Enrichment and characterization of methanogenic hydrocarbon-degrading microbial communities in different ecosystems

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

DOKTORIN DER NATURWISSENSCHAFTEN

Dr. rer. nat.

genehmigte Dissertation

von

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geboren am 27. Februar 1982 in Weimar

2014

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Tag der Promotion: 4. April 2014

Schlagworte: mikrobielle Diversität, anaerober Kohlenwasserstoffabbau, Methanogenese

Key words: microbial diversity, anaerobic hydrocarbon degradation, methanogenesis

Contents

Zusammenfassung

Kohlenwasserstoffe (KW) sind die am häufigsten vorkommenden organischen Verbindungen in der Biogeosphäre unserer Erde. Viele Mikroorganismen besitzen die Fähigkeit Kohlenwasserstoffe abzubauen. Die Folge mikrobieller Abbauprozesse in Öllagerstätten ist eine Akkumulation von schwerem, mit konventionellen Fördermethoden nicht extrahierbaren Rohöls in den Lagerstätten. Vor diesem Hintergrund stellt die mikrobielle Umwandlung von schwer extrahierbarem Rohöl in Methan eine neue Strategie zur besseren KW-Nutzung dar. Auch Grubengas aus aktiven und stillgelegten Kohleminen kann als Energiequelle zur Strom- und Wärmeerzeugung genutzt werden. Desweiteren trägt das erweiterte Wissen über KW-abbauende Prozesse und der daran beteiligten mikrobiellen Gemeinschaften zur effektiveren biologischen Sanierung und Eliminierung von toxischen Substanzen aus kontaminierten Böden, Sedimenten und Aquiferen bei.

Ziel dieser Studie ist der Nachweis mikrobieller KW-Abbauprozesse in anoxischen Ökosystemen. Neuartige anaerobe mikrobielle Gemeinschaften, welche fähig sind Braunkohle- und Rohölbestandteile sowie ausgewählte Alkane und aromatische KW unter methanogenen Bedingungen abzubauen, konnten aus unterschiedlichsten Ökosystemen angereichert werden. Anhand dieser Anreicherungskulturen wurde der Abbau von verschiedenen KW zu Methan und CO_2 nachgewiesen. Es konnte das parallele Vorhandensein von putativ syntrophen Bakterienpopulationen und methanogenen Archaeen beim KW-Abbau aufgezeigt werden. In Braunkohle-assoziierten Sedimentschichten und dem daran angrenzenden tiefen Aquifer wurden putativ KW-abbauende Vertreter der Familie *Syntrophaceae* und die Gattungen *Pseudomonas*, *Acinetobacter* und *Arthrobacter* sowie Sulfat-reduzierende Bakterien identifiziert. In beiden Ökosystemen konnte die Dominanz von hydrogenotrophen und methylotrophen Archaeen durch phylogenetische Analysen der mikrobiellen Gemeinschaft sowie durch die Isotopensignatur des gebildeten Methans nachgewiesen werden.

Die in dieser Arbeit präsentierten geochemischen Untersuchungen von Rohöl-, Fluid- und Gasproben des Dagang Ölfeldkomplexes (China) wiesen auf mikrobielle Abbauprozesse von aliphatischen und aromatischen KW in der Lagerstätte hin. $\delta^{13}C$-Messungen von CH_4 und CO_2 bestätigen, dass das vorhandene biogene Methan durch hydrogenotrophe Archaeen produziert wurde. In den Fluidproben konnten *Methanosarcina, Methanosphaera* und *Methanobacterium* als dominierende methanogene Vertreter identifiziert werden.

Weiter wurden in dieser Arbeit die Einflüsse von verschiedenen abiotischen Faktoren sowie die Verfügbarkeit von Elektronenakzeptoren auf die Zusammensetzung der mikrobiellen Gemeinschaften und syntrophen Interaktionen beim Abbau von unterschiedlich strukturierten Kohlenwasserstoffen untersucht und diskutiert. Zum Beispiel führte die Zugabe von bestimmten Elektronenakzeptoren oder Spurenelementen zu den untersuchten mikrobiellen Gemeinschaften zu einem gesteigerten methanogenen KW-Abbau. Desweiteren zeigten Untersuchungen an KW-abbauenden mikrobiellen Gemeinschaften, dass eine Anpassung an erhöhte Temperaturen und Drücke, wie sie in Lagerstätten vorherrschen, möglich ist.

Erstmalige geochemische Untersuchungen sowie quantitative und phylogenetische Analysen verschiedener Mikroorganismengruppen in Sedimentproben der nördlichen Baffin Bay zeigten die mikrobielle Reduktion von Eisen, Mangan und Sulfat. Zusätzlich zu den stark verlangsamten mikrobiellen Reduktionsaktivitäten an den drei Beprobungsstandorten dieses nährstoffarmen marinen Ökosystems konnte nur im küstennahen Sediment Potential zum KW-Abbau nachgewiesen werden.

Summary

Hydrocarbons (HC) are among the most abundant organic compounds in earth´s biogeosphere. A great number of microorganisms are able to degrade hydrocarbons. In consequence of microbial biodegradation in oil reservoirs, heavy crude oil accumulates within the oil reservoirs and is not extractable with conventional technologies. Because of that, the microbial conversion of heavy and not accessible oil into methane might provide a new strategy for enhanced HC recovery. Additionally, coalbed methane from active and abandoned mining areas is usable for heat and power production. Furthermore, the knowledge of HC-degrading processes and involved microbial communities can lead to more efficient bioremediation of toxic petroleum compounds in contaminated soils, sediments, and aquifers.

The goal of this study is the detection of microbial HC-degradation processes in anoxic ecosystems. A broad range of novel anaerobic microbial communities from samples of different ecosystems which are able to degrade coal, oil or selected alkanes and aromatic HC under methanogenic conditions could be enriched. These enrichment cultures showed the degradation of different HC to methane and CO_2. Additionally, the concurrent presence of HC-degrading putative syntrophic bacterial communities and methanogenic Archaea is established.

In coal-associated sedimentary layers and adjacent deep aquifer, potential HC-degrading members of the family *Syntrophaceae* and relatives of *Pseudomonas*, *Acinetobacter* and *Arthrobacter* as well as sulfate-reducing bacteria were identified. The isotopic signature of the produced methane as well as phylogenetic analyses of the microbial communities showed the dominance of hydrogenotrophic and methylotrophic Archaea in both ecosystems.

The presented geochemical investigations of samples from crude oil, fluids and gasses collected from the Dagang oil field complex (China) indicated microbial degradation processes of aliphatic and aromatic HC. $\delta^{13}C$ measurements of CH_4 and CO_2 confirmed that the measured biogenic methane is of hydrogenotrophic origin. The methanogenic community found in the fluids is dominated by relatives of *Methanosarcina, Methanosphaera* and *Methanobacterium* species.

Furthermore, the influence of different abiotic factors on, as well as the availability of electron acceptors to the microbial community composition and syntrophic interactions during the degradation of differently structured HC was investigated and discussed in this study. For example, the addition of certain electron acceptors or trace elements to examined microbial communities resulted in an enhanced methanogenic HC-degradation. Moreover, HC-degrading microbial communities showed an adaption to increased temperatures and pressures like those found in reservoirs.

First-time geochemical investigations as well as quantitative and physiological analyses of different microbial groups occurring in sediment samples from the northern Baffin Bay showed the microbial reduction of iron, manganese and sulfate. In addition to the slowed microbial reduction activity at the investigated three sampling sites of this poor nutrient marine ecosystem, only at the Shelf site a HC-degradation capability could be attested.

List of abbreviations

BES	2-bromoethanesulfonate
bp	base pair
Bss	benzylsuccinate synthase
BTEX	benzene, toluene, ethene and xylene
cmbsf	centimeters below seafloor
DNA	deoxyribonucleic acid
Dsr	dissimilatory sulfite reductase gene
et al.	et alii / et aliae (“and others”)
FID	flame ionisation detector
FISH	fluorescence *in situ* hybridisation
GC	gas chromatography
HC	hydrocarbon
IC	ion chromatrography
Mcr	methyl-coenzyme M-reductase gene
MS	mass spectrometry
n. d.	not detectable
OTU	operational taxonomic unit
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
ppmv	parts per million and volume
Q-PCR	quantitative *real time* PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	revolutions per minute
SIP	stable isotope probing
SMOW	standard mean ocean water
SRB	sulfate-reducing bacteria
TIC	total inorganic carbon
T-RF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
TOC	total organic carbon
VPDB	vienna pee dee belemnite
ΔG°	Gibbs free energy change under standard conditions

Chapter I

Introduction

1. Introduction

1.1. Hydrocarbons

1.1.1. Structure and natural occurrence

Hydrocarbons (HC) are among the most abundant organic compounds in the earth´s biogeosphere. They consist exclusively of carbon and hydrogen and play an important role in the global carbon cycle (Wilkes and Schwarzbauer, 2010). According to their structures they can be divided into four different types: saturated HC (alkanes), unsaturated HC (alkenes, alkynes), cycloalkanes and aromatic HC.

Saturated and unsaturated HC exist as different structural types: linear, branched and cyclic (non-aromatic) compounds (Figure 1.1). Then, these compounds are termed aliphatic hydrocarbons. Alkanes (C_nH_{2n+2}; paraffin series) are the simplest form of hydrocarbons; the linear chains with single bonds are saturated with hydrogen. Alkane molecules are chemically stable and therefore very inert. Mid-chain length *n*-alkanes (C_{23} - C_{25}) occur as major constituents of leaf waxes of aquatic plants; terrestrial vegetation is typically dominated by long-chain length homologues (>C_{29}) (Ficken et al., 2000). Consequently, *n*-alkanes are the main components of undegraded crude oil. Examples for branched alkanes are isobutane, isopentane, 3-methylhexane, and phytane - common constituents of fossil fuels.

Unsaturated HC have double or triple bonds between the carbon atoms, called alkenes (C_nH_{2n}; olefin and di-olefin series) or alkynes (C_nH_{2n-2}; acetylene series), respectively, and are of great structural diversity. These structures allow the addition of H_2 and make them more reactive. The majority of alkenes in the nature is formed by living organisms and includes the enormous variety of monoterpenes found in higher plants. Monoterpenes may function as deterrents, inhibitors of fungal or bacterial growth or attractants, but often their role is unknown (Widdel and Rabus, 2001). Two highly abundant alkenes occurring in higher plants are ethylene, released as a ripening hormone in the life cycle of higher plants, and some carotenoids, plant photosynthetic pigments involved in energy transfer.

Cycloalkanes (also called naphthenes) contain one or more carbon rings, attached with hydrogen atoms. They are relatively stable.

Aromatic HC contain alternating double and single bonds between carbon atoms forming an aromatic ring. The simplest aromatic HC is benzene. Benzene consists of a hexagonal ring and three delocalized π-orbitals, which make the molecule inert. Aromatic HC can be monocyclic (MAH) or polycyclic (PAH) and often occur with aliphatic hydrocarbon chains as alkyl-substituted aromatic HC. The natural occurrence of aromatics is widespread. For example, steroids, hopanoids, and other eukaryotic and prokaryotic lipids are based on annulated cyclohexane and cyclopentane rings. These biomass constituents in turn are source of the structurally diverse mixtures of naphthalenes found in fossil fuels (Wilkes and Schwarzbauer, 2010). Moreover, toluene, ethylbenzene, and xylene are benzene derivatives and highly abundant components of fossil fuels, as well.

In addition to hydrocarbons only consisting of carbon and hydrogen, carbon atoms may also form covalent bonds with functionalized organic compounds such as the halogens fluorine, chlorine, bromine, iodine, or with the hetero elements nitrogen, sulfur, and oxygen (Wilkes and Schwarzbauer, 2010). The majority of natural halogenated compounds (so-called organohalogens) are produced by living organisms. A diverse collection of organohalogens are synthesized by seaweeds, sponges, corals, terrestrial plants, fungi, lichens, bacteria, and higher animals and humans. Some well-known class-members are morphine, penicillin and quinine (Gribble, 2003). On the other hand, a number of halogenated organic compounds are among important anthropogenic environmental contaminants (dioxins) such as hexachlorobenzene (PCB).

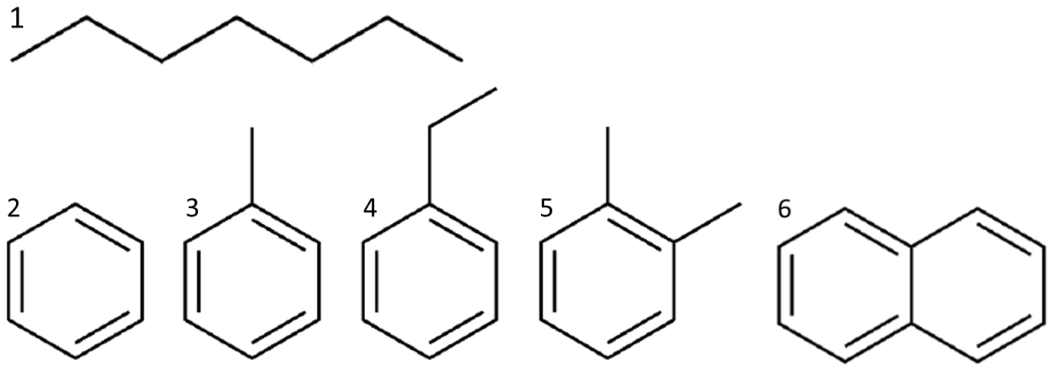

Figure 1.1: Representative hydrocarbon structures. Alkanes: (1) *n*-hexane, monocyclic aromatics: (2) benzene, (3) toluene, (4) ethylbenzene, (5) xylene, polycyclic aromatics: (6) naphthalene.

Hydrocarbons can be present as gases, liquids and solids. Gaseous HC are characterized by low-molecular weights (< C_4), while liquid HC are characterized by molecular weights more than C_5. Naturally occurring gaseous HC (e.g. shale gas, coalbed

methane, gas hydrates) are often dominated by methane and are by far the largest pool of hydrocarbons in the geosphere (Wilkes and Schwarzbauer, 2010).

1.1.2. The formation of crude oil and natural gas

The majority of hydrocarbons found on earth occur in fossil fuels, particularly in crude oil (often referred to as petroleum). Crude oil is a complex mixture of hydrocarbons and other organic compounds.

Two major classes of kerogens exist. Kerogen is the portion of organic matter in sedimentary rocks formed from formerly living organic materials (Dow, 1977). Kerogen, the precursor material of petroleum, originates from marine or aquatic organic matter and has a higher hydrogen content than the humic kerogen. The humic kerogens based on organic matter of terrestrial plants and turn into coal. The amount and the composition of the mixture of generated hydrocarbons strongly depend on the chemical structures of the source of biomolecules and the specific maturation conditions (Libes, 2009).

The formation of crude oil and gas (Figure 1.2) starts with dead marine lower plants and plankton and their remains which become buried under silt, sand or mud. During diagenesis, organic compounds (lipids, proteins, carbohydrates and lignin-humic compounds) are buried at temperatures less than 50°C in a time scale of a few thousand years and converted into kerogen. Kerogen in sediments of various ages is by far the most abundant form of organic carbon on earth (Widdel and Rabus, 2001). Microorganisms are involved in this maturation process by converting the biopolymers into biomonomers under anoxic conditions. During microbial degradation, oxygen and most of the nitrogen and sulfur is removed from kerogen material (Libes, 2009).

As the kerogens get buried by sediments over time, elevated temperature and pressure conditions, the catagenetic process has formed a mixture of hydrocarbons – petroleum, bitumen and gases. The hydrophobic character of kerogen increases by further defunctionalization, parts of the organic carbon are released as aliphatic and aromatic hydrocarbons (Widdel and Rabus, 2001). The resulting HC with different carbon chain lengths are able to migrate out of the source rock into the ambient porous geological layers until they are trapped under a non-permeable cap rock layer to build an oil reservoir or move further on to the surface.

At the end of the maturation process (during the metagenesis) another increase of pressure and temperature up to 150°C cracks the residual kerogen into dry gas, including methane (CH_4) and other gases such as hydrogen sulfide (H_2S), carbon dioxide (CO_2) and nitrogen (N_2). The total maturation process can take from 10 to several hundred million years (Connan, 1974).

The major compounds of petroleum are saturated aliphatic and aromatic hydrocarbons (around 86 %) next to low concentrations of organic compounds containing nitrogen, oxygen, sulfur, chlorine, as well as in the petroleum dissolved gases such as CO_2, H_2S and N_2 and a few trace metals e.g. vanadium (V), zinc (Zn), nickel (Ni) and mercury (Hg) (Tissot and Welte, 1984). Resins and asphaltenes are solid components of petroleum, and are high-molecular-weight polycyclic organic molecules containing nitrogen, sulfur and oxygen atoms (Tissot and Welte, 1984). The composition of petroleum from different global regions or even within geological formations can vary enormously depending on where and how the petroleum was formed.

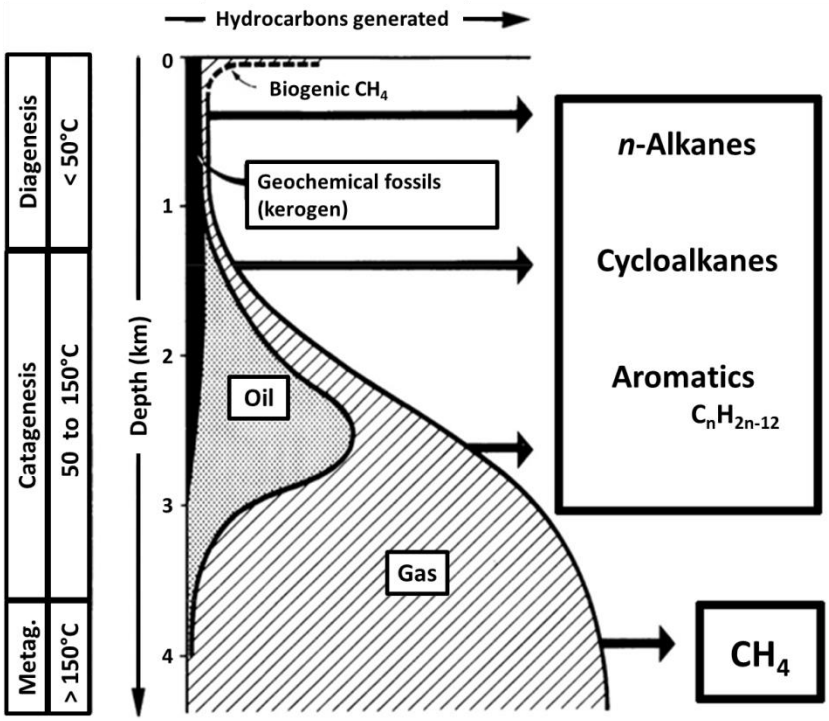

Figure 1.2: General scheme of hydrocarbon formation during diagenesis, catagenesis and metagenesis. Depth and temperature are only indicative and vary according to the particular geological conditions. Figure modified from Tissot and Welte (1984).

1.1.3. The formation of coal

Coal is a solid, brittle, combustible, carbonaceous rock formed by the decomposition and alteration of terrestrial higher plants that grew in swamp ecosystems. The plants biomass was deposited in anaerobic, swamp environments where low oxygen levels prevented its reduction. Coals were formed by huge amounts of such deposits, which were subsequently successively covered by sediments. After processes of microbial action, burial, compaction, geothermal heating and elevated pressure over geologic time the organic matter was transformed into 'higher rank' such as lignite, subbituminous, bituminous or anthracite coal (Speight, 2005). This process from low- to high-rank coals is referred to as coalification, it constitutes diagenesis, catagenesis and metagenesis. High-ranked coals are characterized by an increased heating value, decreased volatile matter and moisture content (Tissot and Welte, 1984) (Figure 1.3).

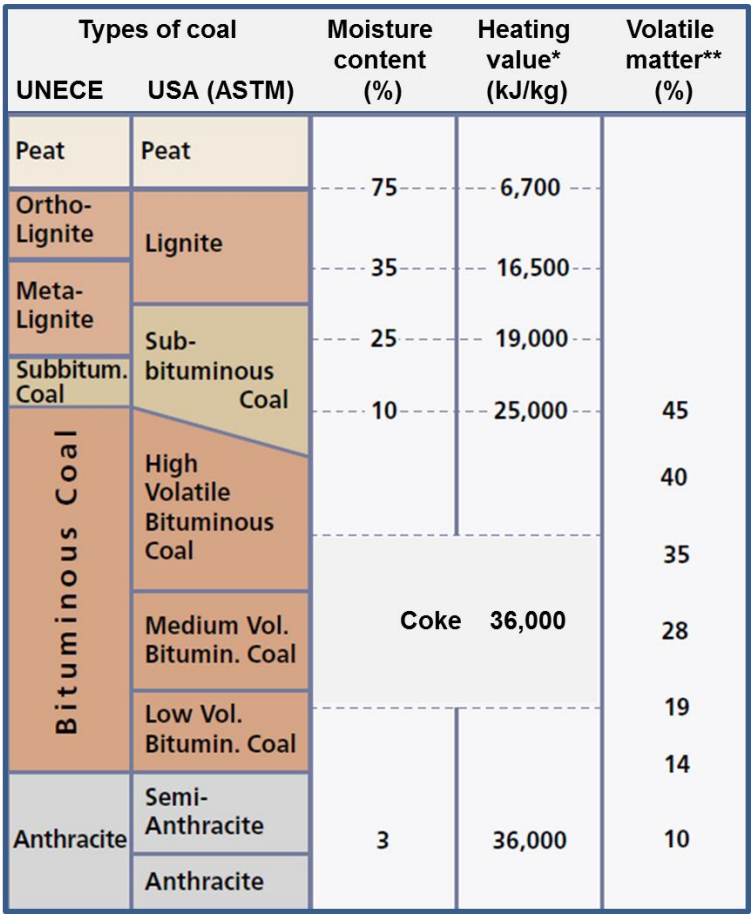

Figure 1.3: Scheme of different types of coal, UN-ECE: United Nations Economic Commission for Europe; ASTM: American Society for Testing and Materials; * ash free; ** water and ash free; modified after Weniger and Krooss (2012) and Bundesverband Braunkohle (DEBRIV; www.braunkohle.de).

Coal is found in all geologic periods and is an extremely complex material that exhibits a variety of physical properties. Different coal types were classified from the American Society for Testing and Materials (ASTM), and the United Nations Economic Commission for Europe (UN-ECE).

Coal consists of more than 50 % by weight of carbonaceous material beside variable quantities of hydrogen, sulfur, nitrogen, and oxygen (Killops and Killops, 2005). Oxygen is present is many functional groups like carboxyl, ketone, hydroxyl and methoxy groups. The distribution of these groups varies in the different burial stadia of coal. Nitrogen is found in amines and pyridyl units of aromatic rings. Thiophenic units of aromatic rings contain sulfur, as well as thiols and sulfides. The inorganic material components of coal comprises pyrites and aluminosilicates, containing sulfur or aluminum. Additionally, coal can contain a variety of metals (Killops and Killops, 2005).

The Westphalian coal of northern Europe is classified as vitrinite coal, the most abundant class of coal. It is formed mainly from woody material (vascular plant remains), thus this kind of coal is extremly rich of complex organic matter which could be a very attractive carbon source for microbial biodegradation. The main molecular structures provided from the terrestrial organic matter are the high-molecular-weight biopolymers cellulose and lignite. In the maturated coal these aromatic structured material is often interlinked by oxygen bridges and includes carboxyl, hydroxyl or ketone functional groups which are metabolizable by different microbial communities.

1.1.4. Methane – the simplest hydrocarbon

Methane is a hydrocarbon containing four hydrogen atoms covalently bond to carbon, and belongs to the alkane family. It is the simplest molecule of all organic compounds. Methane is the most abundant hydrocarbon in the atmosphere and the second most important greenhouse gas after carbon dioxide. The comparative impact of methane on climate change is over 25 times greater than carbon dioxide over a 100 year period (Shindell et al., 2009). The reason is that the CH_4 molecules are much more effective at trapping the infrared radiation reflected from the earth´s surface (Reay et al., 2007). Because methane is a non-polar molecule, it is slightly soluble in water, depending on salinity, temperature and hydrostatic pressure (Yamamoto et al., 1976).

According to its origin methane can be biogenic, thermogenic, or abiogenic. Biogenic methane is produced from organic matter by microbial reduction of organic monomers such as acetate, formate, methanol, or methylamine (acetoclastic methanogenesis) or by CO_2-reduction (hydrogenotrophic methanogenesis) in the absence of oxygen or other oxidants (e.g. nitrate, sulfate, ferric iron) in anaerobic environments. The produced methane is released from anaerobic environments and can re-enter the global cycle (Deppenmeier and Müller, 2008). Most of the methane which is emitted into the atmosphere has a biogenic origin. Wetlands, including bogs, tundra, swamps, alluvial areas, and ponds, are the largest source of microbial methane emission on earth. Furthermore rice agriculture, microbial symbionts in ruminants and termites, and landfills have a large impact on the atmosphere. More than 69 % of the global total of the atmospheric methane is the result of microbial processes (Conrad, 2009; Solomon et al., 2007). On the other hand, it has been noted that microbial methane oxidation has a large influence on the overall atmospheric budget before methane is emitted into the atmosphere. Reeburgh (2007) valuates the amount of microbial oxidation for more than half of the estimated methane production. And Conrad (2009) postulates that the global CH_4 sources are balanced by sinks and sources of similar magnitude.

Thermogenic methane is formed by heat and pressure-induced decay of organic matter. These conditions occur during the metagenesis stage of oil and coal production. About 25 % of all CH_4 sources are associated with mining and combustion of fossil fuels from oil and coal deposits or with biomass burning.

The isotopic signature of CH_4 from biogenic sources is isotopically lighter than CH_4 from geological or thermogenic sources. Carbon or hydrogen stable-isotope signatures in thermogenic methane seem to be controlled by the extent of conversion of organic matter, the timing of gas expulsion and trapping. The different characters of methane in individual sedimentary basins may be a result of the geologic history (Schoell, 1988).

Methane is called abiogenic when it is formed through geological processes within Earth's crust (Horita and Berndt, 1999), i.e. inorganic reactions independent of organic matter. To date, little is known about the mechanisms of abiogenic methane formation or about isotopic fractionation during such processes.

1.1.5. Isotope composition of methane and carbon dioxide and fractionation processes

Most of the chemical elements exist as two or more isotopes. They have the same chemical behavior, but different atomic weights caused by a variable number of neutrons. Carbon, hydrogen, nitrogen, sulfur and oxygen are the main elements of organic biomass with great importance for the biological cycle of life. The isotopes of each element appear naturally in relatively fixed ratios. The ratio of abundances of the stable carbon isotope ^{12}C to ^{13}C is about 99.89 % to 1.11 %, i.e. the light isotope is naturally much more abundant. Hydrogen has the two stable isotopes ^{1}H and ^{2}H (or D for deuterium) and the ratio of abundance is 99.98 % (^{1}H) to 0.015 % (^{2}H) (Boutton, 1996). The standard used for the analysis of the stable isotopes of carbon (^{12}C / ^{13}C) is PDB (Pee Dee Belemnite). It is a fossil of *Belemnitella americana* from the Cretaceous Peedee formation in South Carolina. As a standard for hydrogen the SMOW (Standard Mean Ocean Water) was defined. The ratio of heavy and light isotopes with a particular compound is termed the isotopic signature (δ). The δ-value describes the deviation of isotopic composition of the substance relative to the isotopic composition of a certain standard. The δ-value, expressed in parts per million (‰), is defined in the following equation (Whiticar, 1999):

$$\delta X\,[‰] = \left[\left(R_{sample} / R_{standard}\right) - 1 \right] \times 1000$$

X is the usually less-abundant isotope of the element and R is the ratio of heavy to light isotope of the sample (e.g., $^{13}C / ^{12}C$ or $^{2}H / ^{1}H$) and the international standard, respectively. A positive δ-value means that the substance is isotopically heavy – heavier than the standard. A negative δ-value corresponds to light isotopes – lighter than the standard.

The isotopic fractionation of carbon and hydrogen during the microbial transformation can be used as an indicator for the biodegradation of hydrocarbons. Microorganisms prefer the isotopically lighter stable isotope for their metabolization, because the activation energy for the formation and cleavage of chemical bonds during the initial step of microbial transformation is greater for the heavy isotope. Thus, the substrates become isotopically heavier (Whiticar, 1999).

Therefore, the fractionation of stable isotopes can be used as an indicator for microbial degradation reactions *in situ* e.g. in petroleum reservoirs or contaminated sites, as well as under laboratory conditions (Feisthauer et al., 2011; Meckenstock et al., 1999; Richnow et al., 2003b). Furthermore, the microbial degradation pathway and the stage of biodegradation are determinable by measuring the extent of isotopic fractionation.

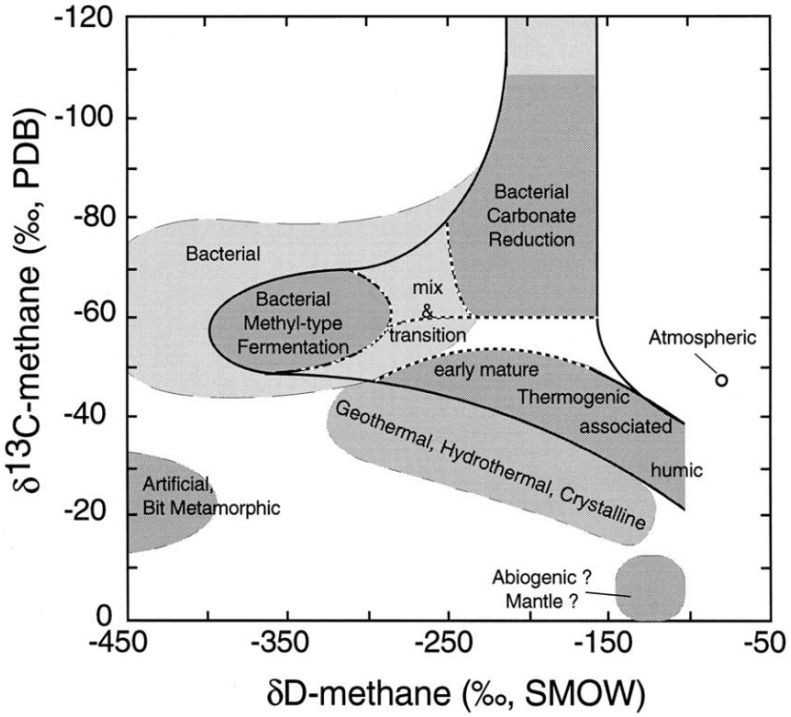

Figure 1.4: Diagram for classification of biogenic and thermogenic methane by the isotopic combination of $\delta^{13}C_{CH4}$ and δD_{CH4} information. The term "bacterial" in the figure replaces the term "biogenic" and means the microbial produced methane (Whiticar, 1999; Whiticar et al., 1986). PDB - Pee Dee Belemnite; SMOW – Standard Mean Ocean Water.

Because of the depletion process during the heat-and pressure-induced decay of organic matter to oil and gas, the isotopic signature of thermogenic methane typically results in $\delta^{13}C$-values between -50 and -35 ‰, while the isotopy of thermogenic hydrogen shows highly varying δD-values of -275 to -100 ‰. Whereas $\delta^{13}C_{CH4}$-values which are more negative than -50 ‰ (-110 to -50 ‰) and in combination with δD_{CH4}-values more negative than about -150 ‰ (-400 to -150 ‰), are strongly indicative of pure microbial methane (Whiticar, 1999). Carbon and hydrogen isotope signatures and ranges are presented in Figure 1.4 for comparison.

1.2. The biodegradation of hydrocarbons

In the history of microbial life, microorganisms have acquired a broad range of pathways to make use of hydrocarbons which are manifold present in the biosphere as growth substrates (Widdel and Rabus, 2001). Since the beginning of the 20th century, the utilization of hydrocarbons in the presence of oxygen has been known. Later, in the 1980s, microorganisms were discovered which were able to degrade hydrocarbons under strictly anoxic conditions. Such microorganisms use biochemical mechanisms for activating hydrocarbons without using oxygen that differs completely from those employed in aerobic hydrocarbon metabolism (Widdel and Rabus, 2001). These microorganisms can couple the metabolism of hydrocarbon molecules to the reduction of soluble anions or metals such as nitrate, Fe(III) and Mn(IV) depending on specific environmental conditions. The consumption of the available electron acceptors follows a predictable metabolic series, according to the free energy change (ΔG°) yielded by their reduction (Table 1.1). The so produced microbially catalyzed oxidant-depletion profile first shows the reduction of O_2, followed by nitrate, Mn(IV), Fe(III), sulfate, and finally CO_2 (DeLong, 2004). In comparison to the other redox-reactions, methanogenesis has the lowest free energy change (ΔG°), so that methanogenic microorganisms live close to the thermodynamic limit (Deppenmeier and Müller, 2008). If the presence of nitrate, Mn(IV), Fe(III), sulfate as electron acceptors is higher, the methanogenic processes are out-competed by anaerobic respiration reactions with higher energy yields (Thauer et al., 2008).

Table 1.1: Microbial reaction pathways and its free energy change modified after McKinley (2001)

Reaction	ΔG°´ (kJ mol^{-1})	Type
$CH_2O + O_2 \rightarrow CO_2 + H_2O$	-475	Aerobic respiration
$5CH_2O + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-448	Denitrification
$CH_2O + 3CO_2 + H_2O + 2MnO^{2+} \rightarrow 2Mn_2+ + 4HCO_3^-$	-349	Mn(IV)-reduction
$CH_2O + 7CO_2 + 4Fe(OH)_3 \rightarrow 4Fe^{2+} + 8HCO_3^- + 3\ H_2O$	-114	Fe(III)-reduction
$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$	-77	Sulfate-reduction
$2CH_2O \rightarrow CH_4 + CO_2$	-58	Methanogenesis

1.2.1. Aerobic hydrocarbon degradation

The utilization of hydrocarbons as growth substrates for bacteria, yeasts and filamentous fungi in the presence of molecular oxygen is well-known for many decades (Harayama et al., 1999; Leahy and Colwell, 1990; Rehm and Reiff, 1981). Under aerobic

conditions HC are always transformed to polar intermediates using molecular oxygen as reactive co-substrate. Aerobic microorganisms initiate the metabolism of alkanes by monooxygenase reactions, while aromatic HC are attacked by monooxygenases or dioxygenases (Heider et al., 1998). Derived from molecular oxygen, these enzymes incorporate hydroxyl groups into the aliphatic chain or the aromatic ring. The alcohols formed from aliphatic hydrocarbons are then oxidized to the corresponding acids; the phenolic compounds generated by ring hydroxylation of aromatic hydrocarbons are direct precursors for oxidative ring cleavage (Harwood and Parales, 1996; Heider et al., 1998). During aerobic biodegradation of *n*-alkanes the enzyme catalyzed terminal oxidation result in an alkanol which further oxidized by dehydrogenase to aldehydes and then to fatty acids. Fatty acids in turn are metabolized through the β-oxidation pathway.

1.2.2. Anaerobic hydrocarbon degradation

In the most hydrocarbon-rich environments such as oil reservoirs, coal deposits, marine sediments, or hydrocarbon-contaminated aquifers in the subsurface, oxygen is not available. Despite of the low aqueous solubility and fairly high toxicity, several studies over the last decade have demonstrated that many different classes of hydrocarbons are degradable by microbial attack coupling with sulfate-, nitrate-, or iron(III)-reduction or methanogenesis under anaerobic conditions (Davidova and Suflita, 2005; Grbić-Galić and Vogel, 1987; Kropp et al., 2000; Townsend et al., 2004; Ulrich et al., 2005; Ulrich and Edwards, 2003; Zengler et al., 1999; Zhang and Young, 1997).

The vast majority of these bacteria couple the biodegradation of a hydrocarbon to the reduction of either nitrate or sulfate (Davidova and Suflita, 2005). Well-studied are the genera *Thauera aromatic* and *Azoarcus toluyticus*, both are able to degrade toluene under nitrate-reduction conditions, whereas *A. toluyticus* also utilizes *m*-xylene and ethylbenzene (Elmén et al., 1997; Shinoda et al., 2004). For complete mineralization of ethylbenzene only few nitrate- or sulfate-reducing bacteria affiliated to *Deltaproteobacteria* were discovered (Ball et al., 1996; Kniemeyer et al., 2003; Rabus and Widdel, 1995). *Pseudomonas balearica* utilizes *n*-alkanes (C_{15}-C_{18}) with the reduction of nitrate (Grossi et al., 2008). Another nitrate-reducer which utilizes C_{18}-alkanes is *Marinobacter* sp. (Bonin et al., 2004). Further, toluene mineralization was also observed in sulfate-reducing cultures including *Desulfobacula toluolica* (Rabus et al., 1993) and *Desulfotomaculum* sp., the latter also

utilizes *m*-xylene and *o*-oxylene (Morasch et al., 2004). *Desulfatiferula olefinivorans* metabolizes *n*-1-alkenes (C_{14}-C_{23}) with sulfate as electron acceptor (Schink, 1985; Widdel et al., 2010). Representative for anaerobic hydrocarbon degradation coupled to iron(III)-reduction is *Geobacter metallireducens,* known to metabolizes toluene (Lovley et al., 1993; Lovley and Lonergan, 1990).

Until today, only limited information could be obtained for the anaerobic biodegradation of benzene. Benzene is, based on its structure, most hardly to degrade, but Coates et al. (2001) described two *Dechloromonas* strains that can completely mineralize anaerobically various mono-aromatic compounds to CO_2 with nitrate as the electron acceptor. However, the mechanism by which anaerobic benzene degradation occurs is unclear (Coates et al., 2002).

According to their potential to carbon-oxidation, sulfate-reducing bacterial communities are categorized into the group of complete oxidizers which are capable of complete mineralization of organic substrates to CO_2 (members of *Desulfobacteraceae*) and the group of incomplete oxidizers which produce acetate as a final by-product (members of *Desulfobulbaceae*) (Canfield et al., 2005; Kuever et al., 2005). Because of their ability to activate and metabolize alkanes and aromatic hydrocarbons these sulfate-reducing bacteria play an important role in the global carbon cycle.

The free energy change of the anaerobic degradation of hydrocarbons with sulfate or nitrate as electron acceptor has been calculated according to the following equations exemplified with hexadecane (Widdel et al., 2010):

$$5\ C_{16}H_{34} + 98\ NO_3^- + 18\ H^+ \rightarrow 80\ HCO_3^- + 49\ N_2 + 54\ H_2O \qquad \Delta G^{\circ\prime} = -9677\ kJ\ mol^{-1}\ C_{16}H_{34}$$

$$4\ C_{16}H_{34} + 49\ SO_4^{2-} + 34\ H^+ \rightarrow 64\ HCO_3^- + 49\ H_2S + 4\ H_2O \qquad \Delta G^{\circ\prime} = -559\ kJ\ mol^{-1}\ C_{16}H_{34}$$

Furthermore, several studies demonstrated the hydrocarbon degradation by methanogenesis. Methanogenesis is performed by strictly anaerobic methanogenic *Archaea* currently classified into the well-established orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales* (Thauer et al., 2008). Methanogenesis is the final methane-producing step of the biodegradation of organic material like hydrocarbon mixtures in anaerobic environments where the concentrations of nitrate, Mn(IV), Fe(III), or sulfate are low (Thauer et al., 2008). Most of

the methanogenic archaea which are not able to degrade hydrocarbons directly use C_1-compounds (e.g. acetate, formate, methylamine, CO_2) as electron acceptors which are provided by manifold prior microbial mineralization processes.

Harder and Foss (1999) have shown that the anaerobic biodegradation of alpha-pinene and 2-carene supported methanogenesis. Moreover, Schink (1985), observed the complete degradation of 1-hexadecene and identified *Methanospirillum hungatei* and *Methanothrix soehngenii* as prevalent methanogenic microorganisms. Additionally, the rapid conversion of hexadecane to methane in the absence of sulfate-reducing bacteria was described by Anderson and Lovley (2000a), while Zengler et al. (1999) showed that the anaerobic conversion of hexadecane to methane was performed by groups of anaerobic hydrocarbon-degrading bacteria in syntrophic associations with methanogens (see following equations).

Syntrophic *n*-alkane oxidation:

$$4\ C_{16}H_{34} + 64\ H_2O \rightarrow 32\ CH_3COO^- + 32\ H^+ + 68\ H_2 \qquad \Delta G = -929\ kJ\ mol^{-1}\ C_{16}H_{34}$$

$$32\ CH_3COO^- + 32\ H^+ \rightarrow 32\ CH_4 + 32\ CO_2 \qquad \Delta G = -385\ kJ\ mol^{-1}\ C_{16}H_{34}$$

$$68\ H_2 + 17\ CO_2 \rightarrow 17\ CH_4 + 34\ H_2O \qquad \Delta G = -282\ kJ\ mol^{-1}\ C_{16}H_{34}$$

Methanogenic hydrocarbon degradation (net reaction):

$$4\ C_{16}H_{34} + 30\ H_2O \rightarrow 15\ CO_2 + 49\ CH_4 \qquad \Delta G = -1596\ kJ\ mol^{-1}\ C_{16}H_{34}$$

1.2.3. Microbiological anaerobic hydrocarbon activation

The anaerobic biodegradation of hydrocarbons requires activation of the certain substrate. The detailed metabolic processes of alkane degradation seem to function differently and are not completely understood so far (Hassanshahian and Cappello, 2013). Widely reported is the activation through the addition to fumarate by glycyl-radical enzymes. Additional known oxygen-independent hydrocarbon activation reactions comprise the hydroxylation with water by molybdenum cofactor containing enzymes, the 'reverse methanogenesis' is a anaerobic methane-oxidizing process involving variants of methyl-coenzyme M reductase - the key enzyme of methanogenesis, the methylation, and the carboxylation catalyzed by yet-uncharacterized enzymes (Boll and Heider, 2010). The available knowledge about the involved enzymes varies greatly.

Their anaerobic degradation and activation mechanisms are manifold and depending on hydrocarbon structures. Several studies investigated anaerobic degradation mechanisms of different hydrocarbons. Biegert et al. (1996), Beller and Spormann (1997) researched in anaerobic toluene biodegradation and revealed the radical-catalyzed mechanism of the addition to fumarate as co-substrate yielding substituted succinates (Figure 1.5.). Biegert et al. (1996) describes that the first step of alkylbenzenes (e.g. toluene or xylene) activation is redox-neutral and coenzyme A-independent formation of benzylsuccinate as first intermediate by addition of the methyl group of an alkylbenzene to the double bond of fumarate. Aromatic molecules such as alkylbenzenes with a methyl group as a side chain undergo an enzyme addition of fumarate, most likely via a radical mechanism, while alkylbenzenes with side chains of two or more carbon atoms are activated by dehydrogenation of the side chain (Hassanshahian and Cappello, 2013).

This fumarate addition is catalyzed by the large subunit of benzylsuccinate synthase (*BssA*) activating toluene and xylene, a fumarate-adding enzyme (FAE) (von Netzer et al., 2013). Benzylsuccinate is further oxidized to benzoyl-CoA and benzoate, depending on the presence of coenzyme A and nitrate.

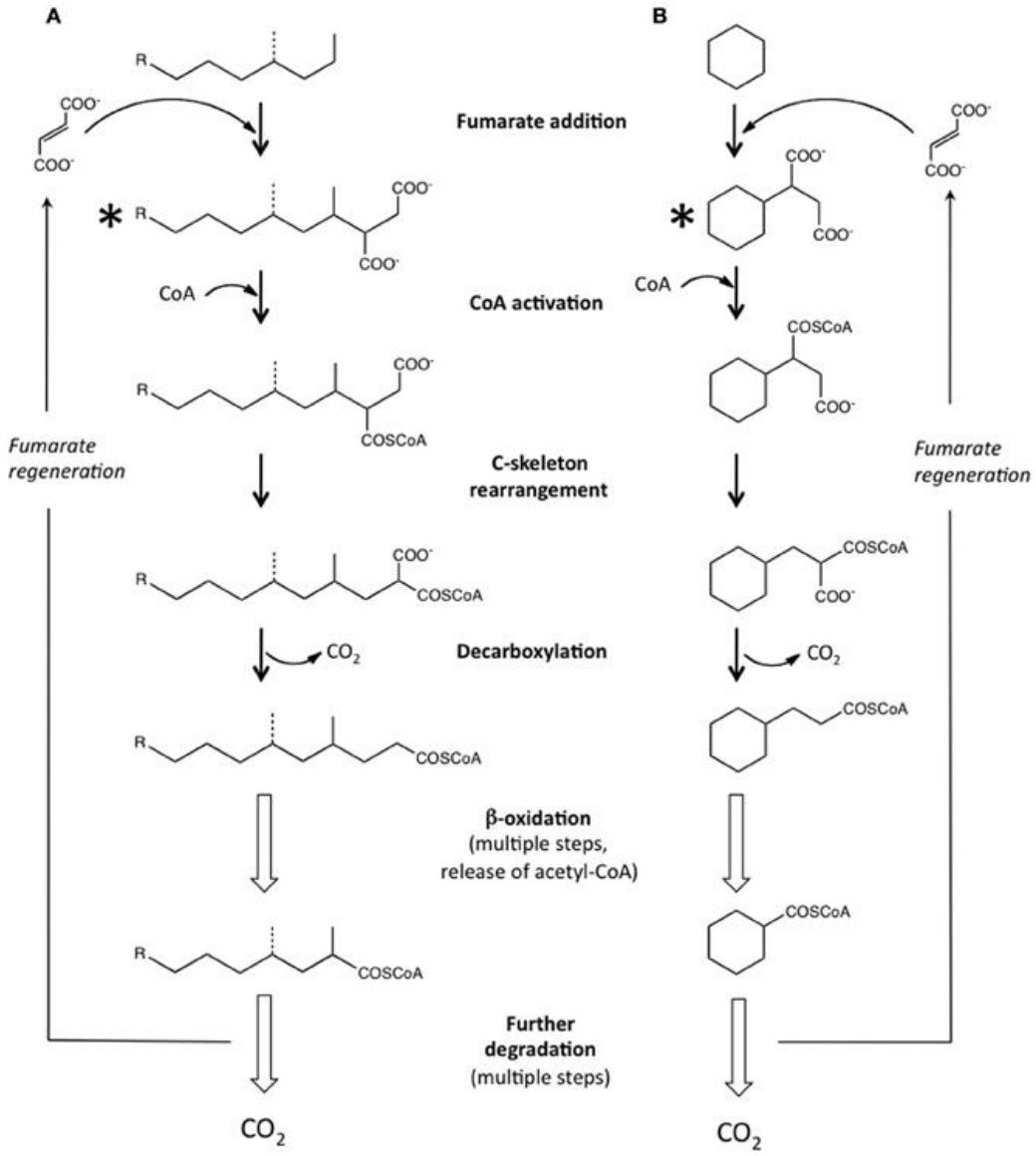

Figure 1.5: Anaerobic hydrocarbon activation of alkanes via fumarate addition and subsequent reactions. **(A)** Pathway for the biodegradation of *n*-alkanes and potentially for isoalkanes (dotted lines) (Widdel and Grundmann, 2010), **(B)** Proposed pathway for the biodegradation of cyclic alkanes (Musat et al., 2010). Compounds marked with asterisks indicate fumarate addition metabolites that are most diagnostic of *in situ* anaerobic biodegradation of alkanes (Gieg and Agrawal, 2013).

Analog to the formation of benzylsuccinate, in the anaerobic activation process of *n*-alkanes, the carbon-carbon bond addition to fumarate activates the alkane and form alkylsuccinates (the alkyl substituents match of the alkane substrate) (Kropp et al., 2000; Rabus et al., 2001). This reaction is catalyzed by the alkylsuccinate synthase (Ass; also referred to as 1-methylalkyl-succinate synthase (Mas)) (Callaghan et al., 2008). Fumarate addition occurs primarily at the subterminal carbon (C-2) atom of the *n*-alkanes (Widdel and Grundmann, 2010). But studies from Rabus et al. (2001) suggested the activation of

n-hexane at the C-3 atom and Kniemeyer et al. (2007) the activation of propane at the C-1 atom, most likely in side-reactions. Further, alkylsuccinates are transformed by decarboxylation yielding branched fatty acids that can be in turn β-oxidized and subsequently mineralized to CO_2 (Callaghan et al., 2006; Gieg and Agrawal, 2013).

This mechanism was observed for sulfate- reducing bacteria (Aitken et al., 2013; Cravo-Laureau et al., 2005; Davidova et al., 2005; Kropp et al., 2000) and for a denitrifying isolate (Rabus et al., 2001; Wilkes et al., 2003; Wilkes et al., 2002). In contrast, the anaerobic transformation of isoalkanes is scarcely reported so far. Pristane and phytane, both are branched alkanes and therefore more recalcitrant than *n*-alkanes, were frequently used as biomarkers indicating the extent of biodegradation of alkanes in crude oils (Huang and Larter, 2005).

The key enzyme of the oxygen-independent, stereospecific hydroxylation (Figure 1.6.) of ethylbenzene to (*S*)-1-phenylethanol is the ethylbenzene dehydrogenase (EBDH), characterized from the denitrifying bacterium *Azoarcus* sp. strain EbN1 (to be renamed *Aromatoleum aromaticum*) (Szaleniec et al., 2007). Then, (*S*)-1-phenylethanol is oxidized to acetophenone by a (S)-1-phenylethanol dehydrogenase (Kniemeyer and Heider, 2001b). Acetophenone is further carboxylated to benzoylacetate by an ATP-dependent carboxylase which is finally activated to benzoylacetyl-CoA and thiolytically cleaved to acetyl-CoA and benzoyl-CoA (Heider, 2007; Rabus et al., 2002).

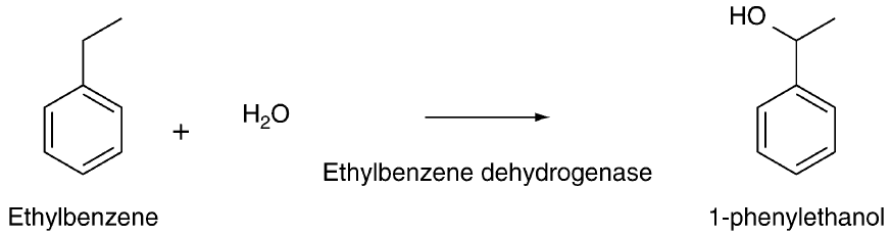

Figure 1.6: Hydrocarbon activation reactions via oxygen-independent hydroxylation (Kaser and Coates, 2010).

The methylation of naphthalene was observed in a sulfate-reducing culture N47 which can metabolize naphthalene or 2-methylnaphthalene as the sole carbon source and electron donor. Initially, naphthalene is methylated to 2-methylnaphthalene which in turn is subsequent oxidized to the central metabolite 2-naphthoic acid. Finally fumarate addition and β-oxidation lead to succinyl-CoA and naphthoyl-CoA (Heider, 2007; Safinowski and Meckenstock, 2006). Three enzymes are involved in anaerobic degradation of

2-methylnaphthalene to 2-naphthoic acid, a naphthyl-2-methyl-succinate synthase, a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase and a naphthyl-2-methyl-succinyl-CoA dehydrogenase (Safinowski and Meckenstock, 2006).

The alkane activation via carboxylation pathway (Figure 1.7.) as alternate mechanism to fumarate addition is proposed by So et al. (2003) in that alkanes are anaerobically oxidized to fatty acids by a sulfate-reducing bacterium strain Hxd3. The alkane activation starts with the subterminal carboxylation step at the C-3 position leading to the elimination of the two adjacent terminal carbon atoms of the alkane by an unknown mechanism. They established further that C-odd and C-even alkanes are indeed transformed to C-even and C-odd fatty acids, respectively. The initially formed fatty acids can be β-oxidized and subsequently mineralized to CO_2 (So et al., 2003). A study from Callaghan et al. (2009) confirmed this mechanism by a *n*-hexadecane-degrading, nitrate-reducing enrichment culture producing similar intermediates. Further, it is conceivable that both alkane degradation pathways, fumarate addition and carboxylation reactions, occur simultaneously in anaerobic ecosystems (So et al., 2003).

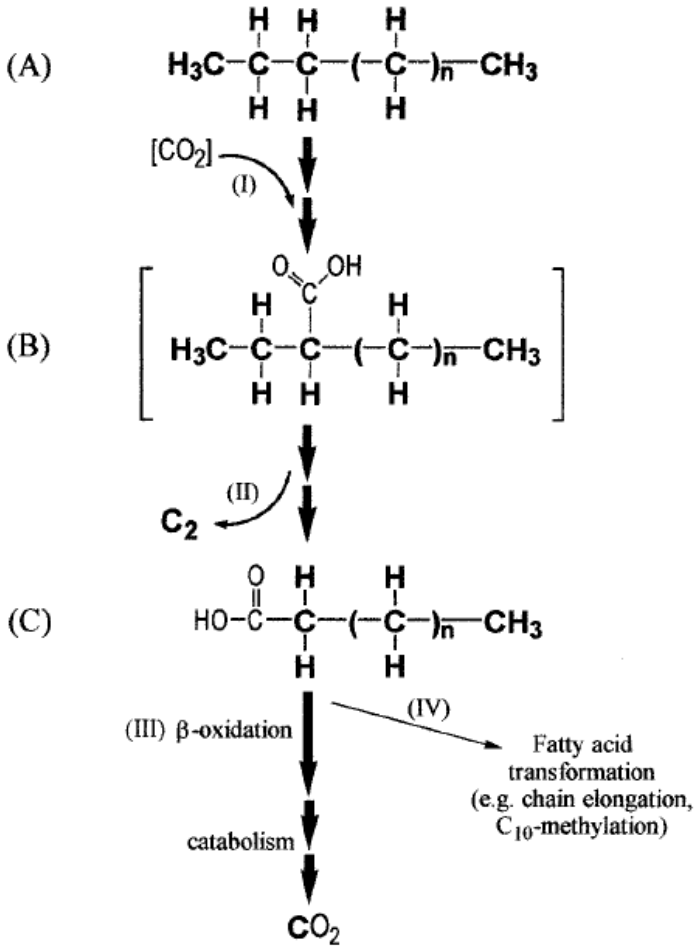

Figure 1.7: Proposed pathway for the alkane-oxidation to fatty acid. An alkane (A) is subterminally carboxylated at C-3 (step I) to form an intermediate (B). Two adjacent terminal carbon atoms are then eliminated (step II) to form a fatty acid one carbon shorter than the original alkane (C). This fatty acid can be beta oxidized (step III) and subsequently mineralized to CO_2 or undergo transformation, such as chain elongation and C-10 methylation (step IV). Compound B (in brackets) is only a hypothetical intermediate. Atoms originating from the alkane are shown in bold type (So et al., 2003).

1.2.4. Syntrophic interactions and *in situ* hydrocarbon biodegradation

Syntrophy is the thermodynamic-based interaction between microbial species, and plays an essential role in the anaerobic degradation of organic matter or hydrocarbons to CH_4 and CO_2, along with the fermentative and methanogenic microorganisms (McInerney et al., 2007) (Figure 1.8.). Even though the understanding of the metabolism of syntrophs is extremely little because so few strains are available in pure co-culture (only harbors the syntrophic partners) it is known that *Syntrophus* sp., a member of the *Deltaproteobacteria* lineage, lives in syntrophy with hydrogen / formate-using methanogens or sulfate-reducing bacteria. The genome sequence from *Syntrophus* sp. suggests the ability of metabolism of aromatic and aliphatic compounds (McInerney et al., 2007). Moreover, members of the

family *Syntrophaceae*, especially *Syntrophus* sp., have been identified as dominant organisms in hydrocarbon-associated systems like biodegraded oil reservoirs (Grabowski et al., 2005), deep coal seam groundwater (Shimizu et al., 2007), freshwater sediment (Zengler et al., 1999), or petroleum-contaminated sediment (Allen et al., 2007).

Jones et al. (2008) found out that in petroleum reservoirs methanogenic hydrocarbon degradation occurs predominantly via syntrophic oxidation of *n*-alkanes to acetate and hydrogen. Then, commonly acetate is oxidized syntrophically to CO_2 and H_2 before the conversion to methane through hydrogenotrophic methanogenesis is performed. The syntrophic oxidation of acetate has been suggested as an alternative to acetoclastic methanogenesis and it converts a large proportion of the acetate generated from hydrocarbon degradation. This process occurs at mesophilic conditions but is more thermodynamically favorable at higher temperatures (Schink, 1997).

The growth of syntrophic bacteria is thermodynamic sustainable only with the efficiently removal of the intermediates by the methanogens (Dolfing et al., 2008). Depending on thermodynamic conditions, homoacetogenesis or syntrophic acetate oxidation are possible reactions within this syntrophic degradation process. With increasing H_2 concentration, increasing pH of more than 7 and low temperature, the free energy change benefits the homoacetoclastic bacteria converting H_2 / CO_2 to acetate. Vice versa, low H_2 concentrations, a pH of less than 7 and high temperature promote the formation of H_2 / CO_2 from acetate by syntrophic acetate oxidation (Thauer et al., 2008).

Consequently, a close contact is essential for the metabolite transfer between the different syntrophic partners (Schink, 1997) and some of them are able to form aggregates or biofilms with each other.

1.2.5. Hydrocarbon biodegradation in petroleum reservoirs

Not only the microbiology and metabolic pathways of the anaerobic utilization of individual single hydrocarbons have been studied so far. Moreover, the ability to metabolize complex hydrocarbons mixtures such as petroleum with or without sulfate or nitrate as electron acceptor are described in numerous studies (Anderson and Lovley, 2000a; Jones et al., 2008; Rabus and Widdel, 1996; Reuter, 1994; Zengler et al., 1999).

Worldwide, crude oil in subsurface petroleum reservoirs is degraded over geological time by anaerobic microorganisms. The consequence of biodegradation is the decline in oil quality and value through the decrease of aliphatic and aromatic hydrocarbons and the relative increase of heavy oil fractions containing heterocyclic sulfur-, oxygen- and nitrogen-rich compounds. Through the increase of viscosity, metals content, total acid numbers and other non-hydrocarbon compounds, the *in situ* biodegradation leads to an increase in the costs of recovery and refining of that oil (Meyer, 1987; Wenger et al., 2001). Based on high alteration level through biodegradation and limits of conventional technology of exploitation, circa 50 % of the initial oil is unrecoverable (Youssef et al., 2009). Examples for highly degraded reservoirs are the Alberta tar sands (Canada) and Eastern Venezuelan Foreland Basins - the largest single accumulations of supergiant tar sands. Tar sands are sandstones saturated with heavy or super-heavy oil (Head et al., 2003).

Wenger and Isaksen (2002) pointed out that the first attack of biodegradation is on the *n*-alkanes in the approximately C_8-C_{15} range, heavy polar and asphaltene are left behind in the residual oil. In non-degraded petroleum reservoirs (possibly prevented through reservoir temperatures above 90°C) the complete range of *n*-alkanes are intact. With increasing biodegradation, the *n*-alkanes are more and more depleted. In heavy and severely degraded oil *n*-alkanes and isoprenoids are completely removed (Figure 1.9.).

According to Head et al. (2003), the general sequence of removal of saturated hydrocarbon types during biodegradation is *n*-alkanes, alkylcyclohexanes, acyclic isoprenoid alkanes and bicyclic alkanes-steranes-hopanes. During the degradation of aromatic hydrocarbons, alkylbenzenes are earlier removed than diaromatic and triaromatic hydrocarbons (Volkman et al., 1984). Aromatic steroid hydrocarbons are biodegradable-resistant until very severe levels of biodegradation are achieved (Head et al., 2003; Peters and Moldowan, 1993). Consequently, methane as well as non-hydrocarbon products like carbon dioxide, sulfide, low-molecular-weight compounds and naphthenic acids, phenols, and high-molecular-weight oxidized compounds are possible products of petroleum biodegradation (Wenger and Isaksen, 2002).

During the last decades several studies investigated the biodegradation of hydrocarbons in many different oil reservoirs all over the world and described general trends and the specific parameters of biodegradation (Aitken et al., 2004; Atlas and Atlas,

1991; Davidova and Suflita, 2005; Gieg et al., 2010; Head et al., 2003; L'Haridon et al., 1995; Lenchi et al., 2013; Magot et al., 2000; Milkov, 2011). But until today, little is known about how the associated microbial populations utilize oil hydrocarbons under extreme conditions and whether oil reservoirs harbor an truly indigenous microbiota (Magot et al., 2000), because it is difficult and costly to obtain uncontaminated samples from deep oil reservoirs where no microbes have been introduced into the reservoir by processes like drilling or water-flooding. (Gray et al., 2010). For example, the injection of marine, meteoric, or natural formation waters is used to preserve the reservoir pressure for oil production (Foght, 2010).

