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Major functional traits of sediment microbiomes exposed to oil for millennia

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“γηράσκω δ' αἰεὶ πολλά διδασκόμενος”

Σόλων ο Αθηναίος (Solon the Athenian)

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Michas A, Vestergaard G, Trautwein K, Avramidis P, Hatzinikolaou DG, Vorgias CE, Wilkes H, Rabus R, Schloter M, Schöler A (2017) More than 2500 years of oil exposure shape sediment microbiomes with the potential for syntrophic degradation of hydrocarbons linked to methanogenesis. Microbiome 5:118

Summary

Various studies have investigated the impact of oil contamination on the environment and the microbial response to sudden inputs of oil, but our knowledge about the long-term effect of oil on the environmental microbiomes is still limited. Ecosystems exposed to natural oil seeps offer the opportunity to study the impact of oil over long time periods of millennia. In the current project, we focused on sediments which are naturally exposed to an asphalt oil spring, obtained from Keri Lake, a coastal fen ecosystem in Zakynthos Island, Greece. The presence of several asphalt oil springs at Keri Lake and the surrounding area is a well-known phenomenon since 2500 years ago.

The structure and the functional potential of the indigenous microbiomes, as well as the available electron acceptors and donors in the sediment, were compared between the oil-exposed site and a reference site. Our analysis showed that sulfate was depleted in the oil-exposed sediment, while the concentration of nitrate was higher compared to the non-exposed. The number of *Desulfuromonadales* and *Desulfobacterales*, as well as the genes of both denitrification and sulfate reduction pathways, were lower at the oil-exposed site, while the abundance of several methanogenic orders (mainly *Methanomicrobiales*) and the potential for methanogenesis was clearly higher. The potential for anaerobic hydrocarbon degradation did not differ significantly between the two sites though, despite the dominant presence of oil hydrocarbons in the oil-exposed sediment. Comparison with other oil-impacted ecosystems revealed that methanogenic orders of the phylum *Euryarchaeota* have higher abundances in the ecosystems characterised by long-term oil exposure, compared to short-term contaminated. These indicate that the potential for the degradation of organic matter via methanogenesis is favoured in the former environments.

The role of sulfate availability on the hydrocarbon degradation activity of the Keri Lake sediment microbiomes was further investigated. The enrichment of *Desulfobacterales* and other orders of *Deltaproteobacteria* in liquid cultures with sulfate confirmed that sulfate reducers thriving in the oil-exposed sediment are able to degrade various hydrocarbons, including the heavy oil mixture that seeps at Keri Lake, despite their low abundance. Subsequently, anoxic microcosms were prepared with seawater media and sediments from either of the two sites at Keri Lake, to test the impact of the possible sulfate input from the adjacent marine environment. After the addition of sulfate, the number of bacterial 16S rRNA and *dsr* (dissimilatory sulfite reductase) genes and transcripts increased in both sediments, but the increase was stronger in the oil-exposed sediments. The number of *bamA* (benzoyl-CoA reductase) genes, as well as the abundance of archaeal 16S rRNA and *mcr* (methyl-CoM reductase) genes and transcripts, significantly increased only in the microcosms with non-exposed sediment at the same time points, suggesting that sulfate reducers compete with methanogens for short-chain compounds in the presence of complex oil hydrocarbons.

Overall, our results significantly contributed to our understanding of the sediment microbiomes chronically exposed to oil, revealing clear signals of complex adaptation to these extreme conditions over long geological time periods.

Zusammenfassung

Obwohl sich bereits verschiedene Studien mit der Auswirkung von Ölkontaminationen auf die Umwelt und mit der Reaktion der mikrobiellen Gemeinschaften auf plötzliche Ölexposition beschäftigt haben, ist unser Wissen über Langzeiteffekte immer noch sehr begrenzt. Ökosysteme, die natürlichen Ölvorkommen ausgesetzt sind, bieten die Möglichkeit, den Einfluss von Öl über den Zeitraum von Jahrtausenden zu untersuchen. In diesem Projekt wurden Sedimente aus Keri Lake untersucht, einem küstennahen Feuchtgebiet auf der Insel Zakynthos in Griechenland mit natürlich vorkommenden asphalthaltigen Ölquellen. Die Existenz dieser asphalthaltigen Ölquellen in Keri Lake und Umgebung ist ein seit 2500 bekanntes Phänomen.

Die Struktur, das funktionelle Potenzial des indigenen Mikrobioms sowie die verfügbaren Elektronenakzeptoren und -donore wurden zwischen Öl-exponierten Sedimenten und Referenzstandorten ohne Öl verglichen. Es konnte gezeigt werden, dass Sulfat in den Sedimenten mit Öl reduziert war, während die Konzentration von Nitrat im Vergleich zum Referenzstandort höher war. Die Anzahl an *Desulfuromonadales* und *Desulfobacterales* sowie der Gene für Denitrifikation und Sulfatreduktion waren an den Öl-exponierten Standorten niedriger, während die Abundanz mehrerer methanogener mikrobieller Gruppen (hauptsächlich *Methanomicrobiales)* und das Potenzial für Methanogenese deutlich erhöht war. Das Potenzial zu anaerobem Abbau von Kohlenwasserstoffen unterschied sich zwischen den Standorten nicht signifikant, trotz des hohen Vorkommens von Kohlenwasserstoffen im Öl. Der Vergleich mit anderen Studien zeigte jedoch, dass Mitglieder der methanogenen Euryarchaeota in Ökosystemen, die durch langfristige Ölexposition gekennzeichnet sind, abundanter sind im Vergleich zu Ökosystemen, die nur kurzzeitig Öl ausgesetzt waren. Dies deutet darauf hin, dass langfristige Ölexposition das Potenzial für den Abbau von organischen Verbindungen durch Methanogenese erhöht.

Des Weiteren wurde der Zusammenhang zwischen Sulfatverfügbarkeit und mikrobiellem Abbau von Kohlenwasserstoffen in Sedimenten von Keri Lake untersucht. Die Anreicherung von *Desulfobacterales* und anderen *Deltaproteobacteria* in Flüssigkulturen mit Sulfat bestätigte, dass Sulfatreduzierer aus dem ölexponierten Sediment trotz ihrer geringen Abundanz viele verschiedene Kohlenwasserstoffe, einschließlich des Schwerölgemisches aus Keri Lake, abbauen können. Um die Auswirkungen eines möglichen Sulfateintrags aus der angrenzenden Meeresumgebung zu testen, wurden Sedimente beider Keri Lake-Standorte in Mikrokosmen mit Meerwassermedium unter anoxischen Bedingungen inkubiert. Obwohl sich durch Sulfatzugabe die Anzahl bakterieller Gene und Transkripte für 16S rRNA und *dsr* (dissimilatorische Sulfitreduktase) in beiden Sedimenten erhöhte, war der Effekt in den ölexponierten Sedimenten stärker. Die Abundanz der Gene und Transkripte für *bamA* (Benzoyl-CoA-Reduktase), archaeale 16S rRNA und *mcr* (Methyl-CoM-Reduktase) stieg zur gleichen Zeit nur in den Mikrokosmen mit nicht exponierten Sedimenten signifikant an, was den Schluss zulässt, dass Sulfatreduzierer in Gegenwart von komplexen Ölkohlenwasserstoffen mit Methanogenen um kurzkettige Verbindungen konkurrieren.

Insgesamt haben unsere Ergebnisse wesentlich zu dem Verständnis des Mikrobioms in langfristig mit Öl belasteten Sedimenten beigetragen und gezeigt, dass diese mikrobiellen Gemeinschaften sich über über lange geologische Zeiträume hinweg sehr gut an diese extremen Bedingungen angepasst haben.

1 Introduction

1.1 Oil hydrocarbons

Hydrocarbons are ubiquitous and abundant compounds in the environment that consist of carbon and hydrogen atoms. They originate from various biological sources, mainly produced by plants in terrestrial ecosystems (Eglinton & Hamilton 1967) or cyanobacteria in marine ecosystems (Coates et al. 2014; Lea-Smith et al. 2015). Many hydrocarbons are also produced when buried biomass is deposited in very high depths and is exposed to extreme underground conditions of high heat and pressure over geologic time periods. The formed hydrocarbons migrate in depths closer to the Earth surface and become trapped in reservoirs (Head et al. 2003).

Crude oil is the complex mixture of geologically formed hydrocarbons and other organic compounds, thus consists mostly of carbon and hydrogen. It is now one of the main energy sources for human activities and has significantly influenced our history (Hall et al. 2003). Oil hydrocarbons and related compounds are grouped in four different classes: saturates, aromatics, resins and asphaltenes, with different characteristics (Harayama et al. 1999). The first two classes contain relatively low-molecular-weight hydrocarbons. Saturates consist of linear and branched alkanes and cycloalkanes, while aromatics consist of non-substituted and substituted monoaromatic and polyaromatic molecules, depending on the number of aromatic rings. Resins and asphaltenes are compounds with higher molecular weight, polar groups and more heteroatoms, distinguished based on their solubility or no solubility in *n*-heptane. The primary crude oil composition greatly varies in different geographical areas depending on the source material and the conditions under which the hydrocarbons are formed. For instance, source rocks rich in land-plant organic matter typically produces lighter oils with less sulfur, polar and asphaltic material, compared to marine-algal material-rich rocks (Wenger et al. 2002). The heterogeneity of oil mixtures due to the complex structures of the heavier fractions and the high number of isomers makes it difficult to resolve their composition (Marshall & Rodgers 2004).

1.2 Environmental exposure to oil

Considering the deep formation of oil, oil hydrocarbons are typically not present in ecosystems other than subsurface reservoirs. In many sites worldwide though, hydrocarbons escape into formerly pristine ecosystems by natural seeps (Kvenvolden & Cooper 2003; Etiope et al. 2009); this process is estimated to contribute an average of 600 thousands metric tonnes per year, which corresponds to 47% of the total oil that enters the marine environment nowadays (Kvenvolden & Cooper 2003). Additionally, human-caused oil spills have increased the hydrocarbon load in the environment during the last century, mainly in marine ecosystems due to the growing offshore drilling activities. Around 1500–2000 accidental spills have been reported since the 1950s (Agunwamba and Mbogu 2013), releasing huge amounts of crude oil, depending on the extent of the accident. Large-scale spills have contributed millions of tonnes of oil; the discharge of the Exxon Valdez supertanker in Prince William Sound (1989,

Alaska), the sinking of the oil tanker Prestige off the Galician coast (2002, Spain) and the blowout of the Deepwater Horizon oil rig in Gulf of Mexico (2010) released more than 30, 50 and 500 thousands metric tonnes of crude oil, respectively (Cohen 1995; Garza-Gil et al. 2006; Crone and Tolstoy 2010; Reddy et al. 2012).

Seeps and spills significantly affect the ecosystems in their vicinity and oil hydrocarbons have a negative impact on natural communities and ecosystem services (Jong 1980; Jackson et al. 1989; Menzie et al. 1992; Duke et al. 1997; Bakker et al. 2000; Peterson et al. 2003; Pérez-Cadahía et al. 2014; Onwurah et al. 2007). Epidemiological studies have also shown that oil spills have short- and long-term effects on human health, including respiratory problems, changes in hormone and heavy metal levels in the blood and even DNA damage (Aguilera et al. 2010). The negative impact of oil to the environment results in great economic damage and restoration expenses. Oil spills significantly affect the production of fisheries and aquaculture in marine environments (Garza-Gil et al. 2006; McCrea-Strub et al. 2011) and the land productivity of agricultural sites (Odjuvwuederhie et al. 2006), leading to serious financial losses. The cost for the clean-up of oil spills adds up to the total expenses depending on many different factors, with location being the most important. While the removal of offshore spills is estimated around 7350 US dollars per tonne of oil, the remediation of shorelines can cost more than 150 thousands US dollars per tonne (Etkin 1999). Indicatively, the caused cost of the Exxon Valdez oil spill was close to 400 million US dollars (Cohen 1995), while the Prestige oil spill resulted a loss of more than 700 million euros for the Galicia coast of Spain only (Garza-Gil et al. 2006). In comparison, the Deepwater Horizon oil spill had estimated costs of more than 35 billion US dollars of economic impact, one third of which was spend for the spill containment (Smith et al. 2011).

The magnitude of these events makes it clear that the complete removal of oil from the environment is impossible in a short period of time. The accumulation of oil, mostly soils and sediments, means that its impact persists in the environment. Despite the negative impact on the macroorganisms, ecosystems are also affected in a micro-scale, but the effect is not always clear. Many microorganisms can directly utilise the oil hydrocarbons, thus many studies have focused on how the presence of oil affects individual microorganisms and microbial communities, in an attempt to improve bioremediation techniques and reverse the effects of oil pollution.

1.2.1 Impact of oil contamination on microbial niches

Other than the increase in toxic compounds (Pickering 1999), oil has many noticeable effects on the chemical and physical matrix-specific properties in water, soil and sediment environments. The clear increase in the total organic carbon content leads to oxygen consumption and depletion of nutrients, like nitrate and phosphorus, by biodegradation (Kisic et al. 2009; Shiller & Joung 2012; Ying et al. 2013). Elevated pH (Ying et al. 2013) and temperature (Balks et al. 2002) values have been recorded, as well as altered grain-size distribution and increased soil compressibility (Brakorenko & Korotchenko 2015). In soil and sediment environments, hydrocarbons adhere to the ecosystem matrix and clog the air- or

water-filled pores. As a result, permeability and porosity decrease (Khamehchiyan et al. 2007; Abosede 2013; Klamerus-Iwan et al. 2015), which limits the penetration of gases in the oil-affected soils and sediments and the replenishment of the consumed oxygen.

1.2.2 Impact of oil contamination on microbiomes

Despite the toxic effect of oil on the viability of many microbes, previous studies have shown contradicting results about the initial effect of oil contamination on environmental microbiomes' structure and activity. Community diversity has been diversely correlated to the contamination. While an expected decrease has been reported by some studies (Sutton et al. 2013; Lamendella et al. 2014; Rodriguez-R et al. 2015), others observed a clear increase in microbial diversity (Juck et al. 2000; Zucchi et al. 2003; Nie et al. 2009; Kimes et al. 2013; Abed et al. 2015). Furthermore, tested enzymatic activities responded differently in experiments with various polluted soils (Margesin et al. 2000; Wyszkowska et al. 2002; Wyszkowska et al. 2006; Labud et al. 2007; Achuba & Peretiemo-Clarke 2008; Guo et al. 2012; Klamerus-Iwan et al. 2015). Overall, these suggest that the impact of hydrocarbon mixtures on microbial functions is not consistent and depends on many factors, such as the composition of hydrocarbons or the soil type. The ability of microbial community members to exploit the oil hydrocarbons should be also taken into account. Experiments with nutrient addition to oil-contaminated soils have proven that natural communities have a good potential for bioremediation (Jackson & Pardue 1999; Röling et al. 2002; Chaineau 2005) and they are able to recover quickly after the contamination (Franco et al. 2004; Bordenave et al. 2007).

Detailed characterisation of the structure of microbiomes affected by oil and the emergence of advanced molecular techniques have offered a better insight into the successional patterns of community members. The response of microbiomes has been studied extensively in marine and coastal oil-contaminated environments, due to increasing number of marine oil spills caused by offshore drilling and marine transportation activities. Many studies have focused on the oil spills that followed the sinking of the Nakhodka (Kasai et al. 2001; Maruyama et al. 2003) and the Prestige tankers (Acosta-González et al. 2013) and the blowout of the Deepwater Horizon drilling rig (Beazley et al. 2012; Lu et al. 2012; Dubinsky et al. 2013; Kimes et al. 2013; Mason et al. 2014; Rodriguez-R et al. 2015). The use of next-generation sequencing has given us a complete overview on the short-term and long-term dynamic responses of microbiota after the Deepwater Horizon oil spill in the Gulf of Mexico (King et al. 2015), demonstrating the effectiveness of this approach to model microbiome responses.

It is now clear that oil contamination enriches opportunistic hydrocarbon-degrading members of the natural microbiomes and genes of hydrocarbon degradation pathways, until the exhaustion of the available nutrients (Stapleton et al. 2000; Evans et al. 2004; Hamamura et al. 2006; Head et al. 2006; Hazen et al. 2010; Kostka et al. 2011; Beazley et al. 2012; Lu et al. 2012; Dubinsky et al. 2013; Sutton et al. 2013). Interestingly, Rodriguez-R et al. (2015) reported decreased taxonomic diversity but increased abundance and functional diversity of copiotrophic taxa related to hydrocarbon degradation in response

to the contamination of beach sands in the aftermath of the Deepwater Horizon oil spill. Blooms of aerobic hydrocarbon-degrading proteobacterial taxa, such as *Oleispira* spp. and *Alcanivorax* spp., were observed in deep-sea oil plumes and coastal sediments shortly after the accident, while anaerobic members of the same phylum were also enriched in anaerobic marine sediments (King et al. 2015).

1.3 Microbial utilisation of oil hydrocarbons

Microorganisms with the ability to degrade hydrocarbons play a significant role in carbon cycle in both oxic and anoxic environments. Chemical bonds between C and H atoms in hydrocarbon molecules are stable and inactive, posing an energetic challenge with regards to hydrocarbon utilisation. The energy to overcome this limitation is provided either by light or by coupling the oxidation of the hydrocarbons to the reduction of alternative molecules (Figure 1). Prokaryotes have the ability to reduce electron acceptors transported into their cytoplasm or directly from their extracellular environment or thrive in syntrophic methanogenic consortia (Widdel & Rabus 2001; Richter et al. 2011).

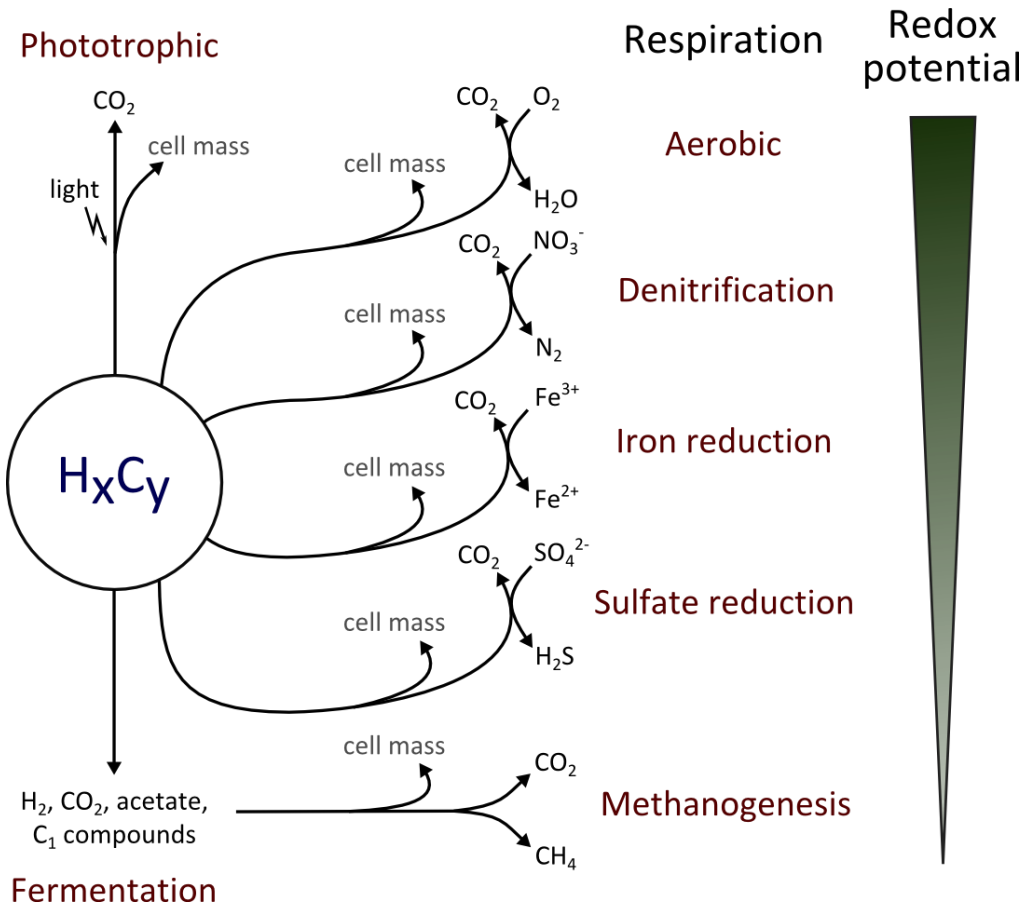

Figure 1. Alternative ways of hydrocarbon degradation (modified by Widdel & Rabus 2001).

1.3.1 Aerobic degradation

The ability of heterotrophic Bacteria and yeasts to exploit hydrocarbons and other oil compounds as sources of carbon in the presence of molecular oxygen (O_2) has been studied extensively and many aerobic hydrocarbonoclastic strains have been detected and isolated so far (Phale et al. 2007; Das & Chandran 2011). The oxygen serves as the terminal electron acceptor and is also directly involved in the activation reactions of the hydrocarbons (Widdel & Rabus 2001). At the first step, oxygenases incorporate oxygen atoms into the hydrocarbon skeleton resulting in hydroxylated intermediates. In most degradation pathways, monooxygenases catalyse the addition of one oxygen atom, but in the case of several alkanes, monoaromatics and polyaromatics the initial attack is performed by dioxygenases, which add two oxygen atoms on the substrate (dihydroxylation). Several pathways of aerobic degradation have been reported which sometimes involve the initial hydroxylation or dihydroxylation on different positions of the same substrate (Harayama et al. 1999).

1.3.2 Anaerobic degradation

During the last four decades, our knowledge on the biodegradation of hydrocarbons has also expanded into anoxic environments (Widdel & Rabus 2001). Under these conditions, microbes use electron acceptors other than O_2 to harness the energy of hydrocarbons, including nitrate (NO_3^-), ferric iron [Fe(III)] and sulfate (SO_4^{2-}) (Figure 1; Rabus et al. 2016); in these cases the oxidation of hydrocarbons is coupled to denitrification, metal reduction or sulfate reduction, respectively. Many Bacteria can also ferment hydrocarbon substrates, yielding intermediate products which are subsequently consumed by nitrate-, iron-, sulfate-reducing partners or methanogens (Gieg et al. 2014). The energy produced by these coupled processes differs (Thauer et al. 1977), therefore the more energy-rich reactions, like denitrification and iron-reduction, are favoured over others. Considering their availability, the respective electron acceptors are utilised sequentially in anoxic environments.

Many anaerobes with the ability to degrade hydrocarbons have been enriched or even isolated and studied in cultures (Rabus et al. 2016). The sequenced genomes of known anaerobic hydrocarbon-degraders, including *Aromatoleum aromaticum* EbN1 (Rabus et al. 2005) and *Desulfobacula toluolica* Tol2 (Wöhlbrand et al. 2013), has shed light on the range of substrates that these organisms can utilise and their metabolic versatility.

Compared to the aerobic hydrocarbon degradation though, our understanding of the enzymatic reactions and the key players of the anaerobic pathways is still limited. A well-studied activation of hydrocarbons is the addition to fumarate, which is catalysed by alkyl-/ arylalkylsuccinate synthases with a glycyl-radical catalytic centre; the reaction involves the addition of methyl and methylene groups of *n*-alkanes or aromatic hydrocarbons. It is used for the activation of a great range of hydrocarbons and can be coupled to all above-mentioned respiratory processes (Heider et al. 2016a). Alternatively, the degradation of hydrocarbons is initiated by O_2-independent hydroxylation (Heider et al. 2016b) or

putative carboxylation (Bergmann et al. 2011). Methylation has been also suggested as the initial step of naphthalene oxidation by the enrichment culture N47 (Safinowski & Meckenstock 2006).

Many peripheral degradation pathways converge at central intermediates, like benzoyl-CoA in the case of aromatic hydrocarbons (Callaghan 2013). The oxidation of benzoyl-CoA proceeds with a reductive dearomatization by class I (ATP-dependent, present mostly in facultative anaerobes) or class II (ATP-independent, present in strict anaerobes) benzoyl-CoA reductases (Kleinsteuber et al. 2012; Rabus et al. 2016). Benzoyl-CoA and the other intermediates are channelled into the central metabolism via acetyl-CoA (Figure 2). Under anaerobic conditions, acetyl-CoA is oxidized completely to CO_2 via two possible pathways: (i) the TCA cycle and (ii) the Wood-Ljungdahl pathway (Thauer 1988; Figure 3). The Wood-Ljungdahl pathway, also known as reductive acetyl-CoA pathway, involves a series of reactions used for carbon fixation and energy production via conversion of CO_2 to acetyl-CoA by anaerobic, mostly acetogenic, Bacteria (Ragsdale & Pierce 2008). It is reductive in the direction of carbon fixation, but can be also used in reverse for the oxidation of acetyl-CoA and the production of reducing power for catabolic reactions during organo-heterotrophic growth (Hattori et al. 2005; Fuchs et al. 2011). The reverse direction has been proposed as the preferred pathway of *Desulfobacula toluolica* Tol2 for the complete oxidation of acetyl-CoA produced by the anaerobic degradation of several aromatics (Wöhlbrand et al. 2013).

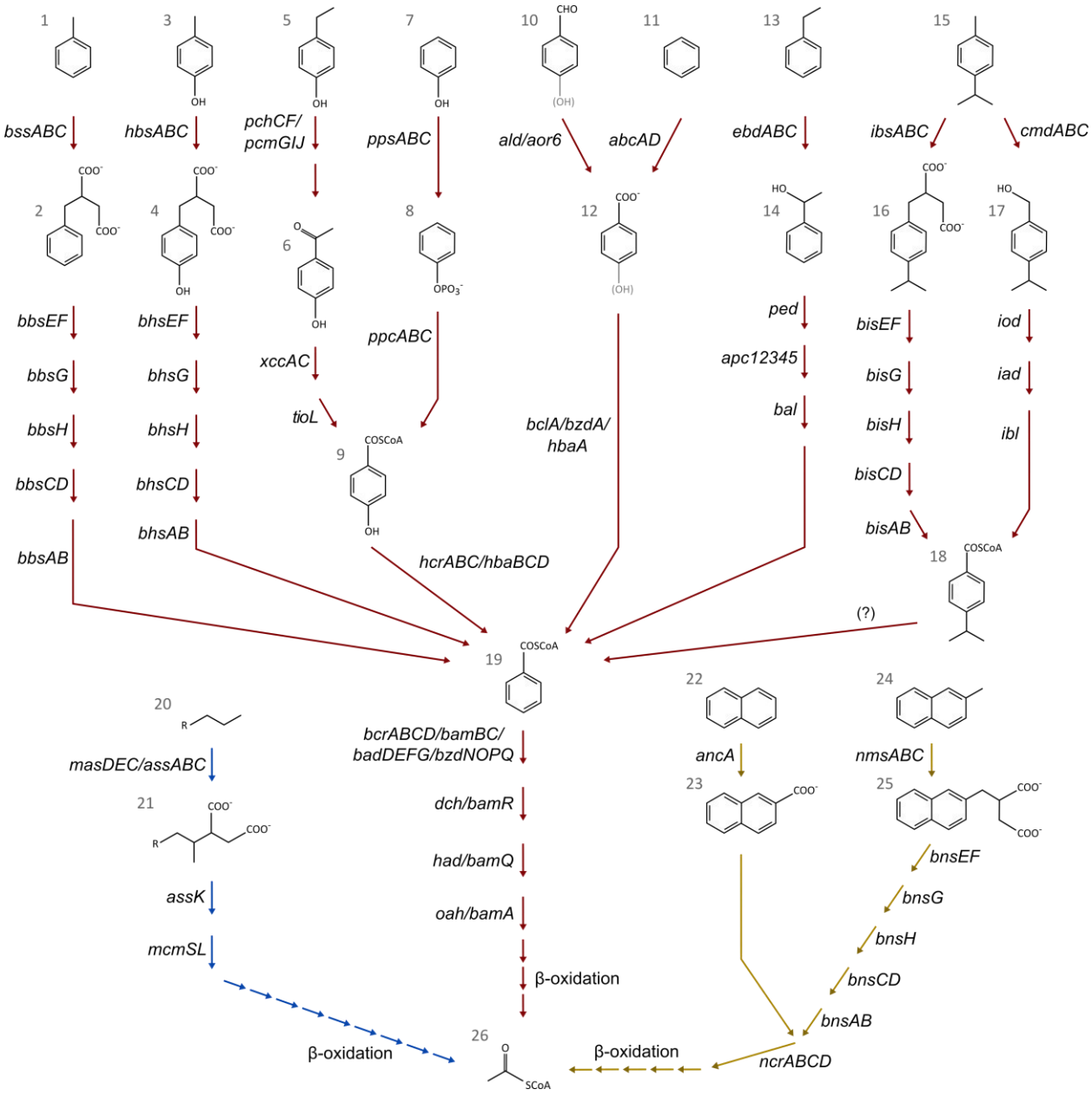

Figure 2. Selected peripheral pathways of anaerobic degradation of alkanes (blue arrows), monoaromatics (red arrows) and polyaromatics (yellow arrows) to acetyl-CoA and the respective genes coding for the enzymes involved in each reaction. The activation products and intermediate compounds are presented. Enzyme names are provided in Appendix Table 1. Compound names: 1, toluene; 2, (*R*)-benzylsuccinate; 3, *p*-cresol; 4, 4-hydroxybenzylsuccinate; 5, 4-ethylphenol; 6, 4-hydroxyacetophenone; 7, phenol; 8, phenylphosphate; 9, 4-hydroxybenzoyl-CoA; 10, benzaldehyde or 4-hydroxybenzaldehyde; 11, benzene; 12, benzoate or 4-hydroxybenzoate; 13, ethylbenzene; 14, (*S*)-1-phenylethanol; 15, *p*-cymene; 16, (4-isopropylbenzyl)succinate; 17, 4-isopropylbenzyl alcohol; 18, 4-isopropylbenzoyl-CoA; 19, benzoyl-CoA; 20, *n*-alkane; 21, (1-methylalkyl)succinate; 22, naphthalene; 23, 2-naphthoic acid; 24, 2-methylnaphthalene; 25, 2-naphthylmethylsuccinic acid; 26, acetyl-CoA.

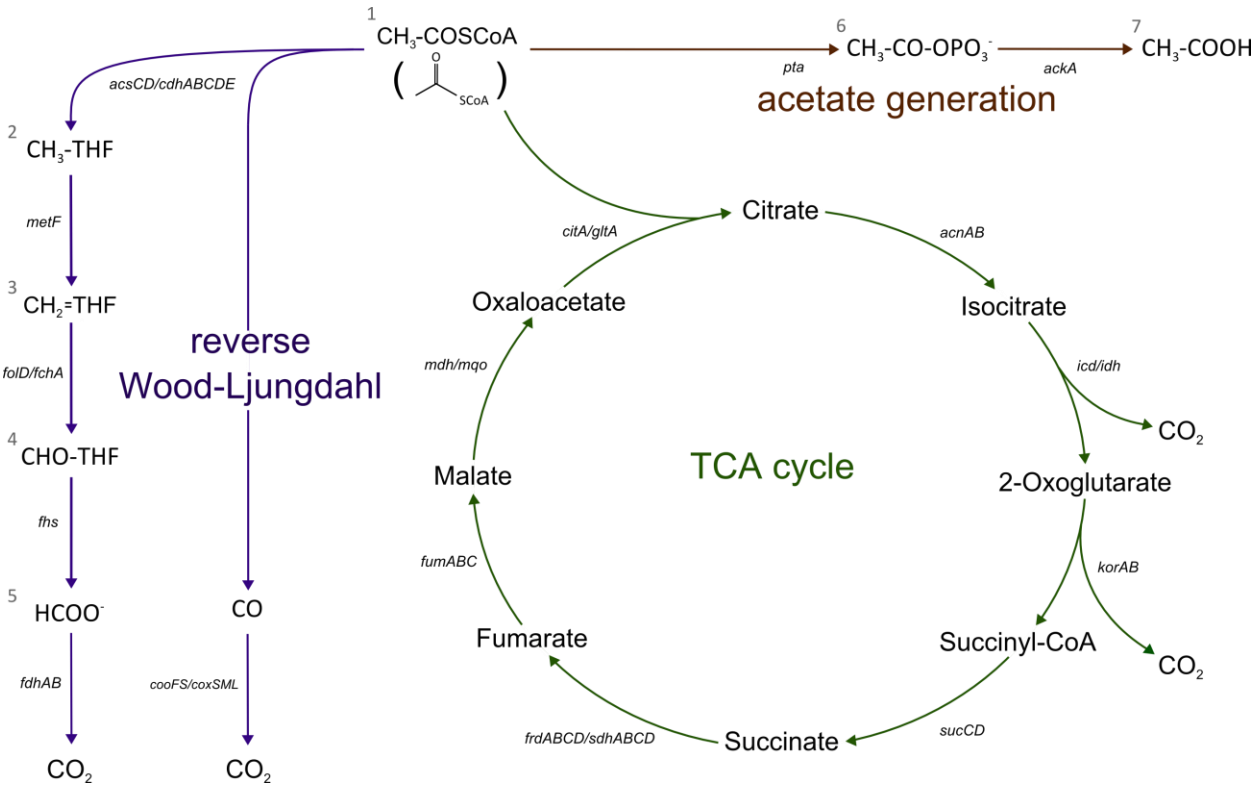

Figure 3. Alternative degradation pathways of acetyl-CoA to CO_2 or acetate. Enzyme names are provided in Appendix Table 1. Compound names: 1, acetyl-CoA; 2, methyl-tetrahydrofolate; 3, methylene-tetrahydrofolate; 4, formyl-tetrahydrofolate; 5, formate; 6, acetyl-phosphate; 7, acetate; THF, tetrahydrofolate.

