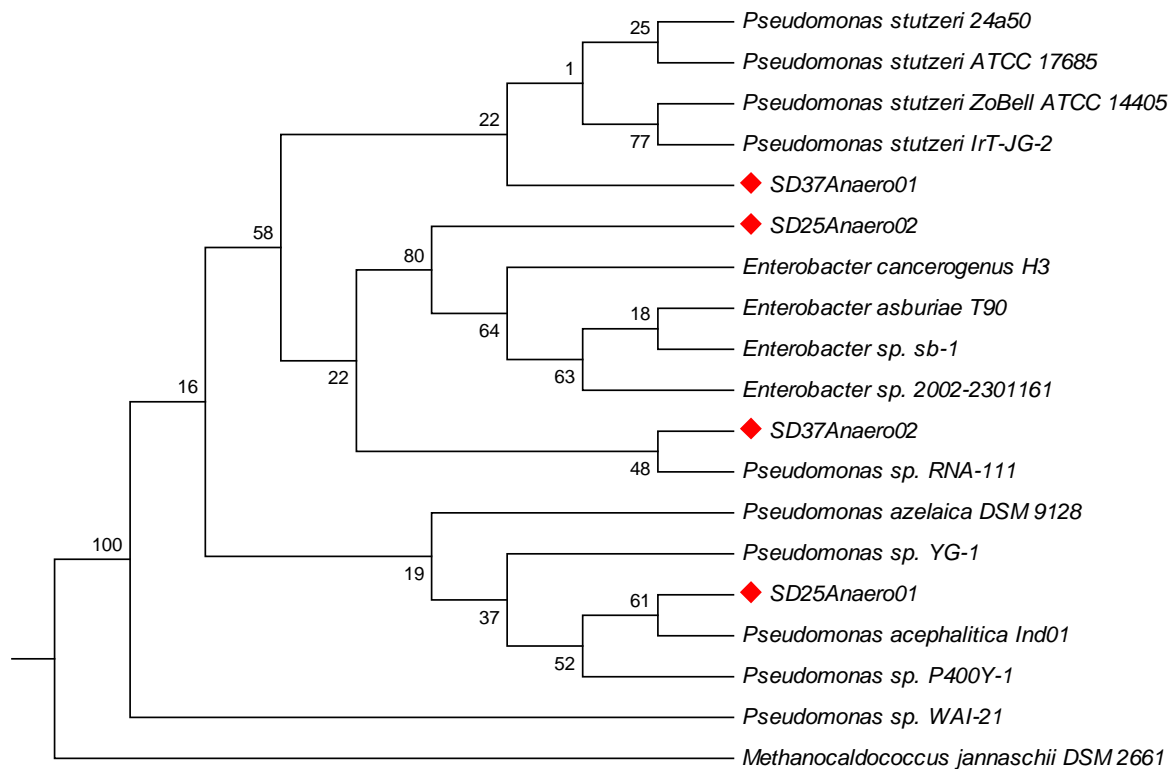


Figure 3.20: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Star Diamond soil 25°C and 37°C anaerobic enrichments and reference sequences from the Ribosomal Database Project (RDP).

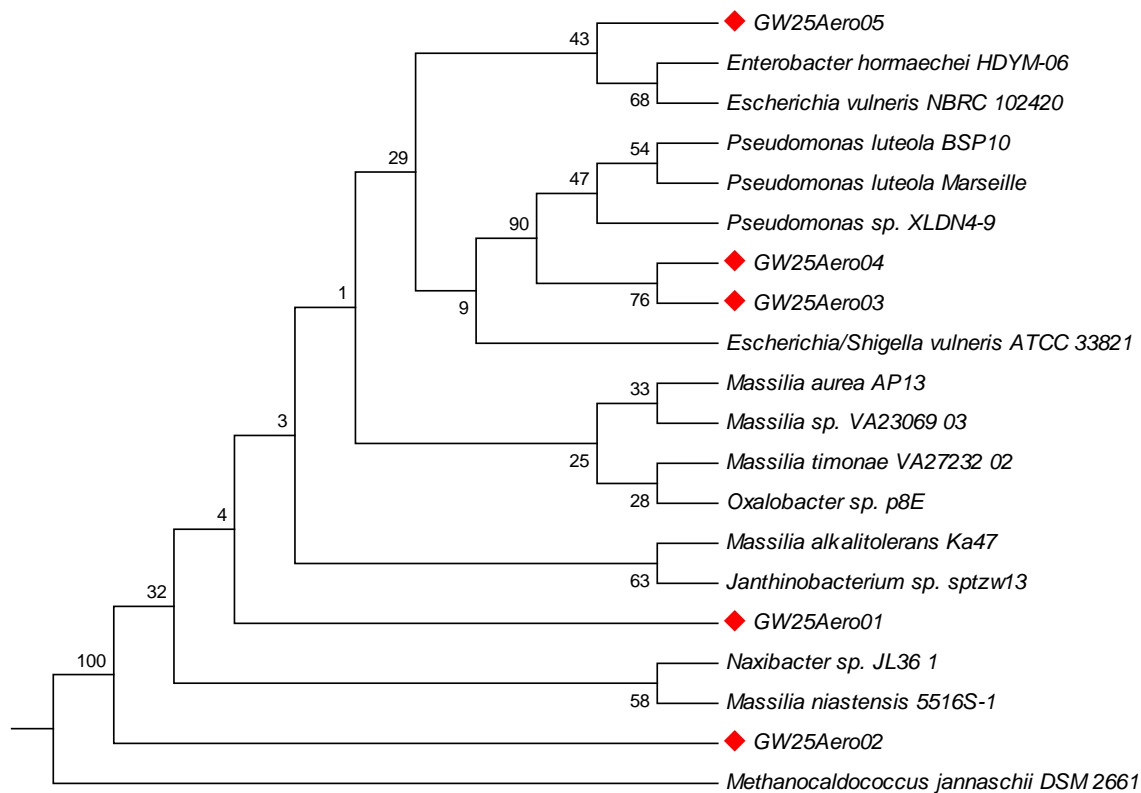


Figure 3.21: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Free State Groundworks soil 25°C aerobic enrichments and reference sequences from the Ribosomal Database Project (RDP).

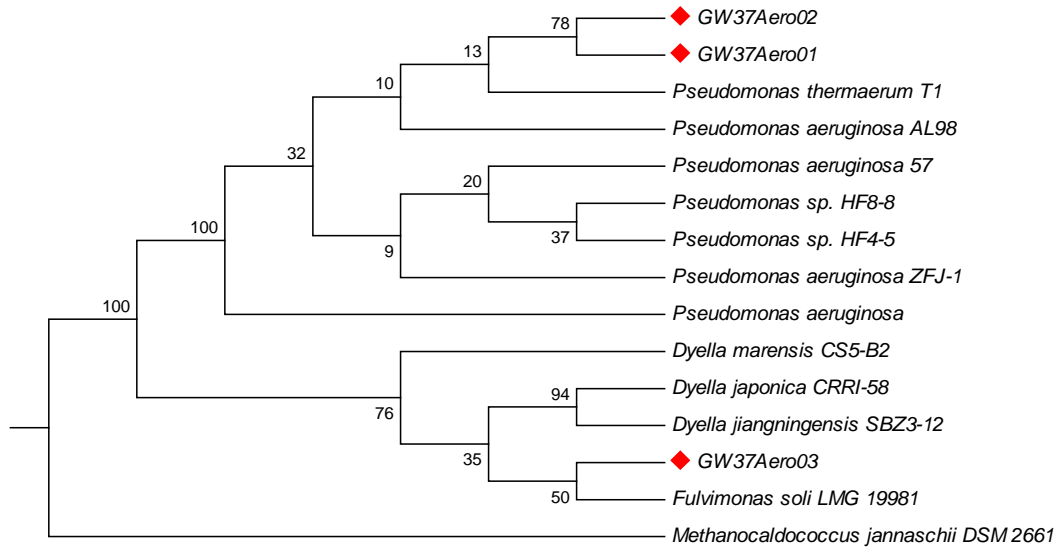


Figure 3.22: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Free State Groundworks soil 37°C aerobic enrichments and reference sequences from the Ribosomal Database Project (RDP).

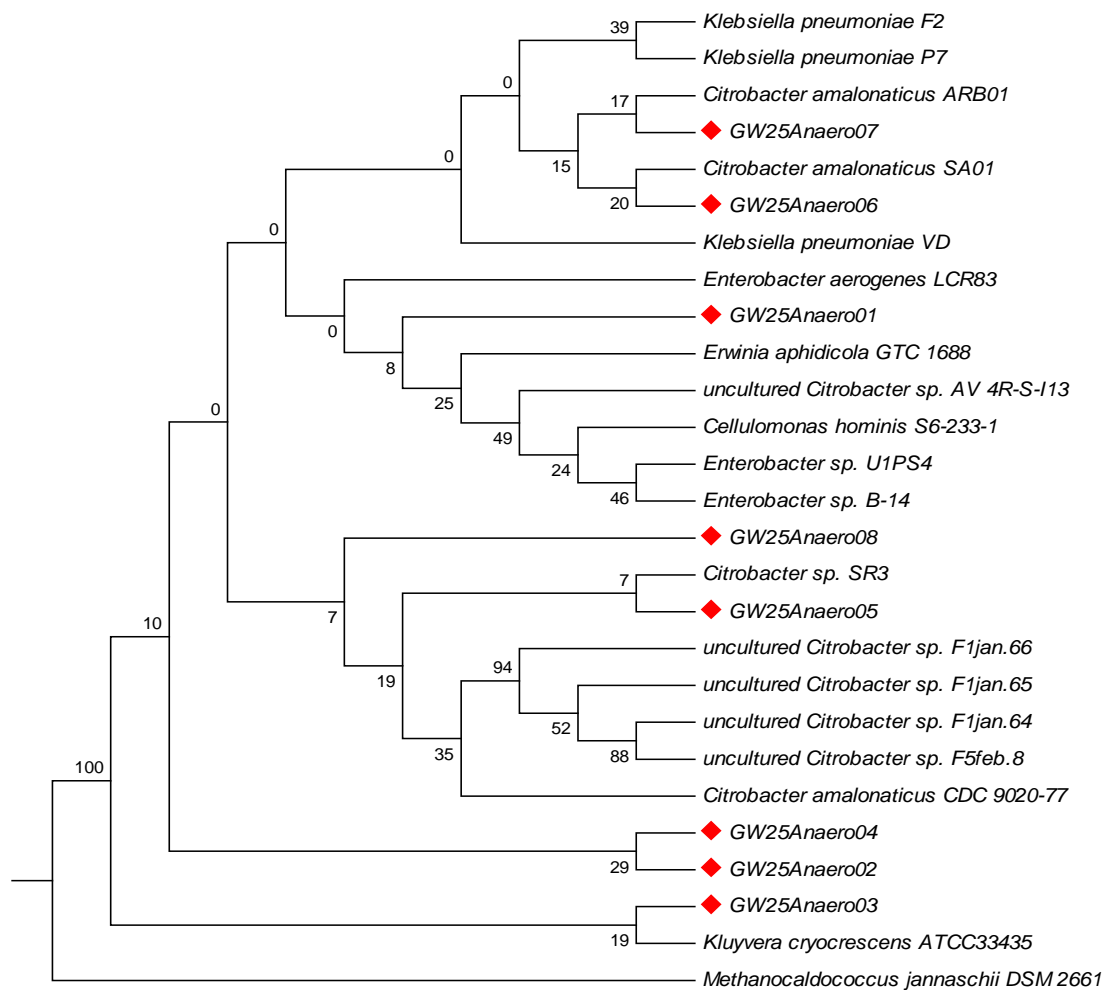


Figure 3.23: Phylogenetic tree of bacterial 16S rRNA gene sequences in the Free State Groundworks soil 25°C anaerobic enrichments and reference sequences from the Ribosomal Database Project (RDP).

Table 3.22: Closest GenBank reference obtained for DGGE band sequences from different enrichment samples.

Consortium	Phylotype	Phylogenetic Affiliation	Closest Relative	Identity
Earthmoving Repair Services Diesel 25°C Aerobic Enrichment	Daero01	γ -Proteobacteria	<i>Stenotrophomonas maltophilia</i> strain BXCC-5	99%
	Daero02	γ -Proteobacteria	<i>Stenotrophomonas</i> sp. JRL-2	99%
	Daero03	γ -Proteobacteria	Uncultured <i>Stenotrophomonas</i> sp. clone GI3-M-7-A09	93%
	Daero04	γ -Proteobacteria	<i>Stenotrophomonas</i> sp. DB-17	100%
	Daero05	β -Proteobacteria	<i>Delftia tsuruhatensis</i> strain DYJL11	93%
	Daero06	α -Proteobacteria	Uncultured bacterium clone SN_OE_82	86%
	Daero07	γ -Proteobacteria	<i>Stenotrophomonas</i> sp. I_28-J6NNFC6A2	96%
	Daero08	α -Proteobacteria	<i>Agrobacterium tumefaciens</i> strain D14	92%
	Daero09	Actinobacteria	<i>Microbacterium</i> sp. OB44-2	99%
Earthmoving Repair Services Diesel 25°C Anaerobic Enrichment	Danaero01	Bacilli	<i>Bacillus licheniformis</i> strain 162	99%
	Danaero02	Actinobacteria	<i>Microbacterium binotii</i> strain R6-367	99%
	Danaero03	β -Proteobacteria	Uncultured <i>Delftia</i> sp. clone 6TA04	100%
	Danaero04	Bacilli	<i>Paenibacillus</i> sp. B16	99%
	Danaero05	Actinobacteria	<i>Microbacterium</i> sp. Ellin175	100%
	Danaero06	Actinobacteria	<i>Sanguibacter antarcticus</i> strain KK13	99%
	Danaero07		Uncultured bacterium clone B2	75%
	Danaero08	β -Proteobacteria	<i>Delftia</i> sp. 3C	99%
Star Diamonds Soil 25°C Aerobic Enrichment	SD25Aero01	γ -Proteobacteria	<i>Acinetobacter baumannii</i> strain M1	91%
	SD25Aero02	β -Proteobacteria	<i>Comamonas odontotermitis</i> strain Dant 3-8	100%
	SD25Aero03	β -Proteobacteria	<i>Achromobacter xylosoxidans</i> strain CD-253	99%
	SD25Aero04	γ -Proteobacteria	<i>Pseudomonas nitroreducens</i> strain T11AT4	99%
	SD25Aero05	γ -Proteobacteria	<i>Dyella</i> sp. M303	99%
	SD25Aero06	γ -Proteobacteria	<i>Pseudomonas nitroreducens</i> strain SN2	99%

Table 3.22 (Continued): Closest GenBank reference obtained for DGGE band sequences from different enrichment samples.

Consortium	Phylotype	Phylogenetic Affiliation	Closest Relative	Identity
Star Diamonds Soil 37°C Aerobic Enrichment	SD37Aero01	α-Proteobacteria	<i>Ochrobactrum intermedium</i> strain TND10	100%
	SD37Aero02	Bacilli	<i>Anoxybacillus</i> sp. IB-BE2-3	100%
Star Diamonds Soil 50°C Aerobic Enrichment	SD50Aero01	Bacilli	<i>Anoxybacillus rupiensis</i> strain :DSM 17127	99%
Star Diamonds Soil 25°C Anaerobic Enrichment	SD25Anaero01	γ-Proteobacteria	Uncultured <i>Pseudomonas</i> sp. clone MKC2	91%
	SD25Anaero02	γ-Proteobacteria	<i>Enterobacter hormaechei</i> strain 1292	99%
Star Diamonds Soil 37°C Anaerobic Enrichment	SD37Anaero01	γ-Proteobacteria	Uncultured bacterium clone BG024	99%
	SD37Anaero01	γ-Proteobacteria	<i>Pseudomonas stutzeri</i> strain KJ-W22	99%
Free State Groundworks 25°C Aerobic Enrichment	GW25Aero01	β-Proteobacteria	Uncultured bacterium partial 16S rRNA gene, clone 13IF-H7	99%
	GW25Aero02	β-Proteobacteria	Uncultured bacterium clone B4-5 16S ribosomal RNA gene	99%
	GW25Aero03	γ-Proteobacteria	Uncultured bacterium clone ncd2520a04c1 16S	95%
	GW25Aero04	γ-Proteobacteria	Uncultured bacterium clone ncd2520a04c1 16S	98%
	GW25Aero05	γ-Proteobacteria	<i>Escherichia vulneris</i> gene for 16S rRNA, partial sequence	100%
Free State Groundworks 37°C Aerobic Enrichment	GW37Aero01	γ-Proteobacteria	Uncultured bacterium partial 16S rRNA gene, clone 16sps17-3f10.w2k	99%
	GW37Aero02	γ-Proteobacteria	Uncultured bacterium partial 16S rRNA gene, clone 16sps17-3f10.w2k	99%
	GW37Aero03	γ-Proteobacteria	Uncultured bacterium clone BG024 16S	96%
Free State Groundworks 25°C Anaerobic Enrichment	GW25Anaero01	γ-Proteobacteria	Uncultured <i>Enterobacter</i> sp. clone F2jun.13 16S	99%
	GW25Anaero02	γ-Proteobacteria	Uncultured <i>Enterobacter</i> sp. clone F2jun.13 16S	98%
	GW25Anaero03	γ-Proteobacteria	Uncultured <i>Enterobacter</i> sp. clone F5jun.16 16S	98%
	GW25Anaero04	γ-Proteobacteria	Uncultured <i>Enterobacter</i> sp. clone F2jun.13 16S	98%

Table 3.22 (Continued): Closest GenBank reference obtained for DGGE band sequences from different enrichment samples.

Consortium	Phylotype	Phylogenetic Affiliation	Closest Relative	Identity
Free State Groundworks 25°C Anaerobic Enrichment	GW25Anaero05	γ-Proteobacteria	<i>Edwardsiella tarda</i> strain CETCK1 16S ribosomal RNA gene	99%
	GW25Anaero06	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain SA01 16S ribosomal RNA gene	99%
	GW25Anaero07	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain SA01 16S ribosomal RNA gene	100%
	GW25Anaero08	γ-Proteobacteria	<i>Edwardsiella tarda</i> strain CETCK1 16S ribosomal RNA gene	99%

3.3.4.2 Enrichment culture hydrocarbon degradation potential

An adapted most-probable-number (procedure) for the enumeration of hydrocarbon degrading microorganism (Haines *et al.*, 1996) was utilized to indicate which, if any, hydrocarbons the enrichment consortia displayed any specificity to degrade. The method uses different hydrocarbons as the selective growth substrate and the reduction of iodinitrotetrazolium (INT) as a growth indicator. INT competes with O₂ for electrons from the respiratory electron transport chain resulting in reduction of INT. The reduced product is an insoluble formazan that deposits as a red precipitate thus indicating actively respiring microorganisms (Figure 3.24) (Relexans, 1996).

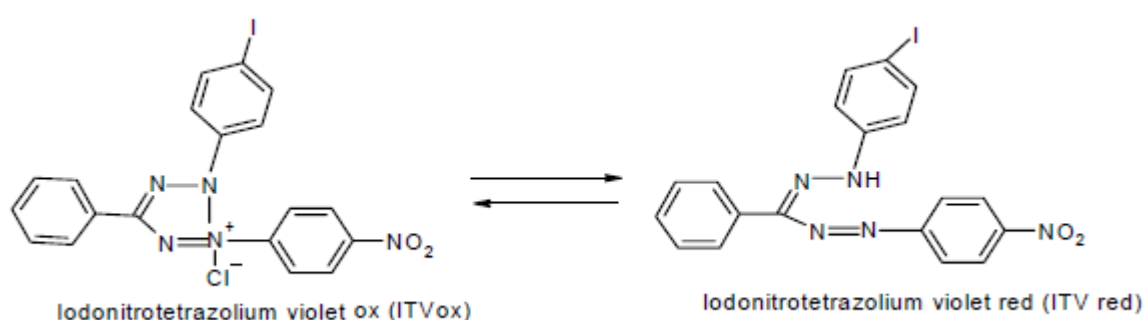


Figure 3.24: Different oxidation states of iodonitrotetrazolium, the reduced state results in a red precipitate being formed.

After 48 hours samples were scored in two ways (Table 3.23), after addition of INT a red or pink sample was scored as positive for metabolic activity (Figure 3.25) with a hydrocarbon as sole carbon source. Samples that showed an increase in optical density at 600 nm (OD₆₀₀) was scored positive for growth. Because some hydrocarbons can be toxic to microorganisms, lower concentrations (than compared to literature) were utilized. Substrates tested included short chain alkanes, monocyclic- as well as polycyclic aromatic hydrocarbons (MAH and PAH).

None of the enrichments displayed the capability to grow on xylene as a sole carbon source, but most of the enrichments were capable to at least display metabolic activity towards the rest of the hydrocarbons. Under aerobic conditions the Star Diamonds soil enrichment was capable of growth with all of the hydrocarbons present at 25°C and 37°C, with the exception of xylene, but was only capable of showing similar capabilities at 25°C under anaerobic conditions. No metabolic activity was observed at 50°C under both anaerobic and aerobic conditions. The Earthmoving Repair Services displayed a small amount of metabolic activity towards most hydrocarbons under aerobic and anaerobic conditions, indicated by a very

small amount of red precipitate being formed. Also growth was almost negligible. The Free State Groundworks soil enrichment did not show any metabolic activity for both xylene and hexane. When comparing aerobic and anaerobic conditions at 25°C one can see that under aerobic conditions the enrichment was only capable of metabolic growth on octane, but under anaerobic conditions activity is now observed for the MAHs and PAHs as well, indicating the possibility of an anaerobic aromatic hydrocarbon degrader being present.

When comparing the diversity to literature, similar results to the observations was obtained (Table 3.24). Both the Earthmoving Repair Services diesel enrichment and the Star Diamonds soil enrichment have rich diversities, consisting mainly of microorganisms which have been found to have facultative anaerobic capabilities, thus the similar observations under both aerobic and anaerobic conditions. Most of them are capable of growing on a vast array of hydrocarbons. The Free State Groundworks soil enrichment's anaerobic enrichment contains facultative anaerobic microorganisms that are not present in the aerobic enrichment. It is also under anaerobic conditions where the most diverse metabolic activity is observed, which could indicate that these microorganisms are responsible for the degradation, however literature does not elucidate definitive conclusions.

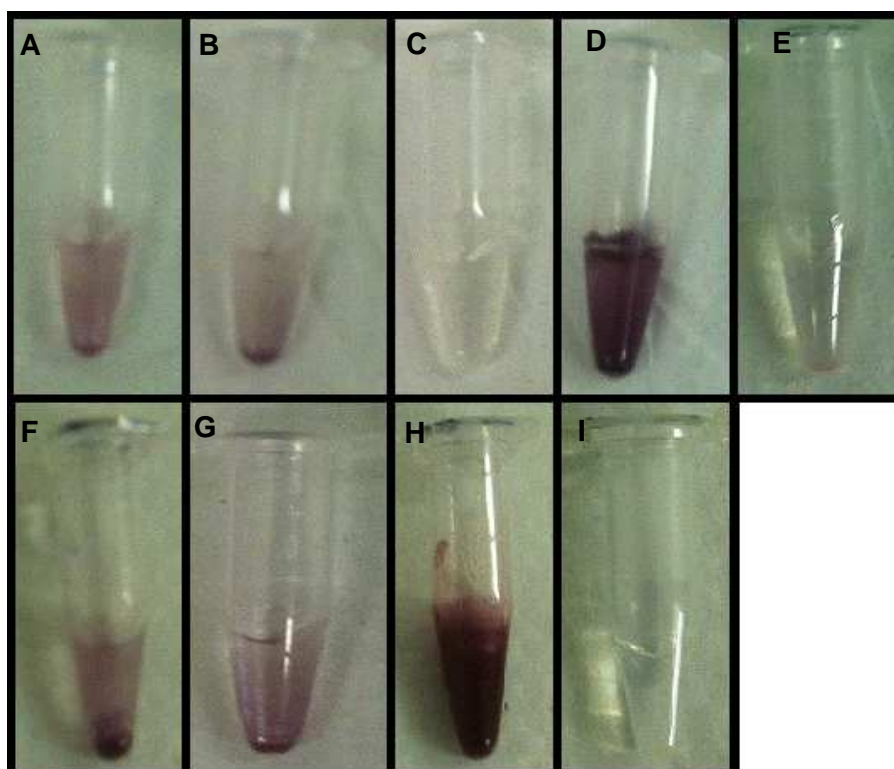


Figure 3.25: Free State Groundworks soil enrichment incubated under anaerobic conditions at 25°C with different hydrocarbons as growth substrate after Iodonitrotetrazolium addition and incubation. (A) Benzene, (B) Toluene, (C) Xylene, (D) Octane, (E) Hexane, (F) Naphthalene, (G) Phenanthrene, (H) Positive Control and (I) Negative Control.

Table 3.23: Enrichment culture hydrocarbon growth potential observed as metabolic activity in the presence of red idonitrotetrazolium precipitate.

Enrichment	Aerobic / Anaerobic	Incubate	Benzene		Toluene		Xylene		Octane		Hexane		Naphthalene		Phenanthrene	
			Utilize	Growth	Utilize	Growth	Utilize	Growth	Utilize	Growth	Utilize	Growth	Utilize	Growth	Utilize	Growth
Earthmoving Repair Services Diesel Enrichment	Aerobic	25°C	Y	N	Y	N	N	N	Y	N	Y	N	Y	N	Y	N
	Anaerobic	25°C	N	N	Y	N	N	N	Y	N	Y	N	Y	N	Y	N
Star Diamonds Soil Enrichment	Aerobic	25°C	Y	Y	Y	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
	Aerobic	37°C	Y	Y	Y	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
	Aerobic	50°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	Anaerobic	25°C	Y	Y	Y	N	N	N	Y	Y	Y	Y	Y	Y	Y	N
	Anaerobic	37°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	Anaerobic	50°C	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Free State Groundworks Soil Enrichment	Aerobic	25°C	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N
	Aerobic	37°C	N	N	N	N	N	N	N	N	N	N	Y	N	Y	N
	Anaerobic	25°C	Y	N	Y	N	N	N	N	N	N	N	Y	Y	Y	Y

Table 3.24: Enrichment culture diversity hydrocarbons degradation potential found in literature.

Enrichment	Organism	Aerobic/Anaerobic	Hydrocarbons utilized	Literature Reference
Earthmoving Repair Services Diesel Enrichment	<i>Stenotrophomonas</i> sp.	Aerobic	Pyrene, fluoranthene, benz[a]anthracene, benzo[a]pyrene, dibenz[a,h]anthracene, coronene, phenol, naphthalene, phenanthrene, BTEX	Arulazhagan <i>et al.</i> , 2010; Boonchan <i>et al.</i> , 2000; Guzik <i>et al.</i> , 2009; Juhasz and Naidu, 2000; Lee <i>et al.</i> , 2003
	<i>Delftia</i> sp.	Aerobic/Facultative anaerobic	Diesel	Alisi <i>et al.</i> , 2009
	<i>Microbacterium</i> sp.	Aerobic/Facultative anaerobic	Diesel, pyrene, phenanthrene, fluoranthene	Heitkamp and Cerniglia, 1989; Lease <i>et al.</i> , 2011; Li <i>et al.</i> , 2008
	<i>Bacillus licheniformis</i>	Facultative anaerobic	Benzene, toluene, phenol, crude oil, diesel	Bayoumi, 2009; Kayode-Isola <i>et al.</i> , 2008; Kebria <i>et al.</i> , 2009; Liu <i>et al.</i> , 2012
	<i>Paenibacillus</i> sp.	Facultative anaerobic	Xylene, diesel, naphthalene, phenanthrene	Daane <i>et al.</i> , 2001; Ganesh and Lin, 2009; Xie <i>et al.</i> , 2010
Star Diamonds Soil Enrichment	<i>Acinetobacter baumannii</i>	Aerobic	Crude oil, diesel, phenol, BTEX,	Abdel-EI-Haleem, 2003; Mishra <i>et al.</i> , 2004
	<i>Achromobacter xylosoxidans</i>	Aerobic	Naphthalene, phenanthrene, anthracene, pyrene, BTEX	Al-thani <i>et al.</i> , 2009; Nielsen <i>et al.</i> , 2006
	<i>Ochrobactrum intermedium</i>	Aerobic	Diesel, benzo[a]pyrene	Owsianiak <i>et al.</i> , 2009
	<i>Pseudomonas</i> sp.	Aerobic/Facultative anaerobic	Various PAHs, BTEX, crude oil	Mazzeo <i>et al.</i> , 2010; Zhang <i>et al.</i> , 2005
Free State Groundworks Soil Enrichment	<i>Enterobacter hormaechei</i>	Facultative anaerobic	Petroleum	Erdogan <i>et al.</i> , 2013
	<i>Edwardsiella tarda</i>	Facultative anaerobic	None	None
	<i>Citrobacter amalonaticus</i>	Facultative anaerobic	Diesel, biphenyl	Grishchenkov <i>et al.</i> , 2002

3.4 Conclusions

The ability of indigenous microorganisms, especially those isolated from contaminated environments, to degrade various hydrocarbons is well known. In the present study we report on the microbial diversity of two hydrocarbon contaminated environmental sites as well as a microbial contaminated diesel sample. The various enrichment techniques were utilized to select for hydrocarbon degraders under aerobic and anaerobic conditions. The cultures were grown with diesel as a sole carbon source in different medium conditions at different temperatures to obtain optimum hydrocarbon degradation conditions. Further growth and metabolic tests on the enriched consortiums incubated with aliphatic, mono-aromatic and poly-aromatic hydrocarbons indicated the presence of hydrocarbon degrading activities.

Based on the analysis of the 16S rRNA gene sequences we have provided evidence indicating that exclusively gram-negative organisms play an important role in all of these environments to degrade hydrocarbons. The Star Diamonds soil and Earthmoving Repair diesel sample, both consist of members that are well studied with regards to their aerobic biodegradable abilities (*Stenotrophomonas* sp., *Microbacterium* sp., *Bacillus* sp., *Paenibacillus* sp. and *Acinetobacter* sp., *Achromobacter* sp., *Ochrobactrum* sp., *Pseudomonas* sp. and *Enterobacter* sp.), frequently found in soils contaminated with hydrocarbons and perform better under aerobic conditions. In contrast, the Free State Groundwork soil enrichment consisted of a diversity that does not have extensive literature coupled to it (*Edwardsiella* sp. and *Citrobacter* sp) and it presents with better degradation potential under anaerobic conditions, when compared to aerobic potential. Degradation is also coupled to nitrate reduction, another field severely lacking in literature when compared to sulphate reduction. Thus, this is the first report, to our knowledge, coupling nitrate reduction to naphthalene and phenanthrene degradation by a *Citrobacter* sp. containing enrichment culture. Out of the three enrichment cultures, the Free State Groundworks soil enrichment is also the only culture containing known anaerobic degradation genes for aromatic hydrocarbons degradation (*bssA*).

Since the greater scope of this project is to elucidate the mechanisms of anaerobic hydrocarbon degradation further study of the Free State Groundworks soil enrichment will significantly contribute to the knowledge base.

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Chapter 4

Isolation and characterization of an anaerobic PAH-degrading enrichment culture

4.1 Introduction

The cycling of relatively small carbon loads by microorganisms can turn aqueous systems anoxic relatively fast. One of the main reasons for this is the low solubility of oxygen (8 mg/L = 250 μ M at 25°C), for example, as little as 33 μ M of the hydrocarbon benzene can result in a system turning anaerobic if microbial cycling is present (Meckenstock and Mouttaki, 2011). If molecular oxygen is absent, which is an essential component of biochemical hydrocarbon activation reactions, biodegradation has to occur by replacing oxygen with other terminal electron acceptors, such as nitrate, sulphate, and iron. Foght (2008) and Heider (2007) have reported on anaerobic degradation pathways for alkylated aromatic hydrocarbons (toluene, xylene, ethylbenzene, methylnaphthalene). In contrast, the enzymatic reactions contributing to the degradation of non-substituted aromatic hydrocarbons are much less understood.

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic compounds consisting of two or more fused aromatic rings. PAHs can be classified into two groups based on the number of aromatic rings present in the structure, low molecular weight PAHs contain 2 or 3 aromatic rings and high molecular weight PAHs contain 4 or more aromatic rings. Even though it has been shown that PAHs can be degraded anaerobically, very little is known regarding the mechanisms and types of microorganisms responsible for this activity. To date, only two reports in literature describe pure cultures that are capable of anaerobically degrading PAHs under nitrate-reducing conditions (McNally *et al.*, 1998; Rockne *et al.*, 2000). Recent investigations into the degradation of PAHs under anaerobic conditions have focussed on naphthalene as a model system to further the understanding of the enzymatic reactions governing the biodegradation process (Meckenstock and Mouttaki, 2011). Naphthalene is the most volatile and least complex of the PAHs, and also the energy of dissociation of the C-H bond of naphthalene in position 1 or 2 (about 480 kJ/mole) is similar to the first bond dissociation energy for benzene (Lardin *et al.*, 2001).

Anaerobic degradation of non-substituted aromatic hydrocarbons such as benzene and naphthalene has been described in several phyla and classes of bacteria. Presently the detailed mechanism involved in the initial activation reactions is still unknown, but the biochemical steps involved are slowly being elucidated.

The aims of this chapter were to:

- Determine hydrocarbon degradation specificity of the enrichment culture
- Determine optimum physicochemical conditions for hydrocarbon degradation
- Assess the role of co-metabolism during hydrocarbon degradation
- Complete a final diversity assessment.
- Start to characterize possible mechanisms of hydrocarbon degradation

4.2 Materials and methods

4.2.1 Enrichment culture selection

It was concluded in Chapter 3 that the Free State Groundworks enrichment culture warranted further investigation. Thus the term 'enrichment culture' in this chapter solely refers to the Free State Groundworks enrichment culture.

4.2.2 Enrichment culture diversity assessment by PCR-DGGE

The culture diversity was assessed as described previously in section 3.2.3. For each 16S rRNA gene sequence, the most closely related sequence and the most closely related cultured bacterial strain was retrieved from the GenBank database by using BLAST and from the Ribosomal Database Project-II (RDP) by using the SEQUENCE MATCH tool. The sequences were aligned and the phylogenetic trees were calculated with the neighbor-joining method using tools on the RDP website (<http://rdp.cme.msu.edu/>) (Cole *et al.*, 2014). Bootstrap values were determined from 100 iterations.

4.2.3 Enrichment culture diversity assessment by clone library

Culture diversity was assessed by sequencing 16S rRNA gene clone libraries. Clone libraries were constructed in similar fashion to the steps performed in section 3.2.3, with the exception that DGGE was not performed and the complete 16S rRNA gene was ligated into the vector system. Briefly, gDNA was extracted from cultures growing under anaerobic conditions at 25°C and 16S rRNA gene amplification performed. The 16S rRNA gene product was excised from the gel, purified and ligated into the pSMART® HCKan vector system and cloned in *E.coli* Top10 competent cells.

Vectors insert were evaluated by restriction fragment length polymorphism (RFLP). Vector inserts were sequenced as described in section 3.2.3.4. The 16S rRNA sequence data was analysed using QIIME (Caporaso *et al.*, 2010), default settings were used unless otherwise specified. Briefly, operational taxonomic units (OTUs) were picked based on sequence similarity using `pick_de_novo_otus.py` (Edgar, 2010) using the clustering algorithm UCLUST (default identity = 97%) and representative sequences for each OTU was picked using `pick_rep_set.py`. Chimera detection was performed using `identify_chimeric_seqs.py` using ChimeraSlayer (Haas *et al.*, 2011). Taxonomy was assigned to the OTU representative

sequences with the RDP Classifier 2.2 using `assign_taxonomy.py` (Wang *et al.*, 2007) and a phylogenetic tree constructed RDP as discussed above.

4.2.4 Functional gene screening

The enrichment culture was evaluated for the presence of only *bssA* genes with primer sets 1 and 2 as described in section 3.2.4, however, multiple reactions were performed at a range of annealing temperatures (from 45°C to 60°C).

4.2.5 Method standarization for hydrocarbon concentration analysis

All hydrocarbon concentrations were determined using head space solid phase micro extraction (HS-SPME) coupled to GC-FID. SPME holders and fibres [100 µm poly(dimethylsiloxane) (PDMS)], sample vials and PTFE-silicone septa were obtained from Supleco. Standard solutions of PAHs were dissolved in acetone and stored at 4°C. A dilution series of working standards were prepared just before extraction and analysis. A GC-2010 (Shimadzu) with a FID detector (Shimadzu) was used for analysis. The capillary column was a FactorFour VF-5ms (60 m, 0.32 mm ID, 0.25 µm film thickness) (Varian) with H₂ as carrier gas at a flow rate of 32,2 cm/sec. A split-splitless injector in the splitless mode was used. The oven temperature was set at 100°C for 1 min, increased to 300 °C at a rate of 10°C/min and held for 15 min. The temperature of the injector and detector were respectively 230°C and 310°C.