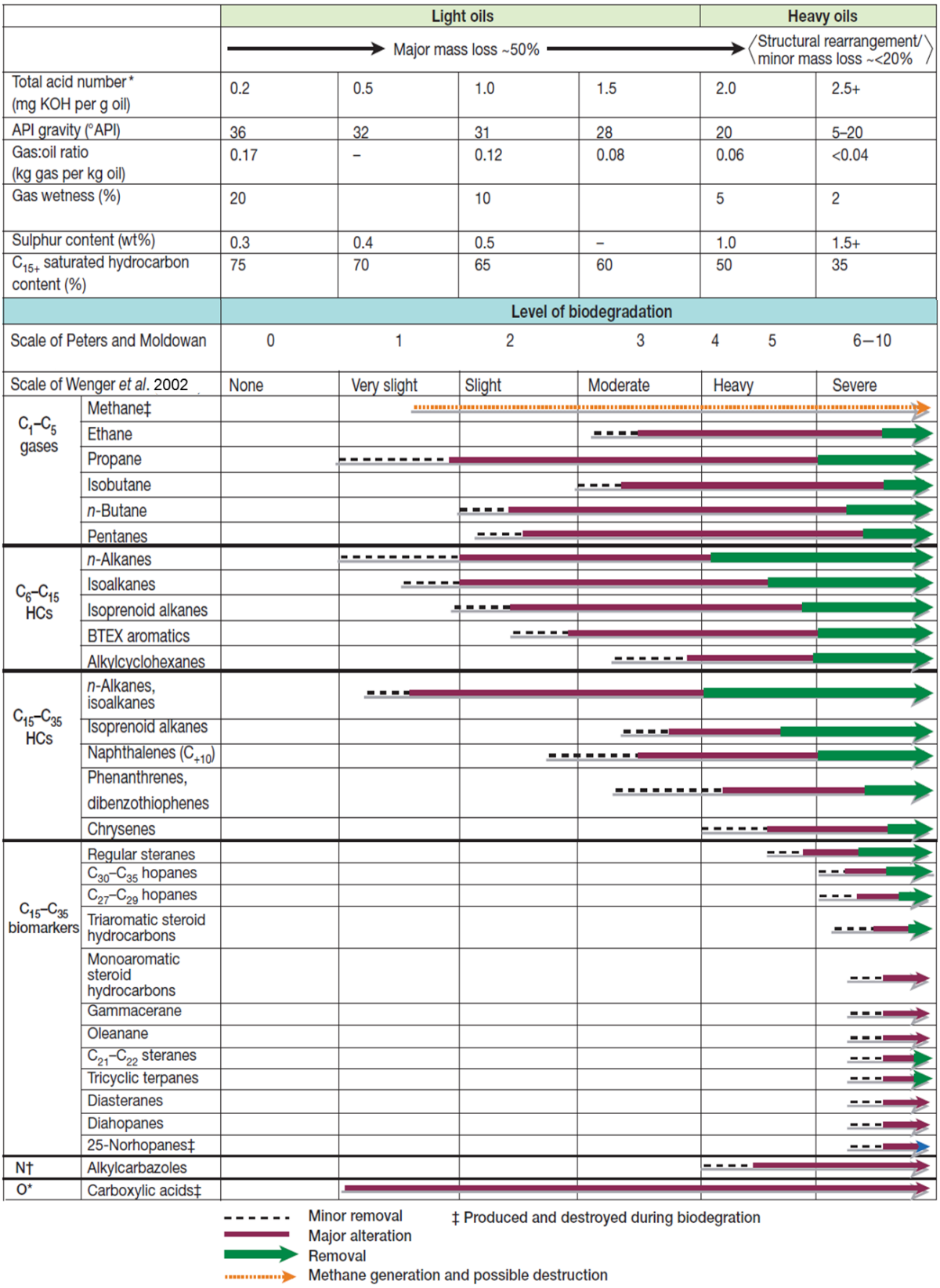

Figure 1.8: Schematic diagram of physical and chemical changes occurring during crude oil and natural gas biodegradation, previously published by Head et al. (2003). Further, data were used for this figure published by Peters and Moldowan (1993); Wenger et al. (2002); * Thorn and Aiken (1998); † Peters and Fowler (2002).

The oil recovery practice of water-flooding with marine waters introduces sulfate, nitrogen and phosphor into the reservoirs and thereby creates a condition for the growth of sulfate-reducing bacteria which leads to the production of hydrogen sulfide (H_2S) and to reservoir souring and/or corrosion problems (Vance and Thrasher, 2005).

Considering the large alteration patterns and deleterious effects on degraded petroleum reservoirs, it is obviously important to better understand microbial hydrocarbon degradation processes in subsurface petroleum reservoirs in order to minimize the negative effects. Moreover, with this knowledge, positive microbial effects can be generated such as the prevention of reservoir souring. Additionally, in many reservoirs a frequent increase of biogenic methane was observed, pointing to the presence and activity of methanogenic microorganisms and the fact that methane is the major end-product of the *in situ* oil biodegradation (Gray et al., 2009; Head et al., 2003; Jones et al., 2008). The microbial conversion of parts of unaccessible residual oil in petroleum systems into methane might provide a new strategy for microbial enhanced oil recovery (MEOR) (Gray et al., 2010; Kaster et al., 2009). Moreover, the anaerobic biodegradation of hydrocarbons is further important for the intrinsic remediation of spilt fuels, due to aged pipelines, storage tank leaking, or natural oil leakage and for the earth´s carbon cycle (Suflita and Duncan, 2011).

In general, the biodegradation of hydrocarbons depends on many factors such as the structures and concentrations of oil and gas, the presence of water, nutrients, oxidants, microbial community and the prevailing temperature, salinity and acidity in the oil reservoir (Wenger et al., 2002). It is to notice that different microbes degrade different kinds of hydrocarbons under various ranges of conditions. For biodegradation in oil reservoirs, water, nutrients and hydrocarbons are most likely available near the oil-water contact in the reservoir (Head et al., 2003). Temperature and salinity are very important parameters, as the microbial degradation activity is greatly higher in reservoirs below 50°C and decreases with increasing temperatures and ceases around 80°C (Larter, 2003). Moreover, the salinity affects the tolerable temperature, higher salt contents lower the maximum temperature at which biodegradation can occur. The maximum limits of salinity and temperature for microbial degradation are difficult to assign (Wenger and Isaksen, 2002).

With the combination of culture-dependent and culture-independent methods a broad diversity of microbes are detected in mesophilic (15-40°C) and thermophilic (45-80°C) petroleum reservoirs as well as in highly saline oil well waters at mesophilic temperatures; a huge part of them are novel bacteria and archaea species (Gieg et al., 2010; Li et al., 2007; Nazina et al., 2006; Orphan et al., 2000). The discovered anaerobic microorganisms include several physiological groups such as diverse fermentative bacteria, nitrite-, iron-, manganese- and sulfate-reducing bacteria, acetogens, and methanogenic archaea are believed to be linked to hydrocarbon degradation but relatively few of them have been isolated (Aitken et al., 2004; Lovley, 1989; Magot et al., 1994; Reuter, 1994; Zengler et al., 1999)

1.2.6. Hydrocarbon biodegradation in coal deposits

Several studies investigated the microbial degradation of coal deposits and formation water, and, moreover, the biogenic gas generation via methanogenesis.

Formolo et al. (2008), Flores et al. (2008) and Ulrich and Bower (2008) showed the natural microbial gas production in the Powder River Basin and San Juan Basin (USA), two of the most productive coalbed methane reserves in the world. The investigated coalbed reservoirs in the Powder River Basin range from lignite to low-volatile subbituminous coal; the San Juan Basin is characterized by higher rank coals. The authors noticed that the biogenic gas generation is linked to incursion of meteoric water.

The influx of water brings in nutrients, essential minerals and microbes through the seam stimulates the microbial biodegradation of hydrocarbons in particular zones depending on stratigraphic variations and fractures in the subsurface of the coal seam system (Formolo et al., 2008). In different zones of biodegradation which are dispersed in particular depths of the Powder River Basin, an extensive removal of short-, medium-, and long-chain *n*-alkanes and acyclic isoprenoids was observed, concurrent with conventional biodegraded petroleum (Formolo et al., 2008).

Furthermore, Flores et al. (2008) emphasized the importance of geological factors like the direction of groundwater recharge, depth of burial, thermal and maturation history, lateral and vertical continuity of stratigraphic units, degree of faulting and fracturing, and coalification processes, affect the generation and composition of microbial hydrocarbon degradation processes in the coal seam.

The aromatic structured high-molecular-weight biopolymers cellulose and lignite, the main compounds of the maturated coal, are fermentable by bacteria to important intermediates such as succinate, propionate, formate, acetate, methanol, CO_2 and H_2. (Strąpoć et al., 2008). Methanogens, all of which are archaea, are able to utilize these intermediates (C_1- and C_2-substrates) for methane production via two common methanogenic pathways. In the acetoclastic pathway, acetate is converted to methane and CO_2. In the hydrogenotrophic pathway (CO_2-reduction methanogensis) CO_2 is reduced using H_2 to produce methane and water. A simplified illustration as a example of lignite structure and the possible biodegradation pathways are shown in Figure 1.10.

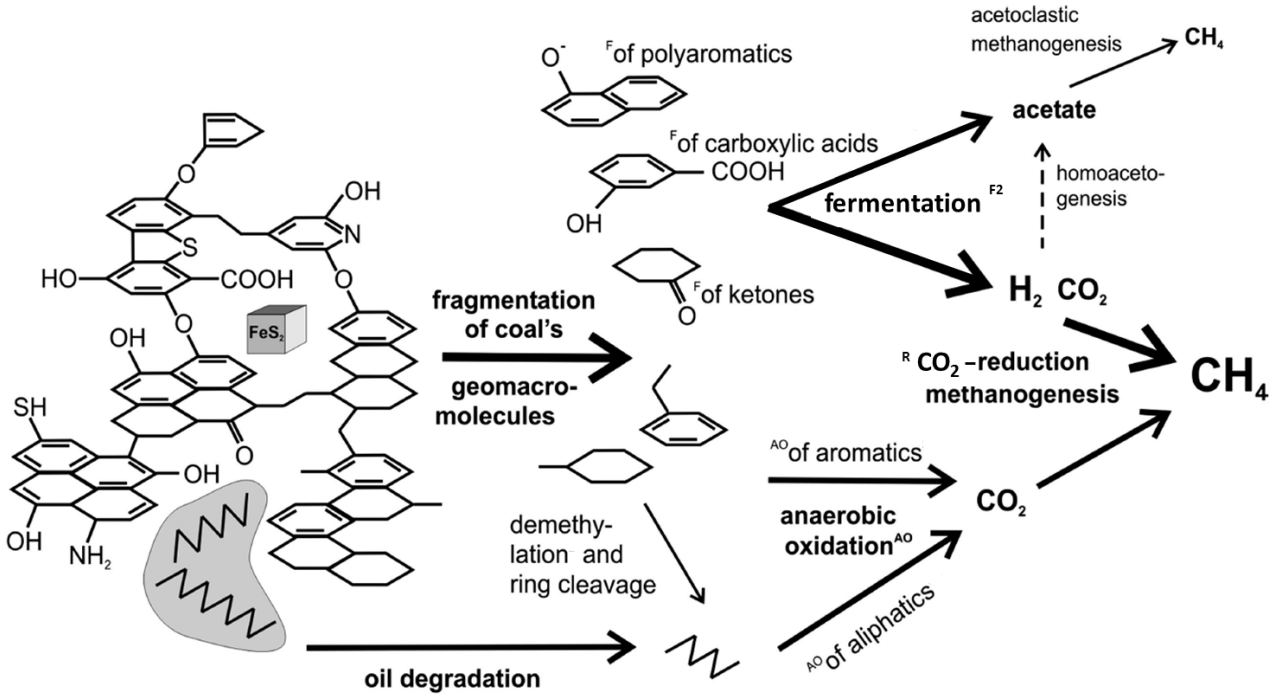

Figure 1.9: Scheme of proposed mechanisms of stepwise biodegradation of organic matter in coal showing potentially capable microbial degradation processes: (F) defragmentation of coal geomacromolecular structure predominately by fermentation targeted at oxygen-linked moieties and oxygen-containing functional groups (this process detaches some of the oxygen-linked aromatic rings and generates some short organic acids); (AO) anaerobic oxidation of available aromatic and aliphatic moieties, derived from coal defragmentation or from dispersed oil; (F2) fermentation of products available from step F described above to H_2, CO_2, and acetate; and (R) methanogenesis utilizing H_2 and CO_2 likely predominating over homoacetogenesis and acetoclastic methanogenesis. The dark area represents a droplet of oil. This scheme is modified after Strąpoć et al. (2008).

Several studies taken around the world based on isotopic measurements, cultivation and molecular biological approaches showed similarities of the origin of coalbed methane. Nowadays, hydrogenotrophic methanogenesis (CO_2-reduction pathway) is generally believed to be the dominant methanogenic pathway in subsurface coal associated sediments and/or formation waters (Adams et al., 2006). Examples supporting this assumption are the Powder River and San Juan Basin (Wyoming and Colorado, USA) (Flores et al., 2008; Formolo et al., 2008), Illinois Basin (Indiana, USA) (Strąpoć et al., 2007), Jharia coal field (Parbatpur, India) (Singh et al., 2012), Gippsland Basin (Victoria, Australia) (Midgley et al., 2010), Sydney and Bowen Basin (Australia) (Ahmed and Smith, 2001), Surat Basin (Queensland, Australia) (Papendick et al., 2011), or Ishikari coal field (Hokkaido, Japan) (Shimizu et al., 2007).

Reasons for the abundance of hydrogenotrophic methanogenic activity in coal seams could be the availability of CO_2 through microbial oxidation and decarboxylation processes during degradation of organic matter and hydrocarbons. The supply of high amounts of CO_2 promotes hydrogenotrophic methanogens. Only few studies described the presence of acetoclastic methanogenesis next to hydrogenotrophic methanogenesis in coal seams. Examples are given by the Alberta coal beds (Alberta, Canada) (Penner et al., 2010), and Powder River Basin (Wayoming, USA) (Green et al., 2008).

Whereas the biodegradation in petroleum reservoirs is well established (Head et al., 2003), the anaerobic biodegradation processes in the coal-bearing sediments and the aquatic systems lying beneath these coal-rich layers are less well known. Moreover, geochemical and isotopic indicators are established to detect and classify microbial methane in the coal basins (Formolo et al., 2008), but the combustion of organic matter leading to methanogenesis and the indigenous microbial consortia which are involved in biodegradation processes remain poorly understood.

Furthermore, the majority of investigated coal reservoirs are composed of a range of subbituminous to low-volatile bituminous coal (e.g. (Beckmann et al., 2011a; 2011b; Dawson et al., 2012; Krüger et al., 2008; Strąpoć et al., 2011b)). The knowledge about anaerobic biodegradation, methanogenic processes and microbial community composition in lignite-containing coal beds is still scarce. A more profound understanding about indigenous coal-associated microbial consortia and methanogenesis processes could help

to control and modify *in situ* conditions in order to enhance microbial methane production resulting in increased methane recovery on economic levels.

1.3. Methanogenic processes

Methanogenic archaea are widespread in anoxic environments, such as freshwater lakes, marine sediments, wetlands, rice field soils, landfills, and intestinal tracts of ruminants and termites (Banning et al., 2005; Brune, 2010; Janssen and Kirs, 2008; Krüger et al., 2005a; Thauer, 1998; Zengler et al., 1999) and play, therefore, an important role in the global carbon cycle.

1.3.1. Hydrogenotrophic methanogenesis

Most of the methanogens are hydrogenotrophs, which reduce CO_2 to CH_4 with H_2 as the primary electron donor (Equation I). Also many of them are able to use formate as the major electron donor (Liu and Whitman, 2008).

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \qquad \Delta G^{\circ\prime} = -135\ kJ\ mol^{-1} \quad (I)$$

Members of the orders *Methanopyrales, Methanobacteriales, Methanococcales* and *Methanomicrobiales* only use H_2 / CO_2 as substrate to conserve energy for growth. An exception is *Methanosphaera stadtmanae;* it is a human intestinal archaeon and depends on methanol and H_2 as energy sources (Thauer et al., 2008). These hydrogenotrophic methanogens grow with a H_2 threshold concentration of 1-10 Pa. Many hydrogenotrophic methanogens are able to grow under hyperthermophilic conditions (Thauer et al., 2008).

The novel order of *Methanocellales*, affiliated with the clone lineage Rice Cluster I (RC-I), was isolated 2008 by Sakai et al. (2008) from an anaerobic, propionate-degrading enrichment culture, which was originally established from rice paddy soil. Members of *Methanocellales* are mesophilic and able to utilize H_2 / CO_2 and formate for growth and methane production.

1.3.2. Acetoclastic methanogenesis

The genera *Methanosaeta* and *Methanosarcina* are the only methanogens which are able to metabolize acetate as substrate for methanogenesis (Kendall and Boone, 2006). These two genera have different affinities to acetate and use different mechanisms for acetate activation. *Methanosaeta,* the only genus within the *Methanosaetaceae* family, uses only acetate as energy source even at concentrations as low as 5-20 µM (Jetten et al., 1992). *Methanosarcina* prefers methanol and methylamine over acetate for methanogenic growth, and many species are able to utilize H_2, as well (Liu, 2010). For acetoclastic methanogenesis *Methanosarcina* needs a minimum concentration of about 1 mM acetate (Jetten et al., 1992). Other relatives of *Methanosarcinaceae* family are *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanolobus*, *Methanomethylovorans*, *Methanosalsum*, and are able to grow by utilization of methylated compounds to produce CH_4 and CO_2 (Liu, 2010).

The free energy yield of the conversion of acetate to methane (Equation II) is considerable lower than that from CO_2-reduction. Moreover, the rate of acetoclastic methanogenesis is considerably slower than the rate of hydrogenotrophic methanogenesis (Blaut, 1994). Remarkably, even as acetate provides the smallest change of free energy of all substrates, these acetoclastic methanogens produce the most methane in anaerobic food chains (Deppenmeier and Müller, 2008). The conversion of H_2 and CO_2 is only possible with a H_2 partial pressure more than 10 Pa (Thauer et al., 2008).

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad \Delta G^{\circ\prime} = -33 \text{ kJ mol}^{-1} \qquad \text{(II)}$$

Furthermore, the species *Methanosarcina acetivorans* is able to use the acetyl-CoA pathway to conserve energy for growth by utilizing carbon monoxide to form acetate and formate, rather than methane. Moreover, with increasing CO partial pressure, the methane production decreased. Thus, CO is an inhibitor of methanogenesis for *M. acetivorans* (Deppenmeier and Müller, 2008).

1.3.3. Methylotrophic methanogenesis

The most versatile methanogenic archaea are the members of the order *Methanosarcinales*, which are capable of growing on H_2 / CO_2, acetate, methanol, methyl

group-containing compounds such as methylamines, and methylthiols as energy substrates (Deppenmeier, 2002). These methylotrophic methanogens are limited to the order *Methanosarcina*, except for *Methanosphaera* sp. as mentioned above. Equations III and IV show the methylotrophic conversion of methanol or methylamine to CH_4 (Liu and Whitman, 2008; Thauer, 1998).

$$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O \qquad \Delta G^{\circ\prime} = -105\ kJ\ mol^{-1} \qquad (III)$$

$$4\ CH_3\text{-}NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3 \qquad \Delta G^{\circ\prime} = -75\ kJ\ mol^{-1} \qquad (IV)$$

1.3.4. The seventh order of methanogens

In addition to the orders of methanogens mentioned above, a seventh order has been proposed. Investigations of Paul et al. (2012) showed robust evidence for the presence of *mcrA* genes, a functional marker for methanogenesis, in environmental clones affiliated to uncultured *Thermoplasmatales*. In enrichment cultures, methanogenesis was stimulated by the simultaneous supply of H_2 and methanol. The provisional name "*Methanoplasmatales*" is suggested (Paul et al., 2012).

1.3.5. Enzymatic reactions during methanogenesis

This is a short overview of the most important steps within the three different methane formation pathways. Since the 1980s, a number of researchers investigated enzyme kinetics, structure and activity of methanogenesis in detail. So far, the concept of the different methanogenic pathways is well understood. Thauer (1998) reported the uniqueness of the metabolic pathways of methane formation and the involvement of a number of unusual enzymes and coenzymes. Some time ago, Deppenmeier and Müller (2008) published a summary about the life close to thermodynamic limits and the specific way of methanogenic energy production. They postulated that the metabolism of H_2 / CO_2, formate, methylated C_1-compounds and acetate proceeds along a central, partly reversible pathway.

Methyl-S-CoM is the central intermediate of all metabolic pathways of methanogenesis. Through the catalytic influence of methyl-coenzyme M-reductase (MCR), methyl-S-CoM is reductively demethylated to CH_4. Electron donor is HS-CoB, providing the two electrons required in this process. The nickel-containing tetrapyrrole F_{430} is the

prosthetic group of MCR and it is necessary for its activity (Ermler, 2005). Finally, a heterodisulfide CoM-S-S-CoB is formed from HS-CoM (Coenzyme M, 2-mercaptoethanesulfonate) and HS-CoB (Coenzyme B, 7-mercaptoheptanoylthreoninephosphate) (Beifuss et al., 2000; Thauer, 1998). The membrane-bound heterodisulfide reductase catalyzes the reduction of the electron acceptor CoM-S-S-CoB back to the thiol-containing cofactors HS-CoM and HS-CoB (Deppenmeier and Müller, 2008).

Different proton-translocating enzyme systems are involved in the membrane-bound electron transfer (Beifuss et al., 2000). In the methanogenesis HS-CoB and coenzyme F_{420} function as electron carries. F_{420}, a central electron carrier in the cytoplasm of methanogens, replaces nicotinamide adenine dinucleotides in many reactions. For example, the F_{420}-nonreducing hydrogenase is part of the electron transport system, the H_2:heterodisulfide oxidoreductase system, and channel electrons to the heterodisulfide reductase in the presence of hydrogen (Deppenmeier and Müller, 2008). Methanophenazine, another membrane integral electron carrier (similar to quinones found in Bacteria and Eukarya), is exclusively found in cytochromes of *Methanosarcinales* and is a hydrophobic cofactor. HS-CoM, methanofuran (MFR), and tetrahydromethanopterin (H_4MPT) (Figure 1.11) work as carriers for C_1-moieties of intermediates in the methanogenic pathway (Deppenmeier and Müller, 2008).

During the hydrogenotrophic methanogenesis CO_2 is bound to MFR and is then reduced to formyl-MFR (Deppenmeier and Müller, 2008). The electrochemical ion gradient across the cytoplasmic membrane provides the energy for this endergonic reaction. Next, the formyl group is transferred to H_4MPT to form a formyl-H_4MPT bond, which is stepwise reduced to methyl-H_4MPT. Afterwards, the methyl-group is transferred to HS-CoM by a membrane-bound methyl-H_4MPT:HS-CoM-methyltransferase. This exergonic reaction is used to generate a electrochemical sodium ion gradient. In the final step the methyl-coenzyme M reductase (MCR) catalyzes the reduction of methyl-S-CoM with the electron donor HS-CoB in the presence of the coenzyme F_{430} (Ermler, 2005). The products are methane and the corresponding heterodisulfide CoM-S-S-CoB (Deppenmeier and Müller, 2008).

In the methylotrophic pathway trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA) are metabolized. By substrate-specific soluble

methyltransferases different cognate corriniod proteins are methylated that transfer the methyl group to coenzyme M (Paul et al., 2000). Consequently, the methyl groups are channeled into the central pathway which is split into a branch of reduction and one of oxidation. Methane is formed via HS-CoB-dependent reduction of methyl-S-CoM catalyzed by MCR, according to the hydrogenotrophic pathway. In the oxidative branch, the methyl group is transferred to H_4MPT catalyzed by the membrane-bound methyl-H_4MPT:HS-CoM-methyltransferase followed by the stepwise oxidation of methyl-H_4MPT to formyl-H_4MPT. Finally, the formyl group is transferred to MFR and the formyl-MFR dehydrogenase catalyzes the oxidation of formyl-MFR to CO_2 and MFR. Thus, the oxidative branch of the methylotrophic pathway is the reversal of CO_2-reduction to methyl-CoM as already mentioned in the hydrogenotrophic pathway (Deppenmeier and Müller, 2008).

At the beginning of the acetoclastic pathway, acetate is activated via phosphorylation and exchange of inorganic phosphate with CoA. Acetyl-CoA is produced. The cleavage of the acetyl-CoA is performed by the nickel-containing carbon monoxide dehydrogenase/acetyl-CoA synthase, yielding enzyme-bound methyl and carbonyl groups (Drennan et al., 2004). During the oxidation of the enzyme-bound CO to CO2, the methyl group of acetate is transferred to H_4MPT. The yielding electrons are used for ferredoxin (Fd) reduction. The resulting methyl-H_4MPT is converted to methane catalyzed by a Na^+-translocating methyl-CoM methyltransferase and the methyl-S-CoM reductase, which is used HS-CoB as the electron donor for the reduction of the methyl group. The CoM-S-S-CoB and the reduced ferredoxin are recycled by an electron transport system referred to as Fd:heterodisulfide oxidoreductase (Welte et al., 2010).

The methyl-coenzyme M reductase (MCR) is exclusive to methanogens, excluding the methane-oxidizing *Archaea* (ANME). Members of the ANME groups are related to each other and to methanogenic *Archaea*, specifically the *Methanosarcinales* (Nunoura et al., 2006). They own a nickel protein similar to methyl-coenzyme M reductase and are possibly able to perform reverse methanogenesis (Krüger et al., 2003; Scheller et al., 2010).

In addition to 16S rRNA genes, the methyl-coenzyme M reductase alpha-subunit (*mcrA*) gene has been applied as a phylogenetic marker for methanogens (Springer et al., 1995).

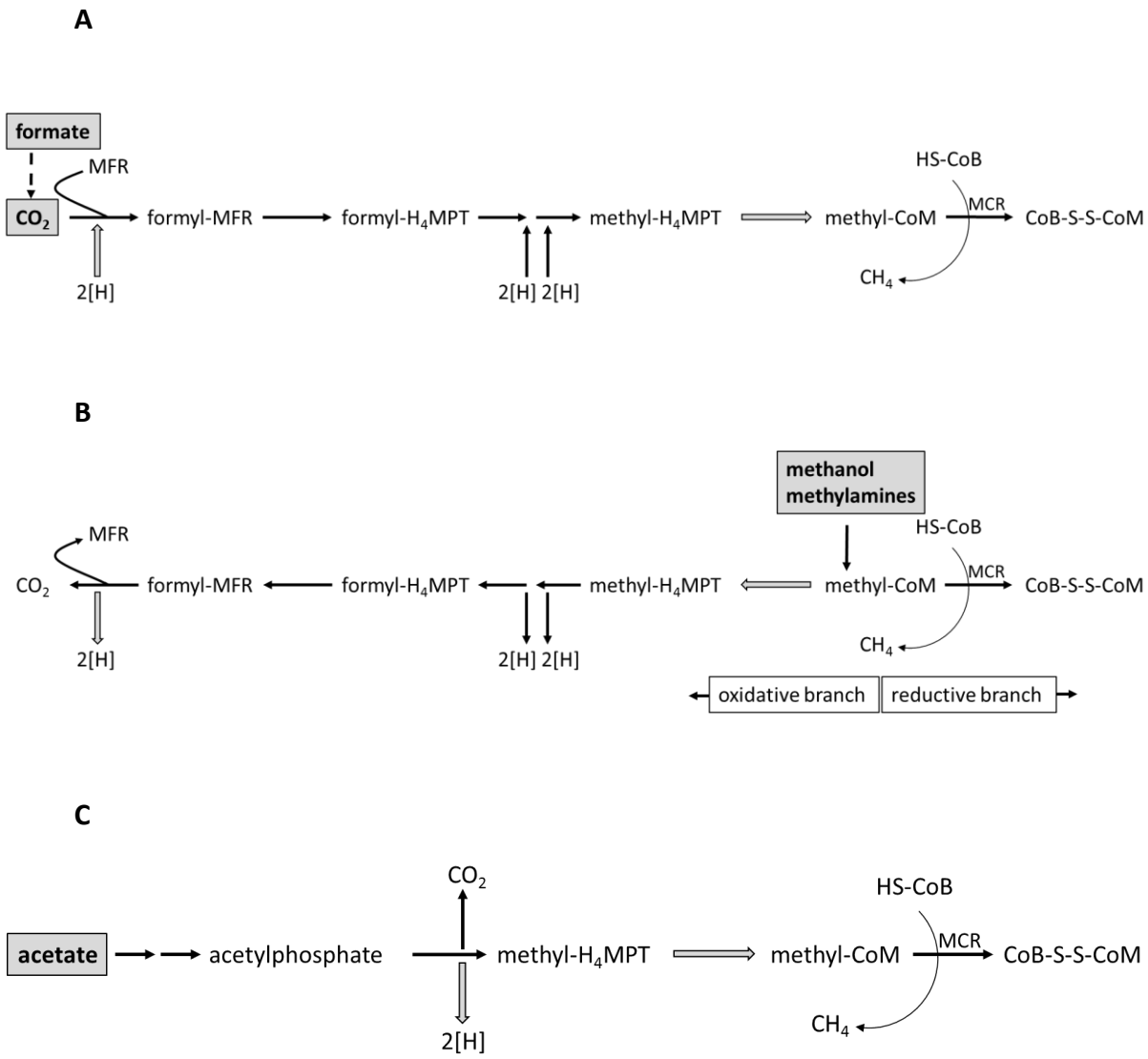

Figure 1.10: Schemes of enzymatic pathways of methanogenesis from formate or H_2 / CO_2 (**A**), methyl group containing substrates (**B**) or acetate (**C**); grey arrows denote reactions catabolyzed by membrane-bound, ion-translocated enzymes; MFR: methanofuran; H_4MPT: tetrahydromethanopterin; CoM: Coenzym M; CoB: Coenzyme B; MCR: methyl-coenzyme M reductase; modified after (Deppenmeier and Müller, 2008).

1.3.6. Cultivation methods and phylogenetic analysis of anaerobic microbes

To study the microbial diversity, the community composition, or individual microorganisms which are able to degrade hydrocarbons several approaches are available. To get a detailed and reliable data set it is recommended to combine the particular information which is offered by each approach.

Based on the different atomic weights, the slightly different physicochemical behaviors of the discovered stable isotopes in the nature or in laboratory microbial cultures lead to a better scientific understanding of how and where these isotopes were formed (see also Section 1.1.5.). Consequently, through the measurement of the isotopic signature of organic compounds such as carbon or hydrogen, the biogenic or geothermal origin can be provided. Furthermore, with the analysis of stable isotope composition of fossils, rocks and minerals the reconstruction of the carbon cycles and prevalent redox reactions during earth´s history can be performed.

With cultivation based approaches, the physiological and environmental parameters which influence a microbial community are simulated or amended with additions like alternative substrates, electron acceptors, trace elements, vitamins or inhibitors. Then, the microbial adaption, growth and activity are studied by measuring alteration of the initial conditions e.g. hydrocarbon composition of crude oil, CH_4 and CO_2 production, sulfate concentration, fractionation factor of methane. Previous studies showed the cultivation of methanogenic microorganisms and their associated (syntrophic) bacterial community. But most of the methanogens are fastidious organisms that are very difficult to cultivate under laboratory conditions, one reason for the nowadays huge number of microorganisms, which are uncultivable and not describable. Moreover, the knowledge about the characteristics and abilities of cultivated microbial communities cannot simply be transferred to complex biological systems, because microbial communities are commonly influenced by environmental factors and other microorganisms harboring an environment.

Molecular biological techniques such as the quantitative *real time* PCR (Q-PCR) or terminal restriction fragment polymorphism (T-RFLP) can be used to quantify the microbial community, within certain environment or laboratory culture, but they give no information about the microbial activity of the different groups or species. Q-PCR is a highly sensitive method, in which universal primers for 16S rRNA genes are applied or functional primers for genes coding for specific key enzyme to identify and quantify groups of microorganisms

like bacteria, archaea, sulfate-reducers or methanogens. T-RFLP is a genetic fingerprinting method used for inferring the relative composition of microbial communities; in combination with 16S rRNA sequence analysis the identification of single T-RFs, i.e. single community members, is possible.

With ^{13}C-labeled compounds as substrates for the laboratory microcosms the hydrocarbon degradation pathways, the kinetics and their conversion to metabolic intermediates can be studied. Moreover, *in situ* experiments with ^{13}C-labeled compounds incubated directly in the environment are possible.

To identify the active hydrocarbon-degrading microorganisms and their benefit from the added substrates, samples from the enriched microcosm or environment are taken in time intervals and DNA, RNA or proteins are extracted and analyzed via stable isotope probing technique (SIP). The incorporation of the ^{13}C-label from the substrate into the microbial metabolism and biomass is shown. The SIP technique is a powerful tool to increase the understanding of the role of specific microbial community members in diverse environments and the key microbes of hydrocarbon degradation (Lueders et al., 2004). Furthermore, the labeled carbon atoms which originate from ^{13}C-labeled substrates which have passed the microbial metabolism and are converted into intermediates (e.g. formate, acetate or CO_2) and end products (e.g. CH_4 or CO_2) are measurable and reveal the microbial hydrocarbon degradation activity and the methane formation pathways.

To identify the phylogentic affiliation of microbial species, the molecular analysis of 16S rRNA is used (Woese et al., 1975). The 16S rRNA sequence analysis allows the rapid description of a phylogenetic variety of microbial communities. Additionally, 16S rRNA sequences obtained from communities that occur in different environments can be clustered into “operational taxonomic units” (OTUs). Sequences from organisms sharing less than 97 % identity of their ribotypes are summarized into clusters, e.g. Rice Cluster, Marine Benthic Group or Miscellaneous Crenarchaeotic Group.

1.4. Motivation and main objectives of this work

This thesis was accomplished in the framework of the DFG priority program SPP 1319 "Biological transformations of hydrocarbons without oxygen: from the molecular to the global scale". The SPP started in July 2008 as a joint interdisciplinary collaboration of research groups in the fields of ecophysiology, microbiology, biogeochemistry, biochemistry, chemistry, structural biology and biophysics of microbial anaerobic hydrocarbon degradation. The biological transformation and degradation of hydrocarbons as well as the formation of methane are playing an important role in the global carbon cycle. In many cases, methanogenesis is a final step of the microbial hydrocarbon degradation to methane under anaerobic conditions, occurring e.g. in oil reservoirs, coal mines, and anoxic sediments and soils.

In particular, the objectives of this thesis are:

(I) The basis of this work is the enrichment of anaerobic microbial communities from samples of different ecosystems able to degrade coals, oils or selected alkanes and aromatic hydrocarbons under methanogenic conditions.

(II) Furthermore, molecular genetic analyses from original and cultured hydrocarbon-enrichment samples will be used to compare the microbial community composition of the enrichments with those in hydrocarbon-rich geosystems (e.g. oil reservoirs and coal-bearing sediments), pristine nutrient-rich (e.g. pristine limnic and marine sediments) and nutrient-poor habitats (e.g. arctic sediments).

(III) The investigation of physiological characteristics of selected hydrocarbon-degrading enrichment cultures will be carried out to determine possible limiting and stimulating factors, and the impact of varying environmental conditions, like the changes of temperature and pressure or the availability of electron acceptors, on the conversion of hydrocarbons to methane.

1.5. Overview of publications and author contributions

In the following chapters the results of the conducted research summarized in articles for scientific journals are given.

Chapter 2: ***Evidence for in situ methanogenic oil degradation in the Dagang oil field***

Nuria Jiménez, Brandon E.L. Morris, Minmin Cai, Friederike Gründger, Jun Yao, Martin Krüger, Hans H. Richnow

Published in Organic Geochemistry 09/2012; 52: 44–54

Author contributions: M. Krüger and H. Richnow designed the project. F. Gründger and M. Krüger planned and performed the cultivations experiments and the analytical measurements. M. Cai assisted in the experiment set up and measurements. F. Gründger carried out the molecular analysis, and together with B. Morris and N. Jiménez evaluated and interpreted the data. M. Cai and N. Jiménez performed the isotopic analysis of reservoir fluids, the chemical analysis of oil, and the subsequent evaluation and interpretation of the data. B. Morris, H. Richnow, M. Krüger and N. Jiménez wrote the manuscript.

Chapter 3: ***Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany***

Friederike Gründger, Hans H. Richnow, Thomas Thielemann, Martin Krüger

Manuscript in preparation (for submission to *Frontiers in Microbiology*)

Author contributions: M. Krüger and T. Thielemann designed the project. F. Gründger planned, performed parts of the cultivation, the analytic measurements, the molecular analyses and the interpretation of the data. H. Richnow accomplished the isotopic measurements. F. Gründger and M. Krüger wrote the manuscript.

Chapter 4: ***Similar features of microbial consortia from different ecosystems degrading hexadecane under methanogenic conditions***

Friederike Gründger, Michael Siegert, Hans H. Richnow, Frederick v. Netzer, Tillmann Lüders, Martin Krüger

Manuscript in preparation (for submission to *FEMS Microbiology Ecology*)

Author contributions: M. Krüger designed the project. F. Gründger planned and performed parts of the cultivation, the analytic measurements, the molecular analyses, the evaluation and interpretation of the respective results. M. Siegert assisted in cultivation. H. Richnow accomplished the isotopic measurements. F. Gründger and F. v. Netzer performed the T-RFLP analysis, the 454-pyrosequences and F. v. Netzer helped with interpretation of the data set under supervision of T. Lüders. F. Gründger and M. Krüger wrote the manuscript.

Chapter 5*: Isotopic fingerprinting of methane and* CO_2 *formation from aliphatic and aromatic hydrocarbons*

Stefan Feisthauer, Michael Siegert, Martin Seidel, Hans H. Richnow, Karsten Zengler, Friederike Gründger, Martin Krüger

Published in *Organic Geochemistry* 05/2010; 41 (5): 482–490

Author contributions: S. Feisthauer prepared the manuscript and was in charge of the field sampling and isotope measurements of the field samples. M. Seidel performed the isotope measurements. K. Zengler and M. Krüger conducted the cultivation approaches and contributed to the preparation of the manuscript. F. Gründger performed parts of the enrichment cultures and a part of the molecular experiments. M. Siegert and H. Richnow contributed to data interpretation and manuscript preparation. H. Richnow and M. Krüger designed the project and reviewed the manuscript.

Chapter 6*: Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron- and sulfate-reducing conditions*

Michael Siegert, Danuta Cichocka, Steffi Herrmann, Friederike Gründger, Stefan Feisthauer, Hans H. Richnow, Dirk Springael, Martin Krüger

Published in *FEMS Microbiology Letters* 02/2010; 315 (1): 6–16

Author contributions: M. Siegert designed the experimental set-up, conducted the experiments except the naphthalene experiment as well as parts of the cloning and wrote the manuscript. D. Chichocka and S. Herrmann conducted the naphthalene experiment. S. Herrmann, S. Feisthauer contributed to the preparation of the manuscript. S. Feisthauer contributed data to an early version of the manuscript. F. Gründger contributed to the preparation of the clone libraries. H. Richnow and D. Springael contributed to the design of the naphthalene experiment and the manuscript. M. Krüger contributed Eckernförde data and reviewed the manuscript.

Chapter 7*: Geochemistry and microbial populations in sediments of the northern Baffin Bay, arctic*

Camelia Algora, Friederike Gründger, Volkmar Damm, Hans H. Richnow, Lorenz Adrian, Martin Krüger

Published in *Geomicrobiology Journal* 01/2013; 30 (8): 690-705

Author contributions: M. Krüger designed the project. F. Gründger and C. Algora collected the samples and performed the Q-PCR analysis. F. Gründger performed parts of the enrichment cultures amended with hydrocarbons. H. Richnow and L. Adrian contributed to data interpretation and manuscript preparation. C. Algora wrote the manuscript with help of F. Gründger and M. Krüger. L. Adrian reviewed the manuscript.

Publications not included in this thesis

Extended gene detection assays for fumarate-adding enzymes allow uncovering anaerobic hydrocarbon degraders in terrestrial and marine systems

Frederick v. Netzer, Giovanni Pilloni, Sarah Kleindienst, Martin Krüger, Katrin Knittel, Friederike Gründger, Tillmann Lüders

Published in *Applied and Environmental Microbiology* 01/2013; 79 (2): 543–552

Chapter II

Evidence for in situ methanogenic oil degradation in the Dagang oil field

2. Evidence for *in situ* methanogenic oil degradation in the Dagang oil field

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Organic Geochemistry 09/2012; 52: 44–54
DOI:10.1016/j.orggeochem.2012.08.009

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Abstract

In situ biotransformation of oil to methane was investigated in a reservoir in Dagang, China using chemical fingerprinting, isotopic analyses, and molecular and biological methods. The reservoir is highly methanogenic despite chemical indications of advanced oil degradation, such as depletion of *n*-alkanes, alkylbenzenes, and light polycyclic aromatic hydrocarbon (PAH) fractions or changes in the distribution of several alkylated polycyclic aromatic hydrocarbons. The degree of degradation strongly varied between different parts of the reservoir, ranging from severely degraded to nearly undegraded oil compositions. Geochemical data from oil, water and gas samples taken from the reservoir are consistent with *in situ* biogenic methane production linked to aliphatic and aromatic hydrocarbon degradation. Microcosms were inoculated with production and injection waters in order to characterize these processes *in vitro*. Subsequent degradation experiments revealed that autochthonous microbiota are capable of producing methane from ^{13}C-labeled *n*-hexadecane or 2-methylnaphthalene, and suggest that further methanogenesis may occur from the aromatic and polyaromatic fractions of Dagang reservoir fluids. The microbial communities from produced oil-water samples were composed of high numbers of microorganisms (on the order to 10^7), including methane-producing Archaea within the same order of magnitude. In summary, the investigated sections of the Dagang reservoir may have significant potential for testing the viability of *in situ* conversion of oil to methane as an enhanced recovery method, and biodegradation of the aromatic fractions of the oil may be an important methane source.

Keywords: oil chemistry, biodegradation, methanogenic Archaea, MEOR, oil biomarkers, qPCR

2.1. Introduction

Microbial activity in oil reservoirs affects the quality and economic value of recovered petroleum products, not only by decreasing the *n*-alkane content of the oil, but also by increasing oil density, sulfur content, acidity, and viscosity (Connan, 1984; Röling et al., 2003). Unlike shallow subsurface reservoirs, deeper petroleum reservoirs are not commonly connected to meteoric water cycles, resulting in low nitrate and oxygen availability. Consequently, oil degradation by aerobic or facultative anaerobic organisms is limited. In addition, the supply of large amounts of Fe (III) or manganese (IV) via the water cycle is also unlikely due to poor solubility and slow water recharge rates in subterranean cycles. Moreover, although, iron and manganese oxides from sandstone could be used as electron acceptors for oil oxidation, they are unlikely to be responsible for significant compositional changes in the oil, due to their limited availability in the reservoir. However, oil degradation linked to sulfate-reduction is possible when sulfate arises from geological sources, such as evaporitic sediments and limestone, or from the injection of seawater for pressure stabilization, and may lead to significant oil degradation and increased residual-oil sulfur content.

Methanogenic oil degradation, on the other hand, does not require external electron acceptors and leads to less overall souring of the oil reservoir. Several studies have described methanogenic degradation of crude oil-related compounds *in vitro* (Gieg et al., 2008; Jones et al., 2008) including *n*-alkanes (Jones et al., 2008; Zengler et al., 1999) and mono- and polyaromatic hydrocarbons (for a review see Gieg et al. (2010)). To sustain the process in an oil-bearing reservoir, the only requirements would be adequate amounts of N and P for biomass production, trace metals and vitamins for enzymes, and a sufficient water supply delivered over geological time scales for biodegradation to occur. This water and nutrient supply may also be provided during secondary production involving waterflood. Methane may be recovered relatively easily using extant production infrastructure and used as a downstream energy source. Therefore, the transformation of residual oil to methane is being considered as a tertiary recovery method for abandoned reservoirs with high water cuts and low oil recovery. According to estimates more than 50 % of the initial oil is unrecoverable by conventional technology (Youssef et al., 2009) and will remain entrenched in the reservoir matrix. Therefore, methanogenesis could be an interesting strategy for microbially enhanced recovery of carbon from exploited reservoirs.

Previous experiments performed in the area of microbial enhanced oil recovery have been intent on increasing oil degradation by stimulating *in situ* production of biosurfactants after addition of an oxidant (O_2 or H_2O_2; e.g. Nazina et al. (2008)). However, these experiments resulted in a slight enhancement in oil recovery upon formation of surfactants by aerobic, thermophilic microbiota, or biomass that helped to indirectly solubilize the oil. However, this strategy requires constant addition of electron acceptors to sustain the metabolic activity of aerobic microbial communities, and may be limited over longer periods of time. Consequently, recent attention has focused on the potential of anaerobic microbial processes for microbially enhanced oil recovery (MEOR) applications.

In light of these situations, the aim of this study was to assess the ability of indigenous microbial communities from a water-flooded thermophilic oil reservoir to produce methane under reservoir conditions using laboratory microcosms and molecular biological analyses. As an incremental step towards this goal, we have conducted a geochemical study employing isotopic analyses of reservoir fluids to characterize microbial methanogenesis. In addition, the oil was analyzed using GC-MS fingerprinting techniques to assess the effects of biodegradation on the distribution of oil constituents.

2.2. Materials and methods/experimental details

2.2.1. Site description

The Dagang oil field complex is located in the Huanghua depression of the Bohai Bay Basin. It comprises a total area of 24 km^2 in Cang County, roughly 200 Km SE of Beijing, and consists of series of sandstone oil-bearing strata of the Paleogene and Neogene (Li and Wu, 1991; Vincent et al., 2009). The sampling campaign for this study included four trips in 2010 and 2011 to the Hebei Province in NE China. The sampled oil complex contains different production blocks ranging in age from approximately 3 to more than 40 years, and well depths from 800 m to 2600 m. Production methods for sampled wells range from primary production to tertiary enhanced-recovery methods (i.e. polymer flooding) (Nazina et al., 2007). The average reservoir temperature ranged from 35 to 80°C at the time of sampling. Within the oil field complex, several production wells are serviced by one injection well during secondary recovery/waterflood and range in distances from 50-300 m to the injection well. Hydraulic residence times for injected waters are typically on the order of 40-80 days,

as previously determined (Nazina et al., 2007). A description of the sampled blocks and wells is in Table 2.1. Three different production blocks were sampled during the campaigns and are labeled as Block I, II and III.

2.2.2. Sampling and establishment of microcosms

Samples were collected directly from the wellhead of production and injection wells (Table 1) in sterile glass bottles, flushed with formation gases, and transported back to the laboratory in Leipzig, Germany. All samples were cooled to around 4°C until further analysis. Anaerobic incubations were established in an anaerobic chamber, using oil field fluids as inoculum. Glass serum bottles containing 10-30 mL of a sulfate-free mineral medium (Widdel and Bak, 1992) were inoculated with 5-10 mL of formation water respectively, and sealed with butyl rubber stoppers and aluminum crimp seals. Controls included autoclaved fluids to assess residual degassing of methane from the fluids, as well as replicates without any added hydrocarbon substrates to determine potential methanogenesis from organic matter present in the fluids. Sulfate was added from a sterile anoxic stock solution to several replicates to a final concentration of 2mM sulfate. The salinity of the microbial medium was adjusted to mimic the conditions in Dagang (Appendix 2.A). Sample headspace was flushed with nitrogen to remove residual hydrogen (from the anaerobic chamber), and replicates were incubated statically at 30°C and 60°C. The enrichments were amended individually with non-labeled components to compare with the labeled ones, [$^{13}C_{16}$] hexadecane, ethylbenzene, [$^{13}C_7$] toluene, 2-[^{13}C]-methylnaphthalene, or 2-carboxynaphthalene as a sole carbon source. The labeled hexadecane was synthesized from uniformly ^{13}C-labeled palmitic acid, (Campro Scientific, Berlin, Germany) by reduction of the carboxyl group to an alcohol (with $LiAlH_4$), conversion to the p-tosylate ester, and reduction to the hydrocarbon (with $LiAlH_4$). 2-[^{13}C]-methylnaphthalene was synthesized by the Institute of Organic Chemistry at the University of Leipzig, using a two-step methylation of naphthalene: acetylation to 2-[^{13}C]-naphthol (analogous to Coombs et al. (2000)), and reduction with $Pd/C/H_2$ (analogous to Ofosu-Asante and Stock (1987)). Purity was confirmed via gas chromatography mass spectrometry (GC-MS).

Methane and CO_2 production rates were calculated by preforming a linear regression of the methane increase with incubation time, and the values were expressed in µmol CH_4 or CO_2 per day^{-1} mL^{-1} sample, as described previously in Krüger et al. (2001).

Table 2.1: Overview of sampling site characteristics and the analyses performed from sampled materials

Block	Sample	Well type[1]	Sampling date	Depth (m)	Type of rock[2]	Temperature (ºC)[3]	Water injection?	Years in operation	Injection/ production distance (m)	Analysis performed[4]				
										qPCR	clone library	oil	gases	isotopes
Block I	1	prod	10/25/2011	2042 (av)	Sandstone (Eocene)	~80	Yes	~3	not applicable	+	NA	+	+	+
	2	prod	10/25/2011	2589 (av)			Yes	<20		+	NA	+	+	+
	3	prod	10/25/2011	2485 (av)			No	<20		+	NA	+	+	+
Block II	4	prod	10/25/2011	1380-1470	Sandstone (Miocene)	56 - 61	Yes	~15	minimum 50, average 100 - 150	+	NA	NA	NA	+
	5	prod	10/25/2011	1370-1395			Yes	~10		NA	NA	+	+	+
	6	prod	10/25/2011	1200 -1450			Yes	~10		NA	NA	NA	+	+
Block III	7	prod	6/1/2010	1100-1500	Sandstone (Pliocene)	~50	Yes	~40	100 to 250	NA	NA	+	NA	+
	8	prod	5/18/2011	1000 -1035		~54	Yes	~38		+	+	+	+	+
		inj	5/18/2011	NA		~54		~20		NA	NA	NA	+	+
	9	prod	5/18/2011	1002 -1032		~52	Polymer flooding	~20		+	NA	NA	NA	+
	10	prod	12/6/2010	948-957		30-40	Yes	~40		NA	NA	NA	+	+
			5/18/2011							+	+	NA	NA	+
		inj	5/18/2011	1002-1024		30-40				+	+	+	NA	NA
	11	prod	12/6/2010	1575-1604	Sandstone (Miocene and Oligocene)	~50	Yes	~3		NA	NA	NA	+	+
			5/18/2011							NA	NA	+	NA	+
	12	prod	12/6/2010	1579-1616		~50	Yes	~3		NA	NA	NA	NA	+
			5/18/2011							NA	NA	+	+	+
	13	prod	12/6/2010	1436-1443		~50	Yes	From 2007 to 2012 (now closed)		NA	NA	NA	NA	+
			5/18/2011							+	+	+	+	+
	14	prod	5/18/2011	1546-1791		30-40	Yes	~2		+	+	+	+	+
	15	Prod	6/1/2010	NA		~50	Yes	NA		NA	+	NA	NA	NA

[1] *Prod*: production well. *Inj*: injection well.

[2] Lithology (and age) from Zhai et al. (1991).

[3] Measured when sampling.

[4] + shows which analyses have been performed for each sample. NA means not analysed.

2.2.3. Analytical methods (isotopes and headspace gases)

Methane production was analyzed by measuring microcosm headspace isocratically at 60°C using a GC-FID equipped with a 6' Hayesep D column (SRI 8610C, SRI Instruments, USA). Carbon dioxide concentrations were determined using a methanizer-equipped FID detector, after reduction of the CO_2 to methane. The stable isotopic composition of methane and CO_2 was measured using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry system (GC-C-IRM-MS). The system consisted of a gas chromatograph (6890 series; Agilent Technology), fitted with a CP-pora BOND Q column coupled to a combustion or high-temperature pyrolysis interface (GC-combustion III or GC/C-III/TC; Thermo Finnigan, Bremen, Germany), and a MAT 252 IRMS for the carbon analysis or a MAT 253 IRMS for hydrogen analysis (both from Thermo Finnigan, Bremen, Germany) (Herrmann et al., 2010). The carbon and hydrogen isotopic compositions (R) are reported as delta notation ($\delta^{13}C$ and $\delta^{2}H$) in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW), respectively (Feisthauer et al., 2011; Richnow et al., 2003a).

Headspace samples were injected directly into the GC using a split mode for the analysis of $\delta^{13}C$ and $\delta^{2}H$ for methane (for details, see Feisthauer et al. (2011)). Water subsamples were used for the isotopic analysis of carbonates and deuterium in the H_2O as outlined below. For the carbonate analysis, an aliquot of each sample was collected with a syringe, transferred to a crimped vial and acidified to < pH 2 using pure HCl. The gas phase was then injected into the GC-IRMS for isotope analysis. The error associated with the system (accuracy and reproducibility) was around 0.5 ‰ and 4 ‰, for carbon and hydrogen, respectively. The standard deviation of at least three independent measurements is reported. For the H_2O analysis, an aliquot (5 mL) of each sample was cleaned with activated carbon to remove any possible organic contamination prior to the determination of isotope ratios.

2.2.4. Gas-chromatography mass-spectrometry of oil

Samples from the reservoir and from degradation experiments were extracted with dichloromethane and dehydrated through a 2-g anhydrous Na_2SO_4 column. The oil content in the sample was determined by gravimetry in 1.0 mL of the eluate, and carefully evaporated until dryness. An aliquot (10 mg) of the eluate was cleaned by passing it through

2 g of Al_2O_3 (5 % w/w deactivated), concentrated and exchanged to hexane (1.0 mL) by a gentle solvent evaporation under a stream of nitrogen gas. GC-MS analysis was performed using a 7890A gas chromatograph (Agilent Technologies), fitted with a capillary column (J&W Scientific, Folsom, CA, USA) HP-5 MS (30 m × 0.25 mm i.d., 0.25 µm film), coupled to a 5975C MS spectrometer equipped with a triple-axis detector (Agilent Technologies) as reported elsewhere (Jiménez et al., 2006).

Table 2.2: Diagnostic ratios for source and weathering assessment for the oil samples

Index[1]	Diagnostic ion m/z	Definition	Structures
%29aaS	217	100*29ααS/(29ααS+29ααR)	29αα: 24-ethyl-14α(H),17α(H)-cholestane (20S and R)
%29bb(R+S)	217	100*29ββ(R+S)/[29ββ(R+S)+ 29αα(R+S)+29ααR)]	29ββ: 24-ethyl-14β(H),17β(H)-cholestane (20S and R)
%27bb	217	100*27ββ/[27ββ(R+S) +28ββ(R+S)+29ββ(R+S)]	27ββ: 14β(H),17β(H)-cholestane (20S and R)
			28ββ: 24-methyl-14β(H),17β(H)-cholestane (20S and R)
%26TA	231	100*26TAS/(26TAS+28TAS)	aromatized cholestane (20S) and 24-ethylcholestane (20S)
%27Ts	191	100*Ts/(Ts+Tm)	Ts: 18α(H)-22,29,30-trisnorhopane
			Tm: 17α(H)-22,29,30-trisnorhopane
%29ab	191	100*29αβ/(29αβ+30αβ)	29αβ: 17α(H),21β(H)-30-norhopane
			30αβ: 17α(H),21β(H)-hopane
%32abS	191	100*32αβS/(32αβS+32αβR)	30αβ: 17α(H),21β(H)-bishomohopane (22S and R)
2-3MD/1MD	198		
D2/P2	212/206	100*D2/(D2+P2)	D2, D3: dimethyl and trimethyldibenzothiophenes
D3/P3	226/220	100*D3/(D3+P3)	P2, P3: dimethyl and trimethylphenanthrenes
D2/C2	212/256	100*D2/(D2+C2)	Py2, Py3: dimethyl and trimethylpyrenes
D3/C3	212/270	100*D3/(D3+C3)	C2, C3: dimethyl and trimethylchrysenes
C2/Py2	256/230	100*C2/(Py2+C2)	
C3/Py3	270/240	100*C3/(Py+C3)	

[1] The indexes %29ab, %32ab, %27Ts, D2/P2 and D3/P3 have been selected for source identification, whereas changes in the other indexes may indicate different degrees of degradation.

2.2.5. Assessment of oil degradation

To assess the extent of microbiological oil degradation, the distribution of *n*-alkanes or polycyclic aromatic hydrocarbons was calculated with relation to 17α(H),21β(H)-hopane (m/z 191), used as internal conservative molecular marker (Prince et al., 1994). Sample 3' was used as a reference to calculate the relative degradation for the rest of the samples. The quantification of *n*-alkanes was determined by using the m/z 85 fragment ion. Linear alkylcyclohexanes, alkylbenzenes and alkyltoluenes were quantified by monitoring their

characteristic ions (*m/z* 82, 92 and 106, respectively). Quantification of individual aromatic compounds was based on the molecular ion for each: N-N4, naphthalenes (*m/z* 128, 142, 156, 170, 184); F-F3, fluorenes (*m/z* 166, 180, 194, 208); P-P3, phenanthrenes (m/z 178, 192, 206, 220); D-D3, dibenzothiophenes (m/z 184, 198, 212, 226); Py-Py3, fluoranthenes and pyrenes (*m/z* 202, 216, 230, 244); C-C3, chrysenes (*m/z* 228, 242, 256, 270). In addition, several molecular markers were used to calculate indexes for fingerprinting and weathering assessment: triaromatic steroids (*m/z* 231), steranes and diasteranes (*m/z* 217 and 218) and triterpanes (m/z 191). The different diagnostic ratios are specified in Table 2.2.

2.2.6. Molecular methods

For molecular characterization and cloning experiments, DNA from the microcosms was extracted after incubation with individual hydrocarbons using a modified protocol from Lueders et al. (2004). For further purification of crude DNA, ethidium bromide was added to 0.6 mg mL^{-1} and ammonium acetate to 2.6 M final concentration (Lovell and Piceno, 1994). Genes of interest were quantified by real-time PCR using an ABI Prism 7000 (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). The 16S rRNA gene copy numbers for Archaea and Bacteria were determined as described previously (Nadkarni et al., 2002; Takai and Horikoshi, 2000). Methanogen abundance was assessed using the methyl-CoM reductase gene A (mcrA) (Steinberg and Regan, 2009). Microorganisms capable of dissimilatory sulfate-reduction were quantified using dsrA gene copy number. This gene codes for the alpha subunit of the dissimilatory (bi)sulfite reductase (Kondo et al., 2004; Schippers and Neretin, 2006). All PCR reactions were run in triplicate at three dilutions. Copy numbers are expressed as DNA copies mL^{-1} sample.

2.3. Results

2.3.1. Oil chemical analysis

The majority of the oil-water samples taken from the reservoir present an elevated content heavy and polar oil fractions (resins and asphaltenes, up to 29 %), whereas the saturated fraction represents 43 to less than 50 % of the bulk oil. These values are slightly higher and lower, respectively, than values previously reported by Nazina and coworkers

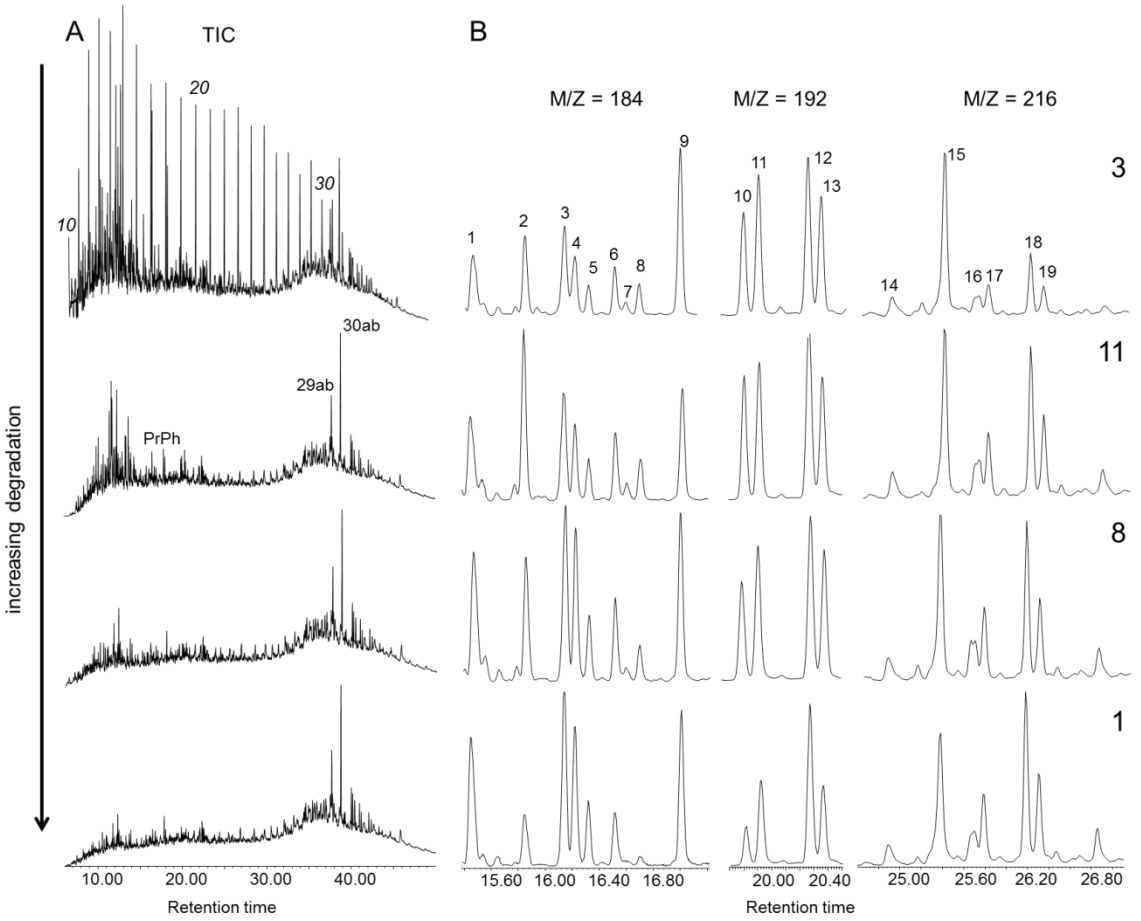
(around 20 % of resins and asphaltenes and >53 % saturated hydrocarbons) and might suggest higher biodegradation levels than previously described (Nazina et al., 2007). In addition, most samples are completely depleted of *n*-alkanes, as revealed by GC-MS analysis (Figure 2.1). The degree of degradation compared to a relatively non-degraded sample from the same reservoir was higher than 90 % (considering C_{10}-C_{35}). Oil profiling revealed that alkylbenzenes and alkyltoluenes are severely degraded. However, branched alkanes and alkylcyclohexanes are still present to a varying extent in most of the samples.