1.3.2.1 Syntrophic interactions with methanogens

In the absence of inorganic electron acceptors, methanogenesis can serve as the final electron acceptor process during hydrocarbon degradation (Rabus et al. 2016). Methanogenesis is performed under strictly anoxic conditions and solely by archaeal phyla; it was once thought that methanogenesis is performed by *Euryarchaeota*, but other archaeal species with the potential to perform methanogenesis were recently discovered as well (Lang et al. 2015; Evans et al. 2015; Borrel et al. 2016).

Methanogens have been detected in many different environments, from anoxic soils and sediments to the intestinal tracts of eukaryotic organisms (Issazadeh et al. 2013). They thrive in syntrophic associations with fermenting Bacteria and are dependent on bacterial metabolic by-products, which serve as intermediate compounds that transfer electrons between the syntrophic partners (Gieg et al. 2014). They are able to use a restricted number of substrates (Liu & Whitman 2008), typically H_2/CO_2, formate (hydrogenotrophic methanogenesis) or acetate (acetotrophic methanogenesis), but also a few other short methylated compounds, like alcohols and methylamines (methylotrophic methanogenesis).

Depending on the conditions, the possible routes of methanogenesis have different range of conditions in which they are thermodynamically feasible (Dolfing et al. 2008). Independent of the substrate utilised, the last two steps of methanogenesis are common and carry out the conservation of energy and the production of methane.

The fermenting Bacteria also profit from this interaction. Without syntrophic partners, the oxidation of hydrocarbons or other substrates is energetically favourable via the production of ethanol, lactate, butyrate or propionate. When methanogenic Archaea are present though, the fermenters switch from the production of these fermentation products to the production of intermediate compounds, due to the higher energy produced per molecule of the substrate (Schink 1997; Sieber et al. 2012). The latter process is thermodynamically feasible only when the intermediates are continuously removed by the syntrophic partners and thus the presence of methanogens (or potentially sulfate reducers) is necessary.

The initial activation mechanism of hydrocarbons is still not clear. The former detection of genes coding for fumarate-adding enzymes and the respective reaction metabolites in methanogenic enrichment cultures amended with saturates or aromatics indicates that fumarate addition is the activation mechanism (Laban et al. 2015; Wawrik et al. 2016; Oberding & Gieg 2018; Toth & Gieg 2018). Contrasting evidence that fumarate addition is rather not performed under methanogenic conditions has been previously provided too (Aitken et al. 2013), suggesting that other mechanisms are also possible.

1.3.3 Impact of microbial degradation on oil composition (weathering)

Following the sudden input of oil to the environment, several processes alter its chemical composition, such as the evaporation of volatile compounds, the dissolution of the water-soluble compounds, the photo-oxidation and the biodegradation (Spaulding 1988). Biodegradation takes place at the oil-water interface and typically results in the sequential removal of selective compounds. Low-molecular-weight compounds are more susceptible to degradation and are consumed first, leading to the enrichment of more recalcitrant molecules. Based on studies of oils obtained from reservoirs and hydrocarbon seeps of different biodegradation levels, straight-chain saturates, branched saturates, cyclic saturates, monoaromatics and polyaromatics are removed in the described order (Wenger et al. 2002; Wolicka & Borkowski 2007; Cheng et al. 2016). High-molecular-weight compounds, like steranes and hopanes are utilised only under severe biodegradation over long time periods, thus they are used as biomarkers to estimate the level of biodegradation of the crude oils. As a consequence, biodegradation can affect many oil quality properties, including the viscosity of the mixture and the sulfur and metal content (Wenger et al. 2002).

1.4 Studies on long-term oil-exposed ecosystems

Due to the high need for the bioremediation of ecosystems impacted by oil spill accidents, studies have focused mainly on the response and the ecological role of microbiomes shortly after marine oil spills (days to a few years), limiting our knowledge about how microbiomes evolve over longer time periods on the geologic time scale. When the oil input is continuous and persists in the environment, like in the case of naturally-occurring hydrocarbon seeps, conditions become more challenging for microbial life. Oil accumulates in the less dynamic soil and sediment environments, in the vicinity of the oil sources, and hydrocarbons adhere to the soil/sediment particles. The oil-water interphase is restricted in the hydrocarbon-saturated environment and moisture is trapped in small isolated cavities with low water activity. Additionally, the long-term effect of biodegradation on the oil composition becomes more significant. The weathered crude oil produced by the above-mentioned processes is a highly viscous liquid, often referred to as asphalt, tar or bitumen. In fact, hydrocarbon degradation can start already in the oil reservoirs and continue during the migration of oil to the Earth surface (Head et al. 2003), but its impact on the oil composition is more noticeable in the surface environments, where electron acceptors are more abundant.

It was once thought that these environments cannot harbour microbial life due to the extremity of the conditions. Culture-based studies on these natural ecosystems have first confirmed that microbes are present in these environments and are able to proliferate when conditions become favourable (Christensen et al. 1992; Stetter et al. 1993; Beeder et al. 1994; Grassia et al. 1996; Takahata et al. 2000). Over the last years, several culture-independent studies, including studies on offshore submarine oil seeps (Hawley et al. 2014a), subsurface oil reservoirs (Orphan et al. 2000; Dahle et al. 2008), terrestrial tar pits (Kim and Crowley 2007) and mud volcanoes (Alain et al. 2006), have helped us uncover the taxonomic profiles of the indigenous microbiomes.

1.4.1 Marine hydrocarbon seeps

In most cases, migrating hydrocarbons escape in marine environments and don’t reach the terrestrial surface. Thus, marine seeps (hydrothermal vents, cold seeps and asphalt volcanoes) have so far received more attention, compared to the respective terrestrial ecosystems, and our knowledge is skewed towards sulfate-rich marine sediments.

The seepage of hydrocarbons results in a complex biotic network that takes advantage of the released compounds and its structure is affected by the hydrocarbon composition. Methane vents, seeps and hydrates worldwide and the adjacent sediments harbour core microbiomes consisting of anaerobic methane oxidizers (Orphan et al. 2001; Teske et al. 2002; Joye et al. 2004; Knittel et al. 2005; Brazelton et al. 2006; Lloyd et al. 2006; Ruff et al. 2015), since methane is the main gas released in these environments. Hydrocarbon seeps and asphalt volcanoes are characterised by mixtures of various oil hydrocarbons and asphalt deposits, respectively, thus their microbiomes are associated with the

breakdown of more complex hydrocarbons (Raggi et al. 2013; Vigneron et al. 2018). The studies agree that microbial cycling of carbon and sulphur substrates are the main active processes in these environments; the available sulfate diluted in seawater boosts the sulfate-reducing activity and degradation of hydrocarbons. Sulfate reducers can directly utilise the provided electron donors or metabolise the by-products of other hydrocarbon degraders, as long as sulfate is available. In niches deeper in the hydrocarbon-impacted sediments or inside the asphalt though, sulfate is restricted and methanogenesis is favoured, possibly coupled to the degradation of hydrocarbons (Schubotz et al. 2011; Cruaud et al. 2017).

1.4.2 Oil sands and tailings ponds

The best-studied accumulation of hydrocarbons in the terrestrial surface are the Athabasca oil sands in Alberta, Canada. The Athabasca oil sands are large-scale natural deposits of heavy oil (commonly mentioned as bitumen), which are extensively used for bitumen extraction (Foght et al. 2017). The mining operation activities produce big volumes of tailings, which contain the by-products of bitumen extraction (sand, water, bitumen and other residues) and are deposited in artificially-made reservoirs/ponds. The sand material of the tailings is gradually deposited at the bottom of the ponds, resulting in oily sediments (fine tailings) with accumulated unextracted bitumen and by-products of the extraction, while the surface water is reused for mining (Foght et al. 2017). The large volume of produced tailings has a big impact in the contaminated environment and oil sands tailings ponds have received a lot of attention for remediation purposes.

The frequent water recycling and the external input of new tailings material (Stasik & Wendt-Potthoff 2014) continuously supply the ponds with new microbial biomass and electron donors and acceptors. Several studies (reviewed by Foght et al. 2017) on various tailings ponds sediments revealed an unexpectedly high diversity of microbiota, despite the low number of labile carbon sources. Methanogens comprised a significant part of the core microbiome of the ponds (Wilson et al. 2016), showing that there is a high potential for the degradation of the available hydrocarbons coupled to methanogenesis. Indeed, Siddique et al. (2006, 2007, 2011 and 2015) have shown that microbiomes in fine tailings are able to degrade many alkanes and monoaromatics, in mixtures with the residual naphtha used during the extraction process, to methane. Members of *Peptococcaceae* (class *Clostridia*) have been repeatedly identified as key hydrocarbon degraders in methanogenic enrichment cultures (Abu Laban et al. 2015; Tan et al. 2015; Mohamad Shahimin & Siddique 2017a; Mohamad Shahimin & Siddique 2017b). Viable nitrate reducers, sulfate reducers, fermenters and methanogens have been also found in the tailings after bitumen extraction (Foght et al. 2017), indicating that they can survive the intense extraction process and end up in the ponds. Shotgun metagenomic analysis revealed that microbiomes of the anoxic oil sands and tailings ponds even harbour many aerobic hydrocarbon degradation genes (An et al. 2013), indicating that even if not actively transcribed, aerobic degradation genes can be potentially stimulated under the right conditions and enhance the removal of hydrocarbons from the ecosystem by aerobes.

1.4.3 Terrestrial asphalt deposits

The extremity of the conditions for microbiomes is especially noticeable in terrestrial hydrocarbon seeps and accumulations, considering the low water activity in the available matrix-specific niches and the high abundance of recalcitrant carbon sources and toxic compounds. Despite that, analysis of clone libraries of samples from the large natural asphalt deposits of La Brea tar pits in Los Angeles, California, and the Pitch Lake in La Brea, Trinidad and Tobago, revealed unique indigenous microbiomes. Many sequences were affiliated to sulfur-metabolizing chemolithotrophic Bacteria (Kim & Crowley 2007; Schulze-Makuch et al. 2011) with the potential to utilise the oil hydrocarbons. Interestingly, a fungal strain with the ability to degrade recalcitrant asphaltenes has been isolated from the natural asphalt Lake Guanoco in Sucre, Venezuela (Uribe-Alvarez et al. 2011).

Meckenstock et al. (2014) recently provided a clear proof that microbiota can survive and remain active even inside the asphalt itself, particularly in minuscule water droplets engulfed in asphalt oil. The microbiomes consisted complex methanogenic communities with the ability to degrade the abundant oil compounds. The study was performed on surface samples obtained from the Pitch Lake and supports the deep subsurface origin of the droplets, showing that microbes survive in the small water-filled cavities, during the ascent of the migrating oil into the terrestrial surface, and contribute to the degradation of oil compounds.

1.5 Study area – Keri Lake

The detection of active microbial communities in these waterproof asphalt-saturated environments has a great impact on our understanding of the extreme conditions that can harbour active microbial life, from the oil-water transition zones to oil-engulfed sediment particles and the oil body itself, opening a new avenue for further investigation of their indigenous microbiomes. In the present study, we focused on microbiomes thriving in sediments which are naturally exposed to an asphalt oil spring found at Keri Lake in Zakynthos Island, Greece, over a long time period in the scale of millennia.

Keri Lake is a coastal ecosystem in the southern part of Zakynthos, which expands over an area of 3 km^2 (Avramidis et al. 2017). The former lake at this site has evolved into a wetland environment with characteristics of a middle-brackish to a freshwater fen. It is located in the transition zone between the land and Laganas Bay and is lying 1 m above the sea level; a low relief sand barrier limits the communication with the open sea. The fen is covered mainly with reeds, which has led to the accumulation of a 5 m thick peat bed in the sediment over time. Hydrocarbon springs are present at several sites at Keri Lake, first mentioned by the ancient Greek historian Herodotus (Book IV, Chapter 195). Herodotus described the methods by which the asphalt was being recovered from one of the springs, therefore the escape of oil at Keri Lake is a well-known physical phenomenon since 2500 years ago. The escaped hydrocarbons originate from an oil source rock which is located in the eastern vicinity of Keri Lake, below the neighbouring Marathonisi Island. They ascent through rock fractures towards the Earth surface; the geological structure of the escapes and their geochemical characteristics has been described by Palacas et al. (1986). As a result, gas and liquid hydrocarbon escapes are observed at several terrestrial and marine sites at the area of Keri and near Marathonisi Island (Dermitzakis & Alafousou 1987). It is estimated that the methane and oil hydrocarbon escapes at Keri Lake and the surrounding area started five million years ago. Former geochemical analyses of oil revealed a marine organic matter origin and confirmed a high level of maturity with abundant aromatic compounds (Palacas et al. 1986; Pasadakis et al. 2016).

2 Scientific questions and hypotheses

Our project aims to expand our knowledge about the ecological role of sediment microbiomes evolved under conditions of heavy oil exposure for long time periods, using the asphalt oil springs of Keri Lake as a model system for terrestrial environments affected by hydrocarbon escapes. In contrast to most previous studies focusing on the taxonomic characterisation of samples obtained from natural oil seeps, we performed a comprehensive comparison of a chronically asphalt-exposed site with a reference site in the same ecosystem, in order to investigate the long-term impact of the exposure on the structure of sediment microbiomes from an ecological perspective ("how the microbiomes are" compared to "how the microbiomes would be"). Furthermore, the relatively recent discovery of novel anaerobic degradation pathways and their respective genes means that past studies were restricted to specific metabolic processes. It was therefore our goal to examine the potential for anaerobic degradation and the associated respiration *in situ*, covering the full range of currently known pathways and identify key players of the complex sediment microbiomes that are potentially involved in these processes. Lastly, information about the anaerobic degradation activities of microbiomes is still limited in natural ecosystems, especially terrestrial hydrocarbon seeps. Thus, the degradation activities of the sediment microbiomes in the context of the extreme conditions of asphalt exposure were studied under well-defined lab conditions and differing redox conditions, in the context of the present thesis.

In this respect, the following scientific questions were investigated and our hypotheses were tested on the oil-exposed sediment microbiomes of Keri Lake:

I. What is the impact of the persistent oil exposure on the available electron acceptors and donors?

Considering the extensive biodegradation in these ecosystems, nitrate and sulfate should be depleted in the oil-exposed sediments. The progressive accumulation of heavy oil in the vicinity of the asphalt spring will result in a chemical fingerprint indicative of the asphaltic nature of the oil. The organic matter in the sediment will be dominated by complex recalcitrant hydrocarbons, covering the imprint of the peat-derived organic material.

II. What is the impact of the persistent oil exposure on the structure and function of the sediment microbiomes?

Following the consumption of nitrate and sulfate in the sediment, denitrifiers and sulfate reducers shouldn’t be abundant at the oil-exposed sediment and the potential for the corresponding functions should be lower compared to the reference site. Taking into account the lack of inorganic electron acceptors and the common agreement that methanogens are involved in the degradation of hydrocarbons in chronically contaminated ecosystems, we hypothesize that methanogens are not just present but their growth is favoured in these environments. Thus, an increased abundance of methanogenic Archaea and high potential for methanogenesis is expected at the oil-exposed site of Keri

Lake, in comparison to the reference site. In contrast, the potential for anaerobic degradation of the oil hydrocarbons should not be higher, since most compounds of the asphalt oil are considered resistant to biodegradation.

III. Is the effect of the persistent oil exposure consistent among ecosystems impacted by terrestrial hydrocarbon escapes?

Since different studies on natural hydrocarbon seeps agree that methanogenic biodegradation of hydrocarbons is prominent, we hypothesize that our observations on the respiratory potential of Keri Lake microbiomes will also apply to other chronically exposed to oil ecosystems. Methanogens are expected to comprise a part of the core microbiome of these environments, which shouldn't be the case for ecosystems with short-term oil contamination. The potential for the known anaerobic degradation pathways of hydrocarbons should differ though, considering the dissimilar hydrocarbon composition of the oil mixtures in the different environments.

IV. Do anaerobic respiring prokaryotes that are present in the oil-exposed sediment of Keri Lake grow on a complex mixture of heavy oil when electron acceptors becomes bioavailable?

Prokaryotes respiring inorganic electron acceptors other than O_2 have been repeatedly reported in oil-exposed environments, i.e. sulfate reducers in marine hydrocarbon seeps, proving that they are able to survive and remain active in these extreme environments. Thus, we hypothesize that their numbers will be limited at the oil-exposed site of Keri Lake, due to the lack of inorganic electron acceptors only and not because they are unable to degrade the oil hydrocarbons. When the respective electrons acceptors will be provided in liquid anoxic cultures, we expect that anaerobic respiring Bacteria will be enriched in the presence of several different hydrocarbon substrates as carbon sources, including oil from Keri Lake, since their ability to utilise a wide range of hydrocarbon substrates is well-proven.

V. What is the effect of input of inorganic electron acceptors to the oil-exposed sediment on the hydrocarbon-degrading activity of the indigenous microbiomes?

We hypothesize that the core energy-conserving metabolism of the microbiomes will switch to an energetically more favourable respiration, with a negative impact on the established methanogenic part of the community. The addition of inorganic electron acceptors to the oil-exposed sediment of Keri Lake will favour the growth of anaerobic respiring prokaryotes and enhance the degradation of hydrocarbons. At the same time, methanogens will be outcompeted by the fast-growing respiring species and their growth will be suppressed. The stimulation of respiring prokaryotes will result in increased activity of the genes of the anaerobic degradation pathways during the time period of active growth.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

Substance	Company
Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
$BaCl_2$ x2H_2O	Merck KgaA, Darmstadt, Germany
BSA (bovine serum albumin)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
$CaCl_2$ x2H_2O	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
CI (chloroform/isoamyl alcohol)	Carl Roth GmbH + Co, Karlsruhe, Germany
$CoCl_2$	Carl Roth GmbH + Co, Karlsruhe, Germany
CTAB (hexadecyltrimethylammoniumbromide)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$CuCl_2$	Carl Roth GmbH + Co, Karlsruhe, Germany
n-decane	VWR International GmbH, Darmstadt, Germany
DEPC	AppliChem GmbH, Darmstadt, Germany
Dichloromethane/methanol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
dNTPs	Fermentas, Vilnius, Lithuania
Ethanol	Merck KgaA, Darmstadt, Germany
Ethidium bromide solution	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$FeCl_2$	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formamide	Fisher Scientific GmbH, Schwerte, Germany
Gelatin	AppliChem GmbH, Darmstadt, Germany
H_3BO_3	Merck KgaA, Darmstadt, Germany
HCl	Merck KgaA, Darmstadt, Germany
HMN (2,2,4,4,6,8,8-heptamethylnonane)	VWR International GmbH, Darmstadt, Germany
Isopropanol	AppliChem GmbH, Darmstadt, Germany
Kanamycin sulfate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
KCl	Serva Electrophoresis GmbH, Heidelberg, Germany
KH_2PO_4	AppliChem GmbH, Darmstadt, Germany
β-mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$MgCl_2$ x6H_2O	Carl Roth GmbH + Co, Karlsruhe, Germany
$MnCl_2$	AppliChem GmbH, Darmstadt, Germany
MspI and Buffer Tango with BSA	Fisher Scientific GmbH, Schwerte, Germany
Na-acetate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
NaCl	Merck KgaA, Darmstadt, Germany
$NaHCO_3$	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Na_2HPO_4	AppliChem GmbH, Darmstadt, Germany
NaH_2PO_4	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Na_2MoO_4	Merck KgaA, Darmstadt, Germany
NaOH	Merck KgaA, Darmstadt, Germany

Na_2S	Merck KgaA, Darmstadt, Germany
Na_2SeO_3	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Na_2SO_4	Acros Organics BVBA, Geel, Belgium
Na_2Wo_4	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
NH_4Cl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
$NiCl_2$	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
PCI (phenol/chloroform/isoamyl alcohol)	Carl Roth GmbH + Co, Karlsruhe, Germany
PEG (polyethylene glycol) 6000	Serva Electrophoresis GmbH, Heidelberg, Germany
Q-Solution	Qiagen GmbH, Hilden, Germany
Resazurin sodium	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
SDS (sodium dodecyl sulfate)	Fisher Scientific GmbH, Schwerte, Germany
TaqI and Buffer TaqI with BSA	Fermentas, Vilnius, Lithuania
TE buffer	AppliChem GmbH, Darmstadt, Germany
Toluene	Acros Organics BVBA, Geel, Belgium
Tris-HCl	Merck KgaA, Darmstadt, Germany
$ZnCl_2$	Fisher Scientific GmbH, Schwerte, Germany

3.1.2 DNA fragments

All PCR primers were supplied by Metabion International AG (Planegg, Germany); purified by HPLC, synthesized with scale 0.02 µmol, quality checked by mass spectrometry and shipped in dry conditions. The DNA fragment with part of the sequence of the *ncr* gene of strain N47 was synthesized and supplied by Integrated DNA Technologies BVBA (Leuven, Belgium).

3.1.3 Kits and master mixes

Kit	Company
Agencourt AMPure XP beads	Beckman Coulter Inc., Brea, California, USA
Agilent DNA 7500 kit	Agilent Technologies GmbH & Co, Waldbronn, Germany
DNase Max Kit	MO BIO Laboratories Inc, Carlsbad, California, USA
Gel and PCR Clean-up kit	Macherey-Nagel GmbH&Co. KG, Düren, Germany
Genomic DNA from Soil Kit	Macherey-Nagel GmbH&Co. KG, Düren, Germany
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Vilnius, Lithuania
High Sensitivity DNA kit	Agilent Technologies GmbH & Co, Waldbronn, Germany
MapMarker 1000	Eurogentec, Köln, Germany
MiSeq Reagent Kit v3	Illumina Inc., San Diego, California, USA
NeBNext Multiple Oligos for Illumina (Index Primers Set 1)	New England BioLabs LtD., Hitchin, UK
NEBNext High Fidelity Master Mix	New England BioLabs LtD., Hitchin, UK
NEBNext Ultra DNA Library Prep kit for Illumina	New England BioLabs LtD., Hitchin, UK
Nextera XT Index kit v2 (Set C)	Illumina Inc., San Diego, California, USA
Nucleospin Bead Tubes Type A	Macherey-Nagel GmbH&Co. KG, Düren, Germany

Nucleospin Gel and PCR Cleanup Kit	Macherey-Nagel GmbH&Co. KG, Düren, Germany
Nucleospin Plasmid kit (No lid)	Macherey-Nagel GmbH&Co. KG, Düren, Germany
OneStep PCR Inhibitor Removal Kit	Zymo Research Corp., Irvine, California, USA
Power SYBR Green PCR Master Mix	Life Technologies LTD, Warrington, UK
Quant-iTTM Picogreen dsDNA assay kit	Life Technologies Corporation, Eugene, Oregon, USA
TA cloning kit	Invitrogen AG, Carlsbad, California, USA
Taq DNA Polymerase kit	Life Technologies, Darmstadt, Germany
TopTaq DNA Polymerase kit	Qiagen GmbH, Hilden, Germany
ZeroBlunt TOPO cloning kit	Invitrogen AG, Carlsbad, California, USA

3.1.4 Instruments and lab equipment

Instrument	Company
ABI 7300 Real Time PCR System	Applied Biosystems Inc, Foster City, California, USA
Agilent 2100 Bioanalyzer	Agilent Technologies GmbH & Co. KG, Waldbronn, Germany
Digital oximeter GMH 3691	Greisinger electronic GmbH, Regenstauf, Germany
E220 Focused-ultrasonicator	Covaris Inc., Woburn, Massachusetts, USA
Fine-Ject needles	Henke-Sass Wolf GmbH, Tuttlingen, Germany
Precellys 24 homogenisator	Bertin technologies, Montigny-le-Bretonneux, France
ICS–1100 ion chromatograph	ThermoFisher Scientific Inc., Waltham, Massachusetts, USA
Millex-GP filter units	Merck Millipore Ltd., Tulagreen, Ireland
MiSeq System	Illumina Inc., San Diego, California, USA
Nanodrop 1000 Spectrophotometer	PeqLab Biotechnologie GmbH, Erlangen, Germany
Omnifix-F single-use syringes	B.Braun, Melsungen, Germany
PeqStar 96x PCR cycler	PeqLab Ltd, Fareham, UK
San++ Continuous Flow Analyzer	Skalar Analytical B.V., Breda, The Netherlands
SpectraMax Gemini EM Spectrofluorometer	Molecular Devices GmbH, Ismaning, Germany
Vibrating corer	Eijkelkamp Soil & Water, Giesbeek, The Netherlands
Victor Microplate reader	Perkin Elmer Inc. Waltham, Massachusetts, USA
Voltcraft PS-200HTP pocket scale	Conrad Electronics SE, Hirschau, Germany

3.2 Methods

The thesis is based on three major parts: (i) an ecosystem study of Keri Lake sediment microbiomes, (ii) the enrichment of sulfate-reducing Bacteria in liquid anoxic cultures and (iii) the stimulation of hydrocarbon degradation with sulfate addition in anoxic microcosms/slurries prepared with sediments from Keri Lake. The results of the ecosystem study have been successfully published in the above-mentioned peer-reviewed publication.

3.2.1 Sampling campaigns

All sampling activities were conducted with permissions from the Greek National Focal Point to the Convention on Biological Diversity/Ministry of Environment, Energy and Climate Change, and the National Marine Park of Zakynthos. Two sites were selected with information from a former geological survey (Avramidis et al. 2017): (i) one non-exposed to oil (NE; 37° 41.112 N, 20° 49.769 E) and (ii) one highly-exposed to natural asphalt oil (HE; 37° 41.169 N, 20° 49.900 E). Both sites are located in the ecosystem of Keri Lake with a similar distance from the sea coast, but not in close vicinity, making NE a good reference site for comparing to the HE site (Figure 4). Unlike the HE site, no apparent presence of oil was observed at the NE site, based on visual and olfactory inspection of the collected sediment.

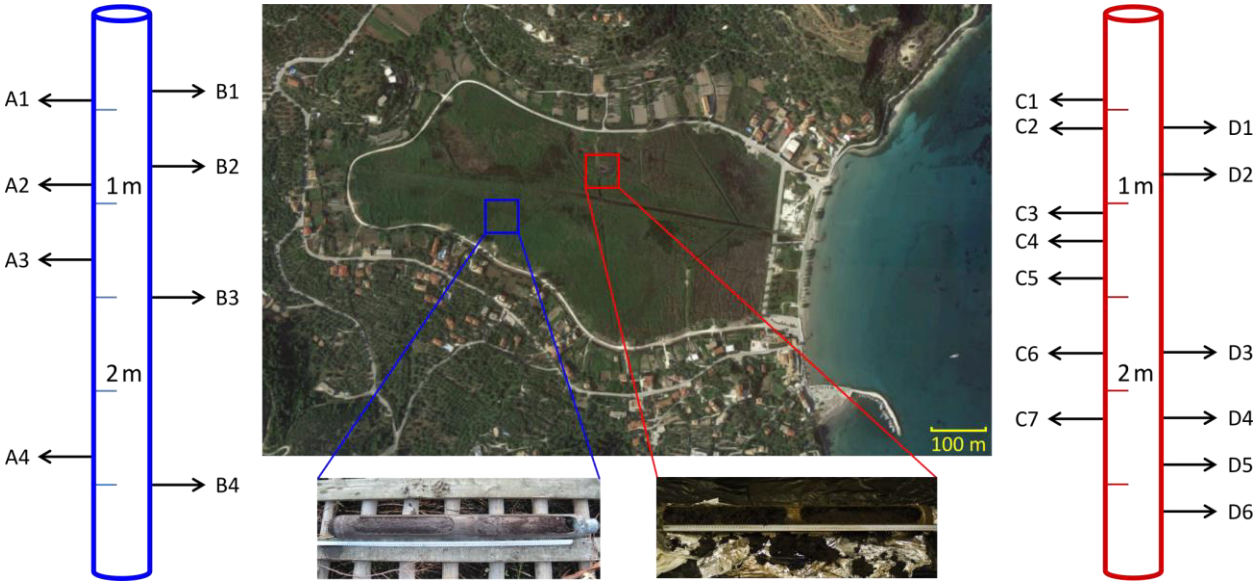

Figure 4. Map of Keri Lake showing the location of the studied sites, the collected cores and the sampled depths. NE site, blue colour; HE site, red colour.

Sediment from both sites was first collected in October 2013 in order to study the indigenous microbial communities (Figure 4). The peaty mud layer of the sediment was sampled (introduced as Unit III by Avramidis et al. 2017); duplicate cores were collected using a vibrating corer and stainless steel tubing (diameter 5–10 cm) to a maximum of 3.6 m depth and 1 m distance between replicates. The top sediment layer was excluded, due to past human interference at the HE site. After removing the outer layer in contact with the sampling equipment, sediment samples from various depths were obtained for the analysis of electron acceptors (IC), organic matter (GC) and the extraction of nucleic acids (NA); samples were weighted with a Voltcraft PS-200HTP pocket scale (resolution 0.01 g; Table 1). Anions were extracted from the IC samples by shaking with 10 ml of KCl (1 M) for 1 hour. The supernatants were collected in falcon tubes, after filtering through Millex-GP filter units (0.22 μm), and stored together with the GC samples at 4°C until further analysis. NA samples were treated with 4.5 ml of sodium phosphate buffer (112.9 mM Na_2HPO_4, 7.1 mM NaH_2PO_4; pH 8.0; DEPC-treated) and 1.5 ml of TNS solution (10% w/v SDS, 0.5 M Tris-HCl at pH 8.0, 0.1 M NaCl; DEPC-treated) in bead tubes immediately after sampling, frozen in liquid nitrogen and stored at −80 °C.

Table 1. Weights of the collected samples from Keri Lake. IC, samples for ion chromatography; GC, samples for gas chromatography; NA, samples for nucleic acid extraction. NE site samples, blue colour; HE site samples, red colour.

Sample name	Site	Depth (cm)	IC samples (g)	GC samples (g)	NA samples (g)
A1	NE	40–50	3.36	4.12	3.44
A2	NE	83–93	3.28	5.10	2.80
A3	NE	125–135	4.90	3.23	3.22
A4	NE	230–240	3.21	3.18	2.03
B1	NE	35–45	3.26	2.05	3.79
B2	NE	75–85	2.18	2.44	2.73
B3	NE	145–155	4.59	3.13	3.47
B4	NE	245–255	4.44	2.91	4.80
C1	HE	42–52	2.38	1.46	2.66
C2	HE	57–67	2.94	1.70	2.13
C3	HE	101–111	2.40	1.79	3.70
C4	HE	113–123	2.21	1.42	2.38
C5	HE	137–147	2.01	2.22	2.84
C6	HE	173–183	2.86	1.99	2.87
C7	HE	209–219	1.92	2.60	2.96
D1	HE	55–65	1.63	2.58	2.59
D2	HE	176–186	1.55	2.56	3.05
D3	HE	212–222	1.68	2.60	3.46
D4	HE	236–246	3.08	2.29	3.68
D5	HE	260–270	2.79	2.32	3.12

For the preparation of enrichment cultures, sediment was collected from the HE site of Keri Lake in May 2015 using a soil auger. The sediment from 0.4–0.6 m depth was transferred in sealed falcon tubes under conditions of minimum oxygen exposure, carefully avoiding the outer layer of the cores in contact with the sampling auger, and stored at room temperature until further use. Approximately 2 grams of the sediment were stored at -20^{o}C to study the structure of the original sediment microbiome. Asphalt oil from the same site was additionally collected in a Schott bottle for later use as a substrate.