Before the SPME device was used and before any sample analysis, the fibre was conditioned by exposing the fibre to the GC injector port at 250°C for 1 hour. Fibre blanks were run at regular intervals to ensure the fibres were fully conditioned and that no interference from the fibres was present in the GC chromatograms.

For analysis, 4 mL of the samples were placed in 10 mL Headspace Screw Neck Vials (Separations) and extractions were performed by exposing the 100 µm PDMS fibre over the heated sample. Extraction was performed for 60 min at 50°C in a Reacti-Therm III Heating Module (Thermo Scientific). After sampling, the fibre was withdrawn into the holder, placed in the GC injector port and thermally desorbed for 2 min at 250°C. Reinserting the SPME fibre after the run did not show carryover. Analyses of obtained peaks were performed using the Lab Solution software (Shimadzu).

4.2.6 Determination of anaerobic hydrocarbon degradation potential of the enrichment culture during short term incubations

A 10% (v/v) inoculum from the enrichment culture was grown in aerobic conditions in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) until late exponential phase (determined experimentally to be at $OD_{600} \sim 0.8$). Cells were harvested and washed three times with 20 mM MOPS buffer, pH 7.0, before being resuspended in Bushnell Haas broth ($OD_{600} \sim 0.5$) containing no carbon source (3.2.5.2). Resuspended cells were transferred to autoclaved serum vials, sealed with a combination of Teflon-faced chlorobutyl stoppers and aluminium crimps (Bellco Glass, Vineland, N.J., U.S.) and purged with O_2 -free N_2 (Air Liquide, Alrode, S.A.) to establish anaerobic conditions. Following purging, the serum vials were transferred into an anaerobic glove box (Coy Laboratories, Grass Lake, M.I., U.S.) with a 10% CO_2 / 10% H_2 / 80% N_2 headspace (Air Liquid, Alrode, S.A.) and subjected to 24 hours of starvation before inoculation (Watanabe *et al.*, 2000).


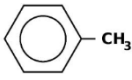
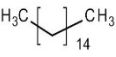

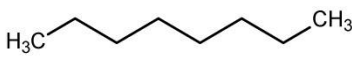
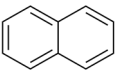
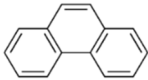
4.2.6.1 Hydrocarbons as sole carbon source in Bushnell Haas broth

Bushnell Haas broth (4 mL) containing cells, prepared above, was added to individual 10 mL Headspace Screw Neck Vials (Separations) containing the various hydrocarbons at set concentrations of 1% (v/v) (Table 4.1) (Greene *et al.*, 2002). Before culture addition naphthalene and phenanthrene was dissolved in acetone (1% m/v), poured into empty serum vials and left in a fume hood until the acetone evaporated. Naphthalene and phenanthrene was also dissolved in 2,2,4,4,6,8,8,-heptamethylnonane (HMN) added as liquid carbon sources (Garcia-Junco *et al.*, 2003). Vials were incubated at 25°C for two weeks in the anaerobic chamber. Analysis was performed on the vials as described in section 4.2.5.

4.2.6.2 Hydrocarbons as additional carbon source in LB-medium

Cells were prepared as described in section 4.2.6 with the exception that washed cell pellets were resuspended in LB medium with the addition and analysis of hydrocarbons as described in section 4.2.6.1 above.

Table 4.1: Hydrocarbons tested for degradation.

Hydrocarbon tested	Structure	Concentration	Aqueous Solubility
Benzene		1% (v/v)	2.053 mL/L*
Toluene		1% (v/v)	0.6 mL/L*
Hexadecane		1% (v/v)	0.1163 µL/L*
Cyclohexane		1% (v/v)	0.08 mL/L*
Octane		1% (v/v)	9.96 µL/L*
Naphthalene		1% (m/v)	30 mg/mL*
Phenanthrene		1% (m/v)	1 mg/mL*

* Yalkowsky *et al.*, 2010

4.2.6.3 Determination of optimum pH and temperature for naphthalene degradation

Cells were prepared as described in section 4.2.6 with the exception that the standardized inoculums ($OD_{600} \sim 0.5$) were resuspended in Bushnell Haas broth adjusted to pH values of 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 with HCl and NaOH depending on the required pH. For optimum temperature assessment a standardized inoculum was resuspended in Bushnell Haas broth (pH 7.5) and incubated at 15°C, 20°C, 25 °C, 30°C and 37°C. Naphthalene concentration analysis was done on the vials as described in section 4.2.5.

4.2.6.4 Effect of increasing concentrations of naphthalene and phenanthrene on the enrichment culture grown

Anaerobic LB medium was prepared as described in section 3.2.5 and cells were prepared as described in section 4.2.6. The medium was supplemented with 0 mg/L, 2000 mg/L, 4000 mg/L and 10 000 mg/L naphthalene and phenanthrene before being inoculated (5%) with the cells. Growth was assessed at 25°C monitoring turbidity by monitoring the optical density of

each sample on a Spectronic® Genesys™ 5 at 600 nm. Turbidity was related to cells/mL using standard curves as described below.

4.2.6.4.1 Relating optical density to amount of cells per millilitre

The enrichment culture was grown overnight in LB-medium at 25°C. Grown cells were harvested and washed three times with 50 mM Tris-HCl (pH 7.0) and resuspended to an OD₆₀₀ of 1.041. Dilutions were made to the following OD₆₀₀ values – 0.078, 0.204, 0.310, 0.413, 0.509, 0.607, 0.697, 0.790 and 0.872. For cell counting, all OD₆₀₀ values from 0.078 to 0.509 were further diluted 10 times and all OD₆₀₀ values above were diluted a further 20 times.

On a Petroff-Hausser Counter (Hausser Scientific), 5 large blocks were counted consisting of 16 smaller blocks. The volume of a small square was calculated as follows:

$$\text{Volume of small square} = 0.02 \text{ mm (height)} \times 0.0025 \text{ mm}^2 \text{ (area)}$$

$$\text{Thus, Volume of small square} = 0.00005 \text{ mm}^3 = 5 \times 10^{-8} \text{ mL}$$

The number of cells in suspension was calculated as follows:

$$\text{Number of cells in suspension} = (\text{Total cells}/\text{total blocks}/\text{volume}) \times \text{dilution factor}$$

(Total blocks = 80 small blocks)

A standard curve of OD₆₀₀ vs Cells/mL was constructed (Figure 4.1).

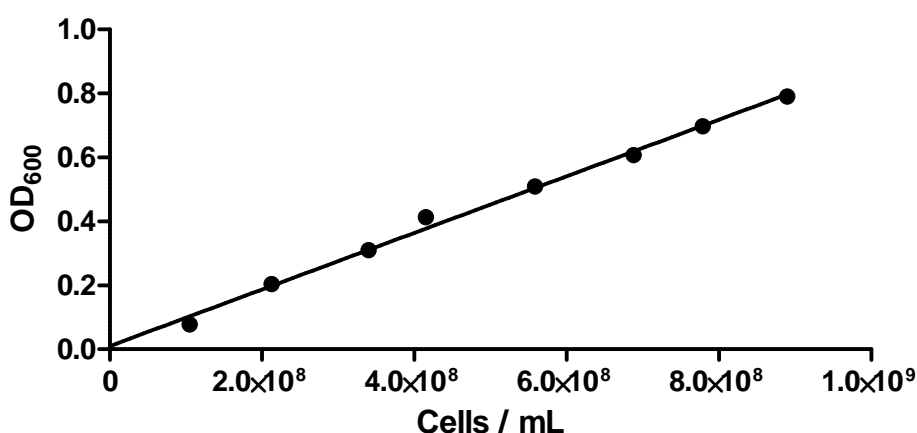


Figure 4.1: Standard curve relating OD₆₀₀ to the amount of cells per milliliter (R²=0.9949).

4.2.6.5 Determination of anaerobic growth on naphthalene and phenanthrene with acetate as co-substrate

Cells were prepared as described in section 4.2.6. The anaerobic Bushnell Haas broth was supplemented with 30 mg/L naphthalene and phenanthrene and 100 mg/L acetate before being inoculated (5%) with the prepared cells. Control vials contained only naphthalene or phenanthrene and a second control contained only cells. Growth was assessed at 25°C monitoring turbidity by measuring the optical density of each sample on a Spectronic® Genesys™ 5 at 600 nm. Turbidity was related to cells/mL using a standard curve as described in section 4.2.6.4.1.

4.2.6.6 Effect of different co-substrates on nitrate reduction and growth of naphthalene grown enrichment cultures

Bushnell Haas broth and cells were prepared as described in sections 3.2.5.2 and 4.6.2 (below) respectively. To each vial, naphthalene (30 mg/L) and an additional carbon source was added to a final concentration of 100 mg/L, carbon sources tested was glucose, pyruvate, fumarate and acetate. Cultures were regularly monitored for turbidity by measuring the optical density of each sample on a Spectronic® Genesys™ 5 at 600 nm (turbidity was related to cells/mL using a standard curve as described in section 4.2.6.4.1) as well as nitrite (as described in section 3.2.6.3) and ATP concentrations (as described below). Naphthalene concentration analysis was performed on the vials as described in section 4.2.5.

4.2.6.6.1 ATP concentration determination

ATP concentrations were determined with the CellTiter-Glo® Luminescent Cell Viability Assay (Pr) according to the manufacturer's specification. Briefly, the CellTiter-Glo® Reagent was assembled by mixing the appropriate volume of room temperature CellTiter-Glo® Buffer with the lyophilized CellTiter-Glo® Substrate. Samples were analysed by adding 100 µL sample per well in a clear walled 96-well plate. To determine background luminescence control wells contained only growth medium and no cells. To each well containing sample, 100 µL of the prepared CellTiter-Glo® Reagent was added and mixed for 2 min followed by incubation for 10 min at room temperature to stabilize the luminescent signal. Mixing of samples, incubation and luminescence determination was performed on the GloMax®-Multi Detection system (Pr) containing the GloMax®-Multi Luminescence Module.

Luminescence was related to moles ATP using a constructed standard curve (Figure 4.2). The standard curve was constructed by measuring the luminescence of varying concentration (10 nM to 1 μ M or 10^{-10} to 10^{-12} moles) of ATP disodium salt (Sigma-Aldrich) as described above.

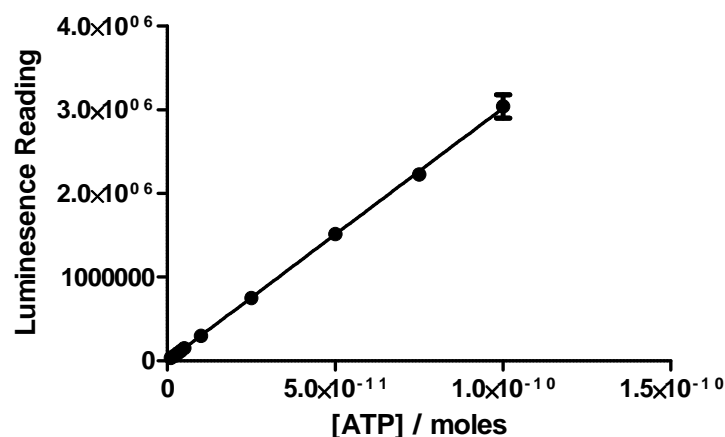


Figure 4.2: Standard curve relating luminescence reading to the amount of moles ATP per 100 μ L ($R^2 = 0.9959$).

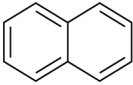
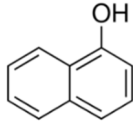
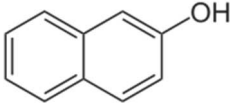
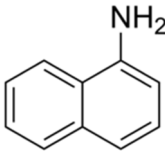
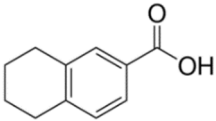
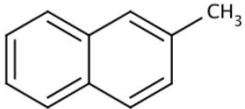
4.2.6.7 Growth on phenanthrene covered agar plates

The spray-plate method was performed as described by Kiyohara and co-workers (1982). Briefly, the enrichment culture was transferred to MacConkey agar plates (17 g/L peptone, 10 g/L lactose, 1.5 g/L bile salts, 5g/L NaCl, 0.03 g/L neutral red, 14 g/L agar). The surface of the plate was then sprayed with a phenanthrene solution in acetone and the acetone was allowed to evaporate from the surface. Following the evaporation of the acetone, a visible opaque phenanthrene coating remains on the surface of the agar plate. Plates were incubated at room temperature in the anaerobic chamber and routinely checked for clear zones.

4.2.7 Growth of enrichment culture on various naphthalene derivatives

Bushnell Haas broth and cells were prepared as described in sections 3.2.5.2 and 4.6.2 respectively. To individual vials various naphthalene based compounds (Table 4.2) were added to a final concentration of 30 mg/L. Cultures were regularly monitored for turbidity by measuring the optical density of each sample on a Spectronic® Genesys™ 5 at 600 nm (turbidity was related to cells/mL using a standard curve as described in section 4.2.6.4.1) as well as nitrite (as described in section 3.2.6.3) and ATP concentrations (as described in section 4.2.6.6.1).

Table 4.2: Naphthalene derivatives and structures assayed for growth potential.

Compound	Structure	Solubility in water at 25°C
Naphthalene		30 mg/L*
1-Naphthol		870 mg/L*
2-Naphthol		740 mg/L*
1-Naphthylamine		1700 mg/L*
2-Naphthoic acid		22.38 mg/L*
2-Methylnaphthalene		24.6 mg/L*

* Yalkowsky *et al.*, 2010

4.2.8 Screening for degradative metabolites of naphthalene derivatives

Bushnell Haas broth and cells were prepared as described in sections 3.2.5.2 and 4.2.6 respectively. Naphthalene was added to a final concentration of 30 mg/L. Vials were incubated at 25°C for 5 days in the anaerobic chamber. Activated naphthalene derivative standards were prepared by dissolving 100 mg/L of each compound listed in table 4.2 in acetone. Analyte extraction by SPME was performed on the vials as described in section 4.2.5, but analysis was performed on GC-MS. A Trace GC Ultra (Finnegan) coupled to a Trace DSQ MS (Finnegan) was used for analysis. The capillary column was a FactorFour VF-5ms (30 m, 0.25 mm ID, 0.25 µm film thickness) (Varian) with He as carrier gas at a flow rate of 2 mL/min. A split-splitless injector in the splitless mode was used. The oven temperature was programmed at 60°C for 2 min, increased to 300 °C at a rate of 15°C/min and held for 10 min. The temperature of the injector was 250°C, the detector gain was set at

1×10^5 . Analyses of obtained peaks were performed using the Lab Solution software (Shimadzu).

4.2.9 Protein profile characterization during growth on aromatic hydrocarbons

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess if there are any differences in the total proteins of the enrichment cultures grown exclusively on naphthalene and phenanthrene when compared to the enrichments grown in the presence of acetate, as discussed in section 4.2.6.5, as well as on the naphthalene derivatives, as discussed in section 4.2.8. Standardized culture samples ($OD_{600} \sim 0.5$) were pelleted by centrifugation ($8000 \times g$, 5 min) and resuspended in $20 \mu\text{L}$ 1 x SDS Loading Buffer. Samples were boiled for 10 min before being loaded on the gel. The relative molecular masses (M_r) of the proteins can also be estimated by comparing the electrophoretic mobility with the masses of known proteins, by using a 10% resolving gel and a 4% stacking gel (Laemmli, 1970). SDS-PAGE was performed using the “Mighty small” miniature slab gel electrophoresis unit, SE 200 (Hoefer Scientific Instruments, MA, USA) according the manufacturers specifications.

The set of pre-stained protein standards (Bio-Rad) included recombinant protein standards with the following molecular masses: 250 000 Da, 150 000 Da, 100 000 Da, 75 000 Da, 50 000 Da, 37 000 Da, 25 000 Da, 15 000 Da and 10 000 Da.

The gel was stained with Coomassie® Brilliant Blue R-250 using the Fairbanks method (Fairbanks *et al.*, 1971).

4.2.9.1 Fragment analysis by MS/MS

After the protein samples were separated by SDS-PAGE, bands of interest (bands present in samples that are absent in controls and also bands that appear more prominent than compared to controls) were excised from the acrylamide gels, digested in-gel with trypsin and the peptides generated were extracted for NanoLC/MS/MS analysis (Shevchenko *et al.*, 2006). Briefly, to the excised gel bands $100 \mu\text{L}$ of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) was added followed by incubation for 30 min with occasional vortexing. After incubation $500 \mu\text{L}$ of acetonitrile was added and samples were incubated at room temperature with occasional vortexing, the acetonitrile was removed. Trypsin buffer (13 ng/ μL trypsin, 10 mM ammonium bicarbonate, 10% (vol/vol) acetonitrile)

was added to cover gel pieces and incubated on ice, after 30 min samples were checked to see if the gel pieces were still saturated with trypsin buffer, if not, more buffer was added. After 90 min 10-20 μ L trypsin buffer was added to cover all gel pieces and samples were incubated overnight at 37°C for digestion. Following digestion 100 μ L of extraction buffer (1:2 vol/vol 5% formic acid/acetonitrile) was added and tubes were incubated at 37°C in a shaker. For NanoLC/MS/MS analysis, 20 μ L formic acid (5% vol/vol) was added into each tube. Tubes were vortexed, incubated for 5 min in a sonication bath and centrifuged (15 min, 10,000 rpm).

Five microliter (5 μ L) of each digestion reaction was individually injected and concentrated on a C18 reverse phase trapping column and eluted onto, and separated by, a custom packed C18 reverse phase column. The peptides were separated and eluted from the column with 0.1% formic acid containing a 10 to 25% acetonitrile gradient over 60 min. The eluted peptides were analyzed on a 4000 QTRAP® System (AB SCIEX) hybrid triple quadrupole ion trap mass spectrometer with a nanospray source at 350nL/min using a Nano HPLC (Agilent). A survey scan between 400 and 1200 Da were performed, scanning for eluting peptides. An enhanced resolution scan was performed on peptides to determine the charge state of each peptide before fragmenting the peptides in the collision cell. Peptides selected for fragmentation were dynamically excluded for 30 seconds after 3 occurrences before it could again be selected for fragmentation. The peptide sequence information obtained from this MS/MS experiment was analyzed by an in-house Mascot Server (Matrix Science) using the Swiss-Prot database (Bairoch and Apweiler, 2000).

4.2.10 Screening for naphthalene degradation gene

An oligonucleotide primer pair (Table 4.3) was utilized to screen for the presence of the gene encoding 2-naphthoyl-CoA reductase (Ncr) (Morris *et al.*, 2014). This gene has been identified in known anaerobic naphthalene degraders. Genomic DNA from the enrichment culture was interrogated with the primer set in 50 μ L reactions containing 5-50 ng of DNA template. PCR conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 45 s, 55°C for 1 min and 72°C for 2 min followed by a final extension step at 72°C for 10 min.

Table 4.3: Primers used for 2-naphthoyl-CoA gene fragment amplification.

Primer	Sequence	Reference
NcrF	5'-CGT TAT WCK CCY TGC CGT G-3'	(Morris <i>et al.</i> , 2014)
NcrR	5'-CGA TAAG CCA TRC ADA TRG G-3'	(Morris <i>et al.</i> , 2014)

4.3 Results and discussions

4.3.1 Enrichment culture diversity and functional screening

Enrichment of the Free State Groundworks soil culture was performed for the period of a year with regular reinoculation into fresh media with diesel as a sole carbon source. During this time culture diversity was assessed at three different time intersections. This final diversity assessment was performed +/- a year after enrichment was initiated.

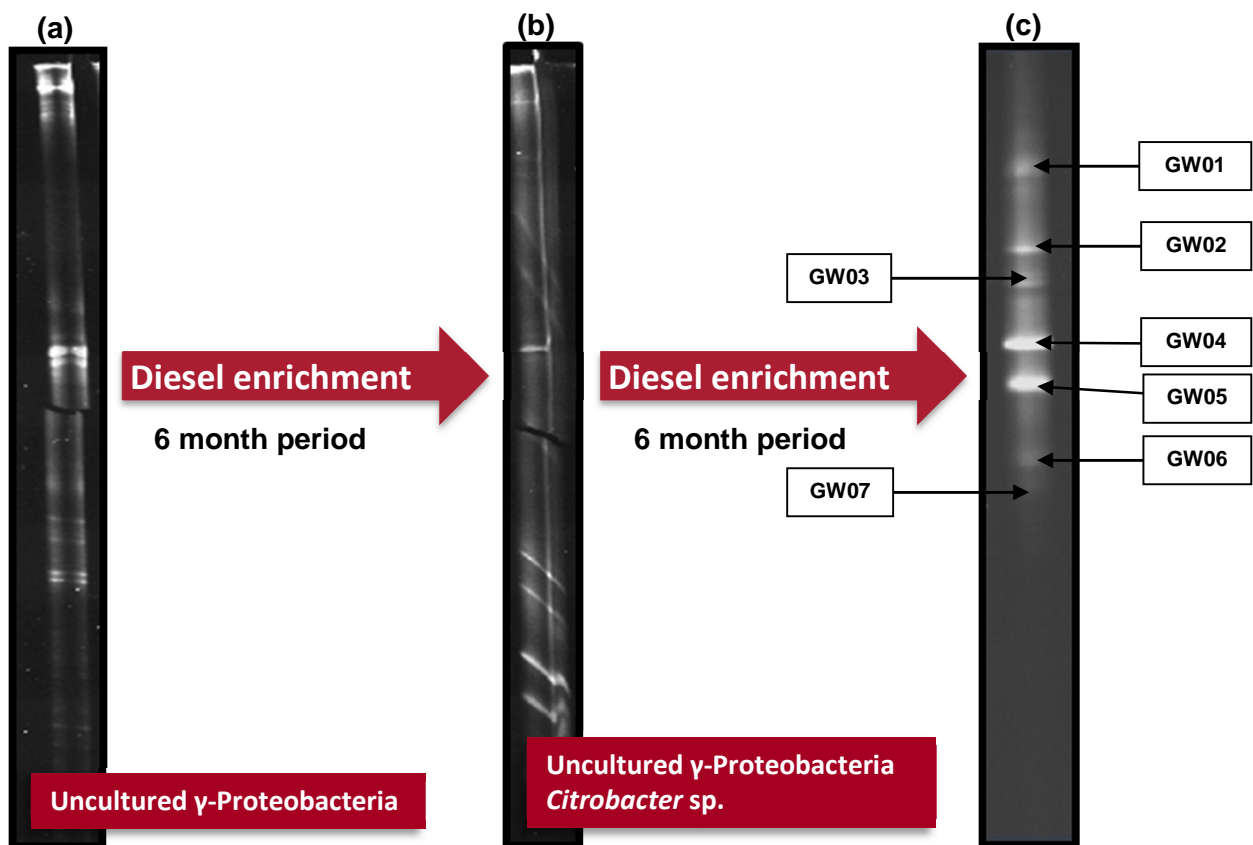


Figure 4.3: Sample diversity assessed over time as enrichment on diesel as sole carbon source occurred. (a) Initial soil diversity. (b) Diversity after 6 month enrichment on diesel as sole carbon source. (c) Diversity after 1 year enrichment on diesel as sole carbon source.

A definite shift in population can be observed (Figure 4.3) in the enrichment culture over this time period. The initial soil diversity was dominated mainly by uncultured γ -Proteobacteria, however, enrichment with diesel as sole carbon source showed a shift in the diversity and the emergence of *Citrobacter* sp. and *Edwardsiella* sp.. Further enrichment passaging appeared to have selected for the predominant presence of *Citrobacter* sp. in the enrichment culture (Table 4.4, Figure 4.4).

Table 4.4: Closest GenBank reference obtained for DGGE band sequences from the enrichment culture.

Phylotype	Phylogenetic Affiliation	Closest Relative	Identity
GW01	γ-Proteobacteria	<i>Citrobacter</i> sp. Z7	100%
GW02	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain: GTC 01552	99%
GW03	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain: GTC 01552	100%
GW04	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain: GTC 01552	100%
GW05	γ-Proteobacteria	<i>Citrobacter amalonaticus</i> strain: SA01	99%
GW06	γ-Proteobacteria	<i>Citrobacter freundii</i> strain: ADS47	99%
GW07	γ-Proteobacteria	<i>Citrobacter freundii</i> strain: ADS47	99%

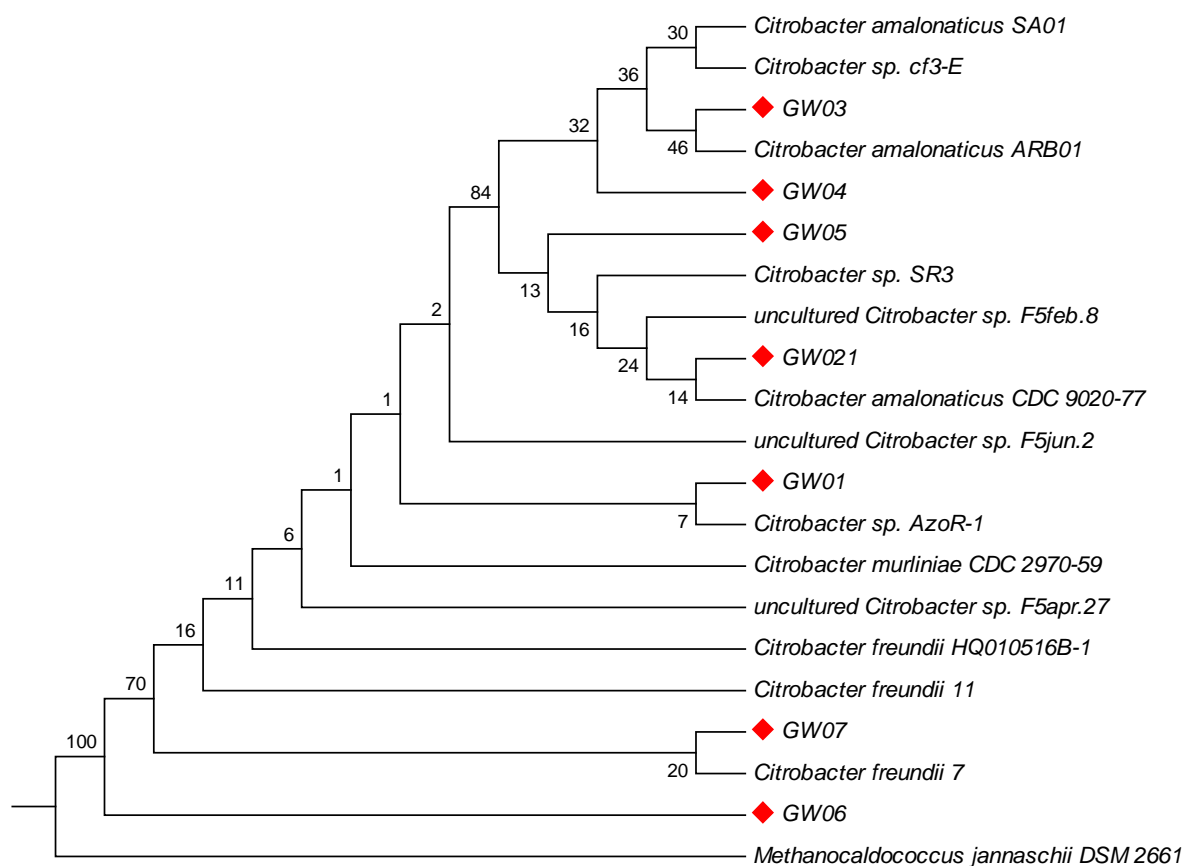


Figure 4.4: Phylogenetic tree of bacterial 16S rRNA gene sequences in the enrichment culture and reference sequences from the Ribosomal Database Project (RDP).

One consideration with diversity assessment using DGGE is that shorter segments of the 16S rRNA gene (+/- 600bp) are analysed. Not shown in table 4.4 is that although top BLAST hits were related to *Citrobacter* sp., however, hits with *Edwardsiella* sp. were also obtained with similar E-values and identity matches. Thus, a 16S rRNA clone library was constructed in an attempt to sequence the entirety of the 16S rRNA genes in the enrichment and analyse the obtained sequence data using QIIME. QIIME (Quantitative Insights Into Microbial Ecology, canonically pronounced 'chime') is software designed for microbial analysis and is

routinely used for the analysis of nucleic acid sequence data from fungal, viral, bacterial and archaeal communities. QIIME provides three high-level protocols for OTU picking, separated into *de novo*, closed reference and open-reference OTU picking. For the analysis of the 16S rRNA gene library, *de novo* was used as no external reference sequence collection is available for the environmental enrichment. The OTU picking step assigns similar sequences to OTUs by clustering sequences based on a similarity threshold where sequences, which are similar at or above the threshold level, represent the presence of a taxonomic unit in the sequence collection. For the analysis a 97% threshold was utilized. A total of 40 colonies were picked, RFLP analysis indicated the presence of the 16S rRNA insert in 27 selected colonies (an example of the *Eco*RI digested plasmids can be seen in figure 4.5), these inserts were subsequently sequenced.

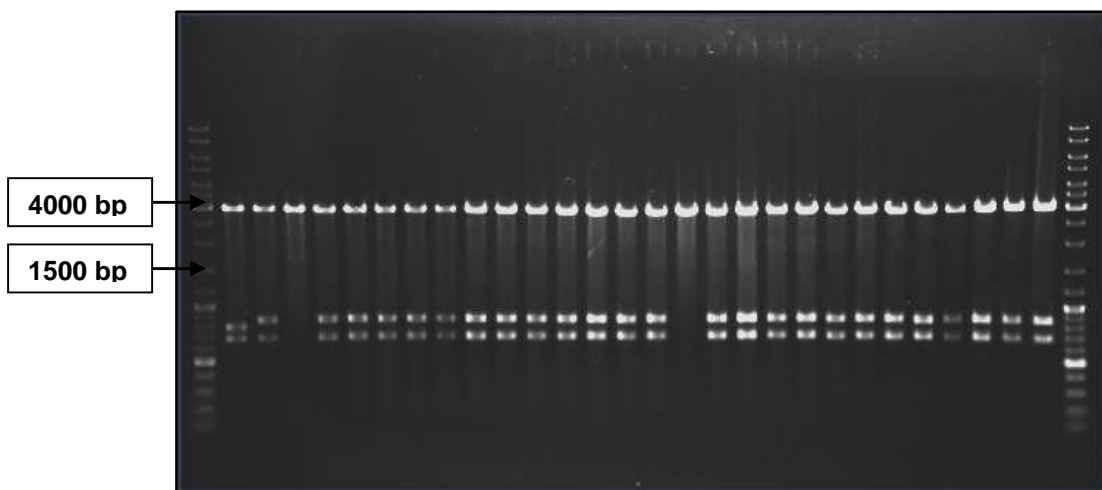


Figure 4.5: Plasmids containing the 16S rRNA gene product digested with *Eco*RI.

The OTU picking software only identified two distinct OTUs in the enrichment, with 86.6% of the sequences clustering together (Figure 4.6 and 4.7). OTUs are comprised out of many related sequences, as such a representative sequence for each OTU was selected for downstream analysis. QIIME assigned the taxonomy of both representative sequences in the genus *Citrobacter* and further BLAST analysis resulted in identifying both the sequences as *Citrobacter freundii* strains (Table 4.5). ClustalΩ alignment of the two representative sequences (Table 4.6) indicates a high sequence similarity between the two sequences, but with some internal differences, as would be expected from different strains, similar results were obtained when alignment was performed in QIIME.

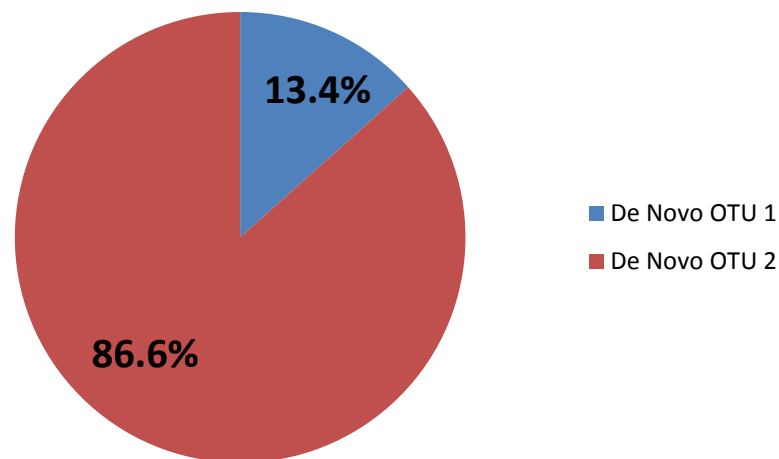


Figure 4.6: Percentage of obtained sequences from 16S rRNA gene library clustering together.

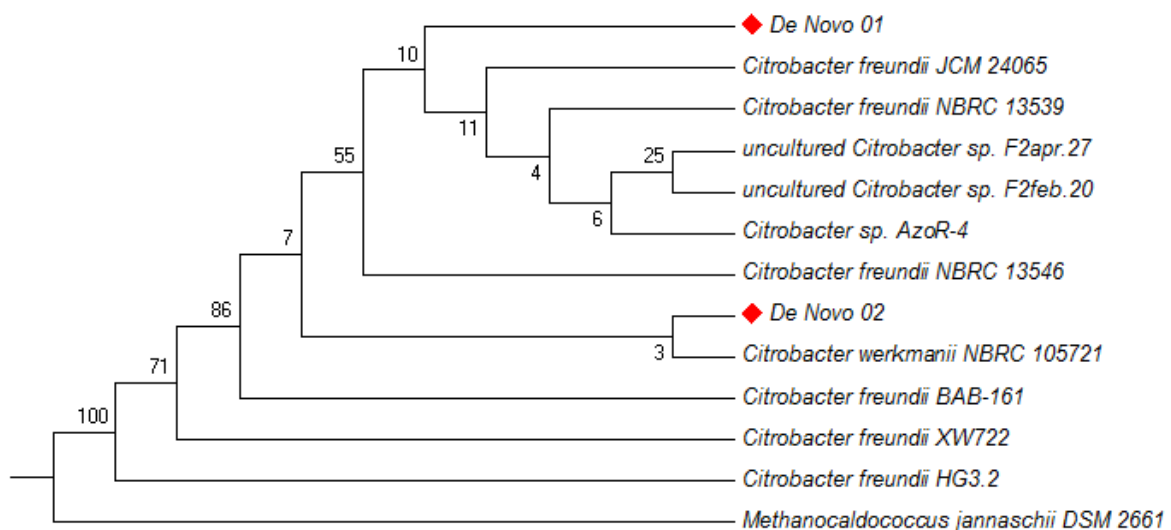


Figure 4.7: Phylogenetic tree of representative bacterial 16S rRNA gene sequences in the enrichment culture and reference sequences from the Ribosomal Database Project (RDP).

In literature Grishchenkov and co-workers (2002) described the bacterial strain *Citrobacter freundii* BS2211 as being capable of mediating the anaerobic degradation of biphenyl, a polycyclic aromatic hydrocarbon, coupled to nitrate reduction. To our knowledge that is the only other report of *C. freundii* involved in anaerobic hydrocarbon degradation.

Table 4.5: Closest GenBank reference obtained for the two representative sequences from the enrichment culture.