Polycyclic aromatic hydrocarbon (PAHs) degradation varied significantly among samples and within compound families, ranging from around 10 % to nearly 100 % for naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes (Table 2.3). Compound removal decreased with increasing molecular weight, as generally expected. Tetra-aromatic hydrocarbons (e.g. pyrenes and chrysenes) were slightly affected, as reflected by the relative decrease of C2- and C3-dibenzothiophenes with respect to C2- and C3-pyrenes (D2/C2 and D3/C3) (Figure 2.2). This observation is consistent with heavy to severe biodegradation (4-7 using the scale of Peters and Moldowan (1993) and Head et al. (2003)).

Table 2.3: Degradation percentages of different hydrocarbon families with respect to the non-biodegraded sample (Block I, sample 3). Samples have been ordered according to the extent of biodegradation

	3	**7**	**11**	**12**	**8**	**10**	**5**	**13**	**1**	**14**
C17/Pr	65	15	NA	NA	NA	15	NA	NA	NA	NA
C18/Ph	63	11	NA	NA	NA	8	NA	NA	NA	NA
					% Degradation relative to sample 3 of block I					
***n*-alkanes**		92	100	100	100	99		100	100	100
N-N3		20	10	15	48	86	92	99	94	100
F-F3		13	15	18	30	56	60	60	67	100
P-P3		9	14	16	16	45	57	40	76	96
D-D3		31	35	39	43	54	52	56	63	100
Py-Py3		-	-	-	-	18	-	14	37	59
C1-C3		-	-	-	-	-	-	-	30	55

NA: Not available due to the lack of *n*-alkanes.



Peak	Diagnostic ion (m/z)	Identification	Peak	Diagnostic ion (m/z)	Identification
Pr	85	pristane	8	184	1,2,3,6-TMN
Ph	85	phytane	9	184	1,2,5,6- + 1,2,3,5-TMN
29 ab	191	17α(H),21β(H)-30-norhopane	10	192	3-MP
30ab	191	17α(H),21β(H)-hopane	11	192	2-MP
			12	192	4+9-MP
1	184	1,3,5,7-TMN	13	192	1-MP
2	184	1,3,6,7-TMN	14	216	2-MF
3	184	1,2,4,6 + 1,2,4,7- + 1,4,6,7-TMN	15	216	BaF
4	184	1,2,5,7-TMN	16	216	B(b+c)F
5	184	2,3,6,7-TMN	17	216	2-MPy
6	184	1,2,6,7-TMN	18	216	4-MPy
7	184	1,2,3,7-TMN	19	216	1-MPy

Figure 2.1: A) GC-MS total ion current (TIC) profiles of representative samples with different degrees of biodegradation. Each sample corresponds to a different sampling well. The non-degraded profile corresponds to sample Bannan 3 from block I. The numbers 10, 20 and 30 refer to *n*-alkane chain lengths. **B)** Ion chromatograms of the C4-naphthalenes (m/z 184), C1-phenanthrenes (m/z 206) and C1-pyrenes and fluoranthenes (m/z 216) for the same samples. Each profile is presented on a scale relative to the largest peak. The identification of the different isomers is given below.

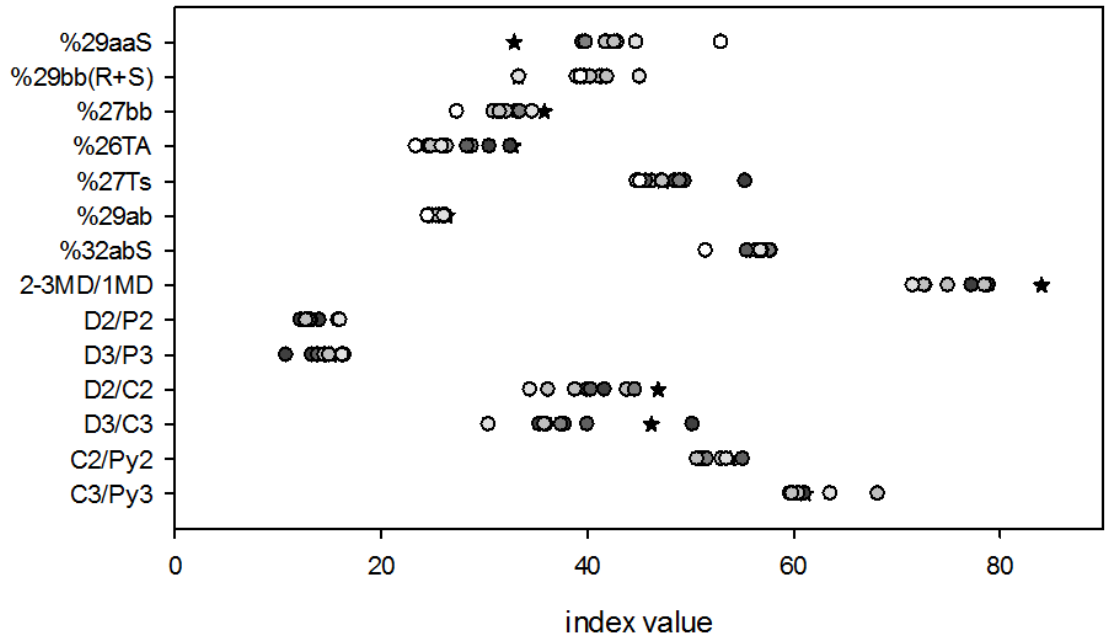


Figure 2.2: Molecular marker indices for source and biodegradation assessment. Stars represent sample 3, Block I, which was used as a control. The other samples are represented as shaded circles, going from dark grey (less degraded) to white (more degraded).

For alkylated PAHs, we have detected changes in the isomeric distributions of tetramethylnaphthalenes (TMN), methylphenanthrenes (MP), methyldibenzothiophenes (MD) and higher molecular weight PAHs like methyl-pyrenes (MPy). This observation suggests that biodegradation of these oil components in this reservoir occurs via stereospecific biological processes. Specifically, 1,3,6,7- and 1,2,3,6-TMN are the most easily degraded isomers and, like 1,2,6,7-TMN, their relative abundance decreases with increasing biodegradation. On the other hand, 1,3,5,7- and 1,2,5,7-TMN seem to be more recalcitrant (Figure 2.1). 3- and 1-methylphenanthrene exhibit higher levels of biodegradation compared to the other isomers, whereas the 4/9-MP relative abundance increased over a wide range (Figure 2.1). This pattern was however not consistent among the most degraded samples, as reported in other studies (Williams et al., 1986). The abundance of 2- and 3-MD decreased in relation to 1-MD (Figure 2.2). Persistence of 4-methylpyrene (4-MPy) was noted, while all other methylpyrenes were significantly degraded (Figure 2.1).

Aliphatic and aromatic biomarker patterns (hopanes, steranes and triaromatic steroid hydrocarbons) varied with the extent of degradation (Figure 2.2). Steranes and triaromatic steroid hydrocarbons followed a similar trend, whereby degradation decreased when

increasing side chain length, as reflected in the ratios of %27bb and %26TA. In addition, a preferential removal of several C-29 steranes was observed: The 14α(H),α(H) and 20R isomers were more readily degraded compared to the 14β(H), β (H) and 20S isomers, respectively (%29aaS and %29bb(R+S)). On the other hand, diagnostic ratios, such as the relative abundance of C2- and C3-phenanthrenes compared to C2- and C3-dibenzothiophenes (D2/P2 and D3/P3) or the distribution of bishomohopane S and R isomers (%32abS), suggest that all oils from this study belong to the same source. In addition, the lack of significant differences in the Tm/Ts index (%27Ts) among the samples indicates that they have a similar thermal maturity and imply a similar genesis, with the lone exception of sample 14.

2.3.2. Gas analysis

Gas phases recovered from the reservoir consisted mainly of CH_4, while C_2 and C_3 were less abundant and other light *n*-alkanes (C_4-C_6) were rarely detected, as reflected by the ratio of $C_1/\Sigma(C_1\text{-}C_5)$ (Table 2.4). However, samples from Block I (wells 2 and 3) were particularly wet, with values below 0.6. The $C_1/(C_2+C_3)$ ratios varied from 2 to nearly 600, and indicate either thermogenic origin, according to the Bernard diagram (Bernard et al. (1976); Appendix 2.B) or a mixture of biodegraded and secondary microbial gas (Milkov, 2011). The ratio of CH_4/CO_2 ranged from 1 to around 200. Methanogenic alkane degradation produces methane to CO_2 ratios of about 3.27:1. However, our values are generally much higher, which could be related to the diversity of carbon sources, a further degradation of CO_2 or the solubility of CO_2 in formation water or precipitation of carbonates in the reservoir.

The isotopic composition of methane carbon ($\delta^{13}C$) from different wells ranged from -39 ‰ to -68 ‰ relative to VPDB, and averaged around -47 ‰ (Table 2.4). These values are consistent with biogenic methane production (Larter et al., 2005; Pallasser, 2000), and similar to values from other degraded reservoirs in China (Dai, et al. 2004). Two of the samples (1 and 14) have a lighter value (below -55 ‰) and, according to Milkov (2011), are typical for primary microbial gas. Carbon dioxide was highly enriched in ^{13}C for most of the samples, with values observed up to +17.2 ‰. The lightest value corresponded to sample 14 from block III (-12.8 ‰), followed by samples from blocks I and II (up to -7.6 ‰). The bulk isotopic discrimination ($\Delta\delta^{13}C$) between methane and CO_2 was between 32 and 65 ‰

(Figure 2.3), in accordance with previously reported results for methane formation during hydrocarbon degradation (Feisthauer et al., 2010; Morris et al., 2012). They are also typical of values found in highly biodegraded subsurface oils from marine systems (Larter and di Primio, 2005) and may indicate that extensive reduction of CO_2 to methane has occurred (Jones et al., 2008; Milkov, 2011). However, no clear correlation can be established between the isotopic discrimination of methane/carbon dioxide and the degree of biodegradation at this time.

Table 2.4: C and H isotopic composition of the fluids (*) from the reservoir, together with their dryness. (*) relative to the VPDB and VSMOW standards

Block	Sample		$C_1/(C_2+C_3)$	$C_1/\Sigma(C_1-C_5)$	$\delta^{13}C$ (‰)		$\delta^{2}H$ (‰)	
					CH_4	CO_2	CH_4	H_2O
Block I	1	prod	585	0.998	-69.8 ± 0.4	-7.6 ± 0.1	NA	-75.2 ± 0.9
	2	prod	3	0.589	-40.8 ± 0.4	-7.6 ± 0.1	-219.4 ± 1.3	-60.0 ± 0.3
	3	prod	2	0.492	-41.3 ± 0.3	-4.5 ± 0.2	-223.3 ± 1.1	-60.5 ± 0.5
Block II	4	prod	NA	NA	-40.2 ± 0.4	-7.6 ± 0.1	NA	-71.7 ± 0.7
	5	prod	25	0.961	-43.7 ± 0.1	-5.1 ± 0.1	-241.9 ± 1.6	-73.0 ± 0.5
	6	prod	31	0.968	-43.8 ± 0.9	9.1 ± 0.1	-237.1 ± 0.8	-68.1 ± 0.4
Block III	7	prod	NA	NA	-39.5 ± 0.3	11.2 ± 0.0	-238.9 ± 0.3	-69.2 ± 0.7
	8	prod	14	0.920	NA	11.2 ± 0.2	NA	-72.8 ± 0.7
		inj	32	0.954	NA	9.3 ± 0.1	NA	-71.7 ± 0.9
	9	prod	NA	NA	-43.1 ± 0.4	15.5 ± 0.2	-224.8 ± 3.3	-69.6 ± 0.5
	10	prod	214	0.995	-48.5 ± 0.2	13.8 ± 0.2	-222.1 ± 1.6	-76.5 ± 1.6
			73	0.986	-48.7 ± 0.2	13.7 ± 0.0	-227.7 ± 0.3	-72.0 ± 0.5
		inj	NA	NA	NA	13.4 ± 0.3	NA	-70.6 ± 1.6
	11	prod	11	0.925	-46.9 ± 0.2	3.6 ± 0.1	-246.3 ± 0.3	-70.4 ± 1.2
			NA	NA	-47.2 ± 0.6	NA	-241.5 ± 0.3	NA
	12	prod	NA	NA	-47.6 ± 0.2	6.7 ± 0.2	-237.9 ± 2.3	-72.1 ± 1.5
			24	0.959	-47.6 ± 0.3	9.9 ± 0.2	-240.0 ± 0.8	-73.1 ± 2.5
	13	prod	NA	NA	-46.9 ± 0.4	17.5 ± 0.2	-235.5 ± 1.5	-72.0 ± 1.6
			176	0.994	-46.9 ± 0.4	16.4 ± 0.2	-229.4 ± 1.4	-69.4 ± 2.9
	14	prod	541	0.998	-59.0 ± 0.2	-12.8 ± 0.2	-241.8 ± 10.5	-72.4 ± 1.0

NA: not available.

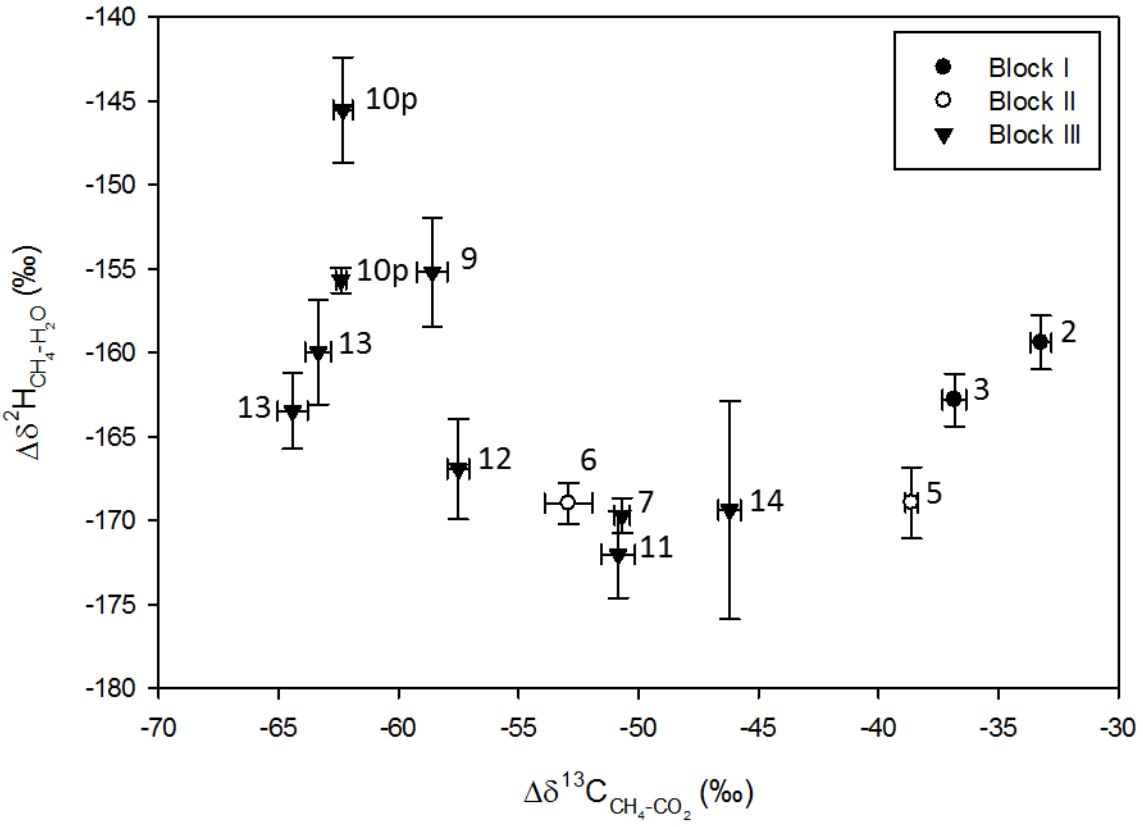


Figure 2.3: Hydrogen isotopic discrimination between methane and water ($\Delta\delta^2H$) vs. carbon isotopic discrimination between methane and CO_2 ($\Delta\delta^{13}C$). Error bars represent the standard deviation of three independent analyses.

The δ^2H values for methane and water ranged from -220 ‰ to -246 ‰ and -69 ‰ to -73‰ relative to the VSMOW, respectively. These values are on the lower range of previously reported statistics from marine sediments (Whiticar et al., 1986) (-250 ‰ to -170 ‰). The δ^2H discrimination between methane and water ($\Delta\delta^2H(CH_4\text{-}H_2O)$) varied between -145 ‰ and -170 ‰, and were found to be significantly lower than values from enrichment cultures studied by Feisthauer et al. (2010) ($\Delta\delta^2H(CH_4\text{-}H_2O)$ -336 ‰ to -257 ‰) (Figure 2.3).

2.3.3. Microbial abundance

Thirteen production wells and three injection wells were sampled intermittently over a period of two years throughout the Dagang field complex. A thorough microbiological survey using qPCR was conducted to characterize the bacterial and archaeal numbers at the site via amplification of the 16S rRNA gene. Further insight into biogeochemical processes

was achieved by determining mean copy number of the dissimilatory sulfate reductase (*dsrA*) and methyl-CoM reductase (*mcrA*) genes. Reservoir temperatures in Dagang usually range from 35-60°C and can reach 80°C in the deeper section (Table 2.1). Average bacterial numbers for injection and production wells, based on 16S rRNA, ranged from 10^5 to 10^8 copies mL^{-1} sample. Archaeal numbers were between 10^3 to 10^7 copies mL^{-1} sample. Generally, bacterial and archaeal copy numbers were on the same order of magnitude for individual wells. The exception to this rule occurred when wells with high water cuts were sampled (above 90 %, Well 13). In this case, bacteria outnumbered archaea by two orders of magnitude. Clone libraries of 16S rRNA genes were constructed to assess the dominant archaeal genera within nine Dagang production/injection wells (Appendix 2.C). Production water samples from wells 10 and 15 were dominated by *Methanosarcina* (100 %) and *Methanobacterium* (47 %) sequences, respectively. The dominant archaea in well 14 were the crenarchaeotal genus *Hyperthermus* (80 %) and the euryarchaeotal genus *Archaeoglobus* (15 %). Interestingly, no methanogenic genera were detected in this sample. *Methanosphaera, Methanosarcina, and Methanobacterium* were the dominant genera in samples from the other wells.

The *dsrA* numbers ranged from 10^4 to 10^7 copies mL^{-1} sample, and seem to indicate that the Dagang field could quickly become sulfidogenic if sulfate-containing injection waters were added to the formation. In addition, if sulfide oxidation is promoted by adding of O_2 or H_2O_2 to the injection water, biogenic sulfate-reduction may occur (Nazina et al., 2008). Copy numbers for *mcrA* were comparable to *dsrA* numbers. In several wells, the *mcrA* copy number was higher than the 16S bacterial number (Figure 2.4), but this pattern was not correlated with higher rates of methane generation. Several wells were sampled during both the November 2010 and April 2011 campaigns. Overall, bacterial gene copy numbers were generally higher in April 2011, especially for injection well 10 (Figure 2.4). This may indicate seasonal variation in reservoir bacterial numbers, most likely associated with the water management of the reservoir, but longer-term study would be needed to validate these trends.

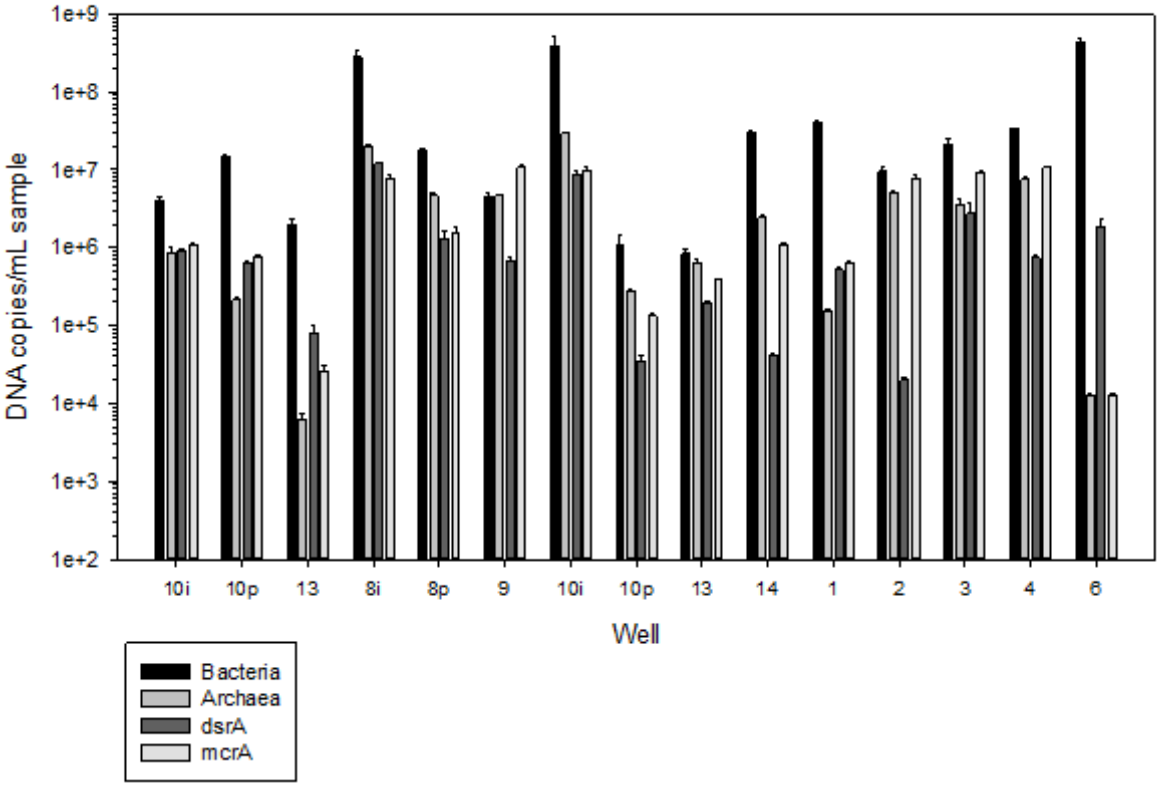


Fig 2.4: Abundance of bacteria, archaea, sulfate-reducing prokaryotes, and methanogens in samples as determined by qPCR. The error bars represent the standard deviation of three replicates.

2.3.4. *In vitro* methane production from labeled hydrocarbons

Microbial enrichment cultures were established from Dagang production fluids and amended with ^{13}C-labeled aliphatic and aromatic hydrocarbons to provide unequivocal evidence of methanogenic hydrocarbon degradation by *in vitro* microbial populations from this reservoir. The isotopic composition (δ^{13}C) of methane and CO_2 from enrichment cultures containing ^{13}C-labeled *n*-hexadecane and ^{13}C-methylnaphthalene (provided as sole carbon sources) became continuously enriched during the incubation. After 90 days of incubation, samples amended with ^{13}C-labeled 2-methylnaphthalene produced methane with δ^{13}C signatures above 30 per mil (versus VPDB, Figure 2.5B). Labeled methane continued to be produced over the investigation time of 150 days (Figure 2.5B). Isotopically heavy methane was also observed in the enrichments containing ^{13}C-labeled *n*-hexadecane, and methane δ^{13}C values were approximately 50 ‰ PDB after 40 days (Figure 2.5A). Although a higher rate of *n*-alkane degradation is expected compared to 2-MN, the *n*-alkanes used in this study were uniformly labeled while the 2-MN was labeled at the methyl position. This in and of itself can account for the earlier emergence of labeled methane in the *n*-alkane amended

samples. Carbon dioxide however became much more enriched in the *n*-alkane samples, with ^{13}C values observed at 40 days of over 60 ‰ (Figure 2.5). After 400 days, methane and CO_2 were both highly labeled (Figure 2.5). Methane production rates were quantified for microcosms inoculated with production fluids from Well 15, and were found to be between 0.83 and 1.02 µmol $mL^{-1}d^{-1}$.

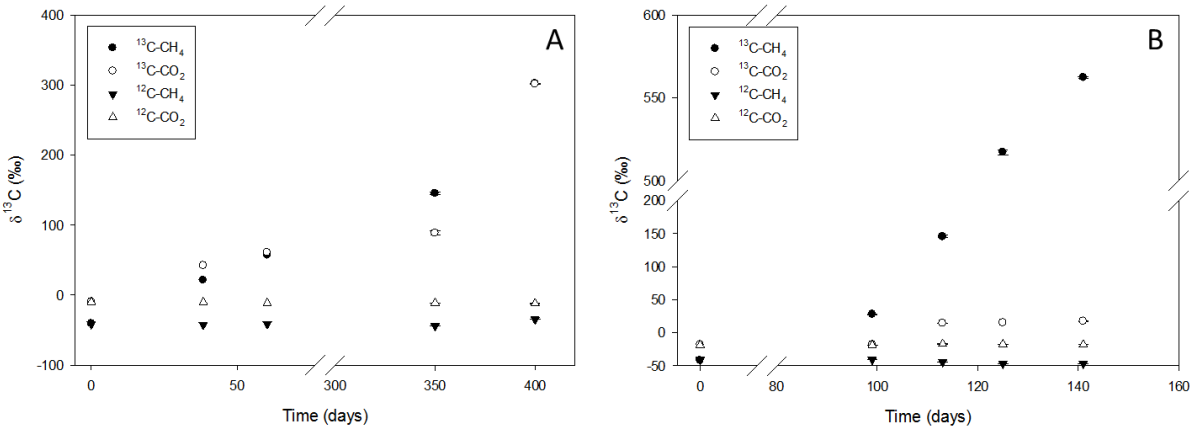


Figure 2.5: Isotopic values for the methane and CO_2 produced from (A) *n*-hexadecane and (B) methyl-naphthalene *in vitro.* ^{13}C-CH_4 and ^{13}C-CO_2 correspond to the gases obtained in the labeled microcosms, whereas ^{12}C-CH_4 and ^{12}C-CO_2 represent the gases obtained in the unlabeled ones. Error bars represent the standard deviation of three independent injections.

2.4. Discussion

The majority of worldwide oil reservoirs show evidence for biodegradation (Jones, 2008). However, we still understand very little about the underlying reservoir microbiology, especially under methanogenic conditions. Understanding these processes might facilitate the development of methods for either reducing biodegradation, or stimulating metabolic processes for microbially enhanced oil recovery (MEOR).

2.4.1. The microbiological potential of the Dagang oilfield for *in situ* methane production

As a way to increase or sustain oil production, secondary recovery by waterflood has been used since its development in the 1930s, and is still a common production method in many countries. This method entails water injection into the oil-bearing strata using a network of injection wells to maintain reservoir pressure (Belyaev et al., 2004). In the Dagang oilfield complex, injection water is separated from production fluids and

continuously reinjected into the formation in a cyclical fashion. The process of oil and water separation before injection takes, on average, two to three hours. Consequently, the chemical composition of the formation and injection waters is very similar at this location (Nazina et al., 2007). This demonstrates that the water cycle is closed and environmental water injection is minimal. Overall geochemical conditions in the Dagang oil field complex represent a favorable environment for the recruitment and sustainability of microbial populations. The sulfate concentrations are relatively low and sulfate is probably not a relevant electron acceptor for biodegradation, as sulfide production was not witnessed in the field during sampling campaigns. Elevated HCO_3 concentrations indicate higher microbial activities in some of the production blocks (Nazina et al., 2007).

We hypothesize that secondary recovery methods including water recycling and reinjection select for, and maintain, microbial populations that can degrade crude oil constituents to methane under *in situ* temperature and pressure. Tracer studies using ^{13}C-labeled aliphatic and polyaromatic hydrocarbons demonstrated that microorganisms in the Dagang formation waters have the intrinsic ability to produce methane from these compounds (Figure 2.5). These results are consistent with those obtained by Wang et al. (2011) using a mixture of *n*-alkanes (C_{15}-C_{20}) and high amounts of methane were detected in samples collected from the oil complex. Large methane accumulations are commonly associated with biodegraded oil (Horstad and Larter, 1997; Milkov, 2010, 2011) and attest to our conclusion of biogenic methane production concomitant with anaerobic hydrocarbon degradation. In this respect, the $\delta^{13}C$ signatures for formation methane are consistent with the mixing of secondary microbial and thermogenic gases (Fuhua, 1987; Larter et al., 2005; Milkov, 2011; Pallasser, 2000; Whiticar et al., 1986). The high $\delta^{13}C$ values for CO_2 found in most of the samples from the reservoir are consistent with the model proposed by Jones et al. (2008) for hydrogenotrophic methanogenesis, although it was established for methanogenesis under mesophilic conditions (below 35°C). The Dagang reservoir has prevailing mesophilic to thermophilic conditions of around 30-60°C, except in the deeper sections, in which the temperature is much higher. Nevertheless, thermophilic hydrogenotrophic methanogens predominate in high-temperature oil reservoirs, for example the Kondiang oil field (Nazina et al., 2006), and our clone libraries suggest that thermophilic, hydrogenotrophic methanogens related to *Methanobacterium* (Smith et al., 1997) are dominant in the Dagang fields. Detected *Methanosarcina*-related organisms also

have the potential to produce methane using electrons from hydrogen. Nazina et al. (2007) observed methane production from acetate from a hydrogen and carbon dioxide mixture by cultures from the same reservoir, paralleling the results obtained by Gray et al. (2009) using samples from a gas field in the North Sea. However Gray et al. (2009) did not detect acetoclastic methanogenesis in their cultures. Thermodynamic considerations also suggest that acetoclastic methanogenesis will become less favored under higher temperatures (Dolfing et al., 2008; Larter, 2005).

The blocks sampled during this study have a production history between 3 and about around 40 years, and clear differences were observed between the total ion chromatogram (TIC) profiles and specific oil biomarkers in the well under primary production (Block I, well 3) and the wells under production by water flood (Figures 2.1 and 2.2). Among the wells under secondary production there are differences in the degradation extent. These results may suggest that changes in oil composition could be observable within the same formation on a human timescale, and can be related at least partly to oil production processes. Nazina et al. (1995a) identified anaerobically active microbial biodegradation processes in water-flooded mesophilic and thermophilic oil reservoirs from Kazakhstan and Western Siberia, and concluded that the injection of surface waters helped to facilitate biogenic oil degradation. In addition, Whelan et al. (2001) reported significant decreases in the C_{11}-C_{19} *n*-alkanes, and to a lesser extent heavier components (up to *n*-C_{32}), in less than 8 years within oil and gas reservoirs along the Gulf of Mexico coastline. However, it cannot be fully excluded that oil mixing and geological complexity in the Dagang region (Fajing and Shulin, 1991) affect these oil profiles to an unknown extent.

Injection and productions waters from Dagang were filtered to collect microbial biomass for DNA extraction, and analyzed using qPCR. Large numbers of bacterial and archaeal 16S rRNA genes were detected in addition to *dsrA* and *mcrA* genes (Figure 2.4). Anaerobic microcosms incubated under temperature regimes of 55-60°C also produced labeled methane within 40 days after inoculation (Figure 2.5). Taken together, this information suggests that a specialized community exists that is capable of completely mineralizing aliphatic, aromatic, and polyaromatic hydrocarbons to methane. Methanogenic hydrocarbon degradation has recently become an area of intense interest from both biological and industrial points of view (Gieg et al., 2008; Gray et al., 2010). From a biochemical perspective, little is understood about the enzymatic pathways involved in

hydrocarbon activation for polyaromatic degradation under reduced conditions, while some progress has been made towards our understanding of *n*-alkane and substituted-aromatic activation (i.e. addition of the hydrocarbon to fumarate, Beller and Edwards (2000); Heider (2007)). Development of enrichment cultures able to degrade PAHs under methanogenic conditions will encourage further study, and a primary understanding of the microbial ecology and diversity involved in oil field systems will help to provide groundwork for further biochemical study.

2.4.2. Chemical evidence for *in situ* oil biodegradation

An extensive fingerprinting of Dagang oil was carried out using GC-MS analyses. These results suggest that the crude oil is heavily degraded within this complex, like in most of the reservoirs at this temperature range (Pepper and Santiago, 2001), and that the degradation of several compound classes is especially severe. For example, *n*-alkanes are almost completely depleted, as normally observed in heavily degraded reservoir. However, we also observed substantial losses of aromatic fractions in the oil, specifically alkylbenzenes, alkyltoluenes, and low-molecular-weight polyaromatic hydrocarbons. Preferential losses of specific alkylated PAH isomers suggest that these compounds are also being degraded biologically. Changes in the distribution of trimetylnaphthalenes or methylphenanthrenes are consistent with previously reported results of oil biodegradation in a reservoir (Huang et al., 2004). The relative abundance of these compounds can be associated with their thermal stability. However, in samples with a common source and similar thermal maturity (reflected by the Ts/Tm indices), as in this case, differences in the chemical distributions are most likely due to biodegradation (Huang et al., 2004). Isomeric specificity for aerobic biodegradation of PAHs has been extensively studied and is well known. For instance, isomers with β-substituents (like 2- or 3-methyl-) are more readily degraded than others. However, trends for PAH degradation under reduced reservoir conditions have not been clearly established. Huang et al. (2004) reported that 2,3,6- and 1,3,6-trimethylnaphthalenes, 1,7- and 2,6- + 3,5-dimethylphenanthrenes, or 1,2,8-trimethylphenanthrene were preferentially degraded. Nevertheless, these trends are likely affected by oil chemistry, formation conditions or oil charging/migration (Larter, 2003), along with microbial variability among wells.

Selective methylphenanthrene and methyldibenzothiophene biodegradation was observed. For example, 4/9-MP and 1-MD, usually the most conserved in biodegraded samples under aerobic conditions (Bayona et al., 1986), were retained in several heavily degraded samples from our study. This parallels the results obtained by Huang *et al*. (2004) for samples from other oil reservoirs. Similarly, preferential degradation of several sterane epimers has been observed here, consistent with results from the previously mentioned study. Our GC-MS results, taken together with the microcosm study, indicate that, although *n*-alkane degradation occurs before any significant degradation of aromatics takes place (Elias et al., 2007; Jones et al., 2008), PAH degradation is responsible for a significant fraction of the methane produced in this oil field complex. Further experiments will contribute to the assessment of metabolic pathways for methanogenic PAH degradation and identify the microorganisms involved, with hope of establishing new indices for the assessment of oil biodegradation under methanogenic conditions.

Acknowledgements

We thank the Robert-Bosch-Foundation for financial support: Grant 32.5.8003.0083.0 "*In Situ* Microbial Degradation of Hydrocarbons in Oil Reservoirs". Furthermore, we thank Daniela Zoch, Holger Probst, Ursula Günther, Matthias Gehre and Falk Bratfisch for help with experiments and Marcel Sickert for synthesis of ^{13}C-labeled 2-methylnaphthalene. Financial support for F. Gründger was provided by DFG Priority Program 1319, Grants KR3311/6-1 and 6-2. N. Jiménez was supported by a Marie Curie Intra-European Fellowship (IEF) within the Marie Curie Mobility Actions of the European Commission's Seventh Framework Program (PIEF-GA-2010-272569). We gratefully acknowledge the contribution of one anonymous review and Alexei Milkov for critiques and improvement of the manuscript.

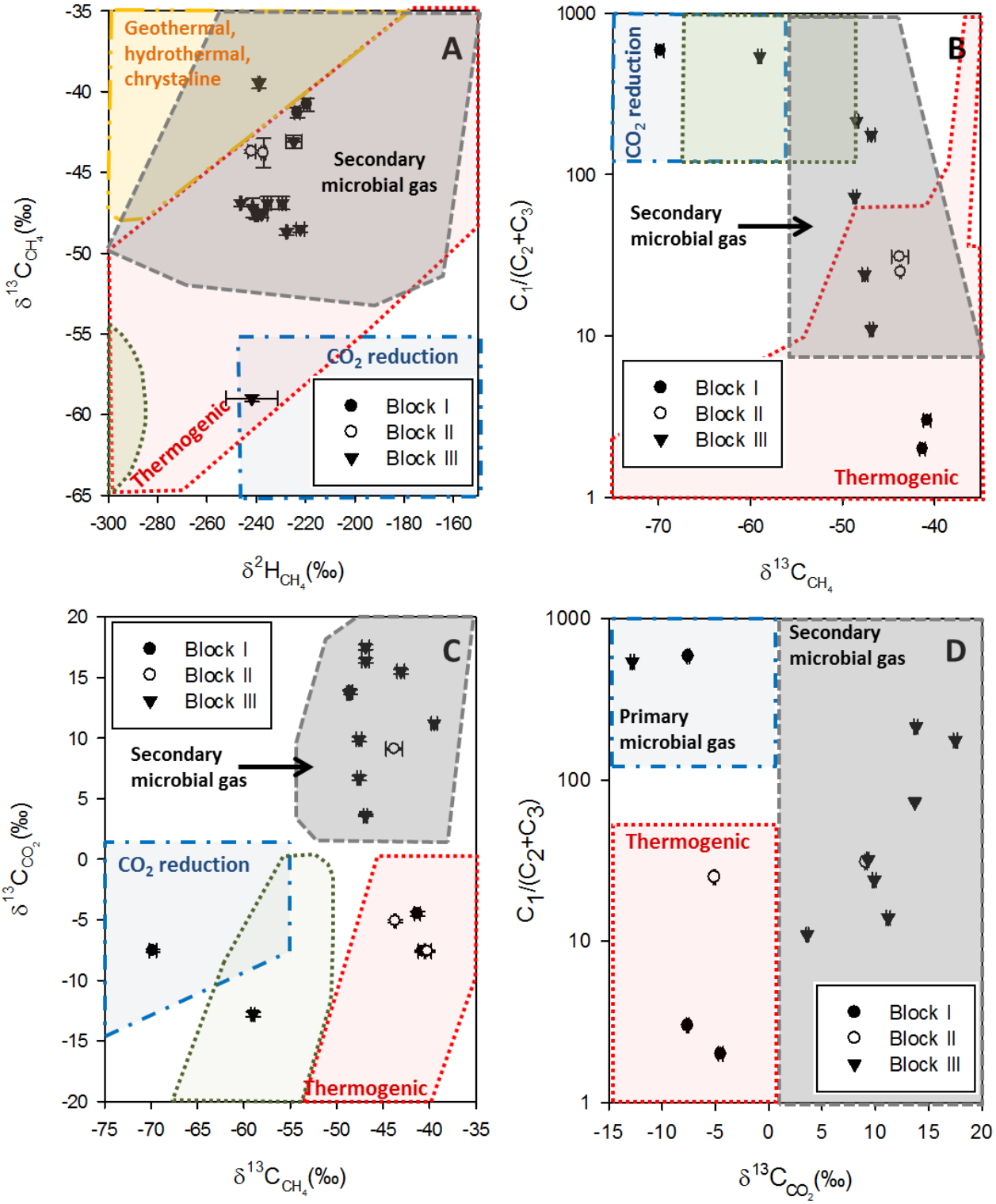
2.5. Appendix

Appendix 2.A: Chemical composition of the formation waters of the Dagang reservoir.

		8p	8i	9	10i		10p		13
pH						8.8		8.8	
EC	**µS/cm**					9610		6190	
K^+	**mg/l**	25.4	35.9	57.8	33.2	47.4	22.9	33.0	12.2
Na^+	**mg/l**	1821	1803	2884	2012	2205	1366	1421	1133
Cl^-	**mg/l**	1161	1152	1027	1494	1841	935	1014	662
Mg^{2+}	**mg/l**	31.4	30.1	21.3	41.4	84.6	60.0	78.8	22.9
Ca^{2+}	**mg/l**	25.9	29.8	23.1	48.3	28.5	46.4	73.1	23.8
SO_4^{2-}	**mg/l**	4.56	12.4	2.24	12.4	41.9	3.01	24.0	2.06
HCO_3	**mg/l**	3120	3117	6451	3174	3012	2570	2509	2048
Fe(II)	**mg/l**	0.11	0.91	1.19	1.03	0.235	0.08	0.089	0.26
Mn^{2+}	**mg/l**	0.01	0.05	0.04	0.07	0.001	-0.01	0.135	0.01
Br	**mg/l**	ND	ND	ND	ND	4.8	ND	2.4	ND
BO_2	**mg/l**	9.42	11.0	7.78	22.4	26.1	3.11	3.68	2.04
Ba	**mg/l**	2.53	1.28	1.01	2.16	0.866	1.30	1.09	1.03
Li	**mg/l**	0.38	0.54	1.07	0.61	0.653	0.39	0.394	0.17
SiO_2	**mg/l**	27.4	36.9	41.1	41.3	62.4	53.8	74.6	35.7
Sr	**mg/l**	1.25	0.83	0.36	1.61	1.27	0.54	0.586	0.66
Total salinity	**g/l**	6.2	6.2	10.5	6.9	7.4	5.1	5.2	4.0

ND: below detection limit.

Appendix 2.B: Genetic diagrams for gases of the different wells. A) Carbon vs. hydrogen isotopic composition of methane. B) A Bernard diagram (Bernard et al., 1976). C) Carbon isotopic composition of methane and CO_2. D) Dryness $C_1/(C_2+C_3)$ vs. isotopic composition of CO_2. All genetic fields have been defined according to Whiticar et al. (1999) and Milkov (2010 and 2011). Error bars represent the standard deviation of replicate samples.



Appendix 2.C. Archaeal clone library community profiles derived from nine Dagang production/injection wells.

Well	8	8	9	10i	10p	11	13	14	15
Methanosphaera	27	25	3	36	0	57	0	0	26
Methanosarcina	0	0	0	0	88	0	0	0	0
Hyperthermus	8	0	1	0	0	0	0	73	0
Methanomethylovorans	19	6	40	14	0	0	0	0	0
Methanobacterium	0	2	0	0	0	27	1	0	41
Methanosaeta	7	0	13	9	0	0	15	3	0
Thermofilum	2	3	0	0	0	2	37	1	5
Methanosalsum	1	1	0	0	0	6	2	0	13
Methanolobus	0	0	0	0	0	0	24	0	0
Thermogymnomonas	11	1	1	2	0	1	8	0	2
Thermococcus	0	0	19	0	0	0	0	0	0
Archaeoglobus	0	1	0	0	0	0	0	14	0
Methermicoccus	13	0	0	0	0	1	0	0	0
Caldisphaera	0	9	0	0	0	0	0	0	0
Methanococcus	0	7	0	0	0	0	0	0	0
Methanocella	0	0	0	0	0	0	6	0	0
Methanothermobacter	0	0	4	0	0	0	1	0	0
Haladaptatus	0	0	0	0	0	1	0	0	0
Fervidicoccus	1	0	0	0	0	0	0	0	0
Total	89	55	81	61	88	95	94	91	87

Chapter III

Microbial methane formation in deep aquifers of a coal bearing sedimentary basin, Germany

3. Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany

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Manuscript in preparation
Running title: Methanogenesis in coal-bearing sediments

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Keywords: methanogenesis, Cenozoic sediments, fluvial deposits, stable isotope fractionation, methanogenic hydrocarbon degradation, methanogenic Archaea

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Abstract

In this study we investigated the specific microbial community inside anoxic cenozoic sediments, in particular its contribution to hydrocarbon degradation processes in a lignite-bearing sedimentary basin located in Germany. The stable isotope signature of methane measured in groundwater and coal-rich sediment samples indicated methanogenic activity. Analysis of 16S rRNA gene sequences showed the presence of methanogenic *Archaea* predominantly belonging to the orders *Methanosarcinales* and *Methanomicrobiales* capable of acetoclastic or hydrogenotrophic methanogenesis. Additionally, members of *Crenarchaeota* belonging to unclassified *Thermoprotei* were detected. Furthermore, we identified fermenting, sulfate-, nitrate- and metal-reducing, or acetogenic *Bacteria* clustering with the phyla *Proteobacteria*, complemented by members of the classes *Actinobacteria, Anaerolineae,* and *Clostridia*. The indigenous community of microbes found in the groundwater as well as in the coal-rich sediments, are able to degrade coal-derived organic components and to produce methane as the final product.

3.1. Introduction

Coal is extremely rich in complex organic matter and therefore a very attractive carbon source for microbial biodegradation (Fakoussa and Hofrichter, 1999). It is a sediment formed from the remains of dead plant material that has been buried under elevated temperature and pressure conditions over geological times. Coal consists primarily of carbon (50-98 % by weight) beside variable quantities of hydrate, oxygen, sulfur, nitrogen (Libes, 2009). Lignite is soft brown sediment with high moisture content and the relatively low heating value. These characteristics put it somewhere between peat and sub-bituminous coal. Based on the high organic matter concentrations, the coal-derived sediments are potential important microbial sources of energy (Fry et al., 2009).

The biodegradation of coal components, containing carbon, hydrogen, sulfur and nitrogen, is primarily performed by bacterial fermentation of polymers and monomers to fatty acids, organic acids, alcohols and/or hydrogen and carbon dioxide. The products can then be used by methanogens via two common methanogenic pathways: the acetoclastic (acetate is converted to methane and carbon dioxide) and the hydrogenotrophic methanogenesis, in which carbon dioxide (CO_2) is reduced using hydrogen (H_2) to the final products methane (CH_4) and water. Thus, the successful conversion of coal to methane depends on the syntrophic interaction of both groups of fermenting bacteria and methanogenic archaea. Furthermore, methylotrophic methanogens take part at the degradation of complex organic matter. These methanogens are able to utilize a wide range of one-carbon compounds like methanol, the methylamines, halomethanes, and methylated-sulfur compounds as the carbon source for their growth. Biogenic methane generation from coal material under anoxic conditions, mostly in association with coalbed methane, has been documented previously by different working groups over the world. Recently, the microbial conversion of hard coal into methane by a complex microbial consortium in reservoirs has been described in numerous previous studies (Beckmann et al., 2011a; Guo et al., 2012; Krüger et al., 2008). The Gippsland Basin (Midgley et al., 2010), the Illinois Basin (Strąpoć et al., 2008), northern Japan (Shimizu et al., 2007) and western Canada (Penner et al., 2010) mostly revealed the presence of the archaeal genera *Methanosarcina*, *Methanolobus*, *Methanobacteria*, *Methanocorpusculum*, *Methanosaeta*, *Methanococci*, *Methanoculleus*, and *Methanoregula*. The dominant *Bacteria*, published by Strąpoć et al. (2011b), include the phyla *Firmicutes*, *Spirochaetes*, *Bacteroidetes* and members of all

subgroups of *Proteobacteria*. In subsurface groundwater from a pristine aquifer with lignite layers Detmers et al. (2001) showed the presence of an anaerobic food chain in the aquifers. They observed that the presence of fermenting *Betaproteobacteria* and the activity of sulfate-reducing bacteria (*Desulfotomaculum spp.*) reached highest population values at the interface between aquifer and lignite seam.

To date, little is known about the microbial diversity and geochemical influences of hydrocarbon biodegradation combined with the interference of putative microbial substrates in ligniteous coal-rich sediments and coal-associated aquifers in the subsurface.

The aim of this work was to study the importance of coal-derived organic substrates for microorganisms present in sediment and water samples, with special focus on methanogenesis. Additionally, investigations of microbial syntrophic interactions and degradation pathways contribute to a better understanding of metabolic processes in coal-associated habitats - known to lead to biogenic gas generation. To study this, we sampled groundwater from an aquifer and coal-rich sediments from a coal-associated sedimentary basin. Isotopic signatures of methane ($\delta^{13}C_{CH4}$ and $\delta^{13}D_{CH4}$) in the groundwater of coal-associated aquifer and in coal-rich sediments were measured. Furthermore, geochemical investigations were combined with microbiological and molecular biological approaches leading to the identification and characterization of the bacterial and archaeal community composition in coal-rich sediment and groundwater samples as well as methanogenic enrichment cultures with hydrocarbons as sole carbon source. Combining these methods we were able to show a close interaction between organic substrates and microbial population in coal-rich sediments with the groundwater aquifer system.

3.2. Materials and methods

3.2.1. Sampling and sample preparation

Groundwater samples and samples of coal-bearing sediments were collected in February 2009. Groundwater samples were taken from ten different fresh water wells in the proximity of an open-cast mine. Coal-rich sediment samples were collected from freshly mined heaps of brown coal (sample 1), air-dried coal from the bottom of the mine (sample 2) and coal slurry from a wet spot (sample 3). Collected groundwater and coal-rich

sediment samples were transferred into sterile glass bottles and immediately flushed with N_2. Directly after collection of groundwater samples, pH-value, temperature, conductivity and salinity of the waters were determined. All samples were stored and transported at 4°C.

3.2.2. Cultivation methods

To prevent the intrusion of air, samples used as inoculum for incubations as well as the medium were handled in an anaerobic glove box.

For first incubations 3 g of coal-rich sediment and 10 mL of groundwater sample were transferred into autoclaved aseptic Hungate vials (19 mL volume) and 5 mL freshwater medium after Widdel and Bak (1992) with 10 mM SO_4 was added. The glass vials were sealed with sterile butyl rubber stoppers and aluminum crimps caps. Cultures amended with acetate (10 mM) or a H_2 / CO_2-mix (80 / 20 %) were prepared to investigate the different methanogenic degradation pathways. To study the methanogenic activity, methane and CO_2 formation in the headspace of the microcosms were measured by gas chromatography repeatedly every month. In addition, cultures were either grown without any additives or with 2-bromoethanesulfonate (BES; 10 mM), a specific inhibitor for methanogenic microorganisms for the detection of possible non-microbial methane emission from the water or coal-rich sediment samples. Cultures with the addition of sodium azide (NaN_3, 50 mM), a strong microbial toxin, were performed to show feasible methane degassing from non-microbial origin.

In a second step of cultivation, cultures were re-inoculated into fresh medium and amended with ^{13}C-labeled substrates to investigate the transformation of selected hydrocarbons into methane and CO_2. ^{13}C-labeled or unlabeled substrates, particularly hexadecane, ethylbenzene (both 0.1 % v/v), toluene or methylnaphthalene (0.5 mg) were added into the anaerobic enrichment cultures, containing 25 mL fresh sterile medium and 5 mL transferred pre-culture from groundwater or coal-rich sediment samples (as described previously) in 56-ml serum bottles. U-^{13}C-hexadecane was synthesized as described by Feisthauer et al. (2010). The other labeled and unlabeled single hydrocarbons (ethylbenzene, toluene and methylnaphthalene) were obtained from Campro Scientific GmbH Germany.

Each incubation was set-up in triplicates and incubated at 30°C in dark. Methane and CO_2 production rates were calculated by linear regression and expressed in µmol day^{-1} mL^{-1} groundwater or µmol day^{-1} gDW^{-1} (dry weight) of the coal-rich sediment (Krüger et al., 2001).

3.2.3. Analytical methods

From ten different groundwater samples, the elemental composition was analyzed using an inductively-coupled-plasma mass-spectrometry instrument (ICP-MS ELAN 5000, Perkin Elmer Sciex, USA) (Dekov et al., 2007). Concentrations of potassium, sodium, chloride, magnesium, calcium, sulfate, bicarbonate, ferrous iron, manganese, nitrate, ammonium, nitrite, phosphate, aluminum, arsenic, borate, barium, cadmium, chromium, lithium, nickel, lead, silica and strontium were measured. Furthermore, the electrical conductivity (EC), the total inorganic carbon content (TIC) and the non-purgeable organic carbon (NPOC) were determined.

Isotopic analyses of δ^{13}C- and δ^{13}D-values from methane and carbon dioxide of the emanation of the gases released from the coal-rich sediment samples in the bottles were performed.

Methane in the headspace of the microcosms was analyzed using a gas chromatograph with flame ionization detector (GC-FID) equipped with a 6´ Hayesep D column (SRI 8610C, SRI Instruments, USA) continuously running at 60°C. Carbon dioxide concentrations were determined by a methanizer-equipped GC with FID detector. The stable isotopic composition of methane and CO_2 was analyzed using a gas-chromatography-combustion-isotope ratio mass spectrometry system (GC-C-IRM-MS) (Feisthauer et al., 2010; Herrmann et al., 2010). The δ^{13}C- and δ^{13}D-values are expressed as ‰ vs. Vienna Pee Dee Belemnite (VPDB) and Standard Mean Ocean Water (SMOW).

3.2.4. Molecular biological methods

Total cell numbers were counted after staining with SYBR Green II under the fluorescence microscope as described by Weinbauer et al. (1998).

Genomic DNA from the coal-rich sediments and from the microcosms amended with hydrocarbons was extracted by bead-beating and a phenol-chloroform extraction using protocols from Gabor et al. (2003) and Lueders et al. (2004). For further purification of crude DNA extracts, ethidium bromide was added to 0.6 mg mL^{-1} DNA extract and ammonium acetate to 2.6 M final concentration. More particular purification steps were carried out following Lovell and Piceno (1994). Groundwater were aseptically filtered with membrane filters (0.22 µm) (Whatman, General Electric Company, Munich, Germany) and DNA was

extracted from the filters using the bead-beating and phenol-chloroform extraction method as mentioned above without the ethidium bromide washing steps.

The quantity of 16S rRNA gene copy numbers of *Archaea* and *Bacteria* were determined as described previously (Nadkarni et al., 2002; Takai and Horikoshi, 2000) using the Q-PCR instrument ABI Prism 7000 (Applied Biosystems, Life Technologies Corporation, USA). Concentrations of methyl-coenzyme M reductase subunit alpha gene (*mcrA*) (using mlas and mcrA-rev primers) and *dsrA* gene coding for the alpha subunit of the dissimilatory (bi)sulfite reductase of sulfate-reducing prokaryotes were determined according to Steinberg and Regan (2008); (2009) and Schippers and Neretin (2006). The quantification of *Crenarchaea* was performed by using method and primer set described by Schleper et al. (1997). All Q-PCR reactions were measured in three parallels and three dilutions. To perform Q-PCR quantification, a StepOne detection system (StepOne version 2.0, Applied Biosystems, USA) coupled with the StepOne v2.1 software was used.

For terminal restriction fragment length polymorphism (T-RFLP) analysis, extracted DNA was used as template for PCR amplification of phosphoramidite fluorochrome 5-carboxyfluorescein (FAM)-labeled amplicons. Amplifications were generated with the use of the primer sets Ar109f and 912rt-FAM, or Ba27f-FAM and 907r. To account for possible inhibitor effects in environmental DNA extracts, a dilution series of each fresh extract was used. T-RFLP analysis of PCR products was done using the restriction endonucleoases TaqI (archaeal assay) and MspI (bacterial assay), respectively. The procedure was described by Winderl et al. (2008); (2010). Capillary electrophoresis and data collection were operated on an ABI 3730 Genetic Analyzer (Applied Biosystems, USA). The electropherograms were processed with sequence analysis software PeakScanner 1.0 and GeneMapper 4.0 (Applied Biosystems, USA). T-RFLP histograms were performed with the use of the T-REX online software using the default settings (Culman et al., 2009). Terminal restriction fragments were compared to theoretical predictions from 16S rRNA gene sequences for a preliminary identification of specific groups of bacteria. The particular T-RF length represents the most abundant microorganisms within the bacterial community.

Clone libraries were created using DNA extract from the original coal-rich sediment samples and the derived microcosms amended with hydrocarbons. 16S rRNA gene fragments were amplified by PCR using the domain specific primer pairs 21f (5`-TTC CGG TTG ATC CYG CCG GA) and 958r (5`-YCC GGC GTT GAM TCC AAT T) for *Archaea* (DeLong, 1992),

and GM3f (5`-AGA GTT TGA TCM TGG C) and GM4r (5`-TAC CTT GTT ACG ACT T) for *Bacteria* (Lane, 1991). Cloning and sequencing of the archaeal and bacterial 16S rRNA amplicons was performed by Microsynth AG (www.microsynth.ch, Switzerland). Sequences were assembled use the Geneious ProTM 5.3 software (www.geneious.com). Prior to phylogenetic analysis, vector sequences flanking the 16S rRNA gene inserts were removed. Chimeric sequences were detected using the DECIPHER´s Find Chimeras online software (Wright et al., 2012) from the University of Wisconsin Madison (http://decipher.cee.wisc.edu/FindChimeras.html) and were excluded from further analysis. Sequences were compared to GenBank BLASTn algorithm from the National Center for Biotechnology Information (Altschul et al., 1990) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project Classifier (Wang et al., 2007) (RDP; http://rdp.cme.msu.edu/classifier/classifier.jsp) to select closely related species. Sequences were aligned with their nearest neighbors in the SSU dataset using SINA Alignment Service (www.arb-silva.de/aligner/) (Pruesse et al., 2012).

Amplicon pyrosequencing, amplicon treatment, and downstream analysis for the domain *Bacteria* was performed as described in Pilloni et al. (2011).

Cloning sequences and contigs originate from pyrosequencing were grouped into operational taxonomic units (OTUs) based on a sequence similarity cutoff of 97 % (Yu et al., 2006) using mother software (www.mothur.org) (Schloss et al., 2009).

3.3. Results

3.3.1. Geochemical characterization of groundwater samples

All groundwater samples showed pH-values of ~7, the salinity ranged between 5 and 9 ‰. The locally measured water temperature was ~29°C, the air temperature ~0°C; only in well site 3 a water temperature of 15°C was detected. Groundwater from sampling sites 2, 4, 5, 10 smelled sulfurous, the other samples were odorless. The dissolved CH_4 concentrations ranged from 10 µM in well 1 to 100 µM in well 5 (Table 3.1). The isotopic signature of carbon from the dissolved methane ($\delta^{13}C\text{-}CH_4$) in the water samples ranged from -71 to -80 ‰. Hydrogen isotopic values ranging from -234 to -376 ‰ (Table 3.1).

Table 3.1: Methane content and stable isotope signatures of natural methane and carbon dioxide in groundwater samples from deep aquifers of coal-rich sedimentary basin. VPDB – Vienna PeeDee Belemnite; SMOW – Standard Mean Ocean Water

groundwater site	CH_4-content [µM]	$\delta^{13}C$-CH_4 [‰, VPDB]	δD-CH_4 [‰, SMOW]	$\delta^{13}C$-CO_2 [‰, VPDB]
well 1	10.237	-79.8	-244.7	-19.9
well 2	24.229	-72.8	-246.2	-16.5
well 3	22.099	-72.0	-233.5	-16.8
well 4	37.518	-74.4	-248.6	-17.3
well 5	99.412	-71.0	-236.8	-14.8
well 6	51.016	-72.5	-239.4	-16.0
well 7	13.368	-79.7	-376.4	-20.1
well 8	71.299	-75.9	-258.6	-14.7
well 9	16.516	-73.4	-300.4	-19.1
well 10	24.890	-76.0	-345.5	-16.8

The geochemical analysis of the groundwater samples revealed that concentrations of sulfate between 0.5 in well 4 to 14.2 mg l^{-1} in well 3 (Table 3.2). Ferrous iron showed concentrations from 0.1 to 2.0 mg l^{-1} and manganese was measured with concentrations of 0.01 to 0.2 mg l^{-1}. Nitrate and ammonium were present in low concentrations from 0.01 to 0.3 mg l^{-1} (NO_3^-) and from 0.4 to 0.8 mg l^{-1} (NH_4^+), respectively. No nitrite was detected. Further, relatively high concentrations of total inorganic carbon (TIC) (64-117 mg l^{-1}) were measured, while the groundwater samples showed a relatively low content of non-purgeable organic carbon (NPOC) (3-11 mg l^{-1}).

3.3.2. Isotopic signatures of gasses from coal-rich sediments

The C and H isotopic signatures of natural gas originated from coal-rich sediments were determined. The geochemical analysis of collected gas samples revealed $\delta^{13}C_{CH4}$-values of -70.8 ‰ (surface location 1), -72.3 ‰ (surface location 2), and -63.4 ‰ (surface location 3). $\delta^{13}C$-values of carbon dioxide showed signatures of -16.8, -15.6, and -24.1 ‰.