For the preparation of microcosms/slurries, sediment from the same sites was collected in September 2016 from 0.4–0.6 m depth in 2 L glass bottles under minimum oxygen conditions. The bottles were transferred to the lab and incubated at room temperature for two weeks, in order to let the microbiota recover from the disturbance during sampling and allow microbial activity to stabilise.

3.2.2 Anoxic enrichment cultures[γ]

In order to enrich anaerobic hydrocarbon-degrading sulfate-reducing Bacteria from the oil-exposed sediment of Keri Lake, liquid cultures and media were prepared under strictly anoxic conditions, using the Hungate technique. Basal freshwater medium for Gram-negative mesophilic sulfate-reducing Bacteria (NaCl, $MgCl_2$ x6H_2O, KH_2PO_4, NH_4Cl, KCl, $CaCl_2$ x2H_2O, trace element mixture 1, selenite-tungstate solution) was made according to Widdel and Bak (1992), with Na_2SO_4 (20 mM) as electron acceptor. The medium was distributed in sealed serum bottles (50 ml final volume) and autoclaved (121^{o}C; 20 min). For each culture (8 in total), approximately 0.4 g of sediment were added in a serum bottle, in the presence of N_2/CO_2 gas, and sealed with butyl stoppers and aluminium caps. Bicarbonate ($NaHCO_3$) solution and vitamin mixture were subsequently added in all bottles as described in the same protocol (Widdel and Bak 1992). Na_2S (1 mM) and resazurin (180 mM) were also included as a reductant and a redox indicator, respectively. The cultures received one of the following carbon sources: *n*-decane (100 mM; cult 1 and 2), toluene (100 mM; cult 3 and 4) or Keri Lake asphalt oil (1:60 v/v; cult 5, 6 and 7). These substrates were first dissolved in an immiscible carrier phase (HMN), to reduce the toxic effects of the pure compounds and facilitate the injection through the butyl stoppers. Culture 8 received *n*-decane, as above, was autoclaved (121^{o}C; 20 min) and used as a negative control, to test if there is any sulfate reduction due to abiotic factors. All cultures were incubated at 28^{o}C and were sampled regularly for 20 months. Sampling was conducted with Omnifix-F single-use syringes (1 ml) and Fine-Ject needles (23Gx1 1/4''; 0.6x30 mm) and samples were stored at -20^{o}C until the measurement of sulfate.

[γ]The anoxic enrichment cultures were prepared with the help of PD Dr. Tillmann Lueders and Anne Himmelberg (Institute of Groundwater Ecology, Helmholtz Zentrum München).

3.2.3 Anoxic microcosms/slurries

Anoxic microcosms/slurries were used to test the effect of sulfate addition on the function of microbiomes in Keri Lake sediments. Solutions were prepared and autoclaved according to Widdel and Bak (1992) as described above[δ], but with several modifications in their composition and the final concentrations of the individual compounds. Basal saltwater medium with NaCl (21 g/l), $MgCl_2$ x6H_2O (3 g/l) and Na_2SO_4 (4 g/l) was made to simulate the input of sulfate by seawater flooding in Keri Lake; medium without sulfate was used for control microcosms. Trace element solution (TES), selenite-tungsten solution (Na_2SeO_3, Na_2Wo_4, NaOH) and $NaHCO_3$ were added as suggested by Widdel and Bak (1992). The composition of TES was modified ($FeCl_2$, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, Na_2MoO_4, H_3BO_3, HCl); sulfate was completely omitted in order to be able to test for fermentative growth or syntrophic interactions with methanogens in the control microcosms. Reduced conditions were maintained with Na_2S (1.6 mM) and pH was adjusted to 7; no resazurin was added to avoid any possible toxic effects on microbial cells.

The dry weight of each sediment collected in 2016 was calculated gravimetrically by incubation of ~5 g of sample at 60°C, until constant weight (two days). Slurries were prepared in an anoxic chamber by mixing 3.5 g of dry sediment (6.66 g of fresh sediment from the NE site or 5.83 g of fresh sediment from the HE site) with 3.5 ml of medium in 15 ml glass vials. This resulted four different types of microcosms: (i) NC, which contained sediment from the NE site and medium without sulfate, (ii) NS, which contained sediment from the NE site and medium with sulfate, (iii) HC, which contained sediment from the HE site and medium without sulfate and (iv) HS, which contained sediment from the HE site and medium with sulfate. The presence of O_2 was monitored using a digital oximeter GMH 3691; concentrations were below the detection limit of the instrument throughout the process. Vials were sealed with butyl septa and screw caps and stored in horizontal position under continuous shaking (75 rounds min^{-1}) at room temperature. Four replicates from each treatment were sampled every week as follows. Microcosms were centrifuged (700×g; 1 min) and 1 ml of the supernatant was transferred in an eppendorf tube, using Omnifix-F single-use syringes (1 ml) and Fine-Ject needles (23Gx1 1/4''; 0.6x30 mm). The tubes were centrifuged again (6000×g, 2 min) to pellet the remaining sediment particles and the supernatant was filtered through Millex-GP filter units (0.22 μm). The filtered supernatants and the original microcosms were stored at –80°C until used to measure sulfate and extract nucleic acids, respectively.

[δ]The anaerobic media were prepared with the help of PD Dr. Tillmann Lueders and Gabriele Barthel (Institute of Groundwater Ecology, Helmholtz Zentrum München).

3.2.4 Nitrate and sulfate measurements

The concentrations of nitrate and nitrite extracted from the IC samples were determined using a San++ Continuous Flow Analyzer[ζ] (60 and 20 µg l^{-1} detection limits), while the concentrations of sulfate were measured using an ICS–1100 ion chromatograph with an AS-18 anion-exchange separation column[θ] (1.5 ml min^{-1} eluent flow rate, 5 µg l^{-1} detection limit).

Sulfate concentrations in the supernatants obtained from the enrichment cultures and the microcosms were determined by a barium-gelatin spectrophotometric assay (Tabatabai et al. 1974). The supernatants were filtered through Millex-GP filter units (0.22 µm), diluted with MilliQ water (1:10) and mixed with barium-gelatin solution ($BaCl_2$ x$2H_2O$, gelatin, HCl). The turbidity due to the formation of $BaSO_4$ precipitate was then measured in duplicates on a Victor microplate reader, after continuous shaking for 10 min, and compared to a linear curve prepared from SO_4^{-2} standard solutions (0, 2, 4, 6, 8 and 10 mM).

0.1–2.5 g (freshweight) from the sediment samples collected in 2013 were also incubated at 60°C and the dry weight was calculated gravimetrically after the complete removal of water. The values were used for the normalization of nitrate, sulfate and the following organic matter measurements per g of dry sediment.

[ζ]Nitrate and nitrite concentrations were measured by Gudrun Hufnagel (Research Unit Comparative Microbiome Analysis, Helmholtz Zentrum München).

[θ]Sulfate measurements by ion chromatography were performed by Prof. Dr. Ralf Rabus, Dr. Kathleen Trautwein and Dr. Marvin Dörries (General and Molecular Microbiology, Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky University Oldenburg).

3.2.5 Organic matter composition[λ]

To confirm that the bioavailable organic material was different between the sites, due to the exposure of the HE site to asphalt oil, the saturated hydrocarbons in the sediments were analysed. Organic matter was first extracted from the GC samples and weighted to determine the extractable organic matter (EOM) yields. Extraction was performed with dichloromethane/methanol (99:1 v/v) in a Soxhlet apparatus for 24 h, followed by evaporation of the solvent. The saturated fractions were then isolated by asphaltene precipitation (Theuerkorn et al. 2008) and subsequent medium pressure liquid chromatography (Radke et al. 1980) and analysed by gas chromatography with flame ionization detection (Hosseini et al. 2017).

[λ]The analysis of hydrocarbons by gas chromatography was conducted by Prof. Dr. Heinz Wilkes, Kristin Günther, and Anke Kaminsky (Organic Geochemistry, Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky University Oldenburg).

3.2.6 Nucleic acid extraction

3.2.6.1 Keri Lake sediment samples

DNA extraction from the stored NA samples collected in the 1st sampling campaign was performed according to Lueders et al. (2004) with modifications. Samples were heated at 65°C for 10 min, in the presence of 1 ml of phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 v/v/v), and cells were destructed by two bead beating steps on a Precellys 24 homogenisator (6.5 m/s; 1 min). Homogenised samples were centrifuged (4000×g; 5 min) to pellet big sediment and cell particles. Nucleic acids were first purified from the supernatants with PCI (1× sample volume) and subsequently chloroform/isoamylalcohol (CI; 24:1 v/v; 1× sample volume). The extracted DNA was precipitated with a mixture of isopropanol (0.8× sample volume) and Na-acetate (0.1× sample volume; DEPC-treated) at –20°C overnight followed by centrifugation (4000×g; 2 h; 4°C). The pellets were washed with 3 ml of ice-cold ethanol (70%) and, after ethanol removal, the pelleted DNA was eluted in TE buffer (1×; pH 7.5). The presence of residual contaminants was assessed with a Nanodrop 1000 Spectrophotometer. To improve the purity of DNA for sequencing, samples were further purified using the OneStep PCR Inhibitor Removal and the Genomic DNA from Soil Kit kits, excluding the protocol's lysis steps. DNA concentrations were measured on a SpectraMax Gemini EM Spectrofluorometer using the Quant-iTTM Picogreen dsDNA assay kit.

3.2.6.2 Enrichment cultures

Cultures obtained after 20 months of enrichment were used for DNA isolation. Microbial cells were collected from 2 ml of each culture in Nucleospin Bead Tubes Type A by centrifugation (13000×g; 15 min). The cell pellets and 300 mg of Keri Lake sediment were used for extraction with the Lueders et al. (2004) protocol as follows. First, 750 µl of buffer $NaPO_4$ (120 Mm; pH 8; DEPC-treated) and 250 µl of buffer TNS (10% w/v SDS, 0.5 M Tris-HCl at pH 8, 0.1 M NaCl; DEPC-treated) were added and cells were destructed by bead beating on a Precellys 24 homogenisator (6.5 m/s; 45 s). After centrifugation (13000 rpm; 5 min; 4°C), the supernatants were purified with PCI (25:24:1 v/v/v; 1× sample volume), followed by another purification with CI (24:1 v/v; 1× sample volume). Purified DNA was mixed thoroughly with polyethylene glycol 6000 (PEG; 30%, 2× sample volume; DEPC-treated) in 1.6 M NaCl, incubated at 4°C for 20 min and precipitated (13000 rpm; 30 min; 4°C). After removal of the supernatant, the pellet was washed with ice-cold ethanol (70%) and centrifuged again (13000 rpm; 5 min; 4°C). Precipitated DNA was eluted in TE buffer (1×; pH 7.5) and further purified with the OneStep PCR Inhibitor Removal kit, according to the manufacturer's protocol. One non-template water control was included in the extraction process to test the contamination by reagents (ntc1).

3.2.6.3 Microcosms/slurries

DNA and RNA were coextracted from the sediment slurries with a modified version of the protocol from Griffiths et al. (2000). For each sample, a total of 2 grams were transferred in Nucleospin Bead Tubes Type A (4 tubes × 0.5 g). The slurries were subjected to cell destruction on a Precellys 24 homogenisator (5 m/s; 30 s), in the presence of 0.5 ml of hexadecyltrimethylammoniumbromide buffer (CTAB, NaCl, K_2HPO_4, KH_2PO_4, β-mercaptoethanol; DEPC-treated) and equal volume of PCI (25:24:1 v/v/v) per tube. After centrifugation (16100×g; 5 min; 4°C), the destruction step was repeated with 0.3 ml of CTAB and PCI per tube. The combined supernatants from both steps were purified with CI (24:1 v/v), followed by centrifugation (16100×g; 5 min; 4°C). Precipitation of nucleic acids was conducted with equal volume of PEG 6000 (30%; DEPC-treated) in 1.6 M NaCl for 2 hours at 4°C and centrifugation (20000×g; 10 min; 4°C). The resulted pellet was washed with ice-cold ethanol (70%) and, after ethanol removal, air dried for 10 min at room temperature. Nucleic acids were resuspended in 50 µl of DEPC-treated water (DEPC-H_2O), 40 µl of which were used for the isolation of RNA by DNA digestion with the DNase Max® Kit, following the manufacturer's protocol. Due to low nucleic acid concentrations, 1 unit of DNase Max Enzyme was used and the reactions were incubated at 37°C for 10 min. The purity of RNA was confirmed by 16S rRNA PCR with the bacterial primers 968f and 1401r (Nübel et al. 1996) and agarose gel electrophoresis (2% agarose) with ethidium bromide staining. The PCR reactions contained Taq Polymerase (2.5 U), 1× Taq PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3% bovine serum albumin (BSA), 5% dimethyl sulfoxide (DMSO), 10 pmol of each primer and 2 µl of RNA template in a final volume of 50 µl. Contaminant DNA was denatured at 95°C for 5 min, and amplified by 30 cycles of 94°C for 1 min, 58°C for 30 s and 72°C for 1 min, followed by final extension at 72°C for 10 min; absence of a DNA band on the gel confirmed that DNA was completely digested.

Complementary DNA (cDNA) was immediately synthesized from the isolated RNA using the High Capacity cDNA Reverse Transcription Kit, following the manufacturer's protocol, and stored at -20°C until further analysis.

3.2.7 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Bacterial and archaeal 16S rRNA genes were first amplified with 6-carboxyfluorescein-labelled primers (6-FAM) as described in Table 2; the presence of the correct PCR products was confirmed with agarose gel electrophoresis (2% agarose) and ethidium bromide staining. The amplicons were purified with the Nucleospin Gel and PCR Cleanup Kit, according to the manufacturer's protocol. DNA concentrations were measured with a Nanodrop 1000 Spectrophotometer and 400 ng of each sample were digested with the restriction enzymes MspI or TaqI, for Bacteria and Archaea respectively (Table 2). The digested products were purified again with the Nucleospin Gel and PCR Clean-up Kit and concentrations were measured with Nanodrop. Double-stranded DNA was diluted to 5 ng/µl and denatured at 95°C for 5 min, in the presence of formamide. The T-RFLP fragments were separated by an ABI 3730 sequencer using MapMarker 1000 as internal standard.

The T-RFLP electropherograms were obtained using PeakScanner v2 (standard: MM1000; size: 50 bp). After the removal of peaks smaller than 50 bp, peak information was exported and analysed on the T-REX online tool (Culman et al. 2009), with Std dev multiplier for fluor B = 0.8 and clustering threshold 2. The resulted data matrices were analysed by calculating Bray-Curtis distances and non-metric multidimensional scaling (NMDS) using the metaMDS command of the vegan R package (Oksanen et al. 2017).

Based on the taxonomic fingerprints of the microbiomes, three samples representing three different depths were selected from each of the four cores for further analysis/measurements.

Table 2. Reaction mixtures and thermal profiles of the PCR and enzymatic digestion steps of T-RFLP.

	Bacteria	Archaea	Bacteria	Archaea
Reagents	PCR mixtures		Amplification protocols	
TopTaq PCR Buffer	1×	1×	95°C – 5 min	94°C – 3 min
CoralLoad Concentate	1×	1×	94°C – 45 s 56.5°C – 45 s 72°C – 45 s ×30	94°C – 1 min 62°C – 1 min 72°C – 1 min ×20
Q–Solution	0.5×	0.5×		
$MgCl_2$	-	3 mM		
dNTPs	0.1 mM	0.2 mM		
FAM–B27 fwd*	0.2 µM	-	72°C – 5 min	94°C – 1 min 52°C – 1 min 72°C – 1 min ×10
B1401	0.2 µM	-		
A109 fwd	-	0.2 µM		
FAM–A934 rev*	-	0.2 µM		
TopTaq polymerase	2.5 U	1.25 U		72°C – 10 min
DNA template	40 ng	40 ng		
DEPC-H_2O	up to 50 µl	up to 50 µl		
	Digestion mixtures		Digestion protocols	
Buffer Tango with BSA	1×	-	37°C – 3 h	65°C – 3 h
MspI	5 U	-	80°C – 20 min	
Buffer TaqI with BSA	-	1×		
TaqI	-	5 U		
DNA template	400 ng	400 ng		
DEPC-H_2O	up to 25 µl	up to 25 µl		

*, 6-FAM-labelled primers at the 5’ end.

3.2.8 High throughput sequencing

Extracted DNA from the sediment samples collected in 2013 (NA samples) was used for the preparation of shotgun metagenomic libraries. First, DNA was fragmented by shearing in an E220 Focused-ultrasonicator for 100 s, using 175 W peak incident power, 10% duty factor and 200 cycles per burst, and purified with Agencourt AMPure XP beads (1.8× sample volume). Up to 1 μg of DNA was used for end repair, adaptor ligation, size selection and library amplification following the protocol of NEBNext Ultra DNA Library Prep kit for Illumina, with the following modifications. For samples with low starting material, the adaptor was diluted 1:2 (for 0.5–0.7 μg) or 1:3 (for 0.3–0.5 μg and the extraction non-template control). Selection of 400-500 bp insert size was achieved during the clean-up step and amplification with NEBNext Multiple Oligos for Illumina (Index Primer Set 1) was performed with 9 PCR cycles. The length distributions of the final libraries were estimated with the High Sensitivity DNA kit and a 2100 Bioanalyzer.

Bacterial 16S rRNA genes were amplified in triplicate reactions from the enrichment cultures' samples and the stored sediment using the primer pair S-D-Bact-0008-a-S-16 and S-D-Bact-0343-a-A-15 suggested by Klindworth et al. (2013)[μ]. The PCR reaction mixture contained 1× NEBNext High Fidelity Master Mix, 0.06% of BSA, 4 pmol of each primer and 20 ng of the DNA template in 25 μl total volume; primers carried the overhangs that needed to incorporate the indices for Illumina sequencing. Genes were amplified with the following conditions: 98^{o}C for 5 min, 30 cycles of 98^{o}C for 10 s, 60^{o}C for 30 s and 72^{o}C for 30 s, followed by final extension at 72^{o}C for 5 min. One non-template water sample was included (ntc2) to test the amplification of contaminant DNA. The presence of the correct amplicon products and primer dimers was tested on an Agilent 2100 Bioanalyzer using the DNA 7500 kit. Triplicate PCR reactions were pooled prior to DNA purification with the protocol of Gel and PCR Clean-up kit; a non-template control was included in the purification process (ntc3). Buffer NTI was diluted 1:4 in order to remove primer dimers. The absence of primer dimers was confirmed by analysis on a 2100 Bioanalyzer using the DNA 7500 kit and DNA concentration was measured with the Quant-iTTM Picogreen dsDNA assay kit on a SpectraMax Gemini EM Spectrofluorometer. Barcoded sequences and Illumina indices from the Nextera XT Index kit v2 (Set C) were incorporated to the DNA by PCR amplification with 1× NEBNext High Fidelity Master Mix, 2.5 μl of each primer and 10 ng of DNA template, with the following conditions: 98^{o}C for 30 s, 8 cycles of 98^{o}C for 10 s, 55^{o}C for 30 s and 72^{o}C for 30 S, followed by 72^{o}C for 5 min. Purification of amplicons and quantification of DNA were repeated as described above.

Shotgun and amplicon metagenomic libraries were diluted to 3.5 and 4 nM final concentrations, respectively. Paired end sequencing was performed on a MiSeq instrument using the MiSeq Reagent Kit v3 for 600 cycles. Sequencing depth differed among shotgun metagenomes; from each duplicate samples, one was sequenced with a depth of >2 million reads (deep sequenced), while the second with a depth of 1–2 million reads (low sequenced).

The metagenomic datasets obtained from shotgun sequencing have been deposited to the Sequence Read Archive (SRA) database under the BioSample accessions SAMN05712722–SAMN05712733 (SRA accession: SRP083127).

[μ]The amplification of bacterial 16S rRNA genes and preparation of libraries were optimized by M.Sc. Katarzyna Czetyrbok under my supervision, as a part of a 3-month internship.

3.2.9 Bioinformatic analysis

3.2.9.1 Annotation of metagenomic datasets

Bioinformatic analysis of shotgun datasets was conducted using a pipeline developed in-house for Illumina paired-end data[ξ], as follows. Paired raw reads were merged and processed with AdapterRemoval v2.1.0 (Lindgreen et al. 2012), to trim adapter sequences, low quality bases (>15) and filter out short reads (>100 bp). Reads identified as phiX by DeconSeq (Schmieder and Edwards 2011) were subsequently removed. The resulted datasets were taxonomically annotated by aligning to sequences of the NCBI non-redundant protein database (October 2015) with DIAMOND v0.5.2.32 (Buchfink et al. 2015). Output data were analysed by the MEGAN5 analyser v5.7.1 (Huson et al. 2011), with Min Score = 50, Top Percent = 10, Min Support = 1, Min-Complexity Filter = 0. Each shell script used for the above steps was executed in parallel for the individual samples with GNU Parallel (Tange 2011). Metagenomic reads were further assigned to specific functions, related to anaerobic hydrocarbon degradation: anaerobic degradation of alkanes and aromatics, TCA cycle, Wood-Ljungdahl pathway, denitrification, sulfate reduction and methanogenesis. Protein databases were compiled for each gene (80 in total) involved in these pathways (Appendix Table 1) by manually selecting protein subunit sequences of representative strains from the UniProtKB database (Magrane and UniProt Consortium 2011). Strains with sequenced genomes and reference proteomes were preferred. Annotations were filtered based on the identity of the aligned reads (≥50%), the alignment length (≥50 bp) and the bit score (≥50). Genes successfully assigned to selected genes from each pathway were further affiliated to taxa, as described above.

The bacterial 16S rRNA gene amplicons from the enrichment cultures were analysed with Qiime 2 v2017.11 (https://qiime2.org/; Caporaso et al. 2010). Low quality bases (>15) and adapter sequences were trimmed from the ends of the sequenced reads using AdapterRemoval v2.1.0 (Lindgreen et al. 2012) and reads shorter than 100 bp were removed. Paired reads were subsequently merged and chimeras were removed using the denoise-paired command of DADA2 R package v1.3.4 with default filtering parameters, after C-terminal trimming of the forward and reverse reads at length 200 and 190 bp, respectively. Amplicon sequence variants (ASVs) were inferred at the same step and taxonomy was assigned using the feature-classifier classify-sklearn command against the SILVA 128 database (Quast et al. 2013; Yilmaz et al. 2014).

Data visualization was performed using the statistical program R v3.4.2 (R Core Team 2017) and RStudio v1.1.383. The absolute counts of the shotgun and amplicon metagenomes from Keri Lake samples were normalized by subsampling all datasets to an equal sequencing depth using the rrarefy command of the vegan package (Oksanen et al. 2017). Rarefaction curves were calculated and NMDS analysis of the Bray-Curtis distances was performed with the rarecurve and metaMDS commands of the same package, respectively. Results were displayed graphically with the following packages: ggplot2 (Wickham 2009) and phyloseq (McMurdie & Holmes 2013) for barplots, pheatmap (Kolde 2015) for heatmaps, ggpubr (Kasambara 2017) and cowplot (Wilke 2017) for plot annotation.

[§]The pipelines for annotation of the shotgun and amplicon metagenomic reads were developed by Dr. Gisle Vestergaard (Department of Biology, University of Copenhagen, Denmark).

3.2.9.2 Comparison of shotgun metagenomes with other public datasets

In order to allow a direct comparison with publicly available datasets, the deep sequenced shotgun metagenomes from Keri Lake were additionally analysed on the MG-RAST server (Meyer et al. 2008) with the default pipeline options. Paired reads were merged, dereplicated and subsequently trimmed with maximum five bases below quality phred score 20. Taxonomy and functions were assigned to the reads of all metagenomes as described above, after the removal of short reads (<100 bp). The final results were normalized and visualized using the R package pheatmap (Kolde 2015). Due to the low sequencing depth of some metagenomes, the datasets were not normalized by subsampling but relative abundances to the total number of reads of each dataset were calculated and compared instead.

The metagenomes selected for comparison represented ecosystems with different histories of oil exposure to study the long-term impact of oil on microbiomes in the environment. These metagenomes originated from: three asphalt-contaminated water samples from the Pitch Lake in La Brea, Trinidad and Tobago, three wastewater samples from oil sands tailings ponds in Alberta, Canada, three marine sediment samples exposed to the Deepwater Horizon oil spill, Gulf of Mexico (Kimes et al. 2013), three soil samples contaminated by polyaromatic hydrocarbon (PAHs) leakage from a former oil refinery in Xaloztoc, Mexico, and one soil sample contaminated by BTEX (benzene, toluene, ethylbenzene, xylenes) leakage from an oil refinery in Cubatao, Brazil (Table 3). Based on the history of oil exposure, these ecosystems were grouped into two distinct categories: (i) ecosystems with continuous and persistent exposure of at least a few decades (stabilised) and (ii) ecosystems with more recent contamination by oil spills or hydrocarbon wastewater leakages.

Table 3. MG-RAST shotgun metagenomic datasets analysed and compared.

MG-RAST id	Name	Collection site	Sample material	Seq. method	Seq. count
mgm4663020.3	Keri Lake A1	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	2096081
mgm4663012.3	Keri Lake A3	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	2006501
mgm4663014.3	Keri Lake A4	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	2128547
mgm4663010.3	Keri Lake C2	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	1778556
mgm4663018.3	Keri Lake C5	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	3005817
mgm4663021.3	Keri Lake C7	Keri Lake, Zakynthos, Greece	asphalt oil-exposed sediment	Illumina MiSeq	8138021
mgm4470906.3	TarLake	Pitch Lake, La Brea, Trinidad and Tobago	asphalt-contaminated water	Roche 454	378465
mgm4470910.3	Pond6_08	Athabasca oil sands tailings ponds, Canada	wastewater/sludge	Roche 454	862499
mgm4470907.3	Pond6_10	Athabasca oil sands tailings ponds, Canada	wastewater/sludge	Roche 455	327084
mgm4492624.3	SyncrudePit	Athabasca oil sands tailings ponds, Canada	wastewater/sludge	Roche 454	543947
mgm4465489.3	GoM023	Gulf of Mexico	deep sea sediment	Roche 454	122703
mgm4465490.3	GoM278	Gulf of Mexico	deep sea sediment	Roche 454	127356
mgm4465491.3	GoM315	Gulf of Mexico	deep sea sediment	Roche 455	144700
mgm4600198.3	SAC1T0	Xaloztoc, Tlaxcala, Mexico	PAH-contaminated soil	Illumina MiSeq	806947
mgm4600199.3	SAC1T1	Xaloztoc, Tlaxcala, Mexico	PAH-contaminated soil	Illumina MiSeq	738035
mgm4600200.3	SAC1T2	Xaloztoc, Tlaxcala, Mexico	PAH-contaminated soil	Illumina MiSeq	780405
mgm4470861.3	Cubatao_C	Cubatao, Brazil	xylene-contaminated soil	Roche 454	598041

3.2.10 Quantification of marker genes

The potential and activity of the microbiomes in the sampled slurries were investigated by measuring the copy numbers of selected genes and their respective transcripts with qPCR on an ABI 7300 Real Time PCR System. Bacterial and archaeal 16S rRNA genes were selected to monitor the changes in the number and activity of prokaryotic cells, while *dsrB*, *mcrA*, *bamA*, *bamB* and *ncr* were quantified as marker genes for sulfate reduction, methanogenesis and anaerobic degradation of aromatics, respectively. The genes were amplified with the Power SYBR Green PCR Master Mix in 25 µl final reaction volume; the reaction mixtures are described in Table 4, primer sets and PCR thermal profiles are presented in Table 5. For the amplification of genes *mcrA*, *bamB*, *bamA* and *ncr*, Taq DNA Polymerase was additionally included in the PCR master mix to improve the PCR efficiency. Samples were diluted 1:100 before measurements, to minimize PCR inhibition. Positive standards for the *dsrB*, *mcrA* and *bamA* genes were prepared using the TA cloning or ZeroBlunt kits, following the manufacturer's protocol. After transformation, cultures grew

overnight in LB medium containing kanamycin (50 µg/ml) and the plasmids were extracted with the Nucleospin Plasmid kit (No lid), following the manufacturer's protocol. The *mcr* gene of *Methanosarcinales* sp. was amplified from extracted DNA from an anaerobic methane oxidizing enrichment culture dominated by Archaea of the *Methanosarcinales* order and *Methylomirabilis oxyfera*-like Bacteria (Ettwig et al. 2016).

Statistical analysis and data visualisation of the gene and transcript numbers quantified by qPCR were also performed with the statistical program R v3.4.2 (R Core Team 2017) and RStudio v1.1.383. Statistically significant differences in the abundance of genes and transcripts, as well as the concentrations of sulfate in the microcosms were detected by Kruskal-Wallis non-parametric tests using the kruskal.test command, after square root transformation of the data. The R packages ggplot2 (Wickham 2009) and cowplot (Wilke 2017) were used to create and annotate the barplots, respectively.

Table 4. Selected genes quantified by qPCR and PCR mixtures used.

Reagents	16S rRNA (Bacteria)	16S rRNA (Archaea)	*dsrB*	*mcrA*	*bamB*	*bamA*	*ncr*
Master Mix	1×	1×	1×	1×	1×	1×	1×
Taq PCR buffer	-	-	-	1×	1×	1×	1×
Taq Polymerase	-	-	-	0.75 U	2.5 U	1 U	2.5 U
$MgCl_2$	-	-	2.5 mM	-	-	-	-
Q-Solution	-	-	-	0.3×	-	-	-
BSA	0.06%	0.06%	0.06%	0.024%	0.06%	0.06%	0.06%
forward primer	5 pmol	5 pmol	10 pmol	6.25 pmol	10 pmol	10 pmol	10 pmol
reverse primer	5 pmol	5 pmol	10 pmol	6.25 pmol	10 pmol	10 pmol	10 pmol

Table 5. Primer sets, thermal profiles and sources of positive standards used for qPCR.