	Sequence	Closest Relative	Identity
<i>De novo 01</i>	<p>gcgttgggagctctcccatatggtcgacctgcaggcggccgcgaattcactagtattaga gttgatcatggctcagattgaacgctggcggcaggcctaacacatgcaagtcgaacggta gcacagaggagctgtctccttgggtgacgagtgccggacgggtgagtaatgtctgggaaa ctccccgatggagggggataactactggaacggtagtaataaccgcataacgctgcaa gaccaaaagggggacctcggcctctgcatcggatgtgccagatgggattagcta gtaggtgggtaacggctcacctaggcagcagatccctagctggtctgagaggatgaccag ccacctggaactgagacacggctccagactcctacgggaggcagcagtggggaatattg cacaatggcgcaagcctgatgcagccatgccgctgtatgaagaaggcctcgggtgtg aaagtaacttcagcaggagggaaggcgttgggttaataaccgcaacgattgacgttactc gcagaagaagcaccggtaactcctgcccagcagccgctgtaatacggagggtgcaa cggttaacggaattactggcgtaaagcgcacgcaggcggctgtcaagtcggatgtgaa atccccggcctcaacctgggaactgcatccgaaactggcaggctagagttctgtagaggg gggtagaattccagggtgtagcggtaaatgcgtagagatctggaggaataccgggtggcga aggcggccccctggacaagactgacgctcaggtgcgaagcgtggggagcaaacag gattagataccctgtagtccacgcgtaaacgatgtcagctggagggtgtgcccttagg cgtggctccggagcctaaccgcttaagtcgaccgctggggagtagcccgcaaggttaa aactcaaatgaattgacggggcccgcaaacgggtggagcatgtggttaattcgatgca acgcaagaacacttactactctgacatccagagaacttagcagagatgctttgtgccttc gggaactctgagacagggtgctgactggctgctcagctcgtgtgtgaaatgtgggtaag tcccgcaagcagcgaaccctatccttgttgcacggttagcgggtaagcgggaactcaagga gactgcccagtgataaactggggaagggtggggatgacgtcaagtcacatggccctacg agtagggctacacacgtgctacaatggcatatacaagagaagcagcctcgcgagagc aagcggaacctataaagtatgtcgtatgctggatggagctgcaactcagcctcaatgaa cggaaatcgtagtaactggtgacagaatgccacgggtaatacgtccggccttgtaaca caccgcccgtcacaccatgggagtggttgcaaaagaagtaggtagcttaacctcggga gggcttaccacttgtgattcatgactgggtgaagtcgaacaaggaaccaatcgaatt cccggcggccatgcccgc</p>	<i>Citrobacter freundii</i> strain HPG143	99%
<i>De novo 02</i>	<p>tgccggccgggaattcgattagattgatcatggctcagattgaacgctggcggcagg cctaacacatgcaagtcgaacggtgacacagagagctgtctcgggtgacgagtgccg gacgggtgagtaattgtctgggaaactccccgatggagggggataactctggaaacggt agtaataaccgcataatgtcgcaagaccaaagggggacctcgggctcttgcctc gatgtgccagatgggattagctagtagtgggttaacggctcactaggcagcagatccct agctggtctgagaggatgaccagccactggaactgagacacggctccagactcctacg ggagggcagcagtgagggaatattgcaaatggcgcaagcctgatgcagccatgccgct gtatgaagaaggcctcgggtgtaagtaacttcagcaggagggaaggcgttgggtta aaccaacagcagattgacgttactcgcagaagaagcaccggctaacctcctgcccagcagcc gggtaatacggagggtgcaagcgttaactggaattactggcgtaaagcgcacgcagg cggctctgcaagtcggatgtgaaatccccgggctcaacctgggaactgcatccgaaactgg caggctagagctctgtagagggggtagaattccagggtgtagcgggtgaaatcgtagagat ctggaggaaatcagggtggcgaaggcggccccctggacaagactgacgctcaggtgcg aaagcgtggggagcaaacaggattagataccctgtagtccacggcgtaaacgatgtcg acttgganggtgtgcccctgaggcgtggctccggagcctaaccggttaagtcgaccgctg gggagtagcggcccaagggttaanactcaaatgaattgacggggcccgcaaacgggt ggagcatgtggttaattcgatgcaacgcgaagaaccttacctactctgacatccagagaa cttagcagagatgcttgggtcctcgggaactctgagacaggtgctgcatggctgctcag ctcgtgtgtaaatgttgggttaagtcggcaacgcagcgaaccttaccttcttggccagc gattcggctcgggaactcaaggagactgccagtgataaactggagggaaggtggggatga cgtcaagtcacatggcccttacgagtagggctacacacgtgctacaatggcatatacaaa gagaagcagcctcgcgagagcaagcggacctcataaagtatgctgtagccggattgga gtctgcaactgactccatgaagtcggagtcgtagtaactgtggatcagaatgccacgggt gaatacgttccccggcctgtacacaccggcctcacaccatgggagtggttgcaaaag aagtaggtagcttaacctcgggaggccttaccacttgtgattcatgactgggtgaagt cgtaacaaggaaccaatcactagtgattcgcggccgctgaggtcgacccatagggga gagctccaacgc</p>	<i>Citrobacter freundii</i> CFNIH1	99%

Table 4.6: ClustalΩ alignment of the two representative sequences.

De_Novo_01	gcgctgggagctctcccatatggtcgacctgcaggcggccgcaattcactagtgattag
De_Novo_02	-----tggcggccgcggaattcgattag
	* ** *****
De_Novo_01	agtttgatcatggctcagattgaacgctggcggcaggcctaacacatgcaagtcaaacgg
De_Novo_02	agtttgatcatggctcagattgaacgctggcggcaggcctaacacatgcaagtcaaacgg

De_Novo_01	tagcacagaggagcttgctccttgggtgacgagtgccggacgggtgagtaatgtctggga
De_Novo_02	tagcacagagagct--tgctctcgggtgacgagtgccggacgggtgagtaatgtctggga
	***** ** *****
De_Novo_01	aactgccgatggaggggataactactgaaacggtagctaataaccgcataacgtcgca
De_Novo_02	aactgccgatggaggggataactactgaaacggtagctaataaccgcataatgtcgca

De_Novo_01	agaccaaagagggggaccttcgggctcttgccatcggatgtgccagatgggattagct
De_Novo_02	agaccaaagagggggaccttcgggctcttgccatcggatgtgccagatgggattagct

De_Novo_01	agtaggtgggtaacggctcacctaggcgacgatccctagctggtctgagaggatgacca
De_Novo_02	agtaggtgggtaacggctcacctaggcgacgatccctagctggtctgagaggatgacca

De_Novo_01	gccacactggaactgagacacggtccagactcctacgggagggcagcagtggggaatattg
De_Novo_02	gccacactggaactgagacacggtccagactcctacgggagggcagcagtggggaatattg

De_Novo_01	cacaatgggcgcaagcctgatgcagccatgccgctgtatgaagaaggccttcgggttgt
De_Novo_02	cacaatgggcgcaagcctgatgcagccatgccgctgtatgaagaaggccttcgggttgt

De_Novo_01	aaagtactttcagcgaggaggaaggcgttgtggttaataaccgcaacgatgacgttact
De_Novo_02	aaagtactttcagcgaggaggaaggcgttgtggttaataaccacagcgatgacgttact
	***** ** *****
De_Novo_01	cgcagaagaagcaccggctaactcogtgcagcagccggtaataaccgaggggtgcaagc
De_Novo_02	cgcagaagaagcaccggctaactcogtgcagcagccggtaataaccgaggggtgcaagc

De_Novo_01	gttaatcggaattactggcgtaaaagcgcacgcaggcggctctgtcaagtcggaatgtaaa
De_Novo_02	gttaatcggaattactggcgtaaaagcgcacgcaggcggctctgtcaagtcggaatgtaaa

De_Novo_01	tcccgggctcaacctgggaactgcatccgaaactggcaggctagagctctgtagagggg
De_Novo_02	tcccgggctcaacctgggaactgcatccgaaactggcaggctagagctctgtagagggg

De_Novo_01	ggtagaattccaggtgtagcggtaaatgcgtagagatctggaggaataaccggtggcgaa
De_Novo_02	ggtagaattccaggtgtagcggtaaatgcgtagagatctggaggaataaccggtggcgaa

De_Novo_01	ggcggccccctggacaaagactgacgctcaggtgcgaaagcgtggggagcaaacaggatt
De_Novo_02	ggcggccccctggacaaagactgacgctcaggtgcgaaagcgtggggagcaaacaggatt

De_Novo_01	agataccctggtagtccacgcgtaaacgatgtcgacttggga-ggtgtgcccttgaggc
De_Novo_02	agataccctggtagtccacgcgtaaacgatgtcgacttgganggtgtgcccttgaggc
	***** *****
De_Novo_01	gtggcttcggagctaacgcgtaagtgcaccgctggggagtaaccgcccgaaggttaa
De_Novo_02	gtggcttcggagctaacgcgtaagtgcaccgctggggagtaaccgcccgaaggttaan

De_Novo_01	actcaaatgaattgacggggcccgcacaaagcggtagcatgtggtttaattcgatgca
De_Novo_02	actcaaatgaattgacggggcccgcacaaagcggtagcatgtggtttaattcgatgca

De_Novo_01	acgcaagaaccttacctactcttgacatccagagaacttagcagagatgctttggtgcc
De_Novo_02	acgcaagaaccttacctactcttgacatccagagaacttagcagagatgctttggtgcc

De_Novo_01	ttcgggaactctgagacaggtgctgcatggctgtcgtcagctcgtgttgaaatggtgg
De_Novo_02	ttcgggaactctgagacaggtgctgcatggctgtcgtcagctcgtgttgaaatggtgg

Table 4.6 (continued):

De_Novo_01	g t t a a g t c c c g c a a c g a g c g c a a c c c t t a t c c t t t g t t g c c a g c g g t t a g g c c g g g a a c t
De_Novo_02	g t t a a g t c c c g c a a c g a g c g c a a c c c t t a t c c t t t g t t g c c a g c g a t t c g g t c g g g a a c t ***** ** * *
De_Novo_01	c a a g g a g a c t g c c a g t g a t a a a c t g g a g g a a g g t g g g g a t g a c g t c a a g t c a t c a t g g c
De_Novo_02	c a a g g a g a c t g c c a g t g a t a a a c t g g a g g a a g g t g g g g a t g a c g t c a a g t c a t c a t g g c *****
De_Novo_01	c c t t a c g a g t a g g g c t a c a c a c g t g c t a c a a t g g c a t a t a c a a a g a g a a g c g a c c t c g c g
De_Novo_02	c c t t a c g a g t a g g g c t a c a c a c g t g c t a c a a t g g c a t a t a c a a a g a g a a g c g a c c t c g c g *****
De_Novo_01	a g a g c a a g c g g a c c t c a t a a a g t a t g t c g t a g t c c g g a t t g g a g t c t g c a a c t c g a c t c c
De_Novo_02	a g a g c a a g c g g a c c t c a t a a a g t a t g t c g t a g t c c g g a t t g g a g t c t g c a a c t c g a c t c c *****
De_Novo_01	a t g a a g t c g g a a t c g c t a g t a a t c g t g g a t c a g a a t g c c a c g g t g a a t a c g t t c c c g g g c
De_Novo_02	a t g a a g t c g g a t c g c t a g t a a t c g t g g a t c a g a a t g c c a c g g t g a a t a c g t t c c c g g g c *****
De_Novo_01	c t t g t a c a c a c c g c c c g t c a c a c c a t g g g a g t g g g t t g c a a a g a a g t a g g t a g c t t a a c
De_Novo_02	c t t g t a c a c a c c g c c c g t c a c a c c a t g g g a g t g g g t t g c a a a g a a g t a g g t a g c t t a a c *****
De_Novo_01	c t t c g g g a g g g c g c t t a c c a c t t t g t g a t t c a t g a c t g g g g t g a a g t c g t a a c a a g g t a a
De_Novo_02	c t t c g g g a g g g c g c t t a c c a c t t t g t g a t t c a t g a c t g g g g t g a a g t c g t a a c a a g g t a a *****
De_Novo_01	c c a a t c g a a t t c c c g c g g c c g c c a t g g c g g c-----
De_Novo_02	c c a a t c a c t a g t g a a t t c g c g g c c g c c t g c a g g t c g a c c a t a t g g g a g a g c t c c c a a c g c ***** * * *

Screening for *bssA* gene products by PCR was performed to determine if the *bssA*-like gene that is identified in the initial soil diversity was still present in the enrichment consortium. As can be seen in figure 4.8, the *bssA* gene (~793 bp) is still amplified, but only with primer set 2, which is specific only for *bssA*, and not with primer set 1, which can produce a positive result in the presence of both *assA* and *bssA* genes. Further sequencing produces the same results as discussed in chapter 3, thus a match with the *bssA* gene from *Thaura aromatica*.

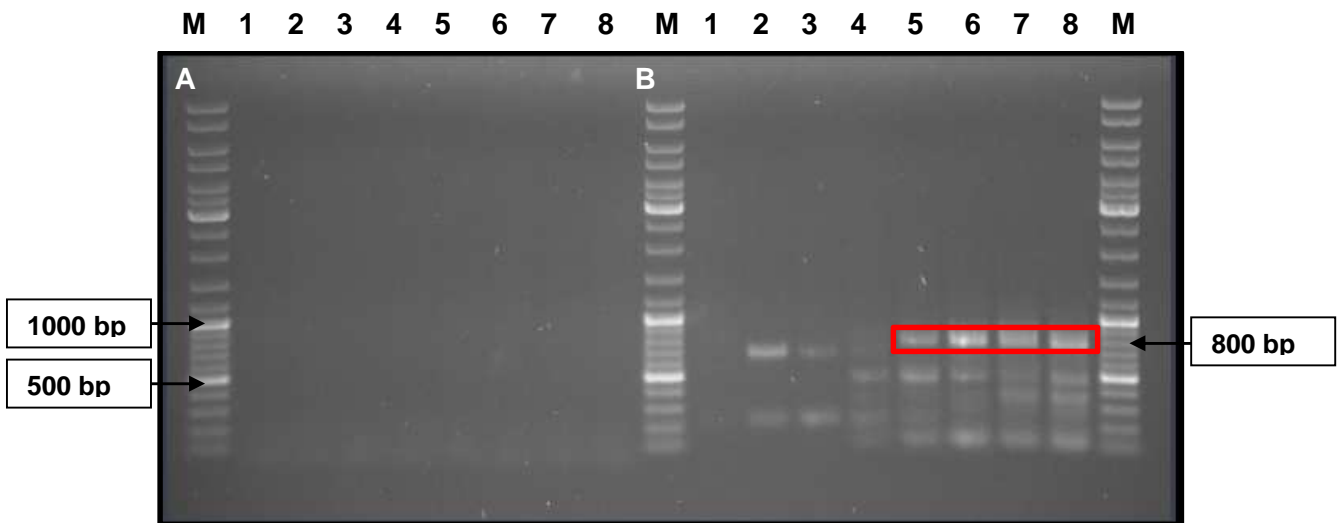


Figure 4.8 : PCR amplification of *bssA* and *assA* genes from the enrichment sample genomic DNA. (A) Primer set 1 and (B) Primer set 2. Lane M, MassRuler™ DNA ladder (Fermentas). Lane 1, 60°C; Lane 2, 59°C; Lane 3, 57°C; Lane 4, 54.3°C; Lane 5, 50.9°C; Lane 6, 48.1°C; Lane 7, 46.1°C; Lane 8, 45°C.

4.3.2 Method standardization for Head Space Solid Phase Micro Extraction (HS-SPME) for the analysis of cyclic and polycyclic aromatic hydrocarbons

Cyclic and polycyclic hydrocarbons, due to their low and varying solubility, are usually present in trace levels in aqueous solutions and as such a suitable and sensitive extraction method is required. Among the various extraction methods developed over the years solid phase micro extraction (SPME) has presented itself as an innovative, sensitive and repeatable extraction technique (Rianawati and Balasubramanian, 2009). SPME has many advantages, it is simple, practical and solventless but sensitive enough for the determination of trace contaminants and at part per trillion levels. SPME also provides integration of multi-stage procedures, extraction, preconcentration and purification into a single step. As hydrocarbons can be highly volatile, SPME provides the perfect solution to avoid analyte loss which can occur with other extraction methods. The optimization of the SPME method for the analysis of cyclic and polycyclic hydrocarbons present in enrichment culture incubations in conjunction with gas GC-FID is presented.

Parameters affecting the hydrocarbon recovery of SPME were investigated using distilled water samples containing known concentrations of the compound under investigation to optimize the SPME method with respect to obtaining high extraction efficiency within a reasonable time frame. Optimal conditions for extraction efficiency were determined by assessing the hydrocarbon peak areas obtained on GC-FID with increased extraction time and temperature.

SPME is an equilibration extraction thus the maximum amount of analyte that can be extracted by the fibre is achieved at the equilibrium time (Rianawati and Balasubramanian, 2009). For this study, SPME extraction was performed on benzene, toluene, naphthalene and phenanthrene. No results could be obtained for phenanthrene using SPME, this might be due to the very low solubility of the compound (1 mg/L) (Aquan-Yuen *et al.*, 1979) and also the very high boiling point (340°C) (Tetko, 2007). For the other three substrates, equilibration was reached after about 50 min.

Temperature plays a very important role in SPME sensitivity as it can affect the migration of the hydrocarbons through the aqueous sample and fibre coating (Louch *et al.*, 1992), thus an increase in temperature can lead to a faster equilibration time. To obtain an optimal sample temperature for extraction the hydrocarbons were extracted under increasing

temperature between 25°C and 65°C. Once again no results could be obtained for phenanthrene but the other three substrates reached equilibration at 50°C. The optimum time experiments were repeated at an incubation temperature of 50°C and at this increased temperature equilibration was reached after only 10 min.

To prevent the carry-over effect, where some analyte is retained on the fibre after injection into the GC-FID, the fibre was exposed to an unoccupied injection port between analyses. The temperature of the injector port was set to 250°C and the fibre was exposed for 20 min. A blank run of the fibre showed no carry-over of compound.

It is of note here that the samples analysed were prepared fresh and analysed immediately. Hydrocarbon samples exposed to the enrichment cultures and incubation for longer periods and will be susceptible to a higher level of compound loss due to evaporation since these compounds are highly volatile and this was corrected by a substrate blank (containing no cells) to determine the amount of degradation. The data in figure 4.9 shows the linearity of the SPME performed on a range of concentrations for benzene, toluene and naphthalene (Figures 4.9 a, b and c).

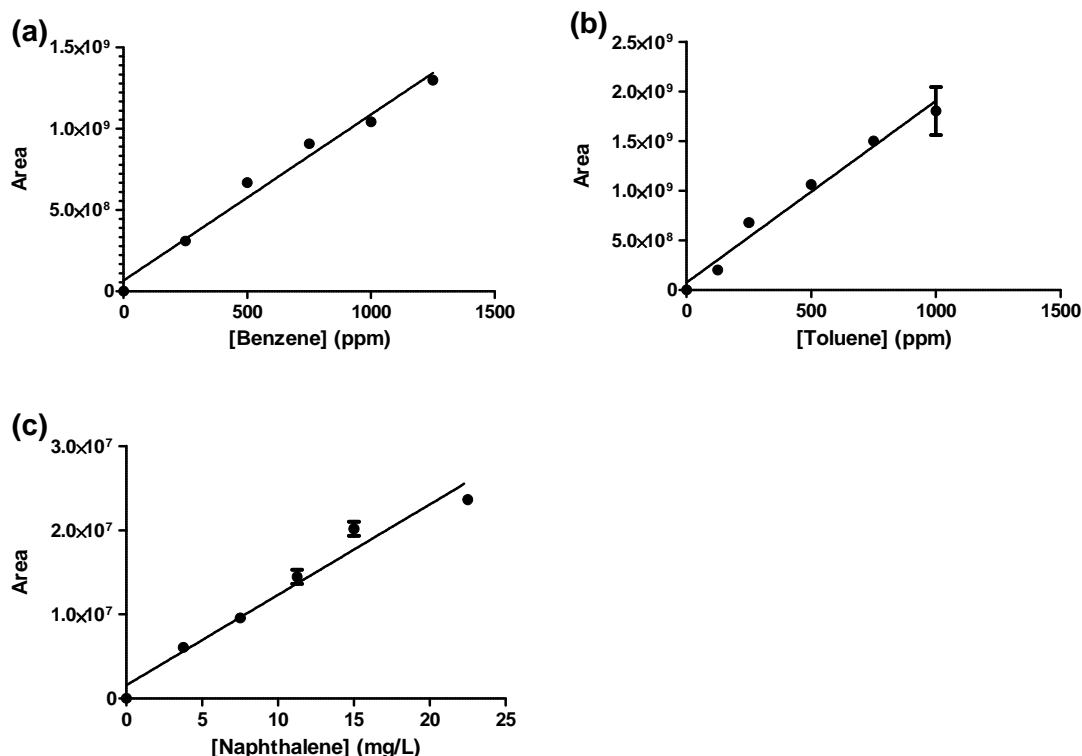


Figure 4.9: Different hydrocarbons analysed using SPME-GC-FID. (a) Benzene ($r^2 = 0.9796$). (b) Toluene ($r^2 = 0.9551$). (c) Naphthalene ($r^2 = 0.9870$).

4.3.3 Anaerobic degradation of hydrocarbons during short term incubations

The enrichment culture was incubated under anaerobic conditions in 10 mL Headspace Screw Neck Vials (Figure 4.10) with the addition of various aromatic, cyclic and straight chain hydrocarbons (Table 4.1). Once the substrate was added, vials were kept closed, no additional substrate or nitrate was added, and an entire vial was used for analysis by SPME and GC-FID after incubation after two weeks, as this was deemed enough time to see any potential hydrocarbon degradation taking place (Weiner *et al.*, 1998).

Out of all the hydrocarbons tested (Table 4.1) the only reduction in hydrocarbon concentration was observed for naphthalene and toluene. An average of ~40% of the provided naphthalene (1%) was degraded within the two week period (Figure 4.11 a), this was only observed with the naphthalene present as a thin film at the bottom of the vials after the acetone evaporated (as described in section 4.2.6.2). Dissolving naphthalene in the non-degradable solvent HMN and adding it as a drop to the surface of the medium, the HMN has a lower density than water, resulted in only 2% of the naphthalene being degraded. This is in contrast to what has been showed in some literature where groups utilize HMN as a carrier for naphthalene that facilitates mass transfer of naphthalene into the medium (Efroymsen and Alexander, 1991). However, dissolving hydrocarbons in HMN has been shown to have enhance hydrocarbon degradation in some strains, but totally inhibit others, especially with the addition of aromatic substrates. The reason for this is uncertain at present uncertain (Allen *et al.*, 1999). Very low levels of toluene degradation was also observed (4% of the 1% added) but no degradation of benzene, hexadecane or octane was observed. Co-metabolism of hydrocarbons was examined in growth experiments using LB medium (Figure 4.11 b) supplemented with the various hydrocarbons (Katsivela *et al.*, 2003) and resulted in an increase in naphthalene degradation as well as the degradation of hexadecane.



Figure 4.10: Anaerobic hydrocarbon incubation in 10 mL screw top vials.

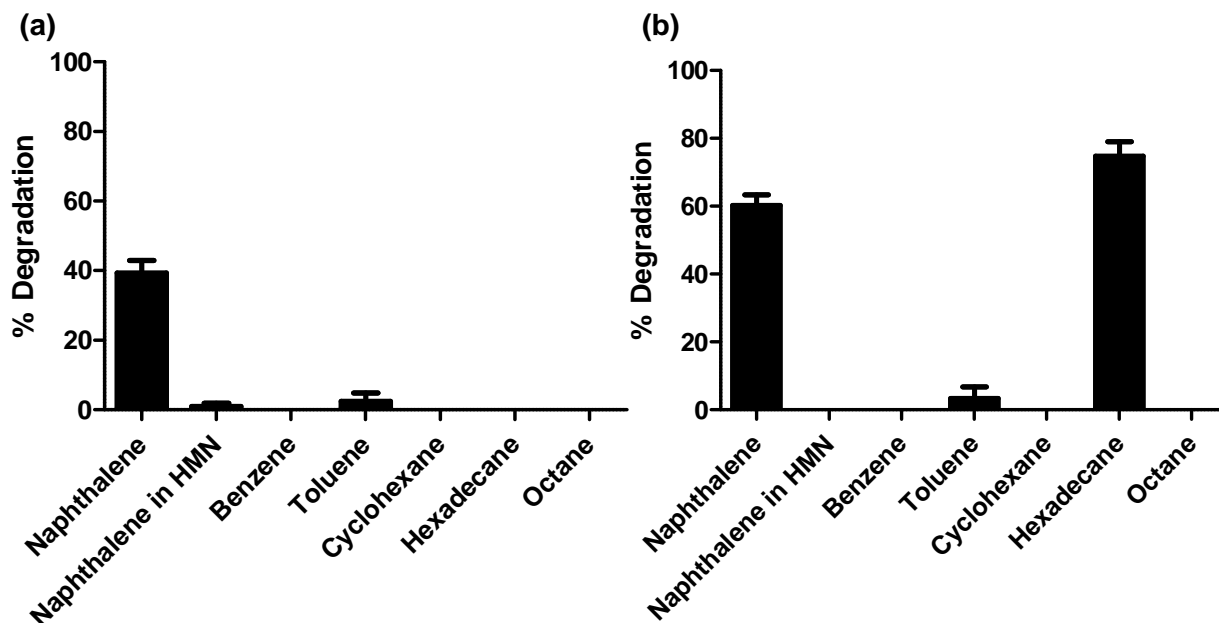


Figure 4.11: Percentage hydrocarbon degradation by the enrichment culture compared to reagent blanks. (a) Hydrocarbons as sole carbon source. (b) Hydrocarbons with LB medium.

To determine if the enrichment culture is capable of growth on phenanthrene, the enrichment culture was plated out on MacConkey agar plates that were covered in phenanthrene. Growing clear zones appearing on the plates was deemed as a positive sign that the culture could degrade phenanthrene (Figure 4.12).

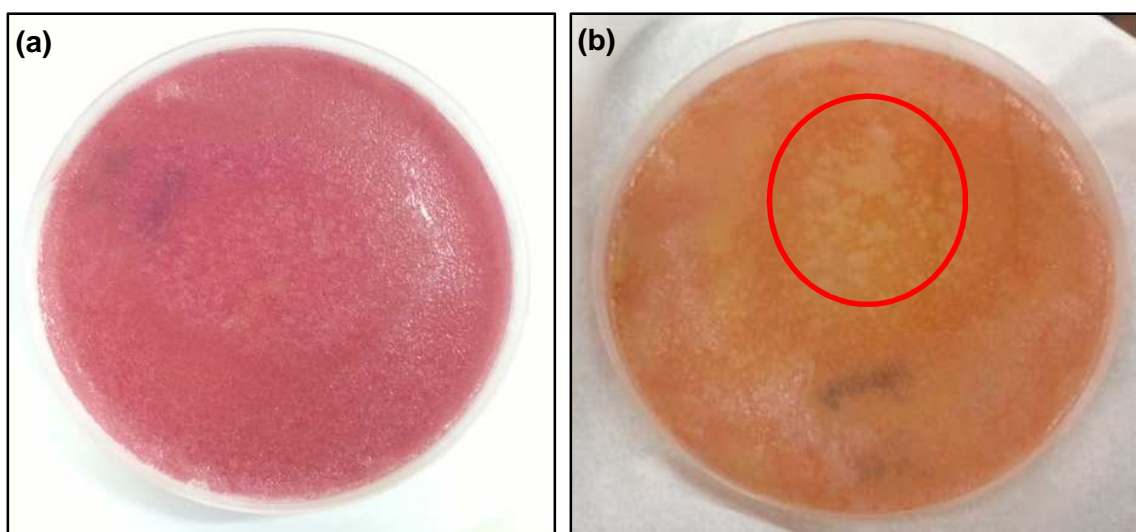


Figure 4.12: Enrichment culture grown on MacConkey plates covered in phenanthrene. (a) Before and (b) after incubation.

Naphthalene being one of the fractions in petroleum has resulted in many groups using naphthalene degrading isolates from petroleum contaminated soils (Vaidya and Kadam, 2011), these results, indicating effective degradation, constitutes the first time a consortium dominated by *Citrobacter* spp. has been described. Previously a *Citrobacter* sp. has only been shown to be capable of degrading biphenyl under anaerobic conditions (Grishchenkov *et al.*, 2002). In literature the rate and degree of naphthalene degradation by cultures can range from very low to complete degradation, with complete degradation usually obtained by the addition of electron acceptor or by reinoculation of fresh cultures. Many studies have found that naphthalene degradation ceases with the depletion of electron donors and Guerin and Boyd found that naphthalene degradation would drop exponentially once their cultures reached the stationary phase (Guerin and Boyd, 1995). As previously stated no additional electron donor was added to the growing cultures due to the volatile nature of the compounds. Other factors influencing the extent and rate of naphthalene biodegradation include pH and temperature (Raïssa *et al.*, 2012). For the enrichment culture, slightly higher levels of degradation were observed at neutral pH values 6.5 to 7.5 (Figure 4.13 a) which is in line with the optimum growth pH for *Citrobacter* sp., 7.2 (Hamilton *et al.*, 2010). The pH may influence the composition of the enrichment culture, resulting in a naphthalene degrading species becoming more dominant (Shiaris, 1989).

Optimum temperature for degradation of naphthalene was observed at 20°C (Figure 4.13 b) with degradation levels dropping as temperatures rise. Optimum temperature can also dictate the rate of naphthalene metabolism by influencing the microbial community, but temperature also has a direct effect on the physical nature and chemical composition of naphthalene (Alquati *et al.*, 2005). Optimal degradation temperatures are usually observed in the range of 30°C to 50°C, but biodegradation of hydrocarbons has been observed at temperatures as low as 0°C and as high as 70°C (R M Atlas, 1981).

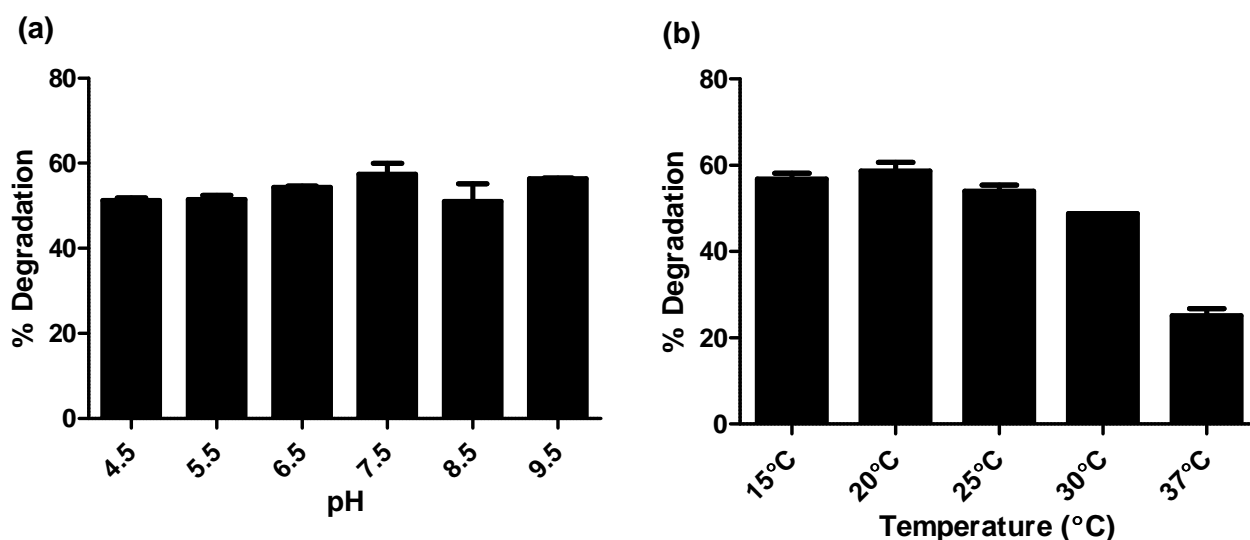


Figure 4.13: Percentage naphthalene degradation by the enrichment culture compared to reagent blanks at (a) different pH values and (b) temperatures.

4.3.4 *In situ* observation during growth on poly cyclic hydrocarbons

4.3.4.1 Toxicity of naphthalene and phenanthrene on the enrichment culture

The ability or inability of microorganisms to survive in hydrocarbon contaminated sites represents a key technical barrier for using microorganisms to study biodegradation of these compounds (van Veen *et al.*, 1997). There have been numerous studies on the potential of degrading hydrocarbons in contaminated sites using microorganisms but survival studies performed on degrading bacteria in contaminated soils are scarce (Park *et al.*, 2004). Naphthalene and phenanthrene toxicity on humans and animals have been investigated extensively (Schreiner, 2003; Stohs *et al.*, 2002) but the knowledge on the effect of these compounds and their intermediates on microorganisms in soils is limited. Naphthalene degrading bacteria are considered to be naturally resistant to the toxicity of naphthalene purely due to their ability to degrade naphthalene (Park *et al.*, 2004). However, Ahn and co-workers (1998) and Garcia and co-workers (Garcia *et al.*, 1998) showed that naphthalene can be toxic to naphthalene degraders under aerobic conditions and nutrient deprivation. Our experimental design included anaerobic conditions, but not nutrient deprived as the enrichment culture was grown in a rich organic medium (LB medium). In contrast to what was shown by Ahn and Garcia, where cultures were grown in a minimal medium, only a slight decrease in growth was observed even with the enrichment grown in the presence of 10 000 mg/L of naphthalene and phenanthrene (Figure 4.14 and Table 4.7) indicating that the upper limits for survival in the presence of these compounds can be overcome quite

easily by providing the enrichment culture with a nutrient rich environment. Naphthalene and phenanthrene crystals were visible in the media throughout the incubation (24 hours) thus the potential for maintaining an equilibrium saturated naphthalene concentration was present (~30 mg/L for naphthalene and ~1 mg/L for phenanthrene) and naphthalene and phenanthrene uptake by viable cells matched the dissolution.

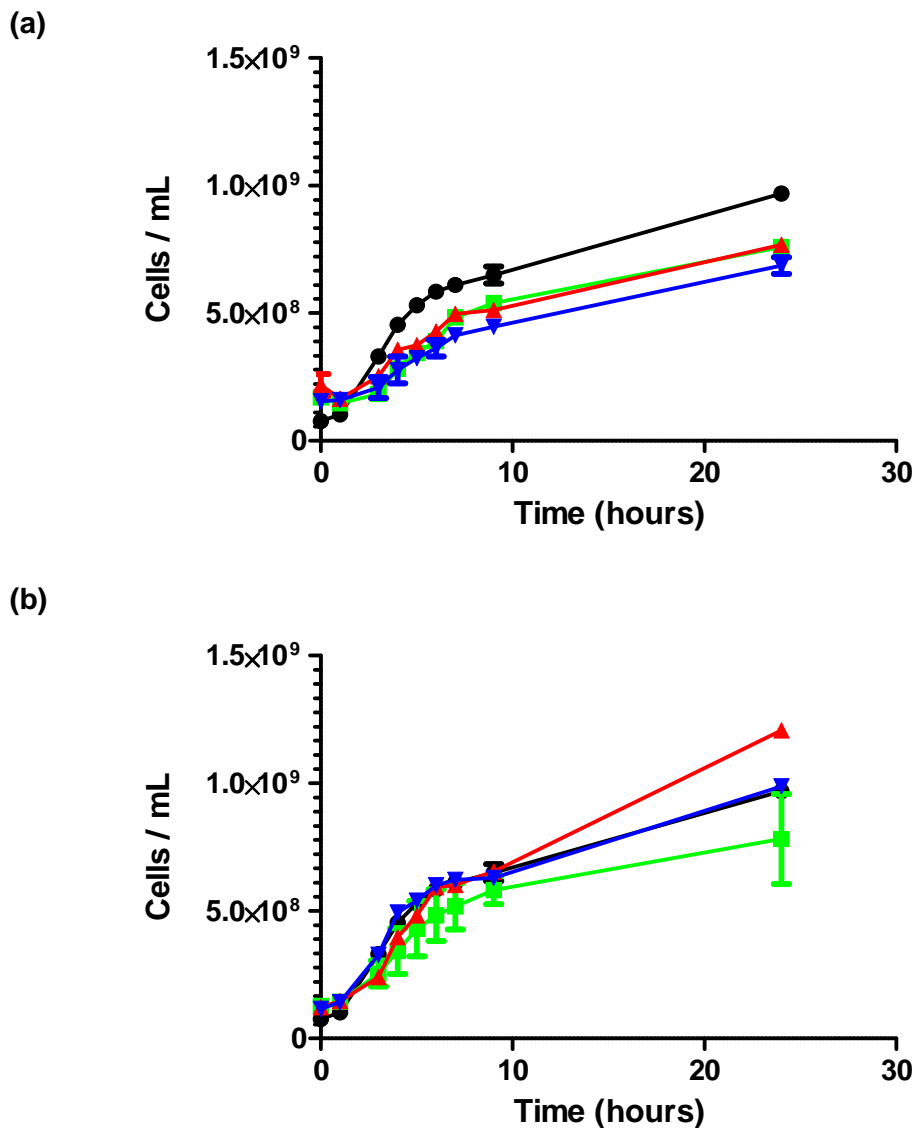


Figure 4.14: Growth of the enrichment culture at various concentrations of (a) naphthalene and (b) phenanthrene). (●) 0 mg/L; (■) 2000 mg/L; (▲) 4000 mg/L; (▼) 10 000 mg/L.

Table 4.7: Doubling time and μ_{max} for the enrichment culture grown on different concentrations of naphthalene and phenanthrene.