Table 3.2: Geochemical properties of groundwater samples collected from wells located in coal-rich sediments. EC-Electrical Conductivity; TIC-Total Inorganic Carbon; NPOC- Non Purgeable Organic Carbon

Water site	pH-value	EC [µS/cm]	K^+ [mg/l]	Na^+ [mg/l]	Cl^- [mg/l]	Mg^{2+} [mg/l]	Ca^{2+} [mg/l]	SO_4^{2-} [mg/l]	HCO_3^- [mg/l]	Fe^{2+} [mg/l]	Mn^{2+} [mg/l]	NO_3^- [mg/l]
well 1	6.8	471	10.4	63.9	31.4	10.8	17.6	1.73	242	0.459	0.039	0.27
well 2	6.9	691	11.1	111	34.8	13.2	21.2	1.04	398	0.282	0.056	0.09
well 3	6.8	558	7.8	55.2	16.9	16.1	41.2	14.2	325	1.95	0.191	0.02
well 4	7.3	616	10.8	90.7	27.3	13.5	21.5	0.54	359	0.302	0.034	0.03
well 5	7.1	848	13.6	150	28.7	17.5	26.9	1.12	523	0.951	0.052	0.01
well 6	7.2	699	11.5	131	27.8	12.2	18.7	0.84	417	0.364	0.053	0.02
well 7	6.9	423	9.3	53.3	19.7	9.84	20.2	2.16	237	0.234	0.071	0.02
well 8	7.1	742	10.6	169	21.2	7.08	8.53	0.95	456	0.299	0.011	0.03
well 9	6.9	485	9.8	61.0	22.0	11.7	22.9	3.24	273	0.272	0.056	0.02
well 10	7.3	551	10.0	81.5	29.8	9.99	19.9	0.87	304	0.124	0.056	0.04

Water site	NH_4^+ [mg/l]	PO_4^{3-} [mg/l]	Al^{3+} [mg/l]	BO_2^- [mg/l]	Ba^{2+} [mg/l]	Cr^{2+} [mg/l]	Li^+ [mg/l]	SiO_2 [mg/l]	Sr^{2+} [mg/l]	TIC [mg/l]	NPOC [mg/l]
well 1	0.64	0.45	0.005	0.57	0.161	0.013	0.130	12.3	0.269	64	3.1
well 2	0.66	0.56	0.003	0.63	0.229	0.011	0.117	14.9	0.246	100	4.9
well 3	0.39	0.90	0.006	0.34	0.093	0.015	0.091	25.3	0.380	89	2.3
well 4	0.66	0.49	0.003	0.61	0.207	0.013	0.120	15.5	0.299	90	4.1
well 5	0.79	0.72	0.005	0.81	0.205	0.013	0.159	17.4	0.331	133	7.1
well 6	0.72	0.69	0.004	0.64	0.215	0.012	0.117	15.4	0.193	108	6.1
well 7	0.5	0.42	0.003	0.34	0.307	0.017	0.087	14.3	0.243	67	2.9
well 8	0.6	1.29	0.006	0.94	0.064	0.017	0.149	13.2	0.093	117	11.4
well 9	0.54	0.36	0	0.41	0.274	0.018	0.100	14.6	0.295	73	2.9
well 10	0.57	0.52	0	0.41	0.270	0.016	0.100	14.7	0.263	82	4.6

3.3.3. Methanogenic and hydrocarbon degradation activity

Groundwater and coal-rich sediment samples were incubated in microcosms under methanogenic conditions and with several additions over a time of 94 days. No methane production was measured in the cultures incubated with the inhibitors BES and sodium azide (data not shown). Microcosms cultivated without any additives showed no significant methane production rates, as well. However, microcosms with the addition of H_2 / CO_2-mix as substrate for hydrogenotrophic methanogenesis showed methane production in all microcosms inoculated with groundwater and coal-rich sediments (Figure 3.1). Further, only in the groundwater sample 8 acetate served as substrate for acetoclastic methanogenesis, while all the other incubations showed no methane production with acetate as substrate.

Additionally, in hydrocarbon amended microcosms exclusively those inoculated with coal-rich sediment and amended with hexadecane showed significantly increased methane production rates.

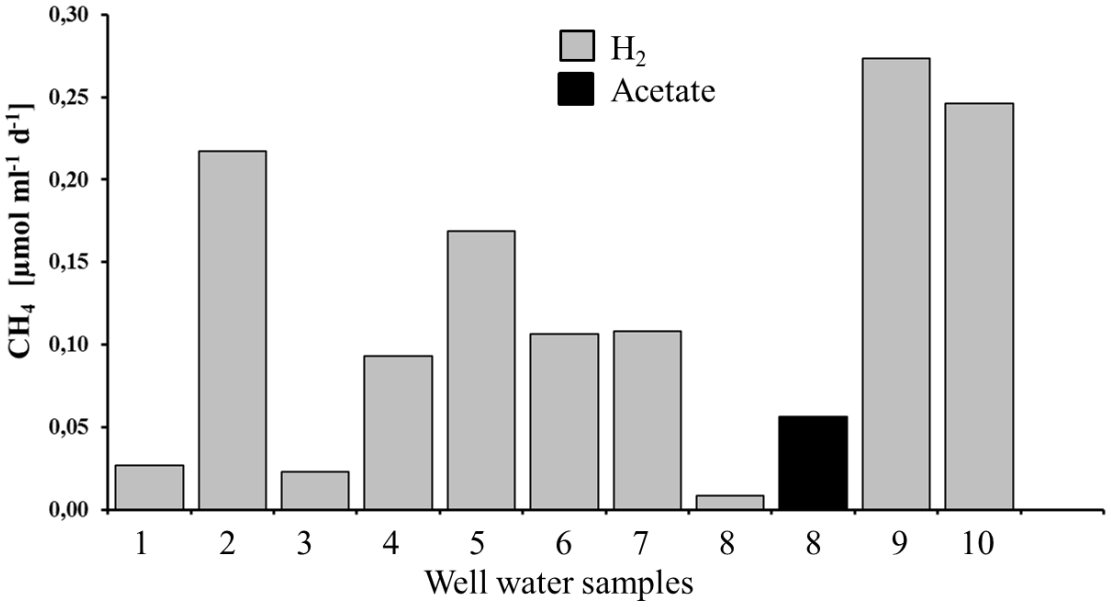


Figure 3.1: Methane production rates from anaerobic cultured groundwater samples 1-10 amended with H_2 (grey) or acetate (black). All cultures were incubated 73 days at 30°C.

3.3.4. Quantification of microbial groups

The total number of cells in the groundwater samples, determined by staining with SYBR Green, was in the samples 4, 5 and 10 around 1×10^7 cells mL^{-1}.

Groundwater sample 3 showed with 1×10^4 cells mL^{-1} the lowest numbers. Groundwater samples 1, 2, 6, 7, 8 and 9 showed intermediate cell numbers ranging from 2 to 9×10^6 cells mL^{-1}.

The abundance of selected microbial groups was determined in the coal-rich sediment samples via quantitative (real time) PCR (Q-PCR) (Figure 3.2). The *Bacteria* were detected with 16S rRNA gene copy numbers ranging between 2×10^9 and 1×10^{10} copies g^{-1}. *Archaea* only were present in a range of 10^4 to 10^5 copies g^{-1}. *Crenarchaeota* were found in all three coal samples in nearly similar 16S rRNA gene copy numbers (6×10^6, 8×10^6, and 1×10^6 copies g^{-1} in coal-rich sediment sample 1, 2, and 3). The quantitative detection of the dissimilatory sulfite reductase gene (*dsrA*) revealed sulfate-reducing prokaryotes in the range of 2×10^6 (coaly sediment 3) and 1×10^7 copies g^{-1} in coaly sediment 1 and 2. The methyl-coenzyme M reductase gene (*mcrA*) as a proxy for methanogenic *Archaea* was quantified in coaly sediment sample 1 with 1×10^5 copies g^{-1} and in coaly sediment 2 and 3 with 2×10^5 copies g^{-1}.

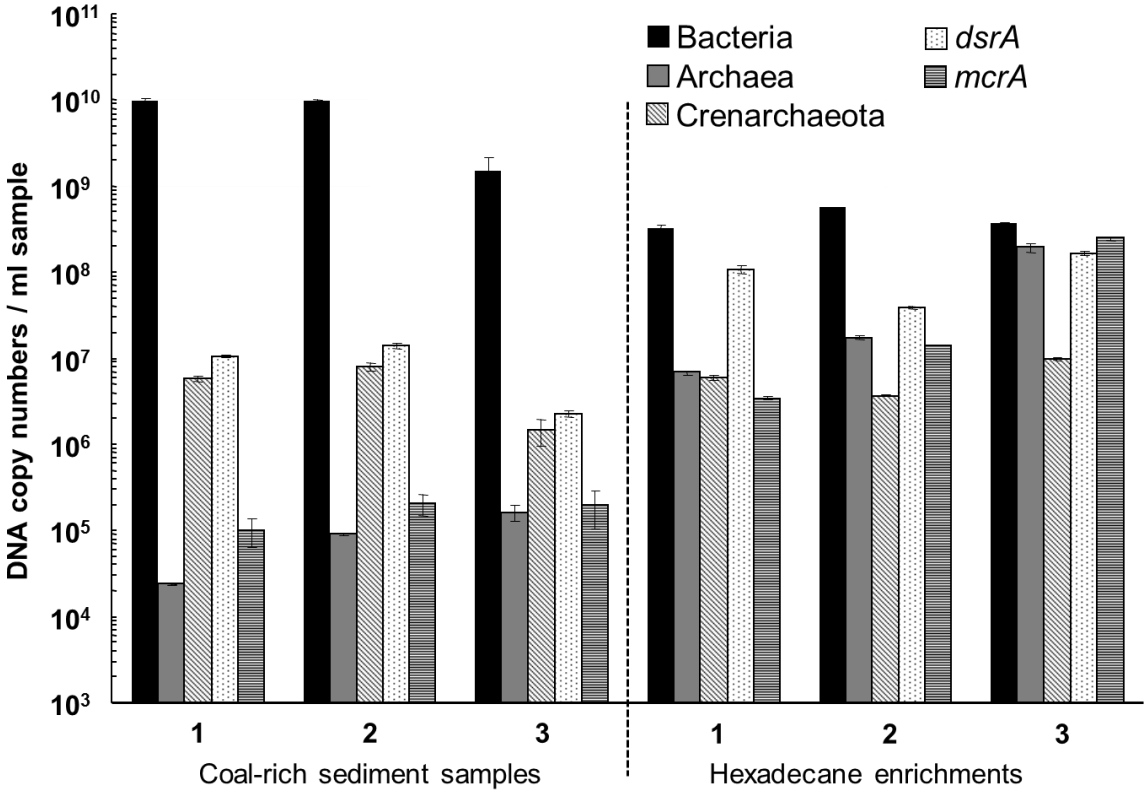


Figure 3.2: Quantitative analysis from the different coal-rich sediment samples 1, 2, 3, and their derived enrichment cultures amended with hexadecane. Relative abundance of *Bacteria*, *Archaea*, *Crenarchaeota* and the functional genes of sulfate-reducing prokaryotes (*dsrA*) and methanogens (*mcrA*) were determined by quantitative PCR. The error bars represent the standard deviation of three replicates.

In comparison to the coal-rich sediments, the bacterial 16S rRNA gene copy numbers in the coal-rich sediment enrichments amended with hexadecane showed a slight decreased quantitative abundance of 3 x 10^8, 6 x 10^8, and 4 x 10^8 copies mL^{-1} in samples 1, 2, 3 (Figure 3.2). Whereas copy numbers of *Archaea* and methanogens were 3-4 orders of magnitude higher than those of the coal-rich sediments. Moreover, the amounts of copy numbers of *Crenarchaeota* as well as sulfate-reducing prokaryotes were nearly similar in all three enrichments and revealed a higher abundance compared to coal-rich sediments.

3.3.5. Phylogenetic analysis of the microbial community composition

The diversity of the microbial community in the groundwater samples was analyzed using T-RFLP and cloning. The bacterial community showed characteristic profiles for each sample, although most groundwater samples showed only one or two predominant peaks (Figure 3.3). Dominant T-RFs were assigned to the bacterial genera *Arthrobacter* (161-bp T-RF), related to the order *Actinomycetales* within the phylum *Actinobacteria*, *Sulfurospirillum* (467-bp T-RF), a member of the epsilonproteobacterial order of *Campylobacterales,* and *Arcobacter* (476-bp T-RF) within the class of *Epsilonproteobacteria*. One of the most abundant T-RF in groundwater sample 8 and 9 is the 492-bp T-RF and correspond to more than one group of bacteria. This T-RF could be assigned to members of *Pseudomonadales* (*Pseudomonas* sp. and *Acinetobacter* sp. within the *Gammaproteobacteria*), as well as to members affiliated to several families within the *Betaproteobacteria* (e.g. *Methylophilaceae*, *Oxalobacteraceae*, and *Comamonadaceae*). Beside these identifiable peaks, the community showed a couple of unknown fragments with varying lengths and quantities (Figure 3.3).

The archaeal community composition was highly reduced in all groundwater samples. Most of the 16S rRNA gene sequences were related to members of the *Methanomicrobia* and unclassified *Crenarchaeota* within the class of *Thermoprotei*. The respective T-RFLP fingerprints (Figure 3.4) showed only in groundwater sample 2 and 3 more than one T-RF peak.

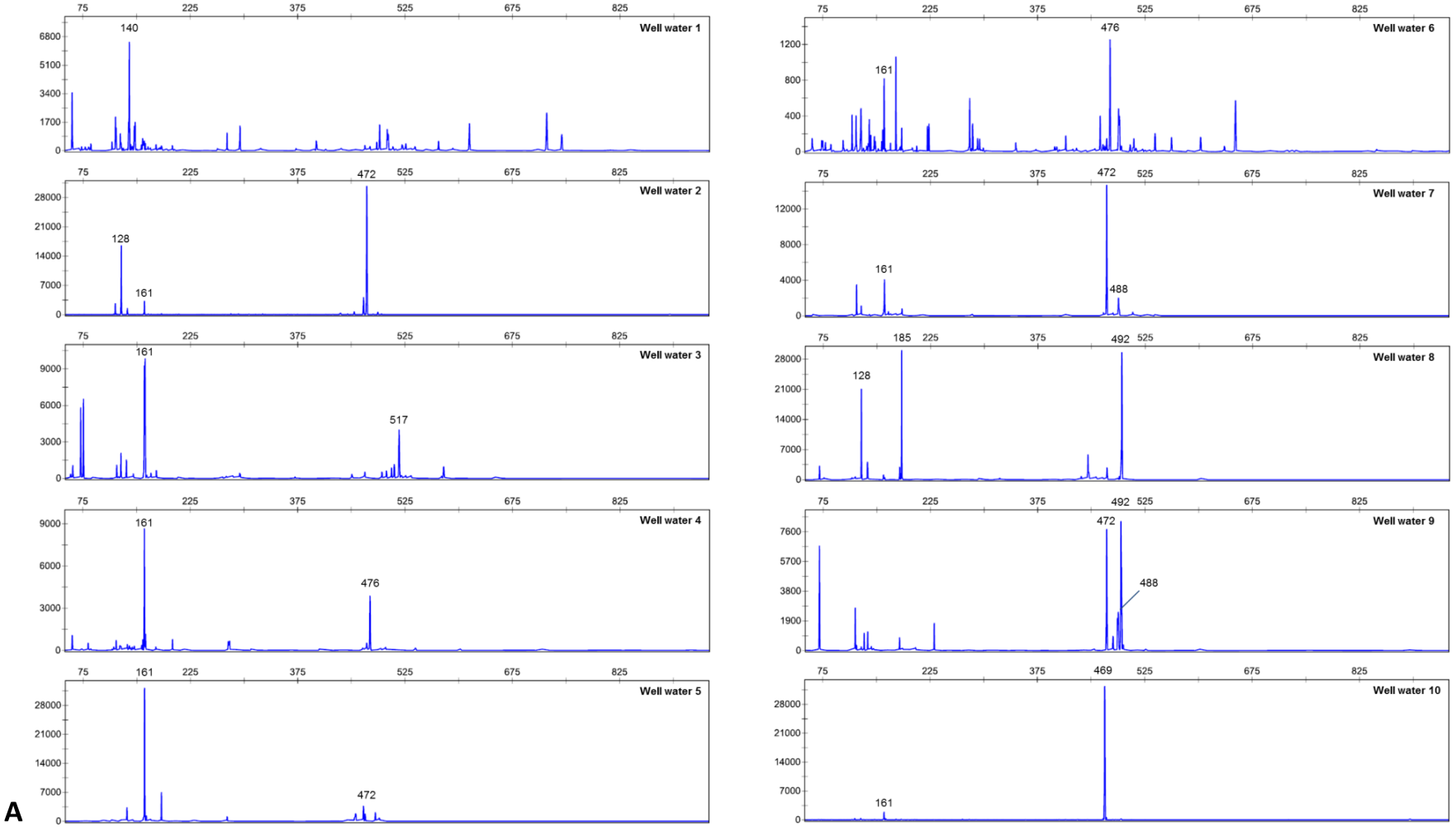
In groundwater sample 2 and 3 the detected T-RFs were assigned to *Methanosarcina horonobensis* (183-bp T-RFs), *Methanosaeta concilii* (282-bp T-RFs)

and *Methanoregula formicicum* (392-bp T-RFs). In sample well 2 and well 3 a not assignable but dominant peak with the fragment length of 227 bp was observed. It is not clear which archaeal species is concealed behind the 87-bp T-RF, the only dominant peak in groundwater samples 1, 4, 5, 7, 8, and 10.

The community composition in the groundwater samples showed high similarity with the coaly sediment community. According to groundwater samples, T-RFLP fingerprints of the three different coal-rich sediment samples (Figure 3.3) revealed a community which consists of species represented by two dominant fragment lengths. The 476-bp T-RF, assigned to *Arcobacter* sp. and the 492-bp T-RF, identified as species belonging to the genus *Methylotenera*, a *Betaproteobacteria*. Additionally, T-RFs with the length of 492 bp also matched with betaproteobacterial species affiliated to relatives of the orders *Burkholderiales* (e.g. *Oxalobacteraceae* and *Comamonadaceae*) and with the gammaproteobacterial order of *Pseudomonales* (mostly *Pseudomonas* sp.). Furthermore, the 467-bp T-RF with less abundance could identified as the genus *Sulfurospirillum*. *Arthrobacter* sp. (161-bp T-RF) was also detected in coal samples and takes part at the bacterial community.

The phylogenetic analysis of archaeal 16S rRNA gene fragments (Table 3.3; Figure 3.4) confirmed the presence of methanogenic *Euryarchaeota* in the coal-rich sediment samples, mainly *Methanosaeta* sp. (24 %). In lower abundance affiliations to *Methanosarcina* (1 %; 183-bp T-RF) and *Methanomethylovorans* (1 %; 790-bp T-RF) were identified. Furthermore, 46 % of sequences were affiliated to unclassified representatives of *Thermoprotei* within the phylum *Crenarchaeota*.

In contrast to the coal-rich sediment samples, the phylogenetic analysis of the bacterial lineages of enrichments amended with hexadecane (Figure 3.3) revealed a higher bacterial diversity and a shift of the bacterial dominance towards the *Deltaproteobacteria* and the *Anaerolineae* (*Chloroflexi)*. Within the *Deltaproteobacteria*, *Desulfobacterales, Desulfuromonadales* and *Syntrophobacterales* were predominantly detected. The remaining sequences fell in the classes of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Clostridia and Actinobacteria.*


A
Well water 1
140
Well water 2
128
161
472
Well water 3
161
517
Well water 4
161
476
Well water 5
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472
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492
Well water 9
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Well water 10
161
469

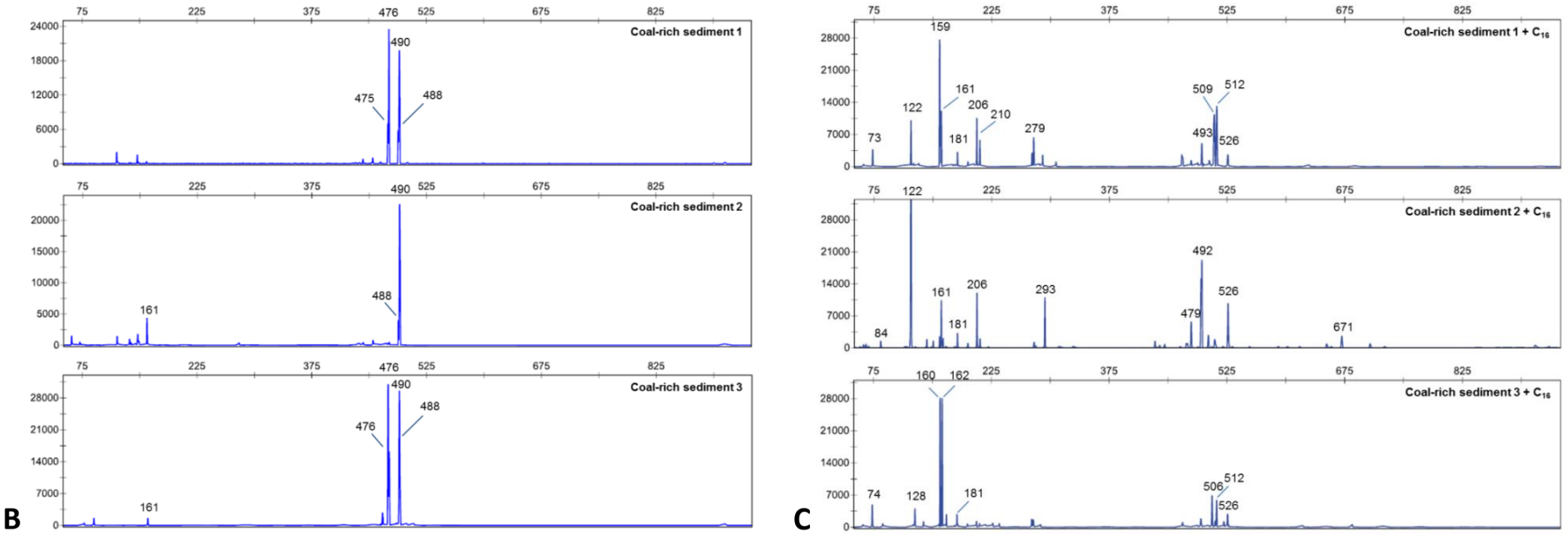
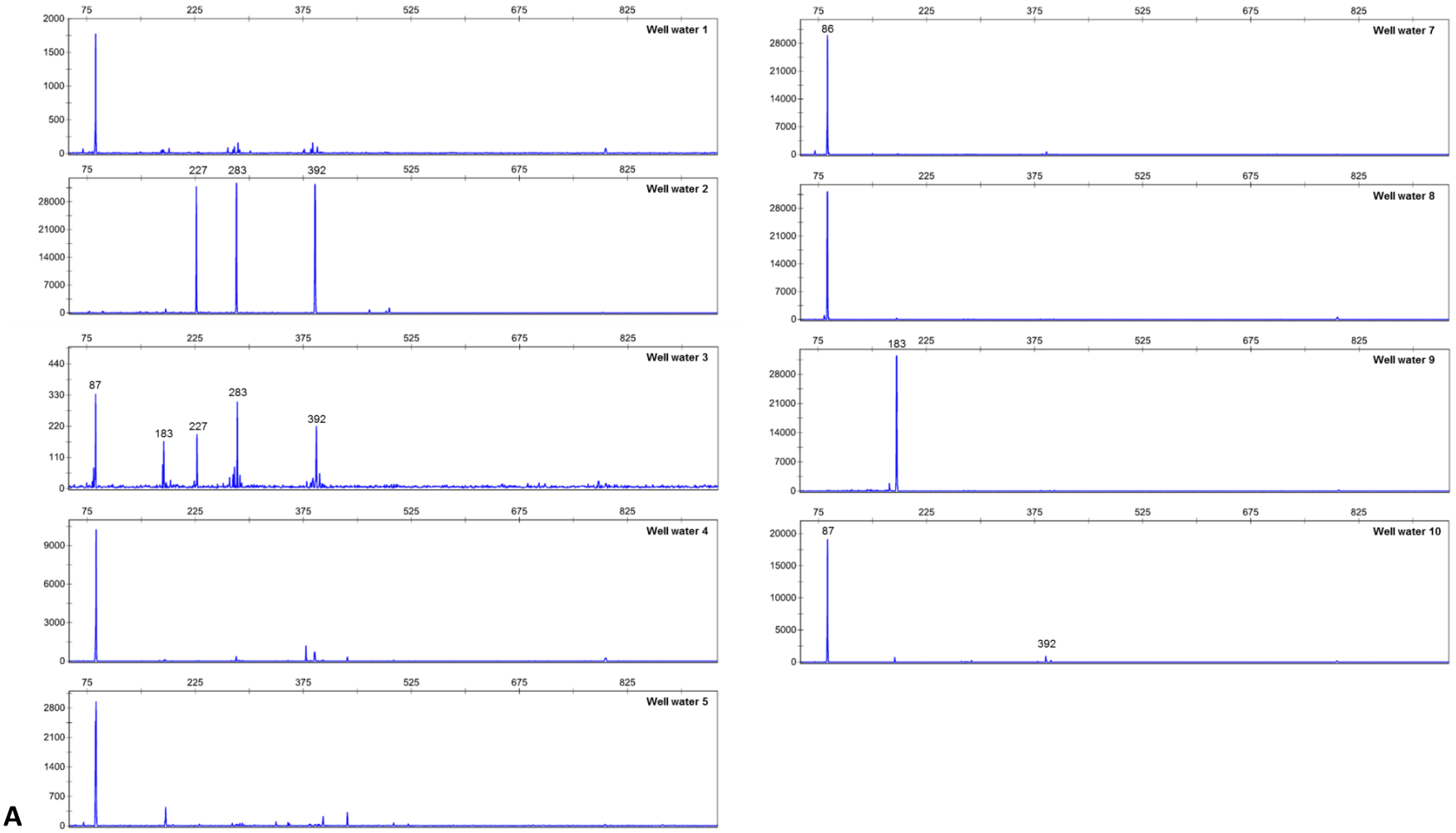


Figure 3.3: Bacterial 16S rRNA gene T-RFLP fingerprints from different groundwater samples collected from deep aquifers of coal-rich sedimentary basin (A), from coal-rich sediment samples (B) and the derived enrichment cultures amended with hexadecane (C). The lengths (bp) of selected T-RFs are given.


Well water 1
Well water 2
Well water 3
87
183
227
283
392
Well water 4
Well water 5
Well water 7
86
Well water 8
Well water 9
183
Well water 10
87
392

A

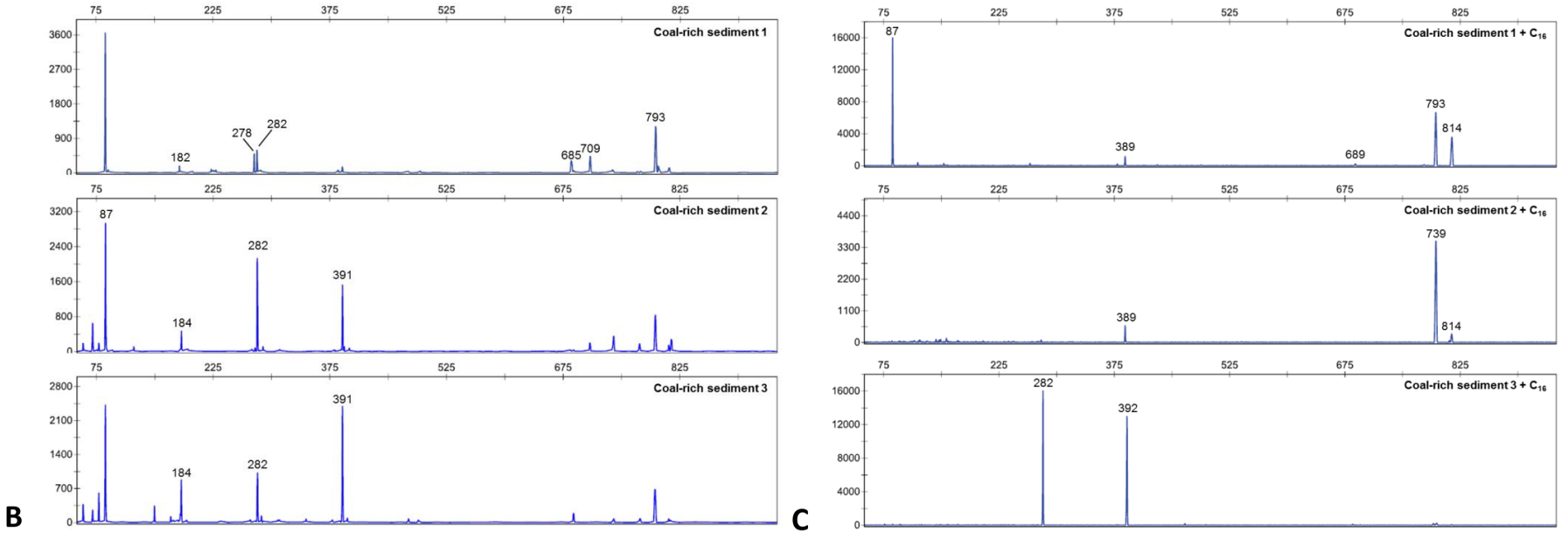


Figure 3.4: Archaeal 16S rRNA gene T-RFLP fingerprints from groundwater samples 1-10 collected from deep aquifers of coal-rich sedimentary basin (A), from coal-rich sediment samples (B) and the derived enrichment cultures amended with hexadecane (C). The lengths (bp) of selected T-RFs are given.

Table 3.3: Phylogenetic affiliation of clone sequences produced via clone libraries of archaeal 16S rRNA gene fragments retrieved from DNA extracted from unamended ligniteous coal-rich sediment and derived enrichment cultures amended with hexadecane. A total of 96 sequences (coal-rich sediments), 92 sequences (enrichment culture 1) and 95 sequences (enrichment cultures 2 and 3) were included in this calculation. The abundance of sequence accordance in percentage was calculated. Numbers in cursive characters show accordance with dominant peaks in the T-RFLP fingerprint plots (Figures 3.3 and 3.4).

	Original coaly Sediment samples			**Long-term enrichment cultures**								
				Coal-rich sed. 1			**Coal-rich sed. 2**			**Coal-rich sed. 3**		
RDP Classifier with confidence threshold of 80 %	No. seq.	%	*T-RFs [bp]*	No. seq.	%	*T-RFs [bp]*	No. seq.	%	*T-RFs [bp]*	No. seq.	%	*T-RFs [bp]*
Archaea	96	100		92	100		95	100		95	100	
Unclassified Archaea	10	10		3	3		1	1				
Euryarchaeota	42	44		2	2		2	2		58	61	
unclassified Euryarchaeota	14	15		2	2					56	59	
Methanomicrobia	28	29					2	2		2	2	
unclassified Methanomicrobia	3	3										
Methanosarcinales	25	26					2	2				
unclassified Methanosarcinales	22	23										
Methanosaetaceae	1	1										
Methanosaeta	1	1	*283*									
Methanosarcinaceae	2	2					2	2				
Methanosarcina	1	1	*183*									
Methanomethylovorans	1	1	*790*				2	2	*831*			
Methanomicrobiales										2	2	
Methanomicrobiales incertae sedis										2	2	
Methanoregula										2	2	*392*
Crenarchaeota	44	46		87	95		92	97		37	39	
unclassified Thermoprotei	44	46		87	95		92	97		37	39	

The phylogenic analysis of *Archaea* (Table 3.3; Figure 3.4) showed that the majority of the generated sequences from enrichment culture 1 and 2 belonged to unclassified *Thermoprotei*. The residual sequences arise from enrichment culture 2 showed a similarity to members of the genus *Methanomethylovorans* (790-bp T-RF). Most of the sequences from the enrichment culture 3 were matched with members belonging to the *Methanomicrobia*. Sequences affiliated to members of *Methanosaeta concilii* (282-bp T-RF) and *Methanoregula formicicum* (392-bp T-RF) were detected. The other part of sequences affiliated to unclassified *Thermoprotei,* as well.

3.4. Discussion

3.4.1. Geochemical links between groundwater and coal-bearing sediments

The isotopic signatures of methane ($\delta^{13}C_{CH4}$) measured in the groundwater samples are indicative for a biogenic origin of methane. Furthermore, the isotopic signature of the gases released from the coal-rich sediment samples and produced in laboratory microcosms showed similar $\delta^{13}C$-values with the methane measured from the groundwater. Whiticar (1999) postulated the isotope ratios for biogenic methane originated from different methanogenic pathways and indicated that the range of carbon and hydrogen isotopic ratios varying from -50 to -110 for $\delta^{13}C$ and from -150 to -400 for $\delta^{13}D$ (Figure 3.5). The examined groundwater fell within the range of methanogenic CO_2-reduction pathway, except three groundwater samples which showed $\delta^{13}C_{CH4}$-values correlated with acetoclastic methanogenesis. The varieties of $\delta^{13}C_{CH4}$-values correlate well with the identified methanogens which are able to use different pathways for methane production. These methanogens might be adapted to their directly affecting environmental conditions - the composition of electron acceptors, organic and inorganic carbon come from the coal-rich sediment, and the hydrolytic fermenting bacterial community, which provides the substrates for the methanogens.

The analysis of the water geochemistry revealed a sufficient presence of trace elements and electron acceptors to support microbial growth. Electron acceptors such as nitrate, manganese, ferrous iron, and sulfate are indicative of potential microbial reaction pathways, i.e. nitrate-, iron-, and sulfate-reduction. Many bacteria can couple the

degradation of hydrocarbon molecules to the reduction of soluble anions under anoxic conditions, and typically representatives are bacteria within the classes *Beta-*, *Gamma-*, and *Deltaproteobacteria* (Kaser and Coates, 2010). We identified several sulfate-, nitrate-, and metal-reducers within the bacterial communities of groundwater and coal-rich sediments which could be involved in hydrocarbon metabolism. Because of the high similarity of the microbial communities in both geosystems, we suppose that the indigenous microbes of coal seam layers were leached out into the groundwater by dewatering processes in the coal mine area or by the movement of meteoric water and thus affected the microbial groundwater community.

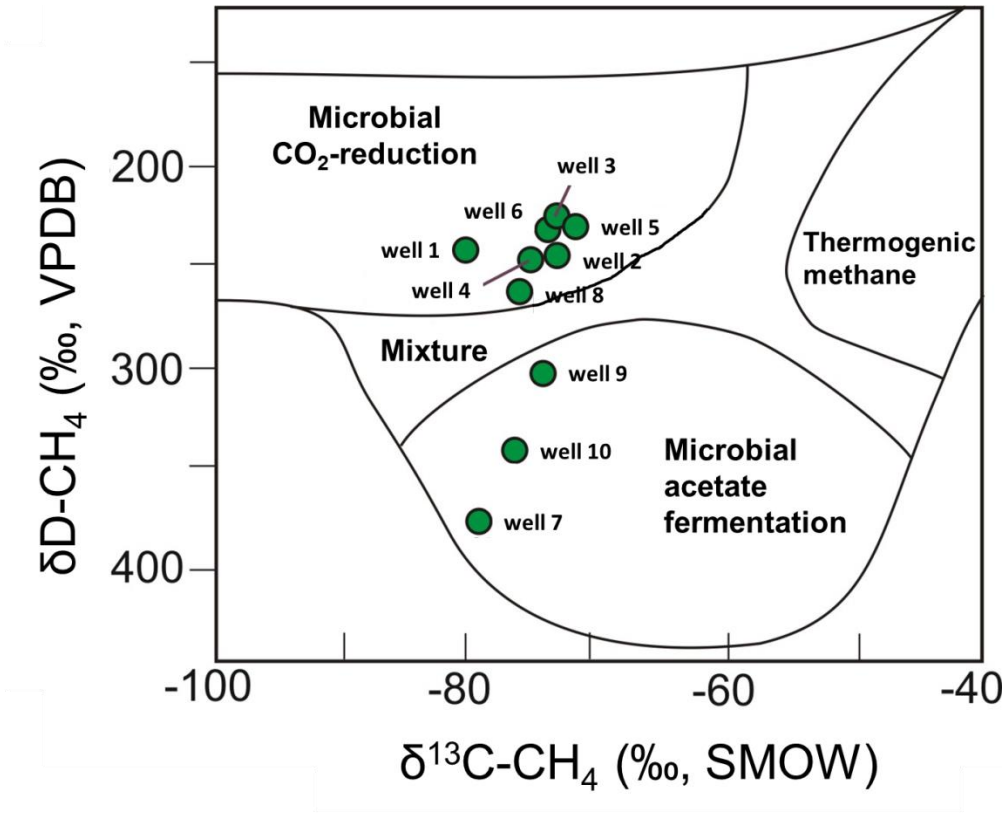


Figure 3.5: Diagram of isotopic composition ($\delta^{13}C_{CH4}$ and δD_{CH4}) of natural methane in groundwater samples from deep aquifers of coal-rich sedimentary basin. Zoning after (Schoell, 1980, 1983; Whiticar et al., 1986). VPDB – Vienna PeeDee Belemnite; SMOW – Standard Mean Ocean Water.

Until today, little is known about microbial metabolisms and the availability of potential substrates in subsurface coal-associated aquifer systems. A study from Vieth et al. (2008) demonstrated that some low-molecular-weight organic acids such as acetate, formate, and oxalate were released from low rank ligniteous coals into surrounding water. The reason for this might be the degree of diagenetic alteration (the impact of heat and

time) of the macromolecular organic material, leading to a depletion in such easily available small molecules in the macromolecular structure. Furthermore, this study also showed that the water soluble acids were transported by diffusion and/or advection from the coal layers to the adjacent carrier lithologies to support the deep biosphere (Vieth et al., 2008). According to Bergmann (1999) the main source of organic carbon in the aquifers are the lignite seams. Available low-molecular-weight organic acids in subsurface aquatic systems have the potential for providing a sufficient energy source for microbial life, especially for methanogenic activity (Vieth et al., 2008). Moreover, low-molecular-weight organic compounds could also be produced by microbial degradation of high organic carbon material from low rank coals (Fakoussa and Hofrichter, 1999; Laborda et al., 1997). Further, several studies have also demonstrated that the natural microbial activity was stimulated at lignite-rich/sediment interfaces (Ulrich et al., 1998) additionally at the interface between aquifer and lignite seam (Detmers et al., 2001). In both studies the microbial fermentation of organic matter in lignite-rich sediments provides low-molecular-weight organic acids that in turn feed the sulfate-reducing bacteria.

In this coal-associated aquifer the input of low-molecular-weight organic compounds produced by biodegradation of organic matter from the underlying lignite-rich sediments seems likely, because fermenting as well as sulfate-reducing bacteria were found in the lignite-rich sediment samples. Their activity was demonstrated by cultivation in enrichment cultures amended with coal or hydrocarbons as sole carbon sources.

The high total inorganic carbon (TIC) content in all groundwater samples, demonstrated the presence of active microbial metabolization processes.

3.4.2. The bacterial community in groundwater

To identify the microorganisms responsible for the degradation of complex organic matter of ligniteous coals to substrates for methanogenesis, we used T-RFLP fingerprinting combined with pyrosequencing of 16S rRNA gene fragments.

We detected many genera and functional groups in the groundwater samples similar to those obtained from other coal- or hydrogen-associated environments. Playing a role in the degradation of organic matter of lignite and sub-bituminous coals (Machnikowska et al., 2002), the typically aerobic *Pseudomonadales*, known as a very diverse and widespread genus capable of utilizing hydrocarbons as carbon and energy sources (Das and Chandran,

2011) was found in the groundwater. Furthermore, *Pseudomonas* species, isolated from marine sediment are well known as nitrate-dependent alkene- and polycyclic aromatic hydrocarbon-degraders (Rockne et al., 2000). This correlates well with the nitrate content, the absence of oxygen, and with the close association of groundwater to coal-rich sediments, where different hydrocarbons are available as substrate for microbial reactions. Additionally, Lemay and Konhauser (2006) found *Pseudomonas* spp. in groundwater associated with coal bed methane wells in Alberta. *Acinetobacter* species has been identified as oil degraders in soil and sediments contaminated with crude oil firstly by Reisfeld et al. (1972). They are also commonly found in hydrocarbon-rich environments like petroleum reservoirs (Li et al., 2007; Tang et al., 2012). *Acinetobacter* stains are able to degrade efficiently short- and long-chain alkanes, branched-chain alkanes, and various aromatics and connect the anaerobic degradation via oxidation with the reduction of nitrate (Li et al., 2012b; Mbadinga et al., 2011).

Betaproteobacteria are also clearly important players in the turnover of hydrocarbons in many environments, but especially in soils and aquifers. The most common genera that are known to degrade hydrocarbons and related substituted molecules under aerobic conditions include *Acidovorax*, *Burkholderia*, *Comamonas*, *Delftia*, *Polaromonas*, and *Ralstonia* (Parales, 2010).

Further, the betaproteobacterial relatives *Acidovorax*, *Massilia*, and *Georgfuchsia* were corresponded to 488-bp T-RF and detected in groundwater sample 9. *Acidovorax* species are hydrogen-utilizing members of the *Comamonadaceae* family and were also detected in studies performed by Li et al. (2008), Midgley et al. (2010) and Penner et al. (2010). But they assume that this facultative anaerobic *Acidovorax* spp. are not directly involved in degradation of hydrocarbons or complex organics found in coal.

The betaproteobacterial hydrocarbon degrader *Georgfuchsia toluolica* (within the *Rhodocyclaceae* family) has been isolated from a BTEX-contaminated aquifer by Weelink et al. (2009) and degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor.

The groundwater well 3 revealed a bacterial community consisting of the predominant 161 and 517-bp T-RFs affiliated to members of *Arthrobacter* sp. and *Desulfatiferula* sp.. Relatives of *Arthrobacter* sp. (161-bp T-RF) are Gram-positive aerobic soil actinomycetes, able to degrade cellulose and other polysaccharides (Li et al., 2008). *Actinomycetales* also

reduces nitrate to nitrite and are able to degrade polycyclic aromatic hydrocarbons. Kallimanis et al. (2009) characterized the novel phenanthrene-degrading bacterium *Arthrobacter phenanthrenivorans*, isolated from a creosote-contaminated soil in Greece. The presence of *Actinobacteria* in coal samples as well as in coal-associated formation water in Australia and Canada has been previously reported by Midgley et al. (2010) and Penner et al. (2010). It is known that several sulfate-reducing bacteria are able to oxidize aliphatic hydrocarbons under anaerobic conditions (Widdel et al., 2007). *Arthrobacter* sp., also predominantly present in several groundwater samples, possibly plays an important role in biodegradation of coal-associated hydrocarbon compounds at the coal-water-boundary layer within the sites investigated here. *Desulfatiferula olefinivorans* (517-bp T-RF), a mesophilic sulfate-reducing bacteria belonging to the family of *Desulfobacteraceae* and isolated from oil-polluted sediment, exclusively oxidizes long-chain alkenes and fatty acids incompletely to acetate; only sulfate is used as electron acceptor (Cravo-Laureau et al., 2007). *Desulfatiferula* sp. was exclusively found in groundwater sample 3 where the sulfate concentration was higher than in the other groundwater samples. The denitrifying *Arcobacter* species was previously found in an activated sludge basin, which is a bioreactor with biomass recycling (Snaidr et al., 1997), and further in petroleum-related environments (Voordouw et al., 1996).

The bacterial community identified in the groundwater samples consists of lot of putative hydrocarbon degraders able to degrade complex organic compounds such as long-chain alkenes, polycyclic aromatic hydrocarbons, BTEX, and other coal-associated compounds (e.g. fatty acids) to lower molecular weight compounds which are utilizable by methanogens. The results show the natural recycling capability for carbon compounds in the sedimentary sequence examined here.

3.4.3. The archaeal community in groundwater

The T-RFLP fingerprints of the groundwater samples showed a reduced archaeal community composition consisting mainly of *Methanomicrobiaceae*. 16S rRNA gene sequences showed strong affiliations to *Methanosarcina horonobensis* (183-bp T-RF), *Methanosaeta concilii* (282-bp T-RF), and *Methanoregula formicicum* (392-bp T-RF). *Methanosarcina* sp. and *Methanosaeta* sp. are the only two genera which can utilize acetate as a substrate for methanogenesis. *Methanosarcina horonobensis* was isolated from deep

subsurface groundwater from a mudstone formation and metabolize methanol, methylated compounds (e.g. di- and trimethylamine, dimethylsulfide) or acetate as sole energy source (Shimizu et al., 2011). *Methanosarcina* is not strongly abundant in the groundwater, with the exception of water sample 9 where *Methanosarcina* was the sole detectable archaeon. The genus *Methanosaeta,* found in water sample 2 and 3, exclusively use acetate as sole source for energy and as substrate for acetoclastic methanogenesis (Patel and Sprott, 1990). The hydrogenotrophic methanogen *Methanoregula formicicum*, detected in nearly all groundwater samples, is able to use H_2 / CO_2 and formate as substrates for growth (Yashiro et al., 2011).

The T-RFLP plots of the archaeal community composition from groundwater sample 2 and 3 showed strong abundance of both acetoclastic and hydrogenotrophic methanogens and were likely to have contributed to methane production. Acetoclastic produced methane was also observed in other coal seams characterized by the movement of meteoric water, which contained nutrients and microbes e.g. by Flores et al. (2008) and Klein et al. (2008).

In contrast to sequence analysis results, the distribution of the δ^{13}C-values of methane (Figure 3.5) indicated a dominance of CO_2-reducing methanogens in most of the groundwater wells, suggesting that the acetoclastic methanogens, observed via sequencing and fingerprinting methods are admittedly present but not or less methanogenic active under environmental conditions. However, in groundwater sample 9, the δ^{13}C-value indicated that the measured methane has an acetoclastic origin and the sequence analysis confirmed the dominance of acetoclastic *Methanosarcina* sp.

3.4.4. The bacterial community in the coal-rich sediments and derived enrichment cultures

In contrast to the groundwater community, the samples from the freshly collected coal-rich sediments showed a bacterial community composition dominated by only two phyla, *Actinobacteria* and *Proteobacteria*, and a minor part consisting of *Clostridia* and *Anaerolineales*. Like in the groundwater samples, most of the *Bacteria* found in the coal-rich sediment samples are classified as hydrogenotrophic archaea or nitrate-reducing bacteria – such as the genera *Acidovorax* (488-bp T-RF) or *Pseudomonas* (490-bp T-RF), *Janthinobacterium* (490-bp T-RF), *Arthrobacter* (161-bp T-RF), *Arcobacter* (476-bp T-RF), and *Methylotenera* (490-bp T-RF). Moreover, relatives of the families *Comamonadaceae*,

Oxalobacteraceae, Rhodocyclaceae and *Geobacteraceae* including sulfate- and iron-reducing hydrocarbon degraders were also found in the coal-rich sediments. A relatively high abundance of relatives of the *Sulfurospirillum* spp. (467-bp T-RF) and *Arcobacter* spp. point to an important role in sulfur and nitrogen cycling through the co-occurrence of these genera. Some *Arcobacter* species are able to reduce nitrate and oxidize sulfide, while *Sulfurospirillum* species have extremely diverse metabolic features and possibly reduce sulfur and oxidize nitrite (Tang et al., 2012*)*. A major part of sequences showed affiliations to *Methylotenera* spp., classified as aerobic Gram-negative bacteria within the family *Methylophilaceae*. The methylotrophic *Methylotenera* spp. are able to utilize methylamine as a single source of energy, carbon and nitrogen. They were isolated from Lake Washington sediment and characterized by Kalyuzhnaya et al. (2006).

The bacterial community composition of the enrichment cultures amended with hexadecane showed resemblance rather with groundwater than with coal-sediment samples. The majority of *Bacteria* were relatives of the *Proteobacteria*, followed by members of *Actinobacteria, Anaerolineae,* and *Clostridia*. *In contrast to coal samples, in the enrichment cultures Deltaproteobacteria* and *Anaerolineae* were present. The detected deltaproteobacterial sulfate-reducers belonging to *Desulfobacterales*, *Desulfuromonadales*, *Desulfovibrionales* and *Syntrophobacterales* are known for their ability to degrade long-chain fatty acids (Sousa et al., 2009). The high abundance of sulfate-reducing bacteria identified via sequence analysis correlated well with the detection of high amounts of *dsrA* genes via Q-PCR in the enrichment cultures. Furthermore, members of the family of *Geobacteraceae,* exclusively identified in the enrichment cultures*,* are known as anaerobic metal-reducing organisms and relatively common coal associated microbes, published by several authors (Beckmann et al., 2011b; Jones et al., 2010; Li et al., 2008). The class of *Anaerolineae* contains a vast number of environmental 16S rRNA gene sequences derived from mesophilic and thermophilic environments, but only a few cultured strains growing under strictly anaerobic conditions (Yamada et al., 2006). The sequence analysis of enrichment cultures only revealed the presence of unclassified relatives of *Anaerolineaceae.* Additionally, sequences affiliated to not specified members within the order *Actinomycetales,* already described in the previous chapter as an aerobic *Bacteria* which can typically degrade cellulose and other polysaccharides (Li et al., 2008) were identified.

3.4.5. The archaeal community in coal-rich sediments and derived enrichment cultures

As a strong similarity to groundwater samples, the microbial community in coal-rich sediments is divided into methanogenic *Archaea* solely affiliated to *Methanomicrobia* and unclassified members of the *Crenarchaeota*. In comparison to the groundwater samples, the community composition of the coal-rich sediments showed a higher diversity of methanogenic *Archaea* extend through the presence of *Methanomethylovorans* species and a few of not identifiable T-RFs. The identified archaeal community composition of all three coal-rich sediment samples consists of acetoclastic and hydrogenotrophic methanogens affiliated to *Methanosarcina horonobensis*, *Methanosaeta concilii, Methanoregula formicicum,* and *Methanomethylovorans* sp. Relatives of *Methanomethylovorans* were described by Lomans et al. (1999) and Jiang et al. (2005) and utilize methanol, methylated amines, dimethyl sulfide and methane-thiol for methanogenic activity. *M. hollandica* was isolated from eutrophic fresh water sediment (Lomans et al., 1999). *Methanomethylovorans* species were also detected in rice field soil (Lueders et al., 2001) and oil contaminated groundwater (Watanabe et al., 2002).

The methanogenic archaeal community including *Methanosaeta concilii, Methanoregula formicicum,* and *Methanomethylovorans* sp. together with their syntrophic bacterial partners as well as unclassified *Crenarchaeota* were successfully enriched in enrichment cultures amended with hexadecane. Because the added hexadecane was the sole carbon source for the enriched microbial community commonly inhabits the coal-rich sediments the microbial ability to degrade hexadecane to methane has been proven. Additionally, in comparison to the quantitative analysis of the coal-rich sediments, the enrichment cultures amended with hexadecane showed a significant increase of the archaeal, crenarchaeal, methanogenic, and sulfate-reducing population in all enrichments. Together with the observed methane production rates, these results revealed the formation of a highly adapted microbial community, whereas the archaeal community consists of the acetoclastic and the more dominate hydrogenotrophic methanogens. Related results regarding methanogenic pathways and involved microorganisms were previously reported in several coalbed methane formations (Faiz and Hendry, 2006; Papendick et al., 2011; Penner et al., 2010; Shimizu et al., 2007; Strąpoć et al., 2011b).

Interestingly, the very strong abundance of 16S rRNA gene sequences affiliated to the unclassified *Thermoprotei,* a subgroup within the phylum *Crenarchaeota,* was found all coal-rich sediment samples and in enrichment cultures. Recently, relatives of hyperthermophilic *Thermoprotei* were detected in production waters from high-temperature petroleum reservoirs in China (e.g. Li et al. (2007); Ren et al. (2011); Tang et al. (2012)) that suggest a capability of hydrocarbon degradation or a close contribution to the degradation process. The fact that these hyperthermophilic unclassified *Thermoprotei* could highly enriched in cultures amended with hexadecane confirm the suggestion of the ability of hydrocarbon degradation. Furthermore, Kemnitz et al. (2007) in turn showed that *Crenarchaeota* are ubiquitous and affect low temperature environments as a common microbial part. But little is known about their physiological characteristics and their ecological importance, because the majority of this group of microorganisms has not been cultivated yet.

3.5. Conclusion

The investigated coal-rich sediments provide an important source of organic material and electron acceptors, essential for natural microbial life in the subsurface biosphere. This organic material possibly includes low-molecular-weight organic acids derived from coal biodegradation, and then partly transferred into the coal-associated aquifer system. The detected fermenting and sulfate-reducing bacterial consortia are able to degrade complex organic material from coal to lower molecular weight compounds which consequently influence directly the microbial interaction in the groundwater system. The low-molecular-weight organic acids are potential substrates for methanogenic archaea. The identified methanogenic archaea point to the simultaneous occurrence of both methanogenic pathways, whereas hydrogenotrophic methanogenesis dominates. This assumption was supported by $\delta^{13}C_{CH4}$- and δD_{CH4}-values in coal-rich sediment and groundwater samples.

Finally, the large number of unclassified bacterial and archaeal sequences showed that the microbial ecosystem in the examined coal-bearing sedimentary basin contains a greater wealth of microbial life than originally thought and the coal-associated aquifers accommodate a lot new and to date not described indigenous microbial species whose metabolic potential is far from being completely understood.

Acknowledgement

This work was supported by funding from German Research Foundation (DFG) within the SPP1319 (projects KR3311 6-1, 6-2). We would like to thank Daniela Zoch, Holger Probst and Michaela Blank for collecting samples and for technical assistance.

Chapter IV

Similar features of microbial consortia from different ecosystems degrading hexadecane under methanogenic conditions

4. Similar features of microbial consortia from different ecosystems degrading hexadecane under methanogenic conditions

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Manuscript in preparation
Running title: Hexadecane degrading microbial consortia

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Keywords: methanogenic hydrocarbon degradation, methanogenesis, bioremediation, Q-PCR, T-RFLP, physiology

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Abstract

Since almost two decades it is known from stable isotope studies that large amounts of biogenic methane are formed in oil and coal reservoirs or contaminated aquifers. However, still only little knowledge has been gained about the metabolic processes and the microorganisms involved in methanogenic hydrocarbon degradation in these and other ecosystems. Consequently, we established enrichment cultures obtained from polluted and pristine brackish water and freshwater sediments as well as from terrestrial mud volcano soil, degrading hexadecane under methanogenic conditions. These showed high methane production rates after the addition of hexadecane, and were also capable of converting added oil or coal. The enrichment cultures showed a broad tolerance against environmental parameters, like salinity, temperature and pressure, even at assumed reservoirs conditions. The addition of specific inhibitors and ^{13}C-labeled hexadecane indicated that acetate was an important intermediate for methane production. Also, low sulfate and elevated trace element concentrations had stimulating effects on methane production. The quantitative community analysis showed equally high abundances of *Archaea* and *Bacteria* in all enrichment cultures. Another common feature independent of the original habitat was the presence of functional genes indicative of sulfate-reduction and methanogenesis in high numbers in all incubations. The diversity analysis via terminal restriction fragment length polymorphism fingerprinting, amplicon pyrosequencing and cloning showed a large bacterial diversity with *Smithella*, *Syntrophomonas* and *Gammaproteobacteria* species dominating. In contrast, the archaeal diversity was always limited to three or four dominant species, mainly acetoclastic *Methanosarcinales*. In conclusion, an active hydrocarbon-degrading community with similar physiological and compositional features could be established from different habitats, independent of former hydrocarbon exposure.

4.1. Introduction

Major world oil reserves, such as the Athabasca oil sands (Yergeau et al., 2012), other foreland basins as well as many offshore reservoirs, contain heavily biodegraded crude oil. The occurrence of biodegraded oil is indicative of indigenous subsurface microbial communities and points to *in situ* biodegradation in petroleum reservoirs as a globally significant biogeochemical process. Around 50-98 % of a typical crude oil consists of readily biodegradable hydrocarbons such as alkanes, cycloalkanes and alkylated aromatic compounds (Townsend et al., 2003). The removal of aliphatic and aromatic hydrocarbons during *in situ* biodegradation enriches heavy oil fractions containing heterocyclic sulfur-, oxygen- and nitrogen-rich compounds, thus decreasing oil quality and value. Today about only ~40 % of existing crude oil can be recovered using conventional technologies, resulting in large amounts of inaccessible oil remaining within the world's oil deposits (U.S. Department of Energy, 2006). Consequently, the microbial conversion of parts of these not accessible resources into methane might provide a new strategy for enhanced hydrocarbon recovery.

Besides in oil, coal and gas reservoirs, hydrocarbons are also important compounds in other environmental systems. Either originating from the degradation of organic material, like plants, or being introduced by human activities, like oil spills, waste removal, traffic, etc. (Coates et al., 1996b; Feisthauer et al., 2012; Gray et al., 2010; Meckenstock et al., 2004), they constitute important substrates for phylogenetically and physiologically diverse microbial communities in freshwater, terrestrial as well as brackish water habitats.

Still, the factors controlling *in situ* biodegradation in the different oil-associated environments and the specific microorganisms responsible remain poorly understood. Since electron acceptors for anaerobic metabolism, like nitrate, sulfate and oxidized metal species are largely absent or sequestered (e.g. as iron silicates), methanogenesis is the most relevant process for *in situ* biodegradation of hydrocarbons. Interestingly, the addition of small amounts of alternative electron acceptors, like sulfate, nitrate, ferric iron or manganese oxide in many cases accelerated the microbial conversion of hydrocarbons to methane (Siegert et al., 2010).

Until today, a broad range of hydrocarbons has been shown to be biodegradable under anoxic conditions and with various electron acceptors. For example, *n*-alkanes comprise a major fraction of most crude oils and have been found to be biodegradable under

methanogenic conditions, both as pure substrates, e.g. hexadecane (Anderson and Lovley, 2000a; Zengler et al., 1999) and in crude oils (Gieg et al., 2008; Jones et al., 2008; Siddique et al., 2006; Townsend et al., 2003). Simple aromatic compounds, such as BTEX and phenols, can also degrade under methanogenic conditions (Head et al., 2003; Schöcke and Schink, 1997; Weiner and Lovley, 1998). Feisthauer et al. (2010) investigated the stable carbon and hydrogen isotopic signature of methane, carbon dioxide and water during microbial formation of methane from alkanes, BTEX and PAH (or during methane oxidation) in order to examine the variability in the carbon and hydrogen isotope signatures of methane. The observed carbon and hydrogen isotope signatures and discrimination factors fell in a relatively narrow range, suggesting common mechanisms independent of the habitat and hydrocarbon source, and involving the coupling of fermentation with acetoclastic and CO_2-reducing methanogenesis. The degradation of oil or coal components under methanogenic conditions requires the interaction of fermenting and methanogenic microorganisms in a microbial community (Beckmann et al., 2011a; Beckmann et al., 2011b; Gray et al., 2011; Head et al., 2003; Jones et al., 2008; Zengler et al., 1999).

Both, methanogenesis and to a lesser extent sulfate-reduction, generally depend on fermentation reactions that degrade complex organic compounds into smaller electron donors (e.g., short organic acids or hydrogen), as catalyzed by syntrophic and fermentative anaerobes often observed in oil and coal reservoir habitats (Beckmann et al., 2011a; Beckmann et al., 2011b; Gray et al., 2011). The presence of methanogens and methanogenesis is well known for water-flooded oil reservoirs (Edwards and Grbić-Galić, 1994; Gieg et al., 2011; Mueller and Nielsen, 1996; Nazina et al., 1995a; Orphan et al., 2000). In contrast to the archaea, the metabolic diversity of the bacterial communities involved in the anaerobic biodegradation of petroleum hydrocarbons both *in situ* and *in vitro* is large, including nitrate-, ferric iron- or sulfate-reducing, acetogenic and fermenting microorganisms, partially living syntrophically with methanogens (Gieg et al., 2011; Gray et al., 2011; Gray et al., 2010; Hubert et al., 2012; Jones et al., 2008).

However, the distribution, structure, and activity of these methanogenic communities capable of hydrocarbon degradation in different geosystems still remain widely unknown. In this study, we performed a combination of physiological, biogeochemical and molecular biological analyses of a set of hexadecane-degrading methanogenic enrichment cultures obtained from a range of different ecosystems, including brackish water, terrestrial and

freshwater habitats. This led to a detailed investigation of the microbial diversity and metabolism of involved microorganisms and to the identification of common patterns and differences across the diverse ecosystem types studied.

4.2. Experimental procedures

4.2.1. Sample description and experimental set-up

Sediment samples for enrichment cultures were obtained from different methanogenic environments, including eutrophic lake (Lake Plußsee), freshwater (Kuhgraben), brackish water (Eckernförde Bay), mangroves (Brazilian mangrove forests) and terrestrial (Romanian mud volcanoes, coal and timber) environments. Details and acronyms are provided in table 4.1.