Target gene	Primer set	Fragment length (bp)	Initial denaturation	Touchdown	Number of cycles	Thermal profile	Number of cycles	Source of positive standard	Reference
16S rRNA (Bacteria)	FP 16S RP 16S	263	95°C – 10 min	–	–	95°C – 45 s 58°C – 45 s 72°C – 45 s*	40	*Pseudomonas putida*	Bach et al. (2002)
16S rRNA (Archaea)	rSAf(i) 958r	617	95°C – 10 min	95°C – 20 s 55°C – 60 s† 72°C – 60 s	5	95°C – 20 s 50°C – 60 s 72°C – 60 s*	40	*Methanobacterium* sp.	Uksa et al. (2015)
dsrB	DSRp2060F DSR4R	350	95°C – 10 min	–	–	95°C – 20 s 55°C – 20 s 72°C – 30 s*	40	*Desulfotomaculum* sp.	Geets et al. (2006)
mcrA	mlas mcrA-rev	470	95°C – 3.5 min	–	–	95°C – 30 s 55°C – 45 s 72°C – 30 s*	40	enrichment culture of *Methanosarcinales* sp.‡	Steinberg & Regan (2009)
bamB	bamBf bamBr	320	94°C – 10 min	–	–	94°C – 30 s 60°C – 30 s 72°C – 30 s 78°C – 30 s*	40	*Geobacter metallireducens* GS-15	Löffler et al. (2011)
bamA	SP9 ASP1	300	94°C – 10 min	94°C – 30 s 64°C – 45 s† 72°C – 60 s	5	94°C – 30 s 59°C – 45 s 72°C – 30 s 78°C – 30 s*	35	*Geobacter metallireducens* GS-15	Kuntze et al. (2008)
ncr	ncr2for ncr2rev	320	95°C – 5 min	95°C – 30 s 60°C – 30 s† 72°C – 60 s	5	94°C – 30 s 55°C – 30 s 72°C – 60 s*	35	strain N47 synthesized DNA fragment	Morris et al. (2014)

†, -1°C/cycle; *, data collection steps; ‡, Ettwig et al. (2016)

4 Results

4.1 Microbiome fingerprints

The investigation of indigenous microbiomes in Keri Lake sediments started with a PCR-based fingerprinting analysis of the communities. The use of different primer pairs allowed the independent

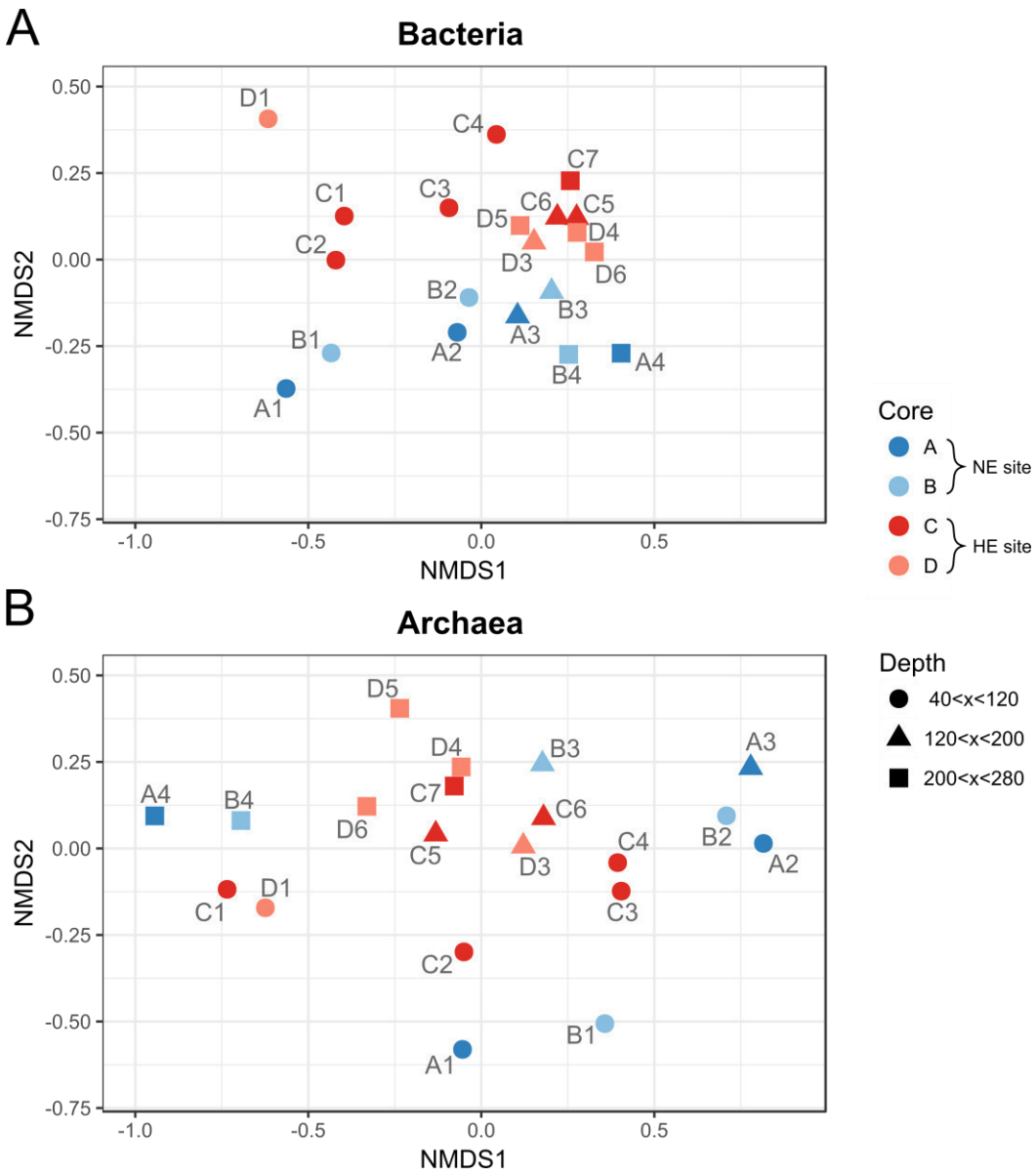

Figure 5: NMDS analysis of T-RFLP data for Keri Lake sediment samples, based on the (A) bacterial and (B) archaeal community fingerprints. Collected cores are presented with different colours and sample depths with distinct shapes. Stress values: 0.1220 for Bacteria, 0.1075 for Archaea.

analysis of Bacteria and Archaea. Based on the structure of the bacterial communities, the samples from the different sites of Keri Lake were separated on the ordination plot (Figure 5A). The communities changed with increasing depth in a way that was similar at both sites; samples transitioned from the left towards the right side of the plot. The samples closer to the sediment surface (A1, B1, C1, C2 and D1) were positioned further away from the deeper ones. Cores A and B were separated from C and D on the y axis. On the other side, archaeal communities from the two sites didn't show a clear separation (Figure 5B). A and B samples were separated with increasing depth, while C and D samples were mainly ordinated at the centre of the plot. Duplicates were generally positioned closely on both NMDS plots with a few exceptions in the archaeal fingerprints. NMDS stress values indicated that ordinations were reliable (stress values ≈0.1) for both Bacteria and Archaea.

Based on the fingerprinting analysis, 12 samples (A1, A3, A4, B1, B3, B4, C2, C5, C7, D1, D3 and D4) were selected for further in-depth analysis, representing three different depths from each site: 40–60 cm, 130–180 cm and 210–250 cm.

4.2 Electron acceptors and organic material in Keri Lake sediments

The concentrations of the available electron acceptors varied between the two sites at Keri Lake (Table 6), especially at depth 40–60 cm. Nitrate concentrations in cores C and D were generally higher compared to A and B (0.33–0.35 mg g^{-1} compared to 0.03–0.08 mg g^{-1} of dry sediment). In the deep sediment samples the concentrations decreased, especially the ones obtained from core C, with the exception of samples A4 and B4, where concentrations were slightly higher compared to the other samples of the same cores. Nitrite was not detected in any of the collected samples. Sulfate was depleted in cores C and D in contrast to the other cores; values were below 0.01 mg g^{-1} of dry sediment in all samples. However, sulfate concentrations were higher in cores A and B, especially at depth 40–60 cm (0.5–1.2 mg g^{-1} of dry sediment).

During the organic extraction process, significantly higher organic material was extracted from the samples of cores C and D as opposed to A and B (Table 6). Values ranged from 250–1460 mg g^{-1} of dry sediment. The difference was more pronounced between the samples close to the sediment surface, due to the very low amount of organic matter in samples A1 and B1 (1.9–5.7 mg g^{-1} of dry sediment). The saturated hydrocarbon fraction extracted from the NE site consisted mainly of long-chain alkanes with 15–35 carbon atoms, particularly *n*-alkanes with odd number of carbon atoms (C_{27}, C_{29}, C_{31}, C_{33}). High abundance of *n*-tetracosane (C_{24}) was also observed. In contrast, the extracted fraction at the HE site consisted of an unresolved complex mixture of hydrocarbons and hopanoid biomarkers with number of carbons in the range of 27–34.

Table 6. Nitrate and sulfate concentrations and EOM content per g of dry sediment in the sediment samples obtained in 2013 from Keri Lake. NE site samples, blue colour; HE site samples, red colour.

Sample name	Depth (cm)	Nitrate (mg g^{-1} dry sediment)	Sulfate (mg g^{-1} dry sediment)	EOM content (mg g^{-1} dry sediment)
A1	45	0.083	0.542	1.9
A3	130	0.038	0.021	34.0
A4	235	0.105	0.100	180.0
B1	40	0.030	1.133	5.7
B3	150	0.031	0.030	11.8
B4	250	0.083	0.152	120.5
C2	62	0.336	0.006	813.9
C5	142	0.014	0.003	979.8
C7	214	0.008	0.001	1208.4
D1	60	0.353	0.007	1460.5
D3	217	0.356	0.000	604.8
D4	241	0.239	0.000	256.9

4.3 Analysis of shotgun metagenomic datasets

The structure and potential function of the sediment microbiomes in the 12 selected samples were studied in more detail by shotgun metagenomic sequencing. The concentration of DNA extracted from samples A1 and B1 of the NE site (14–15 ng/μl) was clearly higher compared to the rest of the samples (3–6 ng/μl). The number of sequenced reads exceeded the 1 million in all samples and most reads had length 301 bp, as provided by the MiSeq platform. After quality control, the deep sequenced datasets (samples A and C) contained 2−7.6 million reads and the low sequenced replicate samples 0.6–2 millions (Table 7). Merging of the forward and reverse reads resulted in average 300–400 bp sequences, with the exception of sample C7, which contained shorter sequences (255 bp average length). In the same sample, only 49% of the merged post-quality reads were taxonomically annotated, while the percentage was higher in the rest of the samples (58–68%). Rarefaction curves of the number of prokaryotic orders and detected genes investigated approached a plateau (Figure 6), especially the curves that correspond to the deep sequenced samples, indicating sufficient coverage of the metagenomic datasets to describe most of the diversity. The low number of sequenced reads (641) obtained from the metagenomic library of the non-template control, compared to the rest of the prepared libraries, indicated very low contamination of the metagenomes by lab handling and reagents.

Table 7. Number of sequencing reads of the shotgun metagenomic datasets before and after the quality control step. The average length after merging and the percentage of reads annotated to the NCBI non-redundant database are also presented. NE site samples, blue colour; HE site samples, red colour; non-template control, green colour.

Sample name	Number of raw read pairs	Number of merged reads after filtering	Average length of merged reads (bp)	Percentage of annotated reads (%)
A1	2472312	2129232	312.14	60.02
A3	2852587	2517450	371.16	65.41
A4	2790844	2208453	370.42	59.31
B1	1519239	1191785	393.01	67.57
B3	1052536	669678	383.23	62.11
B4	1515059	1349317	379.29	58.85
C2	2571722	2232063	375.73	64.83
C5	3843410	3080866	401.47	61.48
C7	8860372	7613773	255.92	49.06
D1	1337873	1140087	385.77	67.12
D3	2020786	1742007	385.42	64.16
D4	1602468	1298635	390.09	61.43
non-template	641	568	358.6	-

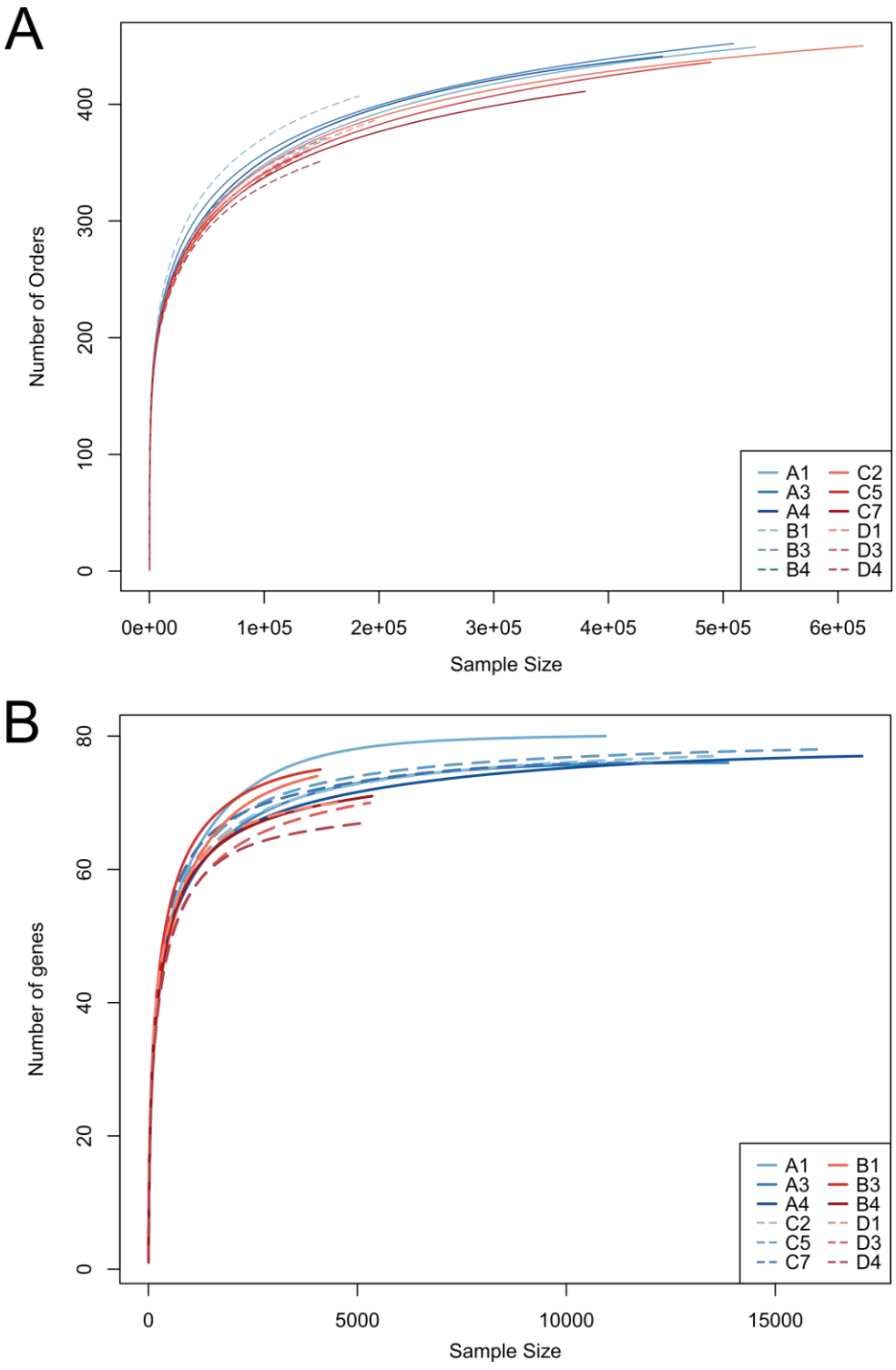

Figure 6: Rarefaction curves of metagenomic data from Keri Lake sediment samples. The numbers of (A) microbial orders and (B) selected genes detected in the metagenomes after rarefaction are presented as a function of the number of sequenced reads. Solid lines, deep sequenced samples; dashed lines, low sequenced samples; NE site samples, blue colour; HE site samples, red colour.

4.3.1 Phylogenetic annotation

All shotgun metagenomic datasets were first rarefied to ~0.66 million reads for direct comparison. Based on the total taxonomic affiliations of the rarefied metagenomes, an NMDS analysis was performed (Figure 7). Duplicate samples were ordinated closely to each other in all cases. Samples from different depths were separated on the x axis. The composition of microbiomes changed with increasing depth, such that the deeper samples from both sites clustered on the right part of the plot. In contrast, samples C2 and D1 were positioned further away from the samples at the same depth of the NE site (A1 and B1), on the left part of the NMDS plot.

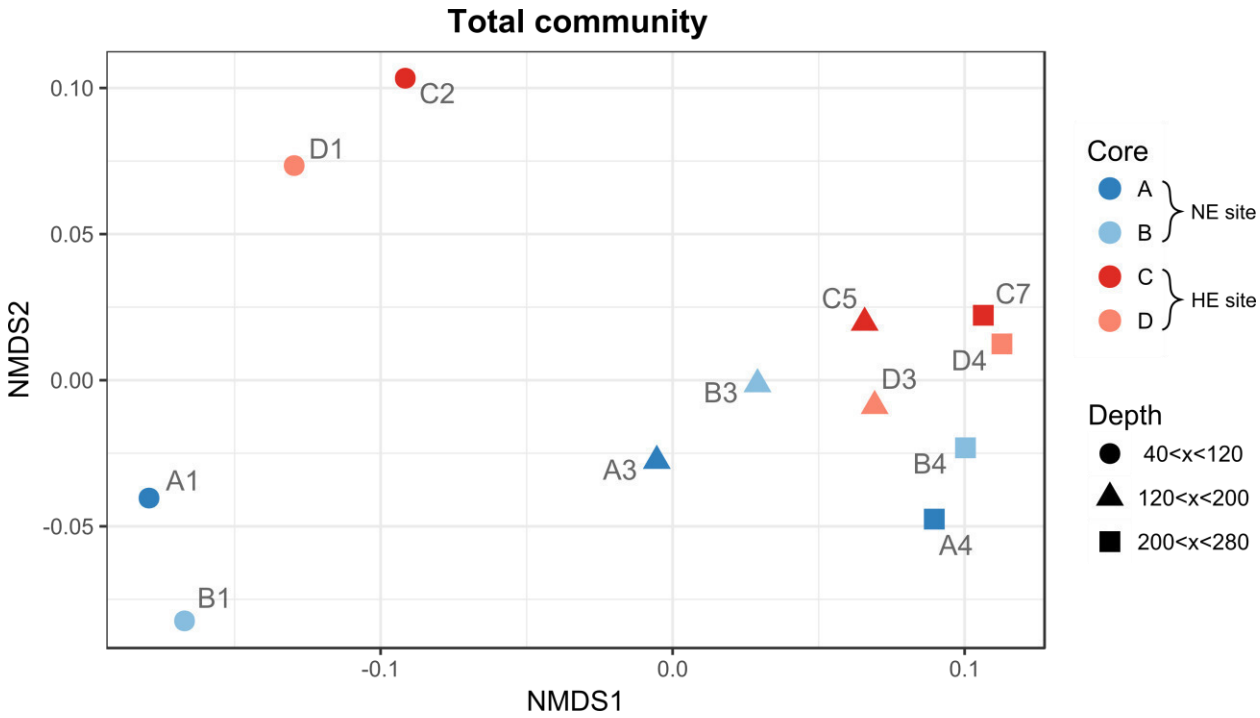

Figure 7: NMDS analysis of shotgun metagenomic data for the selected Keri Lake samples. Collected cores are presented with different colours and sample depths with distinct shapes.

For further analyses, deep sequenced metagenomes were rarefied to ~2.12 million reads, while the low sequenced samples to ~0.66 million. Most reads were assigned to Bacteria, which showed slightly lower abundance at the HE site (Table 8; Figure 8). *Proteobacteria* was the most abundant prokaryotic phylum in the samples closer to the sediment surface (A1, B1, C2 and D1), but became less abundant in the deeper samples. The orders of *Desulfobacterales* and *Desulfuromonadales* were less abundant in samples C and D compared to A an B in all depths. The same was also observed for *Actinomycetales* and *Planctomycetales*. In contrast, *Bacteroidales*, unclassified *Dehalococcoidia*, *Bacillales* and *Clostridiales* had slightly higher abundance in C2 and D1 samples when compared to A1 and B1, while the abundances were similar deeper in the sediments. The numbers of *Rhizobiales* were also higher in the former samples, mainly due to the increased abundance of reads assigned to *Methyloceanibacter caenitepidi*. The *Thermotogales* order was abundant only in sample C2, including mostly the genus *Mesotoga*, but not in the replicate sample D1. The number of Archaea was higher at the HE site at depths 40–60 cm and 130–180 cm, compared to the respective depths at the NE site, while abundances were similar between the cores at the deepest samples analysed (Table 8). The most abundant archaeal orders detected were *Methanomicrobiales* and *Methanosarcinales* of the phylum *Euryarchaeota* (Figure 8). More reads were assigned to these orders in C and D samples, especially a high abundance of *Methanomicrobiales* was observed in samples C2 and D1. In the deeper samples of cores C and D, the abundance of *Euryarchaeota* was lower, while more reads were annotated to unclassified Archaea. Eukaryotes and viruses consisted less that 0.5% of the total reads in samples A1 and B1 and further decreased in the rest of the samples.

Table 8. Percentages of metagenomic reads assigned to Bacteria and Archaea in each sample. NE site samples, blue colour; HE site samples, red colour.

Sample name	Bacteria (%)	Archaea (%)
A1	55.18	1.26
A3	55.96	3.21
A4	44.48	6.94
B1	61.35	1.46
B3	50.76	4.96
B4	43.50	7.52
C2	52.67	6.93
C5	45.24	8.49
C7	34.90	7.42
D1	56.14	5.92
D3	49.60	7.13
D4	45.94	7.96

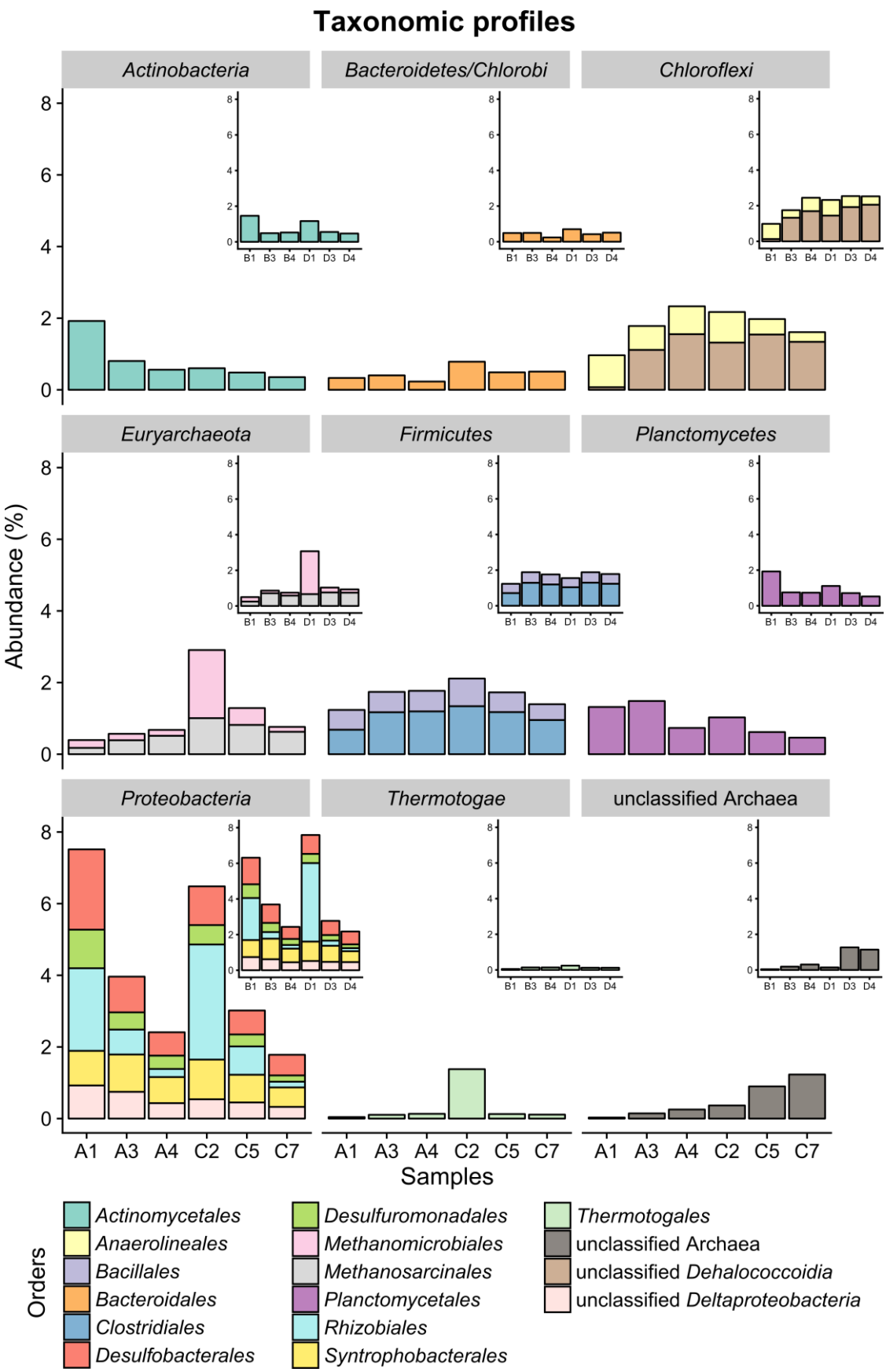
Taxonomic profiles
Actinobacteria
Bacteroidetes/Chlorobi
Chloroflexi
Euryarchaeota
Firmicutes
Planctomycetes
Proteobacteria
Thermotogae
unclassified Archaea
Abundance (%)
Samples
A1 A3 A4 C2 C5 C7
B1 B3 B4 D1 D3 D4
Orders
Actinomycetales
Anaerolineales
Bacillales
Bacteroidales
Clostridiales
Desulfobacterales
Desulfuromonadales
Methanomicrobiales
Methanosarcinales
Planctomycetales
Rhizobiales
Syntrophobacterales
Thermotogales
unclassified Archaea
unclassified Dehalococcoidia
unclassified Deltaproteobacteria

Figure 8: Taxonomic composition of microbial communities from Keri Lake sediment samples. The relative abundances (percentage of annotated reads) of the nine most abundant prokaryotic phyla (>0.5% of the total reads in at least one sample) are presented in separate subplots. Prokaryotic orders are depicted with different colours. The deep sequenced samples (cores A and C) are presented in the main barplots, while the low sequenced samples (cores B and D) are presented at the top right panels of each plot.

4.3.2 Functional annotation

The intrinsic respiratory potential of the microbiomes in Keri Lake sediments was assessed by quantifying the abundance of reads annotated to the genes of the selected reductive pathways (Figure 9). When comparing samples from the same depth, the abundance of genes coding for the sulfate-reducing enzymes was clearly higher in the A and B samples, compared to C and D. Genes of the denitrification pathway were also slightly more abundant in samples A and B, with the exception of *nos* gene in the deep sequenced samples. In contrast, the genes of the methanogenesis enzymes had higher abundances in samples C and D, especially *mtr* and *mcr*. These genes had notably higher abundance in the samples closer to the sediment surface (40–60 cm), while the abundance of the rest of the genes of methanogenesis increased with increasing depth.

The assessment of the metabolic potential of the sediment microbiomes was extended to the quantification of the genes of all the known pathways possibly involved to the complete anaerobic degradation of hydrocarbons to CO_2 (Figure 10). The genes of the hydrocarbon degradation pathways to acetyl-CoA had generally low abundances (<80 reads), even in the deep sequenced samples, with a few exceptions. The most abundant genes were related to the degradation of short-chain *n*-alkanes (*mcm*), phenolic compounds, benzoyl-CoA and polyaromatics (*ncr*). At the NE site, a clear increase in the number of reads assigned to these genes was observed with increasing depth, while in cores C and D the gene abundances were slightly higher in depth 130–180 cm (samples C5, D3) compared to the other sampled depths. The same differences were observed for the genes of the Wood-Ljungdahl pathway, but gene abundances were higher (up to 2000 reads). In contrast, the genes *cit/glt* and *acn* of the TCA cycle, as well as *pta* and *ack* genes of the acetate generation/degradation steps, were clearly less abundant in the deep samples from both sites.

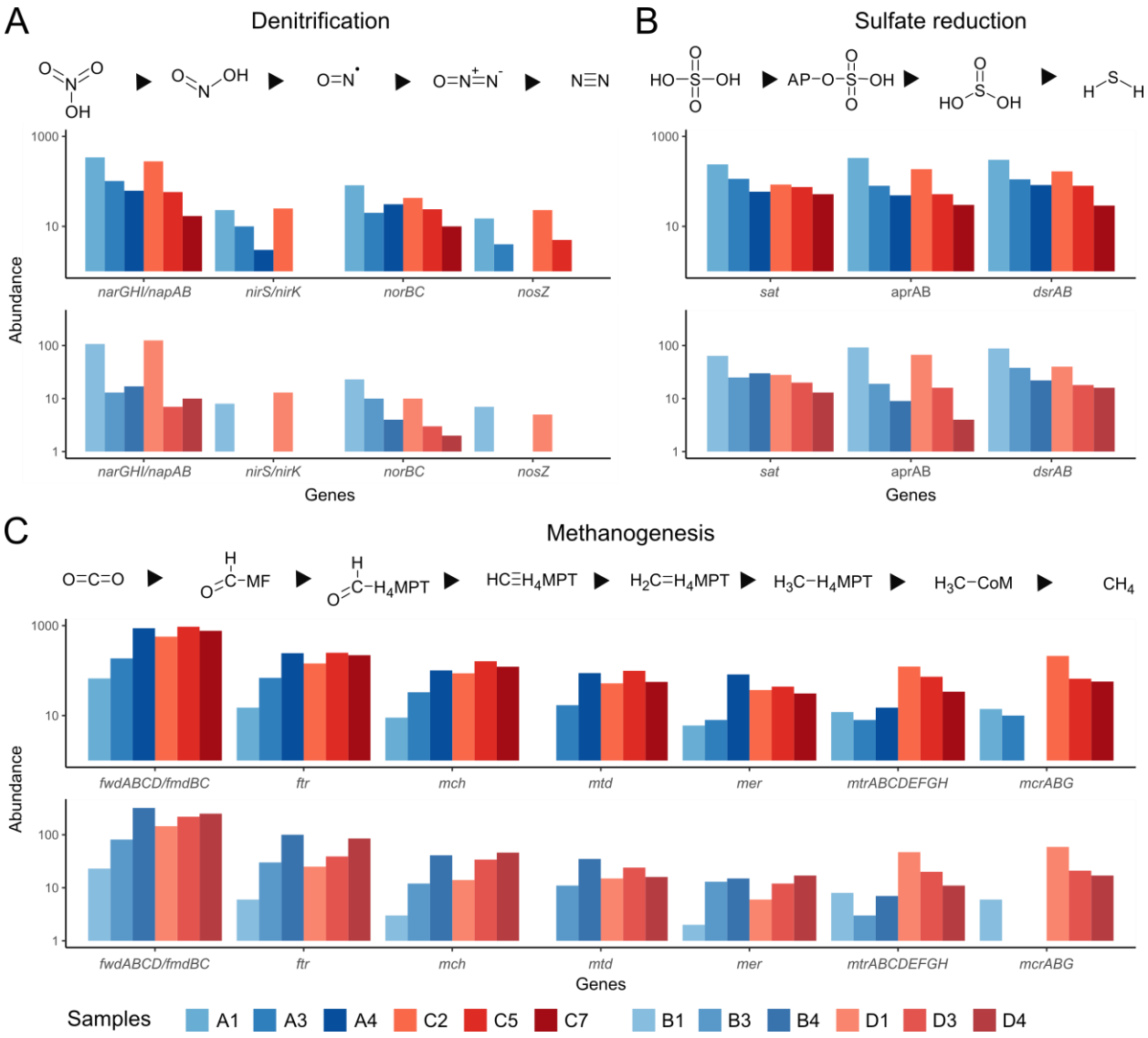

Figure 9: Potential of Keri Lake sediment microbiomes for the utilisation of inorganic and organic electron acceptors. The normalized abundances of the genes coding for the enzymes of (A) denitrification, (B) sulfate reduction and (C) hydrogenotrophic methanogenesis are presented. The abundances are presented in logarithmic scales. Enzyme names are provided in Appendix Table 1. The chemical reactions catalysed by the respective enzymatic products of each gene are presented above each graph. Top panels, deep sequenced samples; bottom panels, low sequenced samples.