	μ_{max} (h ⁻¹)		t_d (h)	
	Naphthalene	Phenanthrene	Naphthalene	Phenanthrene
0 mg	0.121	0.122	5.694	5.694
2000 mg/L	0.096	0.086	7.149	8.039
4000 mg/L	0.082	0.109	8.471	6.373
10 000 mg/L	0.080	0.117	8.694	5.930

4.3.4.2 Growth of enrichment culture on naphthalene and phenanthrene with and without co-substrate

Co-metabolism is the process by which a contaminant, in this case hydrocarbons, can be fortuitously degraded by an enzyme or co-factor produced during the metabolism of another compound, a co-substrate, by a microorganism or culture (Hazen, 2010). Biostimulation by the addition of a co-substrate has been shown to be an effective strategy to enhance the anaerobic degradation of hydrocarbons (Kim *et al.*, 2003; Okolo *et al.*, 2005). Taylor and Jones (2001) as well as Ambrosoli and co-workers (2005) showed that poly cyclic aromatic hydrocarbons can be more effectively removed when using co-substrates as a biostimulation agent, as compared to the addition of electron acceptors and nutrients.

The findings above in section 4.3.3 suggest that the addition of co-substrates, introduced as a nutrient rich medium, improve the degradation of naphthalene and phenanthrene by the enrichment culture. The results of section 4.3.4.1 also suggest that the inherent toxicity of these compounds can be overcome in a minimal medium with the addition of additional substrates. In literature, acetate has been shown to be an effective co-substrate for the enhancement of degradation (Ambrosoli *et al.*, 2005) and a co-metabolism strategy will only stimulate the indigenous microorganism capable of degrading the contaminant and co-substrate, unlike the nutrient rich medium. To verify if the co-substrate has any effect on the enrichment culture, the culture was firstly grown in the presence of naphthalene and phenanthrene with and without a co-substrate, and also with acetate as sole carbon source. The amount of acetate added was stoichiometrically determined as sufficient, so that the enrichment culture would only reduce 10% of the added nitrate, if growing solely on the acetate. In both cases, growth on naphthalene and phenanthrene, the addition of acetate resulted in increased growth rates (Figure 4.5 and Table 4.8).

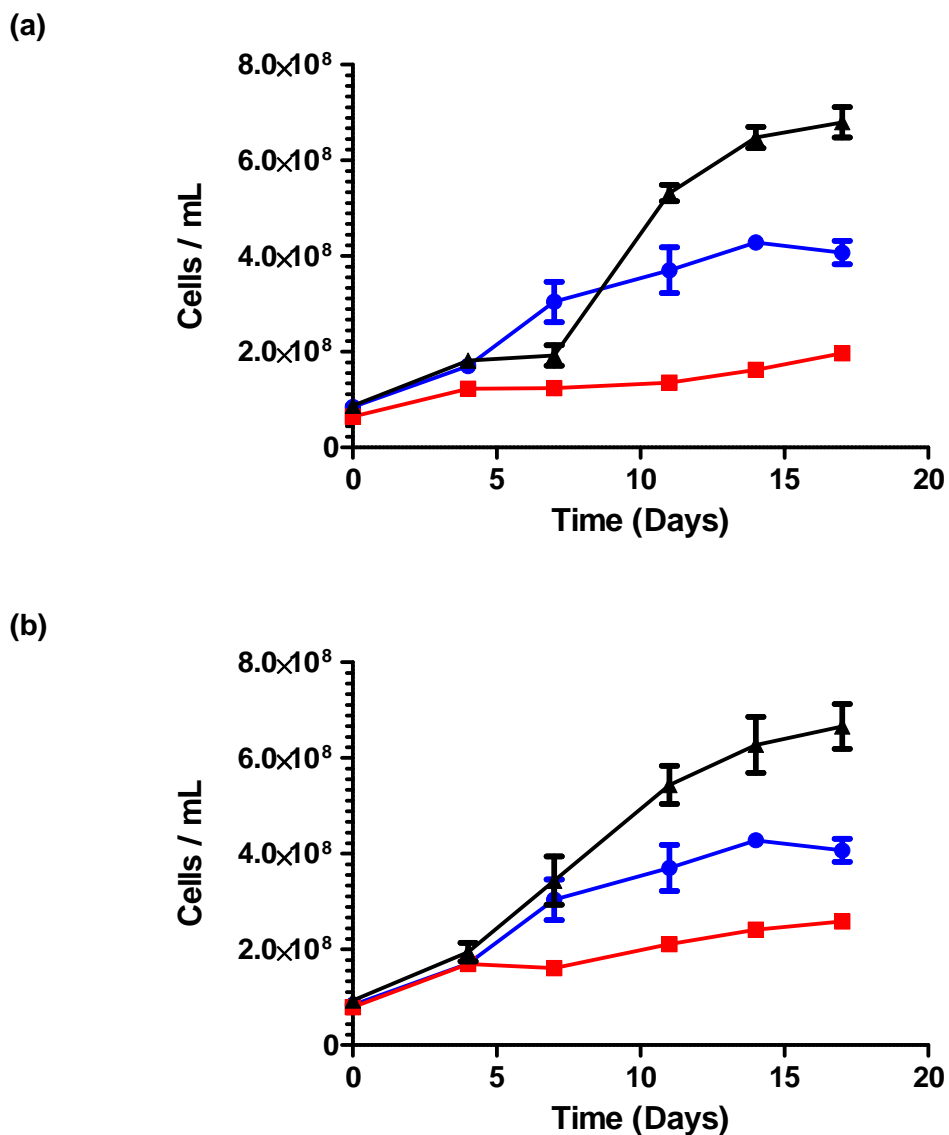


Figure 4.15: Growth of the enrichment culture on (a) naphthalene and (b) phenanthrene with and without the addition of acetate. (▲) PAH + acetate; (●) Acetate; (■) PAH.

Table 4.8: Doubling time and μ_{max} for the enrichment culture grown on naphthalene and phenanthrene with and without acetate.

	μ_{max} (h^{-1})		t_d (h)	
	Naphthalene	Phenanthrene	Naphthalene	Phenanthrene
Acetate	0.059	0.059	11.726	11.726
PAH	0.036	0.035	19.189	19.689
PAH + Acetate	0.089	0.094	7.789	7.397

For effective co-metabolism a suitable substrate is needed to stimulate the appropriate reactions. Other than acetate, methanol, glucose, lactate, sulphate or pyruvate has been shown to serve as substrates during co-metabolic anaerobic reduction (Hazen, 2010). To determine if one of these other co-substrates might increase naphthalene degradation, the enrichment culture was grown in the presence of naphthalene as well as glucose, pyruvate, and acetate. A culture was also grown in the presence of fumarate since fumarate addition for the activation of hydrocarbons for biodegradation has been well described in literature during the degradation of aromatic hydrocarbons (Foght, 2008).

Analysis of the remaining naphthalene in the samples (Figure 4.16) points towards acetate as the preferred co-substrate for naphthalene degradation, with a slight increase in the amount of naphthalene degradation observed, an average of ~40% vs ~47% degraded. Mittal and Rockne (2008) showed an increase in anaerobic naphthalene degradation by *Pseudomonas stutzeri* strain NAP-3 in the presence of acetate, but did not speculate on a mechanism. Both the NAP-3 strain and *Citrobacter* sp. (the dominant member of the enrichment culture) are facultative anaerobic, nitrate reducing Gammaproteobacteria, but the significance of this is currently unknown. Naphthalene degradation also occurred with added glucose, pyruvate and fumarate, but in contrast to acetate, it seemed that the addition of these co-substrates inhibited naphthalene degradation rather than increasing it. Co-substrates have been found to inhibit hydrocarbon degradation in some strains. Ethanol and acetate have been found to decrease toluene degradation in *Pseudomonas putida* TOD102. This was attributed to gene repression by the co-substrates, competitive inhibition of the key enzymes in the degradation mechanism or metabolic flux dilution by the co-substrates (Lovanh and Alvarez, 2004).

When comparing growth, ATP production and nitrate reduction (observed as nitrite production) (Figure 4.16 a, b and c) acetate also seems to be used most effectively as co-substrate. Even though the initial rates were more rapid with glucose, pyruvate and fumarate, a definite lag phase was observed after this initial growth, it could be concluded that they stimulated non-naphthalene degrading members of the enrichment, as further growth was not established until after 20 days of growth. The same observations can be made for ATP production, where an initial use of ATP (during the start of growth) is followed by an increase of ATP for the naphthalene and acetate addition cultures, whereas the glucose addition culture starts to have a slight increase in ATP after 20 days, and once again, the fumarate culture was decreasing. The pyruvate addition culture displayed an ATP curve very similar to the acetate addition curve. All cultures appeared to actively reduce nitrate and produce nitrite during the incubation period.

Our results demonstrated that the addition of acetate supported the naphthalene degrading activities. The effect of acetate in batch experiments probably reflects its use as reductant to maintain enzyme activity and to improve degradation rates. McFarland and co-workers (1992) found similar results for formate as a noncompetitive substrate for the methane monooxygenase activity in methanotrophs responsible for trichloroethylene removal. Acetate is just metabolised to result in the production of specific co-factors that are required by enzymes in the undefined anaerobic naphthalene degradation pathway.

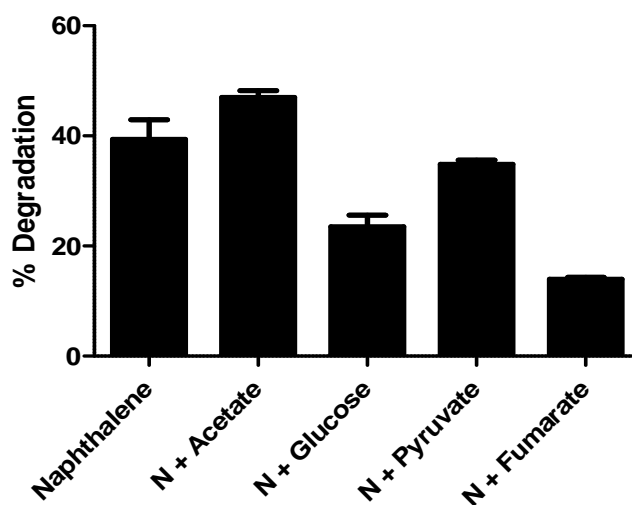


Figure 4.16: Degradation of naphthalene in the presence of different co-substrates.

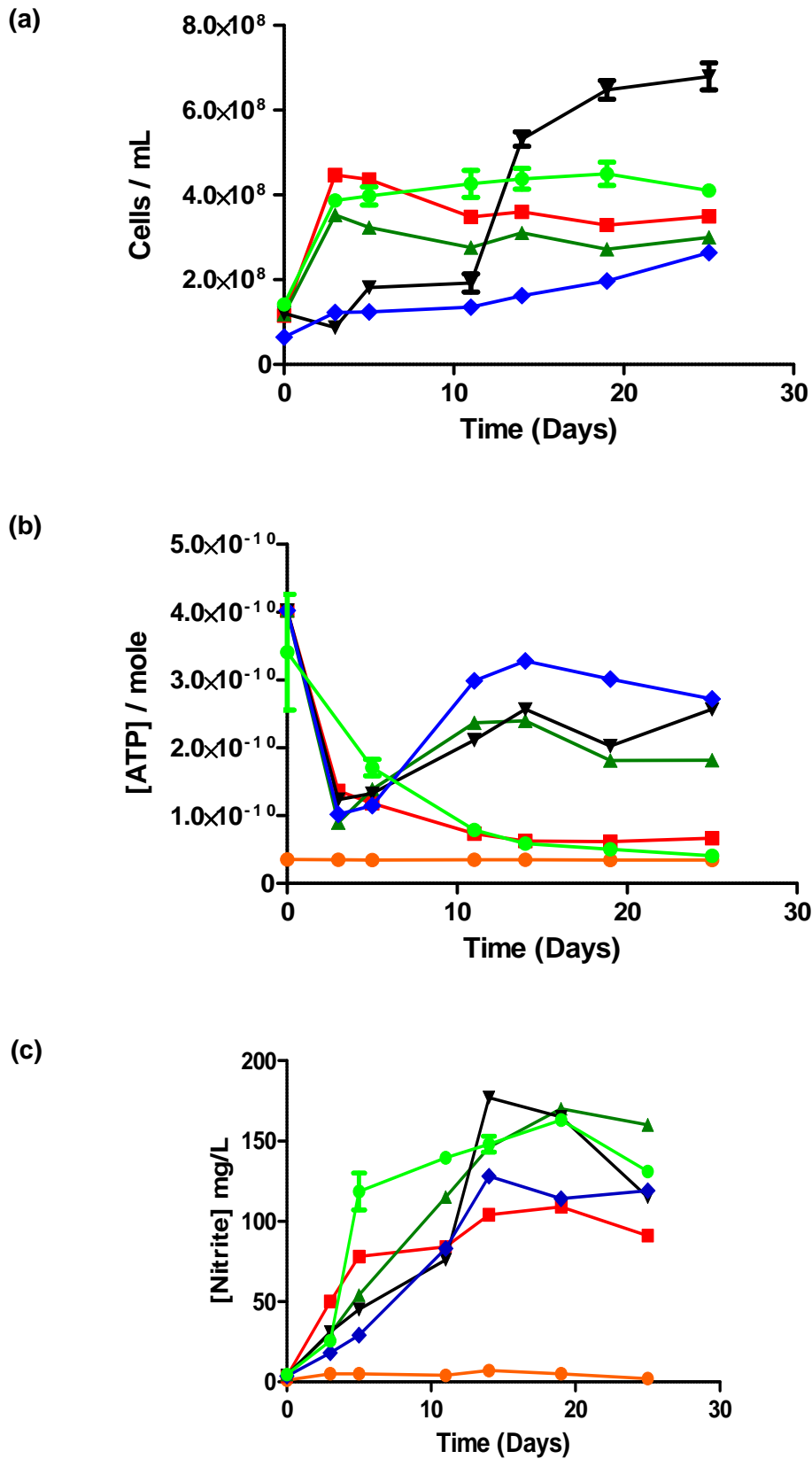


Figure 4.17: Effect of different co-substrates on (a) growth (, control values have been subtracted), (b) ATP production and (c) nitrite production of the enrichment culture. (●) Control; (■) naphthalene + glucose; (▲) naphthalene + pyruvate; (▼) naphthalene + acetate; (◆) naphthalene and (●) naphthalene + fumarate.

4.3.5 Growth and nitrate reduction of various activated naphthalene derivatives

Identification of hydrocarbon degradation metabolites can give an indication of the mechanism employed for degradation, especially if the first intermediate which forms in the initial step in activating the compound can be identified. 2-Naphthoic acid was identified as a metabolite of naphthalene degradation in a sulphate-reducing enrichment culture of marine origin (Zang and Young, 1997) which lead to the assumption that carboxylation was the initial step. Other authors have identified naphthol as a side product in a sulphate-reducing, naphthalene degrading culture suggesting a hydroxylation reaction (Bedessem *et al.*, 1997). In an attempt to identify if the enrichment culture had a preference for any naphthalene degradation intermediates, substrate utilization tests were performed by growing the enrichment culture on various activated forms of naphthalene as the sole carbon source (Figure 4.18a). The enrichment culture could utilize 2-naphthol and 1-naphthylamine after a lag phase of about 10 days. Utilization of 2-naphthol makes sense since *Citrobacter* sp. is a facultative anaerobic bacterium and under aerobic conditions the initial activation of naphthalene would be a hydroxylation step (Seo *et al.*, 2009) resulting in a very similar compound to 2-naphthol, 2,3-dihydroxy-naphthalene. However no oxygen is present in the medium, as indicated by the colourless resazurin, and the hydroxylation reaction would have to occur via some unknown mechanism. Very little, if any, growth was observed with 1-naphthol, 2-naphthoic acid and 2-methylnaphthalene as substrates. ATP production (Figure 4.18b) coupled to growth on 2-naphthol increased steadily for the first 10 days after which it started to decline. ATP production during growth on 2-methylnaphthalene increased without growth correlation, and seems to coincide with nitrate reduction. This might indicate that the enrichment culture is metabolically active on 2-methylnaphthalene even though it does not result in active growth. The anaerobic catabolism of 2-methylnaphthalene has been studied in detail in sulphate reducing microorganisms (Annweiler *et al.*, 2000; Meckenstock *et al.*, 2004), but not in nitrate-reducing cultures. However, the first step in the anaerobic degradation of the unsubstituted compound, in this case the methylation of naphthalene, still remains unclear. Musat and co-workers (2009) found that, when cultures grown on naphthalene were exposed to 2-methylnaphthalene, this compound was only utilized after an extended lag phase and concluded that growth on naphthalene does not automatically induce the capacity for 2-methylnaphthalene degradation. Nitrate reduction occurred in all strains during the incubation time, but from the graph it seems that growth on 1-naphthol and 2-naphthylamine was very actively reducing nitrate and further metabolizing the formed nitrite. Low levels of nitrate reduction was observed in the presence of 2-naphthoic acid and 1-naphthol (Figure 4.18c).

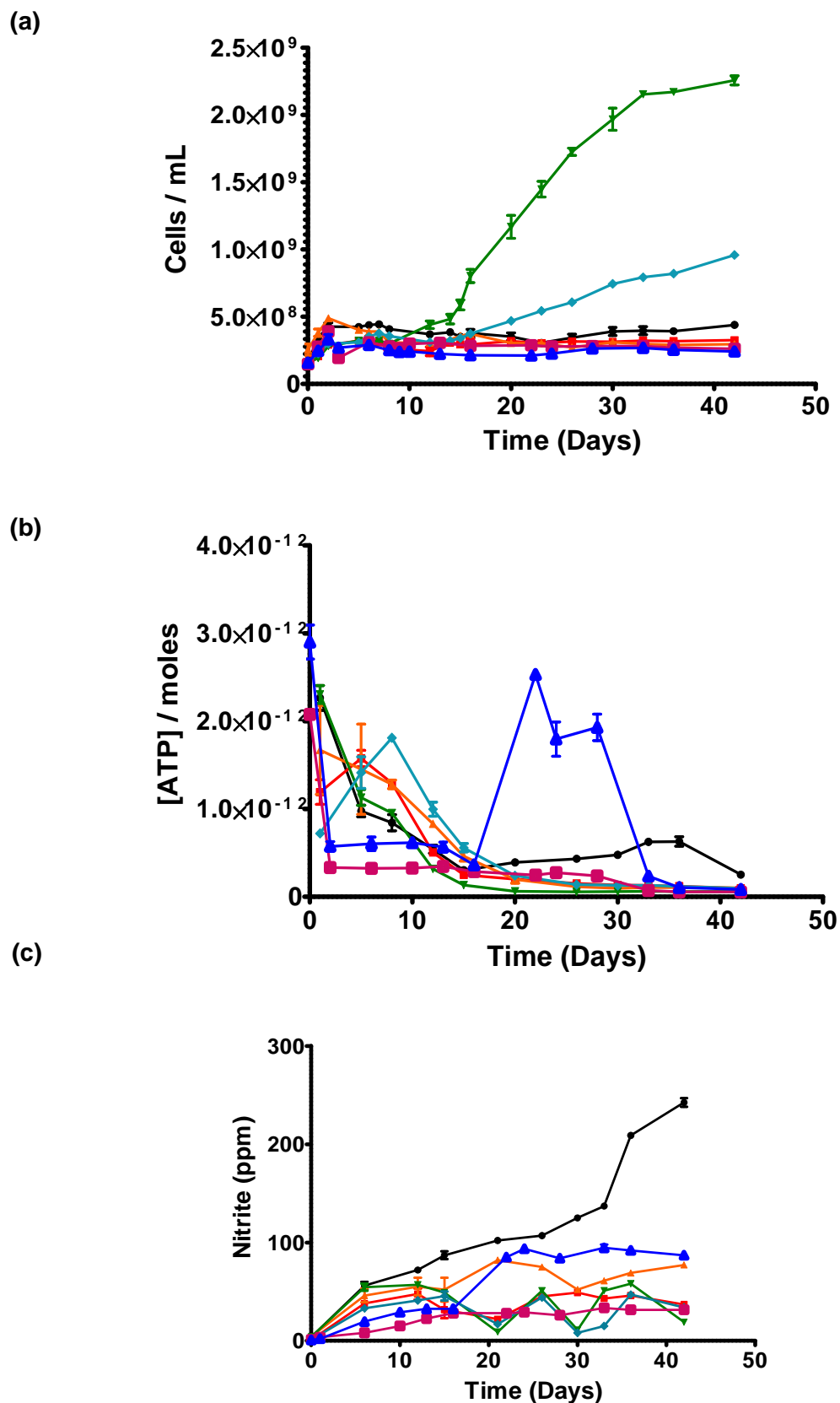


Figure 4.18: Effect of different activated naphthalene derivatives on (a) growth, (b) ATP production and (c) nitrite production, control values have been subtracted. (●) Acetate, (■) 1-naphthol, (▲) naphthalene, (▼) 1-naphthylamine, (◆) 2-naphthol, (■) 2-naphthoic acid and (▲) 2-methylnaphthalene.

To date, the only published pathways for anaerobic naphthalene degradation is coupled to sulphate reduction and proceed via either hydroxylation (Bedessem *et al.*, 1997), carboxylation (Annweiler *et al.*, 2002; Zhang and Young, 1997) or methylation (Safinowski and Meckenstock, 2006a). No published literature has shown metabolic pathways for the biodegradation of naphthalene coupled to nitrate reduction. From our data a few ideas for hydrocarbon degradation mechanisms might be suggested. Bedessem and co-workers (1997) repeatedly found the presence of naphthol in sulphate reducing cultures actively growing on naphthalene and concluded that this could indicate an initial hydroxylation reaction, however they failed to identify a mechanism for this reaction. In the current study no naphthol could be detected in the growth medium of the enrichment cultures while growing on naphthalene when samples were analysed on GC-MS (Figure 4.19). This does not take into account that naphthol might have been present inside of the cells and thus not detected by the headspace analysis. However, the preference of 2-naphthol might also be indicative of a hydroxylation mechanism as it appears that the cells are well equipped to metabolise this compound. Hydroxylation of aromatic hydrocarbons by a nitrate reducing microorganism have been reported for benzene by *Dechloromonas* strain RCB (Chakraborty and Coates, 2005) and ethylbenzene by *Azoarcus* sp. strain EB1 (H A Johnson *et al.*, 2001). The mechanism in *Dechloromonas* strain RCB is unknown but one possible mechanisms might be by the formation of a highly reactive hydroxyl free radical (HO \cdot), which subsequently attacks the aromatic ring (Chakraborty and Coates, 2005), but the enzymes involved in producing this reactive species is unknown. In *Azoarcus* sp. strain EB1, Johnson and co-workers (2001) identified an ethylbenzene dehydrogenase capable of catalysing the anaerobic dehydrogenation of ethylbenzene to 1-phenylethanol, with a water derived oxo or hydroxyl group, as the first step in the anaerobic ethylbenzene degradation pathway.

The same observations can be made regarding the apparent, at least from the ATP and nitrite data, metabolism of 2-methylnaphthalene which might suggest an initial methylation reaction (Safinowski and Meckenstock, 2006a). Methylation to 2-methylnaphthalene was shown in the sulphate reducing enrichment culture N47, but the enzymes involved in this reaction are still elusive. In the current study 2-methylnaphthalene could not be detected by GC-MS.

The growth on 1-naphthylamine, although significant, does more to verify the culture's ability to grown on naphthalene than it does to indicate an intermediate. Babcock and co-workers (1993) showed that their enrichment culture could grow on 1-naphthylamine as sole carbon source, but proposed that the degradation mechanism for this compound would include a

deamination step to naphthalene and then follow the degradation route for naphthalene rather than a direct degradation of the compound.

As such, a degradation pathway could not be elucidated from the experiment reported here, however, it is suggested that the culture may have a preference for 2-naphthol and 2-methylnaphthalene that might indicate hydroxylation or methylation as the initial step of naphthalene degradation. It was decided to attempt to elucidate the activation mechanism via the transcriptome route, which will be discussed in the next chapter.

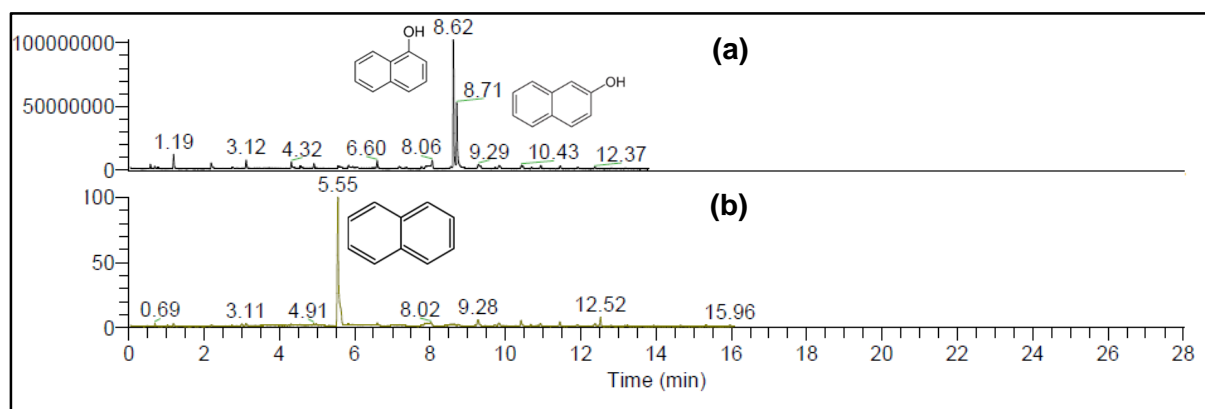


Figure 4.19: GC-MS analysis of (a) 1- and 2-naphthol standard and (b) the enrichment culture growing on naphthalene.

4.3.6 Identification of proteins expressed during growth on polycyclic aromatic hydrocarbons

In order to characterize the molecular interactions involved in the biodegradation process, the proteome profile of the enrichment culture induced by acetate, naphthalene (with and without acetate and fumarate), phenanthrene (with and without acetate), 1- and 2-naphthol, 1-naphthylamine, 2-naphthoic acid and 2-methylnaphthalene were compared as a preliminary screen. The total protein profile of the enrichment culture separated by SDS-PAGE is shown in figure 4.20. Many enzymes involved in the degradation are likely to be expressed constitutively together with house-keeping proteins and are not necessarily induced and as such the proteome profiles and band intensities are largely similar between the different induced groups and the acetate grown control. However, in the presence of naphthalene and phenanthrene 2 proteins bands, ~55 and 45 kDa respectively, were observed. One band, ~37 kDa that is more prominent in the acetate control, two bands present in all the naphthalene derivative samples, ~150 and 130 kDa respectively, and one band, ~25 kDa in the 2-methylnaphthalene grown culture was observed. These bands were excised and subjected to analysis by NanoLC/MS/MS for identification (Table 4.9).

Since various proteins can be approximately the same size it is very unlikely that only one band will be excised from a SDS-PAGE gel. Thus, multiple hits are obtained for each sample. In band 'a', a chaperonin GroEL, quinoprotein alcohol dehydrogenase and export system outer membrane protein TolC were identified. The GroEL belongs to the chaperonin family of molecular chaperones and is required for the proper folding of many proteins (Lee *et al.*, 2002). Quinoprotein alcohol dehydrogenase are common enzymes in alcohol- or alkane degrading bacteria (Groen *et al.*, 1984). Chinnawirotpisan and co-workers (2003) also found that it is involved in catabolic acetate production in *Acetobacter pasteurianus* SKU1108. TolC is an outer membrane protein required for the export of virulence proteins and, of importance to this study, toxic compounds without a periplasmic intermediate (Koronakis *et al.*, 1997). In band 'b', a porin and the elongation factor Tu were identified. Porins span cellular membranes and act as a pore through which molecules can diffuse by passive diffusion. Porins usually facilitate the transport of sugars, ions and amino acids. EF-Tu (elongation factor thermo unstable) is part of the mechanism that synthesizes new proteins by translation at the ribosome.

Considering that naphthalene and phenanthrene are toxic compounds, proteins like the TolC could be related to self-protection of the organism since it plays a common role in the expulsion of diverse molecules including toxins and antibacterial drugs (Koronakis *et al.*, 1997) whilst the alcohol dehydrogenase, EF-Tu and GroEL shows that it is growing in the presence of these compounds. Biodegradation of naphthalene and phenanthrene requires the passage of hydrophobic substrates across the cell membrane and the porins could also be up-regulated for this reason.

Band 'c' and 'd' are the same protein and both are identified as DNA-directed RNA polymerase subunit beta. These bands were present in all samples and may provide evidence that the cells are growing since these proteins are involved in the transcription of DNA into RNA (Igarashi and Ishihama, 1991). The confidence level for the identification of Band 'e' was not very high, but it can possibly be an exonuclease SbcC a gene that affects genetic recombination and the viability of DNA palindromes (Connelly and Leach, 1996). Bands 'f' and 'g' all seem to be up-regulated during the growth on naphthalene and its derivatives. The enzymes identified in these two bands are mostly genes from glycolysis (glyceraldehyde-3-phosphate dehydrogenase A, fructose-bisphosphate aldolase class I and 6-phosphofructokinase), the citric acid cycle (succinyl-CoA synthase subunit alpha, malate dehydrogenase) and the pentose phosphate pathway (deoxyribose-phosphate aldolase, ribose-phosphate pyrophosphokinase and transaldolase B). These bands and their possible gene identities all indicate that the culture is metabolically active. Since this is a mixed

consortium it might be that products formed by one organism are used in glycolysis of one organism but in the pentose phosphate pathway of another. Other enzymes expressed in these two bands once again point towards the cells either protecting themselves or gaining access to the carbon sources (outer membrane protein A, outer membrane porin protein C, outer membrane protein OmpC, outer membrane protein, glutamate/aspartate periplasmic-binding protein, bacterial extracellular solute-binding family protein and dipeptide transporter ATP-binding subunit) or sustaining growth (elongation factor Ts, L-threonine 3-dehydrogenase).

One interesting protein being expressed is the cysteine synthase A, an enzyme that catalyses the production of L-cysteine and acetate, which along with the quinoprotein alcohol dehydrogenase, is the second enzyme identified that might produce acetate for the enrichment culture to use as a co-substrate in naphthalene degradation.

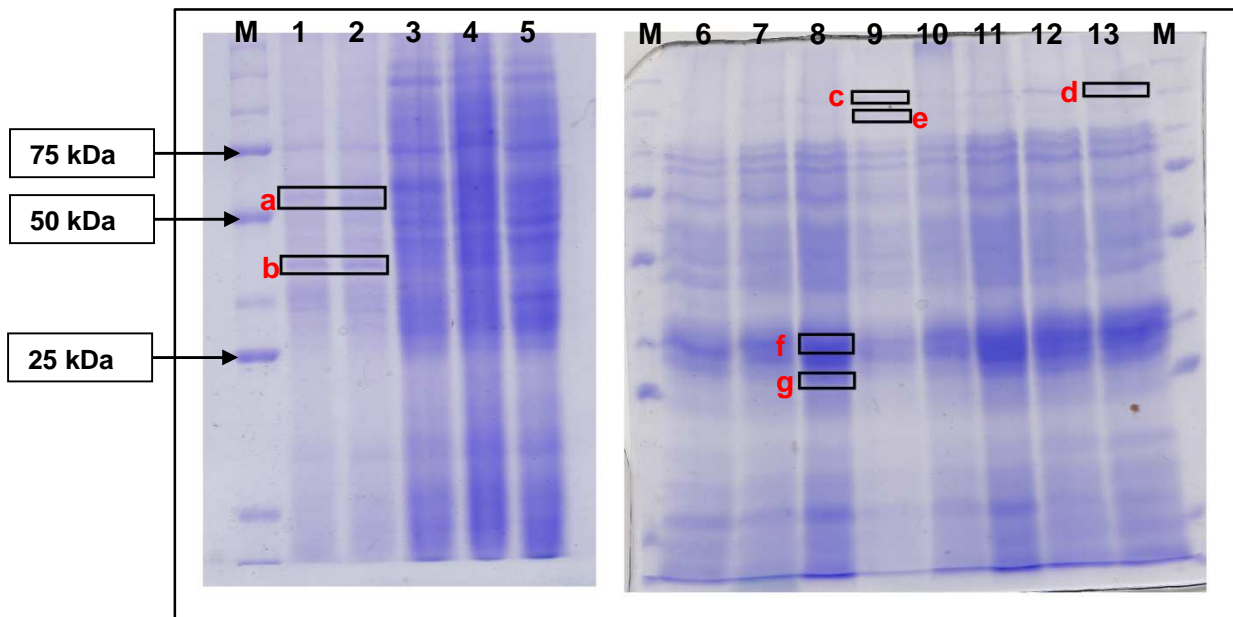


Figure 4.20: SDS-PAGE analysis of die total proteome from the enrichment culture grown in the presence and absence of naphthalene as well as activated naphthalene derivatives. Lane M, Precision Plus Protein™ Standards (Bio-Rad); Lane 1, naphthalene; Lane 2, phenanthrene; Lane 3, acetate; Lane 4, naphthalene + acetate; Lane 5, phenanthrene + acetate; Lane 6, acetate; Lane 7, 1-naphthol; Lane 8, naphthalene; Lane 9, naphthylamine; Lane 10, 2-naphthol; Lane 11, naphthalene + fumarate; Lane 12, 2-naphthoic acid; Lane 13, 2-methylnaphthalene.

Table 4.9: LC/MS/MS identification of excised proteins.

Band	Protein Name	Protein Size (Da)	Species
a	chaperonin GroEL	53080	<i>Pseudomonas stutzeri</i> A1501
	quinoprotein alcohol dehydrogenase	64416	<i>Pseudomonas</i> sp. Chol1
	export system outer membrane protein tolC	53796	<i>Citrobacter</i> sp. 30_2
b	porin	45372	<i>Pseudomonas</i> sp. Chol1
	outer membrane protein (porin)	43120	<i>Pseudomonas</i> sp. Chol1
	elongation factor Tu		<i>Desulfatibacillum alkenivorans</i> AK-01
c	DNA-directed RNA polymerase subunit beta	150376	<i>Citrobacter</i> sp. 30_2
d	DNA-directed RNA polymerase subunit beta	150376	<i>Citrobacter</i> sp. 30_2
e	Exonuclease SbcC	96748	<i>Yersinia enterocolitica</i> IP 10393
f	outer membrane protein A	38321	<i>Citrobacter</i> sp. 30_2
	outer membrane porin protein C	40246	<i>Citrobacter freundii</i> ATCC 8090
	glyceraldehyde-3-phosphate dehydrogenase A	36015	<i>Citrobacter</i> sp. 30_2
	fructose-bisphosphate aldolase class I	38011	<i>Citrobacter</i> sp. A1
	deoxyribose-phosphate aldolase	38034	<i>Enterobacter asburiae</i> LF7a
	outer membrane protein A	38644	<i>Salmonella enterica</i>
	outer membrane protein OmpC	40474	<i>Escherichia coli</i>
	cysteine synthase A	34497	<i>Citrobacter</i> sp. 30_2
	outer membrane porin protein	39811	<i>Citrobacter rodentium</i> ICC168
	succinyl-CoA synthetase subunit alpha	29787	<i>Citrobacter</i> sp. 30_2
	ribose-phosphate pyrophosphokinase	34845	<i>Citrobacter koseri</i> ATCC BAA-895
	L-threonine 3-dehydrogenase	37247	<i>Citrobacter</i> sp. 30_2
	bacterial extracellular solute-binding family protein	42119	<i>Citrobacter</i> sp. A1
g	glyceraldehyde-3-phosphate dehydrogenase A	31421	<i>Citrobacter</i> sp. 30_2
	elongation factor Ts	30381	<i>Citrobacter freundii</i> 4_7_47CFAA
	6-phosphofructokinase	34724	<i>Citrobacter</i> sp. A1
	succinyl-CoA synthetase subunit alpha	29787	<i>Citrobacter</i> sp. 30_2
	malate dehydrogenase	32148	<i>Citrobacter</i> sp. A1
	glutamate/aspartate periplasmic-binding protein	33402	<i>Citrobacter</i> sp. A1
	transaldolase B	37311	<i>Citrobacter</i> sp. 30_2
	dipeptide transporter ATP-binding subunit	35733	<i>Citrobacter freundii</i> GTC 09479

4.3.7 PCR-based screen for the detection of anaerobic naphthalene degradation

The initial carboxylation reaction of anaerobic naphthalene degradation has been elucidated in the highly enriched sulphate-reducing culture N47 (Moultaki *et al.*, 2012). To date Ncr is the only enzyme involved in anaerobic naphthalene degradation that has been characterized (Eberlein *et al.*, 2013). Morris and co-workers (2014) developed a PCR-based functional assay to detect microorganisms that have the ability to degrade naphthalene anaerobically via this pathway. The oligonucleotide probes have shown to be able to amplify a highly

conserved region of the gene encoding Ncr in sulphate-reducing pure cultures and environmental enrichments. Even though our enrichment culture is a nitrate-reducing culture, screening for this related pathway was attempted in the enrichment culture as either a negative or positive result that could provide insight into the anaerobic degradation pathway.