After sampling, sediments were stored at 4°C. Sediment slurries from each sample (1:1 mix of sediment and medium) were transferred under anaerobic conditions into autoclaved serum bottles. Sulfate-free minimal medium after Widdel and Bak (1992) with different salinities similar to environmental conditions of each sampling site was added. Serum bottles were sealed with sterile butyl rubber stoppers and aluminum crimps caps. The headspace of the serum bottles was flushed with nitrogen.

For the investigation of microbial growth with ^{13}C-labeled and unlabeled hexadecane, the substrates were added directly, or demobilized on filter, into the enrichment cultures. 2-5 mg U-^{13}C-hexadecane was added to 100 mL medium. U-^{13}C-hexadecane was synthesized as described by Feisthauer et al. (2010).

For investigation of physiological aspects enrichment cultures amended with different concentrations of sodium chloride, trace element solution, sulfate, and phosphate. The sterile anoxic stock solution of trace elements (SL10) was added as usual during medium preparation in increasing volumes of 1, 2 to 10 mL per liter (normal medium contains 1 mL). As another potentially limited nutrient, phosphate was added in concentrations of 1, 2, 5 and 10 mM. Salinity was changed by varying NaCl concentrations (1 – 100 g l^{-1}). To study the effect of sulfate on the conversion of hexadecane to methane enrichments were established by adding sulfate from a sterile anoxic stock solution to yield initial concentrations between 0-20 mM. Elevated hydrostatic pressures (from atmospheric to 200 bar) to mimic reservoir

conditions were applied using a high-pressure incubation device described by Nauhaus et al. (2002). In the same line, incubation temperatures were varied between 20 to 70˚C.

Cultures were incubated at 30°C or room temperature in darkness. Methane and CO_2 formation in the headspace of the cultures were measured regularity. Methane and CO_2 production rates were calculated by linear regression of the methane increase with incubation time, and expressed in µmol day^{-1} mL^{-1} sample (Krüger et al., 2001).

4.2.2. Analytical methods

Methane in the headspace of the microcosms were analyzed using a gas chromatograph with flame ionization detector (GC-FID) equipped with a 6´ Hayesep D column (SRI 8610C, SRI Instruments, USA) continuously running at 60°C. Carbon dioxide concentrations were determined by a methanizer-equipped FID detector after reduction of the CO_2 to methane using the same instrument as for methane measurements. Sulfide concentrations in the cultures were measured with a Nicolet evolution 100 photometer (Thermo electron corporation, Madison, WI, USA) by the Cord-Ruwisch-method (Cord-Ruwisch, 1985). The stable isotopic composition of methane and CO_2 was measured using a gas-chromatography-combustion-isotope ratio monitoring mass spectrometry system (GC-C-IRM-MS, MAT252, Thermo Fisher Scientific Inc., USA) (Herrmann et al., 2010). The $\delta\ ^{13}C$-values are expressed in ‰ vs. Vienna Pee Dee Belemnite (VPDB).

Concentrations of acetate in the liquid phase of the sediment incubations were analyzed by a HPLC Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA), consisting of a diode-array detector, a micro-vacuum degasser, and a binary pump. The analytes were separated on an Agilent Eclipse Plus C8 4 column (4.6 x 250 mm) kept at 20°C and using 5 mM H_2SO_4, as eluant at a flow rate of 1.0 mL min^{-1}. A 10 µL injection of each sample was loaded on to the column.

Table 4.1: Overview of sampling sites for sediments used in enrichment cultures amended with hexadecane.

Sample site	ISO 3166	ID	In situ temp.	Sampling depth	Reference
Eckernförde Bay, semi enclosed bay, western Baltic Sea, brackish water, methane rich pristine sediment	**DE**	**brackish water**	16°C	2-20 cm	Treude et al. (2005), Whiticar (2002)
Gulf of Mexico, sediment from natural gas and oil seeps		**mexico**	8-12°C	560 m	(Joye et al., 2004)
Brazilian mangrove forests, brackish water, saline coastal sediment	**BR**	**mangrove**	29°C	intertidal sediments	Samples from Prof. Dr. P. Frenzel, MPI Marburg (2005), Feisthauer et al. (2010)
Lake Plußsee, stable anoxic hypolimnion, high CH_4 concentrations in the water column, eutrophic lake	**DE**	**eutrophic lake**	12°C	28 m	Overbeck and Chrost (1994), Eller et al. (2005)
Kuhgraben, freshwater ditch, non-contaminated	**DE**	**freshwater**	25°C	2 m	Zengler et al. (1999)
Terrestrial mud volcano field, located in Carpathian Mountains (Paclele Mici), naturally occurring oil seepage	**RO**	**mud volcano**	25°C	upper 20 cm of the sales	Alain et al. (2006)
Coal and Timber, underground mining, sealed compartments of coal mines	**DE**	**coal** or **timber**	35°C	surface	Beckmann et al. (2011a)

4.2.3. Molecular biological methods

Genomic DNA from hydrocarbon enriched microcosms was extracted by bead-beating and a phenol-chloroform extraction using protocols from Gabor et al. (2003) and Lueders et al. (2004). For molecular analysis and cloning experiments, crude DNA extracts were purified by Wizard® DNA Clean-Up Resin (Promega Corporation, Madison, WI, USA). Recovery was routinely >80 %.

Via quantitative real-time PCR genes of interest were quantified using the Q-PCR instrument ABI Prism 7000 (Applied Biosystems, Life Technologies Corporation, CA, USA). 16S rRNA gene copy numbers of *Archaea* and *Bacteria* were determined as described previously by Takai and Horikoshi (2000) and Nadkarni et al. (2002). By using functional primer sets for detection of the methyl-coenzyme M reductase gene (*mcr*A) the quantity of methanogenic microorganisms was collected (Nunoura et al., 2006). The abundance of sulfate-reducers was determined by quantifying the copy number of the *dsr*A gene coding for the alpha subunit of the dissimilatory (bi) sulfite reductase (Neretin et al., 2007). All PCR reactions were measured in three parallels and three dilutions. To perform Q-PCR quantification, a StepOne detection system (StepOne version 2.0, Applied Biosystems, CA, USA) coupled with the StepOne v2.1 software was used. 16S rRNA gene copy numbers are expressed as copies mL^{-1} sample.

For terminal restriction fragment length polymorphism (T-RFLP) analysis, extracted DNA was used as template for PCR amplification of phosphoramidite flourochrome 5-carboxyflourescein (FAM)-labeled amplicons. Amplifications were generated by using the primer sets Ar109f and 912rt-FAM, or Ba27f-FAM and 907r. To account for possible inhibitor effects in DNA extracts, a dilution series of each fresh extract was prepared. T-RFLP analysis of PCR products was done using the restriction endonucleases TaqI and MspI, respectively. The procedure was described by Winderl et al. (2008), (2010). Capillary electrophoresis and data collection were operated on an ABI 3730 Genetic Analyzer (Applied Biosystems, CA, USA). The electropherograms were processed with sequence analysis software PeakScanner 1.0 and GeneMapper 4.0 (Applied Biosystems, CA, USA). T-RFLP histograms were performed with the help of the T-REX online software using the default settings (Culman et al., 2009).

Clone libraries were created using DNA from enriched microbial communities amended with hexadecane. 16S rRNA gene fragments were amplified by PCR using domain specific primer pairs 21f (5`-TTC CGG TTG ATC CYG CCG GA) and 958r (5-YCC GGC GTT GAM

TCC AAT T) for *Archaea* (DeLong, 1992), and GM3f (5-AGA GTT TGA TCM TGG C) and GM4r (5-TAC CTT GTT ACG ACT T) for *Bacteria* (Lane, 1991). Cloning and sequencing of the archaeal and bacterial 16S rDNA amplicons was performed by Microsynth AG (www.microsynth.ch, Switzerland). Sequences were assembled use the Geneious ProTM 5.3 software (www.geneious.com). Prior to phylogenetic analysis, vector sequences flanking the 16S rDNA gene inserts were removed.

Amplicon pyrosequencing was performed as described in Pilloni et al. (2011). With the primer pair Ba27f and Ba519r (Lane, 1991) extended as amplicon fusion primers with respective primer A or B adapters, key sequence and multiplex identifiers (MID) bar-coded amplicons for multiplexing were prepared. Emulsion PCR, emulsion breaking and sequencing were performed applying the GS FLX Titanium chemistry following protocols and using a 454 GS FLX pyrosequencer (Roche). With the use of the automatic amplicon pipeline of the GS Run Processor (Roche) coupled with the valley filter (vfScanAll-Flows false instead of TiOnly) quality filtering of the pyrosequencing reads was performed.

After quality-trimming using the TRIM function of GreenGenes software (DeSantis et al., 2006), the retrieving of forward and reverse reads with inferior read length (> 250 bp) with BIOEDIT software (Hall, 1999).

Chimeric sequences were detected using the DECIPHER´s Find Chimeras online software (Wright et al., 2012) from the University of Wisconsin Madison (http://decipher.cee.wisc.edu/FindChimeras.html) and were excluded from further analysis. Sequences were compared to GenBank BLASTn algorithm from the National Center for Biotechnology Information (Altschul et al., 1990) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project Classifier (Wang et al., 2007) (RDP; http://rdp.cme.msu.edu/classifier/classifier.jsp) to select closely related species. Unique patterns were considered to be operational taxonomic units (OTUs) of at least 97 % distance matrix identity using mother software (http://www.mothur.org) (Schloss et al., 2009). The phylogenetic tree was constructed with the ARB software package (Ludwig et al., 2004) with Maximum-Parsimony (version 102) correlation provided by Pruesse et al. (2007).

4.3. Results

4.3.1. Microbial activities

In all enrichment cultures significant elevated methane production rates in microcosms amended with hexadecane compared to substrate-free and sterile conditions were observed. With increasing numbers of transfers during the cultivation procedure, methane production rates increased.

The comparison of methanogenic activity between the different enrichment cultures obtained from sediments originated from brackish water, mangrove, eutrophic lake and freshwater environments as well as from a terrestrial mud volcano, revealed an increased potential to hexadecane degradation performed by mangrove and freshwater ditch sediment enrichments.

To comprehend the microbial conversion of hexadecane as sole carbon source to methane and CO_2 microcosms were amended with U-^{13}C-hexadecane. The stable isotope composition of CH_4 and CO_2 in the headspace of the Brazilian mangrove enrichment cultures amended with U-^{13}C-hexadecane showed $\delta^{13}C_{CH4}$-values changed from -98.0 ‰ to -40.2 ‰ after 44 days and at last to final -4.9 ‰ after 205 days incubation time. The isotopic value of $\delta^{13}C_{CO2}$ increased from -18.8 ‰ to 8.2 ‰. In the control experiment with unlabeled hexadecane the $\delta^{13}C_{CH4}$-value showed -49.6 ‰ over total incubation time and the $\delta^{13}C_{CO2}$-value ranged from -11.1 ‰ to 21.3 ‰. Extended isotope fractionation data have already been published at Feisthauer et al. (2010).

4.3.2. Physiological aspects

Increasing the amounts of trace elements in the enrichments lead to an increase in methane production rates with highest rates observed for 10 times the initial concentrations. A methane production rate of 5 µmol mL^{-1} d^{-1} was measured in microcosms containing medium with 1 mL TE solution. Methane production changed after addition of 2 x TE (8 µmol mL^{-1} d^{-1}) and 5 x TE (16 µmol mL^{-1} d^{-1}). In incubations with 10 x TE the rates were doubled compared to the normal medium (1 mL TE). Higher amounts were not tested. These findings were the same for all three enrichments tested, i.e. freshwater, brackish water and mexico.

The rates of methane production from hexadecane increased with increasing phosphate concentration in the medium (data not shown). To indicate whether this process functions also under reservoir conditions, the effects of increased salinity, pressure and temperature were tested. An increase of the salinity in the incubation leads immediately to a decrease in methane production (data not shown). The variation of incubation pressure or temperature in contrast showed a relatively broad tolerance of the selected enrichment cultures against these important parameters (data not shown).

The addition of increasing concentrations of the potential alternative electron acceptor sulfate lead in the incubations to a shift from methane to CO_2 production, indicating a change in the community composition (Figure 4.1).

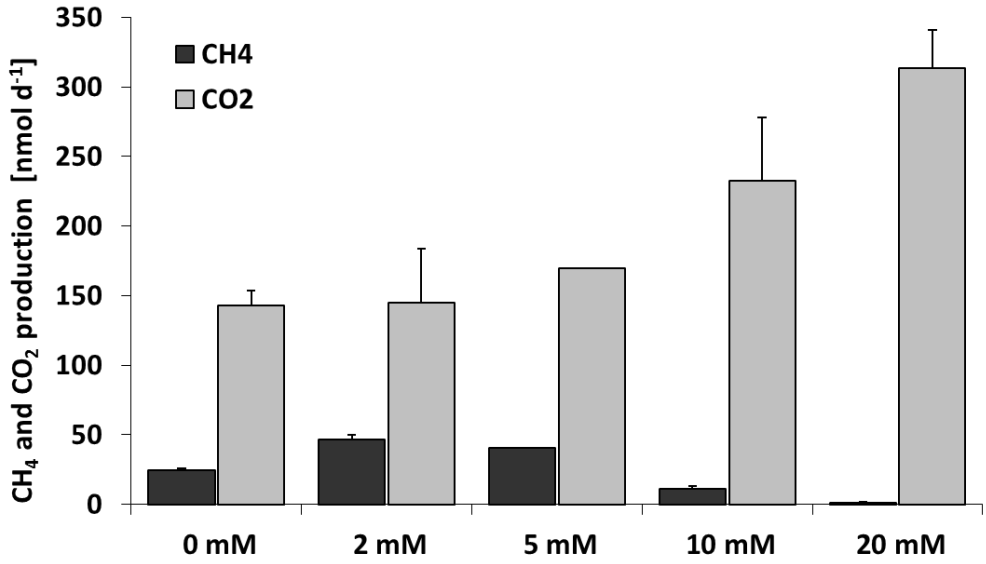


Figure 4.1: Methane and CO_2 production rates from the brackish water enrichments (Eckernförde Bay) amended with hexadecane and different sulfate concentrations. The error bars represent the standard deviation of three replicates (Siegert et al., 2010).

4.3.3. Quantitative community composition of enrichment cultures

The quantitative analysis of the community composition showed high abundances of *Bacteria* and *Archaea* in all enrichment cultures amended with hexadecane (Figure 4.2). After 480 day of incubation the enrichment cultures inoculated with brackish water and mangrove sediments contained 6.1×10^6 and 1.3×10^7 (brackish water) and 4.1×10^5 and 7.6×10^6 DNA copies mL^{-1} sample (mangrove) for *Bacteria* and *Archaea*. The microcosms with eutrophic lake and freshwater sediments showed values of 1.4×10^7 and 1.1×10^7

(eutrophic) and 3.7×10^6 and 3.5×10^6 DNA copies mL^{-1} sample (freshwater), and microcosms incubated with terrestrial sediments contained 2.6×10^7 and 7.9×10^7 (mud volcano), 2.7×10^8 and 9.6×10^7 (coal), 5.0×10^7 and 1.9×10^7 DNA copies mL^{-1} sample (timber) for *Bacteria* and *Archaea*. Furthermore, a significant amount of functional genes of methanogens (*mcrA*) and sulfate-reducers (*dsrA*) were detected and showed a high variation between the different cultures. In enrichment cultures amended with hexadecane incubated with freshwater and brackish water sediments the detectable methanogens slightly exceeded the presence of sulfate-reducers. In enrichment cultures containing mangrove and terrestrial sediments the amounts of sulfate-reducers were higher than the amounts of methanogens. Enrichments from mangrove sediments showed the highest distinction between sulfate-reducers (1.0×10^6 DNA copies mL^{-1} sample) and methanogens (3.92×10^3 DNA copies mL^{-1} sample).

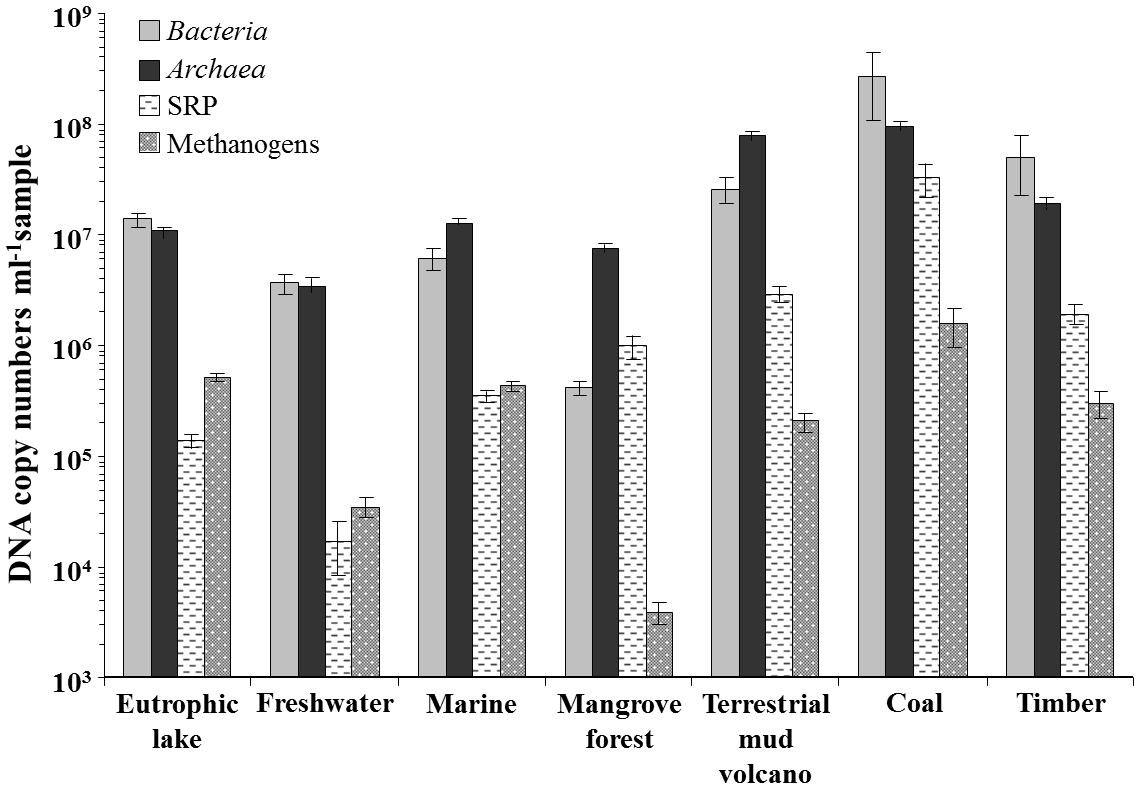


Figure 4.2: Quantitative community composition of eutrophic lake, freshwater, brackish water, mangrove forest, terrestrial mud volcano, and coal & timber enrichment cultures amended with hexadecane. *Bacteria* and *Archaea* were determined via universal 16S rRNA genes. Functional genes of methanogens (*mcrA*) and sulfate-reducing prokaryotes (*dsrA*) were determined by quantitative PCR. Error bars were calculated from standard deviations of three parallel PCR reactions.

Phylogenetic affiliation #	eutrophic			freshwater			brackish water			mangrove			mud volcano		
	reads*	%	*T-RF*	reads*	%	*T-RF*	reads*	%	*T-RF*	reads*	%	*T-RF*	clones†	%	*T-RF*
Bacteria	3490	100		6952	100		7162	100		3218	100		159	100	
Proteobacteria	1981	56,8		5373	77,3		3065	42,8		1390	43,2		133	83,6	
Betaproteobacteria	639	18,3		1833	26,4		4	0,1							
Burkholderiaceae	635	18,2													
Ralstonia sp.	**635**	**18,2**	***436***												
Rhodocyclaceae				1802	25,9										
Azospira sp.				**1802**	**25,9**	***492***									
Gammaproteobacteria	853	24,4		376	5,4		2348	32,8		767	23,8				
Ectothiorhodosinus sp.										306	9,5	*490*			
Pseudomonas sp.	**392**	**11,2**	***490***				**2348**	**32,8**	***490***						
Acinetobacter sp.	36	1,0	*491*	317	4,6	*491*									
Thiorhodospira sp.	**425**	**12,2**	***139***												
Marinobacter sp.										**461**	**14,3**	***145***			
Deltaproteobacteria	489	14,0		3146	45,3		627	8,8		375	11,7		133	83,6	
Syntrophobacterales	370	10,6		2983	42,9					222	6,9				
Sytrophus sp.	5	0,1	*132*	52	0,7	*132/510*									
Smithella spp.	**365**	**10,5**	***164***	**2592**	**37,3**	***164***				222	6,9	*164/509*			
Syntrophorhabdaceae	45	1,3		39	0,6		5	0,1		2	0,1				
Syntrophorhabdus sp.	45	1,3	*162*	39	0,6	*162*	5	0,1	*162*	2	0,1	*167*			
Desulfobacterales													11	6,9	*164/168*
Desulfosalsimonas													9	5,7	*168*
Desulfovibrionales	53	1,5		99	1,4		616	8,6		30	0,9		39	24,5	
Desulfohalobium													23	14,5	*133*
Desulfovibrio sp.	53	1,5	*131*	71	1,0	*125*	**596**	**8,3**	***164***	25	0,8	*164*	14	8,8	*133*
Desulfuromonadales													66	41,5	
Desulfuromonas													**58**	**36,5**	***125/511***
Firmicutes	1234	35,4		479	6,9		1856	25,9		939	29,2		10	6,3	
Clostridia	1166	33,4		363	5,2		1751	24,4		877	27,3		8	5,0	
Clostridiaceae	4	0,1		32	0,5		385	5,4		30	0,9				
Syntrophomonadaceae	574	16,4					98	1,4							
Syntrophomonas sp.	**574**	**16,4**	***305***				95	1,3	*399*						
Thermoanaerobacteraceae	35	1,0		33	0,5		566	7,9		750	23,3		6	3,8	
Thermanaeromonas sp.	7	0,2					566	7,9	*314*	**740**	**23,0**	***315***			
Chloroflexi	87	2,5		616	8,9		134	1,9		172	5,3		1	0,6	
Anaerolineaceae	46	1,3	*508*	613	8,8	*508*	74	1,0	*508*	60	1,9		1	0,6	
Bacteroidetes	78	2,2		119	1,7		1201	16,8		113	3,5		19	11,9	
Prolixibacter sp.	13	0,4	*93*	41	0,6	*93*	**1125**	**15,7**	***93***				3	1,9	
Others		3,2			5,3			**12,7**			18,8			8,8	

Table 4.2: Phylogenetic affiliation of trimmed reads produced in pyrosequencing and clones from clone libraries of bacterial 16S rRNA gene fragments retrieved from DNA extracted from several hexadecane enriched microcosms. Taxonomic assignments were based on the phylogenetic analysis and the results obtained from the RDP Classifier. Characteristic T-RF lengths in bp were predicted for important lineages via assembled amplicon contigs. # Phylum- or division-level read abundances include genus- or lineage-specific read abundances, %

Table 4.3: Phylogenetic affiliation of clone sequences produced in clone libraries of archaeal 16S rRNA gene fragments retrieved from DNA extracted from several enrichment cultures amended with hexadecane. Taxonomic assignments were based on the phylogenetic analysis and the results obtained from the RDP Classifier with confidence threshold of 80 %. Characteristic T-RF lengths in bp were predicted for important lineages via assembled forward and reverse sequences. # Phylum- or division-level read abundances include genus- or lineage-specific read abundance, % Abundance of sequence accordance in percentage, ‡ Number of clones. Bold numbers show accordance with dominant peaks in the T-RFLP plots shown in figure 4.3.

	eutrophic			**freshwater**			**marine**			**mangrove**			**volcano**		
Phylogenetic affiliation#	clones‡	%	*T-RF*	clones‡	%	*T-RF*	clones‡	%	*T-RF*	clones‡	%	*T-RF*	clones‡	%	*T-RF*
Archaea	183	100		93	100		93	100		92	100		174	100	
Euryarchaeota	179	97,8		93	100,0		87	93,5		54	58,7		170	97,7	
Methanomicrobia	179	97,8		92	98,9		87	93,5		23	25,0		170	97,7	
Methanomicrobiales	8	4,4					9	9,7		8	8,7		92	52,9	
Methanomicrobiaceae	5	2,7					9	9,7		5	5,4		61	35,1	
Methanoculleus	4	2,2	*183/390*				2	2,2	*183*						
Methanogenium	1	0,5	*390*				**6**	**6,5**	***390***				**6**	**3,4**	***390/492***
Methanoplanus													**48**	**27,6**	***185***
Methanomicrobiales	3	1,6								2	2,2		31	17,8	
Methanocalculus	**3**	**1,6**	***185/390***							1	1,1	*183*	**25**	**14,4**	***185***
Methanosarcinales	171	93,4		92	98,9		78	83,9		8	8,7		78	44,8	
Methanosaetaceae	171	93,4		92	98,9		2	2,2		8	8,7		61	35,1	
Methanosaeta concilii	**151**	**82,5**	***282***	**86**	**92,5**	***283***	2	2,2	*283*						
Methanosaeta harundinacea	20	10,9	*492*	5	5,4	*225*				**8**	8,7	***494***	61	35,1	***494***
Methanosarcinaceae							75	80,6					4	2,3	
Methanosarcina mazei							**75**	**80,6**	***183***						
Thermoplasmata				1	1,1					28	30,4				
Thermoplasmatales				1	1,1					28	30,4				
Thermogymnomonas				**1**	**1,1**	***380***				**1**	1,1	***380***			
Crenarchaeota	4	2,2					6	6,5		38	41,3		4	2,3	
unclassified Thermoprotei	4	2,2					6	5,5		38	41,3				

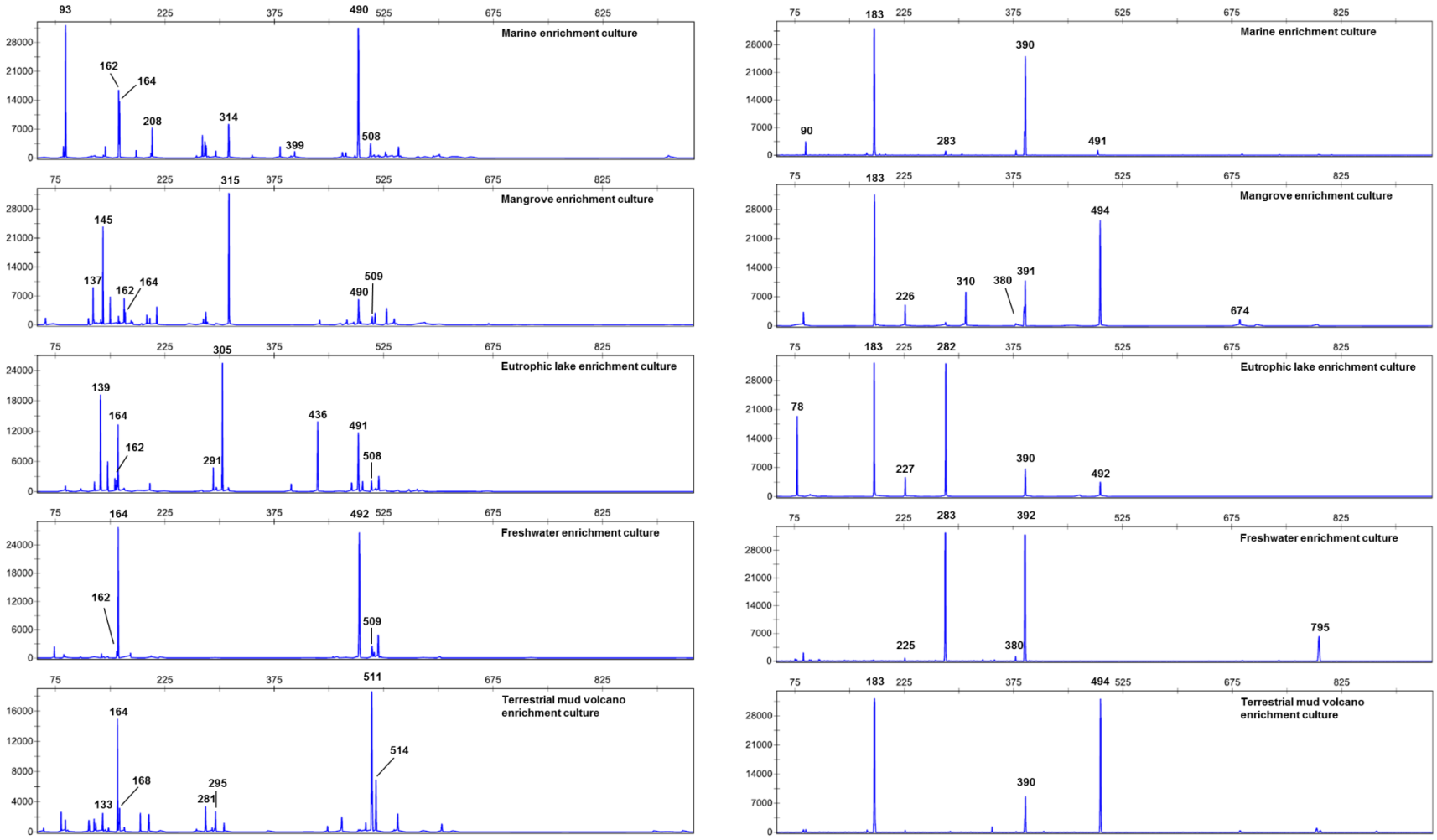


Figure 4.3: Bacterial (left) and archaeal (right) 16S rRNA gene T-RFLP fingerprints from the different enrichment cultures amended with hexadecane. The lengths (bp) of selected T-RFs are given.

4.3.4. Phylogenetic analysis of the enriched hexadecane degrading microbial communities

With the use of amplicon pyrosequencing and the construction of clone libraries a more detailed community analysis was conducted. Further to sequencing data sets, investigations of terminal restriction fragments (T-RFs) were performed to get a second qualitative, but not strictly quantitative overview of the diversity of the present microbial community. Almost all significant peaks present in the electropherograms (Figure 4.3) showed concurrences to sequencing data (Tables 4.2 and 4.3).

The bacterial 16S rRNA gene T-RFLP fingerprints from DNA extracted from enrichment cultures inoculated with eutrophic lake, brackish water and mangrove sediments as well as terrestrial mud volcano samples exhibited a broad diversity, while enrichment cultures derived from freshwater sediments showed only two dominant T-RF peaks. The enrichment communities were mainly composed of sequences related to *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Clostridia*, *Anaerolineaceae* (*Chloroflexi*) and *Bacteroidetes* incertae sedis. The most dominant T-RFs in freshwater enrichment cultures were related to deltaproteobacterial *Smithella* (both 164- and 509-bp T-RF) and betaproteobacterial *Azospira* sp. (492-bp T-RF). Furthermore we identified the 162-, 491- and 508-bp T-RFs as members of *Syntrophorhabdus*, *Acinetobacter* and relatives of the family *Anaerolineaceae*. The same fragment lengths and affiliations to *Smithella, Syntrophorhabdus, Acinetobacter* and *Anaerolineaceae* were found in T-RFLP fingerprints of eutrophic lake enrichment cultures, but they were overtopped by relatives of *Thiorhodospira* (139-bp T-RF), *Syntrophomonas* (305-bp T-RF), *Ralstonia* (436-bp T-RF) and *Pseudomonas* (490-bp T-RF).

As distinct from the fingerprinting results and sequence data from the eutrophic lake and freshwater sediment enrichments, sequences affiliated to *Pseudomonas* species (490-bp T-RF) and *Bacteroidetes* incertae sedis (93-bp T-RF) formed the dominant part in the brackish water sediment enrichment cultures. Furthermore, T-RFs affiliated to *Desulfovibrio* sp. (164-bp T-RF), *Thermanaeromonas* sp. (314-bp T-RF) and *Syntrophomonas* spp. (399-bp T-RF) were identified as members of the bacterial community composition. In mangrove sediment enrichment cultures, the 315-bp T-RF, identified as *Thermanaeromonas* sp. played the main role in the community, followed by *Marinobacter* sp. (145-bp T-RF), *Ectothiorhodosinus* sp. (490-bp T-RF) and *Desulfovibrio* sp. (164-bp T-RF).

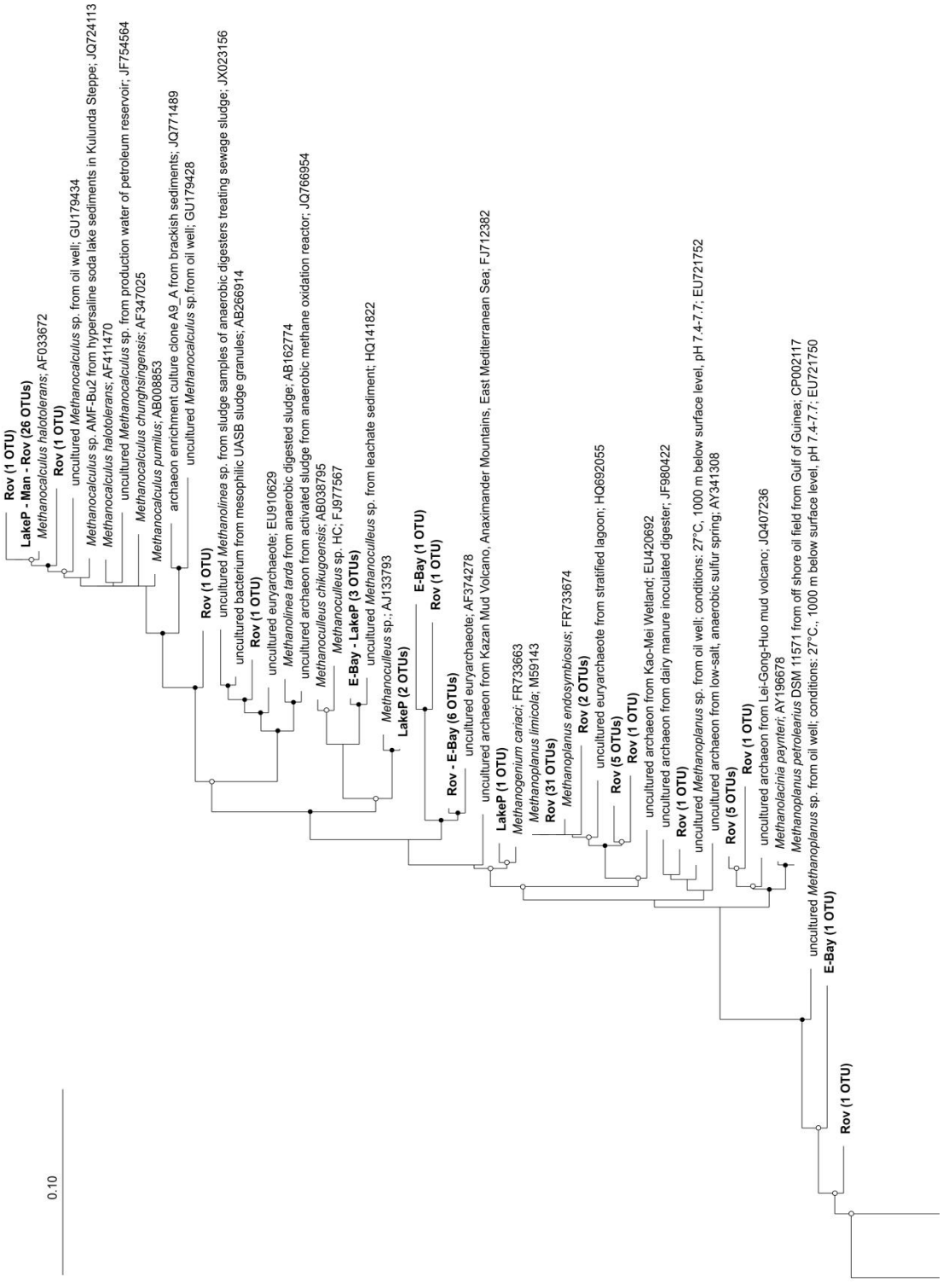
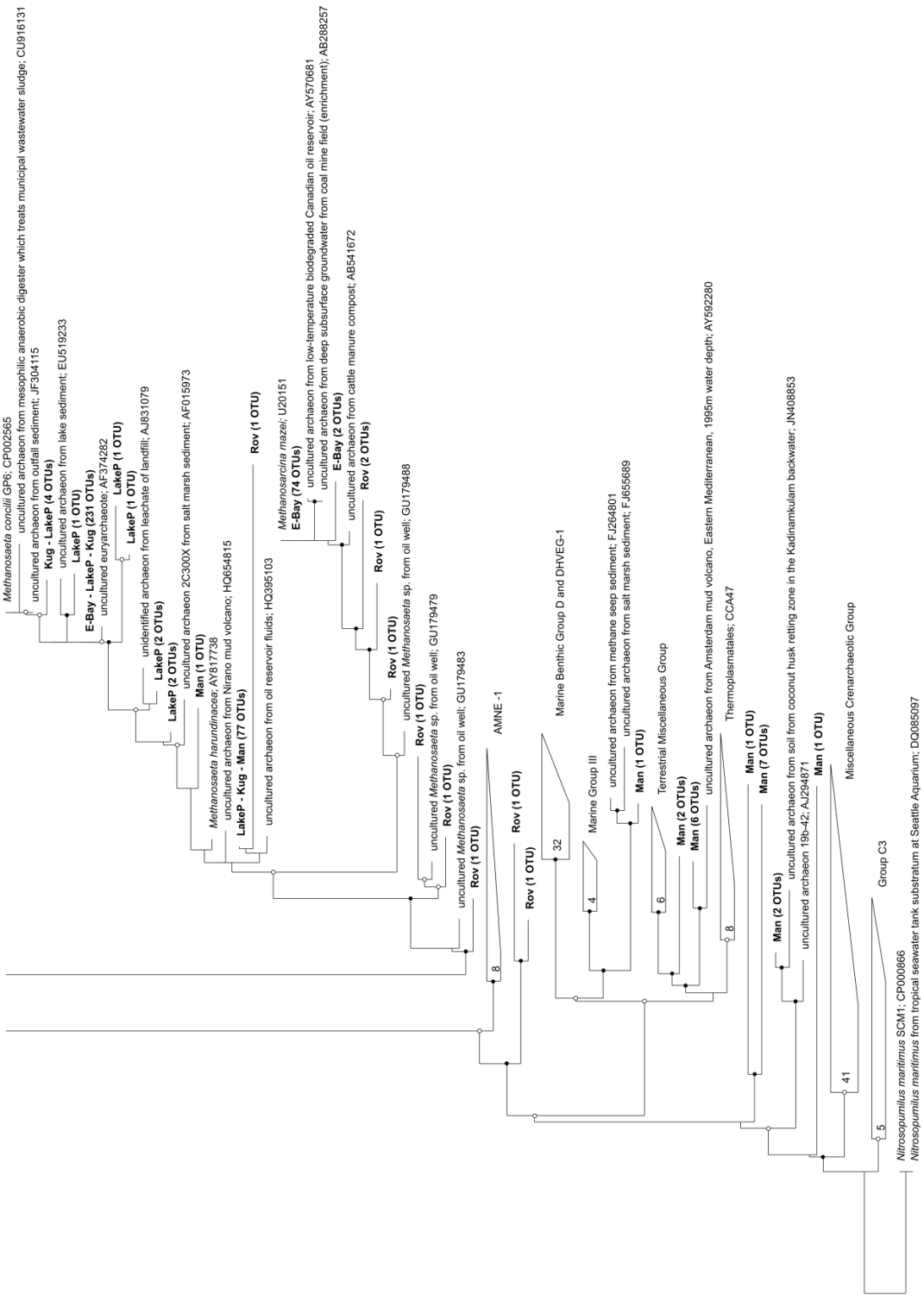


Figure 4.4: Parsimony tree assembled for *Archaea* from 16S rRNA gene sequences summarizing all sampling sites. Sampling sites are: Eckernförde Bay (E-Bay), Brazilian mangrove forests (Man), Lake Plußsee (LakeP), Kuhgraben (Kuh) and Terrestrial mud volcano (Rov). The tree is based on the SILVA database version 102 provided by Pruesse et al. (2007).

Bootstrap support (in %, 1000 replicated trees) is indicated at each branching point. Bootstrap values less than 50 % are not shown, between 50-75 percent are marked with blank circle and between 75-95 percent are marked with filled circle. Reference species are italic and clone sequences are bold. Numbers of OTUs for each phylotype are shown in parentheses. Numbers in boxes indicate numbers of sequences which were assigned to the respective cluster. The tree was rooted using *Nitrosopumilus maritimus* as an out-group **(Figure 4.4, continued from previous page)**.



Quite different from these analyzed enrichments the enriched samples from the terrestrial mud volcano revealed high accounts of relatives to *Desulfuromonas* sp. with 125 and 511-bp T-RFs, which corresponded with one of the dominant peak in the fingerprint plot. *Desulfohalobium* sp., *Desulfovibrio* sp. (both 133-bp T-RF) and *Desulfosalsimonas* sp. (168-bp T-RF) were identified and formed a minor part of the enriched mud volcano community.

As compared with the bacterial 16S rRNA gene T-RFLP fingerprints analysis the archaeal one revealed a community limited to two or four dominant families, mainly methanogens. In eutrophic enrichment cultures the 282 bp fragment identified as *Methanosaeta concilii* was one of two dominant peaks. Additionally, members of *Methanocalculus* sp. (185 / 390-bp T-RF) were identified, the archaeal fingerprint showed the 185-bp T-RF as the second dominant peak. The community was complemented by *Methanoculleus* sp. (183 / 390-bp T-RF), *Methanogenium* sp. (390-bp T-RF) and *Methanosaeta harundinacea* (493-bp T-RF). The archaeal community of the freshwater enrichment cultures was dominated completely by members of *Methanosaeta* sp. Both highest T-RFs, 283 and 392-bp T-RF were identified as *Methanosaeta concilii*. The species *Methanosaeta harundinacea* (225-bp T-RF) and *Thermogymnomonas* sp. (380-bp T-RFs) complemented the community composition. The terrestrial mud volcano enrichment cultures were dominated by members of *Methanoplanus* sp., *Methanocalculus* sp. (both 185-bp T-RF) and *Methanosaeta harundinacea* (494-bp T-RF). The 391-bp T-RF was identified as *Methanogenium* sp. and correspond with the fourth peak of the fingerprinting plot.

The 183-bp T-RF and 390-bp T-RF, the highest peaks in brackish water enrichment cultures were assigned to *Methanosarcina mazei* and *Methanogenium* sp. In cultures enriched with mangrove sediments the main members of the community were identified as members of *Thermoprotei,* dedicated to the *Crenarchaeota* phylum, and *Methanosaeta harundinacea* (494-bp T-RF). The sequence analysis showed minimum amounts of members of *Thermoprotei* in all enrichment cultures amended with hexadecane, excluding freshwater enrichments. The phylogenetic placement of significant archaeal sequences recovered from the hexadecane degrading enrichment cultures is illustrated in Figure 4.4.

4.4. Discussion

4.4.1. Physiological aspects of hexadecane degradation under methanogenic conditions

The aim of this study was the enrichment and characterization of hydrocarbon-degrading methanogenic communities in different environmental habitats such as eutrophic lake, freshwater, brackish water, mangrove and terrestrial mud volcano sites, independent on their hydrocarbon contamination history. Furthermore, we studied the influence of sulfate on hexadecane-dependent methanogenesis in enrichment cultures inoculated with brackish water sediment. These enrichment cultures showed that the addition of 2 mM sulfate stimulated the growth of methanogens. But sulfate is not obligatory for the growth of methanogenic microorganisms. However, higher sulfate concentrations of more than 5 mM influenced the conversion of hexadecane to methane negatively. The sulfate-reducing hexadecane-degrading community was stimulated in growth, resulting changes in the community composition lead to the displacement of the methanogenic part of the community.

The bacterial reduction of sulfate in anoxic sediments is the most important process in the mineralization of organic matter. The process is mediated by a phylogenetically and physiologically diverse group of microorganisms which all use sulfate as the terminal electron acceptor. Sulfate-reducing prokaryotes are able to oxidize all the major fermentation products completely to CO_2 and H_2O and inhibit the methanogens by competing with them for common substrates (Leloup et al., 2009). This suggests that these groups of microorganism found in these different habitats are widespread within hydrocarbon degradation and may have significant impact on the carbon cycle in anoxic sediments worldwide.

The tested effects of increased salinity, pressure and temperature simulating reservoir conditions showed that the enriched microbial communities *in vitro* are adapted to these special reservoir conditions. Salinity was the limiting factor in this composition of physiological effects. The increase of the salinity leads immediately to a decrease in methane production, an adverse effect likely caused by an increase in maintenance energy demand of the microorganisms to cope with the higher salt concentrations. The increased phosphate

concentration in the medium during methane production from hexadecane indicates an important role for this nutrient.

This shows an abundant potential of the laboratory microbial communities to degrade hydrocarbons under reservoir conditions. The knowledge of hydrocarbon-degrading methanogenic communities in different environments can lead to promote efficient bioremediation processes in soils and sediments after oil spills or tanker accidents. Or to develop more effective treatments to prevent biodegradation in oil reservoirs.

4.4.2. Bacterial community composition in enrichment cultures amended with hexadecane

The bacterial community found in the enrichment cultures amended with hexadecane consists of members related to *Proteobacteria, Firmicutes, Chloroflexi,* and *Bacteroidetes.* Their quantitative contribution differs strongly among the cultures enriched from several environments. Relatives of the classes *Actinobacteria* and *Synergistetes* were also found, but only in minor amounts.

The syntrophic bacteria of the genus *Smithella* were identified as dominant species in freshwater and eutrophic enrichment cultures, and are also present in mangrove cultures. The abundant presence of species belonging to *Syntrophaceae* was frequently observed in hydrocarbon-associated environments such as oil-contaminated soils, sediments, oil tailings ponds and aquifers (Allen et al., 2007; Gray et al., 2011; Shimizu et al., 2007). Zengler et al. (1999) gave the first evidence for a direct role of relatives of *Syntrophaceae* in hydrocarbon degradation, in particular in the activation and oxidation of hexadecane to lower molecular fatty acids or acetate and hydrogen which is then metabolizable by acetoclastic methanogens. Thus, they form a syntrophic partnership with the methanogenic archaea.

Additionally, other syntrophic candidates (*Syntrophorhabdus* sp. (162-bp T-RF) and *Syntrophomonas* sp.) were detected in freshwater and eutrophic lake enrichment cultures. The obligatory anaerobic mesophilic *Syntrophorhabdus* is able to oxidize aromatic compounds, such as benzoate in syntrophic association with hydrogenotrophic methanogens. The genus is not able to use sulfate, sulfite, thiosulfate, nitrate, nitrite, elemental sulfur, or ferric iron as an electron acceptor (Qiu et al., 2008). Moreover, sequences affiliated to *Syntrophomonas* sp.*,* adapted for syntrophic growth with

methanogens and other hydrogen- and formate-using microorganisms were predominantly detected in eutrophic lake and, in minor quantity, in brackish water enrichment cultures.

Detected additional in freshwater and eutrophic enrichment cultures the *Acinetobacter* species, known as oil-degraders in soil and sediments contaminated with crude oil, were firstly described by Reisfeld et al. (1972). *Acinetobacter* stains are able to degrade efficiently short- and long-chain alkanes, and even aromatics, connect the anaerobic oxidation of hydrocarbons with the reduction of nitrate (Li et al., 2012b; Mbadinga et al., 2011). They are commonly found in hydrocarbon-rich environments like petroleum reservoirs (Li et al., 2007; Tang et al., 2012). Another *Gammaproteobacteria*, found predominantly in eutrophic and brackish water enrichments, are members of the typically aerobic *Pseudomonadales*, known as a very diverse and widespread genus capable of utilizing hydrocarbons as carbon and energy sources (Das and Chandran, 2011). *Pseudomonas* species were observed and isolated from brackish water sediment and are well known as nitrate-dependent alkene- and polycyclic aromatic hydrocarbon-degraders (Rockne et al., 2000). Moreover, specific bacterial community structures consisted of both aerobic and anaerobic hydrocarbon-degraders including *Alteromonadales*, *Pseudomonadales*, *Burkholderiales*, *Rhodobacteriales*, and *Rhodocyclales* were mainly detected in sediment sites with high level of hydrocarbon pollution (Greer, 2010). These findings correlate well with the identified relatives of the *Proteobacteria* in the freshwater and eutrophic enrichments.

In brackish water and mangrove enrichment cultures members of the species *Desulfovibrio*, an incompletely oxidizing and H_2-utilizing sulfate-reducing bacterium (Widdel and Bak, 1992), *Thermanaeromonas*, an thermophilic, anaerobic thiosulfate-reducing bacterium (Mori et al., 2002), and different members of the class *Gammaproteobacteria* were detected and formed the specific community composition of both enrichments. The presence of sulfate-reducing bacteria (primarily *Desulfovibrio* spp. and *Desulfobacterium* spp.) in the absence of sulfate in the cultures may be explained by their ability to function as proton-reducing acetogens and/or fermenters, as described by Raskin et al. (1996).

In enrichments inoculated with mangrove sediment members belonging to gammaproteobacterial *Marinobacter* species (145-bp T-RF) take part in the community composition. *Marinobacter* species were detected previously in several anoxic hydrocarbon contaminated and subsurface environments (Dunsmore et al., 2006; Gray et al., 2011;

Inagaki et al., 2003b; Orphan et al., 2000) and a number of *Marinobacter* isolates were characterized as facultative anaerobes, which were able to grow fermentative and participate in syntrophic interactions (Köpke et al., 2005). Gray et al. (2011) argued for some *Marinobacter* the capability of anaerobic oxidation of minor components of crude oil in partnership with methanogens. This could explain the comparatively high presence of *Marinobacter* sp. in mangrove hexadecane enriched microcosms. The mangrove enrichment community was completed by the presence of *Thermanaeromonas* sp. (315-bp T-RF), *Smithella* sp. (164-bp T-RF), and other members of *Gammaproteobacteria.*

In contrast to the other enrichment cultures described above, the bacterial community of the terrestrial mud volcano enrichments were exclusively represented by members of *Deltaproteobacteria*, affiliated to *Desulfuromonadales, Desulfobacterales* and *Desulfovibrionales*. The genera *Desulfosalsimonas* sp., *Desulfohalobium* sp., *Desulfovibrio* sp., and *Desulfuromonas* sp. were identified, while the latter of whom showed the highest abundance within the bacterial community. The genus *Desulfuromonas* was first described by Pfennig and Biebl (1976). They isolated the marine species *Desulfuromonas acetoxidans,* which oxidize acetate with elemental sulfur or Fe(III) as terminal electron acceptor (Roden and Lovley, 1993). The most abundant sequences in the clone library showed strong affiliations to *Desulfuromonas palmitatis,* oxidize multicarbon organic compounds completely to CO_2 with Fe(III) as an electron acceptor, and long-chain fatty acids coupled to Fe(III)-reduction (Coates et al., 1995). Moreover, members of the sulfate-reducing *Desulfobacteraceae* family are known for their ability to oxidize carbon comlete to CO_2 (Kuever et al., 2005). Relatives of *Desulfobacteraceae* family were also found in samples from terrestrial mud volcanoes of Azerbaijan (Green-Saxena et al., 2012). This produced CO_2 built the substrate for the hydrogenotrophic methanogens which takes part of the community of the terrestrial mud volcano enrichment culture.

The presence of members of *Prolixibacter* affiliated to *Bacteroidetes* showed a similarity between the eutrophic lake, freshwater, brackish water and mud volcano enrichment cultures. *Prolixibacter bellariivorans,* available in minor quantity in the bacterial community of eutrophic lake, freshwater, mud volcano and dominant of the brackish water enrichment cultures, represents a unique phylogenetic cluster within the phylum *Bacteroidetes*. *Prolixibacter bellariivorans* is a facultative anaerobe that ferment sugars by using a mixed acid fermentation pathway and grow over a wide range of temperatures

(Holmes et al., 2007). Under anaerobic conditions *Prolixibacter* isolates cannot utilize acetate, benzoate, ethanol, formate, fumarate or hydrogen and several other compounds. Li et al. (2012a) described the distribution of *Prolixibacter* in microbial BTEX-enrichments with a wide range of salinity.

In summary, our results showed the presence of bacteria related to *Proteobacteria, Firmicutes, Chloroflexi,* and *Bacteroidetes* in all enrichments. The community composition of eutrophic lake and freshwater enrichments showed closely resemble to each other, while the brackish water and mangrove enrichment community in turn was also similar. The terrestrial mud volcano community composition differs from all other enrichments, because their bacterial community consists nearly exclusive of sulfate-reducers affiliated to the *Desulfuromonadales, Desulfobacterales* and *Desulfovibrionales* families*,* supplemented through minor abundant members of *Bacteroidetes* and *Firmicutes* which were also found in the enrichments from the other sites. These different bacterial community structures found in our environments deviated from each other especially through their salinities and further though their input of organic matter and nutrients, besides abiotic factors like temperature.

The high abundance of sulfate-reducers found in all enrichments showed the importance of this bacterial group in these very different habitats. We suggest that the main degradation of organic matter and hydrocarbons in these observed sediments were performed by sulfate-reducing bacteria. Furthermore, the presence of syntrophic bacteria found in eutrophic lake / freshwater (*Smithella* sp. and *Syntrophorhabdus* sp.) and brackish water / mangrove (*Syntrophomonas* sp. / *Marinobacter* sp.) environments point to a close association to methanogenic archaea, thus to an efficient conversion of hydrocarbons to methane and CO_2.

4.4.3. Archaeal community composition in enrichment cultures amended with hexadecane

The quantitative contribution of archaea and bacteria was nearly similar within a culture, but vary gently in their copy numbers between the different enrichment cultures. In the eutrophic lake, freshwater and brackish water enrichment cultures the detected quantities of sulfate-reducers showed higher copy numbers as the methanogens (Figure 4.3). In the case of the mangrove enrichments the numbers of bacteria are outnumbered by the amounts of sulfate-reducers. An explanation for this could be the presence of sequences

affiliated to sulfate-reducing archaea. The low copy numbers of *mrcA* genes in the mangrove enrichments showed a contrast to the phylogenetic results in which the abundant presence of methanogens and other archaea could be determined. The majority of the archaeal community composition of the enrichment cultures incubated with the variety of sediment samples was decrypted by the combination of T-RFLP data and investigation in clone libraries. Methanogenic archaea were present in all enrichment cultures amended with hexadecane.

All hexadecane enrichments inoculated with eutrophic, freshwater, and mangrove sediments and with samples from the terrestrial mud volcano contained *Methanosaetaceae* as prevalent archaeal members. Most of them were related to the species *Methanosaeta concilii* and *Methanosaeta harundinacea.* Enrichment cultures inoculated with brackish water sediment were dominated by the species *Methanosarcina mazei*, but contained also a small amount *Methanosaeta concilii*. Both *Methanosarcinaceae* and *Methanosaetaceae* are known as obligate acetoclastic methanogens. The dominant presence of members of the *Methanosarcinales* (*Methanosaeta* sp. and *Methanosarcina* sp.) in all enrichments suggests acetoclastic methanogenesis and a close interaction with syntrophic fermenting bacteria. Zengler et al. (1999) demonstrated the conversion of hexadecane via syntrophic anaerobic alkane-degraders to acetate and hydrogen, coupled to acetoclastic and hydrogenotrophic methanogenesis in a freshwater ditch. In this study we showed the hexadecane degradation in all enrichment cultures derived from different ecosystems. The absolute key intermediate during the anaerobic degradation of hydrocarbons used by a variety of different groups of microorganisms is acetate. More than 70 % of biological methanogenesis in flooded soil results from acetate consumption (Conrad and Klose, 1999). Syntrophic interactions between microorganisms are essential in anaerobic biodegradation of complex organic matter, in this case of hydrocarbons, into CH_4 and CO_2 and for the carbon flux in methanogenic ecosystems (Schink, 1997). The isotopic measurements of $\delta^{13}C_{CH4}$-values revealed the conversion of the ^{13}C-labeled hexadecane to methane. Simultaneously, the isotopic value of $\delta^{13}C_{CO2}$ showed a strongly increase over the time of incubation, and indicated in this way that CH_4 was mainly formed by acetoclastic methanogenesis. Isotope fractionation values from enrichment cultures inoculated with freshwater and brackish water sediments analyzed in this study were published by Feisthauer et al. (2010).

The fact that further archaeal genera closely related to the order *Methanomicrobiales* (*Methanoculleus* sp., *Methanogenium* sp., *Methanocalculus* sp. and *Methanoplanus* sp.) were detected in all enrichment cultures amended with hexadecane, except freshwater microcosms, gave a hint to CO_2-reduction to methane. The first proposition of the genus *Methanocalculus* sp., with *Methanocalculus halotolerans* as the type species for a hydrogenotrophic halotolerant methanogen, came from Ollivier et al. (1998). The genus was isolated from an oil-producing well, produced methane from H_2 / CO_2 or formate, and acetate is required in addition for growth (Lai et al., 2004; Ollivier et al., 1998). *Methanogenium,* the proposed genus, and *Methanogenium cariaci,* the type species, catabolize formate or H_2 / CO_2 and were isolated for the first time from Black Sea sediments by Romesser et al. (1979).

The exemption formed the enrichment cultures derived from terrestrial mud volcano samples. These cultures were dominated by the acetoclastic *Methanosaeta harundinacea* and the hydrogenotrophic members of *Methanocalculus* sp., *Methanolinea sp.,* and *Methanoplanus* sp. Two species of *Methanoplanus* were identified in the terrestrial mud volcano community - *M. petrolearius* and *M. limicola. M. petrolearius* was isolated from an African offshore oil field and produced methane from H_2 / CO_2, formate, and CO_2 / 2-propanol, could not utilize acetate, but acetate is required for growth (Ollivier et al., 1997). *M. limicola* was isolated from swamp by Wildgruber et al. (1982), used H_2 / CO_2 or formate as source for methane production and acetate was required for growth. Consequently, the archaeal community of the terrestrial mud volcano cultures amended with hexadecane was the only one of our sample collection which was dominated by acetoclastic and hydrogenotrophic methanogens to equal shares. This findings correlates with the previous work by Zengler et al. (1999). They showed the possibility that methane was formed by acetoclastic and CO_2-reducing processes simultaneously. Additionally, the phylogenic tree revealed close affiliations to uncultured archaea clustering in the methanomicrobial group of ANME 1 and into the *Thaumarchaeota* group C 3 for sequences found in the archaeal community of cultures enriched from terrestrial mud volcano.

Moreover, the sequences found in mangrove enrichments showed a high ratio of uncultured archaea clustering in the Marine Benthic Group D and Deep-sea Hydrocarbon Vent Environment Group 1 (DHVEG 1), Marine Group 3, Terrestrial Miscellaneous Group (TMEG), or accordance with other uncultured archaea (CCA47) all affiliated to

Thermoplasmatales. Sequences revealed high affiliations to the uncultured *Thaumarchaeota* cluster Miscellaneous Crenarchaeotic Group were found in mangrove, brackish water and eutrophic lake enrichments. This high number of uncultured archaea conceals a lot new and to date not described microorganisms with completely unknown metabolic potential.

In conclusion, laboratory microcosms from different habitats showed high methane production rates after the addition of hexadecane. Quantitative analysis of these enrichment cultures revealed equally large numbers of bacterial and archaeal 16S rDNA genes in all cultures. Phylogenetic analyses of the archaeal community showed the abundant presence of methanogenic archaea related to the orders *Methanomicrobiales* and *Methanosarcinales*, whereas each enrichment culture in turn showed phylogenetic characteristics in their specific archaeal community composition on the genera level. The eutrophic lake, freshwater, brackish water and mangrove communities were dominated by acetoclastic methanogens. An exceptional position was found in the community composition of the terrestrial mud volcano enrichment cultures that was composed of equal shares of hydrogenotrophic and acetoclastic methanogens. Another similarity showed the detection of *Thermoplasmatales* and uncultured *Thaumarchaeota* in all these enrichments, except of the freshwater enrichments, whereas the mangrove enrichments were dominated by these both uncultured groups. The exclusive detection of *Methanocalculus halotolerans* in mangrove and mud volcano enrichment cultures indicated the special adapting to saline environmental conditions, in comparison to the other observed sites where this genus was not detectable. The mud volcano sampling site showed high salinity around 40 ‰ (Alain et al., 2006).