A1	A3	A4	C2	C5	C7			B1	B3	B4	D1	D3	D4
31	55	52	33	65	38	*masCDE/assABC**	short-chain *n*-alkane degradation	5	20	11	8	17	28
45	35	34	28	29	50	*assK*		2	15	8	11	9	9
114	192	230	141	243	173	*mcmSL*		35	68	81	57	71	68
11	20	25	13	18	20	*bssABC**	BTEX degradation benzene, toluene, ethylbenzene, xylenes	6	10	2	1	11	10
5	14	7	3	12	20	*bbsEF*		4	6	6	1	9	5
6	3	3	4	1	4	*bbsG*		2	0	1	1	3	0
20	19	33	9	24	49	*bbsH*		10	8	14	6	11	2
25	79	66	41	57	75	*bbsCD*		8	23	19	11	19	22
7	4	17	9	8	8	*bbsAB*		6	4	10	4	0	2
3	17	12	10	22	6	*hbsABC**	p-cresol degradation	3	9	5	4	5	8
4	10	3	1	8	15	*bhsEF*		1	6	3	1	2	5
6	1	1	3	1	1	*bhsG*		0	1	0	0	1	0
5	5	17	4	9	23	*bhsH*		4	6	4	0	0	3
7	12	22	9	16	21	*bhsCD*		4	7	5	1	5	7
7	2	7	2	4	4	*bhsAB*		0	1	1	2	0	1
52	70	48	28	20	9	*ebdABC*+	ethylbenzene degradation	23	4	6	11	2	4
4	1	7	3	3	2	*ped*		0	2	0	4	0	3
28	59	57	19	50	57	*apc12345*		4	11	13	5	21	20
4	0	2	1	5	1	*bal*		0	2	1	2	0	0
6	0	0	4	0	0	*pchCF/pcmGIJ*+	degradation of phenolics phenol, 4-ethylphenol	0	0	0	2	0	0
170	183	156	192	168	92	*xccAC*		86	57	48	71	52	37
42	59	61	44	76	69	*tioL*		10	21	35	16	17	23
61	119	256	127	284	193	*ppsABC*		29	40	85	44	54	88
47	64	78	43	42	31	*ppcABCD*		14	26	23	19	9	9
403	652	596	477	515	346	*hcrABC/hbaBCD*		173	150	181	148	202	178
135	119	106	94	67	61	*ald/aor6*	degradation via benzoate benzene, benzaldehyde, benzoate	32	36	32	26	24	21
8	6	8	16	5	17	*abcAD*~		0	3	6	3	0	2
52	51	41	93	81	68	*bclA/bzdA/hbaA*		26	13	15	28	36	31
11	21	23	13	21	20	*ibsABC**	p-cymene degradation	1	9	9	4	7	8
4	1	2	3	1	4	*bisEF*		2	2	0	0	2	2
2	0	0	1	0	1	*bisG*		0	0	0	0	2	0
11	8	21	8	14	36	*bisH*		4	3	9	1	4	5
8	17	23	20	19	39	*bisCD*		5	4	12	8	7	6
6	2	5	0	13	5	*bisAB*		1	2	3	4	0	0
56	65	50	23	16	3	*cmdABC*+		25	3	16	10	3	0
24	22	32	9	23	12	*iod*		7	11	20	2	5	6
15	8	2	13	4	5	*iad*		4	3	1	9	1	1
15	10	10	15	18	19	*ibl*		2	4	2	9	2	4
304	624	961	398	721	481	*bcrABCD/bamBC/badDEFG/bzdNOPQ*	benzoyl-CoA degradation	106	227	279	110	273	261
36	54	67	38	53	58	*dch/bamR*		11	20	25	16	22	22
53	192	291	71	210	135	*had/bamQ*		29	64	97	33	100	89
65	177	348	105	231	132	*oah/bamA*		23	75	98	24	96	53
9	17	20	18	17	15	*ancA*~	degradation of polyaromatics naphthalene, 2-methylnaphthalene	1	11	5	9	4	5
13	20	17	14	16	17	*nmsABC**		5	9	9	4	6	9
5	7	3	0	7	4	*bnsEF*		1	2	3	0	2	1
4	2	1	1	1	1	*bnsG*		0	0	2	0	2	0
4	6	17	5	12	27	*bnsH*		2	4	0	1	0	3
8	20	27	13	19	19	*bnsCD*		8	10	5	2	5	5
7	1	5	4	8	0	*bnsAB*		1	2	0	2	0	2
93	250	622	151	409	229	*ncrABCD*		41	98	176	64	138	134
330	230	118	321	117	87	*citA/gltA*	TCA cycle	132	42	33	122	37	28
722	595	441	695	507	320	*acnAB*		292	141	131	276	165	125
478	576	502	567	505	412	*icd/idh*		196	149	144	221	194	217
765	894	1175	756	1055	778	*korAB*		264	271	341	254	292	255
473	668	763	500	727	515	*sucCD*		213	189	233	194	209	172
570	499	692	531	591	432	*frdABCD/sdhABCD*		246	135	216	202	139	152
504	621	685	580	745	514	*fumABC*		224	188	203	226	229	269
331	390	493	477	456	391	*mdh/mqo*		136	128	158	160	148	152
581	1311	1999	1015	1750	1357	*acsCD/cdhABCDE*	Wood-Ljungdahl	197	394	611	292	561	687
271	450	462	365	404	309	*metF*		97	142	135	124	154	183
228	319	284	310	262	221	*folD/fchA*		81	90	99	122	98	100
322	684	623	572	620	494	*fhs*		115	192	192	206	272	260
1130	1685	1966	1697	2016	1653	*fdhAB*		368	507	622	564	747	712
399	548	550	380	447	419	*cooFS/coxSML*		120	120	153	136	202	211
154	88	34	157	77	49	*pta*	acetate generation	44	21	11	54	31	24
158	102	63	172	97	74	*ackA*		40	39	24	63	43	23

Figure 10: Potential of Keri Lake sediment microbiomes for the complete anaerobic degradation of *n*-alkanes, monoaromatics and polyaromatics to CO_2. The normalized abundances of the genes coding for the enzymes involved in the degradation pathways are presented. Enzyme names are provided in Appendix Table 1. Left heatmap, deep sequenced samples; right heatmap, low sequenced samples. *, fumarate adding activation reactions; +, hydroxylating activation reactions; ~, carboxylation activation reactions.

4.4 Connection of functions to taxonomy

Further analysis of the taxonomic profiles of metagenomic reads annotated to selected genes revealed taxa with the potential to perform specific metabolic processes. Due to high diversity of the sediment samples, resulting in low number of reads assigned to the metabolic genes studied, only the deep-sequenced datasets were considered for this analysis, which have a better coverage.

Based on the taxonomic analysis of the respiratory genes *nor*, *dsr* and *mcr*, taxa with the potential to conduct denitrification, sulfate reduction or methanogenesis were identified (Figure 11). The taxonomic profiles of genes *nor* and *dsr* didn't not differ significantly between the two sites. *Desulfuromonadales* were the most abundant potentially denitrifying Bacteria in the samples, with the exception of C7. Slight differences in the number of *Spirochaetales* and *Nitrospirales* reads were mainly observed. Several *dsr* reads were assigned to *Desulfobacterales*, *Clostridiales* and *Syntrophobacterales* and had slightly higher abundances in the samples from core A, but the majority of reads were not assigned to specific bacterial orders. In contrast, the annotation of the reads assigned to *mcr* gene was clearly different due to the very low read numbers in the A samples. *Methanomicrobiales* and other methanogenic Archaea were present in high abundance in samples C2 and C5. Many *mcr* genes from unclassified taxa were identified as well in all sediment depths.

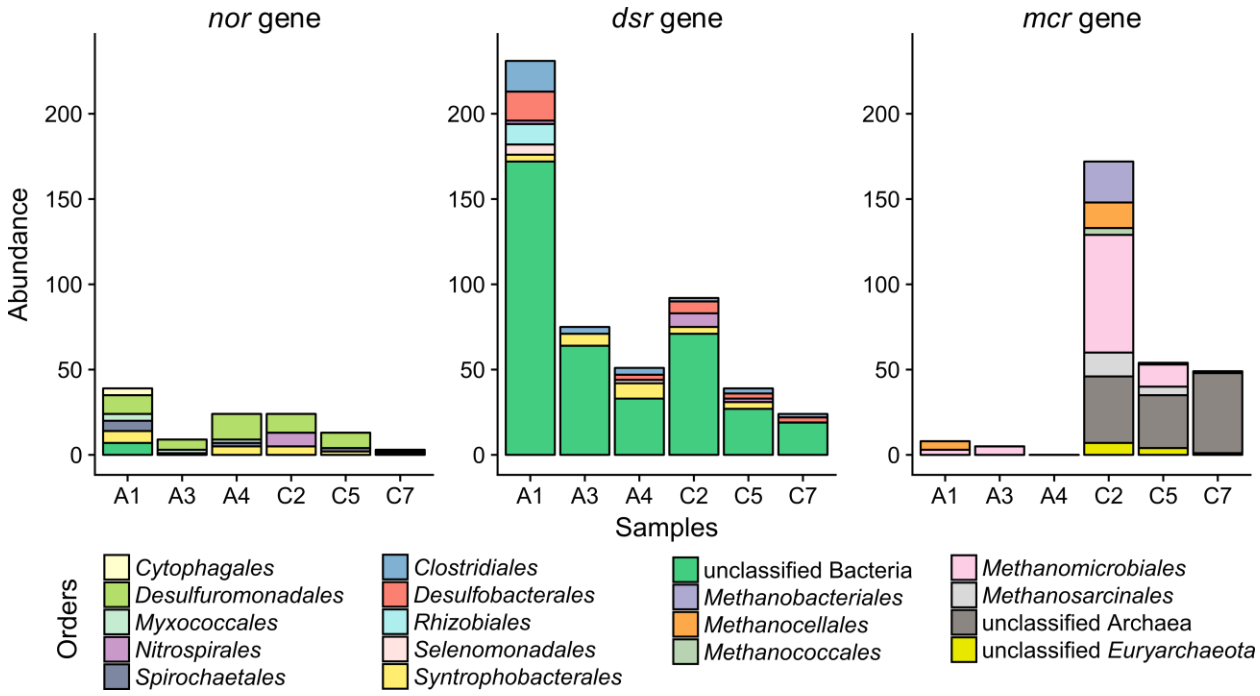

Figure 11: Taxonomic affiliation of reads annotated to selected genes of the respiratory pathways: *nor* (nitric oxide reductase), *dsr* (dissimilatory sulfite reductase) and *mcr* (methyl-CoM reductase). The 7 most abundant orders for each gene are presented in the deep sequenced samples (>10, >6 and >1 reads in at least one sample, respectively).

Many Bacteria with the ability to degrade hydrocarbons under anaerobic conditions were detected as well in Keri Lake sediments. In general, the taxonomic profiles of the reads assigned to these genes did not differ between the two sites; mainly slight changes in the abundance of the detected taxa were observed (Figure 12). *Clostridiales* species carried all of the studied genes, with the exception of the *acs/cdh* genes of the Wood-Ljungdahl pathway. In most cases, genes assigned to *Clostridiales* were more abundant in the deep sediment samples. High number of reads of the monoaromatic degradation genes (*hcr*, *bcr/bam/bad/bzd*) and the Wood-Ljungdahl pathway (*acs/cdh*) were annotated to *Desulfobacterales*, *Syntrophobacterales* and other *Deltaproteobacteria* and were abundant in all samples. Many *mcm* gene reads were assigned to *Thermoanaerobacterales*, while *Zixibacteria* and other unclassified Bacteria were identified as abundant carriers of genes *bcr/bam/bad/bzd* and *ncr*, especially in sample A4. Archaea carrying the genes *acs/cdh* were only detected in the samples from core C. The profile of the acetate generating/degrading *ack* gene didn't change notably between the two sites, only a slightly higher abundance of *Rhizobiales* and *Bacteroidales* species was observed in sample C2.

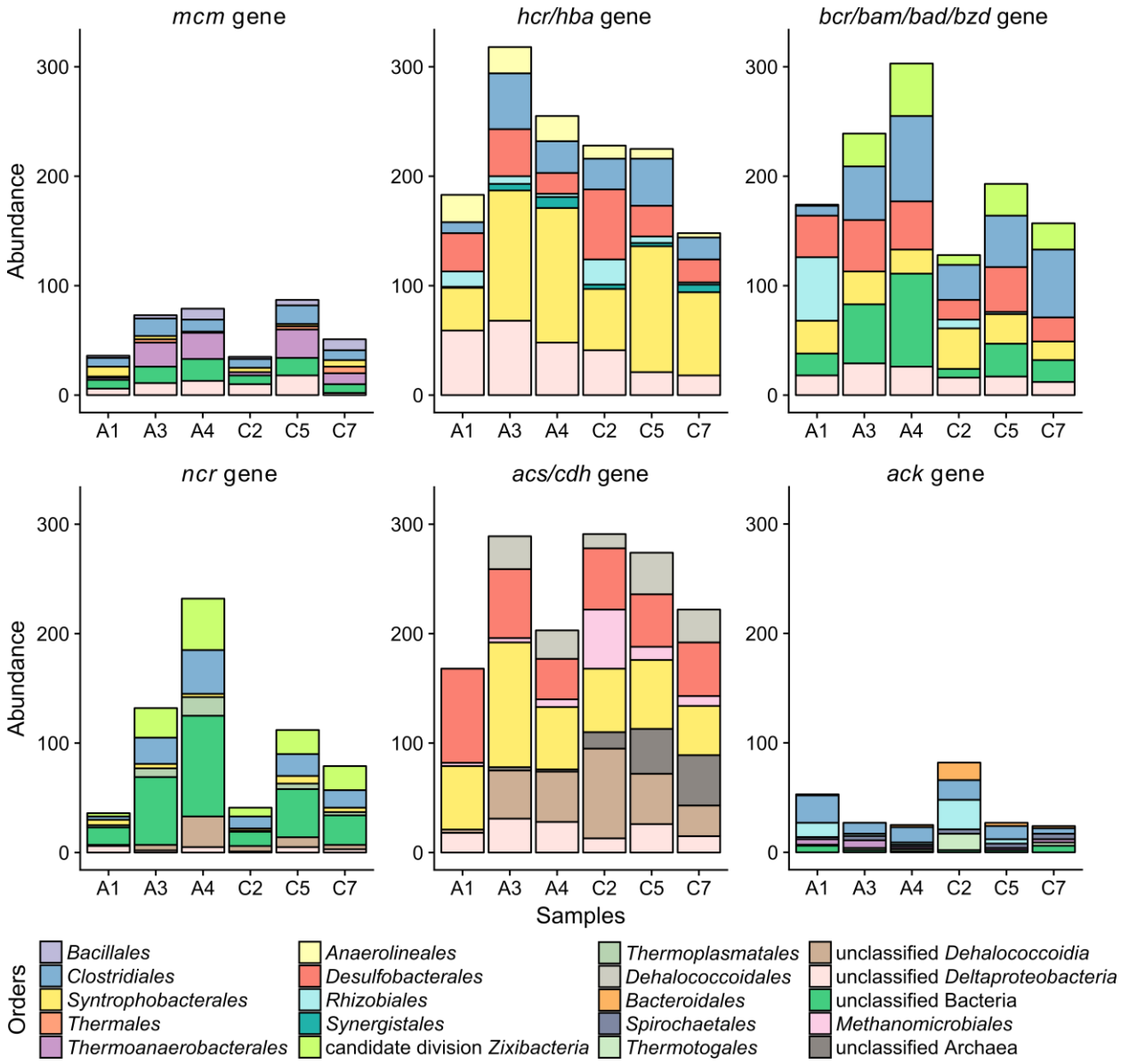

Figure 12: Taxonomic affiliation of reads annotated to selected genes of the hydrocarbon degradation pathways: *mcm* (methylmalonyl-CoA mutase), *hcr* (4-hydroxybenzoyl-CoA reductase), *bcr/bam/bad/bzd* (benzoyl-CoA reductase), *ncr* (2-naphthoyl-CoA reductase), *acs/cdh* (carbon monoxide dehydrogenase/ acetyl-CoA synthase) and *ack* (acetate kinase). The 7 most abundant orders for each gene are presented in the deep sequenced samples (>20, >20, >50, >13, >57 and >10 reads in at least one sample, respectively).

4.5 Cross-study comparison of microbiomes

The direct comparison of metagenomic data allowed us to directly compare the microbiomes harboured in the different studied ecosystems. Most of the bacterial orders had higher abundances in the stabilised ecosystems, compared to the perturbed (Figure 13). These included *Dehalococcoidales* and other *Dehalococcoidia*, *Synergistales*, *Syntrophobacterales*, *Thermoanaerobacterales*, *Thermoplasmatales* and *Thermotogales*. Many archaeal reads from the Keri and Pitch Lake samples were not classified to a lower taxonomic level, but all assigned methanogenic orders were clearly more abundant in the stabilised ecosystems. In contrast, *Actinomycetales*, *Planctomycetales* and *Rhizobiales* had higher abundances in the metagenomic datasets of some perturbed ecosystems. *Desulfobacterales* and *Desulfuromonadales* were detected in high abundances in the samples from the oil sands tailings ponds and the deep sea sediments of the Gulf of Mexico, while *Rhizobiales* were more abundant in low-depth terrestrial samples (Keri Lake C2, Xaloztoc and Cubatao). Reads assigned to the order of *Clostridiales* were abundant in most environments, except for the Pitch Lake and Cubatao samples.

With the exception of Keri Lake oil-exposed samples (core C), the genes of the denitrification pathway had higher abundances in the studied ecosystems, especially the oil sands tailings ponds, compared to Keri Lake sample A1 (Figure 14). The genes coding for the sulfate-reducing enzymes were generally less abundant; the samples from tailings ponds as well as Gulf of Mexico 278 and 315 had abundances closer to the ones of sample A1. The genes of the methanogenesis pathway had clearly higher abundance in the samples from the stabilised ecosystems in comparison to the perturbed. The same was observed also for most of the genes of the degradation pathways of *n*-alkanes and aromatics, although their abundances did not differ notably among samples (Figure 14). The abundances of *ebd* and *xcc* genes were slightly high in the perturbed ecosystems, but had low abundances in the Keri Lake sediment samples, while genes of the central degradation of monoaromatics (*bcr/bam/bad/bzd* and *oah/bamA*) were elevated in Keri Lake and some samples of Gulf of Mexico. Gene *acs/cdh* of the Wood-Ljungdahl pathway had high abundances only in Keri Lake samples from core C, while gene *fdh* was elevated in all stabilised ecosystems and two samples from Xaloztoc (SAC1T0, SAC1T1).

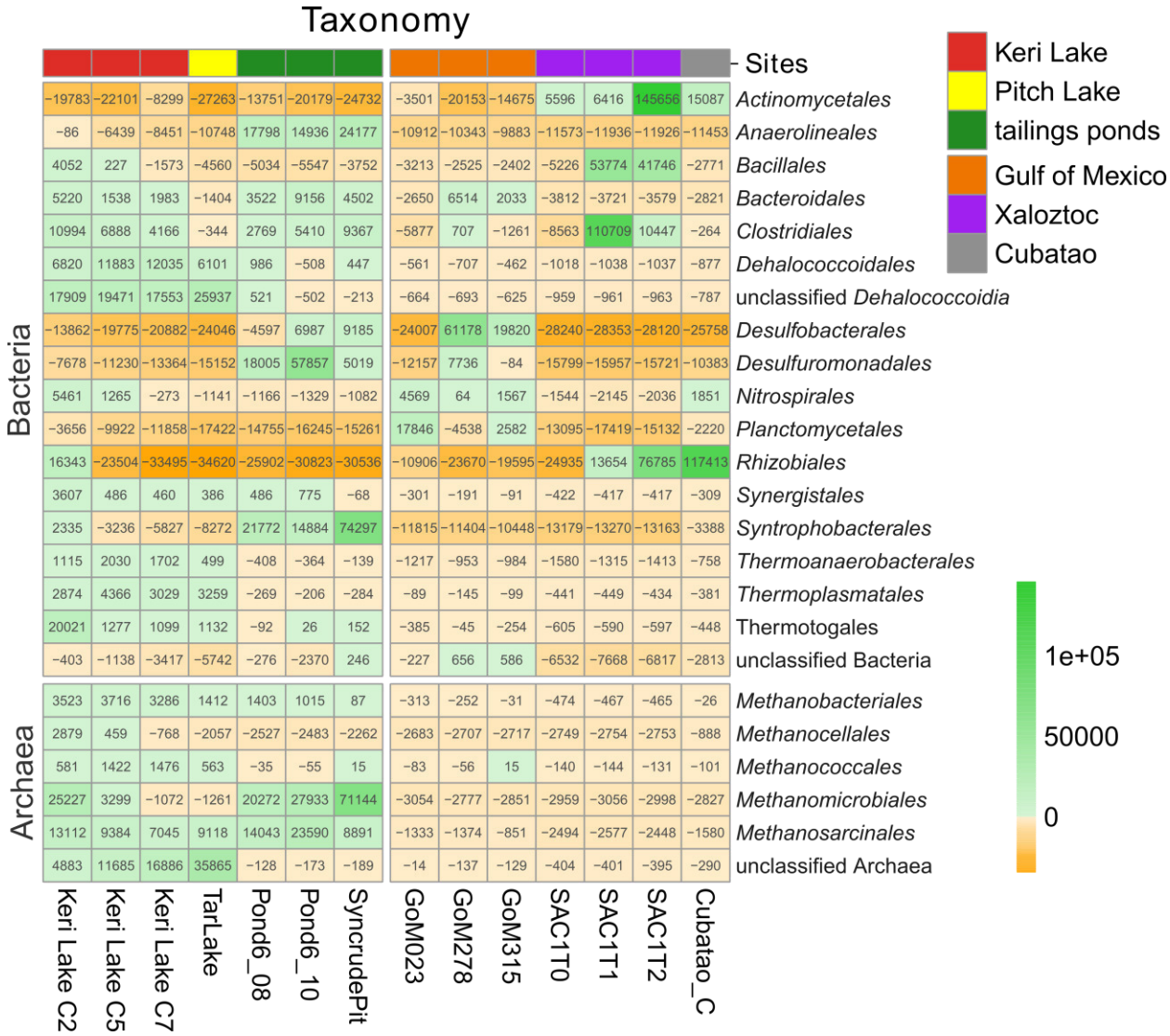

Figure 13: Comparison of the taxonomic profiles of Keri Lake datasets with the profiles of metagenomes from other oil-impacted environments. Cell numbers indicate the normalised abundances of the bacterial and archaeal orders, after subtracting the respective abundance in sample A1 from Keri Lake.

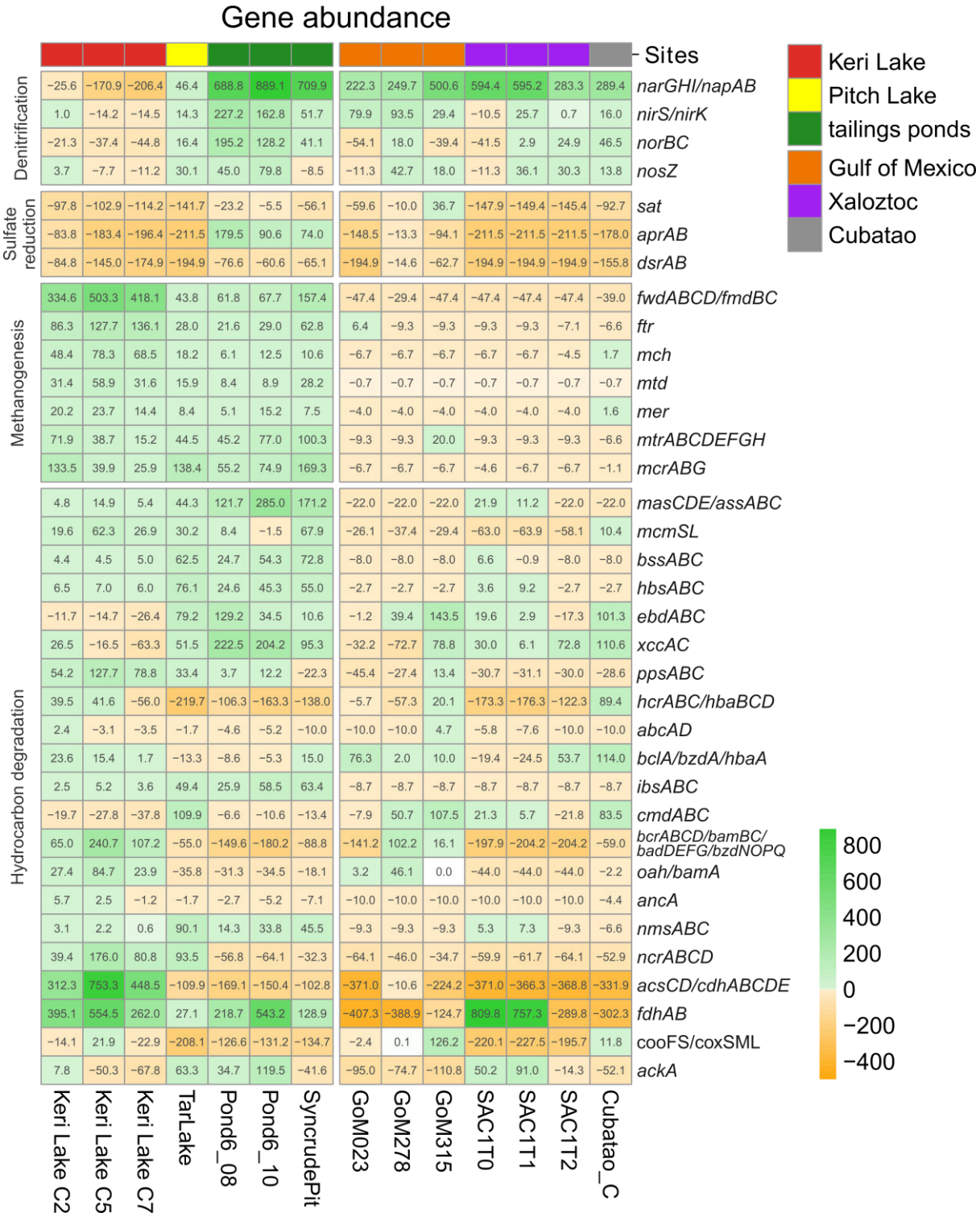

Figure 14: Comparison of gene abundances in Keri Lake datasets and metagenomes from other oil-impacted environments. Cell numbers indicate the normalised abundances of the genes, after subtracting the respective abundance in sample A1 from Keri Lake. Enzyme names are provided in Appendix Table 1.

4.6 Microbial enrichment in liquid cultures

The concentration of sulfate was monitored over the course of the experiment, until its consumption by the enrichment cultures. The initial concentrations measured were around 20 mM in all cultures and did not decrease notably until day 457. A clear decrease in sulfate was observed approximately 20 months after the beginning of incubations (day 616; Table 9). The concentration of sulfate decreased to less than 1 mM also in the autoclaved culture 8. All cultures remained colourless throughout the incubation period, thus conditions remained anoxic during the whole experiment.

Table 9. Concentrations (mM) of diluted sulfate in the anoxic enrichment cultures.

Day / Culture	0	21	49	195	322	457	616
cult 1	21.15	20.12	20.65	14.68	16.80	17.62	0.25
cult 2	21.15	19.62	21.62	13.74	17.18	15.92	0
cult 3	20.14	20.30	17.16	16.85	20.54	18.22	0
cult 4	21.78	20.57	21.74	15.66	18.24	17.92	0
cult 5	20.90	20.90	21.25	17.18	18.57	16.87	0.03
cult 6	21.22	19.74	19.47	18.19	20.74	19.26	0.69
cult 7	24.02	21.32	21.90	19.21	20.34	18.62	0.09
cult 8	18.86	18.69	18.32	16.76	21.27	21.54	0.98

At day 616, DNA was extracted from the enrichment cultures and the inoculum sediment in order to identify the enriched microbial taxa. DNA concentrations extracted from cultures 1–7 ranged from 2.5–9 ng/µl, while less amount of DNA was extracted from culture 8 and the extraction control ntc1 (0.77 and 1.03, respectively). Extraction from 300 mg of the inoculum sediment sample resulted almost 40 ng/µl of DNA.

The number of sequence reads varied from ~150 thousands (culture 17) to ~36 thousands (ntc2). Many raw reads were filtered out during the bioinformatic analysis and the amplicon datasets were finally rarefied to 9255 reads, for direct comparison of the samples. Despite the significant decrease in the number of reads, the rarefaction curves of the number of orders detected in all samples reached a clear saturation plateau (Figure 15A). Based on the bacterial community structure, the enrichment cultures 1–7 clustered closely and further away from the other samples (Figure 15B). The autoclaved culture 8 was ordinated closer to the inoculum sediment sample, compared to the other cultures.

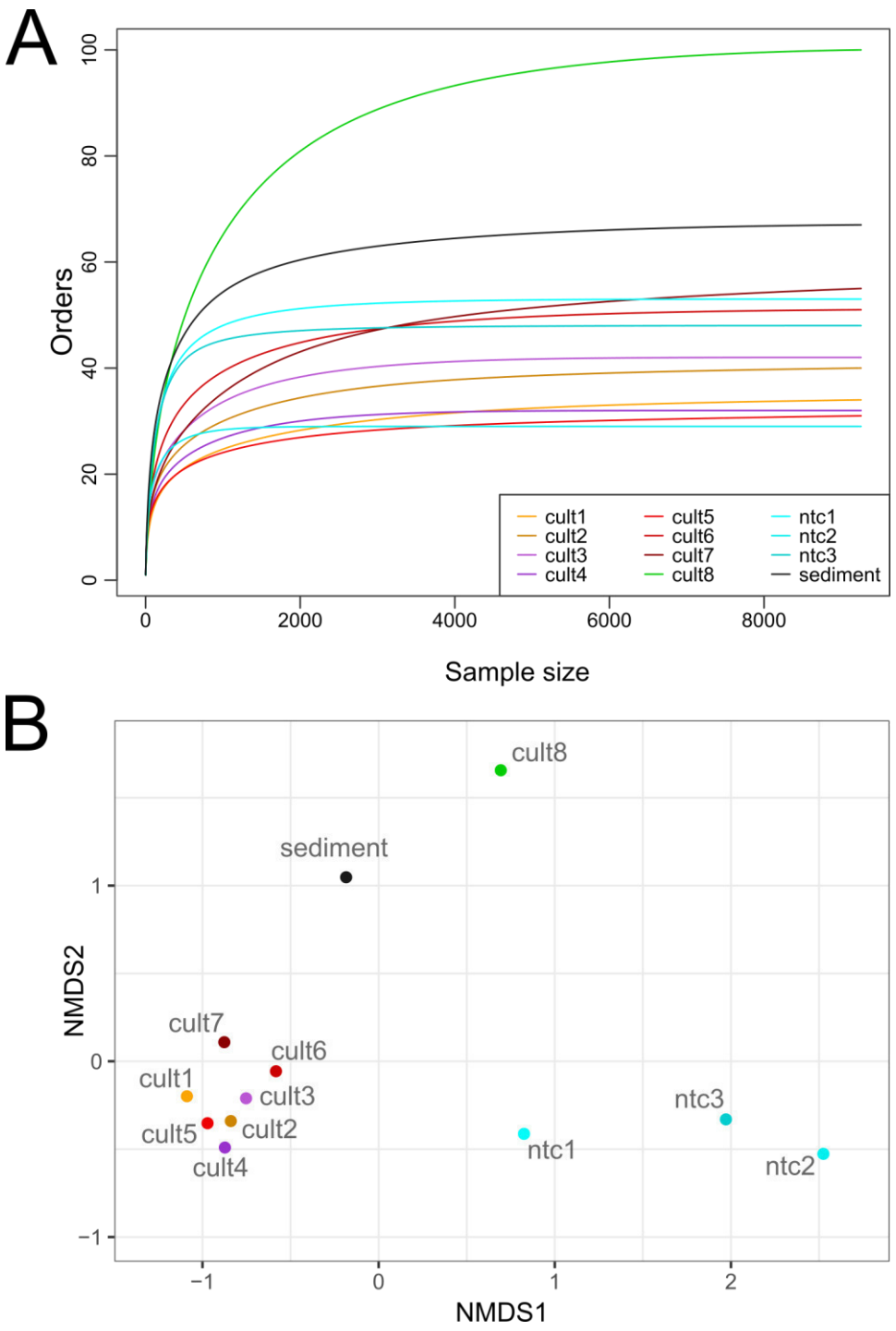

Figure 15: (A) Rarefaction curves of the sequenced bacterial 16S rRNA gene libraries of the sulfate-containing enrichment cultures, the sediment from Keri Lake and the respective non-template controls. The number of microbial orders detected in the amplicon metagenomic datasets after rarefaction are presented as a function of the number of sequenced reads. (B) NMDS analysis of the same datasets.