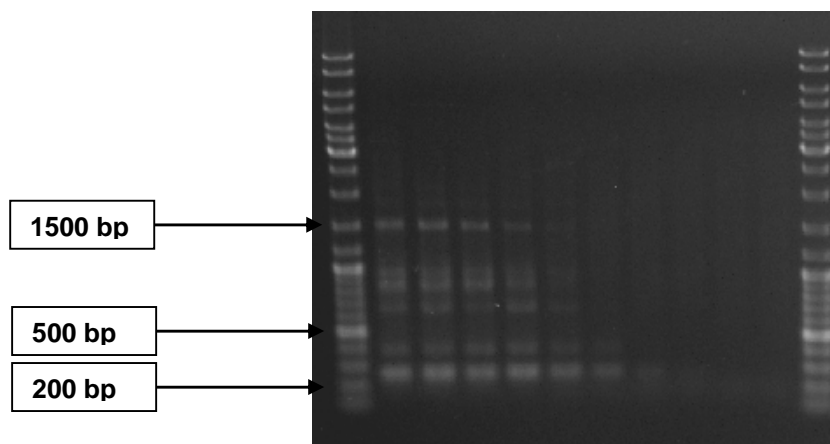


Figure 4.21: PCR amplification of the Ncr gene from the enrichment sample genomic DNA. (A) Primer set 1 and (B) Primer set 2. Lane M, MassRuler™ DNA ladder (Fermentas). Lane 1, 60°C; Lane 2, 59°C; Lane 3, 57°C; Lane 4, 54.3°C; Lane 5, 50.9°C; Lane 6, 48.1°C; Lane 7, 46.1°C; Lane 8, 45°C.

The probes should amplify a 321-bp fragment of the Ncr gene, but no amplicon of that size was observed in the resulting gel. Although amplicons of ~400 bp and ~250 bp was observed (Figure 4.21), these should not be the genes in question since Morris and co-workers were very pertinent on the accuracy of their probes as controls performed by with nitrate-reducing bacteria, *Thauera aromatica* strain K172 and *Azoarcus evansii*, that also resulted in no corresponding fragments. These results, along with previous observations, confirm the fact that the enrichment culture is not degrading naphthalene with an initial carboxylation step.

4.4 Conclusions

The ability of various indigenous bacteria, especially those isolated from contaminated areas, to degrade hydrocarbons are well known. In this chapter it was shown that an enrichment culture, consisting of *Citrobacter* spp., can utilize hydrocarbons as a sole carbon source under dissimilatory nitrate reducing conditions in the absence of molecular oxygen. The interactions of anaerobic hydrocarbon degrading microorganisms coupled to sulphate reduction have been well documented, but the interactions with nitrate reducing consortiums less so. The enrichment culture appeared to have an affinity for the aromatic and polycyclic aromatic hydrocarbons with the best degradation observed for the PAH naphthalene. Growth on phenanthrene was also observed but degradation could not be satisfactorily shown. In the present investigation the optimum temperature for naphthalene degradation appeared to be around 20°C and the optimum pH between 6.5 and 7.5. These values are normal for the denitrification process. Thermophilic bacteria species are apparently absent from the enrichment culture as the rate of degradation drops considerably near 40°C.

Naphthalene is inherently toxic to cells especially under oxygen and nutrient deprived conditions, but our results indicate that the upper levels of tolerance can be pushed, when compared to literature, by providing cells with co-substrates such as a nutrient rich medium. Even more so, naphthalene degradation capabilities can be improved by adding additional substrates with the enrichment culture presenting improved naphthalene degradation rates with only the addition of acetate. However, the exact location of the effect excited by acetate addition in the anaerobic naphthalene degradation pathway is still uncertain.

Since no oxygen is present, the degradation of naphthalene probably proceeds along a different metabolic pathway from that followed under aerobic conditions. In particular, the mechanism by which the aromatic nucleus is ruptured has to differ from that of the aerobic process, in which oxygen and dioxygenases are involved. In an attempt to elucidate the pathway of naphthalene degradation, different lines of experimentation were carried out. Firstly, growth was observed for the enrichment culture with various metabolites of naphthalene. Growth on 2-naphthol and an increase in ATP after an initial lag phase, while growing on 2-methylnaphthalene, indicates the possibility of either a hydroxylation or methylation mechanisms for the initial activation of naphthalene degradation. Further research is needed to elucidate which of these two mechanisms is being utilized by the enrichment culture, as well as the enzymes involved as literature pertaining to the activation of naphthalene by nitrate reducing microorganisms is severely lacking. Secondly, total proteome analysis was performed via SDS-PAGE but it did not produce any clear answer

pertaining the activation of naphthalene. It did however clearly indicate that the cells are actively growing on the substrate with the up-regulation of the TCA-cycle as well as the pentose phosphate pathway. Many membrane bound porin proteins were also induced, indicating that the enrichment culture is actively allowing the substrate into the cellular space.

Our experiments demonstrated that the enrichment culture is perfectly capable of degrading and metabolizing naphthalene as a sole carbon source under anaerobic conditions. Future studies are necessary to elucidate the degradation pathway involved.

4.5 References

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Chapter 5

Metagenomic and -transcriptomic insights into the naphthalene degradation pathways of the enrichment culture

5.1 Introduction

During the last 25 years the knowledge that the uncultured world far outsized the cultured world (Torsvik *et al.*, 1998) has brought about a definitive shift in how the scientific community processes environmental samples. Metagenomics has proven extremely useful as a molecular tool to overcome the limitations imposed by more traditional culturing techniques, enabling a broader perspective of the taxonomic and metabolic potential of environmental organisms (Handelsman *et al.*, 1998) and for the first time, enabling researchers to study the uncultured world in depth. No longer bound by the dated viewpoints stipulated by the *Bergey's Manual* that no organism could be classified without being cultured (Society of American Bacteriologists, 1923) the number of metagenomics projects has dramatically increased with hundreds of environmental samples studied by shotgun sequencing (Ivanova *et al.*, 2010) in recent years from numerous exotic and ordinary environments – ocean surfaces, deep sea vents, hot springs, the deep subsurface, human intestine, etc. Many new lineages are being classified now based solely on their molecular signatures. Today the focus of these studies have started to shift from a mainly diversity focus to more functional analysis, attempting to not only answer the question “Who is there?” but also the more difficult question of “What are they doing?” (Handelsman, 2004).

A metagenome is defined as the genomic information of a community of organisms obtained by sequencing the community as a whole. All genomes of the organisms within this community are then collected in a single metagenomics data set. To determine biome composition of a community it is usually sufficient to only sequence a small region of the ribosomal 16S rRNA gene and compare the obtained results to the existing ribosomal databases. From a complete metagenome sequencing, valuable information pertaining to the functional genetic composition of the community can be obtained by identifying functional genetic composition from the sequence data (Handelsman, 2004). Using this method one can then evaluate the proposed functional composition of a community without any actual knowledge of the individual genomes.

Metatranscriptomics offers the opportunity to reach beyond the communities' genomic potential, as assessed by metagenomics, and provides insights into its *in situ* activity by investigating the actively transcribed ribosomal and messenger RNA. A metatranscriptome can also give information on the "key players" with regard to metabolic activity within a community. Metatranscriptomics has been developed to gain an understanding of how communities respond to external changes in their environment by providing a snapshot of the genetic composition of the community at any given time (Gilbert and Hughes, 2011).

Hydrocarbon contaminated environments contain a high diversity of microorganisms, yet the metabolic activity and the ecological functions of the microbial communities remain largely unexplored, especially under anaerobic conditions. Thanks to advances in sequencing technologies, molecular microbial diversity studies of hydrocarbon impacted environments have made significant progress in understanding the diversities and features of these microbial communities (He *et al.*, 2013), yet still, the enzymes involved in the initial steps in anaerobic hydrocarbon catabolism are not known.

The aims of this chapter were to:

- Assess the enrichment culture diversity by targeted next generation sequencing
- Sequence the complete metagenome of the enrichment culture
- Assemble high quality sequencing reads into usable contigs
- Analyse metagenome sequences and contigs
- Screen metagenome for hydrocarbon degradation metabolic potential
- Sequence the complete transcriptome of the enrichment culture grown with and without naphthalene as carbon source
- Analyse metatranscriptome reads using online and local resources with respect to differentially expressed genes
- Gather insights into the possible activation mechanisms of naphthalene using data obtained from both the metagenome and metatranscriptome

5.2 Materials and methods

5.2.1 Genomic DNA extraction

Genomic DNA was extracted from the enrichment culture using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturers' specifications. The concentration and quality of the extracted gDNA was assessed using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific) and visualized on a 1% (w/v) agarose gel as described in section 3.2.3.1.

5.2.2 16S rRNA metagenome sequencing

The gDNA, from section 5.2.1, was sent for sequencing library construction and sequencing to Inqaba Biotech (Pretoria, South Africa). The 16S rRNA metagenome sequencing was performed on the Illumina MiSeq platform. Briefly, the quality and quantity assessment for the extracted gDNA was performed using the 2100 Bioanalyzer Instrument (Agilent Technologies, Inc.), using a DNA 12000 Chip. Sample quantitation was performed using the Picogreen assay (Invitrogen). The sequencing library was prepared by amplifying a ~460 bp region in the hypervariable V3/4 region of the 16S rRNA gene and adding the Illumina adapter sequences to the amplicons using the primer pair in table 5.1. A second round of amplification will add barcodes to different samples. Finally the amplified PCR products, after gel purification and recovery using the Agencourt AMPure XP Bead Clean-up kit (Beckman Coulter, Inc.), was normalized, pooled and denatured before being subjected to the MiSeq system for deep sequencing using paired 301 bp reads and MiSeq v3 reagents. The primer construct and amplicon can be seen in figure 5.1 and the workflow for the entire process can be seen in figure 5.2 below

Table 5.1: Primers used during Illumina library preparation for 16S rRNA sequencing.

Primer	Sequence	Reference
16S Amplicon PCR Forward	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG -3'	(Klindworth <i>et al.</i> , 2013)
16S Amplicon PCR Reverse	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC -3'	(Klindworth <i>et al.</i> , 2013)

*The sequence highlighted in bold print constitutes the Illumina adapter sequence



Figure 5.1: The primer construct and amplicon used in Illumina sequencing (taken from “Qiime,” 2015).

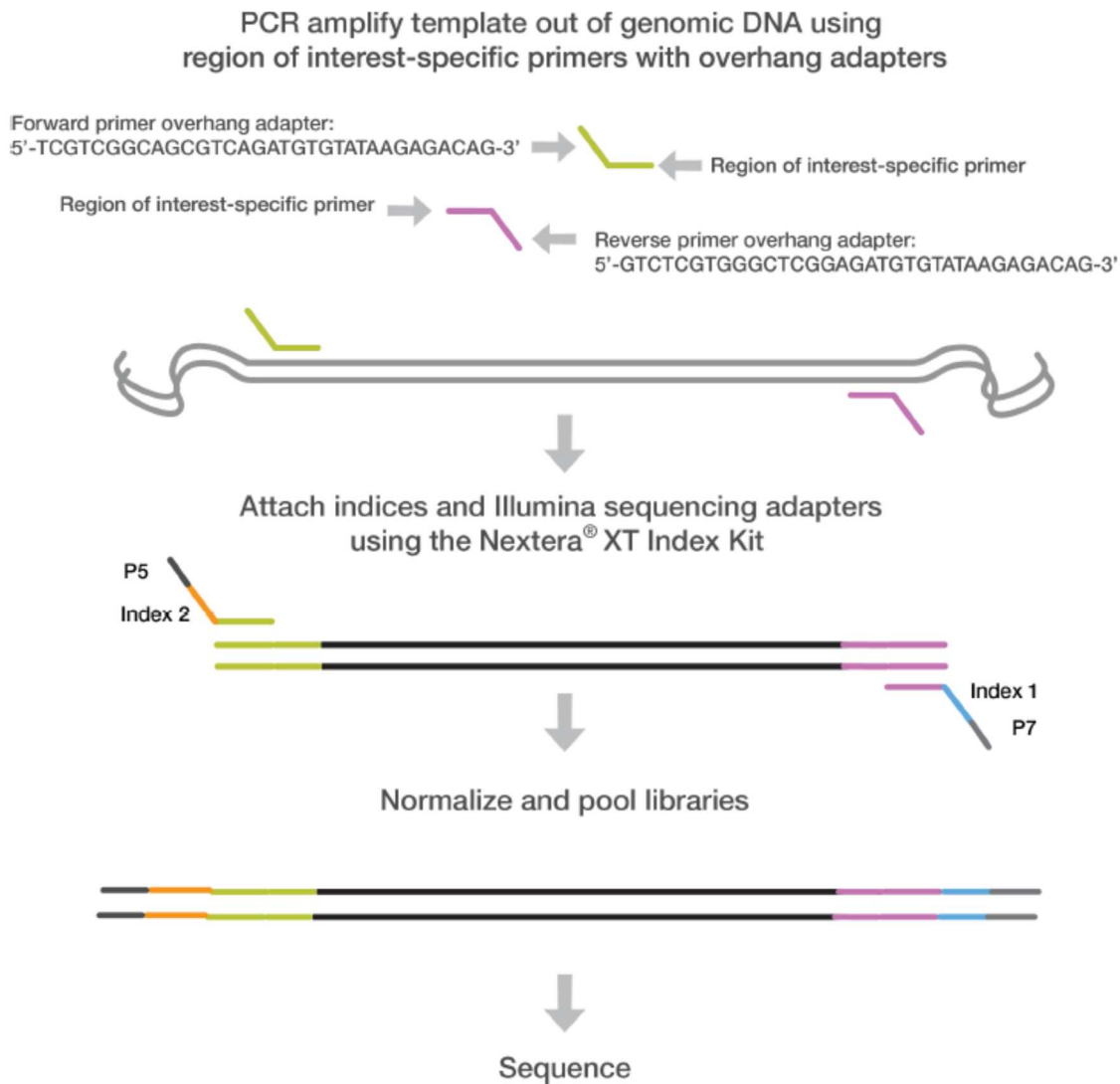


Figure 5.2: Library preparation workflow for sequencing of the V3/4 hypervariable region of the 16S rRNA gene using the Illumina MiSeq platform (taken from “16S Metagenomic Sequencing Library Preparation,” 2015).

5.2.2.1 16S rRNA metagenome sequencing data analysis

The obtained 16S rRNA metadata was analysed using QIIME (Caporaso *et al.*, 2010) as described in section 4.2.3, with the following alterations. Before running the QIIME pipeline the quality of the sequencing was assessed and quality control performed using PrinSeq-lite, version 0.20.4 (Schmieder and Edwards, 2011). All datasets were pre-processed to remove duplicates and sequences were trimmed until the average quality score was ≥ 30 using a 7

nt window with a 3 nt step, all sequences shorter than 200 bp were filtered out. The demultiplex and quality filtering script in QIIME, `split_libraries_fastq.py` was run without any additional inputs to obtain a FASTA output file that could be analysed in the QIIME pipeline. A complete workflow for the step involved in the QIIME pipeline can be seen in figure 5.3 below.

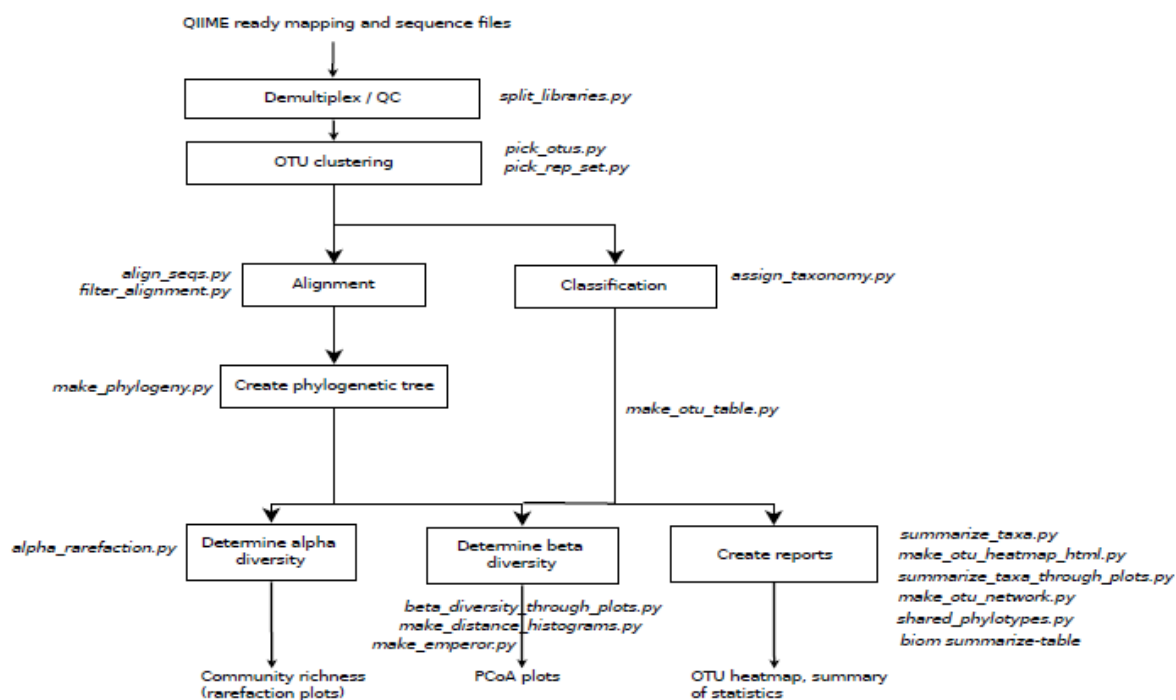


Figure 5.3: Workflow for the steps involved during the analysis of 16S rRNA gene data in QIIME (taken from “Qiime,” 2015).

5.2.3 Total metagenome shotgun sequencing

The gDNA, from section 5.2.1, was sent for sequencing library construction and sequencing to the Singapore Centre on Environmental Life Sciences Engineering (Nanyang Technological University, Nanyang, Singapore) for total metagenome shotgun sequencing on the Illumina MiSeq. Briefly, the quality and quantity assessment for the extracted gDNA was performed using the 2100 Bioanalyzer Instrument (Agilent Technologies, Inc.), using a DNA 12000 Chip. Sample quantitation was performed using the Picogreen assay (Invitrogen). Next-generation sequencing library preparation was performed according to the Illumina TruSeq® Nano DNA Sample Preparation protocol. The goal of the protocol is to add adapter sequences onto the ends of DNA fragments to generate indexed paired-end sequencing libraries. The sample was sheared on a Covaris S220 Focused-ultrasonicator to ~550 bp, following the manufacturers’ specifications. Library quantitation was performed using the Picogreen assay (Invitrogen) and the average library size was determined by

running the libraries on a Bioanalyzer DNA 7500 chip (Agilent). Library concentration were normalized to 4 nM and validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol, and Illumina's PhiX control library as standard. The library was then sequenced on an Illumina MiSeq sequencer at a read-length of 301bp paired-end with MiSeq v3 reagents.

5.2.3.1 Total metagenome sequencing data analysis with MG-RAST

The quality of the sequencing was assessed and quality control performed using PrinSeq-lite, version 0.20.4 (Schmieder and Edwards, 2011). Low-quality reads were disregarded by trimming sequences until the average quality score was ≥ 25 using a 7 nt window with a 3 nt step, additional trimming and filtering was performed as needed.

Post quality filtered paired-end reads were joined, additional quality controlled and annotated using the standard MG-RAST (Metagenomic Rapid Annotation using Subsystem Technology) metagenomics pipeline (<http://metagenomics.anl.gov>; Meyer *et al.*, 2008). MG-RAST is an online annotation service that uses the SEED algorithm in a standardized pipeline analysis of metagenomics DNA sequences.

5.2.3.2 Complementary total metagenome sequencing data analysis

Quality filtering was performed as described above in section 5.2.3.1. The best kmer length for *de novo* assembly was determined using KmerGenie 1.6976 (Chikhi and Medvedev, 2014). Post-quality filtered reads were assembled using Ray Meta 2.3.1 (Boisvert *et al.*, 2012) *de novo* metagenome assembly. The functional annotation was carried out by uploading the obtained contigs to MG-RAST. Reads were mapped to assembled contigs using Bowtie 2 (Langmead and Salzberg, 2012) with alignment sensitivity set on 'very sensitive'.

5.2.3.3 *In silico* screening for known hydrocarbon degradation genes

A local BLAST (Altschul *et al.*, 1990) database was created from a FASTA file containing sequences of known hydrocarbon degradation genes (Table 5.2). The obtained contigs from section 5.2.3.2 was used as query with an E-value cutoff of $1e-5$ in the BLASTx (2.2.31) program.

Table 5.2: Hydrocarbon degradation genes included in the BLAST database.

Gene (Accession number)	Abbreviation	Organism	Substrate	Reference
Alpha subunit of alkylsuccinate synthase (ADJ51097.1)	assA	<i>Desulfoglaeba alkanexedens</i> ALDC	Alkanes	(Callaghan <i>et al.</i> , 2010)
Alpha subunit of benzylsuccinate synthase (AAK50372.1)	bssA	<i>Azoarcus</i> sp. T	Toluene	(Achong <i>et al.</i> , 2001)
Alpha subunit of naphthylmethyl succinate synthase (CAO72222.1)	nmsA	Delta proteobacterium NaphS6	2-Methylnaphthalene	(Selesi <i>et al.</i> , 2010)
Putative anaerobic benzene carboxylase (ADJ94001.1)	AbcD	<i>Clostridia</i> bacterium enrichment culture clone BF	Benzene	(Abu Laban <i>et al.</i> , 2010)
Putative anaerobic benzene carboxylase (ADJ94002.1)	AbcA	<i>Clostridia</i> bacterium enrichment culture clone BF	Benzene	(Abu Laban <i>et al.</i> , 2010)
Methane monooxygenase protein C (BAE86885.1)	pmoC	<i>Methylococcobium japonense</i>	Methane	(Nakamura <i>et al.</i> , 2007)
Alkane 1-monooxygenase (YP_003809668.1)	alkM	Gamma proteobacterium HdN1	Alkanes	(Musat, 2015)
Alpha subunit of ethylbenzene dehydrogenase (YP_158333.1)	ebdA	<i>Aromatoleum aromaticum</i> EbN1	Ethylbenzene	(Kniemeyer and Heider, 2001b)
Beta subunit of ethylbenzene dehydrogenase (YP_158332.1)	ebdB	<i>Aromatoleum aromaticum</i> EbN1	Ethylbenzene	(Kniemeyer and Heider, 2001b)
Gamma subunit of ethylbenzene dehydrogenase (YP_158331.1)	ebdC	<i>Aromatoleum aromaticum</i> EbN1	Ethylbenzene	(Kniemeyer and Heider, 2001b)
BzdQ (AAQ08809.1)	bzdQ	<i>Azoarcus</i> sp. C1B	Benzoyl	(López Barragán <i>et al.</i> , 2004)
Benzoyl-CoA reductase, putative (YP_006720765.1)	bamB	<i>Geobacter metallireducens</i> strain GS-15	Benzoyl	(Aklujkar <i>et al.</i> , 2009)
Alpha subunit of methyl-coenzyme M reductase (YP_004383383.1)	mcrA	<i>Methanosaeta concilii</i> strain ATCC 5969	Methane	(Barber <i>et al.</i> , 2011)
Molybdopterin oxidoreductase (YP_001528081.1)	Dole_0194	<i>Desulfococcus oleovorans</i> strain Hxd3	Ethylbenzene	(H A Johnson <i>et al.</i> , 2001)

5.2.4 Metatranscriptome sequencing

The enrichment culture was grown anaerobically in Bushnell Haas broth with acetate (100 mg/L) and naphthalene (30 mg/L) until late exponential phase (5 days, see section 4.3.4.2). All media preparations were performed as described in section 3.2.5.2. Cells were harvested by centrifugation (8000 \times g, 5 min) and resuspended in RNeasy Lysis Solution (Life Technologies). Sample processing and sequencing was performed at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa). Briefly, total RNA was extracted from the bacterial cultures using the RNeasy Mini Kit (Qiagen). The quality of the extracted RNA was assessed with the NanoDrop ND-1000 (Thermo Scientific) for $A_{260/230}$ ratio, the Qubit® Total RNA BR (Life Technologies) for the absolute concentration and the Agilent BioAnalyzer RNA 600 Pico assay to assess the sample integrity numbers (RIN value). Sequence libraries were prepared using the ScriptSeq™ Complete Kit (Bacteria) (Illumina). The kit protocol includes rRNA depletion, followed by mRNA fragmentation. The fragmented mRNA is then used for the first and second strand cDNA synthesis followed by 3' adenylation, which introduces primer binding sites for the PCR propagation step. After PCR amplification of the cDNA, the library is purified using AMPure XP Beads (Beckman Coulter). The final library was quantified using the Qubit DNA HS Assay and a fragment length analysis was performed on the BioAnalyzer (Agilent) to obtain the nM concentration required for clustering and sequencing. Sequencing of the libraries was performed on the Illumina NextSeq 500 instrument using NextSeq 500 Mid Output Reagent Kit v2 chemistry (300 cycle).

5.2.4.1 Total metagenome sequencing data analysis with MG-RAST

The quality of the sequencing was assessed and quality control performed using PrinSeq-lite, version 0.20.4 (Schmieder and Edwards, 2011). Low-quality reads were disregarded by filtering out sequences with an average quality score of ≤ 20 , additional trimming and filtering was performed as needed.

Post quality filtered paired-end reads were joined, additional quality controlled and annotated using the standard MG-RAST (Metagenomic Rapid Annotation using Subsystem Technology) metagenomics pipeline (<http://metagenomics.anl.gov>; Meyer *et al.*, 2008) as described in section 5.3.2.1.

5.2.4.2 Differential gene expression analysis of metatranscriptome data with TopHat and Cufflinks

The analysis of differentially expressed genes was performed using the method described by Trapnell and co-workers (2012) using the Tuxedo Suite (Bowtie, TopHat, Cufflinks). Briefly, the post quality filtered reads (described in section 5.2.4.1 above) for each condition, growth on acetate and naphthalene as described in section 5.2.4, were aligned to a reference genome, in this case the assembled metagenome contigs obtained from section 5.2.3.2, using TopHat (<http://tophat.cbcb.umd.edu/>). After the TopHat alignment is completed, the resulting alignments are assembled using Cufflinks (<http://cufflinks.cbcb.umd.edu/>) for each condition. The resulting assemblies are merged using the Cuffmerge utility that forms part of the Cufflinks package. Cuffdiff, also part of the Cufflinks package, calculates expression levels and tests the statistical significance of observed changes. The resulting text files were then analysed using the CummeRbund (v2.0.0) package to plot abundance and differential expression. The FPKM (reads per kb per millions reads) method was used to calculate the rate of differentially expressed genes, with a p value ≤ 0.05 and a fold change value ≥ 1 . Sequences for differentially expressed transcripts in the naphthalene grown culture was subjected to BLASTx analysis against the RefSeq protein database (cut-off set at $1e-20$)

5.3 Results and discussion

5.3.1 Genomic DNA extraction

Quality and quantity of the starting material can have a critical effect on the success of any sequencing project. This is especially relevant to next generation sequencing as each platform and their sequencing library construction protocols has differing requirements in terms of the quality and quantity of the DNA/RNA that each uses. RNA, ssDNA and dsDNA all absorb at 260 nm thus they will all contribute to the total absorbance of a sample during spectrophotometric analysis. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally considered “pure” for DNA. If the ratio is lower it may indicate the presence of protein, phenol or other contaminants that will absorb at 280 nm. The ratio of absorbance at 260 nm and 230 nm is used as a secondary measure of nucleic acid purity. A ratio of ~2.0 is usually considered pure, if the ratio is lower it may indicate the presence of contaminant which absorb at 230 nm, such as EDTA, carbohydrates, phenol and guanidine HCl (Lehninger, 1975). Spectrophotometric analysis of the extracted gDNA showed a peak at 260 nm with a 260:280 ratio of 1.84 and a 260:230 ratio with a value of 1.95, indicating very pure DNA and is in range with what was required (1.8 and 2.0 respectively) for sequencing on the Illumina platform. The gDNA integrity was assessed on an agarose gel which showed very low levels of shearing (Figure 5.4).

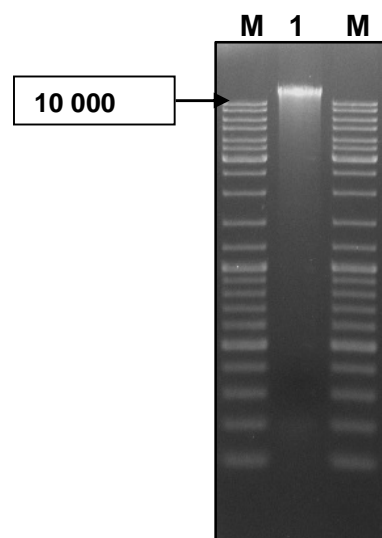


Figure 5.4: Genomic DNA extracted from the enrichment culture using the DNeasy Blood & Tissue Kit (QIAGEN). Lane M, MassRuler™ DNA ladder (Fermentas). Lane 1, gDNA.

5.3.2 Enrichment culture diversity based on 16S rRNA metagenomics

The paired-end library (2x301 bp) resulted in 3195 sequences with a mean sequence length of 285.08 ± 46.46 bp. Quality filtering initially took into account that this was paired-end data, but most of the reads ended up in the 'singleton' file. Paired-end quality control will look at the quality of both the forward and reverse reads and only if both of the sequences pass the quality control filters would both sequences be placed in the 'good sequence' file. Singletons are then the sequences where only the forward or reverse passed the quality control filter. Quality filtering performed on the forward and reverse files separately resulted in 4 (0.16%) of the reverse sequences passing the quality filtering and it was thus decided to disregard the reverse sequences. A total of 1137 (35%) forward sequences passed the quality filtering, with a minimum, maximum and mean sequence length of 200, 301 and 269.67 ± 29.24 bp respectively (Figure 5.5).

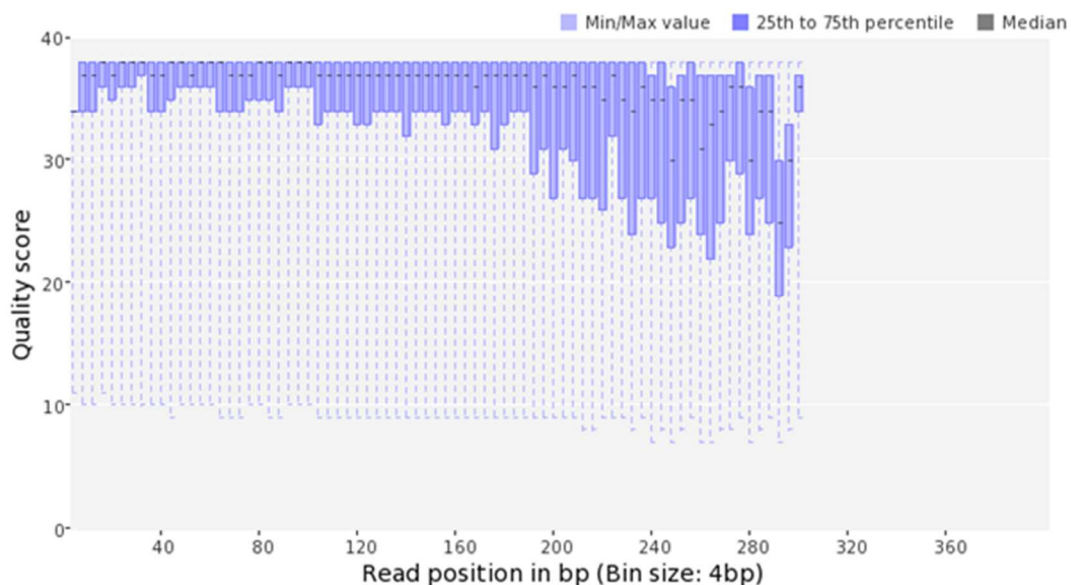


Figure 5.5: Mean of quality score at read position across all obtained sequences.

In what is now seen as the standard approach for tag-sequencing data analysis, it is assumed that sequence similarity of the 16S rRNA hypervariable region, in this case V3/4, can be used as a proxy for phylogenetic relatedness (Tikhonov *et al.*, 2014). Analysis begins by clustering reads by sequence similarity into 'Operational Taxonomic Unit' (OTUs). Sequences assigned to a particular OTU are generally presumed to be close phylogenetic relatives, as a rule OTUs are picked at a standard 97%-similarity level. For the enrichment culture, most of the reads grouped into the OTU for *Citrobacter* sp. (Table 5.3 and Figure 5.6). Enterobacteriaceae and Bacillaceae are commonly found in soils (Degelmann *et al.*, 2009) and thus their presence is not unexpected, however, the Bacillaceae are gram-

positive (Logan and Vos, 2015) microorganisms but thus far no gram-positive organisms have been observed in the enrichment culture (section see 3.3.4). Also the other species observed, *Thalassospira* sp. and *Marinobacter* sp. are most commonly found in marine environments rather than in soil (Gao *et al.*, 2012; Tsubouchi *et al.*, 2014). *Sulfuricurvum* sp. has been isolated from crude-oil contaminated environments (Kodama and Watanabe, 2004).

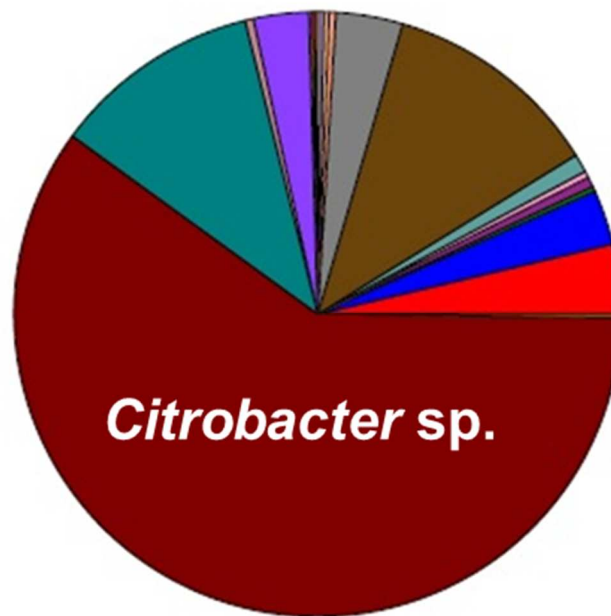


Figure 5.6: Graphical representation of the percentage reads to phylogenetic relatedness for the enrichment culture from 16S rRNA Illumina sequencing data (colours are reference to table 5.3 below).

Removing 'singletons', in this case OTUs with only two or less representative sequences results in the loss of most of the OTUs (Table 5.4 and Figure 5.7) and only the Enterobacteriaceae and Bacillaceae remaining. As mentioned above no gram-positive microorganisms have been observed in the enrichment culture, however their presence might be so low that normal microscopy techniques could have missed it. Thus it would appear that the enrichment culture diversity is dominated by various members of Enterobacteriaceae, the main group amongst these being the *Citrobacter* sp

Table 5.3: Diversity assessment of enrichment culture from 16S rRNA Illumina sequencing data (colours are reference to figure 5.6 below).

Domain	Phylum	Class	Order	Family	Genus
Unclassified					
Bacteria					
Bacteria	Cyanobacteria				
Bacteria	Cyanobacteria	Chloroplast	Chlorophyta		
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Geobacillus</i>
Bacteria	Firmicutes	Clostridia	Peptostreptococaceae		
Bacteria	Proteobacteria				
Bacteria	Proteobacteria	Alphaproteobacteria	Kiloniellales	Kiloniellaceae	<i>Thalassospira</i>
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	<i>Sulfuricurvum</i>
Bacteria	Proteobacteria	Gammaproteobacteria			
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	<i>Marinobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Citrobacter</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	

Table 5.4: Diversity assessment of enrichment culture from 16S rRNA Illumina sequencing data with singletons filtered out (colours are reference to figure 5.7 below).

Domain	Phylum	Class	Order	Family	Genus
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	<i>Geobacillus</i>
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	<i>Citrobacter</i>

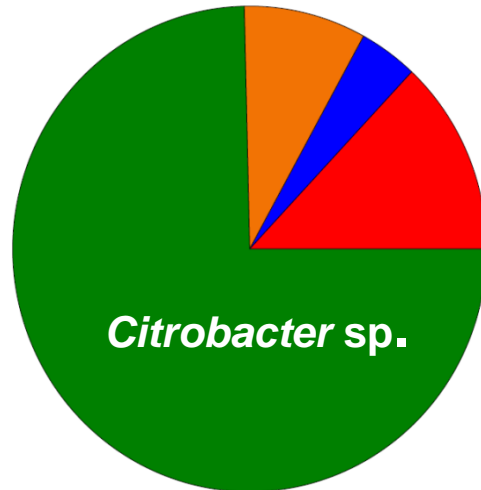


Figure 5.7: Graphical representation of the percentage reads to phylogenetic relatedness for the enrichment culture from 16S rRNA Illumina sequencing data with singletons removed (colours are reference to table 5.4 above).