4.4.4. Ecological environmental aspects of hydrocarbon degradation

Hydrocarbon pollution in such environments we have referred in this study is attributable to human activities like petroleum extraction, transportation, and processing including oil tanker accidents and oil spills, but also to natural oil and gas seeps. We showed that microbial communities inhabit pristine environments such as the freshwater ditch or the eutrophic lake, are able to degrade hydrocarbon. On the other hand, in the investigated sampling site of the brackish water sediments, the Eckerförde Bay, we assumed the presence of hydrocarbons. The semi-enclosed Eckernförde Bay is part of the German Baltic Sea coast there several harbors and marinas are located. Harbors are point sources for

chronic hydrocarbon pollution derived from boat traffic, accidental spills, discharge of ballast water, and bilge oil (Nogales, 2010). In such chronic hydrocarbon-polluted environments, microbes are the key players in hydrocarbon degradation processes, are adapted to the hydrocarbons and form stable communities (Berthe-Corti and Nachtkamp, 2010). Additional to our study, Coates et al. (1996b) demonstrated the high rate hydrocarbon degradation in marine harbor sediments under sulfate- and iron-reducing conditions, comparable to aerobic degradation rates. The rate and the efficiency of hydrocarbon biodegradation depend mainly on the microbial syntrophic interactions. The syntrophic interaction efficiency in turn were influenced by composition, activity, and interspecies cooperation of the hydrocarbon-degrading communities as well as by abiotic factors of the geographical location, such as temperature, concentration of inorganic nutrients, electron acceptors, and presence and structure of hydrocarbons.

4.4.5. Syntrophic interactions

As anaerobic hydrocarbon-degrading microorganisms, a lot of groups were identified including sulfate-reducing, denitrifying, dissimilatory iron(III)-reducing, and syntrophic bacteria that link hydrocarbon degradation to methanogenesis (Kniemeyer et al., 2007; Widdel and Rabus, 2001; Yuan and Chang, 2007). Many studies investigated anaerobic biodegradation of hydrocarbons in aquifers, sediments, soils, oil reservoirs and coal seams (Dojka et al., 1998; Kim and Crowley, 2007; Nazina et al., 2006; Strąpoć et al., 2008; Watanabe et al., 2002; Winderl et al., 2008). The most abundant microorganisms found in all of these hydrocarbon-associated environments were identified as relatives of the fermentative *Firmicutes* (e.g. *Clostridia* and *Thermoanaerobacter*), *Chloroflexi* (class *Anaerolinea*), *Bacteroidetes*, and syntrophic populations of the *Deltaproteobacteria* which includes iron- and sulfate-reducing bacteria. Relatives of these groups were also found in our enrichment cultures. Moreover, relatives of *Betaproteobacteria* are dominant in freshwater environments, in this study in eutrophic lake and freshwater enrichments, but are virtually absent in marine environments including brackish water and mangrove enrichments. That evidence was confirmed by Greer (2010).

Typical syntrophic associations are characterized by strictly syntrophic relationships of two metabolically different types of microorganisms in which both partners depend on each other for energetic reasons and together perform a fermentation process that neither could

run on its own (Schink, 2006). In most syntrophic methanogenic associations the involved microorganisms live close to the thermodynamic limit, because the obtain energy for growth yielding from the conversion of substrates like H_2 / CO_2 or acetate to CH_4 is extremely low (Deppenmeier and Müller, 2008). Mostly hydrogen is the electron carrier between these oxidative and reductive metabolic processes (Schink, 2006). An example for hydrogen transfer is the ethanol-oxidizing sulfate-reducer *Desulfovibrio vulgaris* which oxidize ethanol in the absence of sulfate by hydrogen transfer to a hydrogen-oxidizing methanogenic partner. In the freshwater and eutrophic lake sediment enrichments, described in this study, the organic acid intermediates of anaerobic hydrocarbon degradation are converted syntrophically by members of the family *Syntrophaceae* (*Syntrophus* sp. and *Smithella* sp.) into CO_2, H_2, and formate, which in turn are substrates for hydrogenotrophic methanogens (Schink, 1997) like the members of the family *Methanomicrobiaceae* (e.g. *Methanocalculus* sp.). Furthermore, the syntrophic fermenting bacteria *Syntrophomonas wolfei* oxidizes butyrate and *Smithella propionica* propionate to acetate (and butyrate) in close cooperation with the methanogenic partner organism e.g. *Methanospirillum hungatei,* which has to lower the hydrogen partial pressure to keep the degradation reactions thermodynamically favorable (de Bok et al., 2001; Schmidt et al., 2013).

Despite of manifold research investigations in the field of hydrocarbon biodegradation, the complex interactions of these parameters proceeding under environmental conditions are not yet understood in detail. Wherefore, the complex interactions of these parameters in the environmental systems as well as the composition, structure, and role of hydrocarbon-degrading microbial communities are needs to research, prospectively. This developing knowledge could be improves the potential to bioremediate contaminated environmental sites, could lead to the identification of bioindicator taxa, enhance the dynamic of anaerobic digestion process for biomethane production, and reveal new strategies for highly efficient microbial enhance oil recovery.

In addition, the large number of unknown sequences clustered with uncultured environmental archaea and bacteria sequences indicating new microbial types providing important new biochemical pathways and enzymes with potential biotechnological applications.

Acknowledgements

This work was financed from the German Research Foundation (DFG) within the Priority Program SPP1319 (projects KR3311 6-1, 6-2). We acknowledge Daniela Zoch and Holger Probst for technical assistance.

Chapter V

Isotopic fingerprinting of methane and CO_2 formation from aliphatic and aromatic hydrocarbons

5. Isotopic fingerprinting of methane and CO_2 formation from aliphatic and aromatic hydrocarbons

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Organic Geochemistry 05/2010; 41 (5): 482–490

DOI:10.1016/j.orggeochem.2010.01.003

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Abstract

We investigated the stable carbon and hydrogen isotopic signatures of methane, CO_2 and water during microbial formation of methane from mineral oil-related compounds in order to determine the variability in the methane signatures. The isotopic discrimination for carbon and hydrogen between substrate and methane was calculated and resulted in $\varepsilon C_{DIC\text{-}CH4}$ 26–60‰, $\varepsilon C_{substrate\text{-}CH4}$ 16–33‰, $\varepsilon H_{H2O\text{-}CH4}$ 257–336‰ and $\varepsilon H_{substrate\text{-}CH4}$ 174–318‰, respectively. These carbon and hydrogen isotope signatures fell in to a relatively narrow range, suggesting a coupling of fermentation with acetoclastic and CO_2 reducing methanogenesis. In order to characterize the microbial consortia involved in the methanogenic degradation of hexadecane, a methanogenic enrichment culture was incubated with 1-^{13}C–hexadecane and its biomass was analyzed for the pattern and isotopic signature of carboxylic acids. The highest extent of labeling was detected in the *n*-C17 fatty acid with methyl groups at C-4, presumably indicative of *Syntrophus* sp. To determine if the isotope composition of methane can be used as an indicator for methanogenesis during growth with oil-related compounds in field studies, we analyzed the isotope composition of methane in a confined mineral oil contaminated aquifer. The variability in carbon and hydrogen isotope composition was almost identical to the values obtained from enrichment cultures, thereby providing a tool for screening for microbial methane formation during hydrocarbon exploration.

5.1. Introduction

Methanogenesis is the terminal electron accepting process for degradation of organic matter (OM) after the depletion of other organic and inorganic electron acceptors in a closed environmental system. This unique metabolic pathway may explain the frequently observed hydrocarbon biodegradation in petroleum reservoirs (e.g., Dolfing et al., 2008; Gieg et al., 2008; Gray et al., 2009; Grigoryan and Voordouw, 2008; Head et al., 2003; Jones et al., 2008). Except for CO_2, formed stoichiometrically with CH_4, no external electron acceptors are required to sustain the degradation process over long-term periods. Thus, only water and essential nutrients may be required for methanogenesis in oil reservoirs. CH_4 formation from oil-related compounds in petroleum reservoirs may provide an opportunity for enhanced recovery of fossil fuels from previously exploited oil reservoirs and coal bed strata (Finkelstein et al., 2005; Gieg et al., 2008; Grigoryan and Voordouw, 2008). Even in times of intense exploration for alternative energy sources, the worldwide demand for hydrocarbons, the most readily available energy and carbon source, persists and may even grow as a result of the increase in world population. At the same time, easily accessible petroleum reserves are declining. Roughly 40 % of existing crude oil can be recovered using conventional technologies, resulting in large amounts of inaccessible oil remaining within the world's oil deposits (US Department of Energy, 2006). Around 50–98 % of a typical crude oil consists of hydrocarbons such as alkanes, cycloalkanes and alkylated aromatic compounds (Townsend et al., 2003). Different constituents within the hydrocarbon fraction have been shown to be biodegradable under anoxic conditions with various electron acceptors (e.g., Wilkes et al., 2000, 2003). For example, *n*-alkanes comprise a major fraction of most crude oils and have been found to be biodegradable under methanogenic conditions, both as pure substrates, e.g. *n*-hexadecane (Anderson and Lovley, 2000b; Zengler et al., 1999) and in crude oils (Gieg et al., 2008; Jones et al., 2008; Siddique et al., 2006; Townsend et al., 2003). Converting at least a portion of currently unrecoverable oil biotechnologically into methane may provide an opportunity to increase the recovery of energy from oil reservoirs. This represents an emerging research area for biotechnology and fuel production. Gieg et al. (2008) estimated an additional CH_4 production potential in the USA of up to 2.8×10^{10} m^3 per year from exploited oil reservoirs. This would mean that up to 16 % of the annual gas consumption in the USA could be offset by enhanced methanogenesis (Energy Information

Administration, 2007). In addition, natural gas is a cleaner-burning alternative to gasoline and reduces CO_2 emissions by 25 % (DeLuchi, 1991).

Zengler et al. (1999) showed that methane can be produced from hexadecane by methanogenic microorganisms in a syntrophic consortium with acetogenic bacteria. Acetate, CO_2 and H_2 are intermediates in the process of methanogenic alkane degradation. Acetate is cleaved by methanogenic Archaea to form CH_4 and CO_2. Furthermore, CO_2 reducing methanogenic Archaea maintain a low hydrogen partial pressure, a thermodynamic prerequisite to make the overall reaction energetically favorable. The transformation of hexadecane to CH_4 occurs via the following net reaction (Zengler et al., 1999), showing that ca. 77 % of the carbon can be transformed to CH_4 when neglecting biomass formation and energy demands of the cell:

$$4C_{16}H_{34} + 30H_2O \rightarrow 49CH_4 + 15CO_2 \quad (5.1)$$

The carbon and hydrogen stable isotopic signatures of methane provide clues for characterizing the origin of methane and may allow for a better description of methanogenic processes in the field (Whiticar et al., 1986; Whiticar, 1999). In order to describe the level of conversion of alkane-derived carbon to methane, Jones et al. (2008) linked the stable carbon isotopic composition of CO_2 to biogenic methane from a degraded oil reservoir.

The objective of our study was to elucidate the processes involved in microbial gas generation during growth on oil-related compounds. To analyze the isotopic fractionation processes during CH_4 production from n-alkanes, we incubated several enrichment cultures of anaerobic oil degraders from methanogenic environments with *n*-hexadecane as a model aliphatic hydrocarbon. Also, we described the structure of the syntrophic consortium involved in CH_4 production by tracking the carbon isotope label from 1-^{13}C–hexadecane into microbial biomarkers such as the carboxylic acid fraction. Finally, to characterize the origin of CH_4 *in situ* and *in vitro*, we investigated the isotopic composition ($^{13}C/^{12}C$; D/H) of gases (CH_4 and CO_2) and water at a contaminated aquifer field site. The comparison of *in vitro* and *in situ* results confirmed that the variability in carbon and hydrogen isotope enrichment factors between the CO_2 and CH_4, as well as between H_2O and CH_4, respectively, may be used to monitor biogenic CH_4 formation from crude oil-related compounds *in situ*. The tool

may contribute to a better understanding of methanogenic processes in the field that result in altered oil phases and CH_4 and CO_2 production in petroliferous formations.

5.2. Materials and methods

Reagent grade solvents were obtained from Merck (Darmstadt, Germany). Hexadecane was acquired from Sigma Aldrich; 1-^{13}C– hexadecane and U-^{13}C–hexadecane were synthesized from 1-^{13}C– hexadecanoic and U-^{13}C–hexadecanoic acid, both obtained from Campro Scientific (Berlin, Germany). The hexadecanoic acid samples were: (i) converted to the methyl ester (with Me_2SO_4); (ii) reduced to hexadecanol (with $NaAlH_4$) and (iii) converted to the p-tosylate ester and further reduced to hydrocarbons (with $NaAlH_4$; Zengler et al., 1999). The purity was confirmed using gas chromatography–mass spectrometry (GC–MS).

5.2.1. Experimental setup and growth conditions

For the investigation of microbial growth with labeled and unlabeled hexadecane, the substrate was immobilized onto the surface of Teflon filters (pore size 0.45 µm). The microbial enrichment cultures obtained from Kuhgraben (Bremen, Germany) were cultivated in anoxic mineral medium buffered at pH 7 with HCO_3^- (30 mM) plus CO_2 and reduced with sulfide (1 mM) (Widdel and Bak, 1992). Cultures were incubated at 28°C. Further culture conditions and handling protocols are described elsewhere (Zengler et al., 1999). The unlabeled hexadecane (50 µL, equating to 0.17 mmol) had a carbon isotope signature of -32.4‰. For the experiments with labeled hexadecane, either fully labeled U-^{13}C-hexadecane (100 µL, equating to 0.32 mmol) or 1-^{13}C–hexadecane (50 µL, equating 0.17 mmol) with an isotope composition of 5960 ‰ vs. VPDB (Vienna Pee Dee Belemnite) were applied.

For the two dimensional isotopic analysis of CH_4, several microcosms from different methanogenic environments were set up anoxically using inocula obtained from different methanogenic field sites. Sediment samples for enrichment cultures were obtained from a range of habitats, including freshwater (Lake Plussee, Kuhgraben), marine (Gulf of Mexico, Eckernförde Bay, Mangroves) and terrestrial (Romanian mud volcanoes) environments.

Kuhgraben, a non-contaminated creek in Bremen, Germany, has been described by Zengler et al. (1999). Plussee is a eutrophic lake in northern Germany with relatively stable stratification and significant CH_4 production in the sediment (Eller et al., 2005). Eckernförde Bay is a marine site on the Baltic Sea side of Schleswig–Holstein (Germany) and is characterized by CH_4 production in the sediment (Treude et al., 2005; Whiticar, 2002). Weissandt–Gölzau (Saxony-Anhalt, Germany) represents a mineral oil contaminated aquifer with high rates of microbial CH_4 production within the contamination source zone. The oil phase in the source zone consists mainly of aliphatic hydrocarbons similar to those in diesel fuel, with a low contribution from gasoline-related compounds (benzene, toluene, ethylbenzene and xylenes). Details and acronyms are provided in Table 5.1. The set up and transfer of the enrichment cultures was carried out as described by Zengler et al. (1999). Mineral medium was prepared according to Widdel and Bak (1992) and the salinity was adjusted to the respective *in situ* conditions with NaCl. Hexadecane was added immobilized on Teflon filters (0.5 mL/100 mL medium (Zengler et al., 1999)), methylnaphthalene was dissolved in 5–7 mL of the inert carrier HMN (2,2,4,4,6,8,8-heptamethylnonane, 10 mg methylnaphthalene/ml HMN) as described elsewhere (Musat et al., 2009). Incubation of the enrichments was carried out in the dark at *in situ* temperatures (Table 5.1). CH_4 and CO_2 were analyzed quantitatively in headspace samples using GC-flame ionization detection (GCFID; Kruger et al., 2001). Samples for stable isotope analysis were stored on saturated NaCl. Contaminated groundwater (30 mL) was added to a 50 mL flat glass bottle and sealed gas tight using butyl rubber stoppers and Al screw caps. Hexadecane was added at a final concentration of 0.03 % (v/v). All microcosms were incubated horizontally without shaking. Gas samples from a methanogenic site in Weissandt–Gölzau were taken for the isotopic analysis of methane and CO_2.

Table 5.1: Overview of sampling sites for sediments used in enrichment cultures

Site	Code	Description	Ref.
Lake Plussee	LakeP	Eutrophic lake with stable anoxic hypolimnion. Sample from 28 m depth. In situ temperature ca. 12 °C.	Eller et al. (2005)
Kuhgraben	Kug	Freshwater ditch close to Bremen. Sample from 2 m depth. In situ temperature during sampling 25 °C.	Similar to Zengler et al. (1999)
Eckernförde Bay	E-Bay	Baltic Sea, brackish water, samples from 28 m water depth. In situ temperature during sampling 16 °C.	Treude et al. (2005), Whiticar (2002)
Gulf of Mexico	GoM	Sample from natural gas and oil seeps. Depth 560 m. In situ temperature 8–12 °C.	Joye et al. (2004)
Mangroves	Man	Sample from brackish water Mangroves (Brazil, 2005). Intertidal sediments, in situ temperature during sampling 29 °C	
Romanian mud volcanoes	Rov	Terrestrial mud volcano field with naturally occurring oil seepage. In situ temperature during sampling 25 °C	Alain et al. (2006)
Weissandt-Gölzau	Göl	Gas samples from groundwater aquifer contaminated with crude oil Saxony-Anhalt, Germany. In situ temperature 12 °C.	

5.2.2. Isotope analysis

An isotope ratio mass spectrometry system (Finnigan MAT 253, Thermofinnigan Bremen) was used. The system was coupled to a gas chromatograph (HP 6890 Series, Agilent Technology, USA) either via a combustion device (for carbon analysis; Richnow et al., 2003b) or via a pyrolysis unit (for hydrogen analysis; Fischer et al., 2007). For the GC separation of CH_4 and CO_2, headspace samples (from 50 to 500 µL) were injected into the GC instrument equipped with a CP-Porabond Q column (50 m x 0.32 mm x 0.5 µm, Varian, USA) held at constant temperature of 40°C and flow rate of He (2 mL min^{-1} for carbon and 1.6 mL min^{-1} for hydrogen).

For the isotope analysis of CO_2 in liquid samples, the culture samples were acidified to pH 2 to avoid fractionation via the carbonate system. For carbon isotope measurement, each sample was analyzed at least two times and the statistical standard deviation of the measurements was always better than 0.5 ‰ STD (1σ). For analysis of the hydrogen isotope composition, at least three measurements for each sample were conducted and the STD (1σ) was reported.

The carbon isotope signature of all substrates (unlabeled hexadecane, methylnaphthalene and toluene) were analyzed with an elemental analyzer (Euro EA, HEKAtech GmbH, Wegberg) coupled to an isotope ratio mass spectrometry system (Finnigan MAT 253, Thermofinnigan, Bremen, Germany). The same system was used for hydrogen isotope analysis. In addition, the hydrogen isotope composition of water from the field site (Weissandt–Gölzau), as well as water used for incubation experiments, was analyzed (Gehre et al., 2004). This analysis required the addition of activated carbon to liquid samples in order to adsorb dissolved OM from the water phase.

5.2.3. Analysis of carboxylic acid fraction

The microbial carboxylic acid fraction was isolated and methylated according to Bligh and Dyer (1969) and Morrison and Smith (1964), respectively. The fatty acid methyl esters (FAMEs) were separated on a BPX-5 column (30 m x 0.32 mm x 0.25 µm, SGE, Germany) using a HP 6890 gas chromatograph coupled to a HP 5973 quadrupole mass spectrometer (Hewlett Packard, Wilmington, USA). The temperature programme for GC–MS was: 120°C (held 4 min) to 250°C at 4°C min^{-1}, to 300°C (held10 min) at 20°C min^{-1}. The injector was set to 280°C and 1 µL of sample was injected splitless. The transfer line was held at 250°C and

the He flow was set to 2 mL min^{-1}. The isotopic composition of individual FAMEs was determined using gas chromatography–combustion-isotope ratio mass spectrometry (GC-C-IRMS; Finnigan MAT 253 Thermofinnigan, Bremen, Germany). The isotopic values of the fatty acids (FAs) have been corrected for the methyl groups introduced by methylation. The FAMEs were separated on a BPX-5 column (50 m x 0.32 mm x 0.25 µm, SGE, Germany) using a HP 6890 gas chromatograph with the following temperature programme: 70°C (held 1 min) to 120°C at 20°C min^{-1}, then to 265°C at 2°C min^{-1} and then to 300°C (held 2 min). Samples (2 µL) were injected with a split ratio of 1:1. The injector temperature was 250°C. The He flow rate was 2 mL min^{-1}. Vienna Pee Dee Belemnite [VPDB, $^{13}C/^{12}C$ = (11237.2 ± 2.9) x 10^{-6}] was the standard for calibration of carbon isotope signatures (Coplen et al., 2006) and Vienna Standard Mean Ocean Water [VSMOW, $^{2}H/^{1}H$ = (155.76 ± 0.05) for calibration of hydrogen isotope ratio values.

5.3. Results

5.3.1. Long term incubation with labeled hexadecane

In a long term incubation experiment (ca. 500 days) with enrichment cultures obtained from Kuhgraben sediment, CH_4 and CO_2 formed during the degradation of U-^{13}C-labeled *n*-hexadecane (100 µL corresponding to 0.32 mmol, in 100 mL of 30 mM carbonate buffer of natural isotopic composition) were monitored to elucidate the active methanogenic pathway in the system. During the first 158 days, only low amounts of labeled CH_4 were formed, indicating a lag phase with low CH_4 production. After the initiation of CH_4 production at day 158, up to 2.7 mmol were produced until day 499. Simultaneously, the carbon isotope composition of CH_4 and CO_2 rose to about 66 and 37 atom% ^{13}C, respectively, at day 499, demonstrating the conversion of labeled substrate to CH_4 and CO_2 (Fig. 5.1). The formation of 2.7 mmol CH_4 was consistent with a yield of ca. 70 %, in relation to a stoichiometric transformation of 5.1 mmol of the *n*-hexadecane carbon into a maximum of 3.9 mmol CH_4. A sterile control incubated under the same conditions did not show labeling of CO_2 or CH_4.

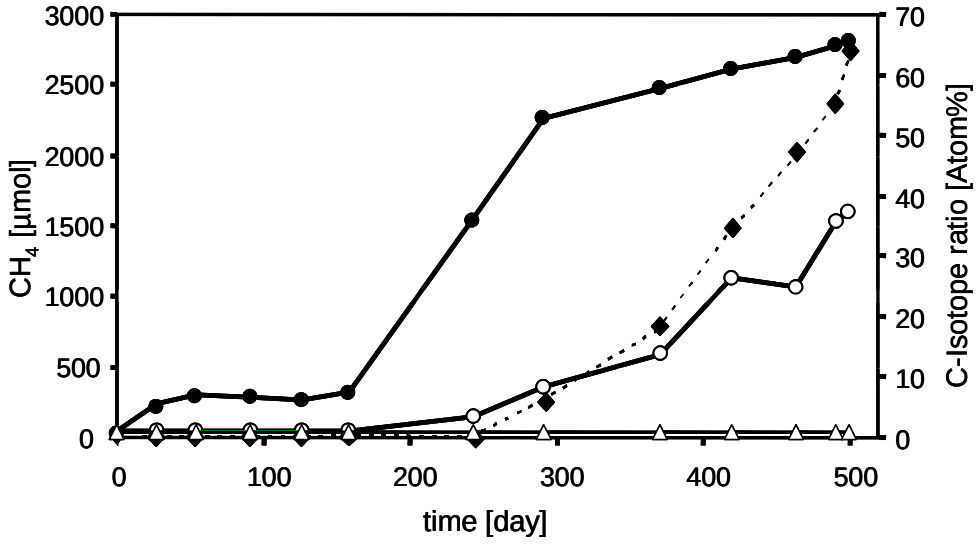


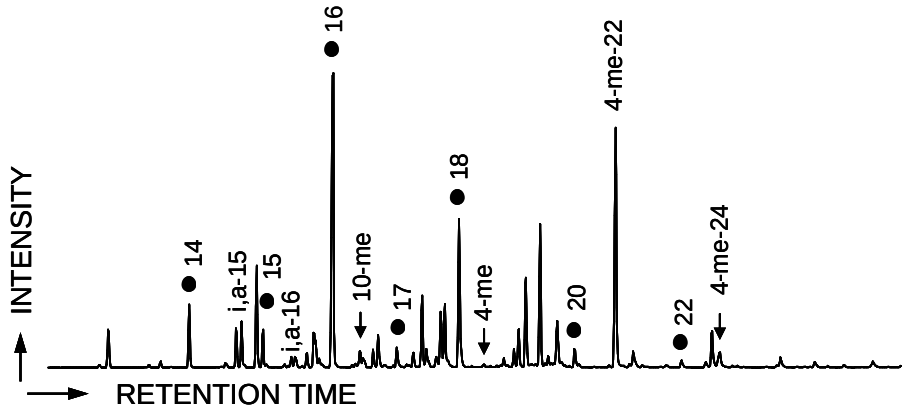
Figure 5.1: Carbon isotope ratio of CH_4 and CO_2 in an oil degrading enrichment culture obtained from Kuhgraben (Bremen, Germany) incubated with ^{13}C labeled *n*-hexadecane (100 atom%). ●, carbon isotope ratio of methane; o, carbon isotope ratio of CO_2; ♦, amount of methane produced; Δ, amount of methane in the sterile control.

5.3.2. Carbon flux in the microbial community

A similar batch experiment with enrichment cultures obtained from Kuhgraben, amended with 1-^{13}C–hexadecane as the sole source for carbon and energy, was conducted to analyze the flux of carbon through the microbial community. The incorporation of an isotopic label into biomarkers such as carboxylic acids might provide evidence of direct usage of the labeled substrate as a carbon source. Such labeled acids might provide information about the structure of the methanogenic microbial community. The same enrichment culture as above was cultivated using 1-^{13}C–hexadecane (5,960 ‰) and unlabeled hexadecane (-32.4 ‰) as carbon substrate and the pattern and isotopic composition of carboxylic acids were analyzed. The isotopic composition of carboxylic acid carbon allowed us to identify three groups of different labeling intensity (Fig. 5.2). The non-specific acids, *n*-C_{16} and *n*-C_{18}, had an isotope value ranging from 50 ‰ to 500 ‰. Values from 2,100 ‰ to 2,700 ‰ combined *n*-C_{15} and *i,a*-C_{15} to a second group. Their isotopic composition indicates that ca. 50 % of the carbon used for the biosynthesis of these FAs was derived from 1-^{13}C–hexadecane. The highest labeling intensities, ranging from 4,700 ‰ to 5,800 ‰, were observed in the *n*-C17 and all 4-methyl FAs. The very extensive label showed that the parent organisms almost exclusively used the 1-^{13}C–hexadecane (5,960 ‰) as a carbon source for the biosynthesis of the *n*-C_{17} and all of the 4-methyl FAs. In contrast to this

labeling experiment, the isotopic values of FAs synthesized with unlabeled hexadecane (-32.4 ‰) ranged from -30 ‰ to -37 ‰.

(A)



(B)

Groups alkanoic acids	Organisms	Labelling experiment (‰ VPDB)	Control (‰ VPDB)
n-C_{16}, *n*-C_{18}	unspecific	50 to 500	-29.8 to -30.3
n-C_{15}, *i,a*-C_{15}	mainly sulfate reducers	2100 to 2700	-32.3 to –34.5
n-C_{17}, 4-methyl-	possibly *Syntrophus* sp.	4700 to 5800	-35.5 to –36.7

Figure 5.2: (A) Carboxylic acid fraction as biomarkers for a methanogenic consortium grown on ^{13}C labeled *n*-hexadecane (5960 ‰ vs. VPDB); ●, *n*-alkanoic acids; 4-me, 4-methyl alkanoic acids; i,a, *iso*- and *anteiso*-alkanoic; 15, etc., carbon numbers. (B) Classification of microbial carboxylic acids according to carbon isotope ratio and biomarker function.

5.3.3. Isotope fractionation during methanogenic hydrocarbon degradation

In order to study the isotopic fractionation during methanogenic growth, the stable carbon isotopic composition of CH_4 and CO_2 was monitored over time in an enrichment culture obtained from Kuhgraben sediments incubated with hexadecane of natural carbon isotope composition (-32.4 ‰) (Fig. 5.3). After a lag phase of ca. 240 days of low CH_4 formation, CH_4 production strongly increased during the following 200 days to more than 1,000 µmol (equivalent to transformation of 36.8 % of the added hexadecane carbon to CH_4). For the remaining 400 days of the experiment, the CH_4 formation rate decreased slightly but resulted in an overall CH_4 production of more than 1,300 µmol after 850 days incubation. This equates to a transformation of 47.8 % of the hexadecane carbon to CH_4. During the lag phase, CH_4 with a carbon isotopic composition of about -40 ‰ was formed. The isotopic signal of CH_4 during the first 50 days was influenced by isotopically light CH_4 (-60 ‰) introduced with the inoculum and the freshly produced CH_4 (-40 ‰). The influence

of the inoculum on the isotope signature of CH_4 disappeared after ca. 80 days. During the main phase of CH_4 formation (days 240–440), the carbon isotopic composition of CH_4 decreased to -65 ‰, while the CO_2 signature increased to -16 ‰, leading to an isotope discrimination of about 33 ‰ and 49 ‰ compared to hexadecane and CO_2, respectively. In the later phase of CH_4 formation, the carbon isotope value of both CH_4 and CO_2 slightly increased, maintaining a discrimination of ca. 54 ‰ at the end of the experiment (850 days). This may be the result of strong isotopic fractionation during CO_2-reduction.

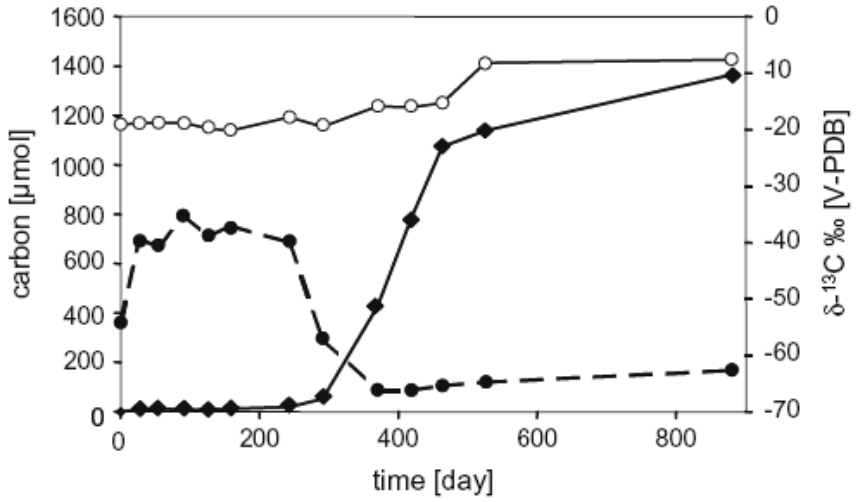


Fig. 5.3: Carbon isotope signature of CH_4 and CO_2 and methane production in an oil degrading enrichment culture obtained from Kuhgraben (Bremen, Germany) incubated with *n*-hexadecane ($\delta^{13}C$, -32.4 ‰ vs. VPDB; ●, carbon isotope ratio of methane; o, carbon isotope ratio of CO_2; ♦, amount of methane produced.

5.3.4. Environmental variability in isotope enrichment factors

In order to examine the variability in carbon and hydrogen isotope enrichment factors for CH_4, we investigated the isotopic signature of CH_4, CO_2 and H_2O during microbial formation of CH_4 from mineral oil-related compounds. The investigation included 41 samples from oil degrading enrichment cultures from diverse methanogenic environments amended with different carbon sources, as well as six gas samples from different monitoring wells within a CH_4-producing, contaminated field site (Table 5.1). Slurry samples from freshwater (Kug, LakeP), marine (Man, GoM, E-Bay) and terrestrial (Rov) sites were used. The carbon and hydrogen isotope values of the biologically produced CH_4 in all enrichment cultures were clustered closely together. The variability ranged from -40 ‰ to -66 ‰ for carbon and

from -317 ‰ to -390 ‰ for hydrogen, respectively (Table 5.2). The corresponding carbon isotope values for CO_2 exhibited a range of -18 ‰ to -1 ‰.

Apart from these ranges, gas samples from a contaminated field site (Göl) ranged from -57 ‰ to -70 ‰for carbon and from -329 ‰ to -373 ‰ for hydrogen, respectively.

In order to calculate the isotope enrichment factors $\varepsilon_{DIC\text{-}CH4}$, $\varepsilon_{substrate\text{-}CH4}$ and $\varepsilon_{H2O\text{-}CH4}$ for carbon and hydrogen, we determined the carbon isotope value for the applied carbonate buffer (-13.4 ± 0.1 ‰ vs. VPDB), hexadecane (-23.2 ± 0.1 ‰ vs. VPDB; -99.5 ± 1.9 ‰ vs. SMOW), toluene (-26.1 ± 0.5 ‰ vs. VPDB; -88 ± 1.0 ‰ vs. SMOW) and methylnaphthalene (-25.3 ± 0.1 ‰ vs. VPDB; -57.5 ± 0.7 ‰ vs. SMOW) as well as the hydrogen isotope value of the H_2O (-59.0 ± 3.3 ‰ vs. SMOW). The calculated enrichment factors ranged from 26 ‰ to 60 ‰ and from 257 ‰ to 336 ‰ for carbon and hydrogen, respectively (Table 5.2). The enrichment factor representing the substrates ranged from 16 ‰ to 33 ‰ for carbon and from 174 ‰ to 318 ‰ for hydrogen.

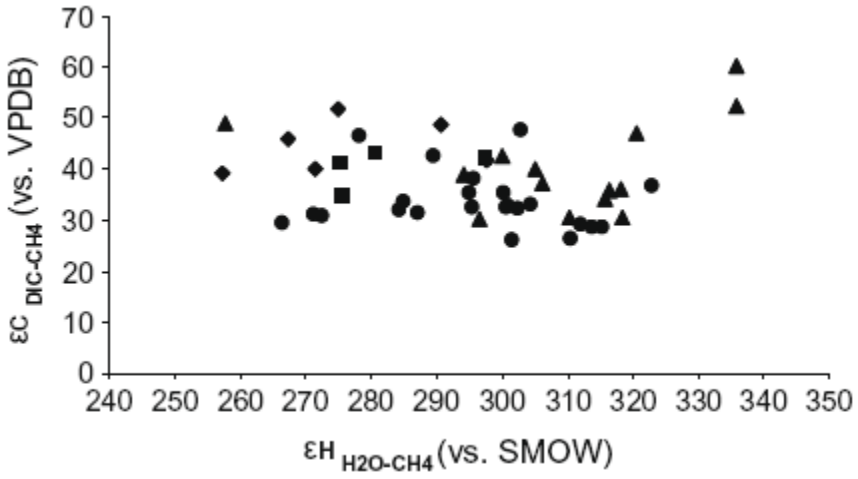


Figure 5.4: Carbon isotope enrichment factor $\varepsilon_{DIC\text{-}CH4}$ and hydrogen isotope enrichment factor $\varepsilon_{H2O\text{-}CH4}$ of 41 oil degrading enrichment cultures from different methanogenic environments and 6 gas samples from a contaminated methanogenic field site (Weissandt-Gölzau, Germany; ●, marine enrichments; ■, terrestrial enrichments; ▲, freshwater enrichments; ♦, gas samples).

Table 5.2: Variability in carbon and hydrogen enrichment factors during methanogenic growth with crude oil (n.d., not detected).[a,b,c]

Code	Substrate	Methane				CO_2		Water		εC DIC-CH_4	εC sub-CH_4	εH H_2O-CH_4	εH sub-CH_4
		$^{13}C/^{12}C$ [‰ vs. V-PDB]		$^{2}H/^{1}H$ [‰ vs. SMOW]		$^{13}C/^{12}C$ [‰ vs. V-PDB]		$^{2}H/^{1}H$ [‰ vs. SMOW]					
Kug1	C16	-53.3	± 0.5	-364.0	± 5.8	-13.4	± 0.1	-59.0	± 3.3	39.9	30.1	305	264
Kug2	C16	-52.3	± 0.5	-353.1	± 17.4	-13.4	± 0.1	-59.0	± 3.3	38.9	29.1	294	254
Kug3	C16	-50.7	± 0.5	-365.1	± 18.2	-13.4	± 0.1	-59.0	± 3.3	37.3	27.5	306	266
Kug4	C16	-49.6	± 0.5	-377.2	± 9.2	-13.4	± 0.1	-59.0	± 3.3	36.2	26.4	318	278
Kug5	C16	-54.3	± 0.5	-316.9	± 3.2	-5.4	± 0.5	-59.2	± 2.6	48.9	21.9	258	174
Kug6	C16	-57.9	± 1.2	-389.7	± 17.3	-5.4	± 0.5	-53.9	± 1.3	52.5	25.5	336	247
Kug7	C16	-65.6	± 0.7	-389.7	± 10.6	-5.4	± 0.5	-53.9	± 1.3	60.2	33.2	336	247
Kug8	toluene	-52.4	± 0.6	-374.5	± 3.0	-5.4	± 0.5	-53.9	± 1.3	47.0	26.3	321	287
LakeP1	C16	-47.2	± 0.1	-365.7	± 11.3	-4.6	± 0.1	-65.7	± 1.1	42.7	24.0	300	266
LakeP2	C16	-44.0	± 0.5	-369.4	± 7.0	-13.4	± 0.1	-59.0	± 3.3	30.6	20.8	310	270
LakeP3	C16	-43.9	± 0.5	-377.4	± 2.7	-13.4	± 0.1	-59.0	± 3.3	30.5	20.7	318	278
LakeP4	C16	-43.6	± 0.5	-355.6	± 3.8	-13.4	± 0.1	-59.0	± 3.3	30.2	20.4	297	256
LakeP5	Me-Naph	-49.3	± 0.5	-375.3	± 6.1	-13.4	± 0.1	-59.0	± 3.3	35.9	24.0	316	318
LakeP6	Me-Naph	-47.5	± 0.5	-374.7	± 7.2	-13.4	± 0.1	-59.0	± 3.3	34.1	22.2	316	317
Göl1	oil contam.	-59.1	± 0.1	-343.1	± 6.5	-19.2	± 0.3	-71.8	± 2.1	39.9	n.d.	271	n.d.
Göl2	oil contam.	-56.9	± 0.1	-328.5	cooling	-17.8	± 0.1	-71.2	± 3.3	39.1	n.d.	257	n.d.
Göl3	oil contam.	-69.8	± 0.1	-359.8	cooling	-21.1	± 0.2	-69.1	± 1.6	48.7	n.d.	291	n.d.
Göl4	oil contam.	-57.1	± 0.0	-373.3	cooling	-15.3	± 0.3	-75.7	± 1.5	41.8	n.d.	298	n.d.
Göl5	oil contam.	-66.3	± 0.2	-340.1	cooling	-20.3	± 0.0	-72.8	± 1.6	46.0	n.d.	267	n.d.
Göl6	oil contam.	-62.9	± 0.0	-346.3	cooling	-11.0	± 0.2	-71.3	± 6.4	51.9	n.d.	275	n.d.
Man01	C16	-42.0	± 0.5	-374.4	± 6.1	-13.4	± 0.1	-59.0	± 3.3	28.6	18.8	315	275
Man02	C16	-42.5	± 0.5	-371.0	± 7.4	-13.4	± 0.1	-59.0	± 3.3	29.1	19.3	312	272
Man03	C16	-48.6	± 0.5	-359.1	± 19.3	-13.4	± 0.1	-59.0	± 3.3	35.2	25.4	300	260
Man04	C16	-44.5	± 0.5	-330.3	± 10.5	-13.4	± 0.1	-59.0	± 3.3	31.1	21.3	271	231
Man05	C16	-50.0	± 0.5	-382.0	± 9.6	-13.4	± 0.1	-59.0	± 3.3	36.6	26.8	323	283
Man06	C16	-45.8	± 0.5	-359.7	± 1.8	-13.4	± 0.1	-59.0	± 3.3	32.4	22.6	301	260
Man07	C16	-42.9	± 0.5	-325.5	± 14.8	-13.4	± 0.1	-59.0	± 3.3	29.5	19.7	267	226
Man08	C16	-48.9	± 0.1	-345.2	± 8.1	-2.4	± 0.4	-67.0	± 0.8	46.5	25.7	278	246
Man09	C16	-48.6	± 0.5	-354.1	± 5.6	-13.4	± 0.1	-59.0	± 3.3	35.2	25.4	295	255
Man10	C16	-44.2	± 0.5	-331.6	± 12.4	-13.4	± 0.1	-59.0	± 3.3	30.8	21.0	273	232
Man11	C16	-46.9	± 0.5	-343.9	± 14.2	-13.4	± 0.1	-59.0	± 3.3	33.5	23.7	285	244
Man12	C16	-39.8	± 0.5	-369.6	± 14.6	-13.4	± 0.1	-59.0	± 3.3	26.4	16.6	311	270
Man13	C16	-46.5	± 0.5	-363.3	± 15.4	-13.4	± 0.1	-59.0	± 3.3	33.1	23.3	304	264
Man14	C16	-39.6	± 0.5	-360.5	± 7.5	-13.4	± 0.1	-59.0	± 3.3	26.2	16.4	302	261
Man15	C16	-45.7	± 0.5	-361.5	± 14.9	-13.4	± 0.1	-59.0	± 3.3	32.3	22.5	302	262
Man16	C16	-42.1	± 0.5	-372.7	± 6.2	-13.4	± 0.1	-59.0	± 3.3	28.7	18.9	314	273
Man17	C16	-45.4	± 0.5	-343.3	± 10.7	-13.4	± 0.1	-59.0	± 3.3	32.0	22.2	284	244
Man18	C16	-48.3	± 0.1	-367.8	± 0.3	-0.8	± 0.2	-65.0	± 1.1	47.5	25.1	303	268
Man19	Me-Naph	-46.0	± 0.5	-354.5	± 6.4	-13.4	± 0.1	-59.0	± 3.3	32.6	20.7	295	297
Man20	Me-Naph	-44.8	± 0.5	-346.1	± 18.6	-13.4	± 0.1	-59.0	± 3.3	31.4	19.5	287	289
Man21	Me-Naph	-46.1	± 0.5	-359.9	± 12.5	-13.4	± 0.1	-59.0	± 3.3	32.7	20.8	301	302
GoM1	C16	-56.1	± 0.5	-348.5	± 2.0	-13.4	± 0.1	-59.0	± 3.3	42.7	32.9	290	249
E-Bay1	C16	-51.5	± 0.5	-354.5	± 11.5	-13.4	± 0.1	-59.0	± 3.3	38.1	28.3	296	255
Rov1	C16	-48.8	± 0.1	-364.6	± 4.3	-6.7	± 0.3	-67.3	± 3.6	42.1	25.6	297	265
Rov2	C16	-49.9	± 0.6	-356.0	± 7.2	-6.8	± 0.3	-75.3	± 0.6	43.1	26.7	281	256
Rov3	C16	-42.2	± 0.1	-355.0	± 5.3	-7.4	± 0.3	-79.5	± 2.2	34.8	19.0	275	255
Rov4	C16	-47.3	± 0.1	-345.9	± 16.7	-6.1	± 0.1	-70.6	± 1.5	41.2	24.1	275	246

[a]The standard deviation of carbon isotope signatures was always better than the analytical accuracy of 0.5 ‰.

[b]The samples for hydrogen analysis were measured at least in triplicate.
[c]C_{16}, hexadecane; Me-Naph, methylnaphthalene; oil contam., crude oil contamination.
[d]Cooling, single injection with cryo focusing at -160°C with liquid N_2.
(**to Table 5.2**)

In order to assess the dependence of enrichment factors on the methanogenic environment from which the samples originated, both carbon and hydrogen enrichment factors were plotted in a box-and-whisker-plot (Fig. 5.5). The overall variability indicated by the whiskers for all habitats together ranged from 26 ‰ to 53 ‰ ($\varepsilon C_{CO2\text{-}CH4}$), 257 ‰ to 336 ‰ ($\varepsilon H_{H2O\text{-}CH4}$), 16 ‰ to 30 ‰ ($\varepsilon C_{C16\text{-}CH4}$) and 228 ‰ to 280 ‰ ($\varepsilon H_{C16\text{-}CH4}$).

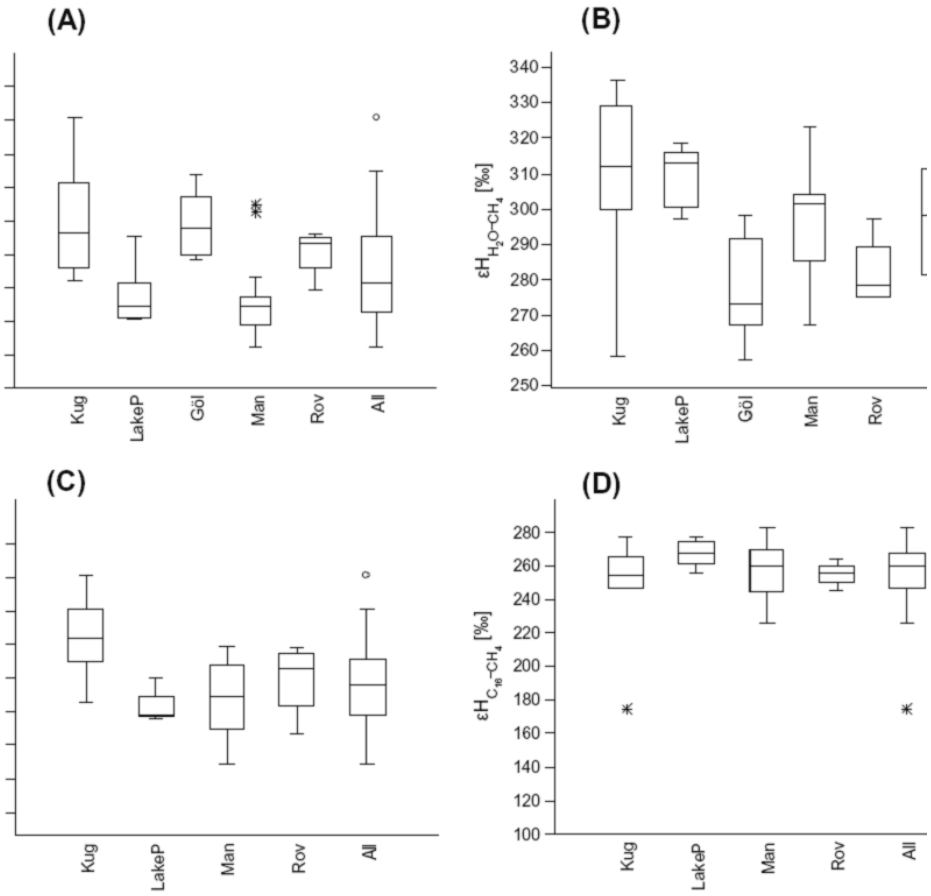


Figure. 5.5: Enrichment factors sorted by methanogenic environments: $\varepsilon C_{O2\text{-}CH4}$ (A), $\varepsilon H_{2O\text{-}CH4}$ (B), $\varepsilon C_{C16\text{-}CH4}$ (C), and $\varepsilon H_{C16\text{-}CH4}$ (D). The 25 and 75 % quartiles are drawn using a box. The median is shown with a horizontal line inside the box. The whiskers are drawn from the top of the box up to the largest data point less than 1.5 times the box height from the box, and similarly below the box. ○, values outside the inner fence; *, values further than 3 times the box height from the box considered as outliers.

5.4. Discussion

We performed a series of experiments designed to investigate CH_4 generation during hydrocarbon degradation. First, we conducted experiments with ^{13}C-labeled hexadecane to elucidate the degradation pathway and to characterize the methanogenic community. In a parallel experiment with unlabeled hexadecane, the carbon isotopic discrimination pattern over time was investigated. In a third set of experiments, we investigated the carbon and hydrogen discrimination pattern in order to characterize the biodegradation of different hydrocarbons in a variety of enrichment cultures from various ecosystems by means of isotopic analysis of CH_4, CO_2, H_2O and the carbon source.

5.4.1. Metabolism of ^{13}C-labeled hexadecane

In the experiment with fully labeled ^{13}C–hexadecane, the ^{13}C label in CH_4 and CO_2 demonstrates the transformation of hexadecane to CH_4 and CO_2 by a consortium consisting of fermenting Bacteria and acetoclastic and CO_2 reducing Archaea, as described previously (Zengler et al., 1999). A stoichiometric calculation performed according to Zengler et al. (1999) would result in a production of 3.9 mmol CH_4, if 0.32 mmol of hexadecane were provided. Thus, a final CH_4 production of 2.7 mmol after 499 days incubation implies a yield of ca. 70 %. The fact that the CH_4 did not show a complete labeling (100 atom%) suggests that a fraction of the CH_4 was formed by the reduction of CO_2. This process lowers the isotope composition of the CH_4 because of the unlabeled carbonate buffer (3 mmol) being used as the main source for CO_2. On one hand, CH_4 formed by CO_2-reduction should therefore exhibit an isotopic signature similar to that of the buffer. On the other hand, the buffer represents a carbon reservoir that becomes enriched in ^{13}C as a result of CO_2 formation during methanogenesis from intermediates, such as acetate, formed during the oxidation of labeled hexadecane.

In order to assess the extent to which $^{13}CO_2$ derived from acetoclastic methanogenesis influences the isotopic label of the carbonate buffer, we assumed that unlabeled $^{12}CO_2$ (0.9 mmol) from the carbonate buffer is used to form unlabeled CH_4 (0.9 mmol) via CO_2-reduction. This reduces the residual carbonate buffer to 2.1 mmol. Based on a 70 % yield, a maximum of 1.8 mmol of labeled $^{13}CO_2$ would be added to the 2.1 mmol of unlabeled carbonate buffer (cf. Eq. 2 in Zengler et al. (1999)). According to the following simple dilution

equation (Eq. (2)), this would result in an isotopic value for the carbonate buffer of 47 atom%:

$$aR_{CO_2} = bR_{cb} + cR_{ac} \quad (5.2)$$

where R_{CO2}, R_{cb} and R_{ac} are the isotope ratio values [atom%] of the measured CO_2, carbonate buffer, and acetate derived CO_2, respectively, and a = b + c, where a, b and c are the amounts [mmol] of the corresponding CO_2 pools.

The difference between the calculated isotope value for the carbonate buffer of 47 atom% and the actually determined isotope value of 37 atom% may be explained by the fact that CO_2 reduction may not exclusively consume unlabeled $^{12}CO_2$ from the carbonate buffer, but may also consume the acetate-derived $^{13}CO_2$, resulting in a ^{13}C-depleted isotopic value for the carbonate buffer in comparison with the theoretical calculated value. Assuming that the $^{12}CO_2$-reduction dominates the reduction of $^{13}CO_2$, the isotopic signature of CH_4 (66 atom%) reflects the stoichiometric calculations of 65 % and 35 % for acetoclastic methanogenesis and CO_2-reduction quite well, respectively. This supports the assumptions of the authors that a cooperative community of fermentative Bacteria and methanogenic Archaea is responsible for CH_4 formation from hexadecane (Zengler et al., 1999).

5.4.2. Microbial consortia involved in hexadecane degradation

In order to characterize the microbial consortia involved in methanogenic degradation of hexadecane, the above enrichment culture was incubated with 1-^{13}C–hexadecane and the biomass analyzed for the pattern and isotopic signature of carboxylic acids. Their isotope composition demonstrates the extent to which the microbial community used the carbon from the 1-^{13}C–hexadecane for the biosynthesis of lipids. Membrane lipids of Archaea consist of isoprenoids ether-linked to glycerol or other carbohydrates rather than ester-bonded carboxylic acids (De Rosa and Gambacorta, 1988; Jones et al., 1987; Langworthy, 1985). These were not detected with the method used. The carboxylic acids could be divided into three different groups on the basis of isotope signatures. The lowest extent of label, 50-500‰, was seen in the *n*-C_{16} and *n*-C_{18} FAs, suggesting that the parent organisms of these taxonomically relatively unspecific biomarkers did not use the amended hexadecane as a major carbon source. These organisms were possibly transferred by the inoculation and may

grow on dead cells or other unlabeled carbon sources. Microbes using detritus from dead cells as a carbon substrate will become labeled in a later phase of the experiment compared to organisms using the 1-^{13}C–hexadecane directly. Since *n*-C_{16} and *n*-C_{18} FAs represent abundant species, despite a relatively low extent of labeling, a significant part of the ^{13}C is bound to this FA fraction. For an estimate of the absolute carbon flux by means of the ^{13}C-label, the concentration and the ^{13}C label in the respective FA have to be considered. The low extent of labeling may therefore be interpreted as a result of relatively low usage of the 1-^{13}C–hexadecane by organisms synthesizing these compounds.

A second group, with more extensive carbon labeling ranging from 2,100 ‰ to 2,700 ‰, consisted of *n*-C_{15} and *i,a*-C_{15} FAs. These FAs may be characteristic for sulfate-reducers like *Desulfovibrio* spp., *Desulfococcus* spp. or *Desulfosarcina* spp. (Kohring et al.,1994). The findings support the results obtained by Zengler et al. (1999). In a similar degradation experiment, the 16S ribosomal RNA gene sequences of the sulfate-reducers affiliated with the delta subclass of Proteobacteria, such as *Desulfovibrio desulfuricans*, *Desulfobacter postgatei*, *Syntrophobacter wolnii* and *Desulfobulbus elongatus*, were detected (Zengler et al., 1999).

The greatest extent of labeling was found in the *n*-C_{17} FA or FAs with methyl groups at C-4 (from 4,700 ‰ to 5,800 ‰). In the investigation by Zengler et al. (1999), some synthrophic bacteria of the genus *Syntrophus* were detected by way of 16S ribosomal RNA gene sequences. In addition, Jones et al. found *Synthrophus* spp. by means of 16S rRNA sequences. These organisms are commonly recovered in methanogenic alkane-degrading systems, whereas controls without oil amendment also lacked detectable levels of *Synthrophus* spp. (Jones et al., 2008). These bacteria likely perform the degradation of hexadecane to acetate and hydrogen. In further steps, the acetate can then be used by acetoclastic archaea for methanogenesis. The extensive incorporation of a ^{13}C-label into the carboxylic acid fraction clearly demonstrates a substantial usage of the labeled hexadecane as carbon source. Methylated branched FAs at C-2, -4 or -6 have been identified as metabolites during growth of sulfate-reducing bacteria with alkanes of different chain length (Grossi et al., 2008; So and Young, 1999; So et al., 2003). However, the highly labeled 4-methyl FAs detected in our investigation had chain length >18 carbons and cannot be considered as intermediates in the degradation of hexadecane. In addition to the cellular FA composition of *Synthrophus aciditrophicus* revealed by Jackson et al. (1999), the highly

labeled 4-methyl and *n*-C_{17} FAs might be suggested as tentative biomarkers for the alkane consuming syntrophic partners in an oil degrading methanogenic consortium, such as *Syntrophus* spp.

5.4.3. Isotope fractionation during methanogenic growth with hexadecane

To further characterize the isotopic fractionation during methanogenic growth with hexadecane, we cultivated an enrichment culture from Kuhgraben with unlabeled hexadecane (-32.4 ‰). The carbon isotope signature for methane of -62 ‰ after 850 days clearly demonstrates that the gas arises from microbial origin (Whiticar, 1999). The isotope value for CO_2 increased in two steps. The first increase (from -18 ‰ to -16 ‰) coincided with the start of CH_4 production after 240 days. The second increase (from -16 ‰to -8 ‰) then signaled the initiation of CO_2-reduction (after 450 days) as a complementary CH_4 production to acetoclastic methanogenesis that predominantly occurred from day 240 to day 450. During the first 240 days, an enrichment factor of 22 ‰ between CO_2 and CH_4 was calculated. The value falls within the range of enrichment factors reported as indicative of acetoclastic methanogenesis (Conrad, 2005; Whiticar et al., 1986; Whiticar, 1999), whereby it is assumed that the isotope fractionation between organic carbon and fermentatively produced acetate (methyl group) is negligible (Blair et al., 1985; Blair and Carter, 1992). With increasing CH_4 production, the influence of CO_2-reduction became more distinct, increasing the enrichment factor to 54 ‰. The discrimination of >40 ‰ between CO_2 and CH_4 may be indicative of CO_2-reduction and supports the thermodynamic assumptions and molecular biological conclusions made by Zengler et al. (1999) that CH_4 was formed by acetoclastic and CO_2 reducing processes simultaneously. These two methanogenic pathways are considered to be associated with two different groups of Archaea. Members of the genus *Methanosaeta* have been found to be involved in the cleavage of acetate to CO_2 and CH_4. The second archaeal genera, *Methanospirillum* and *Methanoculleus*, respectively, reduce the CO_2 to CH_4 (Zengler et al., 1999). The same authors suggested that the acetate for CH_4 generation is provided by bacteria of the *Deltaproteobacterial* genus *Syntrophus* breaking the hydrocarbon chain of hexadecane down to acetate. The carbon enrichment factor e between CO_2 and CH_4 of 54 ‰ lies within a range that suggests that both methanogenic pathways influence the isotopic signal of microbially derived CH_4 in our system. In contrast, a clear dominance of CO_2-reduction in methanogenic enrichment cultures from gas field

formation waters has recently been shown (Gray et al., 2009). The authors reported that methanogenic growth with acetate as a sole carbon source did not occur. The enrichment factor in our enrichment culture between hexadecane and CH_4 resulted in a value of 30 ‰.

5.4.4. Enrichment factors in the environment

These enrichment factors might be influenced not only by the two different major methanogenic pathways, but also by the abiotic conditions in different environments, such as substrate concentration (e.g. Goevert and Conrad, 2009) or energy status (Penning et al., 2005). In order to examine the variability in carbon and hydrogen isotope enrichment factors during microbial formation of CH_4 from crude oil-related compounds, the isotope signatures of CH_4, CO_2, and H_2O were determined in 41 samples from different oil degrading methanogenic environments grown on several substrates (hexadecane, toluene and methylnaphthalene). The two dimensional isotope signature fell in a narrow range for both hydrogen and carbon isotopes (Fig. 5.4). The relative CO_2 isotope signatures were enriched in ^{13}C with regard to the carbon sources (Table 5.2). During aerobic degradation of jet fuel, organic contamination or oil spills in groundwater, CO_2 isotope values close to those of the carbon source have been reported (Aggarwal and Hinchee, 1991; Baedecker et al., 1993; Suchomel et al., 1990; Van de Velde et al., 1995). With decreasing redox potential, CO_2 became enriched in ^{13}C due to CO_2-reduction during methanogenic activity (Baedecker et al., 1993). At sites where methanogenesis occurs, carbon isotope signatures of dissolved inorganic carbon were reported to range from -30 up to +12 ‰ (Conrad et al., 1997; Landmeyer et al., 1996). The model introduced by Jones et al. (2008) predicts a stable carbon isotopic composition for CO_2 of ca. -16 or 21 ‰ for acetoclastic or hydrogenotrophic methanogenesis, respectively. However, the large range of carbon isotope values for CO_2 during methanogenesis lends difficulties to the characterization of the degradation process. In order to distinguish between the two major methanogenic pathways related to freshwater and marine environments, Whiticar et al. (1986) reported carbon and hydrogen signatures for CO_2, CH_4 and H_2O. In freshwater sediments, CH_4 production from acetate is the major methanogenic process, with a contribution of 30–70 % (Koyama, 1955; Takai, 1970; Schutz et al., 1989). In marine sediments, it has been assumed that CH_4 is predominantly produced from CO_2 and H_2 (Whiticar et al., 1986). According to that data set, the isotopic signatures of CH_4 observed in our incubation exhibit a range typical for

acetoclastic methanogenesis in freshwater systems. However, the results from our enrichment culture incubated with fully labeled hexadecane lead to the assumption that the isotopic signature of CH_4 is influenced not only by acetoclastic methanogenesis but also by CO_2 reducing methanogenesis occurring simultaneously. This assumption should be considered for the observed enrichment factor ε for CO_2 and CH_4 ranging between 26 ‰ and 60 ‰ in our incubation. Based on the data set of Whiticar (1999), fractionation factors $\varepsilon C_{DIC\text{-}CH4}$ for acetoclastic methanogenesis and CO_2-reduction can be separated and exhibit a typical range from 40 ‰ to 55 ‰ and from 49 ‰ to 100 ‰, respectively. The range of ε_H between H_2O and methane from 25 7‰ to 336 ‰ is slightly larger than one would expect using regression curves obtained for acetoclastic methanogenesis and CO_2-reduction (e.g. Sugimoto and Wada, 1995), as well as for cultures from landfill material with both methanogenic pathways occurring simultaneously at unknown relative proportions (e.g. Waldron et al., 1988). However, one has to consider a considerable impact of the hydrogen isotope signature for the environmental water and the partial pressure of hydrogen on the stable hydrogen composition of CH_4 (e.g. Burke, 1993). Additionally, one has to take into account that the approximation of the isotopic fractionation factor is feasible for $\Delta\varepsilon$ <100 ‰ (Fry, 2003). Thus, the larger ε for hydrogen found here may imply some uncertainty.

In order to test the influence of different methanogenic communities on e values, we plotted the calculated values in a box whisker plot sorted by locations of the enrichment cultures (Fig. 5.5). In addition to the enrichment factors between CO_2 and CH_4 and between H_2O and CH_4, respectively, this also includes the enrichment factors between hexadecane and CH_4. By comparing the variability in enrichment factors to each other, any influence of the methanogenic environment was apparent. Since the cooperation of fermenting Bacteria and methane producing Archaea was shown by Zengler et al. (1999) and supported by our findings, it can be assumed that similar oil degrading pathways are taking place and result in similar enrichment factors independent of the methanogenic environment.