The most abundant members identified consisted 35% of the total community in the inoculum sample and increased to almost 90% of the total reads of the libraries prepared from the non-sterilised enrichment cultures (Figure 16). The number of reads assigned to *Desulfobacterales*, *Desulfovibrionales* and *Desulfuromonadales* were high in cultures 1–7 (up to 43% of the total number of reads) compared to the sediment sample. In cultures 1 and 2, *Desulfobacterales* reads were annotated to *Desulfatitalea*, while in culture 4 mostly *Desulfobacterium* reads were detected. *Desulfatiglans* (*Desulfarculales*), *Spirochaeta* (order *Spirochaetales*), *Smithella* (*Syntrophobacterales*) and *Thermovirga* (order *Synergistales*) were among the most abundant genera in the sediment sample and further increased in some but not all of the cultures 1–7. Culture 8 and ntc 1 consisted mostly of reads assigned to *Rhizobiales* (*Methylocystaceae* genera and *Xanthobacter*), which accounted for 40–45 % of the total reads. In contrast, the ntcs 2 and 3 contained mainly *Micrococcales* (*Dermabacter* and *Brevibacterium*), *Corynebacterales* (*Corynebacterium*), *Propionibacteriales* (*Propionibacterium*) and *Bacillales* (*Bacillus*) reads (~50% of the metagenomic libraries altogether). These orders were not detected in high abundances in cultures 1–7. Many bacterial reads were not assigned to a lower taxonomic level, which reached 20–28% of the total number of reads in cultures 1–7.

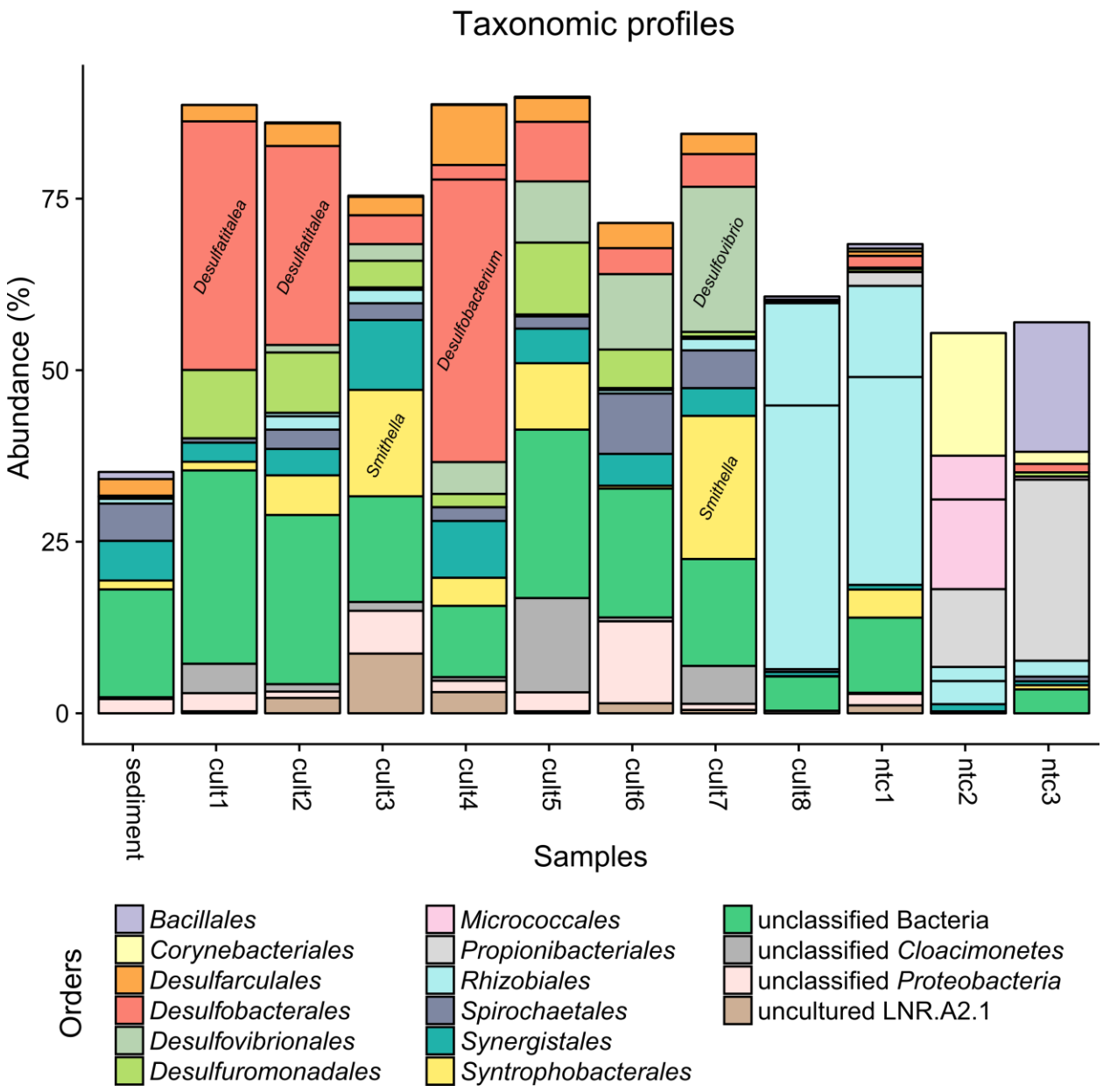

Figure 16: Taxonomic investigation of the samples obtained from the sulfate-containing enrichment cultures, the sediment from Keri Lake and the respective non-template controls (ntcs). The relative abundances (percentage of annotated reads) of the 16 most abundant bacterial orders are presented (>6% of the total reads in at least one sample). Bars with the same colour represent different families that belong to the same order.

4.7 Sulfate consumption in microcosms/slurries

The concentrations of sulfate were quantified once per week in the supernatants of the anoxic microcosms. Initial concentrations (day 0) were ~13 mM in the microcosms which contained the anaerobic medium with sulfate. In the HS microcosms, the concentrations significantly decreased to <2 mM already at day 9, while in the NS microcosms sulfate was depleted after 16 days (p-values =0.003; Figure 17). The concentrations remained low (<5 mM) until day 40. Olfactory inspection of the samples obtained from the microcosms revealed a strong sulfide odour only in the HS microcosms. No sulfate was detected in the samples obtained from the control microcosms (NC and HC) throughout the whole experiment. Based on these measurements, time points 0, 9, 16 and 40 were selected for further analysis, representing (i) the beginning of the experiment (day 0), (ii) the phase of sulfate consumption (days 7 and 14) and (iii) the lack of sulfate (day 38).

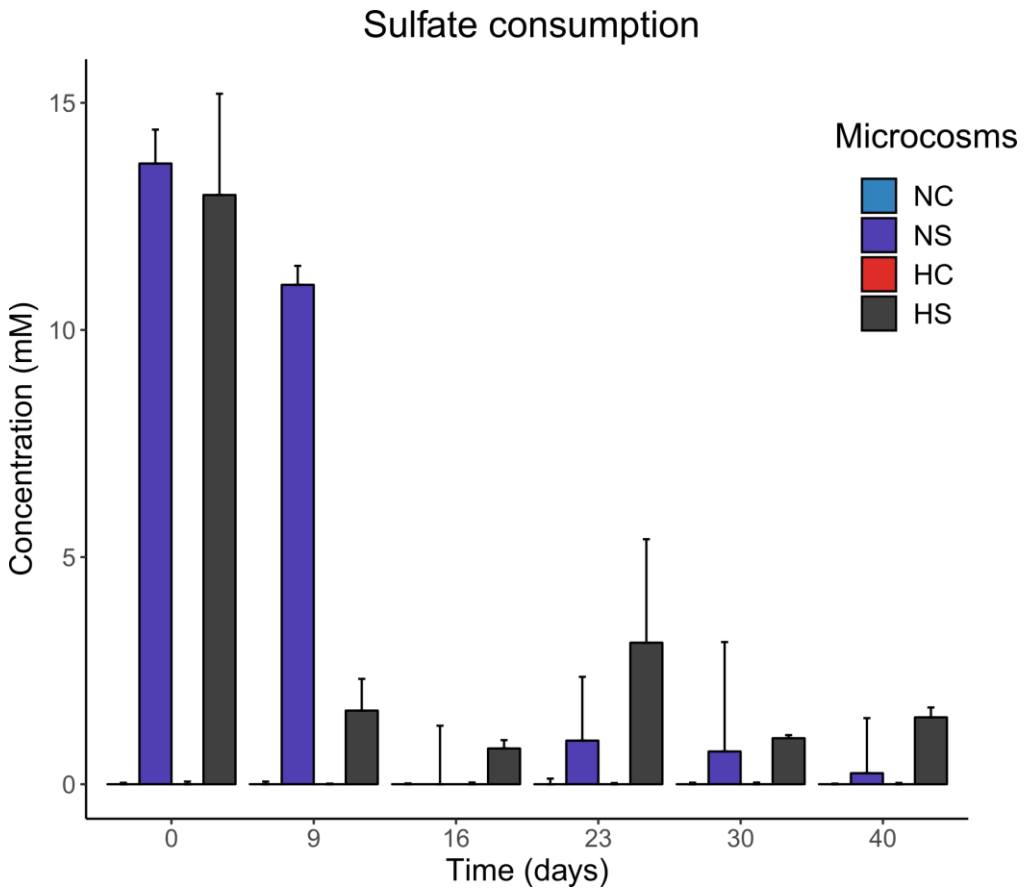

Figure 17: Sulfate concentrations (mM) in the samples obtained from the anoxic microcosms over time. Error bars indicate the standard deviation of the sulfate measurements in the replicate microcosms.

4.8 Functional potential of microbiomes in microcosms/slurries

Changes in the potential of the Keri Lake sediment microbiomes were studied in the anoxic microcosms, by quantification of selected genes in the slurries over the course of the experiment. At day 0, the copy numbers of bacterial 16S rRNA genes were similar in all microcosms, while the number of archaeal 16S rRNA genes was significantly higher at the oil-containing microcosms compared to the NC and NS (p-value =0.002; Figure 18). In the samples containing sediment from the NE site, the number of both bacterial and archaeal 16S rRNA genes gradually increased until day 16 (p-values <0.04 and 0.01, respectively). A higher increase in the number of bacterial 16S rRNA genes was observed in the NS microcosms at day 16 and decreased again to the level of the NC samples. In the HS microcosms, the number of archaeal 16S rRNA genes also increased gradually during the experiment and peaked at day 16, while the bacterial 16S rRNA genes strongly increased already at day 9 and the gene numbers slightly decreased until day 40. In the HC microcosms though, the number of both genes declined significantly during the first nine days (p-values <0.026).

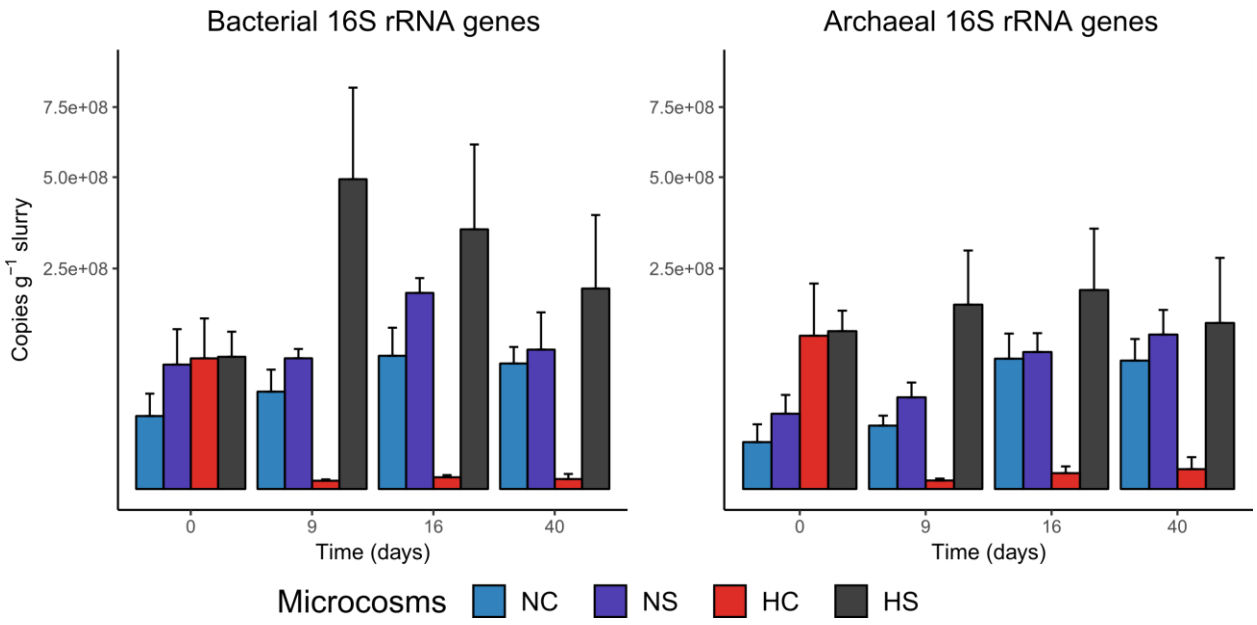

Figure 18: Copy numbers of bacterial and archaeal 16S rRNA genes per g of anoxic slurry. The numbers are presented in logarithmic scale. Error bars indicate the standard deviation of the measurements in the replicate microcosms.

Gene *dsrB* had similar copy numbers in all microcosms at day 0 and increased in microcosms NS and HS at later time points (Figure 19). A significant increase was observed in the NS microcosms after 16 days (p-value =0.009), while gene copies strongly increased (3-fold; p-value =0.044) in the HS microcosms already at day 9. The number of *mcrA* genes gradually increased only in microcosms NC and NS (p-values <0.005), although *mcrA* copy numbers were lower compared to HC and HS at day 0 (p-value =0.001). Interestingly, the number of *bamA* genes was higher at day 0 in the microcosms containing NE sediment (p-value =0.005), compared to the HC and HS, and increased significantly at later time points in the NS samples (p-value =0.006). All three genes decreased in the samples obtained from the HC microcosms already after 9 days of incubation with the anaerobic medium (p-values <0.027). The genes *bamB* and *ncr* were not detected in any of the microcosm samples.

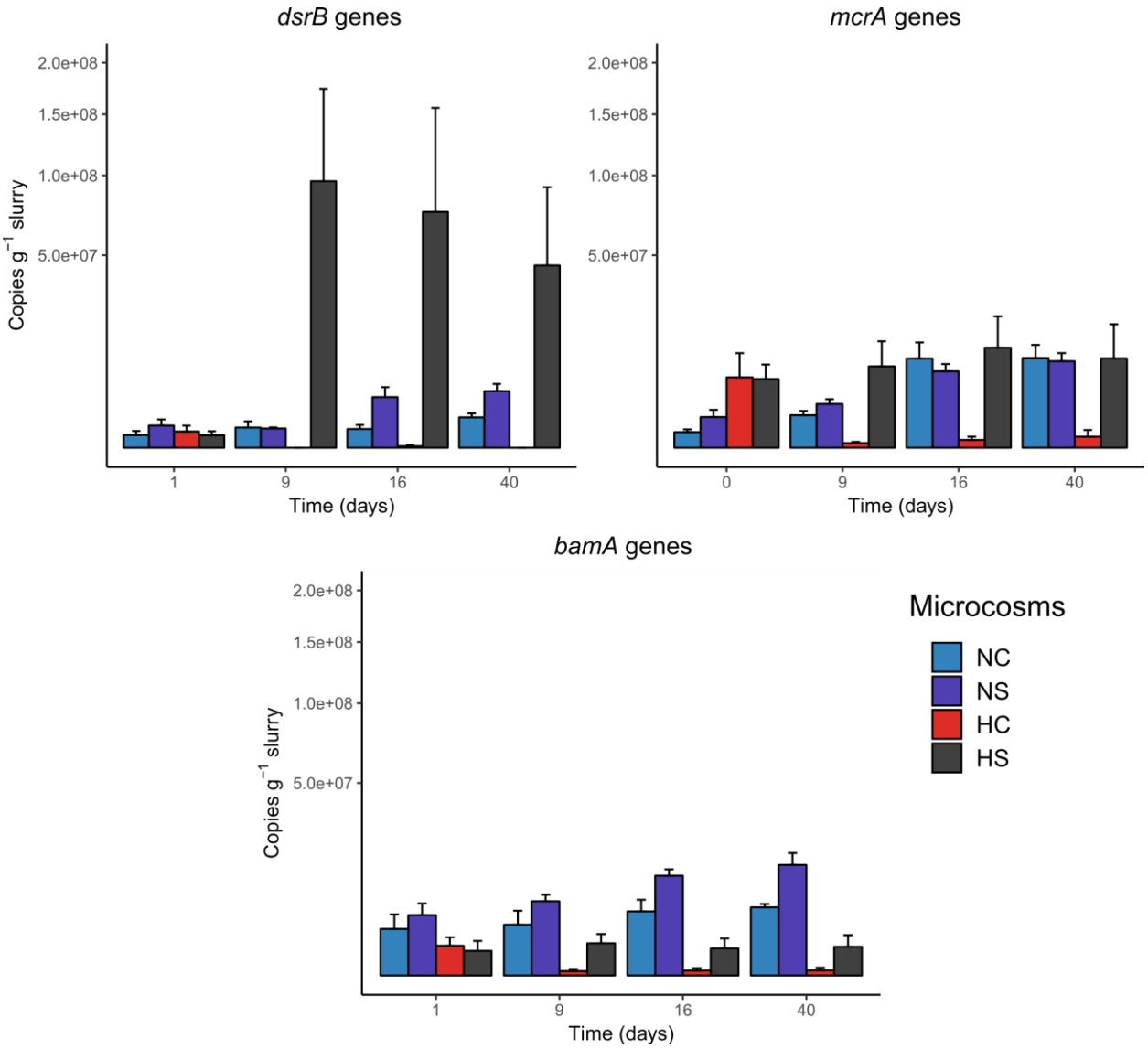

Figure 19: Copy numbers of selected genes per g of anoxic slurry: *dsrB* (dissimilatory sulfite reductase), *mcrA* (methyl-CoM reductase) and *bamA* (6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase). The numbers are presented in logarithmic scale. Error bars indicate the standard deviation of the measurements in the replicate microcosms.

4.9 Microbial activity in microcosms/slurries

The activity of the microbiomes was assessed by quantification of the transcripts of the same genes (Figure 20). Copy numbers of bacterial 16S, archaeal 16S and *mcrA* transcripts were significantly higher in the NC and NS microcosms, compared to HC and HS, at day 0 (p-values <0.028). The transcripts of bacterial 16S rRNA and *dsrB* genes significantly increased in the NS and HS microcosms (p-values <0.042), while archaeal 16S and *mcrA* gene transcripts increased in NC and NS (p-values <0.036). In the NC and NS microcosms, the numbers of the increased gene transcripts peaked at day 16, while in the HS microcosms, the increase in the number of bacterial 16S and *dsrB* transcripts was observed already at day 9. The numbers of these transcripts decreased again in all microcosms until day 40. Only transcripts of gene *mcrA* were measured in the HC microcosms, while no *bamA*, *bamB* and *ncr* transcripts were detected in any of the microcosm samples.

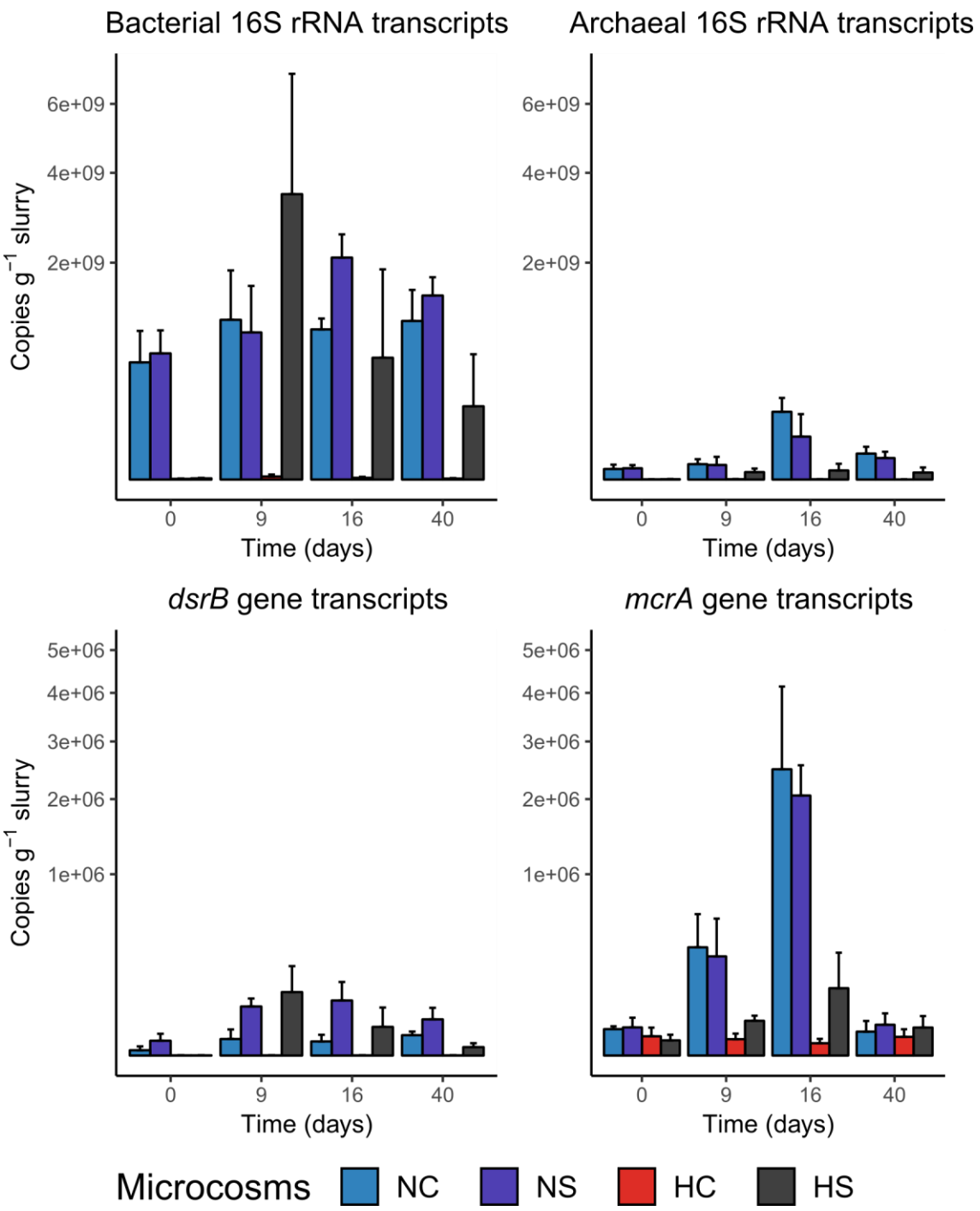

Figure 20: Copy numbers of gene transcripts per g of anoxic slurry: bacterial 16S rRNA, archaeal 16S rRNA, *dsrB* and *mcrA*. The values are presented in logarithmic scale. Error bars indicate the standard deviation of the measurements in the replicate microcosms.

5 Discussion

5.1 The Keri Lake ecosystem

The present study focused on how sediment microbiomes exposed to oil change after a long time period, in relation to their community structure and potential to degrade hydrocarbons in anoxic conditions. The experiments were conducted on oil-exposed sediment samples collected from Keri Lake, a fen environment used as a model for terrestrial land-to-sea transition ecosystems affected by natural oil springs in the order of millennia. The presence of an asphalt oil spring at one site but not in the whole area of Keri Lake allowed the direct comparison with another site at the same ecosystem which is not exposed to asphalt oil. The two studied sites of Keri Lake are in similar proximity to the sea and they are exposed to the same climate conditions and seasonal changes, including seawater surges and rainwater precipitations. Thus, we were able to ascribe the observed differences in the studied physicochemical parameters of the sediment (e.g. concentrations of electron acceptors) and the indigenous microbiomes between the two sites to the long-term presence of oil.

The reference site of our study (NE) had characteristics of a pristine wetland ecosystem. The organic material extracted from the samples was clearly of terrestrial origin; the high-carbon preference index and odd-numbered carbon *n*-alkanes detected in high abundance typically derive from waxes of higher plants (Eglinton et al. 1962; Reddy et al. 2000). The peat mainly originated from partially degraded residues of the reed *Phragmites*, as shown by the strong presence of *n*-tetracosane (Freese et al. 2008). This plant species is generally common in temperate wetland ecosystems (Clevering & Lissner 1999) and widely found in the area of Keri Lake throughout the whole year. The available electron acceptors were abundant only in the samples close to the sediment surface but became scarce in the deeper sediment, as has been observed in unpolluted soils and sediments (Vanderborght & Billen 1975; Sinke et al. 1992; Devereux et al. 1996; Jobbagy & Jackson 2001). Wetlands generally receive many nutrients from various sources, e.g. groundwater, freshwater and/or seawater environments or surface water, due to strong water dynamics. Nitrate and sulfate are generally expected to be sequentially consumed quickly by microbial transformations of the organic matter (Whitmire & Hamilton 2005), but here they seem to be continuously provided to the system of Keri Lake. These electron acceptors most likely provide the necessary reducing force for the active utilisation of the organic matter by anaerobic microbes.

The dominance of plant organic material confirmed that asphalt oil was not present in the sediment collected from the NE site, at least when the first sampling was conducted (October 2013). It is possible that oil compounds are transported from the HE site to other sites at Keri Lake when the ecosystem is flooded, but since our samplings were performed during the dry seasons of the year (May–October), we exclude the fact that our non-exposed samples contained asphalt oil in all conducted experiments.

The taxonomic profiles of the microbiomes of the NE site were also comparable to microbial communities studied in non-contaminated wetland ecosystems. Community structure was significantly affected by the sampled depth. Several prokaryotic orders common in oil pristine environments (e.g. *Actinomycetales*, *Planctomycetales*) had higher abundance at 40–60 cm depth. *Actinobacteria* and

Planctomycetes have been recognised amongst the most abundant phyla in surface soils from various ecosystems worldwide (Delgado-Baquerizo et al. 2018). Members of *Actinobacteria* have been characterised as key players of carbon cycle in diverse environments (Alam et al. 2011) and found repeatedly in non-contaminated soils (Nacke et al. 2011; Peng et al. 2015). *Planctomycetales* are able to catalyse the anaerobic ammonium oxidation (Anammox) and play an important role in the carbon and nitrogen cycles in water, soil and sediment ecosystems (Devol 2003; Tal et al. 2005; Buckley et al. 2006; Elshahed et al. 2007; Hawley et al. 2014b). Potential sulfate reducers were also stratified in the NE sediment (*Desulfobacterales* and *Desulfuromonadales*), following the decreasing concentration of sulfate, as has been previously observed in sediments (Devereux et al. 1996). As these orders decreased in the deeper samples, strictly anaerobic orders were more abundant (unclassified *Dehalococcoidia* and *Methanomicrobiales*), indicating a switch to more reduced conditions.

5.1.1 Sediment matter and microbiome structure due to the long-term exposure to oil

In comparison to the reference site, the site exposed to the asphalt oil (HE) showed several unique features. Sulfate was depleted in all depths, as hypothesized, due to the consumption by anaerobic hydrocarbon degraders. Nitrate accumulated in the sediment though. Since denitrification is thermodynamically more favourable than sulfate reduction and provides more energy to the hydrocarbon degraders (Thauer et al. 1977), nitrate was expected to be preferred as an electron acceptor and thus be consumed faster than sulfate. It seems likely that the asphalt oil restricts the input of nutrients in the sediment and inhibits microbial processes that remove nitrate, e.g. denitrification, or denitrifiers are not able to grow on asphalt oil as a carbon source, resulting in the accumulation of nitrate.

The presence of oil resulted also in a clear increase in the amount of organic material in the sediment, which is generally observed in oil polluted soils and sediments (Ekundayo et al. 2000; Ying et al. 2013). The characteristic signature of plant derived *n*-alkanes observed at the NE site was not apparent, despite the fact that partially-degraded plant material was also present at the HE sediment. Instead, a complex mixture of hydrocarbons was prevalent with strong signals obtained from hopanoid biomarkers. This profile was a clear imprint of the asphalt oil; its complex nature didn't allow us to completely resolve the identity of the extracted organic compounds. The detection of hopanoids was a clear indication for heavy alteration of the original composition of oil (Ourisson & Albrecht 1992). Hopanoids are formed from triterpenoid precursors of bacterial origin (Kannenberg & Poralla 1999) during oil generation and are amongst the most resistant compounds to biodegradation. Thus, they accumulate and persist in the oil. Their presence, together with previous results showing that Keri Lake oil has a relatively low ratio of aliphatic-to-aromatic constituents (Palacas et al. 1986; Pasadakis et al. 2016), confirm the heavy asphaltic nature of Keri Lake oil.

Differences with the microbiomes of the NE site were noticeable. The initial fingerprinting analysis showed that the structure of the bacterial communities was clearly affected by the exposure to oil

throughout the whole sediment column. The structure of the archaeal communities though was not clearly discriminated between the two sites. One possible reason for this is that a major part of the archaeal communities at the HE site consisted of methanogens. Since methanogens are able to use only a limited amount of electron donors (short-chain compounds, H_2, CO_2) provided by their syntrophic partners, the overall composition of the methanogenic community shouldn't be significantly affected by the composition of the organic matter. Shotgun metagenomic data clearly showed that the number of methanogens increased at the HE site though, but the T-RFLP analysis doesn't take into account the abundance of the studied community. On the other hand, Bacteria can directly use the oil compounds as carbon sources and their community structure is directly affected by the available organic matter. This is because there are different carbon sources available in the two studied sites. A clear impact of the oil on the overall structure of the metagenomes was observed because Bacteria comprises the major part of the studied microbiomes. Interestingly, the differences were more pronounced at the samples closer to the sediment surface. This can be reasonably attributed to the fact that concentrations of electron acceptors were especially different at this depth, since nitrate and sulfate are important factors shaping the structure and function of microbiomes.

5.1.2 Microbial diversity and functional potential due to the long-term exposure to oil

In more detail, the orders of *Actinomycetales* and *Planctomycetales* had lower abundances at the site affected by the asphalt oil spring compared to the reference site, similar to previous observations in marine methane and hydrocarbon seeps (Vigneron et al. 2018). Although the opposite has been also observed in some oil-contaminated systems (Abbasian et al. 2016a; Pugovkin et al. 2016), these orders rather not correlate to the presence of oil at Keri Lake. Additionally, less number of metagenomic reads were assigned to *Proteobacteria*, especially to *Deltaproteobacteria* (*Desulfobacterales* and *Desulfuromonadales*). Interestingly, this is in contrast to previous studies investigating the short-term impact of marine oil spills. *Proteobacteria* include many known anaerobic degraders of aromatic hydrocarbons and *n*-alkanes and have been observed to quickly increase in abundance shortly after the spills. Hazen et al. (2010) and Dubinsky et al. (2013) observed a higher abundance of *Gammaproteobacteria* in oil-contaminated seawater samples after the Deepwater Horizon blowout and Mason et al. (2014) and Kimes et al. (2013) suggested that the same accident enriched *Gamma-* and *Deltaproteobacteria* in the impacted sediments. In another oil spill example, three years after the Prestige oil spill, the abundance of *Deltaproteobacteria* decreased in impacted marine sediments (Acosta-González et al. 2013). This suggests that the detected hydrocarbon-degrading *Proteobacteria* are opportunistic species which respond quickly to sudden inputs of oil, while their numbers decrease when oil contamination persists for longer time periods.

The decrease in the total abundance of *Desulfobacterales* at the HE site indicates a lower potential for dissimilatory sulfate reduction, since this order includes many sulfate-reducing hydrocarbon-degrading species. This was confirmed by the decreased number of metagenomic reads assigned to the genes of the sulfate reduction pathway. Although *Desulfobacterales* was one of the most abundant orders in Keri

Lake samples, most of the reads of the *dsr* gene were not closely related to known Bacteria but remained taxonomically unclassified. Despite the taxonomy, all carriers of *dsr* genes had lower abundance at the HE site, compared to the NE site, which shows that all sulfate reducers were affected by the exposure to oil. The above-mentioned differences between the sites were most clear at depth 40–60 cm, where the difference in the concentration of sulfate were more prominent.