5.3.3 Total metagenome shotgun sequence analysis and assembly

The paired-end library (2x301 bp) resulted in two sequence files with 16,951,268 sequences (5,031,760,804 bp) with a mean sequence length of 296.84 ± 19.74 bp and 297.07 ± 19.57 bp for the forward sequences and reverse sequences respectively. A total of 14,012,526 (3,856,376,495 bp) (Figure 5.8 a and b) paired-end sequences as well as 2,256,514 (583,231,977 bp) forward and 88,662 (20,363,300 bp) reverse singleton sequences passed quality filtering. All sequences passing the quality filtering step were utilized for assembly. Various assemblers were utilized in an attempt to assemble the data, including MetaVelvet (Namiki *et al.*, 2012) and Mira (Chevreux, 2004) but Ray Meta consistently produced the best assemblies for this data based on N50 values, amount of contigs and contig lengths. The quality controller sequence data was assembled using Ray Meta with a kmer value of 128 as determined by KmerGenie.

The quality filtered sequences assembled into 27 contigs all larger than 100 bp with a total length of 4,860,257 bp and an average length of 180,009. The assembly had a N50 value of 528,955 bp with a largest contig of 1,647,839. A total of 17 contigs were larger than 500 bp with an average length of 285,716 bp. There are no strict rules for assembly, an assembly with an N50 length that is gene-sized is a decent target for annotation. If the N50 is around the median gene length, then ~50% of the genes will be contained on a single contig and

these complete genes, together with fragments from the rest of the genome, will provide a good resource for downstream analyses (Yandell and Ence, 2012).

Mapping the sequences to the assembled contigs resulted in 99.56% of all reads mapping to the contigs, so almost all of the sequences were utilized during assembly. This also showed ~700x coverage for most of the contigs.

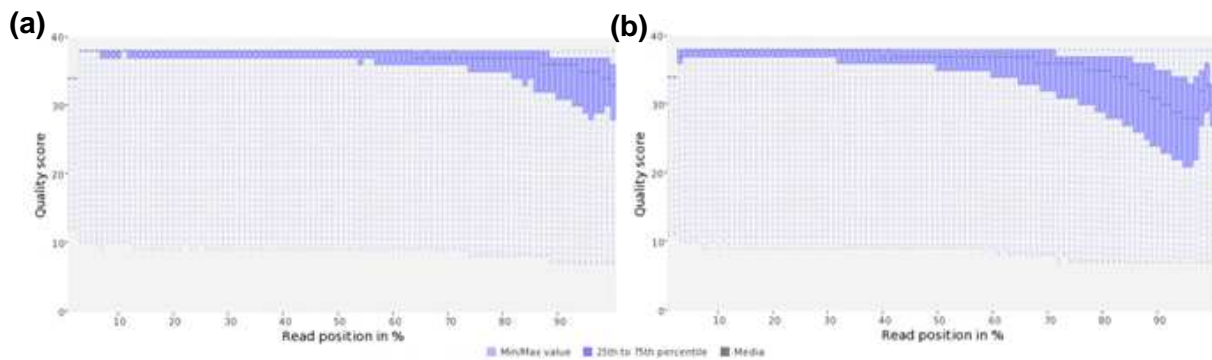


Figure 5.8: Base quality distribution for post-quality filtered reads. (a) Forward sequences; (b) Reverse sequences.

5.3.4 MG-RAST analysis

Quality filtered sequences and assembled contigs were uploaded to the MG-RAST server for annotation, all metagenomics data is accessible on MG-RAST under the ID number 4569511.3 and 4642300.3 for the quality filtered reads and contigs respectively. The analysis statistics for the raw sequence reads are shown in table 5.5 below. MG-RAST was used to determine taxonomic and functional annotations by looking for similarities in the sequences compared to the M5NR database. The M5NR is a protein database comprised of non-redundant protein and rRNA sequences obtained from the following databases:

- Functional and organism classification: GenBank, IMG, KEGG, PATRIC, RefSeq, SEED, SwissProt, TrEMBL and eggNOG
- Functional hierarchy annotation: COG, KO, NOG and Subsystems
- rRNA: Greengenes, SILVA LSU, RDP and SILVA SSU (Wilke *et al.*, 2012)

KEGG (Kyoto Encyclopaedia of Genes and Genomes) (Kanehisa and Goto, 2000) pathway analysis was done in MG-RAST using KeggMapper with an E-value cut-off of $1e-5$ and 60% identity, with a minimum alignment length of 15 bp or 15 a.a.

Table 5.5: Statistical measure of the sequence data uploaded to MG-RAST.

Total starting sequences	Post MG-RAST QC sequences	Post QC mean GC content	Post QC mean Sequence length	MG-RAST identified protein features	MG-RAST identified rRNA features
22,431,253	9,501,748	52 ± 5 %	351 ± 137 bp	412,506	2,147

The raw dataset contained 22,431,253 sequences totalling 8,282,754,426 bp with an average length of 369 bps. Of the 13,157,007 sequences that failed quality control, 11,808,386 were identified as artificial duplicate reads. Of the 9,274,246 sequences, totalling 3,262,848,456 bps, that passed quality control 95.3% produced a predicted 586,037 predicted protein coding regions. Of these predicted protein features, 70.4% could be assigned an annotation using at least one of the protein databases in MG-RAST and 86.4% of those features could be assigned into functional categories.

5.3.5 MG-RAST taxonomic analysis

The enrichment culture metagenome data was analysed for taxonomic classification at the levels of domain, phylum, class, order, family and genus using the M5NR database. The raw reads were classified as 99.6% belonging to the domain bacteria, while the assembled contigs were classified as 99.8% bacteria. At the phylum level the raw reads were annotated as 99.2% Proteobacteria (of which 98.5% were Gammaproteobacteria) and 0.1% Firmicutes. The assembled contigs were found to contain 99.4% Proteobacteria (of which 98.9% were Gammaproteobacteria). The most frequently annotated family in both the raw sequencing reads (97.6%) and the assembled contigs (98.2%) were the Enterobacteriaceae with the most annotated genus being *Citrobacter*, 32% and 55% respectively. Many different genera were annotated, but in relatively smaller proportions, as shown in Figure 5.9 and 5.10 below, with *Salmonella* and *Escherichia* making up the bulk of the annotations. High similarity is observed in diversity when compared to the diversity obtained from 16S rRNA metagenomics (section 5.3.2) in terms of the percentage of Enterobacteriaceae, especially *Citrobacter*, in the enrichment sample. The diversity from the total metagenome sequences appear to be in line with observations made in Chapters 3 and 4 with mostly gram-negative organisms, that are known to be prevalent in soils being present, but with a very small percentage of Firmicutes (the Bacillales) as was also indicated in section 5.3.2.

The much larger amount of data analysed from the total metagenome sequence also contributes to the fact that the obtained diversity will be more representative than from the 16S rRNA metagenome sequencing. It is difficult to draw conclusions on the microbiota in

the enrichment culture obtained from a hydrocarbon contaminated soil sample when compared to literature, considering the broad spectrum of possible pollutants and the varying conditions with respect to age of the contamination, humidity of the soil, land use, etc, from samples analysed. However, population of hydrocarbon contaminated environments by predominantly Gammaproteobacteria have been described (Popp *et al.*, 2006) and communities containing members of the genera *Citrobacter* have been identified (Douglas and Green, 2015). In order to compare the diversity of the enrichment culture with other metagenomes, the MG-RAST database was searched for projects containing sample conditions similar to the enrichment culture, eleven metagenomes were selected for comparison. Unfortunately the metadata for these other metagenomes is fairly limited and no literature, as yet, has been published containing the MG-RAST IDs. However, the metadata does reveal that these sequences were obtained from anaerobic bioreactor enrichments grown on aromatic hydrocarbons obtained from water and soil samples from hydrocarbon contaminated environments. Comparing the diversity of the enrichment culture with eleven other metagenomes on MG-RAST, all enriched in anaerobic bioreactors on various aromatic hydrocarbons (Figure 5.11), it appears that the enrichment culture diversity is very similar, at least on a domain level. The enrichment culture does have a larger amount of bacteria related reads than the other metagenomes. The data was compared to M5NR using a maximum e-value of 1e-5, a minimum identity of 60 %.

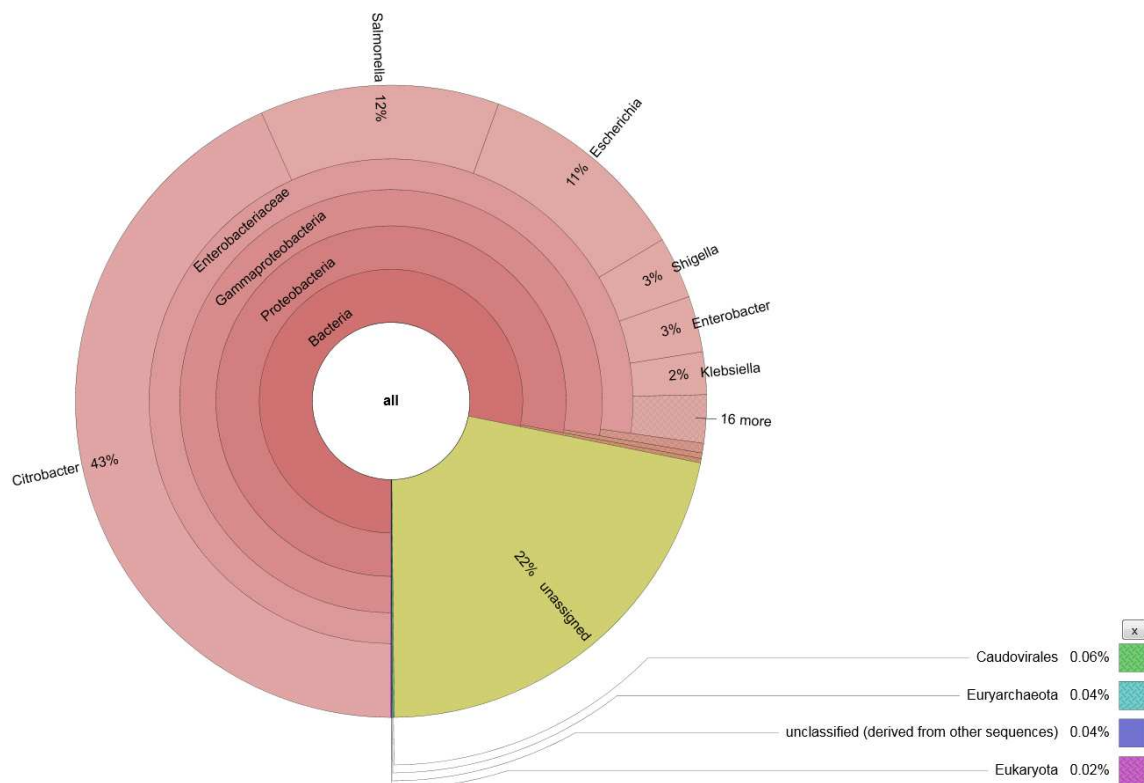


Figure 5.9: Genus-level classification of the quality filtered sequence reads as determined using the M5NR database in MG-RAST.

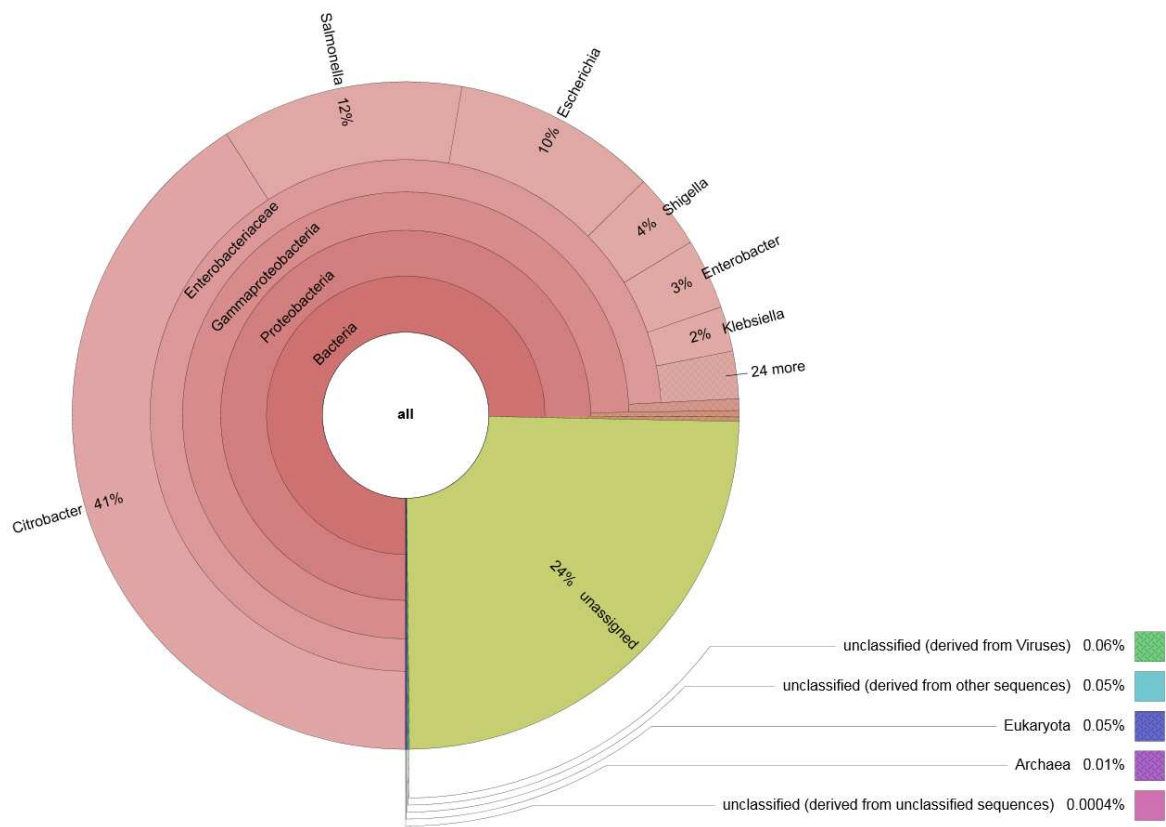


Figure 5.10: Genus-level classification of the assembled contigs as determined using the M5NR database in MG-RAST.

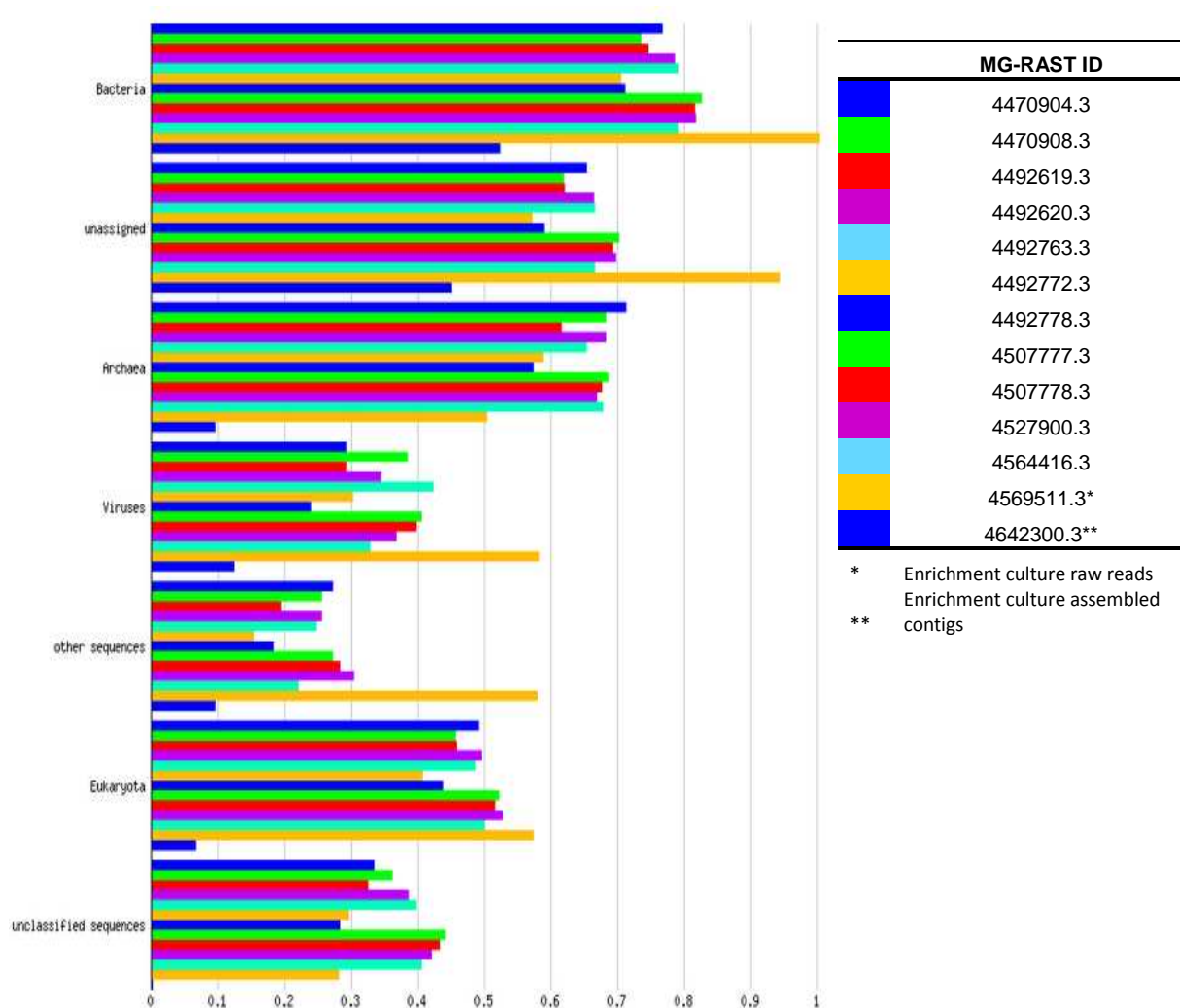


Figure 5.11: Comparison of different metagenomes all from anaerobic sources. The data has been normalized to values between 0 and 1 to allow for comparison of differently sized samples.

5.3.6 MG-RAST analysis of aromatic metabolism

Based on the functional annotations made by MG-RAST (Figure 5.12 and 5.13) the dataset obtained from either the quality filtered sequences or assembled contigs were examined for genes that may be involved in the degradation of aromatic compounds. In the SEED “Subsystems” a category called “Metabolism of aromatic compounds” encompasses a wide variety of metabolic enzymes and subclasses of enzymes, for example “Metabolism of central aromatic intermediates” and most important to this study, “Anaerobic degradation of aromatic compounds”. Very similar, yet slightly varied, proportions of these enzymes were annotated in the quality filtered sequences and assembled contigs (Figure 5.14 and 5.15). In both cases, raw read and assembled contigs, 1% of the annotated sequences contained sequences annotated as “Metabolism of aromatics”. These similar annotations indicate the quality of the assembly but also the definite presence of genes involved in aromatic

metabolism in the enrichment culture. This was further demonstrated by KEGG pathway analysis (Figure 5.16), several enzymes were identified involved in pathways for various known hydrocarbons, such as methane metabolism (Figure 5.17), xylene degradation (Figure 5.18), benzoate degradation (Figure 5.19), toluene degradation (Figure 5.18), ethylbenzene degradation (Figure 5.20), styrene biodegradation (Figure 5.21) and naphthalene degradation (Figure 5.22). None of these pathways are 100% complete, but since this is a metagenome data set, this might be expected, also genes might be miss-annotated or absent.

In terms of naphthalene degradation, even though very few of the enzymes in the known naphthalene degradation pathways are present (Figure 5.22), the presence of all the genes in the benzoate degradation pathway (Figure 5.19) for catechol biotransformation to acetyl-CoA was identified. Catechol is a known intermediate product which is generated through biodegradation of naphthalene (Pawar *et al.*, 2013). Catechol can also be converted to 4-hydroxybenzoate for further degradation in the toluene and xylene degradation pathway (Figure 5.18). The naphthalene degradation pathway (Figure 5.22) describes mainly aerobic degradation, for example *nahC* is a dioxygenase (Peng *et al.*, 2008). As such it is very difficult to speculate on the initial anaerobic activation of naphthalene with this data. However, it would seem that, if the naphthalene is anaerobically activated by one member of the enrichment culture, there exists various pathways for further degradation in the different members of this enrichment culture.

Comparing the KEGG pathway analysis of the quality filtered sequence reads with the other anaerobic hydrocarbons discussed above (Figure 5.23), it can be seen that very similar pathways and genes are being annotated as “Xenobiotic Biodegradation and Metabolism”. It is also interesting that very few genes involved in the naphthalene degradation pathway were identified in all of these metagenomes and also with the entire catechol degradation pathway being present in all the compared metagenomes (Figure 5.24 and 5.25).

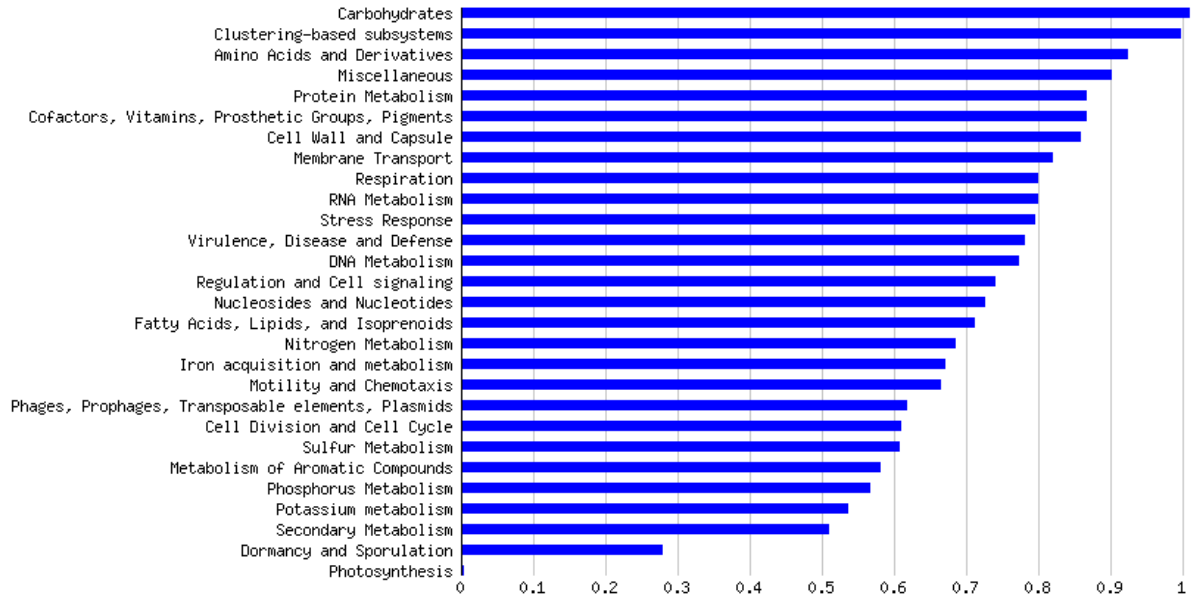


Figure 5.12: Total functional annotation in the SEED Subsystem database using MG-RAST of the quality filtered reads. The data has been normalized to values between 0 and 1.

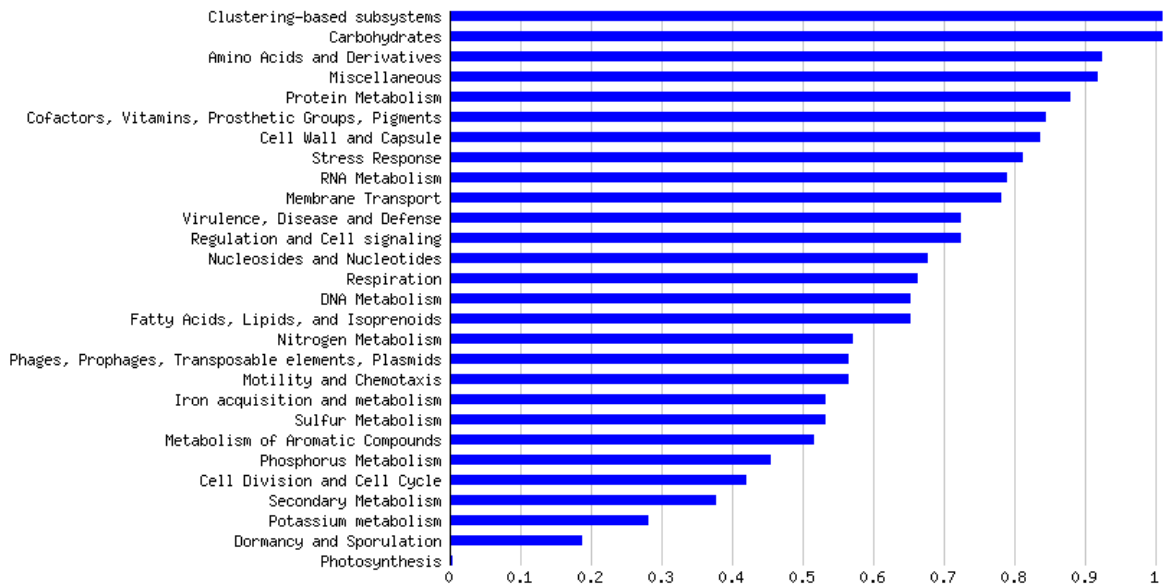


Figure 5.13: Total functional annotation in the SEED Subsystem database using MG-RAST of the assembled contigs. The data has been normalized to values between 0 and 1.

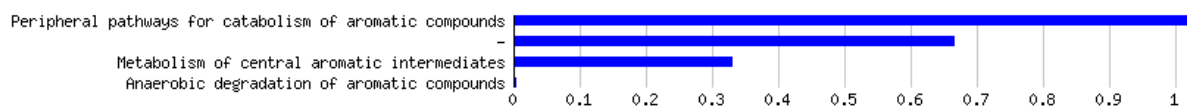


Figure 5.14: Functional category breakdown of reads classified as “Metabolism of aromatic compounds” in the SEED Subcategory database using MG-RAST annotations of the quality filtered reads. Features within the subsystem are found on 1% of the annotated contigs. The data has been normalized to values between 0 and 1.

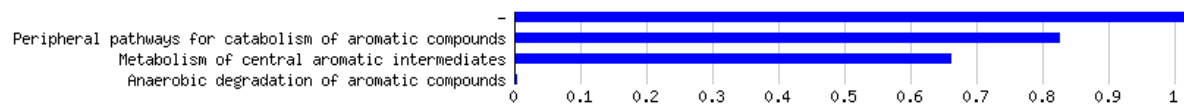


Figure 5.15: Functional category breakdown of reads classified as “Metabolism of aromatic compounds” in the SEED Subcategory database using MG-RAST annotations of the assembled contigs. Features within the subsystem are found on 1% of the annotated contigs. The data has been normalized to values between 0 and 1.

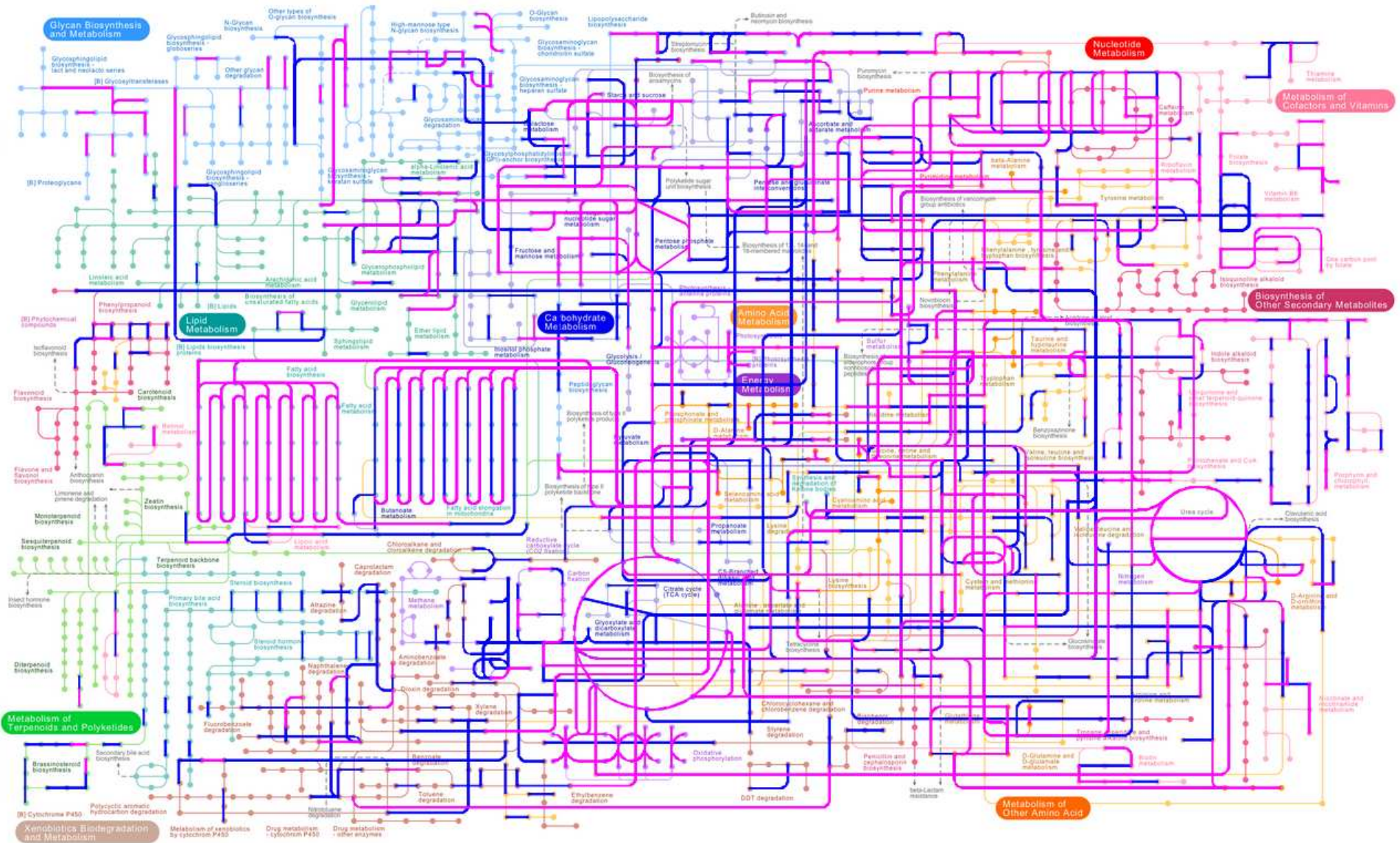


Figure 5.16: KEGG pathway analysis (pathways in blue is obtained from the quality filtered sequence reads, pathways in red from the assembled contigs and pathways in purple are where these two data sets overlap).

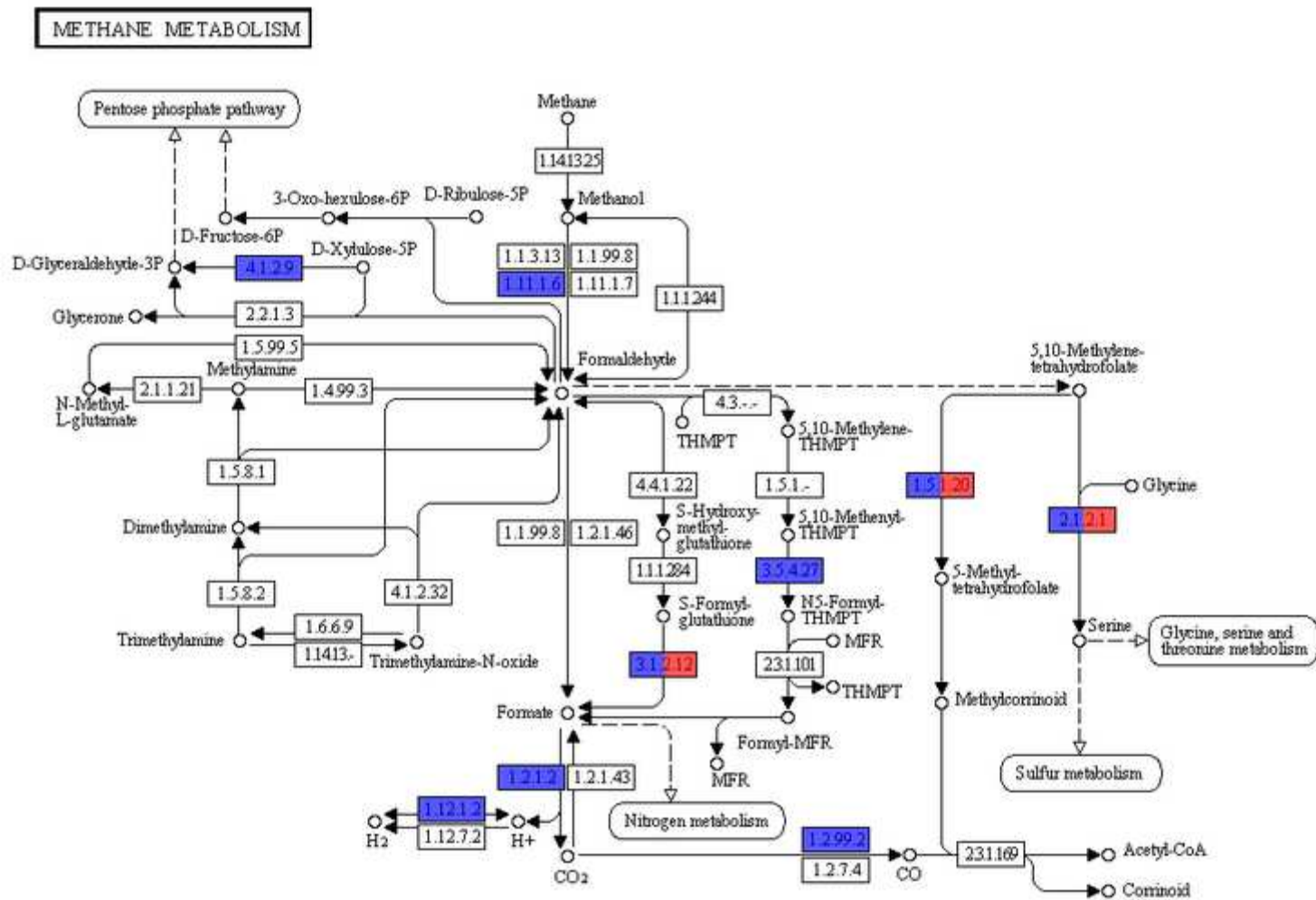


Figure 5.17: KEGG pathway analysis showing the enzymes required for the metabolism of methane. Blue boxes represent enzymes for which genes were found in the quality filtered sequences red; enzymes found in the assembled contigs. No shading indicates that no match was present.