In conclusion, these e values might be used to characterize hydrocarbon-driven methanogenesis during exploration in oil, coal and gas reservoirs, as well as in shale and contaminated aquifers, and may enable researchers to gain a better understanding of methanogenic microbial processes *in situ*.

The microbial conversion of crude oil to methane in mature oil reservoirs may enable recovery of an additional fraction of the large energy pool left behind after conventional

recovery techniques. It has also been shown recently by Gieg et al. (2008) that heavy crude oils stranded in mature reservoirs can be converted to methane by hydrocarbon-degrading methanogenic consortia. This microbially enhanced oil recovery provides a novel biotechnological perspective for enhancing the recovery of hydrocarbons. The data provided here may contribute to a better characterization of methanogenic processes in oil fields and help to develop further tools for tracing methanogenesis *in situ*.

Acknowledgements

This work is integrated into the research and development program of the Helmholtz Centre for Environmental Research. The Landesanstalt für Altlastenfreistellung (LAF) Sachsen-Anhalt is gratefully acknowledged for financial support of the field work. The study is part of the DFG research unit 580 ‘‘electron transfer processes in anoxic aquifers (etrap)” (FOR 580 Grant Ri903/3-2) supporting S.F. M.S. is supported by DFG Grant no. KR 3311 5-1. Further support came from the SPP 1319 of the DFG (Grants KR 3311 6-1 and RI 903/4-1). Finally, we gratefully acknowledge D. Zoch, C. Haveland and H. Probst for technical assistance, and S. Joye, G. Eller, P. Frenzel and F. Musat for providing sediment samples for enrichment cultures. F. Widdel is acknowledged for providing the enrichment culture from Bremen. Lastly, we thank two anonymous reviewers for helpful comments as well as B. E. L. Morris for English improvements.

Chapter VI

Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron and sulfate-reducing conditions

6. Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron and sulfate-reducing conditions

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FEMS Microbiology Letters 02/2010; 315 (1): 6–16
DOI:10.1111/j.1574-6968.2010.02165.x

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Running title: Methanogenic hydrocarbon degradation

Key words: methanogenic hydrocarbon degradation, MEOR, metal-reduction, bioremediation, *Geobacter*, anaerobic oxidation of methane, *Methanosarcina*, BTEX

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Abstract

The impact of four electron acceptors on hydrocarbon induced methanogenesis was studied. Methanogenesis from residual hydrocarbons may enhance exploitation of oil reservoirs and may improve bioremediation. The conditions to drive the rate-limiting first hydrocarbon-oxidizing steps for conversion of hydrocarbons into methanogenic substrates are crucial. Thus, the electron acceptors ferrihydrite, manganese dioxide, nitrate or sulfate were added to sediment microcosms acquired from two brackish water locations. Hexadecane, ethylbenzene or 1-^{13}C-naphthalene were used as model hydrocarbons. Methane was released most rapidly from incubations amended with ferrihydrite and hexadecane. Ferrihydrite enhanced only hexadecane-dependent methanogensis. The rates of methanogenesis were negatively affected by sulfate and nitrate at concentrations of more than 5 and 1 mM, respectively. Metal-reducing *Geobacteraceae* and potential sulfate-reducers as well as *Methanosarcina* were present *in situ* and *in vitro*. Ferrihydrite addition triggered growth of *Methanosarcina*-related methanogens. Additionally, methane was removed concomitantly by anaerobic methanotrophy. ANME-1 & 2 methyl coenzyme A reductase genes were detected, indicating anaerobic methanotrophy as an accompanying process. The experiments presented here demonstrate the feasibility of enhancing methanogenic alkane degradation by ferrihydrite or sulfate addition in different geological settings.

6.1. Introduction

Roughly, one third of oil in reservoirs remains inaccessible (U.S. Department of Energy, 2006). Since Zengler et al. (1999) reported the conversion of hexadecane to methane, it has been suggested that remaining energy can be recovered as methane gas (Anderson and Lovley, 2000a; Head et al., 2003). Moreover, the conversion of hydrocarbons to carbon dioxide (CO_2) or methane represents a useful tool for bioremediation of oil-impacted ecosystems. The overall reaction kinetics of hydrocarbon biodegradation are controlled by the initial attack on hydrocarbons, where hydrocarbon biodegradation with oxygen as an electron acceptor is the energetically most favorable process. However, microbial methanogenesis usually requires anoxic conditions and methanogenesis, including the conversion of hexadecane to methane, is a slow process (Zengler et al., 1999).

The initial anaerobic activation of hexadecane may be irreversible and a removal of reaction products is unlikely to accelerate the initial steps or the overall degradation (Callaghan et al., 2006; Cravo-Laureau et al., 2005). However, β-oxidation and the release of electrons are essential steps in hydrocarbon biodegradation pathways (Figure 6.1; (Callaghan et al., 2006; Kniemeyer et al., 2003; Rabus, 2005)). It is commonly accepted that the removal of reducing power from the reaction system drives β-oxidation. Examples for this are fermentative hydrogen (H_2)-releasing microorganisms, which require low H_2 partial pressure to effectively unload electrons from the system. One can deduce that electron acceptors are required to accelerate oxidation of hydrocarbons and their intermediate reaction products to transform them into substrates for methanogens, for example acetate, CO_2 and H_2 (Figure 6.1; (Zhang et al., 2010)). For activation and processing biochemical hydrocarbon degradation, the presence of oxidants is not necessary (Zengler et al., 1999). However, it is plausible to indirectly stimulate the activity of the methanogenic community by providing oxidants other than oxygen for the hydrocarbon-degrading microorganisms (Zengler et al., 1999; Zhang et al., 2010).

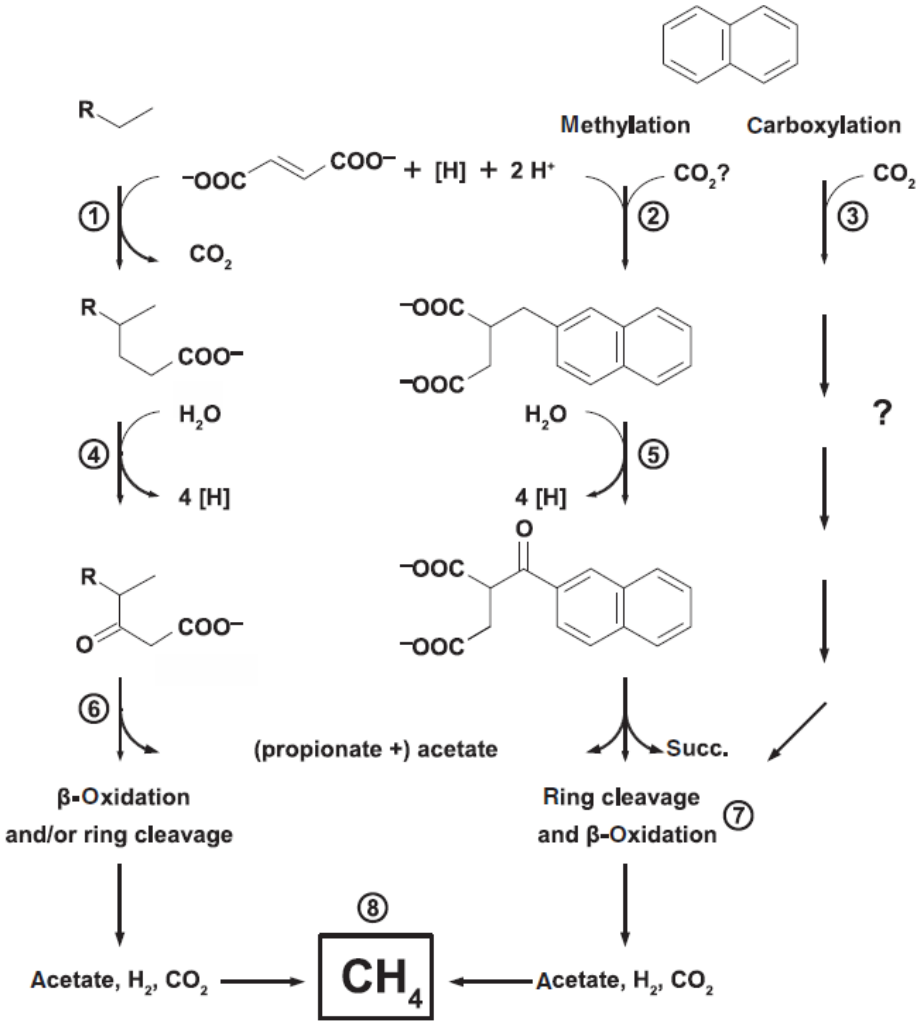

Figure 6.1: Conceptual figure depicting proposed pathways of hydrocarbon degradation. A removal of electrons [H] by adding electron acceptors such as Fe(III), Mn(IV), nitrate or sulfate may accelerate the overall reactions to yield substrates for methanogens. This may accelerate all ß-oxidation reactions, for example at numbers 4-7. R may be an aliphatic or an aromatic residue. Note that besides fumarate addition, hydroxylation was shown for R = phenyl (Kniemeyer and Heider, 2001a). 1, Fumarate addition to the hydrocarbon [e.g. hexadecane (Callaghan et al., 2006; Cravo-Laureau et al., 2005) or ethylbenzene (Kniemeyer et al., 2003)]. 2, Fumarate addition to methylnaphthalene after methylation (Annweiler et al., 2000; Safinowski and Meckenstock, 2005). This may possibly be achieved by CO_2-reduction/acylation in a reversed carbon monoxide dehydrogenase pathway (Safinowski and Meckenstock, 2005). Intermediate succinate adducts and carbon skeleton rearrangements (Callaghan et al., 2006) are not shown because they may be indirectly driven by electron acceptor addition. 3, Carboxylation and further ring reduction (Zhang and Young, 1997). 4 and 5, Proposed ß-oxidation yielding four electrons (Annweiler et al., 2000; Callaghan et al., 2006). 6, ß-oxidation yielding acetate. Propionate would only be released when R = aliphatic. 7, Ring cleavage would precede further ß-oxidation to yield acetate and CO_2 analogous to a proposed ring cleavage of toluene (Boll and Fuchs, 1995). Steps 4-7 and all subsequent ß-oxidations may be accelerated by electron acceptor addition. 8, The substrates acetate and CO_2/H_2 are finally converted to methane by methanogenic *Archaea*. Question marks indicate debated mechanisms. Succ., succinate.

Sulfate-reduction is well described in oil spills and oil field souring, where the latter can result in substantial economic losses (Sunde and Torsvik, 2005). Research on trivalent iron-reduction by hydrocarbon oxidation emerged during the last 20 years (Kunapuli et al., 2007; Lovley, 2000; Rabus, 2005), but was not studied in detail in conjunction with hydrocarbon-induced methanogenesis. Hydrocarbon-associated manganese-reduction has only been described in few reports so far (Greene et al., 1997; Greene et al., 2009; Langenhoff et al., 1997a; Langenhoff et al., 1997b). Alkane biodegradation to methane is well documented and some reports for methanogenesis from aromatics and polyaromatics are available (Chang et al., 2006; Feisthauer et al., 2010; Grbić-Galić and Vogel, 1987; Herrmann et al., 2010; Jones et al., 2008; Kazumi et al., 1997; Townsend et al., 2003; Zengler et al., 1999). However, detailed research on the impact of electron acceptors on hydrocarbon-dependent methanogenesis remains elusive. Our central hypothesis is that electron acceptors can accelerate hydrocarbon-dependent methanogenesis. Thus, we tested their stimulating effect on the rates of hydrocarbon-dependent methanogenesis in different sediments.

6.2. Materials and methods

6.2.1. Site descriptions and sampling

Sediment samples were obtained from two different sites. One sampling site was contaminated by hydrocarbons (Zeebrugge) and the other site was pristine (Eckernförde Bay, Supporting Information, Appendix S1).

The sea port of Zeebrugge (Belgium; NW: 51°19'59 N 3°11'57 E, SE: 51°19'55 N 3°12'12 E, approx. 0.1 km^2) comprised several sediment sections with anoxic conditions and was contaminated with hydrocarbons and heavy metals (Ministerie van de Vlaamse Gemeenschap, 2002). The water depth was 3 m during ebb. A constant freshwater influx was maintained by the irrigation system of Brugge. In September 2008, samples were obtained from three locations within the harbor basin using a manual sediment grabber. Sample bottles were filled completely and closed using butyl rubber stoppers and screw caps. Surface water samples were also collected.

Chemical analyses were performed by SGS, Mol, Belgium. Typical contaminants in the harbor mud originated from protective boat paints and fuel leakages. Besides metals such as nickel, zinc, lead, copper, mercury and chromium, the concentrations of mineral oil ranged from 5 to 400 µg cm^{-3} sediment. Iron, manganese and sulfate were detected in concentrations of up to 85, 0.1 or 2 µmol cm^{-3}, respectively. The pH was between 8.0 and 8.5 and the *in situ* water temperature was 14°C.

6.2.2. Preparation of microcosms

For incubations established from the Zeebrugge samples, filter-sterilized harbor water (using 0.2-µm membrane filters) served as a medium to mimic *in situ* conditions. However, the harbor water naturally contained 2 mM sulfate and sediment microcosms without electron acceptors were therefore impossible to prepare. Basal salts were not added. Dissolved oxygen was removed by nitrogen gassing of 1 L filtered water. All additional manipulations were performed in an anaerobic glove box. To homogenize the sediment sample, a 1/1 mix of sediment and medium was stirred. The slurry was sampled for DNA extraction and 20 mL were used to inoculate 40 mL medium in 120-mL serum bottles. These were sealed with butyl rubber stoppers and aluminum crimp caps. Triplicate microcosms were incubated under a nitrogen headspace at atmospheric pressure at 25°C.

Before inoculation, 2.5 mM ferrihydrite, 1.25 mM manganese dioxide, 1 mM potassium nitrate or 20 mM sodium sulfate were added to the medium. Ferrihydrite was precipitated by neutralization of an $FeCl_3$ solution (Lovley and Phillips, 1986) and manganese dioxide was obtained by oxidation of an $MnCl_2$ solution with $KMnO_4$ (Lovley and Phillips, 1988). To determine indigenous methanogenesis, controls without additional hydrocarbons and electron acceptors were prepared. Controls without hydrocarbons, but with electron acceptors were set up as single incubations.

The final hexadecane or ethylbenzene concentrations were 0.1 % v/v in 60 mL total liquid volume. To test polyaromatic hydrocarbon (PAH) degradation, 1.6 mg 1-^{13}C-naphthalene or ^{12}C-naphthalene were added to 100 mL medium containing 20 mL sediment in 120-mL serum bottles sealed with butyl rubber stoppers and aluminum crimp caps. Manganese dioxide was not used in case of naphthalene. To examine the activity of anaerobic methanotrophs, the headspace of separate microcosms was flushed with a 1/1 methane-nitrogen mix without additional higher hydrocarbons..

6.2.3. Methane and CO_2 measurements

Methane and CO_2 in headspace samples were analyzed using a GC-FID (+ nickel catalyst methanizer, SRI 8610C, SRI Instruments) equipped with a 6-foot Hayesep D column (SRI Instruments) running continuously at 60°C. Methane and CO_2 formation from ^{12}C- and 1-^{13}C-naphthalene was also measured using a Thermo Fisher MAT252 GC-IRMS (Herrmann et al., 2010). The rates were calculated based on the formation of $^{13}CH_4$ measured in the headspace and subtracted from the $\delta^{13}C_{CH4}$ of indigenously produced methane. $\delta^{13}C$ values are expressed as ‰ vs. Vienna Pee Dee Belemnite (VPDB)

The rates in unamended control experiments, hexadecane, ethylbenzene, and methane incubations were calculated for a timeframe of 178 days with an intermediate measurement at day 155. For naphthalene incubations, the rates were calculated in a timeframe of 435 days without an intermediate measurement.

6.2.4. DNA analytical methods

Sediment DNA was extracted using a FastDNA Spin Kit for Soil DNA extraction kit (MP Biomedicals). Genes of interest were quantified using an Applied Biosystems StepOne thermocycler. 16S rRNA gene copy numbers of *Archaea* and *Bacteria* were determined as described previously (Nadkarni et al., 2002; Takai and Horikoshi, 2000). Concentrations of *mcrA* and *dsrA* genes were investigated according to Nunoura et al. (2006) and Schippers and Nerretin (2006), respectively. Members of the *Geobacteraceae* were quantified using the method described by Holmes et al. (2002). Copy numbers are expressed as copies cm^{-3} sediment.

Members of the microbial community in the Zeebrugge sediment were identified by the incorporation of 16S rRNA gene sequence fragments of a clone library into an existing maximum-parsimony tree (version 102) provided by Pruesse et al. (2007). Fragments of 16S rRNA genes were obtained using the modified primer sets Ar109f (5'-ACKGCTCAGTAACACGT) and Ar912r (5'-CTCCCCCGCCAATTCCTTTA) for *Archaea* and 27f (5'-AGAGTTTGATCCTGGCTCAG) and 907r (5'-CCATCAATTCCTTTRAGTTT) for *Bacteria* (Liesack and Dunfield, 2004). Subsequently, cloning was performed using the pGEM-T vector system according to the manufacturer's instructions (Promega). All sequencing was conducted at Seqlab Göttingen (Germany). Sequences were deposited at the GenBank online database under accession numbers HM598465 to HM598629

6.3. Results

6.3.1. Aliphatic hydrocarbon dependent methanogenesis and CO_2 release

Methanogenesis was observed in all Zeebrugge microcosms after 178 days. Without added hydrocarbons, the methanogenesis rates were 2.9, 0.8, 0.6, 0.3 or 0.8 nmol methane cm^{-3} day^{-1} for ferrihydrite, manganese dioxide, nitrate, 2 or 22 mM sulfate-amended microcosms, respectively. The respective CO_2 release rates in these controls ranged from 35.5 nmol CO_2 cm^{-3} day^{-1} for ferrihydrite to 73.8 nmol CO_2 cm^{-3} day^{-1} for nitrate.

In microcosms containing Zeebrugge sediment with hexadecane, a significant increase of methanogenesis was observed compared to control experiments without hexadecane (Figure 6.2a). Moreover, hexadecane-dependent methanogenesis rates were significantly different between microcosms with and without added electron acceptor (Figure 6.2a). Most prominently, ferrihydrite accelerated hexadecane-dependent methanogenesis to 87.3±2.3 nmol methane cm^{-3} day^{-1} compared with 37.8±6.6 nmol methane cm^{-3} day^{-1} in 2 mM sulfate incubations (natural harbor water). The increase of methanogenesis in manganese dioxide incubations to 45.9±1.9 nmol methane cm^{-3} day^{-1} was insignificant compared to 2 mM sulfate incubations (Figure 6.2a). Adding 20 mM sulfate decreased methanogenesis to 2.1±1.1 nmol methane cm^{-3} day^{-1}. Nitrate inhibited methanogenesis completely. However, the addition of hexadecane triggered CO_2 release from the microcosms (Figure 6.2a). The CO_2 release rates ranged from 64.6±5.8 nmol CO_2 cm^{-3} day^{-1} for 2 mM sulfate to 139.6±3.0 nmol CO_2 cm^{-3} day^{-1} for 22 mM sulfate.

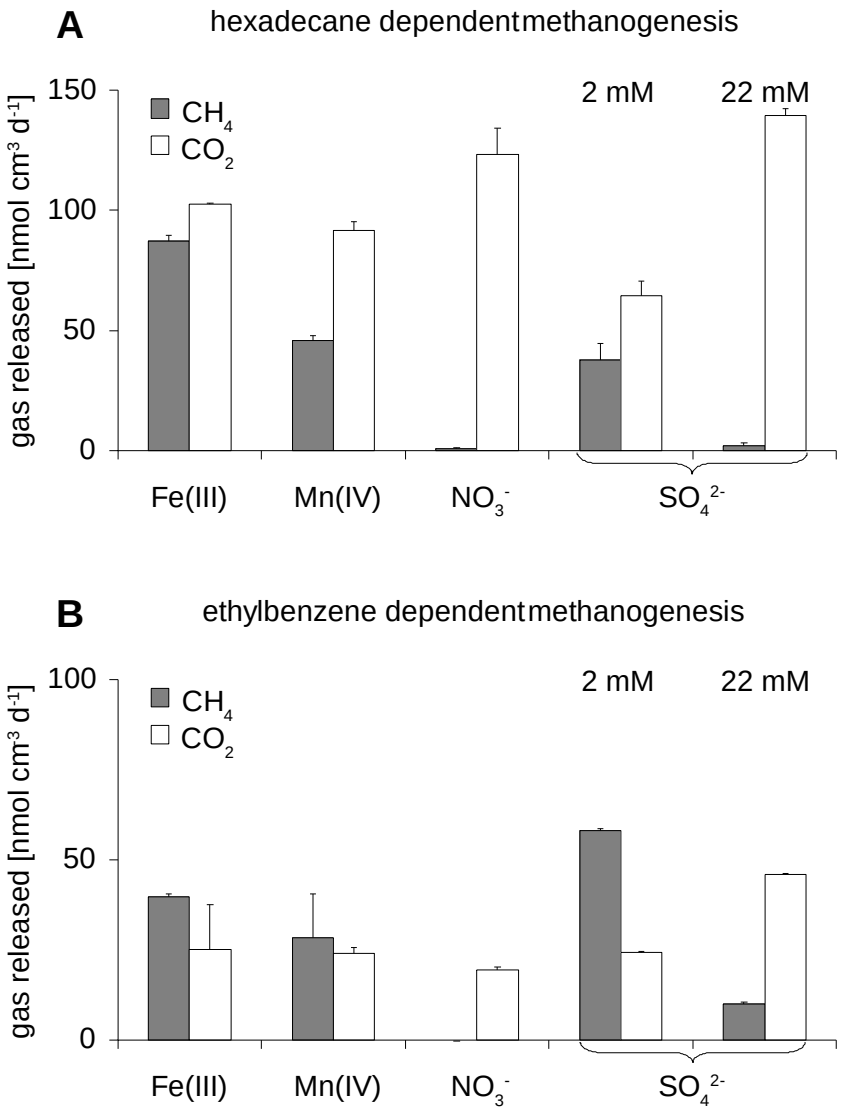

Figure 6.2: Effect of the type of electron acceptor on hydrocarbon-dependent methanogenesis in Zeebrugge sediments. Hexadecane (a) and ethylbenzene (b) were used as substrates. Ninety-five percent confidence intervals of the triplicate regression slopes against time were calculated. SEs within this confidence limit are shown.

The addition of 1 mM nitrate or 10 mM sulfate almost completely inhibited methanogenesis in Eckernförde Bay microcosms (Figure 6.3a). Hexadecane-dependent methanogenesis (46.5±3.5 nmol methane cm^{-3} day^{-1}) was higher than naturally occurring methanogenesis without hexadecane of no more than 10 nmol methane cm^{-3} day^{-1} in the sediment layer of the highest methanogenesis (Figure 6.3a; (Treude et al., 2005)). While hexadecane-dependent methanogenesis occurred without additional electron acceptors at a rate of 24.5±1.7 nmol methane cm^{-3} day^{-1}, the process was significantly slower than in incubations with 2 mM sulfate concentrations 46.5±3.5 nmol methane cm^{-3} day^{-1} (Figure 6.3b).

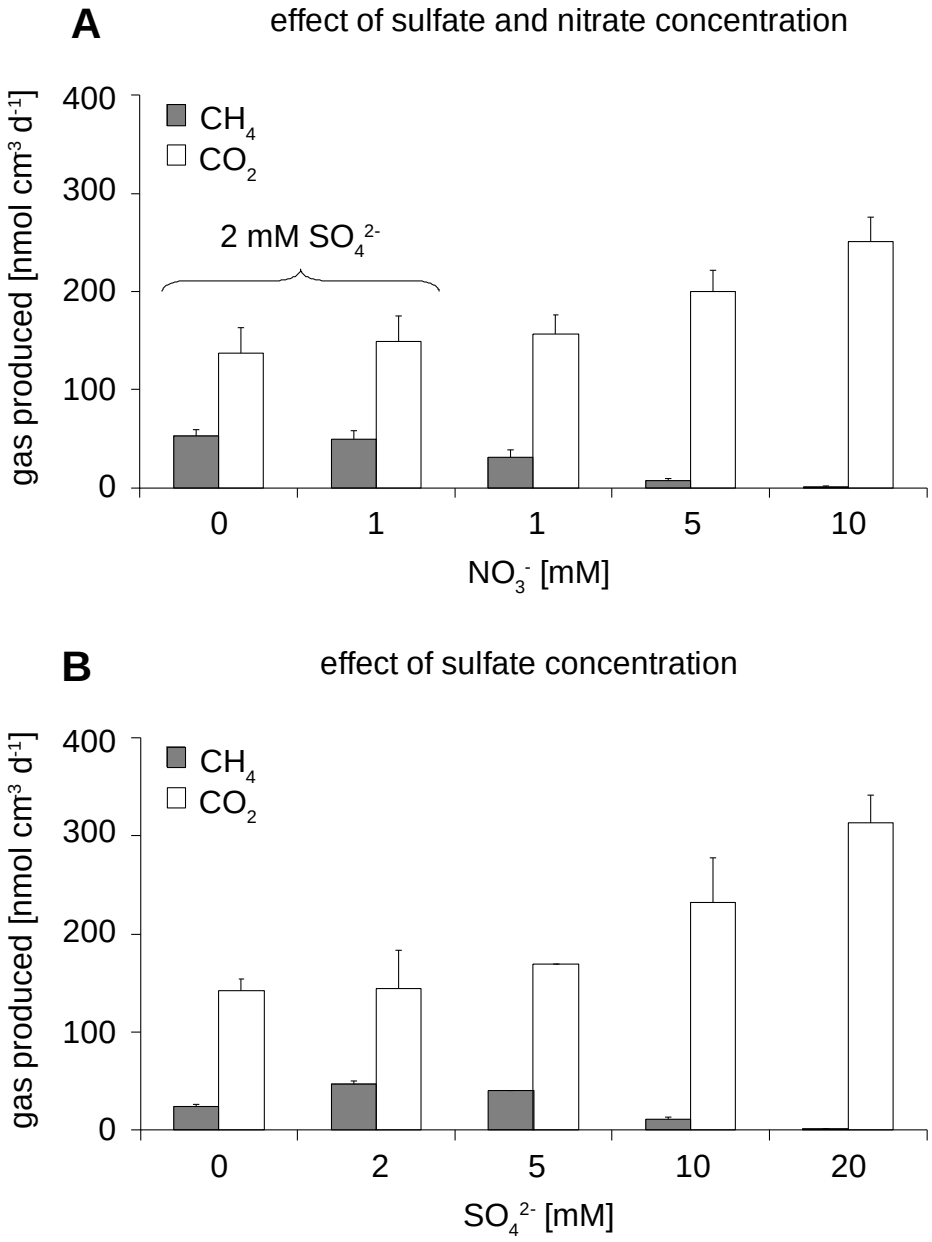

Figure 6.3. Shift from methane production towards CO_2 upon hexadecane addition depending on increased sulfate and/or nitrate concentrations in Eckernförde Bay microcosms. Error bars indicate SEs of three incubations. (a) Nitrate concentrations from 1 to 10 mM are displayed on the x-axis. Additionally, 0 mM and 1 mM nitrate were tested with 2 mM sulfate present (left). All other microcosms were incubated without nitrate. (b) Sulfate concentrations are displayed on the x-axis. Nitrate was not added.

6.3.2. Aromatic hydrocarbon-dependent methanogenesis and CO_2 release

Also, the addition of ethylbenzene significantly increased methanogenesis in microcosms containing Zeebrugge sediment (Figure 6.2b). Compared to 2 mM sulfate, the addition of ferrihydrite or manganese dioxide reduced methanogenesis from 58.1±0.6 to 39.6±0.9 or 28.2±12.1 nmol methane cm^{-3} day^{-1}, respectively (Figure 6.2b). Like in hexadecane incubations, an increase of sulfate concentrations to 22 mM decreased the methanogenesis rate to10.0±0.5 nmol methane cm^{-3} day^{-1}. Nitrate inhibited methanogenesis completely. The addition of ethylbenzene inhibited CO_2 release (Figure 6.2b) compared with unamended controls. The lowest CO_2 production rate was detected with nitrate (19.5±0.6 nmol CO_2 cm^{-3} day^{-1}), while 22 mM sulfate led to an increase in CO_2 release to 45.9±0.3 nmol CO_2 cm^{-3} day^{-1}.

Methanogenesis depending on 1-^{13}C-naphthalene commenced between days 124 and 235 in 2 mM sulfate incubations, with maximum rates of 12.5±0.3 pmol methane cm^{-3} day^{-1} (Table 6.1). At the same time, the $\delta^{13}C_{CH4}$ was -37.1±1.6 ‰ (unamended control: $\delta^{13}C_{CH4}$ = -43.2±1.1 ‰; Figure 6.4d). At day 435, 1-^{13}C-naphthalene-derived $^{13}CH_4$ formation was also detected as indicated by the elevated $\delta^{13}C_{CH4}$ values compared with unamended controls. Methanogenesis rates were, however, within the same order of magnitude in all microcosms (Table 6.1). Furthermore, a strong enrichment in $^{13}CO_2$ was observed already after 42 days of incubation in all setups amended with 1-^{13}C-naphthalene (Figure 6.4e-h). The $\delta^{13}C_{CO2}$ values ranged from +34.9±2.6 ‰ (nitrate addition) to +68.4±23.5 ‰ (iron addition), which was significantly different from the $\delta^{13}C_{CO2}$ values produced in microcosms amended with unlabeled naphthalene (total mean -26.6±0.2 ‰). In the 1-^{13}C-naphthalene-degrading cultures, the $\delta^{13}C_{CO2}$ values further increased to a maximum at day 235 (total mean $\delta^{13}C_{CO2}$ +419 ± 21 ‰; Figure 6.4e-h). The CO_2 release rates were at least 200 times higher than the methane formation rates (Table 6.1). Ferrihydrite addition resulted in relatively low CO_2 formation rates from 1-^{13}C-naphthalene of 236.7±3.4 pmol CO_2 cm^{-3} day^{-1}, while the highest rate was observed with nitrate (499.4±0.54 pmol CO_2 cm^{-3} day^{-1}).

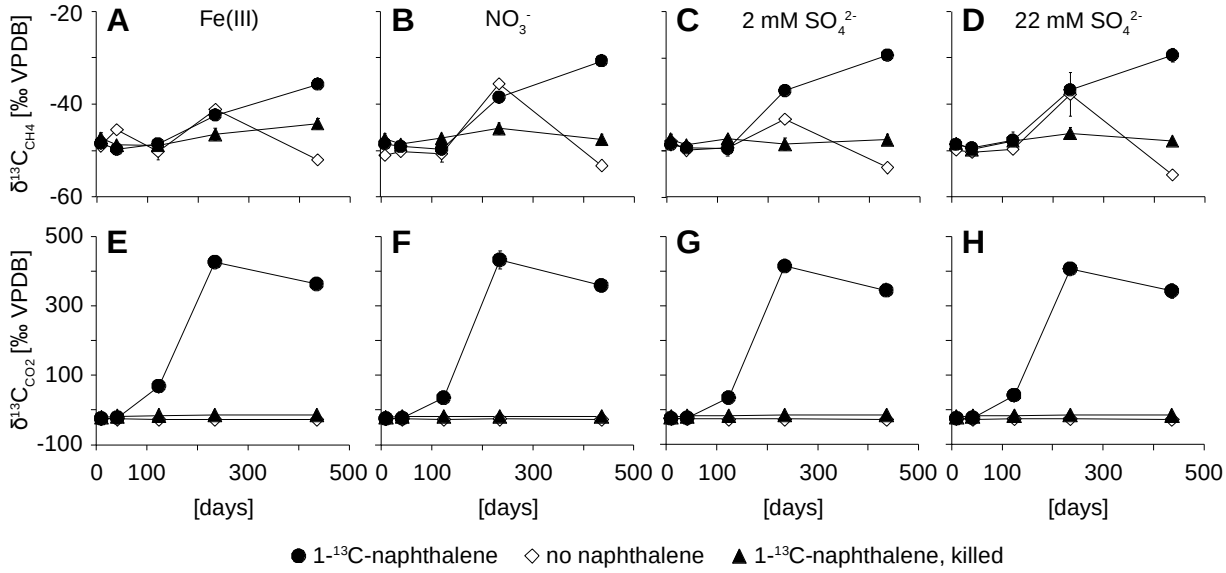

Figure 6.4: Time course of $^{13}CH_4$ (top, a-d) and $^{13}CO_2$ (bottom, e-h) formation upon 1-^{13}C-naphthalene addition to microcosms prepared from contaminated Zeebrugge harbor mud. Error bars are SDs from the mean of three parallel microcosms. Error bars of control experiments (no naphthalene, dead controls) are SDs from the mean of two parallel microcosms. Dead controls were killed with 8 % final concentration formaldehyde.

6.3.3. Anaerobic methanotrophy

In parallel experiments, anaerobic oxidation of methane (AOM) was observed in Zeebrugge microcosms. Incubations with 22 mM sulfate showed the highest AOM rates (1216.0±135.3 nmol methane cm^{-3} day^{-1}), while cultures with ferrihydrite or manganese dioxide displayed slightly lower rates (1117.3±0.2 and 1070.9±37.8 nmol methane cm^{-3} day^{-1}, respectively). The AOM rates were lower with nitrate (881.3±0.7 nmol methane cm^{-3} day^{-1}) or with 2 mM sulfate (479.0±6.4 0.0 nmol methane cm^{-3} day^{-1}).

6.3.4. Hydrocarbon-degrading microbial community

The original Zeebrugge sediment contained 16S rRNA gene copy numbers of 2.6 x 10^9 copies cm^{-3} for *Bacteria* and 3.1 x 10^8 copies cm^{-3} for *Archaea* (Figure S1 in Appendix S1). Compared to the sediment used as inoculum, a significant increase of the methanogenic (*Methanosarcina mcrA*) and the methanotrophic (ANME-1 and -2 *mcrA*) populations was observed in microcosms with ferrihydrite and hexadecane (Figure 6.5). With sulfate and methane, only the number of ANME-2 copies increased. The growth of *Geobacteraceae* – although present in significant numbers – was not initiated by addition of hexadecane or electron acceptors compared to the inoculum (Figure 6.5). In contrast, the

addition of sulfate and/or ferrihydrite increased the growth of the sulfate-reducing community in the microcosms. Enrichments with ethylbenzene, naphthalene, nitrate or manganese were not monitored by real-time PCR.

Table 6.1. Change of the $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$ values during 435 days of incubation with 1-^{13}C-naphthalene, ^{12}C-naphthalene or without naphthalene. Errors are SDs from the mean of samples within 95 % confidence intervals. Methane formation rates (MFR, top) and CO_2 formation rates (CFR, bottom) were calculated based on the difference between the isotopic ratios of day 0 and day 435 related to the total amount of methane in the headspace measured by GC-FID. Of the two $\delta^{13}C_{CH4}$ errors (day 0 and 435), the greater error was selected for the calculation of rate errors. NA, not available.

	$\delta^{13}C_{CH4}$ [‰ VPDB]								
	day 0		day 435					MFR	
electron	^{13}C1-naphthalene		^{13}C1-naphthalene		^{12}C-naphthalene		without	[pmol cm^{-3} d^{-1}]	
acceptor	mean	error	mean	error	mean	error	naphthalene	mean	error
ferrihydrite	-48.4	±1.5	-35.5	±0.1	-52.4	±2.1	-52.1	8.3	±1.0
nitrate	-48.6	±0.4	-30.7	±0.4	-50.1	±1.0	-53.5	11.8	±0.3
2 mM sulfate	-48.7	±0.5	-30.0	±0.1	-58.5	±4.0	-53.6	12.5	±0.3
22 mM sulfate	-48.7	±0.1	-30.5	±0.4	-50.2	n/a	-55.3	12.4	±0.3
	$\delta^{13}C_{CO2}$ [‰ VPDB]							CFR	
ferrihydrite	-24.8	±0.3	374.5	±5.8	-30.3	±0.0	-27.7	236.7	±3.4
nitrate	-24.8	±0.1	363.4	n/a	-28.9	±0.1	-26.6	499.4	±0.5
2 mM sulfate	-24.5	±0.2	336.9	±3.6	-29.4	±0.3	-27.2	285.0	±2.9
22 mM sulfate	-24.3	±0.5	317.3	n/a	-28.7	±0.0	-27.8	338.6	±0.1

16S rRNA gene clone libraries of *Bacteria* (*n*=82) and *Archaea* (*n*=93) of the Zeebrugge sediment revealed a broad microbial diversity (Figures S2-S4 in Appendix S1). Among *Bacteria*, *Alpha-*, *Gamma-* and *Deltaproteobacteria* 16S rRNA gene sequences were recovered as well as sequences associated with *Campylobacterales*, *Planctomycetes*, *Clostridia*, *Actinobacteria* and *Chloroflexi*. 16S rRNA gene sequences associated with potential pathogens, such as *Neisseria* and *Coxiella*, were also found as well as sequences associated with *Geobacteraceae*. Seven potential aerobic iron oxidizers of the family *Acidithiobacillaceae* and another seven of the *Acidimicrobinea* could be identified. Some sequences were closely related to sequences recovered in other potentially hydrocarbon influenced environments such as the Victoria Harbour in Hong Kong, China (Zhang et al., 2008), the Belgian coast off Zeebrugge (Gillan and Pernet, 2007), the Milano mud volcano (Heijs et al., 2005) as well as the Gullfaks and Tommeliten oil fields of the North Sea (Wegener et al., 2008c) (Figure S2 in Appendix S1). The phylogenetic diversity of *Archaea*

comprised *Crenarchaeota* and *Euryarchaeota*. In the latter, members of the *Methanosarcina* prevailed.

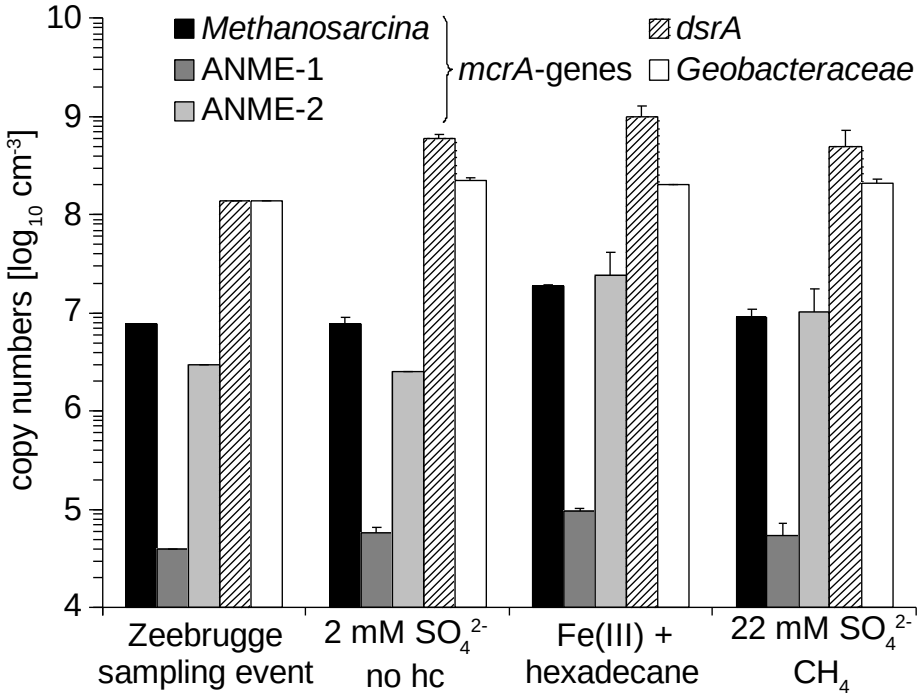

Figure 6.5: Logarithmic plots of community composition in microcosms of a contaminated harbor mud of Zeebrugge. DNA was extracted from the sediment microcosms after 178 days of incubation with 2 mM sulfate without any additional hydrocarbon (hc), ferrihydrite and hexadecane or methane and 20 mM sulfate. ANME-1, ANME-2 and *Methanosarcina*-specific *mcrA* genes were quantified. Sulfate-reducers were detected targeting their *dsrA* gene and *Geobacteraceae* were quantified by amplification of their 16S rRNA genes. When given, error bars were calculated from SD of the mean of two extracted incubations, each determined in three parallel PCR reactions.

6.4. Discussion

Electron acceptors may accelerate hydrocarbon degradation, thus providing an increased substrate supply for methanogenesis. In this work, we evaluate the hypothesis that the addition of electron acceptors leads to accelerated hydrocarbon-dependent methanogenesis. This process may be useful to stimulate the recovery of oil-related carbon as methane from reservoirs or for bioremediation of contaminated sites. Our aim was to stimulate the initial steps in hydrocarbon degradation and thus the formation of methanogenic substrates such as acetate, CO_2 and H_2. Four different electron acceptors were added to sediment microcosms. Two different ecosystems – contaminated harbor mud and pristine marine sediment – were investigated to show that this approach is generally applicable.

6.4.1. Hydrocarbon-dependent methanogenesis

Methane evolved upon hexadecane, ethylbenzene or naphthalene addition in different sediment microcosms (Figure 6.2 and Table 6.1). In most cases, conversion of hexadecane to methane was faster compared with aromatic hydrocarbons (Figure 6.2, Table 6.1). Exceptions were ethylbenzene microcosms with 2 mM sulfate, in which conversion to methane was faster (58.1±0.6 nmol methane cm^{-3} day^{-1}) than that in the respective hexadecane incubation (37.8±6.6 nmol methane cm^{-3} day^{-1}). The observed rates were approximately one order of magnitude lower than those observed in a study of an inoculated oil field sediment core (Gieg et al., 2008). Apparently, inoculation using an enriched consortium was more efficient that the stimulation of indigenous hydrocarbon degraders. In another study of a sediment-free methanogenic hexadecane-degrading enrichment culture, hexadecane-dependent methanogenesis was lower (13 nmol methane mL^{-1} day^{-1}) than the rates observed in our experiments (Feisthauer et al., 2010). Presumably, a sediment-free enrichment culture never reaches cell densities of sediments (approximately 10^9 cells cm^{-3} sediment, Figure S2 in Appendix S1), resulting in lower volume-related rates.

Methanogenesis from naphthalene was in a picomolar range while other hydrocarbons induced methane release in nanomolar ranges (Figure 6.2 and Table 6.1). The time lag between $^{13}CO_2$ and $^{13}CH_4$ evolution as well as the significant difference in $\delta^{13}C$-signature shifts (Figure 6.4) indicate that methanogenesis played a minor role in

naphthalene-degrading microcosms. Primarily, naphthalene seems to have been mineralized to CO_2. Anaerobic oxidation of naphthalene and subsequent formation of CO_2 was demonstrated under nitrate- (Bregnard 1996) and sulfate-reducing conditions (Coates et al., 1996a; Hayes et al., 1999; Langenhoff et al., 1989; Musat et al., 2009). Nevertheless, methanogenesis occurred in our naphthalene-degrading microcosms, a process that was suggested (Chang et al., 2006; Sharak Genthner et al., 1997), but hitherto never confirmed.

Sharak Genthner et al. (1997) observed an inhibition of methanogenesis after naphthalene addition and concluded that naphthalene may be toxic to methanogens. In our microcosms, this seems unlikely because they were naturally exposed to various mineral oil compounds found in the sediments (Ministerie van de Vlaamse Gemeenschap, 2002). Regardless of naphthalene toxicity, methanogens possibly had better access to degradation products of hexadecane and ethylbenzene than not those of naphthalene. We therefore postulate that methanogens themselves were directly involved in the degradation chain of hexadecane and ethylbenzene degradation, but not of naphthalene degradation. The observed increase in the methanogenic population and the finding of a rich methanogenic community in 16S rRNA gene clone libraries support this assumption (Figure 6.5; Figure S4 in Appendix S1).

6.4.2. Impact of electron acceptors on hydrocarbon-dependent methanogenesis

We studied the impact of ferrihydrite, manganese dioxide, nitrate and sulfate on hydrocarbon-dependent methanogenesis. Ferrihydrite accelerated hexadecane-dependent methanogenesis compared to sulfate or nitrate. Nitrate almost completely inhibited methanogenesis from hexadecane and ethylbenzene (Figure 6.2 and Figure 6.3a). This is not surprising because nitrate is a well-known inhibitor of methanogenesis (Klüber and Conrad, 1998). Furthermore, nitrate and high sulfate concentrations negatively influenced conversion rates of hexadecane to methane (Figure 6.2 and Figure 6.3a). However, in the presence of 2 mM sulfate, nitrate was not inhibitory (Figure 6.3a), indicating that a sulfate-reducing hexadecane-degrading community prevailed.

Adding sulfate in concentrations up than 5 mM to the sediment microcosms of Eckernförde Bay resulted in a significant increase of hexadecane-dependent methanogenesis (Figure 6.3b). In contrast, concentrations higher than 5 mM strongly inhibited hexadecane-dependent methanogenesis. Possibly, sulfate addition stimulated the growth of

new or other sulfate-reducers, dominating substrate competition for intermediates with methanogens. In contrast, a previous study reported no inhibition of methanogenesis by sulfate of up to 10 mM (Gieg et al., 2008). The inhibitory effect of 22 mM sulfate on ethylbenzene-dependent methanogenesis was less pronounced compared to hexadecane. For naphthalene, neither inhibition nor stimulation of methanogenesis was found with either electron acceptor (Figure 6.4 and Table 6.1). This agrees with a recent study of contaminated sediments, where no stimulating effect of Fe(III) on PAH degradation was observed (Li et al., 2010).

The impact of electron acceptors on hydrocarbon-dependent methanogenesis demonstrates that (1) the concentration of the added electron acceptor is crucial for hexadecane-fed methanogenesis and (2) the solubility of the electron acceptor appears to be important. Indeed, insoluble electron acceptors such as ferrihydrite or manganese dioxide had a stimulating effect on hexadecane-dependent methanogenesis (Figure 6.2a). However, these electron acceptors are only locally bioavailable, which may result in microscale compartment formation. In contrast, theoretically possible products of hexadecane degradation, such as carbonate, acetate and H_2, can freely diffuse and become available for methanogens in niches where other electron acceptors are depleted.

In Zeebrugge microcosms, the observed increase of the total archaeal community and *mcrA* gene copies suggests that *Methanosarcina* species account for iron-reduction as demonstrated by (van Bodegom et al., 2004) (Figure 6.5 and Supporting Information). Moreover, neither ferrihydrite or sulfate nor hexadecane or methane addition triggered the growth of *Geobacteraceae*. In conclusion, members of this family are probably less important for the respective processes (Figure 6.5). This is not surprising because *Geobacteraceae* are known for their aromatic metabolism, while alkane degradation has not been reported. Instead, other members of the *Proteobacteria,* known for hosting many known hydrocarbon degraders (Widdel and Rabus, 2001), were identified (Figure S2 in Appendix S1). One sequence was closely related to a clone identified at the Gullfaks and Tommeliten oil field methane seeps of the North Sea (Wegener et al., 2008a).

6.4.3. Methanogenesis vs. AOM

AOM rates were determined to assess potential methane losses during incubation time. The rates were in good agreement with those observed typically in methane-fed

environments (Knittel and Boetius, 2009). However, methane seepage was apparently not the major energy source of Zeebrugge sediments. Therefore, *in situ* AOM possibly depended on hydrocarbon-derived methane, as indicated by the growth of the AOM community in hexadecane-amended microcosms (Figure 6.5). Based on the methane partial pressure-dependent and cell-specific AOM rate constant reported by Thauer and Shima (2008), we calculated a loss of no more than 12 % of the produced methane in hydrocarbon-amended microcosms.

6.5. Conclusions and possible practical implications

To fully exploit exhausted oil reservoirs, the conversion of residual oil to methane seems to be a viable technique to recover energy that would otherwise be lost. As a possible contribution for this application, our experiments demonstrated that additional sulfate or trivalent iron accelerated methanogenesis in aliphatic and aromatic hydrocarbon (e.g. BTEX) -degrading communities. In contrast, the inhibitory effect on nitrate, commonly used to suppress sulfate-reducers in oil fields, most likely prohibits its application for oil recovery as methane. Additionally, we present convincing evidence for the conversion of a PAH to methane.

Consequently, our results also provide novel insights for bioremediation, where the conversion of hydrocarbon contaminants to volatile methane seems to be an option. Nevertheless, methane is a much more potent greenhouse gas than CO_2. Therefore, the addition of high amounts of nitrate or sulfate may be preferred to stimulate biodegradation when methanogenesis is unwanted and oxygen treatment is impossible.

Acknowledgements

Funding was partially provided by the Deutsche Forschungsgemeinschaft (grants KR 3311/5-1 & 6-1), the Bundesministerium für Bildung und Forschung (grant 03G0189A), the Landesanstalt für Altlastenfreistellung Magdeburg and the Flemish Environmental and Technology Innovation Platform (MIP, project "*In situ* conditioning of dredged and mineral sludge"). We thank Dr. Axel Schippers for fruitful discussions and improving the manuscript.

6.6. Appendix S1

Eckernförde Bay:

Samples of Eckernförde Bay were taken to demonstrate that hydrocarbon dependent methanogensis accelerated by electron acceptor addition is not restricted to the Zeebrugge site.

Materials and methods:

Site description

The Eckernförde Bay is located at the German coast of the western Baltic Sea. Its pristine sediment was methane rich and was sampled in autumn 2001 (Treude*, et al.*, 2005). This site and sampling procedures were described in detail by Treude *et al.* (2005). Briefly, this marine site was characterised by the absence of tides, a stratified water column and a resulting thermocline and halocline. Sediment samples were derived from the sediment surface at 28 m water depth. Sulfate concentrations ranged from 16 mM to 21 mM at the sediment-water interface.

Sampling

After sampling in September 2001, sediment microcosms of Eckernförde Bay were maintained by several transfers in sulfate containing artificial sea water medium amended with hexadecane as described previously (Feisthauer et al., 2010; Treude et al., 2005; Widdel and Bak, 1992). 1/10 transfers were prepared after methane evolved from the previous incubation. To study the effect of nitrate and sulfate concentrations on hexadecane dependent methanogenesis, nitrate concentrations ranged from 0-10 mM KNO_3 and sulfate concentrations from 0-20 mM $MgSO_4$. In case of 1 mM nitrate, the stimulating effect of sulfate was compared to microcosms without sulfate by adding 2 mM $MgSO_4$. All Eckernförde Bay microcosms were incubated for 70 days at room temperature.

Gas chromatography

Methane and carbon dioxide in Eckernförde Bay microcosms were determined in weekly intervals. Methane and CO_2 were detected by GC-FID (+ nickel catalyst methanizer) headspace measurements (SRI 8610C, SRI Instruments, USA) equipped with a 6 foot Hayesep D column (SRI Instruments, USA running continuously at 60°C.

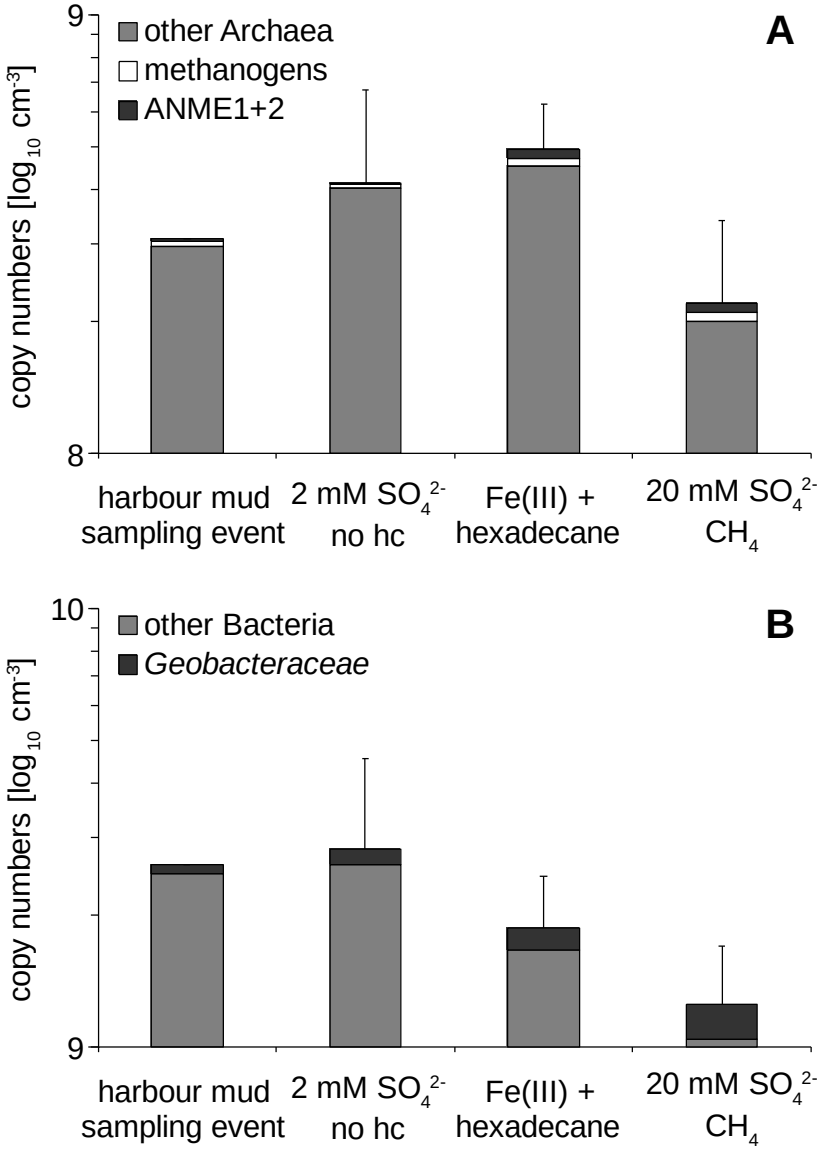

Figure S1: Archaeal (**A**) and bacterial (**B**) 16S rDNA copy numbers of Zeebrugge harbour mud *in situ* (sampling event) and after incubation of 178 days. Error bars represent the error of two parallel incubations. Each incubation was determined in three parallel PCR reactions. Only error bars of the domains (*Archaea*, *Bacteria*) are displayed.

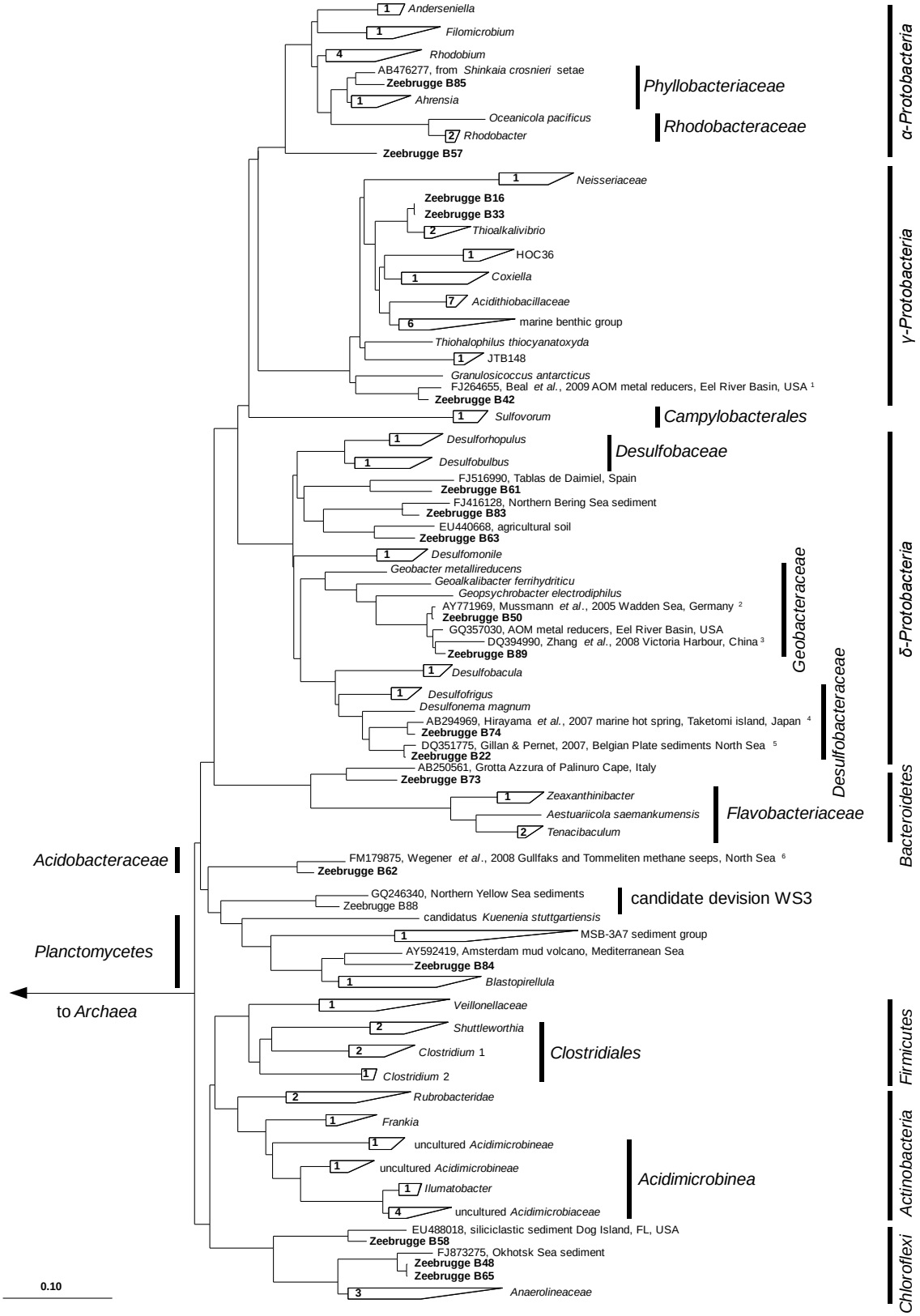

Figure S2: Parsimony tree assembled for *Bacteria* from a 16S rDNA clone library (n=87) of Zeebrugge harbour mud according to Pruesse *et al.* (2007). Duplicate sequences of 100 % identity were removed before constructing the final tree. The SILVA database version 102 was used. Numbers in boxes indicate the number of clones which could be affiliated to the respective group. Numbers in clusters indicate numbers of Zeebrugge 16S rRNA gene sequences which were assigned to the respective cluster. References are [1](Beal*, et al.*, 2009), [2]*(Mußmann et al., 2005)*, [3](Zhang*, et al.*, 2008), [4](Hirayama et al., 2007), [5](Gillan & Pernet, 2007) and [6](Wegener et al., 2008b).