Following the observed depletion of sulfate, methanogenic Archaea clearly increased in the samples from the HE site, indicating that methanogenesis could be the main respiratory process. The abundant presence of methanogens was accompanied by a high abundance of the methanogenesis genes, especially gene *mcr*. This increase was mainly observed at 40–60 cm depth, mainly due to the high numbers of reads assigned to *Methanomicrobiales* and *Methanobacteriales*. These orders consist mainly of hydrogenotrophic species (Garcia et al. 2000), suggesting that CO_2 and/or formate are preferred over acetate as carbon source during methanogenesis. A few of *mcr* reads were also assigned to *Methanosarcina* and *Methanosaeta* at depth 40–60 cm at the HE site and coincided with the highest abundance of the gene *ack* of the acetate generating/degrading pathway. Since these methanogenic orders can utilise acetate (Kendall & Boone 2006; Ferry 2015), there is also some potential for acetoclastic methanogenesis as well, but lower compared to the hydrogenotrophic process. Additionally, a high number of reads was assigned to unclassified Archaea with unknown functions, thus the total role of Archaea in the Keri Lake ecosystem is currently not well understood. Both the abundance of the respiratory genes and the detected prokaryotic taxa, support the idea that the zone of transition from denitrification and sulfate reduction to methanogenesis was closer to the sediment surface at the HE site compared to the NE. Especially the availability of sulfate seems to be a critical environmental driver of the respiratory potential in our study, underlining again the importance of the availability of sulfate for the structure and function of sediment microbiomes.

The high abundance of the *mcr* gene could also mean that there is a potential of anaerobic oxidation of methane at the HE site. One of the proposed mechanisms for anaerobic degradation of methane involves reverse methanogenesis by Archaea (Hallam et al. 2004; McGlynn 2017) and methanotrophs of the *Methanomicrobiales* order have previously been detected in sediments close to a marine methane/oil seep (Hawley et al. 2014a). The anaerobic methane oxidation in the sulfate-depleted sediment could be possible by coupling to nitrate reduction (Raghoebarsing et al. 2006), since nitrate is still present at the HE site, although not many reads related to *Candidatus Methylomirabilis oxyfera* were detected at Keri Lake (Deutzmann et al. 2014).

Since methanogens utilise the metabolic products of their partners in syntrophic associations, fermenting Bacteria should be also present in the same samples. Indeed, the high abundance of methanogens coincided with the increase in abundance of other anaerobic prokaryotes that may potentially provide them with electron donors. Many metagenomic reads were assigned to *Dehalococcoidia* in the samples at depth 40–60 cm at the HE site, which were also abundant in the deeper samples from both sites at Keri Lake. Interestingly, the majority of *Dehalococcoidia* were assigned to one species, bacterium SCGC AB-539-J10. Unlike most known *Dehalococcoidia*, this species is missing genes encoding homologues of reductive dehalogenase enzymes (Wasmund et al. 2014),

indicating that there is no potential for the respiration of organohalides at Keri Lake. Bacterium SCGC AB-539-J10 is a strict anaerobe able to degrade various organic substrates to CO_2 and/or acetate, including aromatic compounds. Genes of the Wood-Ljungdahl pathway from *Dehalococcoidia* were also detected in the samples from Keri Lake, indicating a possible pathway for degradation of the organic matter. *Clostridiales* could also be important exploiters of the organic matter and interact with methanogens in Keri Lake sediments, especially in the samples from the deep layers, despite their abundance was not clearly higher at the HE site. Members of this order have been reported to anaerobically degrade aromatic compounds coupled to the reduction of all main electron acceptors (Kunapuli et al. 2007; Taubert et al. 2012; van der Zaan et al. 2012), which might be important if the concentrations of electron acceptors (nitrate, sulfate) in Keri Lake sediments change. *Clostridiales* also have the potential to generate acetate, which may be utilised by the methanogens. Additionally, *Bacteroidales* and *Syntrophobacterales* showed a slight enrichment at depth 40–60 cm at the HE site. *S*pecies of these orders have been previously detected in oil-contaminated sediments and oil-amended microcosms, respectively (Abbasian et al. 2016b; Gray et al. 2011). Although the involvement of *Bacteroidales* in the degradation of hydrocarbons is not clear, Syntrophobacterales have been suggested to degrade alkanes in the study by Gray et al. (2011). All the above-mentioned orders had higher abundances at the HE site, compared to the NE site, as observed at depth 40–60 cm. In the deeper samples, the abundances were similar between the two sites. It is also possible that these orders proliferate at the HE site because they take advantage of the highly reduced conditions, but they are not involved in syntrophic interaction with the methanogens.

In contrast, the genera of *Mesotoga* (order *Thermotogales*) and *Methyloceanibacter* (order *Rhizobiales*) were present only at depth 40–60 cm at the HE site, indicating that they are favoured by the increased methanogenic potential in this depth. The most abundant *Mesotoga* species detected at Keri Lake, *Mesotoga prima* and *Mesotoga infera*, have been previously enriched in anoxic microcosms and were shown to utilise sugars as carbon source and elemental sulfur as electron acceptor (Nesbø et al. 2012; Hania et al. 2013). On the contrary, *Methyloceanibacter caenitepidi* has been described as an aerobic, facultative methylotroph (Takeuchi et al. 2014). Coexistence of strictly aerobic and anaerobic species is frequently observed in metagenomes from several hydrocarbon-contaminated ecosystems (An et al. 2013). Although methanogenesis seems to prevail at the HE site and sulfate is not available, it is possible that sulfur is present and its reduction is also active at depth 40–60 cm, as has been also observed in other organic-rich sediments (Holmer and Kristensen 1994). Since natural microbiomes are complex communities, especially in soil and sediment environments, we expect that many processes are simultaneously active to some extent and dynamically alter based on the changes on the environmental conditions.

Despite the fact that Keri Lake is a surface terrestrial environment and more dynamic in terms of element cycles (N, S), the taxonomic composition of the studied metagenomes at the HE site resembles the microbiomes found in deep subsurface petroleum reservoirs. These ecosystems are saturated by hydrocarbons and depleted of electron acceptors (Head et al. 2003), where methanogenesis occurs in the absence of sulfate (Bonch-Osmolovskaya et al. 2003). The syntrophic methanogenic consortia are

considered responsible for the weathering of oil (Jones et al. 2008). Particularly interesting is the prevalence of hydrogenotrophic methanogenesis by CO_2 reduction (Head et al. 2014), as has been also suggested for the oil-exposed sediment of Keri Lake. Thermophilic representatives of *Clostridiales* and *Thermotogales* or phylogenetically related orders have been repeatedly isolated or enriched from reservoir production fluid samples and were related to hydrocarbon degradation in syntrophic interactions with methanogens (Orphan et al. 2000; Bonch-Osmolovskaya et al. 2003; Li et al. 2007; Pham et al. 2009; Head et al. 2014; Vigneron et al. 2017). *Bacteroidales* taxa have also been reported in such cultures (Orphan et al. 2000). In fact, a comparative analysis of oil reservoirs suggested the orders of *Clostridiales*, *Bacteroidales* and *Methanomicrobiales* as part of the core microbiome of subsurface petroleum reservoirs (Sierra-Garcia et al. 2017).

The high abundances of all the above strict anaerobes at depth 40–60 cm at the HE site strongly confirm that the zone of transition from denitrification and sulfate reduction to methanogenesis is elevated and conditions become anoxic even in the low depth sediment. Oxygen is a major driver of microbiome structure, since it can directly affect the survival and function of many microbial species (Imlay et al. 2003). Soils and sediments are complex systems containing many different niches that can be occupied by distinct microorganisms (Totsche et al. 2010), which means that at the same depth oxic and anoxic niches can coexist. This is probably the case at the NE site, but the high abundance of methanogenic Archaea already at depth 40–60 cm at the HE site is a very strong indication that the concentration of oxygen is completely depleted; methanogens have been detected in various environments and always under strict anoxic conditions (Issazadeh et al. 2013). Oxygen is quickly consumed in sediments after the contamination of oil by aerobic degradation of hydrocarbons (Genovese et al. 2014), while the thick asphalt coating covering the sediment surface doesn't allow the oxygen from air to penetrate and reach the deeper layers. Thus, the high organic load in the polluted sediments results quickly in anoxic conditions, especially in long-term contaminated environments like Keri Lake. Even at the non-exposed site though, the prevalence of anoxic conditions at depth 40–60 cm is also possible. Despite the absence of oil, the changes on the groundwater level can make conditions anoxic by restricting the penetration of oxygen in the deep sediment layers. The presence of partially decayed peat is a good indication that conditions are at least anoxic during some of the seasons at the NE site.

Under these conditions the anaerobic degradation of hydrocarbons is of relevance and the potential for hydrocarbon degradation by the indigenous microbiomes has been suggested as a bioremediation strategy in natural environments (Head & Swannell 1999). Thus, we focused on the anaerobic potential of microbiomes to degrade the available hydrocarbons. Our focused sampling in depth of up to 3 m ensured that anoxic sediment was collected from both sides.

Most of the genes coding for enzymes of the peripheral pathways of anaerobic hydrocarbon degradation were only detected in low abundance at both sites, which can be attributed to the low bioavailability of the individual hydrocarbons in the site-specific sediment matrix. This may suggest that only a small fraction of the microbiomes at both sites is able to utilise the available hydrocarbons and the intrinsic potential for the activation of many hydrocarbons is not higher at the oil-exposed site. One possible explanation is that the availability of the substrates of these enzymes is not higher in the oil-

exposed samples compared to the non-exposed ones, as expected due to the presence of oil. Most of the tested genes of the anaerobic hydrocarbon degradation pathways code for enzymes involved in the degradation of monoaromatics, which are expected to be available at the NE site as well, considering the detected terrestrial organic matter. Taking into account the asphaltic nature of the oil at Keri Lake, its composition has shifted during its migration from the source rock to the surface due to biodegradation and evaporation of low molecular weight hydrocarbons (Wenger et al. 2002; Thibodeaux et al. 2011) and the majority of the organic matter at the HE site consists of high molecular weight constituents. These compounds typically show very low water solubility and adsorb easily to sediment particles, a fact that reduces their bioavailability and increases their resistance to biodegradation. It is still possible that there might be unknown enzymes of unknown pathways with the capability to degrade these recalcitrant compounds. Considering the relatively recent discovery of most of the hydrocarbon degrading genes and the wide diversity of activation mechanisms under anoxic conditions, our knowledge about the full anaerobic potential of microbiomes is restricted. Many relevant enzymes are probably still missing from the public databases and, as a result, a significant part of the hydrocarbon degradation potential of the communities may have been hidden in the reads that could not be assigned to functions.

At the NE site, the abundances of many genes clearly increased in relation to increasing depth but varied less in the different depths at the HE site (Figure 21). This suggests more conserved metabolic capabilities throughout the samples at the HE site. Among the most abundant genes that showed this pattern in the samples from Keri Lake are the genes of the phenol degradation pathway, i.e. *pps*, *ppc*, *hcr/hba*, and the degradation of the central intermediate benzoyl-CoA. Lignin-derived phenolic compounds are expected to be abundant particularly at the deeper sediment layers at the NE site, due to the accumulation of peat, which is confirmed by the higher potential for their degradation in the deeper sediments. The presence of phenolics though is completely overprinted by the complex hydrocarbons of the asphaltic oil at the HE site. Considering that the depth-related pattern changes at this site, we assume that under these conditions the degradation of oil hydrocarbons and organic material in the deeper sediment layers is suppressed in the oil-exposed sediment.

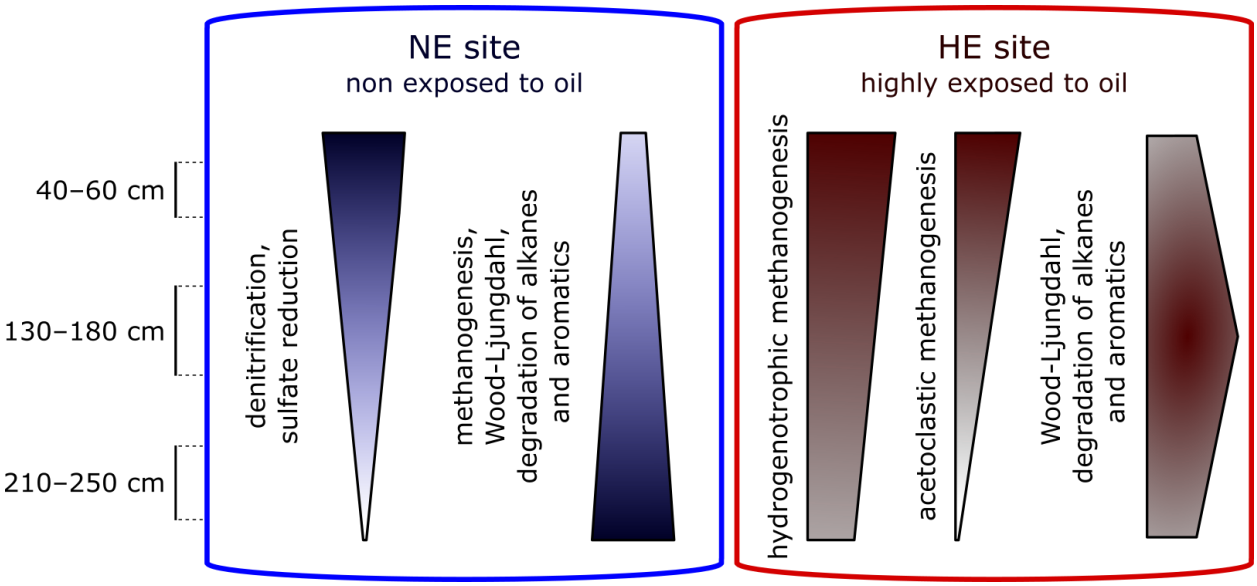

Figure 21. Patterns of the abundances of genes coding for enzymes involved in the anaerobic degradation of hydrocarbons at the two studied sites.

The connection of function to taxonomy allowed us to identify potential members of the microbial community that is involved in the degradation of hydrocarbons derived from the peat or the oil in the two sites at Keri Lake. Syntrophic anaerobic communities are complex and include the activity of primary and secondary fermenters in close interactions with methanogens, to fully convert the hydrocarbons to CH_4 and CO_2 (Schink 1997; Jiménez et al. 2016).

The most abundant carriers of gene *hcr/hba* were *Syntrophobacterales* and *Clostridiales*. The abundance of the gene *hcr/hba* of *Syntrophobacterales* showed the depth-related patterns described in Figure 21, suggesting that members of this order can be the main primary degraders of phenol and related compounds at the NE site. *Syntrophobacterales* were previously detected in slightly higher abundance at the HE site, but their *hcr/hba* genes were more abundant at the NE site, which lets us assume that members of this order are also involved in the degradation of other organic compounds at the HE site. The *Clostridiales* order, especially the family *Peptococcaceae*, possibly also contributes to the degradation of lignin-derived compounds at Keri Lake. Kato et al. (2015) proposed that *Peptococcaceae* are able to utilise degradation intermediates of vanillate in methanogenic cultures containing rice paddy field soil. The depth profile of genes of the degradation of other aromatic compounds were also affected as described in Figure 21 at the HE site, mainly reflected by the changes in the abundance of genes from candidate division *Zixibacteria*, *Clostridiales* and other unclassified Bacteria. Genes of the alkane and polyaromatic degradation pathways were also assigned to *Clostridiales* emphasizing their potential to utilise a wide range of organic substrates in both Keri Lake sediments.

Since hydrogenotrophic methanogens were detected throughout the sediment column at the HE site and in higher abundances compared to acetoclastic methanogens, more syntrophic interactions should involve CO_2-producing fermenters. Indeed, the genes of the Wood-Ljungdahl pathway didn't not show a clear change with increasing depth at the HE site, as observed for the first two genes of the TCA cycle (*cit/glt* and *acn*) and the genes of the acetate generating/degrading reactions (*pta* and *ack)*. This suggests that the reverse Wood-Ljungdahl pathway is the main mechanism of CO_2 production from alkanes and aromatics in all depths. *Dehalococcoidia* species have a high potential for CO_2 production via reverse Wood-Ljungdahl pathway, although no genes of the peripheral hydrocarbon degradation pathways were detected in the samples from Keri Lake. This doesn't exclude though the involvement of *Dehalococcoidia* to the complex oil-degrading community, as secondary fermenters of more simple compounds derived from the degradation of oil hydrocarbons. Last, *Desulfobacterales* and other *Deltaproteobacteria* were also identified as carriers of the genes *hcr/hba*, *bcr/bam/bad/bzd* and *acs/cdh*, which can indicate a possible switch of some deltaproteobacterial members from sulfate reduction to fermenting metabolism at the HE site.

Acetoclastic methanogenesis should be also possible to some extent at depth 40–60 cm at the HE site and could partly explain why the peaks of methanogenesis and hydrocarbon degrading pathways are not observed at the same depths (Figure 21). Interestingly, the abundance of the *ack* gene also peaked at depth 40–60 cm at the HE site, confirming that the potential for acetate production and use as an intermediate for interspecies transfer of electrons to the methanogens was reduced in the deeper samples. *Clostridiales*, *Bacteroidales* and *Thermotogales* were identified as the most abundant acetogens, potentially providing acetate to the acetoclastic methanogens.

5.1.3 Enrichment of sulfate-reducing hydrocarbon-degrading Bacteria

Taking into consideration the low abundance of prokaryotic species with the potential for sulfate reduction and the prevalence of methanogenic microbial communities at the oil-exposed site of Keri Lake, the next aim of our project was to test if the growth of the sparse sulfate reducers is possible in the presence of sulfate. To this end, sediment microbiomes from the HE site were transferred in anaerobic liquid cultures, containing media that allow the growth of freshwater mesophilic sulfate-reducing Bacteria. Hydrocarbon substrates were provided as carbon sources, representing two different groups of hydrocarbons, i.e. alkanes (*n*-decane) and monoaromatics (toluene), and a complex mixture of heavy-molecular-weight hydrocarbons (Keri Lake crude oil). The decrease in the concentration of sulfate in the cultures was monitored over time and the change in the structure of the bacterial communities compared to the inoculum was checked by amplicon metagenomic sequencing, when the added sulfate decreased below 1 mM in the medium.

The consumption of sulfate and correspondingly the growth of sulfate reducers delayed in the enrichment cultures, despite the fact that the anaerobic medium contained all the necessary nutrients for microbial growth. This long delay indicates that the sediment microbiomes might need a long time to adapt, due to the lack of sulfate in their natural environment. Based on the previous shotgun metagenomic analysis, which strongly supports the hypothesis that microbiomes at the oil-exposed sediments are characterized by syntrophic associations of hydrocarbon degraders and methanogens, this is not in line with our data. Since sulfate reduction is thermodynamically favoured over methanogenesis, sulfate reducers should be able to quickly outcompete the established methanogenic communities. Considering the decreased numbers of sulfate reducers at the HE site and the low amount of the inoculated sediment though, it is more possible that the initial inoculum added in the cultures was not enough to allow a rapid grow of the sulfate reducers to a level that the consumption of sulfate is noticeable. The rapid depletion of sulfate during the last five months of the experiment, suggests that once sulfate reducers grow to a certain threshold, they can consume the available substrates relatively fast.

The concentration of sulfate was additionally reduced in the sterilized culture 8, so it seems possible that autoclaving was not enough to completely sterilize this culture. Less amount of DNA was extracted from the sampled medium of culture 8 though and no sulfate reducers were detected as in the non-sterilised cultures. Thus, the decrease in the concentration of sulfate due to abiotic processes cannot be excluded in this case and most likely these processes contributed to the depletion of sulfate in the other cultures as well. Since a clear enrichment of typical sulfate-reducing Bacteria was observed in the other cultures though, we can conclude that sulfate was mainly depleted due to biotic sulfate reduction.

The selection of certain prokaryotic species was clear in the cultures. Many low-abundance taxa present in the oil-exposed sediment, comprising the rare biosphere, became scarce after 20 months of incubation with sulfate, due to the enrichment of hydrocarbon degraders. The sequencing results showed that sulfate reducing species were enriched in the presence of all three carbon substrates, suggesting that the microbiomes at the HE site of Keri Lake have the potential to degrade a wide range of hydrocarbons. Bacteria of the orders of *Desulfarculales*, *Desulfobacterales*, *Desulfovibrionales*, *Desulfuromonadales* and *Syntrophobacterales* were highly abundant in the cultures, compared to the oil-exposed sediment. All of these orders have been previously detected in oil polluted sediments (Acosta-González et al. 2013; Kimes et al. 2013) and their enrichment in our cultures suggests that they are involved in the degradation of the added hydrocarbons. Members of *Desulfarculales*, *Desulfobacterales* and *Desulfovibrionales* are able to grow in enrichment cultures with various electron donors (alkanes, aromatics or products of their biodegradation) by reducing sulfate (Aeckersberg et al. 1991; Rabus et al. 1993; Galushko et al. 1999; Heidelberg et al. 2004; Ommedal & Torsvik 2007; Sun et al. 2010; Suzuki et al. 2014). In contrast, the enriched *Desulfuromonadales* and *Syntrophobacterales* genera are generally not able to reduce sulfate (Widdel & Bak 1992; Liu et al. 1999) and most likely are primary fermenters in sulfate-reducing or methanogenic hydrocarbon-degrading cultures (Harmsen et al. 1993; Berlendis et al. 2010; Tan et al. 2014). The orders of *Desulfobacterales* and *Desulfuromonadales* were also the most abundant *Deltaproteobacteria* in the sediment samples from both sites of Keri Lake.

Their abundance was low at the HE site, but their enrichment in the sulfate reducing cultures indicates that their growth can be stimulated by external sulfate sources, e.g. seasonal seawater flooding events.

A preference of some sulfate-reducing species for specific carbon substrates was noticed. Different *Desulfurobacterales* genera were enriched with *n*-decane and toluene; *Desulfatitalea* species were the main potential degraders of *n*-decane, while the genus *Desulfobacterium* showed a preference for toluene. The growth of *Deulfobacterium* was not replicated though; ≈40% of the sequenced reads were assigned to *Desulfobacterium* in culture 4, while the same genus was undetected in culture 3. The enrichment of *Desulfovibrionales* was also observed mainly in the crude oil-containing cultures. *Desulfovibrio* sp. have been isolated from several oil sources (Magot et al. 1992; Tardy-Jacquenod et al. 1996; Feio et al. 2004), indicating that they are commonly present in environments exposed to complex hydrocarbon mixtures.

The enrichment of the prokaryotic orders was not complete; in the best case the numbers of reads assigned to sulfate reducers consisted ≈40% of the total datasets. Further enrichment of the sulfate reducers could be achieved by transferring the enriched communities into fresh anaerobic sulfate-containing media and given more time, at least in the cultures with *n*-decane or toluene. Taking into account the complexity of the crude oil substrate though, further enrichment would probably not be possible in the oil-containing cultures. Sherry et al. (2013) also observed only a modest enrichment of sulfate reducers in microcosms amended with crude oil and sulfate, due to the complexity of the hydrocarbon mixture.

The amount of DNA extracted from the sample of the autoclaved culture 8 was significantly lower compared to the DNA of the samples obtained from the other cultures. This is clearly the outcome of no microbial growth in this culture due to sterilization. The high similarity of the taxonomic profile of culture 8 to the non-template control 1 (extraction control) shows that the bacterial 16S rRNA genes sequenced originated from the amplification of contaminant DNA. The most abundant taxa detected were not apparent in the samples obtained from the sulfate-reducing cultures and the sediment from Keri Lake. The abundant Rhizobiales genera *Xanthobacter* and *Methylocystaceae* detected in both culture 8 and non-template control 1, are found in various soil and sediment environments (Bowman 2006, Wiegel 2006), including peat bogs and crude oil tank sludge (Hirano et al. 2004), suggesting possible cross-contamination of these samples from Keri Lake sediment during the DNA extraction steps. Non-template control samples 2 and 3 contained also genera that were not present in the other samples reflecting the contamination from the lab. *Corynebacterium*, *Dermabacter*, *Brevibacterium*, *Propionibacterium* and *Bacillus* species are typical residents of the skin (Chiller et al. 2001), which clearly shows the impact of contamination by skin microflora on the structure of these non-template controls. The absence of these orders from the other samples is a sign of good laboratory handling. Considering the high total number of PCR cycles during the preparation of the amplicon metagenomic libraries (30 cycles of gene amplification + 8 cycles incorporation of indices), it is not surprising that even low amounts of starting DNA produced an amplified metagenomic library.

5.1.4 Stimulation of microbial activities

Considering that the addition of sulfate successfully enriched sulfate reducers with the ability to degrade hydrocarbons in liquid cultures, their enrichment was attempted also in the sediments obtained from Keri Lake. Sulfate reducers are important degraders of oil hydrocarbons in highly reduced anoxic environments and a lot of potential for the bioremediation of the oil polluted sites is lost if they are not active. Thus, the next aim of our project was to enhance the growth of the sparse sulfate reducers by sulfate addition to the system of Keri Lake. The goal was to test if the enrichment of sulfate reducers will further result an increase in the expression of the hydrocarbon degrading genes in chronically contaminated ecosystems. Since conditions become anoxic quickly after oil contamination in the environment, there is a growing interest about the use of anaerobic degradation of oil contaminants for bioremediation of polluted sites by the indigenous microbiomes (Wolicka & Borkowski 2007).

One potential source of sulfate at Keri Lake is the neighboring marine environment of Laganas Bay. Seawater is generally rich in sulfate (Capone & Kiene 1988) and could therefore provide sulfate to the system of Keri Lake during the annual surface surges or intrusion in groundwater. For this reason, sulfate-containing saltwater media were prepared in order to mimic the possible seawater input into the sediments of Keri Lake. Sediments from both studied sites were mixed with the media resulting in anoxic microcosms with non-exposed and oil-exposed slurry material; control microcosms contained no sulfate. Our experiment aimed to investigate if the annual sea level changes have an impact on the degradation of the sediment organic matter by the indigenous microbiomes at both sites of Keri Lake.

At the beginning of the experiment the two slurries differed in the copy numbers of the different genes. While bacterial 16S genes had similar abundances, archaeal 16S was higher in the oil-exposed slurries compared to the non-exposed, which shows that the slurries differed in the total number of Archaea but not Bacteria. This is in accordance with our initial metagenomic study which suggested that the microbial communities in the sediment from the HE site harbours more methanogenic Archaea. Despite this difference, the activity of both Bacteria and Archaea was much lower in the oil-exposed slurries compared to non-exposed, based on the detected 16S rRNA transcripts. This confirms the negative impact of the asphalt oil on the activity of sediment microbial communities.

The addition of sulfate clearly promoted the growth of Bacteria in both slurries. Changes in the number of bacterial 16S rRNA genes were coincided with the observed decreases in the concentration of sulfate, proving that they are driven by the use of sulfate. Furthermore, the number of bacterial 16S rRNA and *dsrB* genes increased similarly compared to the control microcosms at the same time points, indicating that the observed increase of Bacteria was attributed to the growth of dissimilatory sulfate reducers. In the NS microcosms, increased numbers of these genes were observed at days 16 and 40, while in the HS microcosms they increased on day 9 and gradually decreased till the end of the experiment. The same changes were observed also in the number of transcripts, with the exception of the transcription of *dsrB* gene already on day 9 in the NS microcosms.

The consumption of sulfate in the microcosms was relatively fast and without a long lag phase, relative to previous studies (Townsend et al. 2003). Sulfate-reducing microbes have generally low growth rates, due to the limited amount of energy offered by dissimilatory reduction of sulfate to sulfide (Thauer et al. 1977). The decrease in sulfate concentration was faster compared to the decrease observed in the liquid enrichment cultures too, despite the fact that extra nutrients, like vitamins, were excluded from the saltwater media. Two weeks after the beginning of the experiment, sulfate was already depleted in all slurries, which suggests that Keri Lake sediment contains elements and nutrients missing from the media that allow the rapid growth of sulfate reducers.

No sulfate was detected in the samples from the control microcosms throughout the whole experiment, although a slightly higher concentration of sulfate was expected in the non-exposed slurries at day 0. The NE site contains more sulfate than the HE site at Keri Lake, according to our first experiment, but this was not reflected on the microcosm's samples. It seems that either the difference in the sulfate concentrations between the two sediments was very low that couldn't be detected by the barium-gelatin assay or sulfate was completely consumed after sampling until the preparation of the microcosms, since the samples were transferred to the lab under anoxic conditions. In any case, the complete absence of sulfate from the NC and HC microcosms supports their use as controls for the scope of our experiment.

Despite the equal amount of sulfate added, a fastest and greater growth of sulfate-reducing Bacteria was observed in the HS microcosms, compared to NS. This suggests that in the prevalence of methanogenic conditions and high organic load in the HE sediment, opportunistic sulfate reducers respond quickly and use up the whole amount of sulfate, due to the thermodynamic advantage of the dissimilatory sulfate reduction over methanogenesis. Indeed, the sudden drop in sulfate concentration resulted in a strong sulfide odor in these microcosms, which was not apparent in any of the other microcosms. Despite the low abundance of sulfate reducers at the HE site, it is possible that some sulfate reducers are active in syntrophic associations with methanogens even when sulfate is absent. Fermentative behaviour of sulfate-reducing Bacteria has been previously observed in sulfate-free co-cultures with methanogenic Archaea (Bryant et al. 1977; Meckenstock 1999). Thus, they can respond fast by shifting from fermentation to the more favourable dissimilatory sulfate reduction, as has been previously discussed (Oude Elferink et al. 1994). In contrast, the advantage of the dissimilatory sulfate reducers is lost in the NS microcosms and they cannot outcompete the rest of the microbial community of the NE sediment. Thus, part of the sulfate is possibly used up by other prokaryotes for different processes, e.g. assimilatory sulfate reduction, in this case.

The fast consumption of sulfate, as well as the increase of *dsrB* genes and transcripts in the HS microcosms, show that sulfate-reducing Bacteria have the potential to utilise the available oil hydrocarbons in the oil-exposed sediment. Vigneron et al. (2017) also observed a quick growth of sulfate reducers among other opportunists in an oil reservoir after the addition of sulfate. Their growth at the HE site at Keri Lake though is restricted by the absence of sulfate; when sulfate was not added in the HC microcosms, sulfate reducers didn't have the opportunity to grow.

No increase of *mcrA* genes and transcripts was observed after the addition of sulfate meaning that the quick growth of sulfate reducers disrupted the syntrophic interactions in the HS microcosms. Salloum et al. (2002) also detected no methanogenic activity after sulfate addition in microcosms containing mature fine tailings sediment. Only a slight increase in the activity of methanogens was observed in the HS microcosms after the complete depletion of sulfate at day 16. These suggest that the presence of recalcitrant oil compounds at the HE sediment makes dissimilatory sulfate reducers compete with methanogens for the use of more easily degradable compounds, like H_2 and acetate. As a result, methanogens are outcompeted due to the low energy yield of methanogenesis (Schink 1997) and their growth is inhibited. Interestingly, this doesn't seem to be the case in the NS microcosms; the copy number of *mcrA* genes followed the increase of *dsrB* gene after the addition of sulfate. Methanogens increased in all non-exposed to oil slurries independent of the sulfate treatment, which means that sulfate did not inhibit the growth and activity of methanogens or at least only a minor inhibition was observed. Previous studies have observed that sulfate reducers and methanogens can coexist in the same environment (Holmer & Kristensen 1994; Bonch-Osmolovskaya et al. 2003) and that sulfate reducers outcompete methanogens in the presence of hydrogen and/or acetate, but they do not compete with them for methanol, trimethylamine or formate (Oremland & Polcin 1982). These suggest that methanogens and sulfate reducers do not compete for the same resources (H_2, acetate) in the non-exposed slurries, but sulfate reducers rather prefer to oxidise different components of the available organic matter.

Despite the decrease in the activity of sulfate reducers after the complete consumption of sulfate, the number and activity of methanogens was not favoured at the end of the experiment (day 40) in the sulfate-treated microcosms. This was especially noticeable in the HS microcosms and can be probably attributed to the accumulation of the products of dissimilatory sulfate reduction, since high concentrations of sulfite and sulfide have an inhibitory effect on methanogenesis (Balderston & Payne 1976; Karhadkar et al. 1987).

The activity of methanogens did not change also in the HC microcosms throughout the experiment, although the oil-exposed sediment is expected to carry a methanogenic microbial community. In fact, only transcripts of the *mcrA* gene were detected in these microcosms, while any other activity, as well as the abundance of both Bacteria and Archaea, significantly decreased. This indicates that the changes in the redox conditions, due to the addition of the anaerobic medium, together with the absence of sulfate inhibited the growth of the indigenous microbial communities.

The detection of *bamA* genes in the microcosms indicates that degraders of monoaromatic compounds are present in the samples, though not highly abundant. The consumption of sulfate and the growth of sulfate reducers was correlated to an increase in the potential for the degradation of monoaromatics only in the NS microcosms. This is in line with our suggestion that the growth of dissimilatory sulfate reducers with the potential to degrade hydrocarbons, at least monoaromatics, is favoured in the non-exposed to oil slurries, while in the oil-exposed slurries the enriched dissimilatory sulfate reducers utilise H_2 and/or short-chain compounds.