TOLUENE AND XYLENE DEGRADATION

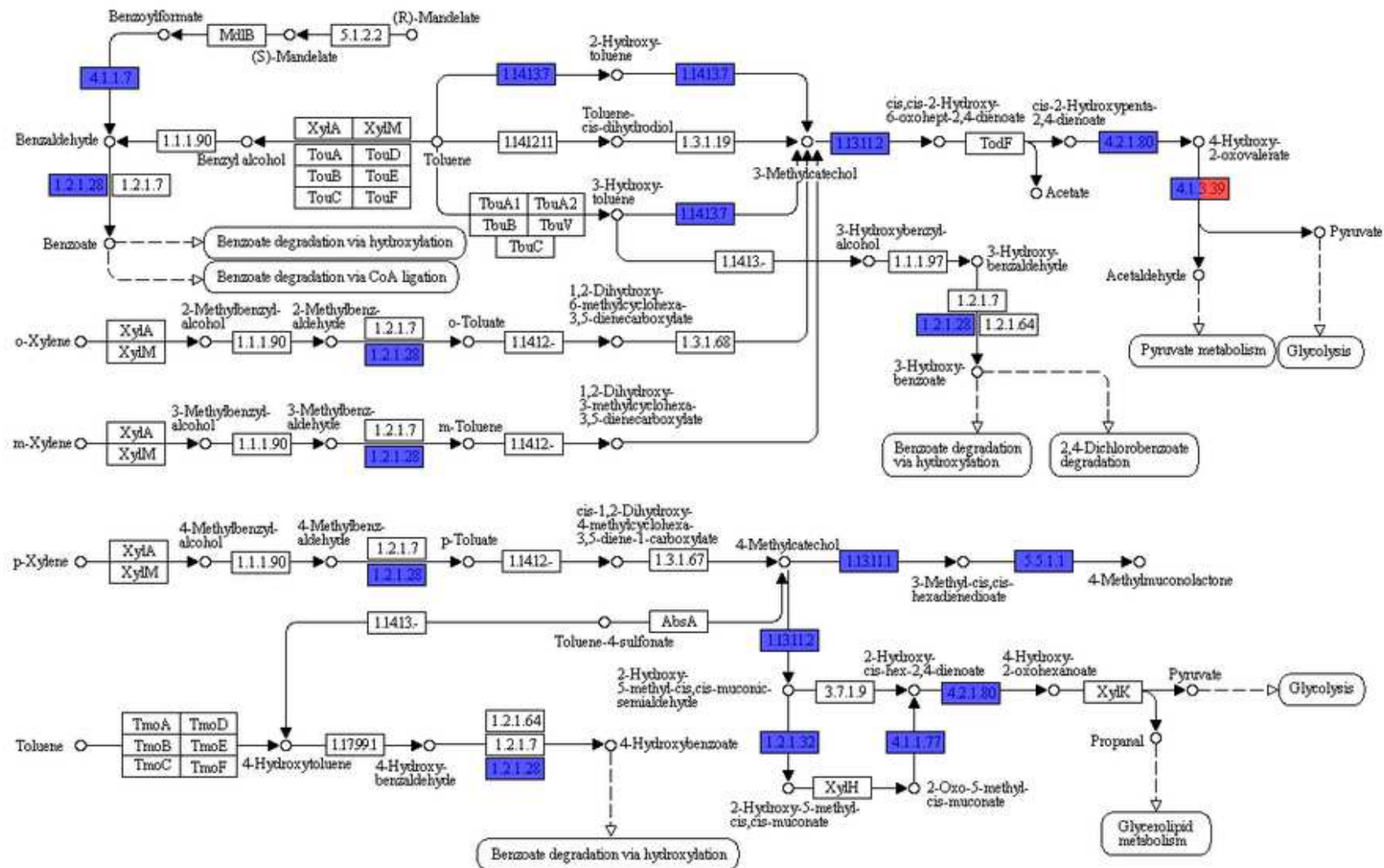


Figure 5.18: KEGG pathway analysis showing the enzymes required for the degradation of toluene and xylene. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the assembled contigs. No shading indicates that no match was present.

BENZOATE DEGRADATION VIA HYDROXYLATION

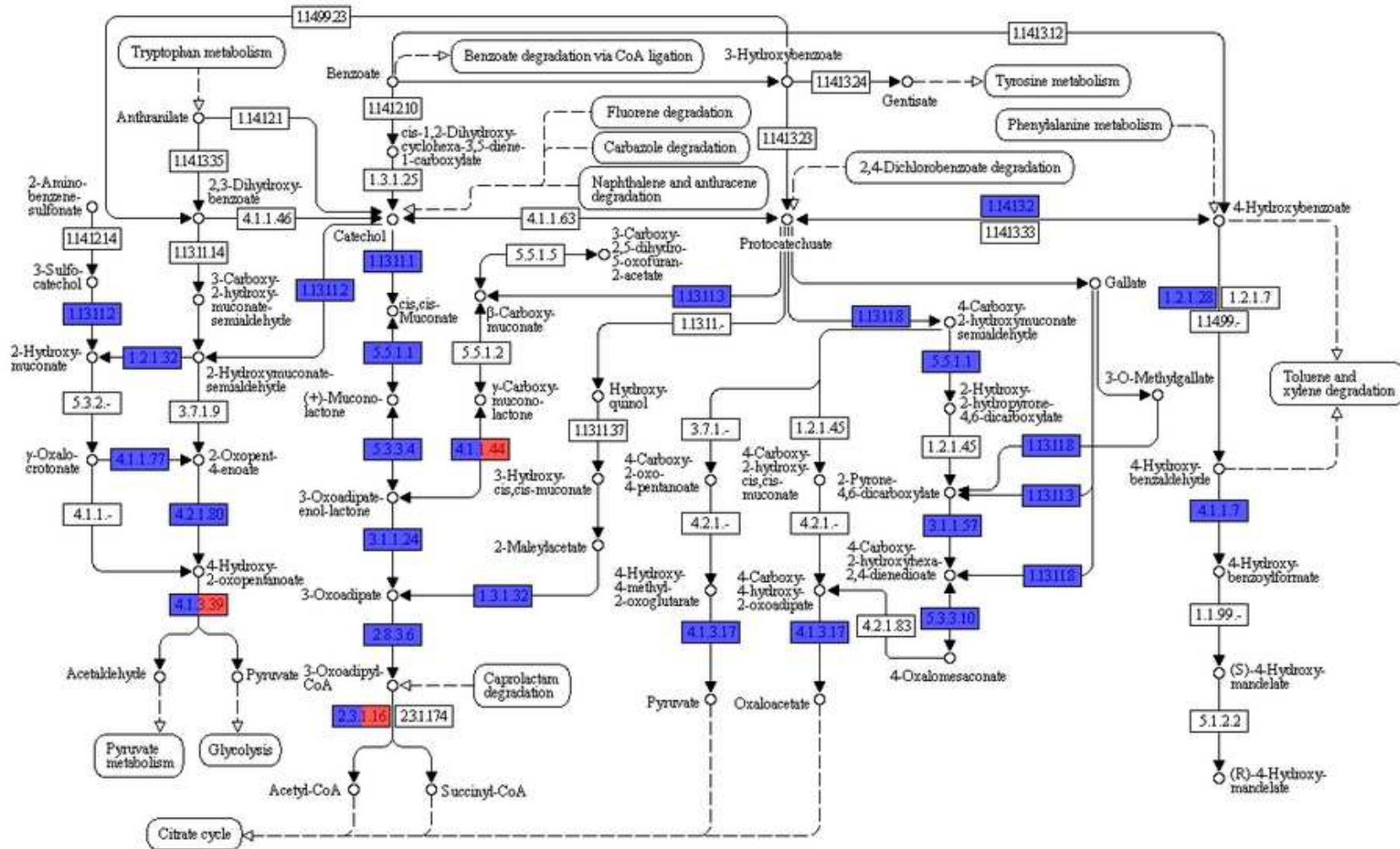


Figure 5.19: KEGG pathway analysis showing the enzymes required for the degradation of benzoate and catechol. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the assembled contigs. Almost all enzymes required for the conversion of catechol to pyruvate and all for the conversion to acetyl-CoA are present. No shading indicates that no match was present.

ETHYLBENZENE DEGRADATION

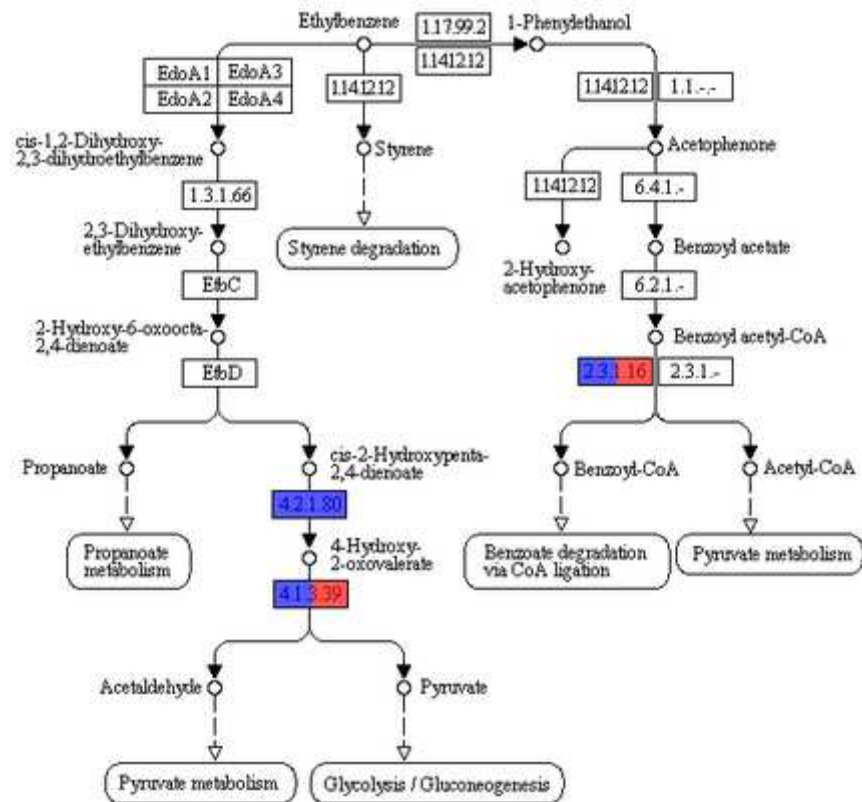


Figure 5.20: KEGG pathway analysis showing the enzymes required for the degradation of ethylbenzene. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the assembled contigs. No shading indicates that no match was present.

STYRENE DEGRADATION

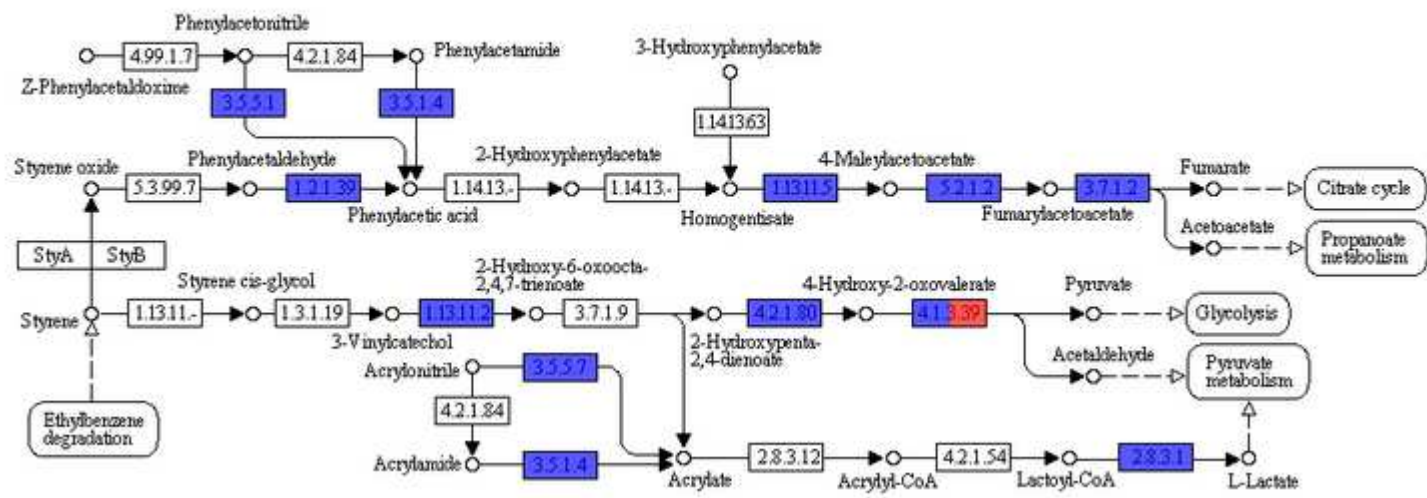


Figure 5.21: KEGG pathway analysis showing the enzymes required for the degradation of styrene. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the assembled contigs. No shading indicates that no match was present.

NAPHTHALENE AND ANTHRACENE DEGRADATION

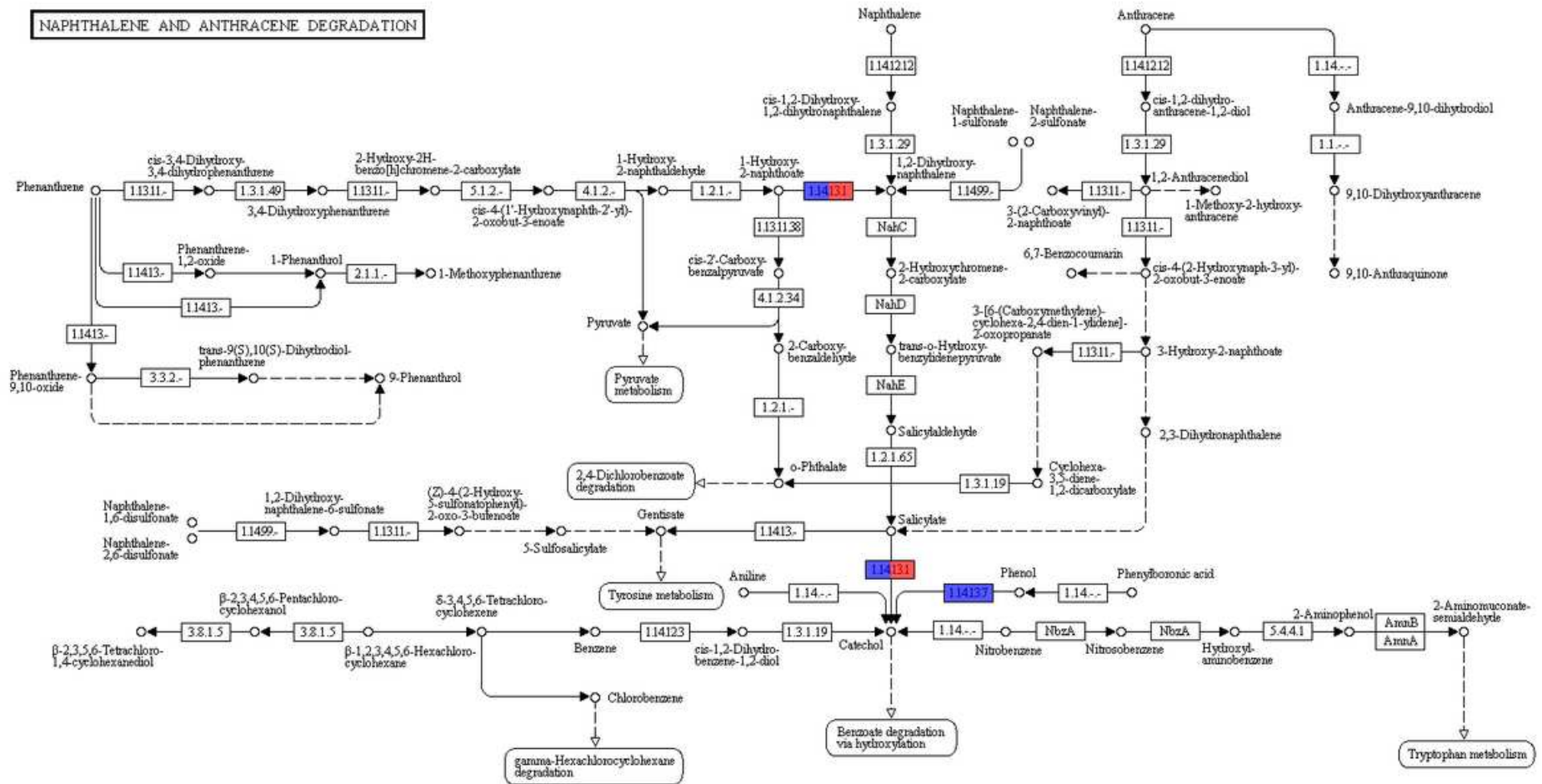


Figure 5.22: KEGG pathway analysis showing the enzymes required for the degradation of naphthalene and anthracene. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the assembled contigs. No shading indicates that no match was present.

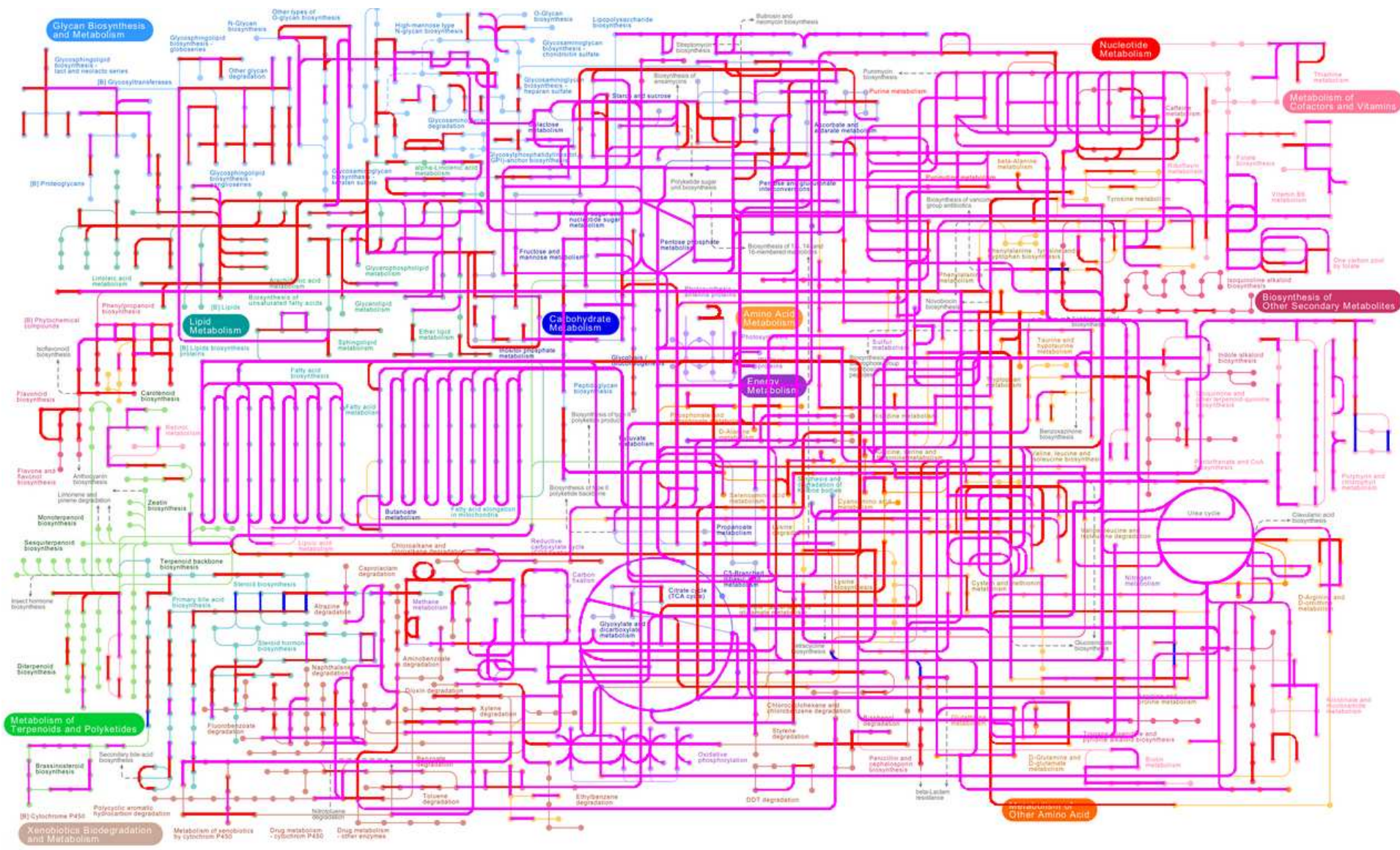


Figure 5.23: KEGG pathway analysis comparing the enrichment culture to other anaerobic hydrocarbon degrading metagenomes (pathways in blue is obtained from the quality filtered sequence reads, pathways in red from the other metagenomes and pathways in purple are where these two data sets overlap).

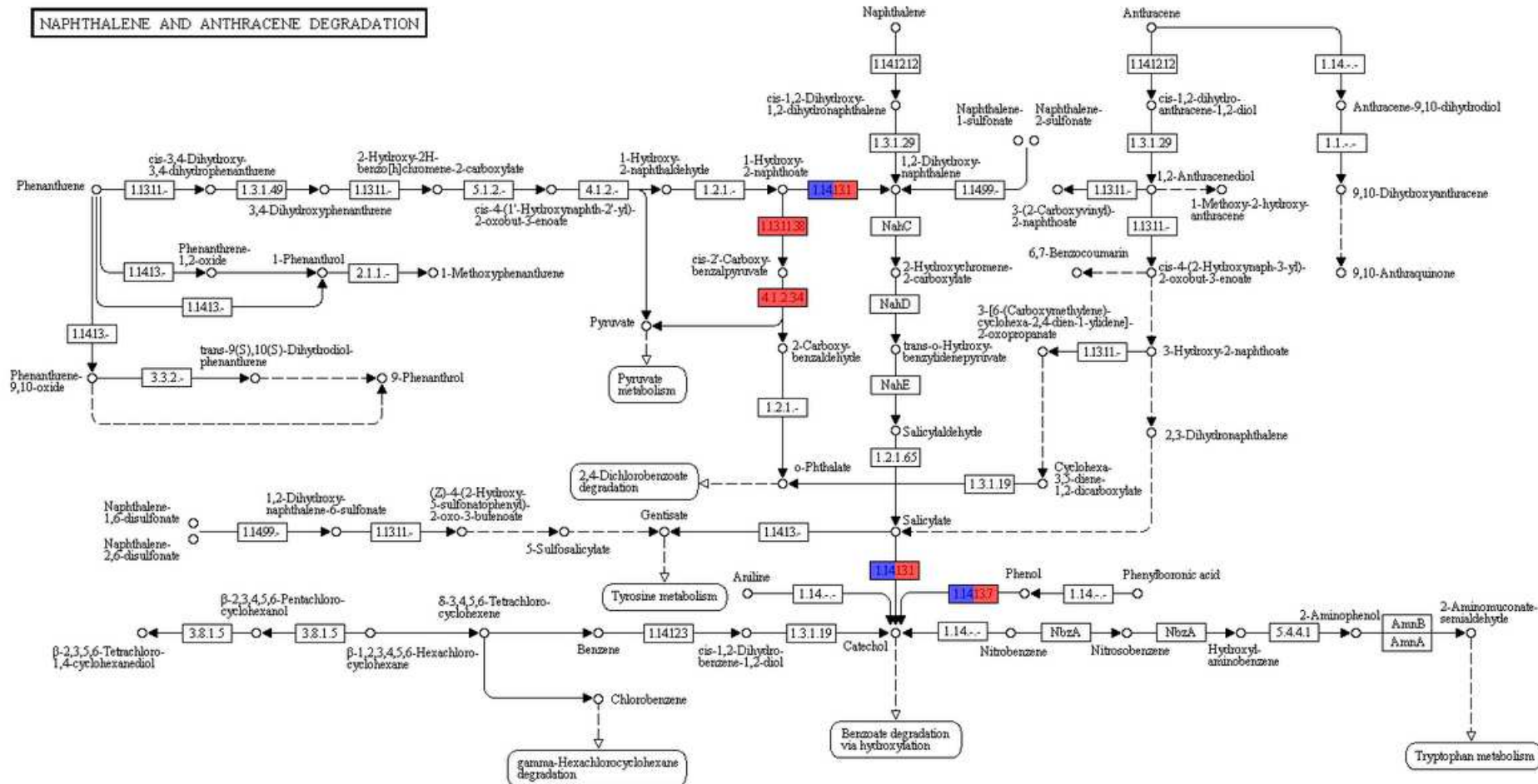


Figure 5.24: KEGG pathway analysis showing the enzymes required for the degradation of naphthalene and anthracene for the quality filtered sequences and the other anaerobic hydrocarbon degrading metagenomes. Blue boxes represent enzymes for which genes were found in the quality filtered sequences ;red enzymes found in the other metagenomes. No shading indicates that no match was present.

BENZOATE DEGRADATION VIA HYDROXYLATION

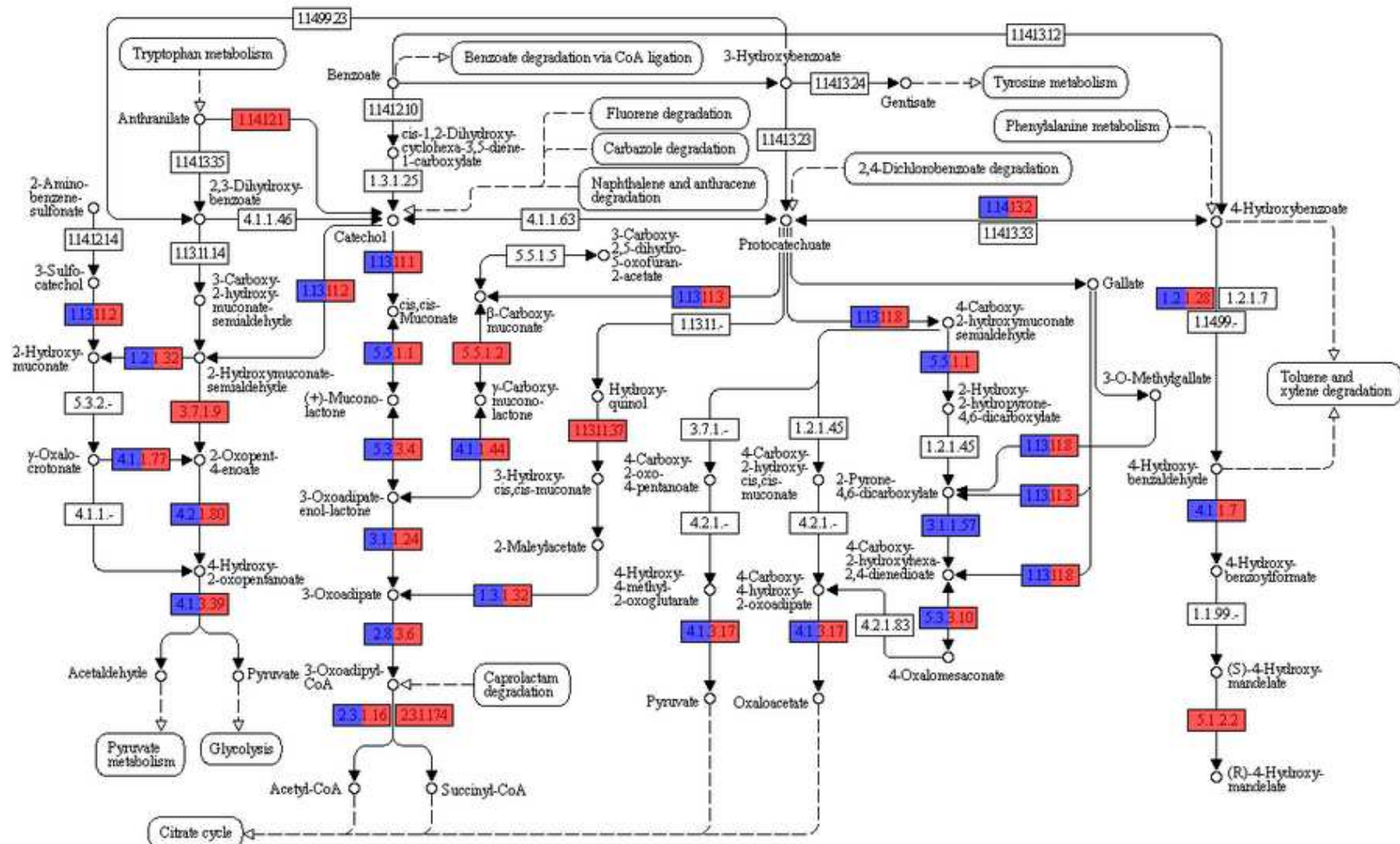


Figure 5.25: KEGG pathway analysis showing the enzymes required for the degradation of benzoate and catechol. Blue boxes represent enzymes for which genes were found in the quality filtered sequences; red enzymes found in the other anaerobic hydrocarbon degrading metagenomes. Almost all enzymes required for the conversion of catechol to pyruvate and all for the conversion to acetyl-CoA are present in both the enrichment culture sequences and the other anaerobic hydrocarbon degrading metagenomes. No shading indicates that no match was present.

5.3.7 Identification of genes involved in hydrocarbon biodegradation

Various genes coding for proteins that are involved in the anaerobic degradation of hydrocarbons, such as *assA/bssA/nmsA* homologues and *ebdA* share a high sequence homology with other phylogenetically related genes. For example, the *assA/bssA/nmsA* genes are often miss-annotated as pyruvate-formate lyases when using the annotation pipelines found in MG-RAST (Tan *et al.*, 2013). Therefore, in addition to the MG-RAST annotation output, reference sequences of genes of interest (Table 5.2) were used in tBLASTn searches against the assembled contigs from the enrichment culture. This tBLASTn query (cut-off set at 1e-20) enabled rapid identification of any potential hydrocarbon degradation genes in the assembled contigs. Table 5.6 summarizes all the positive hits from the tBLASTn search with figure 5.26 giving a broad indication of where in the biodegradation pathways the enzymes are located.

The enzymes involved in aerobic pathways were not included in the BLAST database but were obtained from the MG-RAST annotation, since it is mostly the anaerobic proteins that are miss-annotated. Out of the 14 reference genes (Table 5.2) positive hits were obtained for 7, but there is a large amount of sequence homology between the *assA/bssA/nmsA*. These genes all encode for enzymes involved in the addition of fumarate to straight chain, aromatic or polycyclic aromatic hydrocarbons (these genes and their functions have been discussed in depth in chapter 1). From work done in chapter 3 and 4, it would seem that the positive BLAST hit would be more related to *nmsA*, involved in naphthalene degradation (Meckenstock *et al.*, 2000) (Figure 5.27), than to *assA* (Callaghan *et al.*, 2008b) (alkane degradation) or *bssA* (Callaghan *et al.*, 2010) (benzene or toluene degradation). Genes *ebdA* and *ebdB* are α - and β -subunits of the same enzyme, ethylbenzene dehydrogenase, which is involved in the initial activation step of ethylbenzene by hydroxylation (Kniemeyer and Heider, 2001b). Similarly, for gene *abcD* a benzene carboxylase which activates benzene by carboxylation (Luo *et al.*, 2014). Benzoyl-CoA reductase, *bamB*, forms an important part in the metabolism of many aromatic compounds since most proceed via the common intermediate benzoyl-CoA which is dearomatized by this enzyme (Boll and Fuchs, 1995). None of these genes were identified during MG-RAST annotation against the M5NR database with a more lenient cut-off of 1e-5. These results again indicate the genomic potential for aromatic hydrocarbon degradation of the enrichment culture.

Table 5.6: Genes resulting in positive tBLASTn results.

Gene name	Abbreviation	Substrate
Alkane monooxygenase	AM	alkanes
Non-heme iron monooxygenase	alkB	alkanes
Rieske non-heme iron oxygenases	RNHO	aromatic hydrocarbons
Extradiol dioxygenases	EXDO	aromatic rings
Alcohol dehydrogenase	AD	alcohols
Aldehyde dehydrogenase	ALDH	aldehydes
Alkylsuccinate synthase, α -subunit	assA	alkanes
Benzylsuccinate synthase, α -subunit	bssA	toluene
Naphthylmethylsuccinate, synthase, α -subunit	nmsA	2-methylnaphthalene
Putative benzene carboxylase, δ -subunit	abcD	benzene
Ethylbenzene dehydrogenase, α -subunit	ebdA	ethylbenzene
Ethylbenzene dehydrogenase, β -subunit	edbB	ethylbenzene
Benzoyl-CoA reductase, α -subunit	bamB	benzoyl-CoA

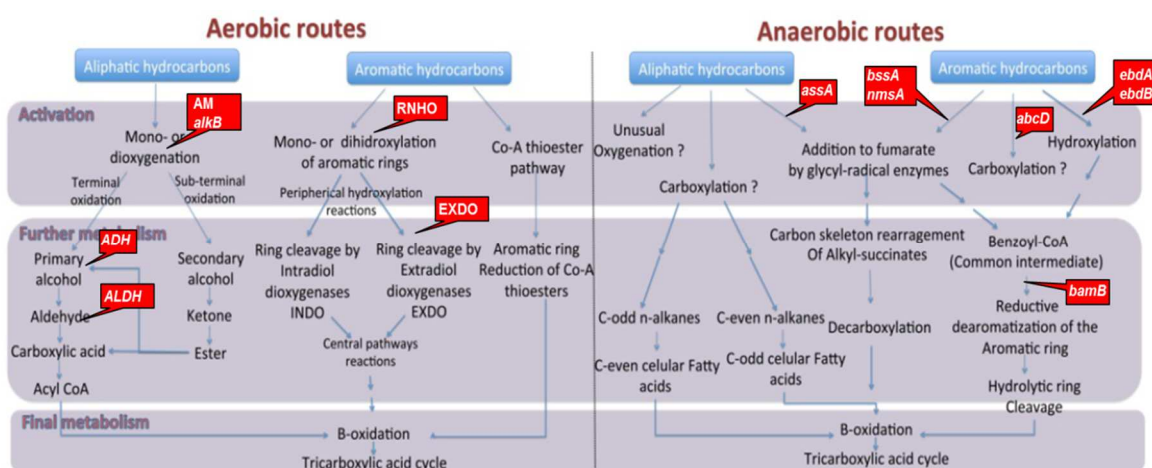


Figure 5.26: Aerobic and anaerobic hydrocarbons degrading genes identified by MG-RAST (aerobic) and tBLASTn query (adapted from Sierra-Garcia and Oliveira, 2013)

5.3.8 Metatranscriptome analysis

5.3.8.1 Sequencing results

A total of four samples were submitted for sequencing. The samples labelled 'Acetate' and 'NAPH' were grown on acetate and naphthalene respectively, as described in section 5.3.2.5, and harvested in late exponential phase, resuspended in RNeasy Protect Bacteria Lysis Buffer (Thermo Fisher) and stored at -80°C. RNA was extracted as described in section 5.3.2.5.

Three quality control steps were performed to assess the quality and quantity of the nucleic acids for the library preparation and sequencing. Firstly, NanoDrop ND1000 (Thermo Fisher) measurements were performed to assess the $A_{260/230}$ ratios. This ratio provides an indication of the presence of the impurities such as chaotropic salts, polyphenols and polysaccharides in the samples. Polyphenols and polysaccharides will entrap the RNA making it unavailable for downstream enzymatic reactions, while the salt lowers the melting temperature threshold for fragmented DNA and biases the sequencing library towards GC-rich base content. The results from the NanoDrop quality determination can be seen below (Table 5.7). For high quality RNA samples the $A_{260/230}$ should be 2.0 (+/- 0.2).

Table 5.7: NanoDrop ND1000 analysis performed on the extracted RNA samples.

Sample ID	A_{280}	$A_{260/280}$	$A_{260/230}$
Acetate_2	0.302	1.34	1.03
Acetate_3	1.212	1.64	1.09
NAPH_2	0.727	1.96	2.29
NAPH_3	0.917	2.01	2.31

Secondly the Qubit® RNA assay kit (Thermo Fisher) uses specific RNA binding probes, thus providing accurate RNA specific concentration values in the sample isolates. The reactions are then analysed on the Qubit® 2.0 Fluorometer (Thermo Fisher) to obtain concentration readouts. The determined RNA concentration for the different samples can be seen below (Table 5.8).

Lastly, the BioAnalyzer (Agilent) and the prokaryote RNA Pico Assay kit (Agilent) were used to assess the integrity of the four bacterial RNA samples prior to downstream applications. For the high quality sequencing a RNA Integrity Number (RIN) between 7 and 10 is needed with an rRNA Ratio (23S/16S) between 1.0 and 2.6. Even though the values obtained for the extracted samples were below the optimum (Table 5.9), it was decided to continue with the

sequencing since these values are a very good guideline, but good sequencing can still be obtained.

Table 5.8: RNA concentration analysis performed on the Qubit® 2.0 fluorometer.

Sample ID	Concentration (ng/μL)	Purity level (A _{260/280})
Acetate_2	77.2	1.03
Acetate_3	1240	1.09
NAPH_2	68.2	2.29
NAPH_3	120	2.31

Table 5.9: RNA integrity analysis performed on the BioAnalyzer.

Sample ID	RIN	rRNA Ratio (23S/16S)
Acetate_2	5.6	0.3
Acetate_3	5.4	0.3
NAPH_2	7.2	0.6
NAPH_3	6.6	0.7

Interpretation of library concentrations and fragment size distribution profiles are very important quality control results for post library preparation and prior to sequencing. These were assessed using the Qubit® HS DNA Assay Kit (Thermo Fisher) and BioAnalyzer High Sensitivity DNA Assay Kit (Agilent). Results are shown in table 5.10 below.

Table 5.10: Library construction and fragment size distribution quality control.

Sample ID	From (bp)	BioAnalyzer		Qubit® (ng/μL)	Conversions nM
		To (bp)	Ave Size (bp)		
Acetate_2	145	601	310	34.8	172.7
Acetate_3	77	7113	398	29.8	115.2
NAPH_2	126	623	292	41	216.0
NAPH_3	126	712	295	33.2	173.1

A total of 100.5 GB of data was generated for the four libraries with an average of 12.6 GB per sequence read cycle per library and 25.1 GB of paired-end data per library. BaseSpace (Illumina) onsite adaptor trimming was used and thus sequence read lengths range from 35 to 151 nucleotides after adapter trimming. The average number of reads per sample (Table 5.11) is about 45 million single-ended reads totalling about 90 million paired-end reads per sample. A total of 80% of the data was at least Q30 and above.

Table 5.11: Sequencing data output.

Sample ID	Read 1	GB	Read 2	GB
Acetate_2	43,688,288	11.9	43,688,288	12.1
Acetate_3	47,182,716	15.1	47,182,716	15.2
NAPH_2	31,344,738	8.7	31,344,738	8.9
NAPH_3	56,679,252	14.1	56,679,252	15.5

5.3.8.2 MG-RAST analysis of aromatic metabolism

Based on the functional annotations made by MG-RAST (Figure 5.27) the dataset obtained from the sequenced transcriptome was examined for genes that may be involved in the degradation of aromatic compounds. All metatranscriptome data is accessible on MG-RAST under the ID numbers 4662020.3, 4662019.3, 4662278.3, 4662270.3, 4662018.3, 4662023.3, 4662266.3, 4662021.3, 4662175.3. As with the metagenomics annotations, described in section 5.3.6, reads were annotated in the SEED “Subsystems” category “Metabolism of aromatic compounds”. However, the metagenome annotations only indicate a presence of these genes in the available gene pool. These results now indicate that mRNA is being actively transcribed for genes in these pathways. Genes for the “Metabolism of central aromatic intermediates” and most important to this study, “Anaerobic degradation of aromatic compounds” were identified by MG-RAST annotation (Figure 5.28).

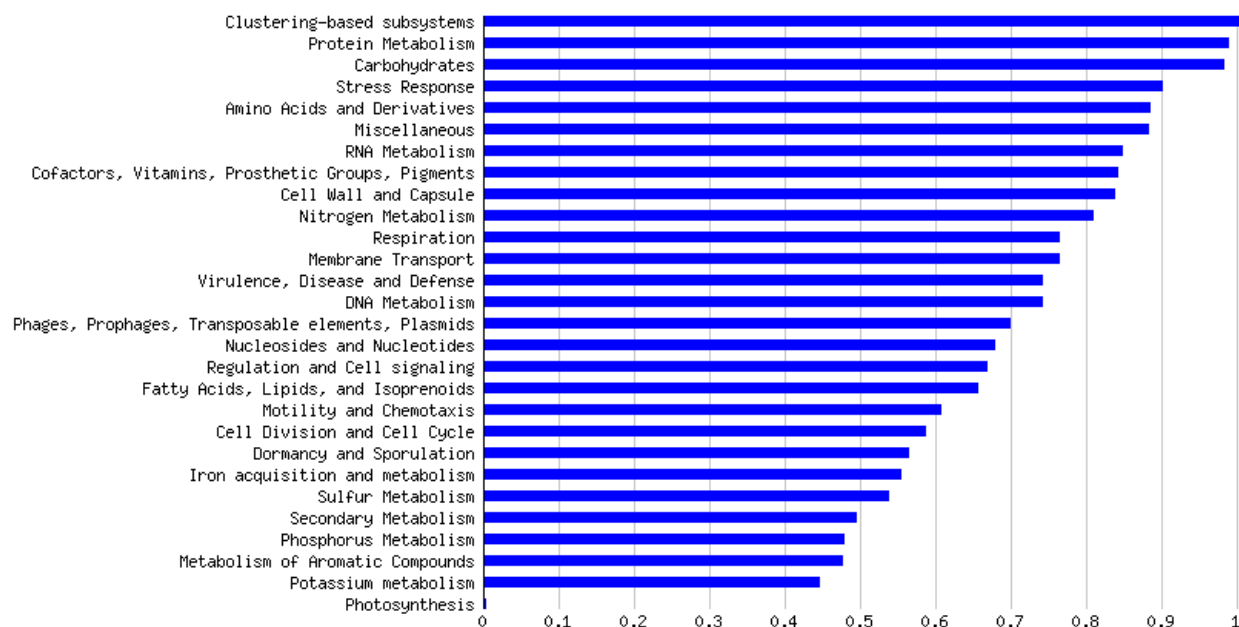


Figure 5.27: Total functional annotation of the transcriptome in the SEED Subsystem database using MG-RAST. The data has been normalized to values between 0 and 1.

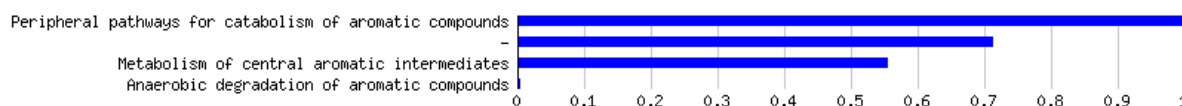


Figure 5.28: Functional category breakdown of reads classified as “Metabolism of aromatic compounds” in the SEED Subcategory database using MG-RAST annotations of the transcriptome. The data has been normalized to values between 0 and 1.

KEGG pathway analysis (Figure 5.29) does not indicate any unique pathways expressed due to growth on naphthalene as carbon source. When considering the pathway for acetate metabolism and some of the proposed pathways employed during anaerobic naphthalene degradation (Figure 3.30) this lack of unique pathway expression can be explained by the fact that naphthalene will initially be activated by novel pathways, as discussed in chapter 1, however, activated naphthalene will be degraded to acetyl-CoA (Kummel *et al.*, 2015) for further metabolism in the citric acid cycle, thus following the same metabolic pathways as acetate.

The pathway for tyrosine metabolism does however contain some mRNA transcripts only present in the naphthalene grown culture (Figure 5.31). One such enzyme is isomerase 5-carboxymethyl-2-hydroxymuconate Delta-isomerase (EC 5.3.3.10) that has been identified in benzoate degradation (Paliwal *et al.*, 2014), which forms part of the meta pathways for naphthalene intermediate degradation (Williams *et al.*, 1975). A methyltransferase (EC 2.1.1.6) involved in various reactions (Figure 5.31) is also only transcribed in the naphthalene grown culture. Methylation of naphthalene to 2-methylnaphthalene is a known activation mechanism (Figure 5.29) in anaerobic naphthalene degradation coupled to sulphate reduction (Safinowski and Meckenstock, 2006b) and it follows that similar reactions would be utilized by nitrate reducing microorganisms. However, to our knowledge, this is the first documentation for this activation mechanism by a nitrate reducing consortium. Further, under sulphate reducing conditions it has been shown that under anaerobic conditions 2-methylnaphthalene is activated by fumarate addition to the methyl group (E. V. A. Annweiler *et al.*, 2000), as is the case in anaerobic toluene degradation (Beller and Spormann, 1997) by the enzyme benzylsuccinate synthase (Birgitta Leuthner *et al.*, 1998). From the hydrocarbon degradation gene database searches performed in section 5.3.7, it is clear that various sequences homologous to the *bssA* sequence are present in the metagenome data (Table 5.5) one of which is the naphthylmethylsuccinate synthase gene (*nmsA*). Similar to *bssA*, *nmsA* is also involved in fumarate addition to a methyl group, in this case to the methyl group on 2-methylnaphthalene (Acosta-González *et al.*, 2013). Although the presence of this gene is not evident from the MG-RAST analysis, as explained in section 5.3.7, these sequences could have been miss-annotated. From this data, the genetic and transcriptomic

possibility exists that naphthalene could be activated via methylation by a methyltransferase followed by the addition of fumarate. The added fumarate will be supplied by the enzyme fumarylacetoacetase (EC 3.7.2.1), which is also only expressed in the naphthalene grown culture.

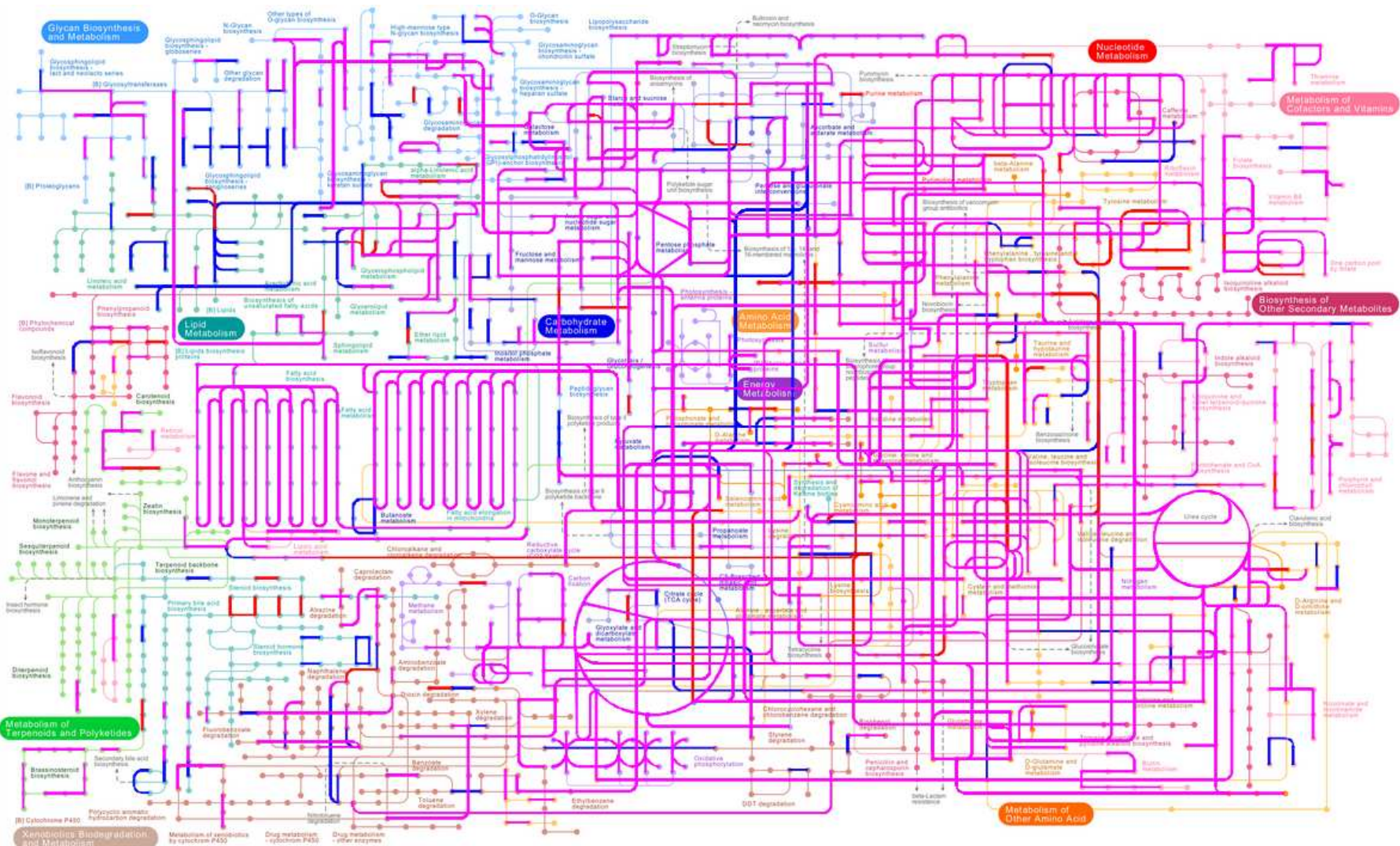


Figure 5.29: KEGG pathway analysis (pathways in blue is obtained from the acetate grown sequence reads, pathways in red from the naphthalene grown sequence reads and pathways in purple are where these two data sets overlap).

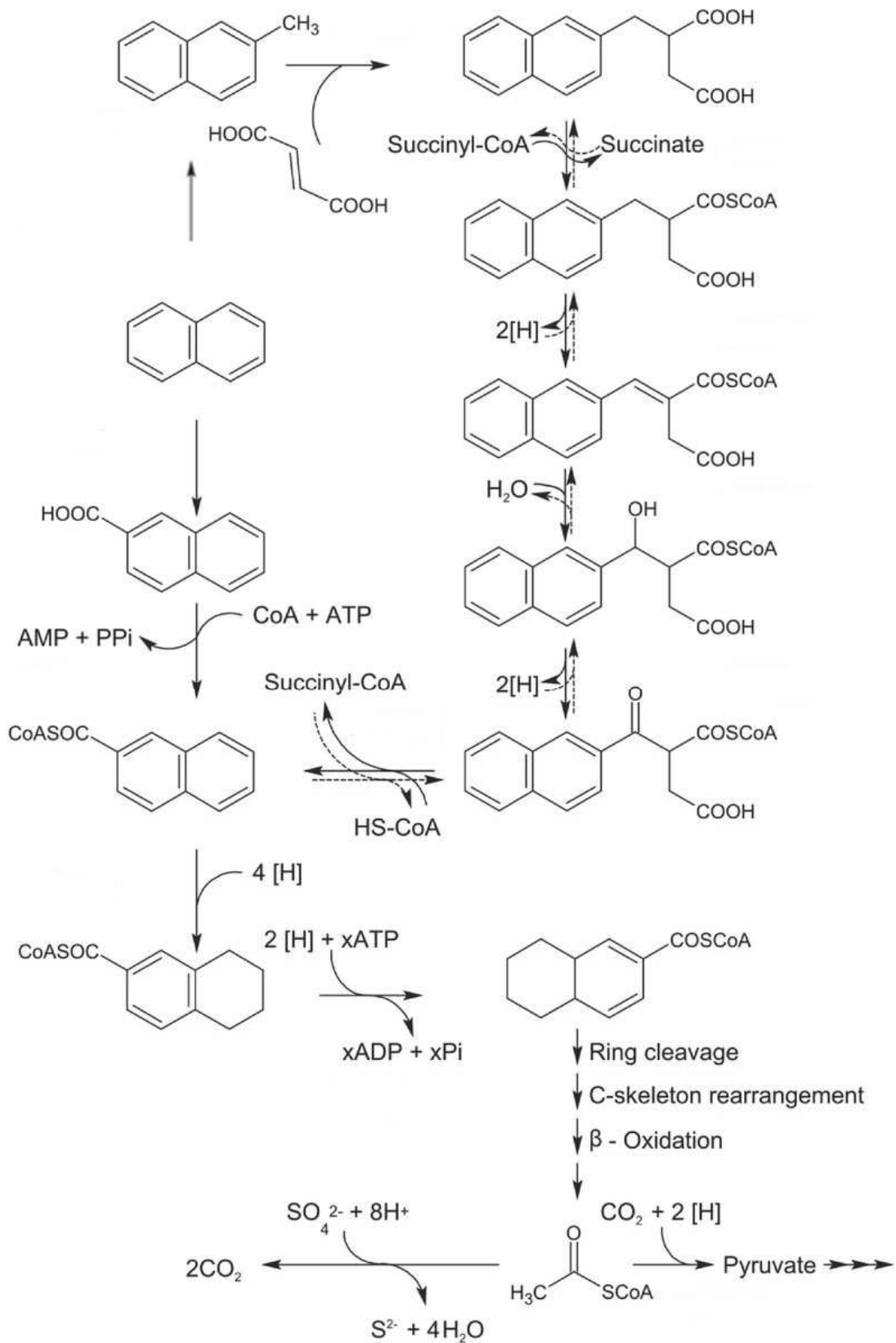


Figure 5.30: Reconstructed naphthalene degradation pathway based on the proteome analysis in sulphate reducing naphthalene-degrading enrichment cultures (adapted from Kummel *et al.*, 2015).

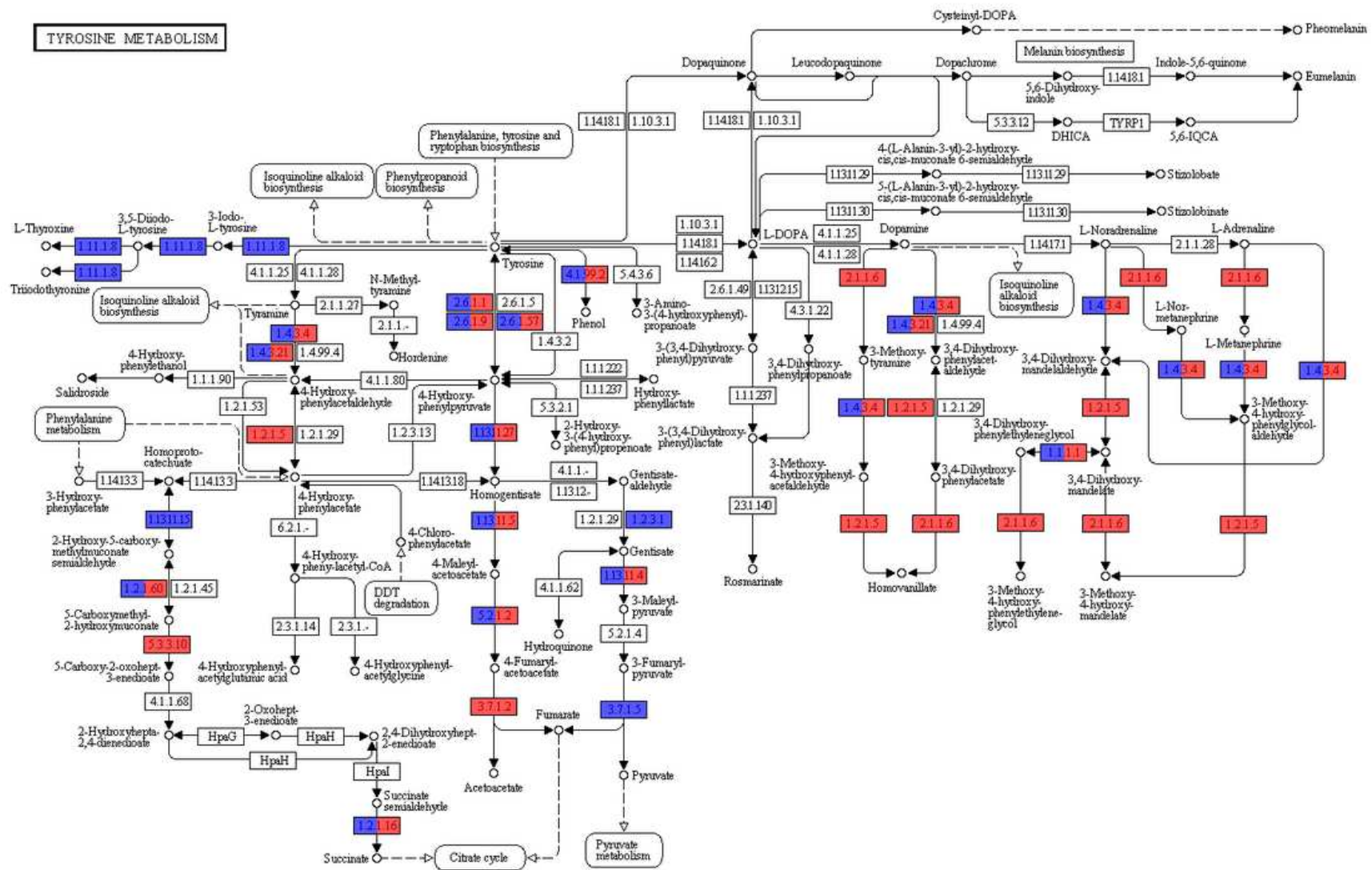


Figure 5.31: KEGG pathway analysis showing the enzymes required for tyrosine metabolism. Blue boxes represent enzymes for which genes were found in the acetate grown sequence reads and red boxes represent enzymes found in the naphthalene grown sequence reads. No shading indicates that no match was present.

5.3.8.3 Differential gene expression analysis

High-throughput mRNA sequencing (RNA-seq) on next generation platforms offers the ability to discover new genes and transcripts. Furthermore, the large number of reads produced by transcriptome sequencing can be utilized as a function of that transcripts' abundance by using read density to measure transcript and gene expression (Trapnell *et al.*, 2012). The sequencing experiment was designed to generate relatively long 151 bp sequences (paired-end) to improve specificity of the mapping. Gene expression was quantified using the FPKM (Fragments Per Kilobase of transcript per Million Mapped reads) normalization method. This method uses the number of reads mapped to a given gene to estimate transcript levels (Trapnell *et al.*, 2012). In order to analyse these large datasets robustly, efficient and statistically principled algorithms need to be employed. RNA-seq analysis tools have to be able to align reads to a reference, assemble transcripts and quantify transcripts and genes. The Tuxedo Suite serves all three these roles and also includes a tool for the visualization of the results and was thus selected for FPKM quantitation.

The paired-end reads were mapped against the assembled metagenome contigs, as this was deemed the best genomic representation of the enrichment culture. Cuffdiff, included in the Cufflinks package, was used to assess biases in read distribution across each transcript and to estimate the statistical significance of gene expression changes between samples. The Cufflinks results were then accessed in the R statistical computing environment using CummeRbund (v2.0.0). A generated scatter plot of the Cufflinks results show the averages of \log_2 transformed expression values between the two growth conditions (Figure 5.32). The selection criteria for selecting differentially expressed genes was based on a \log_2 fold change > 1 to ensure that only genes with at least a two-fold increase in expression in the naphthalene grown culture were considered for downstream analysis. Using these criteria, a total of 49 differentially expressed genes in the naphthalene grown culture were identified, represented in a barplot generated by CummeRbund (Figure 5.33). Unfortunately, the annotated genome feature file (.GFF) generated for the assembled metagenome contigs was incompatible with the TopHat alignment pipeline. Analysis was completed, however, the identities of the 49 differentially expressed genes were annotated as Cufflink identifiers. These identifiers correspond to nucleotide ranges within the contigs, which was extracted and subjected to BLASTp analysis against the RefSeq protein database.

Similarly to what was observed when the total proteome was analysed, as described in section 4.3.6, most of the differentially expressed proteins are homologues of membrane transport proteins such as efflux systems, transporters, porins and permeases which, as previously discussed, could be involved in either transporting these large compounds into

the cells for metabolism or helping to regulate the toxic effect naphthalene might induce. Also various proteins involved in DNA replication are being differentially expressed, such as the DNA primase, which would ensure that DNA that might be damaged due to exposure to naphthalene is repaired before replication (Arezi and Kuchta, 2000). Most important to this study is the sole expression of a methyltransferase in the naphthalene grown culture, once again hinting at a methylation reaction for the activation of naphthalene to 2-methylnaphthalene as discussed above in section 5.3.8.2. Another possible activation mechanism present in the differentially expressed gene data is carboxylation of naphthalene to 2-naphthoic acid (Figure 5.30) by a carboxylase which is also being differentially expressed. Since this is an enrichment culture comprising of various organisms, it is difficult to judge if both these mechanisms are being performed by a single or different organisms.

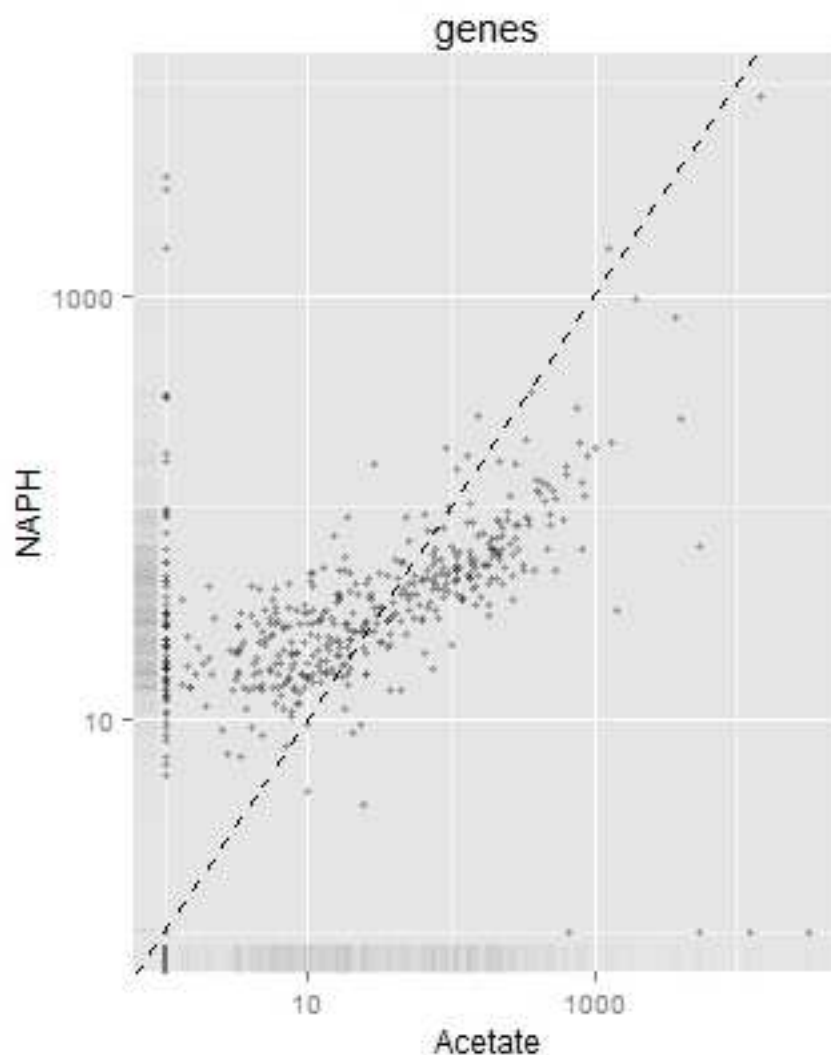


Figure 5.32: Scatterplot comparing genes across the two growth conditions generated by CummeRbund.

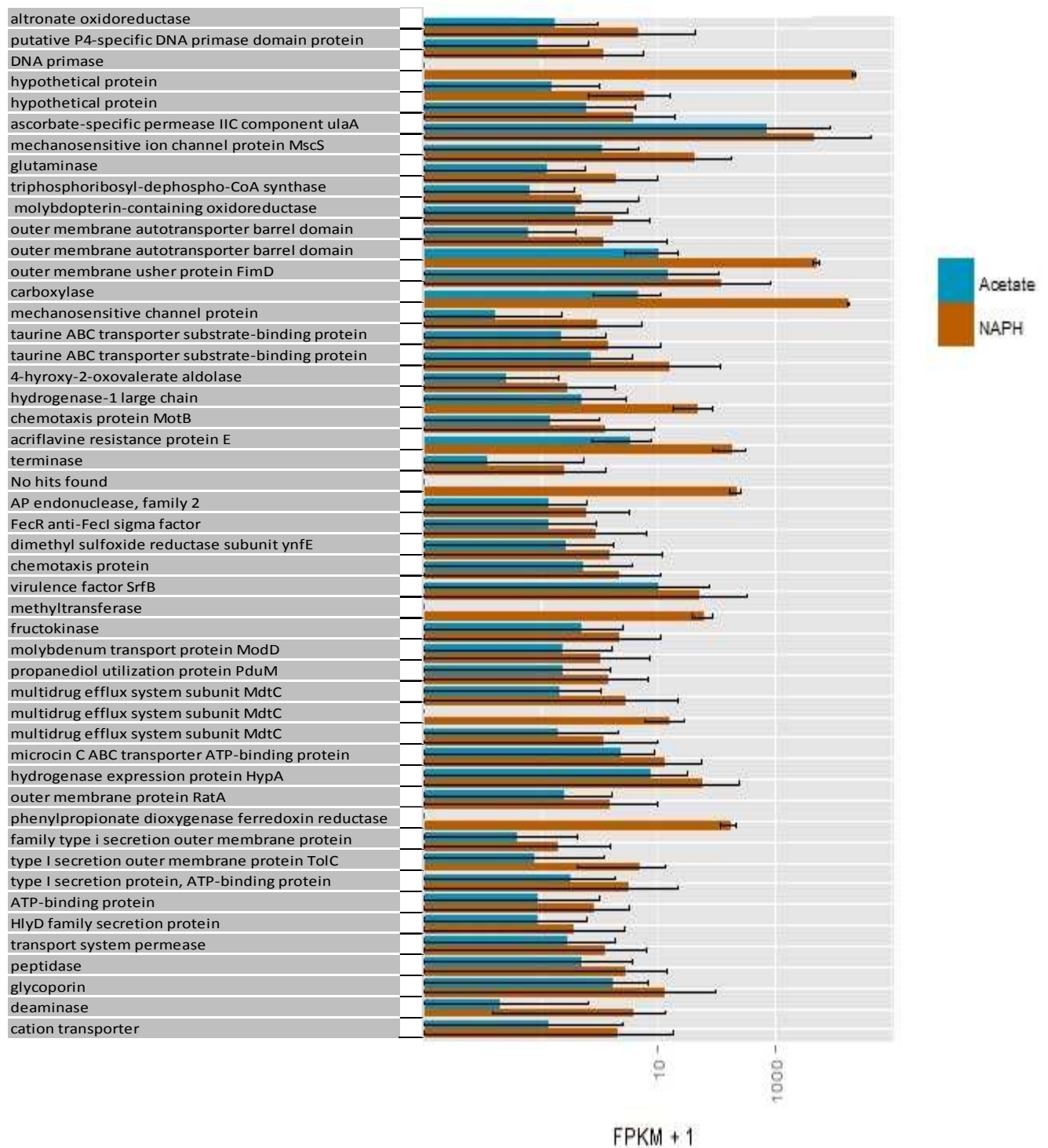


Figure 5.33: Expression barplot showing genes expressed with a log₂ fold change > 1 in the naphthalene grown culture.

5.4 Conclusions

Our understanding regarding anaerobic biodegradation of hydrocarbons have advanced considerably in recent years, but the organisms responsible and the mechanisms employed by nitrate reducing organism for the *in situ* activity remain far from complete. Advances in sequencing technologies have made microbial diversity as well as metabolic potential studies easier and more accurate. A significant increase in the taxonomic classification was observed in the targeted as well as total metagenome sequencing when compared to the results obtained by DGGE and 16S library sequencing. From this data, estimations for the diversity of the enrichment culture indicate dominance by Gammaproteobacteria, specifically the Enterobacteriaceae. Concurrent with the DGGE and 16S library results is the high percentage of *Citrobacter* sp. in the enrichment culture.

Metagenomic analysis of the enrichment culture facilitated the prediction of the metabolic potential of this microbial community. Various pathways are present in the genomic composition of the enrichment culture that would facilitate the degradation of naphthalene, as well as other hydrocarbons. Although MG-RAST analysis was not able to identify any of the anaerobic hydrocarbon activating genes in the sequencing reads or assembled contigs, several genes homologous to known hydrocarbon activation enzymes were present in the enrichment culture as indicated by BLAST analysis. Many of these identified enzymes, *bssA/assA/nmsA*, would facilitate the activation of various hydrocarbons via fumarate addition. However the presence of alternative activation mechanisms, such as hydroxylation and carboxylation, was also detected.

Knowing the genetic potential, particularly for genes related to anaerobic hydrocarbon activation, provided a basis for metatranscriptomics interpretation. Combining the metagenome and metatranscriptome observations indicate that there is definite evidence that the enrichment culture has the potential to, and is actively, degrading naphthalene. We are thus able to propose a mechanism for the nitrate reducing culture similar to that which has been identified in sulphate reducing organisms, where naphthalene is activated by methylation to 2-methylnaphthalene by a methyltransferase, followed by fumarate addition. However, further experimental research is needed to explore the full extent of a naphthalene activation and degradation pathway under anaerobic conditions coupled to nitrate reduction.

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Chapter 6

Conclusions and summary

6.1 General conclusions

Despite the availability of naphthalene degrading enrichment cultures, the knowledge about phylogenetic and biochemical characteristics of naphthalene degrading organisms is still lacking. This study describes the isolation and characterization of a nitrate reducing naphthalene degrading enrichment culture, but more importantly, the identification of the role of a methylation and fumarate addition for the initial activation of naphthalene for further metabolism by the enrichment culture.

To isolate the anaerobic naphthalene degrading culture, initial enrichments were performed on three samples obtained from hydrocarbon contaminated environments. Diesel was provided as the sole carbon source in various mediums. Both aerobic and anaerobic cultures were enriched during this process. The first objective of this study was to perform a detailed molecular and physiological characterization of these enrichment cultures. Molecular characterization of these initial enrichment cultures via 16S rRNA gene analysis resulted in very different microbial diversities between the three environments, even though all three come from diesel contaminated sources, and even between aerobic and anaerobic conditions. This showcases the microbial population elasticity as the redox conditions fluctuate when oxygen is introduced into the environment and is subsequently consumed during metabolism, thus resulting in the degradation of hydrocarbons under both these conditions. Based on the analysis of the 16S rRNA gene sequences, the enrichment culture containing microorganisms phylogenetically related to the genus *Citrobacter* and other Enterobacteriaceae was selected for further study. This enrichment culture was deemed the most novel of the three since it was able to grow on various hydrocarbons and coupled this metabolism to nitrate reductions. Very little literature is available pertaining to *Citrobacter* sp. and their interactions with hydrocarbons, but the knowledge base for hydrocarbon interactions under nitrate reducing conditions is also severely lacking when compared to sulphate reducing enrichment cultures. Even though several hydrocarbon substrates were tested, the enrichment culture appeared to only significantly degrade naphthalene under anaerobic conditions. However, naphthalene is a model compound for anaerobic degradation studies since it is the simplest of the polycyclic aromatic hydrocarbons. As such this study helped to understand the role and importance of Gram-negative

Enterobacteriaceae, especially Gammaproteobacteria, as better key players in enrichment cultures with naphthalene.

The second objective of this thesis was to elucidate the mechanism of anaerobic naphthalene degradation using the characterized nitrate-reducing cultures. The analysis of the total proteome by SDS-PAGE did not hint at any proposed pathways, as no specific naphthalene-expressed peptide bands were identified, and only managed to showcase that the enrichment culture was actively growing on the hydrocarbon substrate. However, growth studies on various activated forms of naphthalene, 2-naphthoic acid, 2-methylnaphthalene, 1- and 2-naphthol and 2-naphthylamine, suggested the possibility of two different mechanisms. Hydroxylation, due to the growth on 2-naphthol, via a hydroxyl free radical, a mechanism that has been described in literature for denitrifying bacteria. Also methylation of naphthalene followed by fumarate addition to form 2-methylsuccinate, due to high ATP production and nitrate reduction after an extended lag phase while grown on 2-methylnaphthalene, a phenomenon previously described in literature for sulphate reducing bacteria.

To resolve the biochemistry of anaerobic degradation, metagenomics and metatranscriptomic approaches were performed. The microbial community analysis from the total metagenome as well as the targeted 16S metagenomics sequencing once again indicated dominance of Enterobacteriaceae in the enrichment culture, especially the Gammaproteobacteria related to *Citrobacter* sp. From the metagenomics analysis various genes coding for putative anaerobic hydrocarbon activating enzymes were identified, such as *assA/bssA/nmsA* homologues that are all active in the activation of hydrocarbons via fumarate addition. The metagenome also contained genes and pathways for the anaerobic degradation of aromatics once they are activated. The identification of putative proteins and enzymes involved in the initial activation of anaerobic naphthalene degradation in the nitrate reducing enrichment cultures was studied by metatranscriptomics. The total transcriptome expressed in naphthalene and acetate grown cells were compared and correlated to the high-throughput sequenced metagenome information. Metatranscriptome analysis indicated that the transcription of genes encoding a methyltransferase only occurred in the naphthalene grown culture. Combining the results from the metabolism of activated naphthalene derivatives (especially the growth on 2-methylnaphthalene), the metagenomics analysis (the presence of the *nmsA* gene), and the evidence based on the metatranscriptome approach (the expressed methyltransferase), this study supports a mechanism that involves the methylation of naphthalene to 2-methylnaphthalene followed by

the addition of fumarate as the initial activation mechanism of the enrichment culture coupled to nitrate reduction.

In summary, the presented data provide comprehensive information to extend our knowledge concerning anaerobic degradation of naphthalene. The results obtained indicated that methylation coupled to fumarate addition is the initial activation mechanisms for naphthalene degradation by the nitrate reducing enrichment culture. Such mechanisms have previously been suggested for anaerobic naphthalene activation but only in sulphate reducing cultures. However, more research is needed to establish the entire pathway and proteins involved in the degradation.

6.2 Summary / Opsomming

In summary, the presented data provide comprehensive information to extend our knowledge concerning anaerobic degradation of naphthalene. The results obtained indicated that methylation coupled to fumarate addition is the initial activation mechanisms for naphthalene degradation by the nitrate reducing enrichment culture. Such mechanisms have previously been suggested for anaerobic naphthalene activation but only in sulphate reducing cultures. However, more research is needed to establish the entire pathway and proteins involved in the degradation.

Ter opsomming, die data wat aangebied is verskaf omvattende inligting om ons kennis van anaërobiese afbraak van naftaleen aan te vul. Die resultate wat verkry is dui aan dat metilering gekoppel aan fumaraat aanvulling die aanvanklike aktivering meganismes vir naftaleen afbraak deur die nitraat reduserende verrykde kultuur is. Sulke meganismes was voorheen voorgestel vir anaërobiese naftaleen aktivering maar slegs in sulfaat reduserende kulture. Daar is egter meer navorsing nodig is om die hele metabolise weg en proteïene betrokke by die afbraak te bevestig.