Our attempts to detect and successfully quantify the genes *bamB* and *ncr* in the slurries failed. The detection of gene *bamA* indicates that *bamB* should be present in the slurries as well, since the enzymatic products of both genes are involved in the same metabolic pathway, i.e. the degradation of benzoyl-CoA. This might be a bias of the chosen primer pair used for the quantification of this gene. It is possible that the coverage of the primers might be low and hinders our ability to detect and quantify the complete range of benzoyl-CoA degraders in the slurries. Gene *ncr* was not detected in any of the slurries as well, but this can be attributed to the absence of naphthalene and derivative compounds in the sediments from Keri Lake or the delayed response of naphthalene degraders. Townsend et al. (2003) showed that even in the absence of other preferred hydrocarbons, the degradation of naphthalene requires long incubation times.

5.2 Comparison to other oil-impacted ecosystems

The comparison of the two sites at Keri Lake allowed us to investigate the impact of oil on the indigenous sediment microbiomes. By comparing with other published studies, we were also able to extract some conclusions about how the history of oil exposure influences the structure and function of the microbiomes. Unfortunately, there is no short-term oil-contaminated site at Keri Lake that would allow us to directly compare long-term and short-term exposed to oil sites and investigate the sediment microbiomes in different stages of their evolvement under these conditions. Thus, in order to study the long-term impact of oil and generalise our results to other ecosystems, we performed a comparative metagenomic analysis with datasets from other ecosystems with different histories of oil exposure. The grouping into stabilised and perturbed ecosystems facilitated the distinction between these environments. In the stabilised ecosystems, microbiomes have fully established and gradually evolved in the conditions of oil exposure over a long chronological scale, while the relatively recent oil contamination in the perturbed ecosystems hasn't allowed the indigenous microbiomes to recover completely from the oil disturbance.

5.2.1 Stabilised versus perturbed ecosystems

The taxonomic profiles of the microbiomes of the stabilised ecosystems had similar compositions, including strict anaerobic Bacteria and methanogenic Archaea. All tested methanogens were especially abundant in these environments, which is in line to our hypothesis that methanogenic consortia are established in sediments exposed to oil after long exposure to oil. Methanogens have been already identified as key players in the anaerobic hydrocarbon degradation at the Pitch Lake and the oil sands tailings ponds (An et al. 2013; Meckenstock et al. 2014). The process of methanogenesis is favoured when inorganic electron acceptors (e.g. nitrate and sulfate) are depleted or their bioavailability is decreased, as typically observed in oil reservoirs, coal deposits, groundwater aquifers and deep sediments. Members of *Anaerolineales*, *Dehalococcoidia*, *Synergistales*, *Syntrophobacterales*, *Thermoanaerobacterales*, *Thermoplasmatales* and *Thermotogales* were among the Bacteria which are potentially associated with the methanogens. *Clostridiales*, particularly *Peptococcaceae*, were detected in high abundance in most datasets, supporting their role as primary or secondary fermenters in various conditions (Gieg et al. 2014). The rest of the bacterial orders had high abundances in specific environments and were not correlated to the group, suggesting that their presence is affected by environmental factors other than the oil. In total, the structure of the sediment microbiomes obtained from the HE site at Keri Lake (particularly sample C7) showed greater similarity with the profile of the microbiome of the contaminated water sample from the Pitch Lake, despite the fact that different material was sampled. This underlines the resemblance of these two ecosystems, in terms of the natural occurrence of the oil, its asphaltic composition and the history of exposure.

Following the structure of the communities, the abundances of the genes of the respiratory pathways confirmed that microbiomes in the stabilised ecosystems have a high potential for methanogenesis

compared to the perturbed. Oil sands tailings ponds samples showed relatively high potential for denitrification and sulfate reduction as well, which can be explained by the several sources of tailing microbiota (initial ore microbiota, process water, airborne propagules) and the continuous input of external material in the ponds, resulting in complex microbial communities. The potential for dissimilatory sulfate reduction was also relatively high in the oil-contaminated samples of Gulf of Mexico, which is the only included ecosystem with a continuous availability of sulfate diluted in seawater.

Many of the peripheral genes of hydrocarbon degradation had slightly higher abundances in the stabilised metagenomes, but overall they didn't show big differences in their abundance between the different environments. As a result, we can conclude that the potential for the degradation of hydrocarbons is not strongly related to the history of oil exposure. In most cases, a site-specific effect was noticed, meaning that gene abundances were similar in samples originated from the same ecosystem. Considering that the composition of oil from different sources can greatly vary (Pasadakis et al. 2016), we believe that the abundances of the genes are affected by the available organic material in each site. Vigneron et al. (2018) also suggested that the composition of hydrocarbons is a critical factor that shapes the functional potential of microbial communities in marine hydrocarbon seeps.

5.3 Methodological issues

Our initial approach to assess the long-term impact of oil exposure to the sediment microbiomes was based on the investigation of the microbiome potential by cultivation independent methods. While T-RFLP revealed the fingerprints of the structure of the microbial communities, shotgun sequencing provided detailed characterisation of the microbiome potential in the selected samples. The sequenced reads were phylogenetically annotated against the NCBI non-redundant protein database and the genes of interest were quantified by comparison to manually compiled protein databases. In order to extrapolate our observations to other oil-impacted environments, the shotgun metagenomic datasets from the Keri Lake samples were compared to publicly available datasets by parallel analysis using the pipeline of the MG-RAST server. Additional sequencing of amplified 16S rRNA gene libraries from sulfate-containing anoxic cultures, allowed us to detect the enriched hydrocarbon-degrading Bacteria taxa with the ability to utilise selected hydrocarbon substrates, including heavy oil from Keri Lake. Last, the microbiome potential for specific processes in sulfate-treated microcosms/slurries, as well as the actual microbial activity, were determined by quantification of representative genes and their respective transcripts with qPCR.

5.3.1 Data reproducibility

Our initial metagenomic study at Keri Lake started with the collection of sediment samples from various depths, in order to examine the depth variability of the microbiomes and get a complete picture about the impact of oil in the sediment column. The number of drilling cores we could collect (especially cores located next to each other) and the sediment depths we could sample was limited though. Human activities are restricted in Keri Lake due to protective regulations that apply in the area of Laganas Bay; the whole area is a Natura 2000 site mainly (but not only) due to the presence of nesting sites of the endangered loggerhead sea turtle (*Caretta caretta*).The second coring event allowed us to replicate our samples, but more sampling points would be necessary for a statistical interpretation of the results.

5.3.2 Metagenome construction and sequenced libraries

The presence of compounds in the sediment that can inhibit enzymatic reactions, including humic substances and the asphalt oil at the HE site, can challenge the use of the extracted nucleic acids for PCR-based techniques. Thus, sufficient purity of the final products is required. The choice of the organic-based extraction protocols used for DNA isolation and DNA/RNA coextraction from our samples, developed by Lueders et al. (2004) and Griffiths et al. (2000), respectively, optimised the extraction yield and purity of nucleic acids. In some cases, the use of an additional purification step was necessary to ensure high-quality products for the preparation and sequencing of the metagenomic libraries.

Following the preparation of libraries, the number of sequenced reads by the MiSeq platform was adjusted in the different experiments, depending on the scope of each application. Over 150 thousands reads were sequenced from the amplicon libraries, but the total number of reads obtained after quality control was less than ten thousands. The rarefaction analysis clearly revealed that this sequencing depth was sufficient to investigate the majority of the diversity of the enriched microbial communities (Schöler et al. 2017), even for the highly diverse sediment sample. For shotgun metagenomic sequencing though, more sequencing effort is always necessary to describe the full potential of the microbiomes (Vestergaard et al. 2017), so our shotgun libraries were sequenced to a deeper level. More than two million reads were obtained for the deep sequenced replicates, while the number of reads sequenced from the the replicates ranged between 1–2 millions; the number of reads that passed the quality trimming was lower. This approach was followed in order to secure sufficient sequencing depth for at least one replicate and ensure that low abundance genes will be detected, while the other replicates were used to test for the heterogeneity of the results. Rarefaction analysis confirmed that the sequencing efforts were sufficient to confirm the full coverage of the genes we are interested in. Many of the interrogated pathways, such as the respiratory pathways, were expected to be highly abundant in the sediment samples. The genes of the anaerobic degradation pathways are part of the genomes of restricted microbes though, thus deeper sequencing would probably reveal more significant differences between the samples. The use of the MiSeq platform was preferred because it provides longer read length, compared to other platforms. Longer read lengths improve the accuracy of the annotations (Singer et al. 2016) and the discrimination of the genes of interest, especially when parts of the genes have close similarities, which was of greater significance for the scope of our study.

5.3.3 Bioinformatics

The bioinformatic pipelines used for the analysis of the sequenced datasets are well-established and has been previously evaluated. For the amplicon sequencing datasets, the open-source software pipeline Qiime was used (Caporaso et al. 2010). Qiime 2 has replaced the clustering of reads into operational taxonomic units (OTUs) with amplicon sequence variants (ASVs), offering higher-accuracy diversity measurement among other advantages (Callahan et al. 2017). For the shotgun metagenomic datasets, the taxonomic and functional annotations were performed with the DIAMOND program by alignments to protein databases, in order to ensure that degenerative sequences are detected in the datasets. DIAMOND determines all significant alignments of a given nucleotide query to the protein database, similar to BLASTX, but is optimised for the analysis of high-throughput sequencing data of short reads in order to increase the process speed without a loss in sensitivity (Buchfink et al. 2015). Functional annotations were performed for all the known genes coding for enzymes involved in the complete anaerobic degradation of hydrocarbons to CO_2 and the linked respiratory pathways. Since many of the genes of the hydrocarbon degradation pathways have been recently discovered and are thus not found in the commonly used databases, custom databases had to be manually compiled. In an attempt to quantify all genes with the same protocol/approach and produce comparable results, individual

databases were created for each of the 80 genes of interest. The comparison of gene abundances and the reconstruction of nutrient cycles, which was the scope of our study, requires relatively low sequencing depth (Vestergaard et al. 2017). The quality of annotations could improve by assembling draft genomes, but this requires the production of more sequencing data, especially in the case of high diversity sediment/soil samples, thus was not possible in our case.

Last, the metagenomic comparison offers a significant insight into how the history of oil exposure shapes the natural microbiomes but has to be faced with caution. When comparing metagenomic datasets from separate studies, bias due to the differences in the studies could arise. The available datasets selected for the needs of our questions had many differences in the DNA isolation, library preparation and sequencing technology, which can introduce a significant bias to the resulted comparison (Delmont et al. 2013). However, the fact that the different metagenomes cluster depending on the history of oil exposure clearly argues that pronounced changes in the microbial communities are still visible, despite the above-mentioned bias. Furthermore, complete metadata are not available for these metagenomes and only the metagenomes from the Gulf of Mexico have been published (Kimes et al. 2013), which hinders our ability to directly relate our results to specific factors, e.g. the concentration of sulfate. The low sequencing depth and the absence of replication of the publicly available metagenomes still make us question the sufficiency of the coverage of the metagenomic datasets for the quantification of the genes of interest and the reproducibility of the results. Unfortunately, the limited availability of shotgun metagenomic datasets from oil impacted environments, especially ecosystems with long-term and persistent oil presence and natural oil seeps, restricts our analysis. An attempt to increase the number of deep sequenced available metagenomes is needed for more comprehensive future comparisons.

6 Conclusions and Outlook

The natural asphalt oil seeps found at the ecosystem of Keri Lake offer a unique opportunity to study sediment microbiomes in a terrestrial coastal ecosystem, which are exposed to asphalt oil for at least 2500 years. Our study sets the basis for investigating the ecological role of these microbiomes, helps us predict the fate of microbial communities in long-term oil-contaminated environments and highlights some knowledge gaps for future research.

Our results gave us a clear insight on the evolution of the sediment microbiomes at the oil-exposed site at Keri Lake. Sulfate was completely consumed at the oil-exposed site, most probably used for the conservation of energy provided by the oxidation of oil hydrocarbons, while nitrate was unexpectedly accumulated. Despite that, the initial metagenomic analysis confirmed that the potential for both denitrification and sulfate reduction was lower at the oil-exposed site. In fact, it revealed a shift in the structure and functional potential of the microbiomes towards syntrophic consortia of fermenting Bacteria and methanogenic Archaea, emphasizing the complexity of the evolved microbiomes. In the extreme conditions of dominant presence of carbon compounds, microbiomes rely on organic compounds for both the production and conservation of energy. Especially the heavy-molecular-weight oil hydrocarbons most likely don't get completely oxidised to CO_2 by individual microbiome members, but rather converted to products that can be further oxidised. Only a few members exploit the asphalt oil hydrocarbons, which are generally resistant to biodegradation, while a big part of the community takes advantage and scavenges the metabolic by-products of the primary fermenters. The high amounts of energy stored in the chemical bonds of hydrocarbons, especially the high-molecular-weight molecules, can sustain several levels of primary and secondary fermenters and scavengers of by-products.

Our observations on the structure of the indigenous microbiomes were based on DNA sequencing, which allowed us to investigate the general potential of the microbial communities, but doesn't provide information about the activity of the microbiomes. A metatranscriptomic and/or metaproteomic approach will shed light on the processes that are active *in situ* at the two studied sites of Keri Lake.

The cross-study comparison of metagenomic datasets extended our main observations of the microbiome potential at Keri Lake to other ecosystems with chronic oil exposure. The presence of methanogens was favoured in all stabilised environments, which harbour well-established microbiomes to the presence of oil, indicating that they can be assumed as part of the core microbiome of asphalt oil seeps. This further supports the use of Keri Lake as a model ecosystem for the study of terrestrial sediment microbiomes exposed to oil over long time periods.

Despite the fact that stabilised oil-impacted ecosystems show similar respiratory potential, differences on the hydrocarbonoclastic members of the microbiomes and the abundances of the genes of the hydrocarbon degradation pathways are noticeable. While complete sets of metadata are not available for the other metagenomic datasets used for the purpose of this study, differences in the organic material and the oil hydrocarbon composition at these environments can be assumed. At Keri Lake, the

presence of peat-derived phenolic compounds and oil with abundant aromatics in the sediment resulted in a high potential for the utilisation of monoaromatic compounds. Additional experiments with microbiomes that have been chronically exposed to different hydrocarbon mixtures, including oil mixtures with a different degree of biodegradation, are necessary in order to investigate how the different electron donors shape the structure and the functional potential of the microbiomes and explain the variation between these datasets.

Since sulfate is depleted at the oil-exposed sediment but can be supplied by the seawater surges at Keri Lake, our last experiment attempted to examine the possible impact of flooding on the structure and function of sediment microbiomes at Keri Lake. The quantification of the above-mentioned genes and their transcripts in the anoxic slurries gave us a valuable insight about the response of the sediment microbiomes to the addition of sulfate, but raised several new questions at the same time. The results showed that when sulfate is externally provided, sulfate reducers grow quickly and their dissimilatory reduction activity increases in the sediment. It is suggested that in the presence of asphalt oil hydrocarbons, sulfate is almost exclusively consumed by sulfate reducers and used up for the oxidation of less complex compounds, which also comprise the preferred electron donors of methanogens. The clear competition between sulfate reducers and methanogens leads to limited methanogenic activity, due to their energetic advantage of the former. The preference for less complex compounds means that sulfate reducers do not degrade the natural organic matter and the degradation of hydrocarbons is not enhanced, which is in contrast to our original hypothesis. Seems reasonable that once the electron acceptors are bioavailable, the oil-exposed microbiomes will first turn towards more readily biodegradable compounds instead of the complex high-molecular-weight hydrocarbons of the oil, compared to the non-exposed ones. Further analysis of the structure and the function of the microbiomes is needed to confirm our new hypotheses. Analysis of the taxonomic profiles of the microbial communities by amplicon metagenomic sequencing will test the idea that distinct sulfate-reducing taxa grew in the NE and HE slurries, as a result of the differences in the preferred substrates for dissimilatory sulfate reduction or show that the different activity originates from the same taxa. Metatranscriptomic and/or metaproteomic analysis may also test the competition of sulfate reducers and methanogens for the same substrates in the HS microcosms, detect more differences in the functions of the microbiomes that are related to the degradation of hydrocarbons and possibly even help us identify new genes and enzymes of novel degradation pathways.

Considering the well-known ability of sulfate reducers to degrade various hydrocarbons, we assume that after the depletion of the easily biodegradable substrates, they will turn towards the oil hydrocarbons, as long as sulfate is still provided. This would mean that the “long-term” absence of inorganic electron acceptors, in “long-term” oil-exposed environments, requires the “long-term” input of electron acceptors for the stimulation of the degradation of recalcitrant compounds. Experiments with a continuous supply of sulfate in oil-exposed microbiomes would confirm or reject this hypothesis at Keri Lake and assist the remediation of other chronically polluted sites.

Overall, our study highlights the impact of oil contamination to the degradation of the organic material in natural ecosystems and the degradation of the available organic matter in the oil-exposed sediment

of Keri Lake. Based on the potential for the anaerobic degradation of the organic matter in the Keri Lake sediments, the degradation of the lignin-derived phenolic compounds is expected to be suppressed at the HE site due to the presence of asphalt oil, especially in the deeper sediment layers where conditions are possibly anoxic. Bargiela et al. (2015) also found that the active microbial metabolism in chronically polluted marine sediments is not directly related to the degradation of oil compounds and concluded that the biodegradation of hydrocarbons is significantly suppressed. These results underline the impact of the chronic asphalt oil exposure to the function of sediment microbiomes and shows how microbiomes are structured under these extreme conditions.

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List of abbreviations

ASVs	amplicon sequence variants
bp	base pairs
BSA	bovine serum albumin
BTEX	benzene, toluene, ethylbenzene, xylenes
CI	chloroform/isoamylalcohol
CTAB	hexadecyltrimethylammoniumbromide
cult	culture
DEPC-H_2O	distilled water treated with DEPC
EOM	extractable organic matter
6-FAM	6-carboxyfluorescein
H_4MPT	tetrahydromethanopterin
HE site	highly-exposed site
MF	methanofuran
NE site	non-exposed to oil control site
NMDS	non-metric multidimensional scaling
ntc	non-template control
OTUs	operational taxonomic units
PAHs	polyaromatic hydrocarbons
PCI	phenol/chloroform/isoamylalcohol
PEG	polyethylene glycol
qPCR	quantitative PCR
TCA cycle	tricarboxylic acid cycle or citric acid cycle
THF	tetrahydrofolate
T-RFLP	Terminal Restriction Fragment Length Polymorphism

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Consent for publication

The genetic information provided in this study may be considered to be part of the genetic patrimony of Greece. Users of this information agree to (1) acknowledge Greece as the country of origin in any country where the genetic information is presented and (2) contact the CBD website (http://www.cbd.int/information/nfp.shtml) if they intend to use the genetic information for commercial purposes.

Appendix

Appendix Table 1. UniprotKB entries contained in the protein databases compiled for the functional annotation of the shotgun metagenomic reads.

Gene name	Enzyme name	UniprotKB Entries
Denitrification		
narGHI/napAB	nitrate reductase	I3R9M8–9, Q5NYZ4–5, Q5NYZ7, Q53176–7, Q89EN4–5, Q92Z36–7, P39185, P42175–7, P9WJQ3, O06560, O06562, Q39YV1–2, Q39YU9, Q39WW0–2, C0QE69, C0QE71–2
nirS/nirK	nitrite reductase	D0RAY2, Q5P7W3, Q53239, Q89EJ6, Q92Z29, Q0JYW5
norBC	nitric oxide reductase	I3R6L7, Q5P8Y4–5, A4WR58, O06844, H7C7W3, Q89QB4, Q92Z15–6, Q7WX97, Q0JYR9, Q39PX6
nosZ	nitrous oxide reductase	I3R9L6, Q5NZ01, A4WXT8, Q89XJ6, Q59746, Q59105
Sulfate reduction		
aprAB	adenylylsulfate reductase	Q59115–6, Q72DT2–3, K0ND09, K0NCP7, F6B3Q3–4
dsrAB	dissimilatory sulfite reductase	Q59109–10, P45574–5, K0ND70, K0NCW7, F6B660–1
sat	sulfate adenylyltransferase	O28606, Q72CI8, K0N4I8, F6B3Q5
Methanogenesis		
ftr	formylmethanofuran-tetrahydromethanopterin formyltransferase	P55301, Q49610, D9PYC7, Q57766, Q49168
fwdABCD/fmdBC	formylmethanofuran dehydrogenase	Q46CZ0, Q46CY7–8, Q48943, D9PU53–4, D9PU56, Q59579, D9PXE7, P61937, Q58569, Q58571, P61154, Q8TV68–9, Q8TV71, Q8TYN5, Q1MVQ4, E3GW09–2
mch	methenyltetrahydromethanopterin cyclohydrolase	P94954, P51616, P94919, Q59030, E3GXM5
mcrABG	methyl-CoM reductase	P07962, P07955, P07964, Q49605, F1SVF3, Q8TXL0, P11558, P11560, P11562, P58815, D9PXZ6, P58816, Q58256, Q58252, Q58255, Q60391, Q60390, Q60387, Q49174, Q49171, Q49173
mer	5,10-methylenetetrahydromethanopterin reductase	Q46FV4, Q8TXY4, Q50744, Q58929, E3GXP5
mtd	methylenetetrahydromethanopterin	Q46DH9, P55300, Q58441, P94951, E3GZ78

	dehydrogenase	
mtrABCDEFGH	tetrahydromethanopterin S-methyltransferase	Q9Y8K0–6, O93716, O32864–9, Q49606, Q8TVA7, Q59584, P80183–6, Q50773–4, P80187, Q58257–64, E3GZ43–50
Anaerobic degradation of alkanes and aromatics		
abcAD	anaerobic benzene carboxylase	D8WWP7–8
ald/aor6	benzaldehyde dehydrogenase	Q5P030, K0NHX8
ancA	anaerobic naphthalene carboxylase	E1YIX0
apc12345	acetophenone carboxylase	Q5P5G2–5
assK	AMP-dependent CoA ligase/synthetase	B8FFM9
bal	benzoylacetate-CoA ligase	Q5P5G7
bbsAB	benzoylsuccinyl-CoA thiolase	Q5P683–4, K0NF62–3, K0N2E7, Q39VG1–2, I6LHV6–7, Q9KJF4
bbsCD	2-[hydroxyphenylmethyl]succinyl-CoA dehydrogenase	Q5P685–6, K0N2F4, K0NCD9, Q39VG0, Q39VF9, I6LHV4–5, Q9KJF1–2
bbsEF	benzylsuccinate CoA-transferase	Q5P687–8, K0NC23, K0NH37, Q39VG8–9, I6LHV2–3, Q9KJF0, Q9KJE9
bbsG	benzylsuccinyl-CoA dehydrogenase	Q5P690, K0NF66, Q39VG7, I6LHV0, Q9KJE8
bbsH	phenylitaconyl-CoA hydratase	Q5P691, K0N2F8, Q39VG6, I6LHU9, Q9KJE7
bclA/bzdA/hbaA	benzoate-CoA ligase	Q5P4T8, Q5P0M7, K0NIT4, Q68VK7, Q53005, Q8GQN9, Q39TQ2,
bcrABCD/bamBC/ badDEFG/bzdNOPQ	benzoyl-CoA reductase	Q5P0N7–9, Q5P0P0, K0N372, K0NFR7, Q39TV8–9, O87874–7, Q6NC14, Q6NC17, G3XCQ6–7, Q68VM0, Q68VL7–9
bhsAB	hydroxybenzylsuccinyl-CoA thiolase	K0NFX5, K0N3G4
bhsCD	2-[hydroxyphenylmethyl]-succinyl-CoA dehydrogenase	K0ND32, K0NCS5
bhsEF	hydroxybenzylsuccinate CoA transferase	K0NI64, K0NH37
bhsG	hydroxybenzylsuccinyl-CoA dehydrogenase	K0N3H1
bhsH	E-phenylitaconyl-CoA hydratase	K0ND34
bisAB	4-isopropylbenzylsuccinyl-CoA thiolase	A0A096ZNX8, A0A096ZNY0
bisCD	2-[hydroxy-4-isopropylphenylmethyl]succinyl-CoA dehydrogenase	A0A096ZNY5, A0A096ZP06
bisEF	succinyl-CoA:R-4-isopropylbenzylsuccinate-CoA transferase	A0A096ZNY4, A0A096ZNY6

bisG	R-4-isopropylbenzylsuccinyl-CoA dehydrogenase	A0A096ZNY3
bisH	E-4-isopropylphenylitaconyl-CoA hydratase	A0A096ZP09
bnsAB	naphthyl-2-oxomethylsuccinyl-CoA thiolase	D2XBJ0–1
bnsCD	naphthyl-2-hydroxymethylsuccinyl-CoA dehydrogenase	D2XBI8–9
bnsEF	naphthyl-2-methylsuccinate-CoA transferase	D2XBI6–7
bnsG	naphthyl-2-methylsuccinate dehydrogenase	D2XBI5
bnsH	naphthyl-2-methylenesuccinyl-CoA hydratase	D2XBI4
bssABC	benzylsuccinate synthase	Q5P6A1–3, K0NCD2, K0N2E2, K0NC20, Q39VF0–2, I6LHT8–9, O87942–4
cmdABC	p-cymene dehydrogenase	A0A096ZNW8, A0A096ZNX0–1
dch/bamR	(di)enoyl-CoA hydratase	Q5P0N1, K0NPB9, Q39TP5, O87873, D8EZY7
ebdABC	ethylbenzene dehydrogenase	Q5P5I0–2
had/bamQ	6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase	Q5P0N0, K0NFR9, Q39TP4, O87871, D8EZY5
hbsABC	hydroxybenzylsuccinate synthase	K0ND30, K0NFX4, K0N3F9, K0NCS2
hcrABC/hbaBCD	4-hydroxybenzoyl-CoA reductase	Q5P3F2–4, K0N6X6, K0NM32, K0NNT3, K0NF27, K0N710, K0NQ86, K0NFN4, K0NIW2, K0NCP9, G3XCP9, Q6NC04, G3XCQ8, O33818–20
iad	4-isopropylbenzaldehyde dehydrogenase	A0A096ZNZ3
ibl	4-isopropylbenzoate-CoA ligase	A0A096ZNX7
ibsABC	4-isopropylbenzylsuccinate synthase	A0A096ZNX3, A0A096ZP03, A0A096ZNX6
iod	4-isopropylbenzyl alcohol dehydrogenase	A0A096ZNX2
masCDE/assABC	1-methylalkylsuccinate synthase	B8FEM2–4, B8FF74–6, A9J4K2, A9J4K4, A9J4K6
mcmSL	methylmalonyl-CoA mutase	B8FEL7–8
nmsABC	naphthyl-2-methylsuccinate synthase	B0CLU5, D2XBH7–9
ncrABCD	2-naphthoyl-CoA reductase	D2XBJ6–9
oah/bamA	6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase	Q5P0M9, K0NCL4, Q39TV7, O87872, A0A0K1J3U9, D8EZY6
pchCF/pcmGIJ	4-cresol dehydrogenase	P09787–8, Q39TS0, Q39TS2, Q39TR9
ped	(S)-1-phenylethanol dehydrogenase	Q5P5I4
ppcABCD	phenylphosphate carboxylase	Q5P478–81, A0A0K1J728, A0A0K1J7P1, A0A0K1J751, A0A0K1J7P8
ppsABC	phenylphosphate synthetase	Q5NZV6–7, Q5P477, K0NIV5, K0N6Z6, K0N705

tioL	predicted thiolase	Q5P8S8
xccAC	4-hydroxyacetophenone carboxylase	Q5P8S2–3
TCA cycle		
acnAB	aconitate hydratase	Q2RNJ0, Q2RHA7, Q46EK0, Q97Z42, Q9I2V5, Q9I3F5, O53166, Q39UD3, Q39WW6, K0NI94, Q5P0Q1, Q5P0Q2, Q6NDA7, A0A0K1J7R6, A0A0K1J8D5
fumABC	fumarate hydratase	Q2RSG6, Q2RS89, Q3AG00–1, Q2RG69, Q2RJ02, Q46FD4, Q469V4–5, P39461, Q51404, Q9I587, P9WN93, Q39SI5, K0ND45, Q5P387, Q5P8Y9, Q6NA57, Q6N440, Q6N319, A0A0K1J3H7, A0A0K1JB94
citA/gltA	citrate synthase	Q2RTZ5, Q2RJF3, Q46EJ9, P80148, P14165, I6Y9Q3, P9WPD5, P9WPD3, Q39S67, K0NKI0, K0N7F6, Q5NYB7, Q6N5R4, A0A0K1J5E4
icd/idh	isocitrate dehydrogenase	Q2RXI4, Q3AD31, Q2RJT1, Q46ET9, Q97WN0, Q9I0L5, P9WKL1, O53611, Q39VY1, K0NFD7, Q5P803–4, Q6N360, A0A0K1J986, A0A0K1J8V1
korAB	2-oxoglutarate ferredoxin oxidoreductase	Q2RQS6–7, Q2RH05–7, O53181–2, K0N453, K0NEW0, K0NQE4, K0NMM4, K0NGD4, K0N617, K0NIB4, K0NAU1, O87870, Q8RJQ9, Q6NAF8–9, A0A0K1J7T1, A0A0K1J873
mdh/mqo	malate dehydrogenase	Q2RV34, Q2RLF4, Q46BQ2, Q97VN4, Q9HYF4, Q9HVF1, P9WK13, P9WJP5, Q39VY0, K0NJD8, K0NHZ0, Q5NYA9, P80458, Q6NA55, A0A0K1J4S8
frdABCD/sdhABCD	succinate dehydrogenase	Q2RV39–42, Q3AFZ8–9, Q3ACD2, Q97W76–9, Q9I3D4–7, P9WN91, P9WN89, P9WNB7, P9WNB5, O53368–71, Q39YX0–1, Q39T02–4, K0NH45, K0NFY5, K0NF70, K0N3I4, K0NC28, K0NI76, Q5NYB2–5, Q5P1U8, Q5P1V0, Q6ND90–3, A0A0K1J4S4, A0A0K1J5D5, A0A0K1J4Z9, A0A0K1J5E9
sucCD	succinyl-CoA ligase	Q2RV32–3, Q3ADM2–3, Q2RLF6, Q2RLF8, Q97VX1–2, Q51567, P53593, P9WGC5, P9WGC7, Q39XQ2–3,, K0ND41, K0NQ58, K0NCT4, K0NLV9, Q5P7A7–8, Q6NDB7–8, A0A0K1J1Z0, A0A0K1J1A4
Wood-Ljungdahl		
ackA	acetate kinase	Q2RQ02, Q3AC47, Q5P5P7, Q46BI1, Q2RJY3, B6JDS2
acsCD/cdhABCDE	carbon monoxide dehydrogenase/acetyl-CoA synthase	Q3ACS0, Q3ACS3–4, K0N5L0, K0NHK3, K0NEA6, Q46G04–6, Q46G08, P27988–9
cooFS/coxSML	carbon monoxide dehydrogenase	P59934, Q9F8A8, Q3AFX6, Q3AE45, Q3AB39, P31894, P31896, K0NSJ7, K0NPB0, A0A0K1Y2Q4, A0A0K1Y182, P19919–21
fdhAB	formate dehydrogenase	P77907–8, Q46C83–4, Q5P4I8–9, Q3AE47, K0NJN2, K0NM42, Q2RXL6, F8BWF5, B6JGD1, F8BUM2, B6JGY9

fhs	formate-tetrahydrofolate ligase	K0NKT5, Q3A9K2, P21164
folD/fchA	methenyltetrahydrofolate cyclohydrolase	Q2RWY4, Q46A53, K0NHK9, K0N5L4, Q5P922, Q3AAY5–6, Q3AE84, Q2RIB4, B6JAU5
metF	methylenetetrahydrofolate reductase	Q46A56, Q2RU65, K0N9S6, K0NL76, Q5P6B6, Q3ACR3, Q2RJ84, B6JJE7
pta	phosphate acetyltransferase	Q46BI0, Q2RQ03, Q5P5P8, Q3AC46, B6JDS1