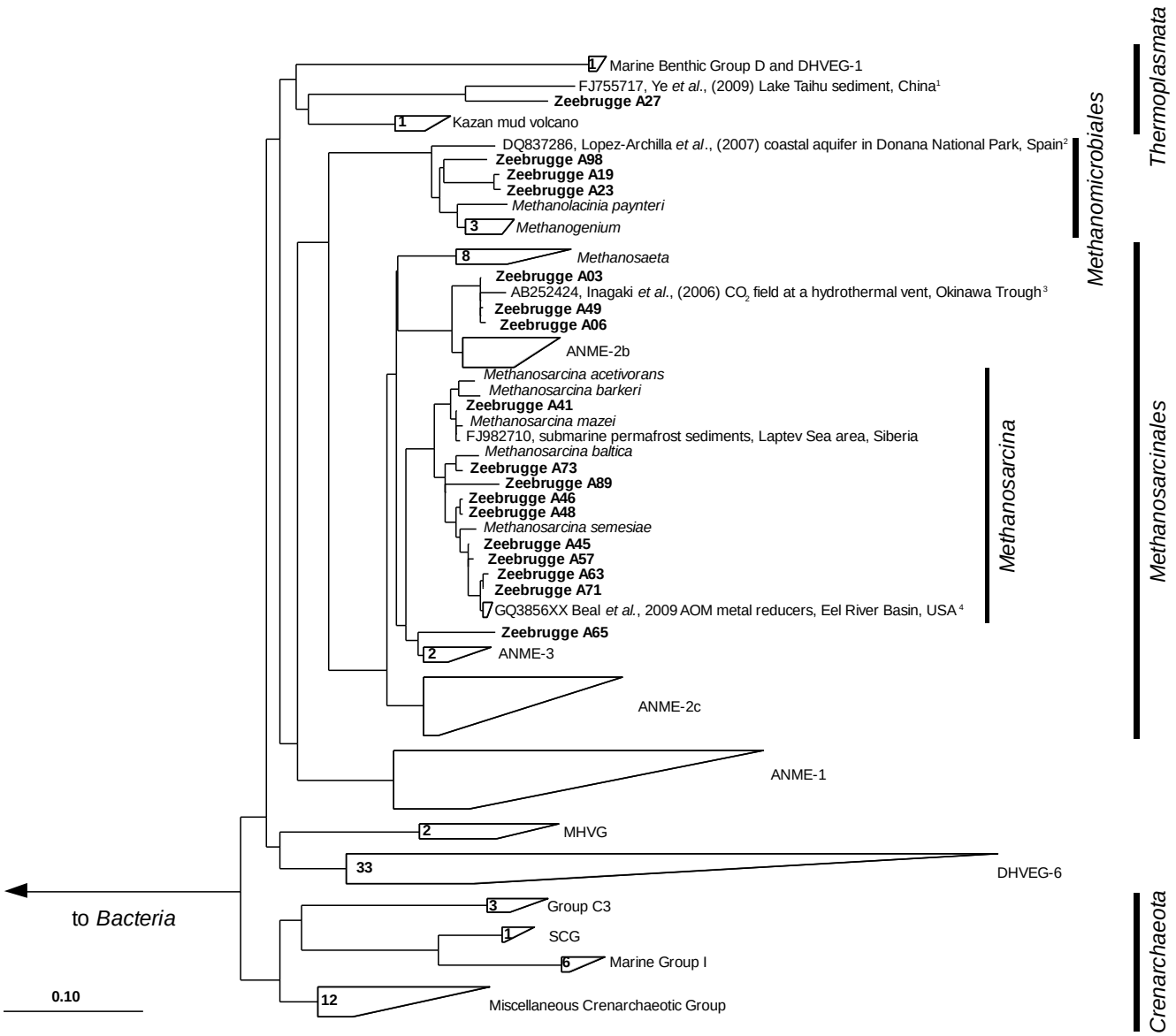

Figure S3: Parsimony tree assembled for *Archaea* from a 16S rDNA clone library (n=98) of Zeebrugge harbour mud according to (Pruesse et al., 2007). Duplicate sequences of 100 % identity were removed before constructing the final tree. The SILVA database version 102 was used. Numbers in boxes indicate the number of clones which could be affiliated to the respective group. Numbers in clusters indicate numbers of Zeebrugge 16S rRNA gene sequences which were assigned to the respective cluster. References are [1](Ye et al., 2009), [2](LOPEZ-ARCHILLA et al., 2007), [3](Inagaki et al., 2006a) and [4](Beal et al., 2009)

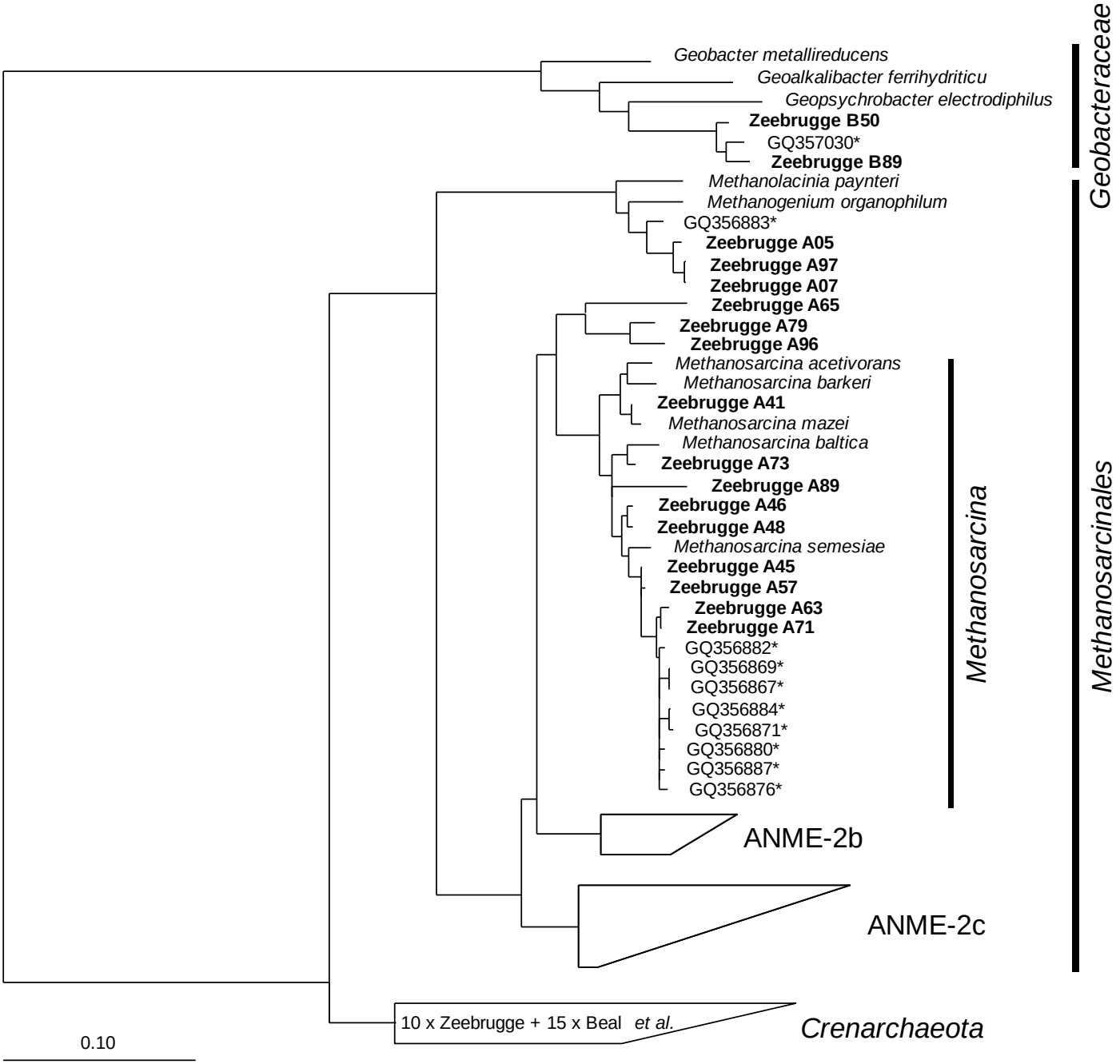

Figure S4: 16S rRNA gene Maximum Likelyhood tree excerpted from the SILVA database 102. The excerpt contains sequences of a proposed metal reducing AOM community sampled in the Eel River basin (Beal*, et al.*, 2009). OTUs of at least 80 % distance matrix identity with clones obtained from Zeebrugge sediment are displayed in the tree. Reference species are *italic*, Zeebrugge clones **bold** and Eel River clones are asterisk (*) marked.

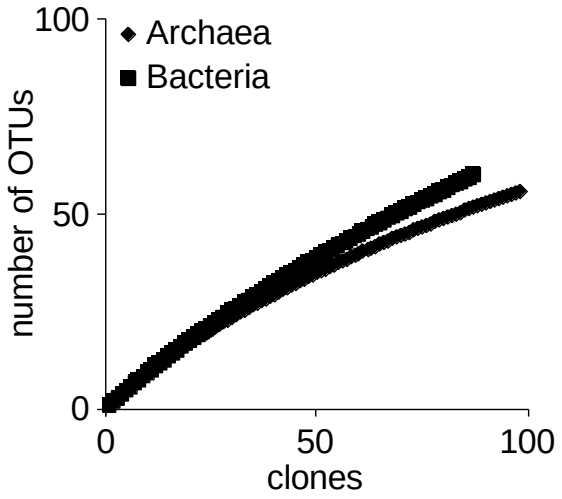

Figure S5: Rarefaction curve of the diversity coverage of a 16S rDNA clone library with primers specific for *Archaea* (Ar109f & Ar912r) or *Bacteria* (27f & 907r; Liesack & Dunfield, [2004]). The number of operational taxonomic units (OTUs) with a sequence identity of less than 97 % is plotted versus the number of sequenced clones. The Mothur software package was employed for the calculation of the rarefaction curve (Schloss et al., 2009).

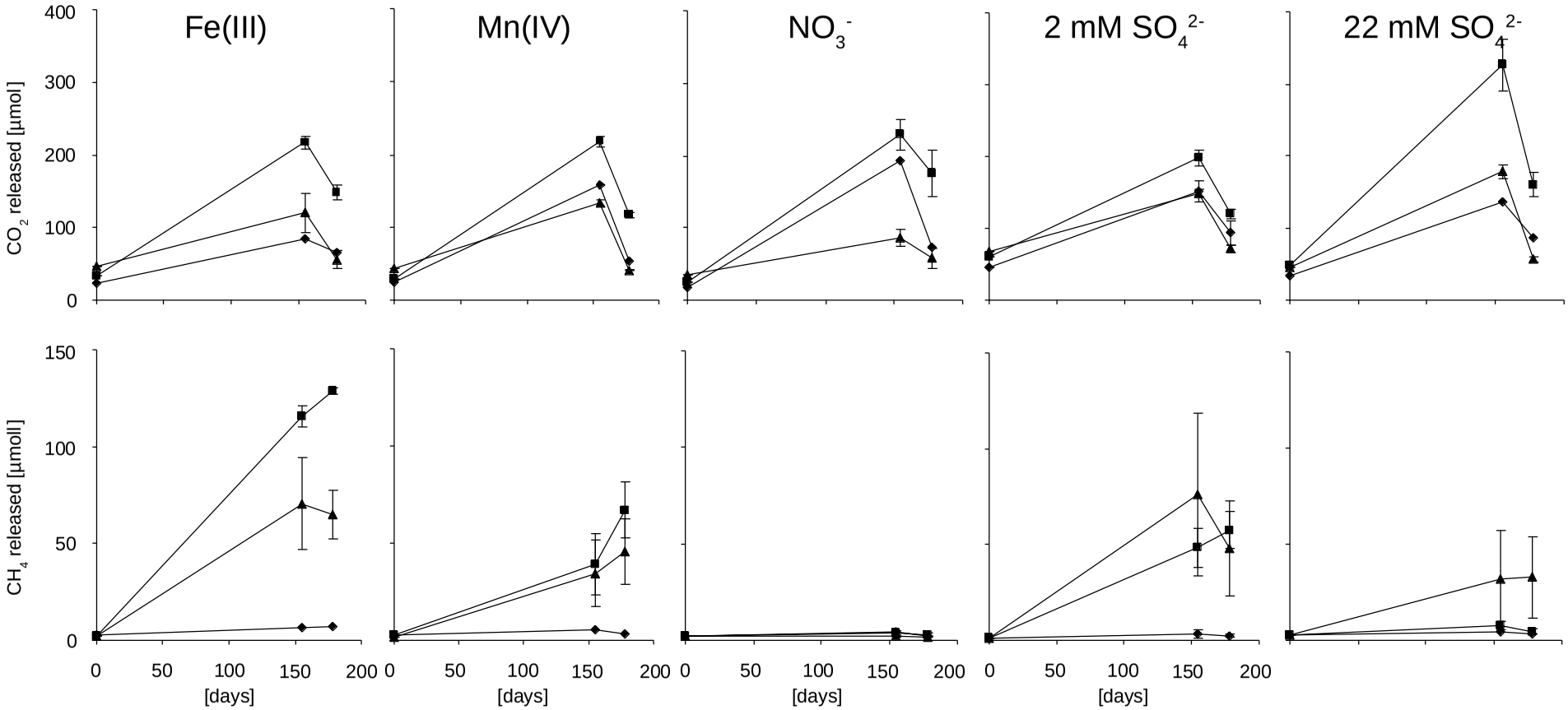

Figure S6: Absolute gas release in Zeebrugge microcosms. Upper row: evolution of CO_2 versus time [days] when ■ hexadecane or ▲ ethylbenzene were added. ● Controls without additional hydrocarbons. Bottom row: corresponding methane release.

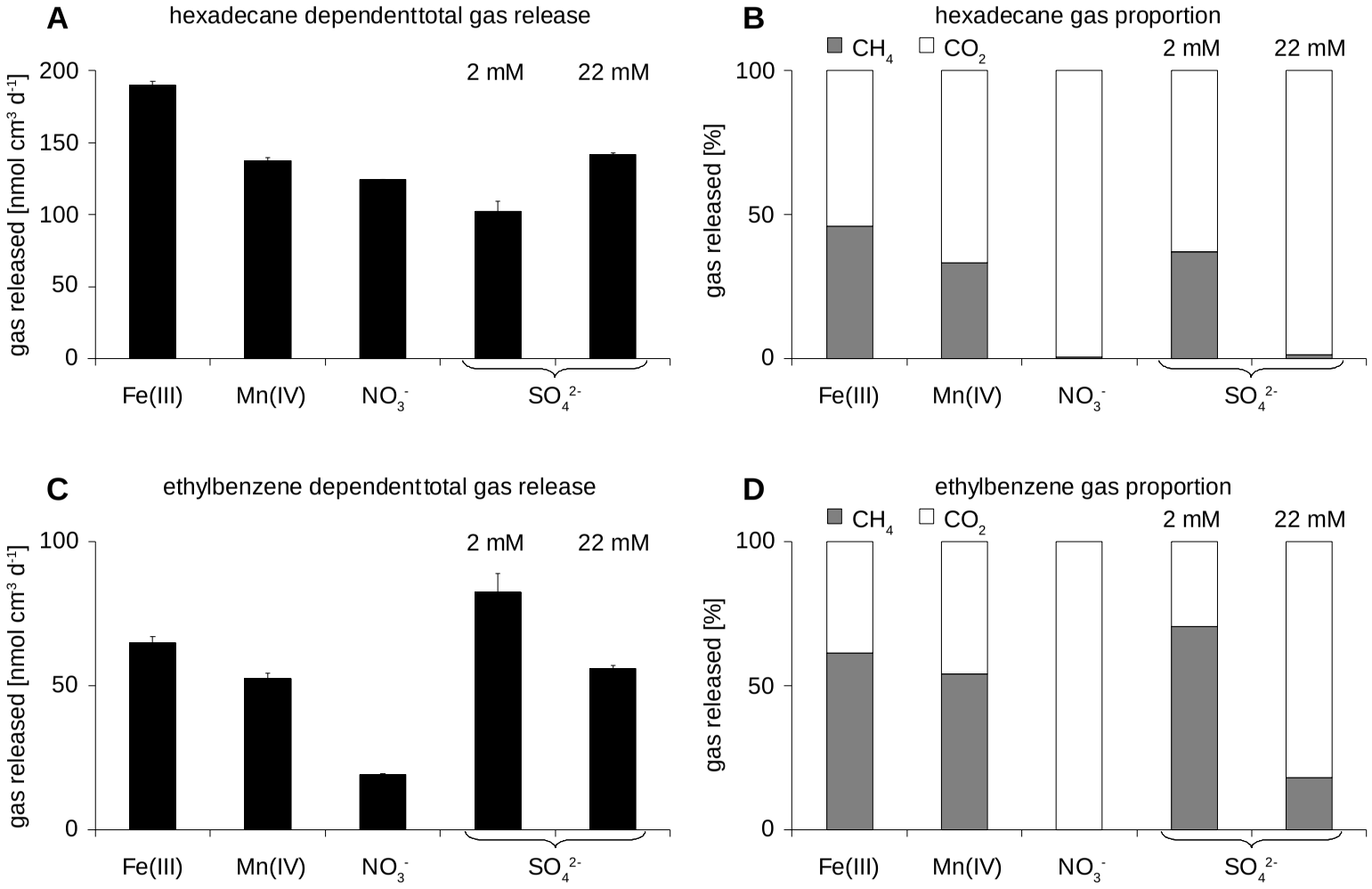

Figure S7: Zeebrugge microcosms: A: Total gas release (CO_2 + CH_4) upon hexadecane addition B: Proportions of CO_2 + CH_4 C: Total gas release upon ethylbenzene additions D: Proportions of CO_2 + CH_4 upon ethylbenzene addition

Chapter VII

Geochemistry and microbial populations in sediments of the Northern Baffin Bay, Arctic

7. Geochemistry and microbial populations in sediments of the Northern Baffin Bay, Arctic

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Geomicrobiology Journal 01/2013; 30 (8): 690–705

DOI:10.1080/01490451.2012.758195

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Keywords: microbial activities, microbial ecology, quantitative PCR, pore water analysis, sediment cores, subsurface microbiology

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Abstract

The Northern Baffin Bay between Greenland and Canada is a remote Arctic area restricted in primary production by seasonal ice cover, with presumably low sedimentation rates, carbon content and microbial activities in its sediments. Our aim was to study the so far unknown subseafloor geochemistry and microbial populations driving seafloor ecosystems. Shelf sediments had the highest organic carbon content, numbers of *Bacteria* and *Archaea*, and microcosms inoculated from Shelf sediments showed highest sulfate-reduction and methane production rates. Sediments in the central deep area and on the southern slope contained less organic carbon and overall lower microbial numbers. Similar 16S rRNA gene copy numbers of *Archaea* and *Bacteria* were found for the majority of the sites investigated. Sulfate in pore water correlated with *dsrA* copy numbers of sulfate-reducing prokaryotes and differed between sites. No methane was found as free gas in the sediments, and *mcrA* copy numbers of methanogenic *Archaea* were low. Methanogenic and sulfate-reducing cultures were enriched on a variety of substrates including hydrocarbons. In summary, the Greenlandic shelf sediments contain vital microbial communities adapted to their specific environmental conditions.

7.1. Introduction

Microorganisms in marine sediments are important drivers of global elemental cycles (Fuhrman, 2009; VanCappellen and Wang, 1996). This is not only the case in temperate or warm climates, but also in cold environments, like the Arctic or Antarctic regions, where sun energy is low resulting in a characteristic climatic, geological and biological situation. Arctic regions experience highly contrasting sun irradiance changes throughout the year in addition to extremely low air temperatures resulting in sea-ice formation. Both sun irradiance and sea-ice restrict primary production during prolonged time periods, and therefore the organic matter supply to the seafloor (Hulth et al., 1996).

In the last three decades, numerous studies have demonstrated the existence of active microbial processes and active prokaryotes in shallow and deep marine sediments all over the world (D'Hondt et al., 2002; Hamdan et al., 2011; Inagaki et al., 2003a; Jørgensen et al., 2006; Nunoura et al., 2009; Schippers et al., 2005; Webster et al., 2009; Wilms et al., 2006). Evidence of ongoing microbial processes in deep sediment cores from hundreds of meters below the seafloor has also been provided by pore water analysis of released and consumed dissolved metabolites in depth profiles (D'Hondt et al., 2004; D'Hondt et al., 2002; Engelen et al., 2008; Heuer et al., 2009; Lever et al., 2010).

Because sulfate-reduction is one of the main processes of organic matter degradation in marine sediments due to high sulfate concentration in seawater, it has been intensively studied in marine sediments worldwide (D'Hondt et al., 2002).

In permanently cold Arctic sediments organic matter mineralization is catalyzed by cold-adapted microorganisms (Knoblauch et al., 1999; Sahm et al., 1999). However, the metabolic processes are not temperature-limited, since substantial activities have been observed even under thermophilic conditions (Hubert et al., 2009). Rather, the availability and reactivity of the present organic matters seems to determine *in situ* microbial activities (Arnosti et al., 1998; Hubert et al., 2010). Apart from sulfate-reduction and methanogenesis, Mn^{4+} and Fe^{3+}-reduction are important geochemical processes as respiratory pathways for the carbon mineralization in Arctic sediments (Vandieken et al., 2006), contributing 70-90 % to mineralization of organic matter in surface sediments of the Barents Sea.

The area investigated in the present study, the Baffin Bay, is an arctic oceanic basin located between the coasts of Baffin Island/Ellesmere Island (Canada) and Greenland, connecting the Arctic Ocean to the Labrador Sea and the North Atlantic Ocean (Srivastava et

al., 1989). The Baffin Bay is sea-ice covered most of the year and completely free of sea-ice only in August and September (Tang et al., 2004), restricting access for sampling. Consequently, until today only few geochemical and microbiological studies have been performed in this region.

In 1985, the Ocean Drilling Program (ODP) Leg 105 sampled the southern Baffin Bay subsurface at site 645 down to depths of 1,147 mbsf for geological and geochemical purposes. The analyzed cores indicated sedimentation rates with an average of 60 m Ma^{-1} and Total Organic Carbon (TOC) values from 0.1 % to 2.8 % increasing with sediment depths to 900 mbsf (Srivastava et al., 1989). In 2007, Galand and co-workers sampled the southern Baffin Bay water column at 1,000 m water depth to study how the hydrography affects bacterial community distribution in deep Arctic water bodies (Galand et al., 2010).

Since 90 % of the seafloor worldwide is – as in the Baffin Bay - exposed to temperatures below 4˚C (Levitus and Boyer, 1994), it is important to gain insights in microbial life in such permanently cold environments. Especially important are its ecology, abundance, spatial distribution, as well as regulatory factors, like porewater chemistry, organic matter availability, sedimentology, etc.

Therefore, we conducted a comprehensive study on the sediment characteristics and the microbial populations within the Baffin Bay using sediment cores collected in three areas of the Northern Baffin Bay during the ARK XXV/3 expedition with the research vessel Polarstern in the year 2010. We investigated the depth- and site-dependent distribution of microbial populations as well as activities using molecular biological and microbiological methods, together with a detailed porewater and solid phase biogeochemical analysis. This combined study contributes to the understanding of the microbial and geological nature of this and other remote Arctic areas and provide data for the evaluation of the global distribution and importance of microorganisms and their role in major elemental cycles.

7.2. Materials and methods

7.2.1. Sampling locations

The present study was conducted as part of the research cruise ARK XXV/3 to the Northern Baffin Bay covering an area from the Kane Basin in the North to Disko Island in the South in Greenland territorial waters. The expedition took place with the research vessel Polarstern from August to mid October 2010 (Damm, 2010). Near-surface sediments were cored along seismic refraction and reflection profile lines. These profile lines extended from Greenland coastal areas into the central part of the Baffin Bay. Water depths at the sampling sites ranged from 598 m to 2,300 m (Table 7.1).

Table 7.1: Overview of the oceanographic data of the sampling sites in the Baffin Bay

Site	Area	Latitude	Longitude	Water Depth (m)	Core depth (m)
363	Shelf	76° 52.92' N	71° 34.01' W	938	4.69
371	Shelf	75º 58.24' N	70° 34.86' W	598	4.05
389	Central Deep Basin	74° 37.05' N	69º 13.75' W	1,716	4.24
391	Central Deep Basin	74° 23.36' N	69º 01.22' W	1,864	4.27
453	Central Deep Basin	73° 19.37' N	64° 58.11' W	2,300	4.69
486	Southern Slope	72° 24.51' N	60° 48.85' W	645	4.69
488	Southern Slope	72° 08.80' N	60° 58.86' W	1,493	4.69

To investigate how the different bathymetric and physiographic characteristics may affect the sediment geochemistry and microbiology, we collected sediment cores in three distinct areas of the Baffin Bay. These three areas were referred to as (a) “Shelf” which includes sites 363 and 371, located in the Northern Greenlandic continental margin with less than 1,000 m of water column; (b) the “Central Deep Basin” which includes sites 389, 391 and 453, all located in the central Baffin Bay basin with water depths of more than 1,500 m; and (c) the “Southern Slope” with the sites 486, and 488 with variable water depths (see Figure 7.1 and Table 7.1).

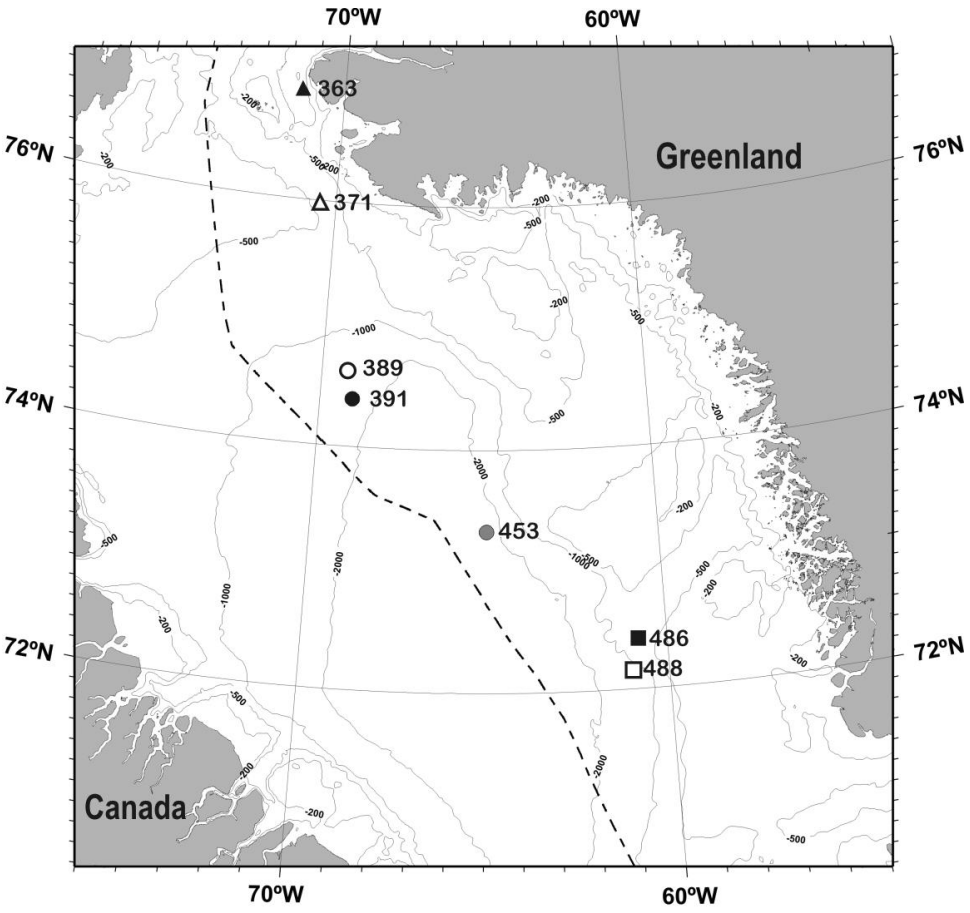

Figure 7.1: Map of the Northern Baffin Bay indicating sites where sediments were cored. The symbols shown for the different sites are used for all graphs in this study. Sites are grouped into three areas on the basis of their bathymetric and physiographic characteristics: sites 363 and 371 were grouped to the 'Shelf Area'; sites 389, 391 and 453, to the 'Central Deep Basin Area'; and sites 486 and 488 to the 'Southern Slope Area'.

7.2.2. Core sampling and processing

Core sampling at the different sites was performed with a gravity corer, using a 4.70 m core barrel (Rehau AG & Co.) equipped with a 90 mm (outside diameter) PVC liner. Collected cores were cut into 1 m sections and kept at 4°C until further analysis and subsampling. From all sediment cores, four different types of samples were taken: (a) sediment samples for analysis of free gases, (b) pore water samples, (c) sediment samples for molecular biological analyses and (d) sediment samples for solid phase analysis of elemental composition and organic carbon content.

Samples for sediment gases were collected at intervals of 50 cm along each core starting at 25 cm below the sediment surface. Five mL sediment samples were placed in

50 mL glass serum vials pre-filled with 20 mL 1 M NaOH and immediately closed with gastight rubber stoppers and aluminum crimp caps. The vials were then vortexed until the sediment was completely suspended and stored at 4°C until GC analysis.

Immediately after gas sampling, 8 − 10 mL pore water were extracted from all cores at 4°C at the same sample ports used for gas sampling with a rhizon sampler (CSS-F 5 cm or 10 cm porous length, 2.5 mm tip diameter, Rhizosphere Research Products, Wageningen, NL). The pore water samples were stored in polypropylene vials preconditioned by rinsing with 1 % (vol/vol) nitric acid solution and washing with distilled water, preserved with 1 % (vol/vol) of a 65 % (vol/vol) concentrated nitric acid, and stored at 4°C.

One half from each core was used for stratigraphic studies and stored as archive while the other half was used for microbiological sampling. All samples for microbiological analysis were taken from the interior of the core immediately after slicing. Molecular biological samples were taken with cut sterile 5 mL syringes every 25 cm along the core. Samples were placed in 15 mL Falcon tubes that were immediately frozen and kept at -80°C until analysis. Sediment for incubations was collected at 50 − 100 cm intervals and stored in sterile glass bottles sealed with butyl septa and screw caps under an N_2 atmosphere at 4°C.

7.2.3. Geochemical characterization

Sediment samples from sites 363, 389 and 486 were selected for solid phase analyses to identify the mineral material. These samples (1 − 2 g) from several depths along the core were freeze-dried and homogenized in an Agate mortar. X-Ray Powder Diffraction (XRD) patterns were recorded with a PANalytical X'Pert PRO MPD θ-θ diffractometer (PANalytical B.V., The Netherlands) (Cu–Kα radiation generated at 40 kV and 30 mA), equipped with a variable divergence slit (20 mm irradiated length), primary and secondary soller slit, Scientific X´Celerator detector (active length 0.59°), and a sample changer (sample diameter 28 mm). The qualitative evaluation of the powder patterns was performed using the search/match software HighScore Plus (PANanalytical B.V.) and the ICDD PDF-2 database.

The elemental compositions of sediment samples were determined by wavelength dispersive x-ray fluorescence spectrometry (WD-XRFS) using PANalytical AXIOS WD-XRF (PANanalytical B.V., The Netherlands), with Rh anode x-ray tubes. Samples were prepared by milling to less than 40 µm particle size. Then, 1,000 mg per sample were mixed with 5.0 g lithium meta-borate and 25 mg lithium bromide, and fused at 1,200°C for 20 minutes. Loss

on ignition (LOI) was determined by heating to 1,030°C for 10 minutes. For samples with a LOI greater than 25 %, 2.5 g lithium meta-borate and 2.415 g lithium tetraborate were used. Pt95-Au5 crucibles and a commercial automatic fluxer (Herzog 12/1500) were used for the fusion. The calibration was constructed using 130 certified reference materials and corrections applied to correct for matrix and spectral interferences.

Total sedimentary sulfur (TS) and total organic carbon (TOC) were determined on a LECO CS-200 elemental analyzer (LECO Corporation, USA). Determination of organic carbon requires the removal of carbonate carbon from the samples through adding a 10 % solution of hydrochloric acid to the sediment, and subsequent drying at 80°C for 24 hours. Reproducibility of S- and C-measurements was ± 0.01 %. All values are reported as weight percentages.

For ^{13}C analysis of organic carbon, 5 mg freeze-dried sediment was decarbonated, transferred in zinc (3.5 x 5 mm, HEKAtech, Germany) or silver caps for solids (5 x 9 mm Lüdi, Switzerland) and measured with an elemental analyzer (EuroEA3000, Euro Vector, Italy) coupled via a ConFlo III (Thermo Fisher Scientific, Germany) to a MAT 253 isotope ratio mass spectrometer (Thermo Fisher Scientific) (Badea et al., 2011). Stable carbon isotopic values are expressed in δ-notation (‰) relative to the Vienna PeeDee Belemnite (VPDB) standard.

$$\delta^{13}C = [((^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{VPDB\text{-}standard})-1] \times 1000$$

Methane concentrations in fixed sediment samples and in incubations were analyzed by measuring headspace samples at 60°C using a GC-FID equipped with a 6’ Hayesep D column (SRI 8610C, SRI Instruments, USA). Carbon dioxide concentrations were determined using a methanizer-equipped FID detector, after reduction of the CO_2 to methane. The stable isotopic composition of methane and CO_2 was measured using a gas chromatography-combustion-isotope ratio monitoring mass spectrometry system (GC-C-IRM-MS). The system consisted of a gas chromatograph (6890 series; Agilent Technology), fitted with a CP-pora BOND Q column, coupled with a combustion or high-temperature pyrolysis interface (GC-combustion III or GC/C-III/TC; Thermo Finnigan, Bremen, Germany) to a MAT 252 IRMS for the carbon analysis and to a MAT 253 IRMS for hydrogen analysis (both from Thermo Finnigan, Bremen, Germany) (Herrmann et al., 2010).

To analyze the elemental composition of the pore water, samples were measured using an inductively-coupled-plasma mass-spectrometry (ICP-MS) instrument (Perkin Elmer Sciex Elan 5000, USA) as previously described (Dekov et al., 2007).

7.2.4. DNA isolation from sediment samples

For molecular analyses, sediment samples frozen at -80°C were slowly thawed on ice. DNA was isolated from 0.86 g of sediment using the FastDNA Spin Kit for Soil (MP Biochemicals, Solon, OH, USA) with a FastPrep instrument (FastPrep FP120; Savant Instruments, Holbrook, NY, USA) according to the manufacturer's instructions with minimal modifications (silica matrix was allowed to settle for 30 min, yields were increased by incubation for 15 min at 42°C in an incubator). DNA was eluted in 100 µL DNase/pyrogen-free ultra-pure water supplied with the kit. For each sediment sample, triplicate DNA isolation was done. Triplicates were pooled and analyzed for DNA concentration using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., USA) and stored at -20°C.

7.2.5. Quantitative analysis of microbial populations via Q-PCR

For quantitative PCR (Q-PCR) analysis, DNA template (1 µL) was diluted 10-fold to prevent interference of co-extractable organic material from the sample. The Q-PCR mix for the quantification of *Bacteria* 16S rRNA gene copy numbers (20 µL final reaction volume) consisted of 10 µL of SensiMix SYBR (Bioline, UK), which included DNA polymerase, dNTPs, SYBR Green I dye, stabilizers and ROX, 7 µL of PCR-grade water, and primers. The primers 341f and 534r (1 µM each) (Muyzer et al., 1993) were used to amplify variable regions of 16S rRNA genes of *Bacteria* in a touchdown PCR program with the following parameters: initial denaturation of 15 min at 95°C; denaturation of 30 s at 95°C, annealing temperature of 65°C decreasing every cycle by 0.4°C for 5 cycles and the remaining 33 cycles at 63°C, elongation of 45 s at 72°C. After amplification a melting curve was run from 60°C to 95°C with the following parameters: initial denaturation for 15 min at 95°C, renaturation for 1 min at 60°C followed by a gradient until 95°C was reached, followed by 15 min at 95°C. The Q-PCR for quantification of *Archaea* and *Geobacteraceae* 16S rRNA gene copy numbers was performed as previously described (Holmes et al., 2002; Schippers and Neretin, 2006; Takai and Horikoshi, 2000). Standard curves to determine the 16S rRNA gene copy numbers of total *Bacteria* were constructed by 10-fold serial dilutions of PCR-amplified 16S rRNA genes

of the *Dehalococcoidetes* group amplified from sediment samples and cloned into a pGEM-T vector. Linear calibration curves were produced by triplicate serial dilutions ranging over six orders of magnitude, from 10^7 to 10^2 copies. Standard curves for the determination of *Archaea* and the *Geobacteraceae* family were done as described (Schippers and Neretin, 2006). Q-PCR amplifications were performed on triplicate samples using a StepOne detection system (StepOne/ StepOnePlus version 2.0, Applied Biosystems, Foster City, CA, USA) and analyzed with the StepOne v2.1 software. Amplification efficiencies were calculated from the slope of each calibration curve according to the formula $10^{(-1/slope)}$. Amplification efficiencies ranged between 90 and 100 %. All SYBR Green I assays were followed by a melting curve stage checking for amplicon specificity.

The quantitative amplification of dissimilatory sulfite reductase gene of sulfate-reducers (*dsrA*) was carried out as described (Schippers and Neretin, 2006). For the quantification of the methyl coenzyme M reductase subunit α gene (*mcrA*), the ME1F and ME3R primer set was used (Hales et al., 1996). The PCR mix for the quantification of *mcrA* copy numbers (10 µL final reaction volume) consisted of 5 µL SYBR Green Real-Time PCR Master Mix (Invitrogen, Life Technology, USA) which included DNA polymerase, dNTPs, SYBR Green I dye, and ROX, 0.5 µL of Bovine Serum Albumin (3 µg/µL stock solution) (BSA; VWR International, Germany), 2.7 µL of PCR-grade water and 0.4 µL of each primer (200 nM of each). The standard curve was done by triplicate 10-fold serial dilutions of *Methanosarcina* DNA.

The presence of anaerobic methanotrophs (ANME) was examined by Q-PCR using specific primers for the *mcrA* genes of ANME-1 and ANME-2 organisms as described (Nunoura et al., 2006).

7.2.6. Microcosm incubations and measurement of potential microbial activities

To determine potential rates of selected microbial processes, sediments from different cores (363, 389, 486) and three different depths (roughly top, middle and bottom of each core) were selected.

For the preparation of the microcosms, autoclaved Hungate tubes were filled with 7 mL of sterile anoxic sulfate-free minimal medium (Widdel and Bak, 1992). Sediment slurries from each sample (1:1 mix of sediment and anaerobic medium) were anaerobically prepared and three mL were added to the Hungate tubes. Then the tubes were purged with

N_2 (100 %) and sealed with sterile butyl rubber stoppers and screw caps. Microcosms were set up at least in triplicate and incubated vertically in the dark with gentle shaking at 4°C.

Anaerobic enrichments were set up under methanogenic conditions without the addition of electron acceptors, and under sulfate-reducing conditions, where sulfate was added from an anoxic stock solution to a final concentration of 20 mM. Without additional carbon sources added, these incubations were used to measure potential rates of methane production and sulfate-reduction of the indigenous microorganisms.

In a second set of incubations under both conditions, combinations of typical sedimentary substrates were added as monomer or polymer mix (Batzke et al., 2007) to enrich methanogenic or sulfate-reducing microorganisms, respectively. In a separate set of vials with sulfate, the headspace was flushed with 100 % methane to measure sulfate-dependent anaerobic oxidation of methane (AOM).

Third, the potential for hydrocarbon degradation under methanogenic conditions was studied in incubations with hexadecane, hexadecanoic acid, ethylbenzene or methyl-naphthalene, which were all added directly to the medium in concentrations of 0.1 % (v/v). Finally, a number of parallels were incubated under aerobic conditions to assess the potential for aerobic methane oxidation (air:methane, 95:5 %).

Sulfide concentrations were determined photometrically by the formation of copper sulfide (Cord-Ruwisch, 1985). Rates for anaerobic oxidation of methane (AOM) were measured and calculated as previously described (Krüger et al., 2005b; Nauhaus et al., 2002; Treude et al., 2005). Aerobic methane oxidation rates (MOR) in microcosms were measured following the depletion of methane in the aerated slurry samples (Krüger et al., 2002). Methane production and oxidation rates were calculated by linear regression of the increase or decrease of these gases in the headspace over incubation time (Krüger et al., 2001). All rates are given in µmol per day^{-1} g^{-1} of dry sediment.

7.3. Results

7.3.1. Sediment description

Sediment stratigraphy differed among the three areas. Dropstones from glacier transport were commonly found in the sediments in the majority of the cores. Shelf sites

363 and 371 were mainly composed of homogeneous olive grey silty mud except in the deepest core sections where color changed into greyish brown or dark yellowish brown and the grain size became coarser. Central Deep Basin Area sites 389 and 391 showed layers of diatomaceous silty mud in the first 60 centimetres below surface (cmbsf). The lithology downwards of the diatomaceous silty muds for site 389 was composed of silty muds of from olive grey to dark yellowish brown and brownish color with intercalations of sandy mud and various coarser or finer grained intervals of dropstone granules.

For site 391 and downwards of the diatomaceous layer, the sediment was composed of silty muds with a pale brown coarser grained intercalated layer of sandy and silty muds from 120 to 145 cmbsf. The rest of the site 391 core was brownish silty muds. In the Central Deep Basin site 453, the whole 100 first cmbsf had a diatom influence in the silty mud lithology of the core. The rest of the core was silty muds until 273 – 300 cmbsf which was silty marl until 430 cmbsf that was composed of silty mud again. Southern Slope Area sites 486 and 488 lithology was featureless olive grey silty mud changing color to brownish grey or greenish grey along the core.

7.3.2. Geochemistry of sediments

The sites 363, 389 and 486 were selected for detailed depth profile analyses of total carbon (TC), total organic carbon (TOC) and bulk organic carbon stable isotopic composition. Of all the measured sites, the Shelf site 363 had the highest organic carbon content with mean TOC values of 1.2 % (Figure 7.2). At this site, TOC values decreased with depth gradually from 1.7 % at 10 cmbsf to 0.9 % at 415 cmbsf and a pronounced decrease to 0.2 % at 460 cmbsf was observed. The Central Deep Basin site 389 and the Southern Slope site 486 contained similar amounts of TOC around 0.5 % but no trend with depth. TC values demonstrated an important contribution of carbonates in the Central Deep Basin site 389, especially in layers deeper than 160 cmbsf and in the Shelf site 363 at its deepest sediment point at 460 cmbsf.

Stable isotope values of organic carbon through depth profiles of the sediment revealed values lighter than -24 ‰ in all cases. Shelf site 363 $\delta^{13}C_{org}$ values were on average -25 ‰, and became lighter in sediments deeper than 400 cmbsf. Both Central Deep Basin and Southern Slope sites showed lighter overall $\delta^{13}C_{org}$ values compared to the Shelf. Central Deep Basin site 389 showed δ^{13} C_{org} values of -28 ‰ which decreased to -31 ‰ by

130 cmbsf and increased to -24 ‰ at 400 cmbsf. In the Southern Slope site 486 $\delta^{13}C$ values were -29 ‰ and became lighter with depth.

The solid phase chemistry of major sediment components showed remarkably high contents of manganese oxide, which were one order of magnitude higher in near-surface sediments from 40 – 63 cmbsf compared to deeper sediments at the Central Deep Basin Area site 389. Total sulfur (TS) values increased with depth for the Shelf site 363 peaking at 200 cmbsf and prominently decreasing in sediments deeper than 400 cmbsf. TS values at the Central Deep Basin site 389 were the lowest measured in all cores with mean values of 0.15 % and showed no clear trend. TS values at the Southern Slope site 486 were patchy.

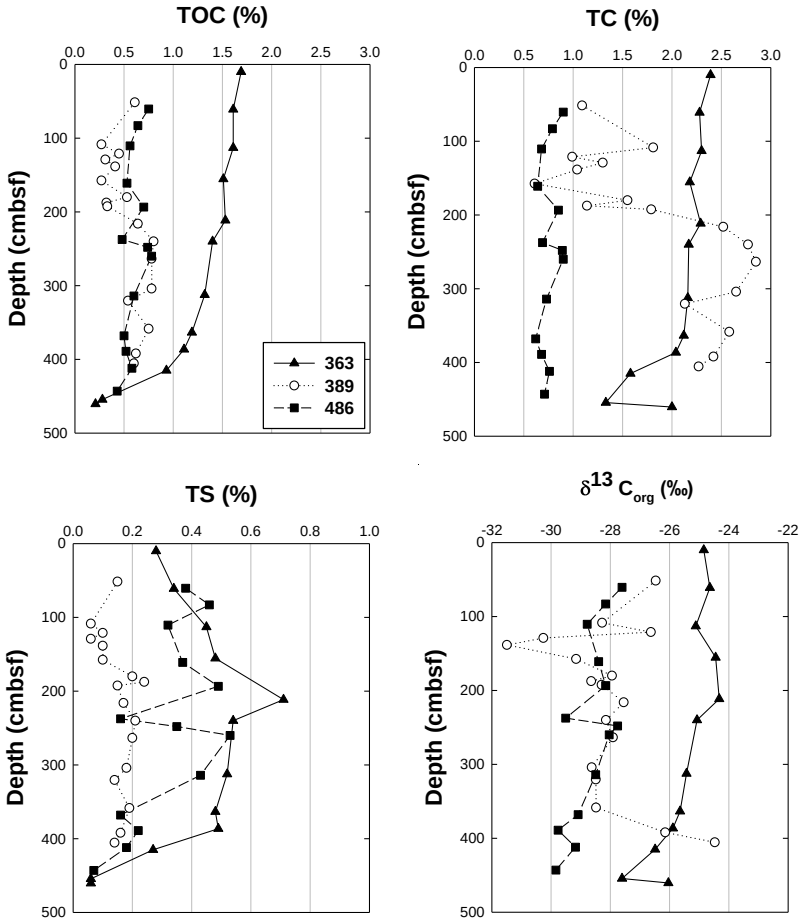

Figure 7.2: Depth profiles of carbon (TOC – total organic carbon, TC – total carbon) and sulfur (TS – total sulfur) content (wt %) and carbon isotopic composition (‰ vs. VPDB) at three selected sites.

7.3.3. Porewater geochemistry

Depth-dependent porewater concentrations of sodium (Na^+), chloride (Cl^-) (data not shown for Na^+ and Cl^-), potassium (K^+), magnesium (Mg^{2+}), sulfate (SO_4^{2-}), ferrous iron (Fe^{2+}) and manganese (Mn^{2+}) (Figure 7.3) were measured for all sites in the selected areas. Na^+ and

Cl^- concentrations were stable with increasing depth for all sites with mean values of 461 mM ± 10 mM and 534 mM ± 13 mM, respectively. K^+ concentrations were around 10 mM in near-surface samples from all sites and decreased by 1 – 2 mM with increasing depth. Mg^{2+} concentrations were 45 – 50 mM near the surface in all sites. While in some samples Mg^{2+} concentrations decreased with depth, in other sites Mg^{2+} concentrations were stable. Major decreases in Mg^{2+} with increasing depth were observed for the Shelf site 363 and the Central Deep Basin site 453.

Near-surface sediment concentrations of SO_4^{2-} of 25 – 27 mM gradually declined with increasing depth for all sites except the Central Deep Basin sites 389 and 391. Shelf sediments showed a gradual decrease of SO_4^{2-} concentrations in the first 225 cm of the profiles. Southern Slope SO_4^{2-} depth profiles showed slighter but steadier SO_4^{2-} decreases with depth compared to Shelf SO_4^{2-} depth profiles. Only the Central Deep Basin site 453 showed a strong decrease in SO_4^{2-}, reaching concentrations close to depletion at 450 cmbsf.

Fe^{2+} concentration profiles differed substantially among the different study areas. Shelf depth profiles showed considerably increased Fe^{2+} concentrations at the deepest measured core sections. Fe^{2+} profiles in the Central Deep Basin sites increased slightly with depth reaching levels of about 20 µM at about 225 – 275 cmbsf. However, single measurements reached highest profile Fe^{2+} concentrations in samples from sites 389 and 391 at depths of 75 and 175 cmbsf, respectively.

The Southern Slope Area showed near the surface Fe^{2+} concentrations of 28 µM and 47 µM for sites 486 and 488, respectively, subsequently decreasing with depth down to 275 cmbsf. In deeper sediment layers, Fe^{2+} concentrations increased at site 486 while it further decreased at site 488. Similarly, Mn^{2+} concentration profiles showed different trends among the three study areas. Shelf sediment Mn^{2+} concentration profiles slightly increased with depth. Central Deep Basin sites 389 and 391 sediment profiles showed pronounced increased concentrations between 75 to 175 cmbsf, decreasing to 20 µM at 275 cmbsf which remained steady with depth. Site 453 showed a gradual increase in Mn^{2+} with depth reaching 20 µM at 375 cmbsf. In the Southern Slope, Mn^{2+} depth profiles for site 486 showed stable concentrations of 20 µM. Site 488 depth profiles decreased from 50 µM to 20 µM at its deepest section.

Concentrations of dissolved methane in porewater generally were low, ranging between 0.05 and 0.1 mM at the sediment surface, then slightly increasing to 0.1 to 0.3 mM

in deeper parts of the cores, and finally decreasing at the bottom of the cores (Figure 7.3). The isotopic composition of methane and CO_2 in the porewater was between -62 to -78 ‰ and -18 to -21 ‰, respectively, indicating a biogenic origin of the gases.

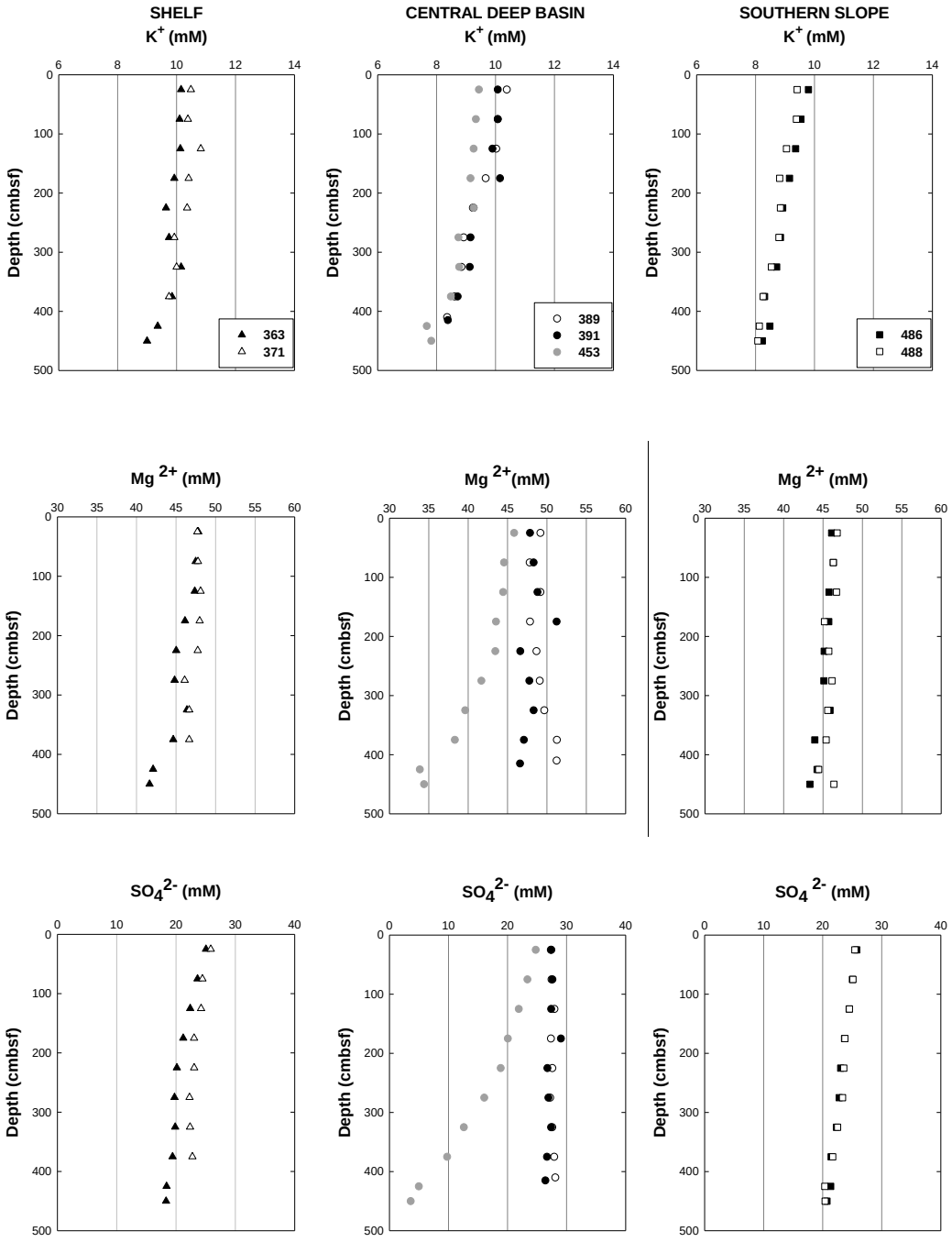

Figure 7.3 (continued on next page)

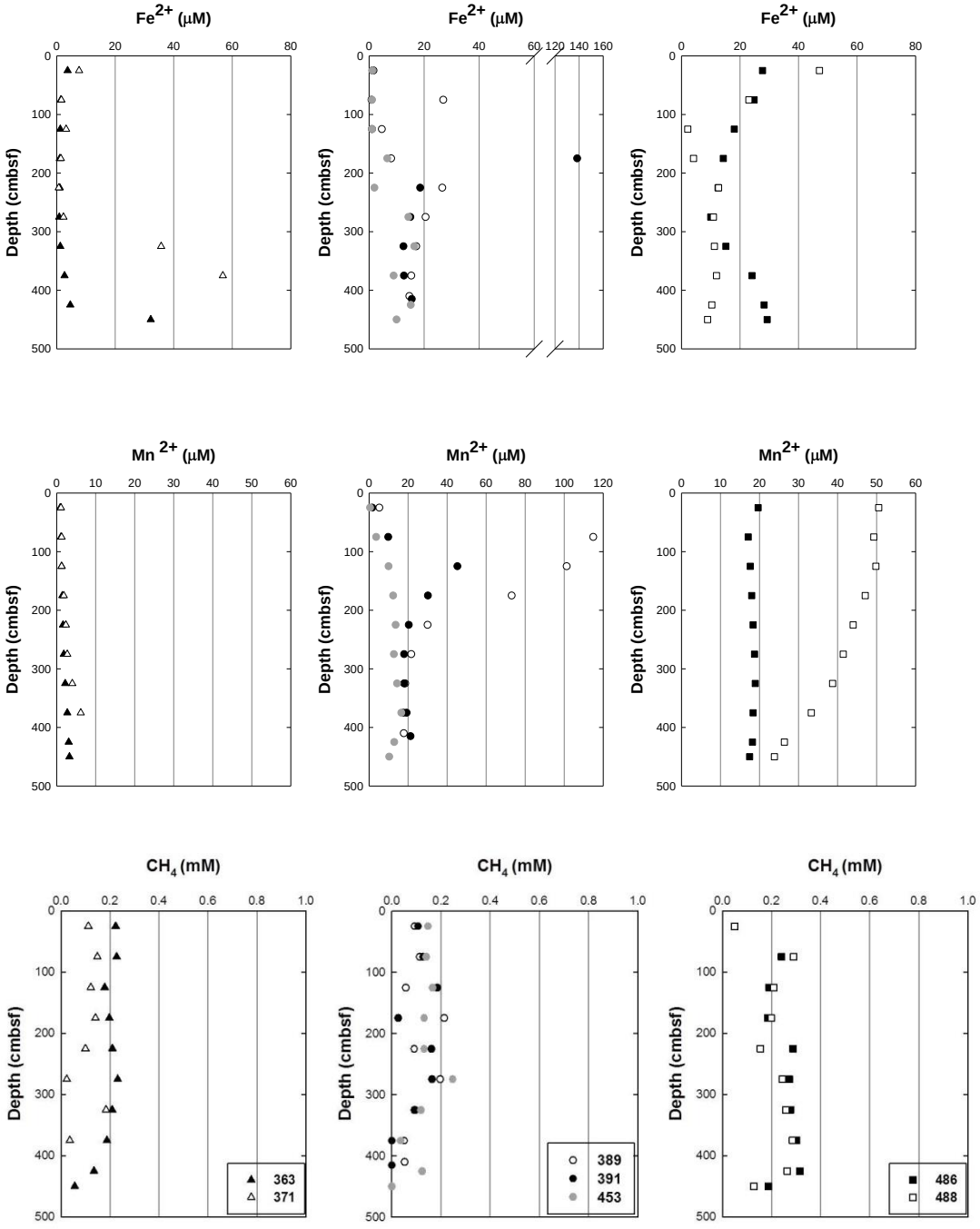

Figure 7.3: Depth profiles of major porewater constituents, for the Shelf Area (left), the Central Deep Basin Area (middle), and the Southern Slope Area (right).

7.3.4. Potential microbial activities in sediment microcosms

Sediments from the sites 363, 389 and 486 collected at various depths were used for the setup of microcosms under diverse cultivation conditions and incubated for up to 160 days to determine potential rates of selected activities, and to study the potential of indigenous microorganisms to react to a range of added substrates.

The potential for sulfate-reduction (SRR) without additional carbon sources was detected in all microcosms inoculated with Shelf site 363 and Southern Slope site 486 sediments, while Central Deep Basin site 389 microcosms showed no sulfide production. In both cores the SRR were highest in surface samples and decreased with increasing sediment depths. At site 363 the SRR decreased from 2.38 ± 0.59 at the top to 0.84 ± 0.28 µmol day^{-1} g_{dw}^{-1} at the bottom of the core, at site 389 from 1.69 ± 1.25 to 0.32 ± 0.27 µmol day^{-1} g_{dw}^{-1}, respectively. In all sediment samples from the three cores the addition of carbon sources in the form of the monomer and polymer mix led to an increase of the SRR by a factor of roughly 5-7 in surface and 2-3 in bottom samples.

Also, the addition of hexadecanoic acid and hexadecane stimulated SRR in the surface samples of cores 363 and 389. The SRR increased after hexadecane addition to 4.86 ± 0.47 and 3.24 ± 0.55 µmol day^{-1} g_{dw}^{-1}, respectively. The stimulation after the addition of hexadecanoic acid was in a similar range. However, no increased sulfate-reduction was observed with ethylbenzene or methyl-naphthalene.

From all microcosms, only those inoculated with Shelf sediments from site 363 at depths 6 – 14 cmbsf showed methanogenic activity without the addition of C-sources. Potential methane production rates without sulfate reached 0.75 ± 0.26 nmol day^{-1} g_{dw}^{-1}. The methane production rates in microcosms containing an initial concentration of 20 mM of sulfate were around 0.47 ± 0.16 nmol day^{-1} g_{dw}^{-1}. Microcosms amended with the polymer and the monomer mix as substrates were monitored over a time period of up to 350 days. If amended with sulfate, methane production rates in these microcosms were 3.26 ± 0.96 and 6.61 ± 2.35 nmol day^{-1} g_{dw}^{-1} for the polymer and the monomer mix, respectively. Without sulfate, the rates were 3.24 ± 0.91 and 7.47 ± 2.59 nmol day^{-1} g_{dw}^{-1} for the polymer and the monomer mix.

To evaluate the potential of Baffin Bay sediment microorganisms to use hydrocarbons as an electron donor for methanogenesis, microcosms were amended with hexadecane, hexadecanoic acid, ethylbenzene, and methyl-naphthalene, and monitored over a time

period of up to 350 days. Again, only microcosms inoculated with Shelf site 363 sediments from the depths of 6 – 14 cmbsf showed methane production. Methane production rates in microcosms amended with sulfate were 2.71 and 3.7 nmol day^{-1} g_{dw}^{-1} for hexadecane and for hexadecanoic acid, respectively. Those microcosms without sulfate showed methane productions rates of 2.17 and 1.87 nmol day^{-1} g_{dw}^{-1} for hexadecane and hexadecanoic acid, respectively. No increased methanogenesis was observed with ethylbenzene or methylnaphthalene.

Neither aerobic nor anaerobic methane oxidation activity was found in any of the tested sediment samples from these three cores.

7.3.5. Quantification of *Bacteria* and *Archaea*

The depth profiles for bacterial 16S rRNA gene copy numbers showed clear differences among the three study areas (Figure 7.4). In the Shelf Area, bacterial 16S rRNA gene copy numbers were steady with depth showing around 10^7 copies $gram^{-1}$ of sediment. Only core sections deeper than 300 cmbsf for site 371 and deeper than 425 cmbsf for site 363 showed decreased copy numbers. The uppermost sediment depth of Shelf site 371 showed the highest bacterial 16S rRNA gene copy numbers of the Shelf sites with 6×10^7 g^{-1}. In the Central Deep Basin Area, total *Bacteria* copy numbers in samples from 25 cmbsf were 6×10^7 to 4×10^7 copies g^{-1} for sites 389 and 391, respectively, and drastically decreased with depths to $10^5 - 10^4$ g^{-1} at 175 cmbsf. Central Deep Basin site 453 showed a different pattern than sites 389 and 391, with 16S rRNA gene copy numbers of 5×10^6 g^{-1} at 25 cmbsf which only slightly decreased with depth reaching 10^6 g^{-1} at 425 cmbsf. In the Southern Slope Area, highest 16S rRNA gene copy numbers were not observed at the shallowest depths like in the Shelf and Central Deep Basin areas but in samples from 125 cmbsf at site 486 with 8×10^7 g^{-1}. Sediment of site 486 deeper than 125 cmbsf had 10^7 g^{-1} 16S rRNA gene copies, which decreased in samples deeper than 375 cmbsf. Southern Slope site 488 showed near surface 16S rRNA gene copy numbers of 3×10^6 g^{-1}, which increased to 6×10^6 copies g^{-1} by 75 cmbsf to subsequently decrease for 10^5 and remain around 10^6 copies g^{-1} for the rest of the depth profile.

Archaeal 16S rRNA gene Q-PCR analysis revealed a distribution of *Archaea* in all sediment samples similar to those of *Bacteria* (Figure 7.4). In the Shelf Area, archaeal 16S rRNA gene copy numbers were about 10^7 g^{-1} of sediment and decreased at the same core

depths where bacterial 16S rRNA gene copy numbers also declined. In the Central Deep Basin Area, highest archaeal 16S rRNA gene copy numbers of 10^8 g^{-1} were observed at site 389 at 25 cmbsf and declined to 10^7 g^{-1} at 75 cmbsf and 10^6 g^{-1} at 125 cmbsf. Central Deep Basin's site 391 had highest *Archaea* 16S rRNA gene copy numbers of 5 x 10^6 g^{-1} at 25 cmbsf and lowered dramatically down in depths of 125 cmbsf and deeper core sections. *Archaea* in Central Deep Basin site 453 decreased from about 10^6 to 5 x 10^4 over the measured core.

In general, *Archaea* were very rare in layers of the Central Deep Basin deeper than 175 cmbsf and often below the detection limit in samples from sites 389 and 391. In the Southern Slope Area, lower numbers of *Archaea* than *Bacteria* were found in all depth samples. In particular, site 488 showed a strong decrease of more than an order of magnitude in 125 cmbsf and deeper sediments.

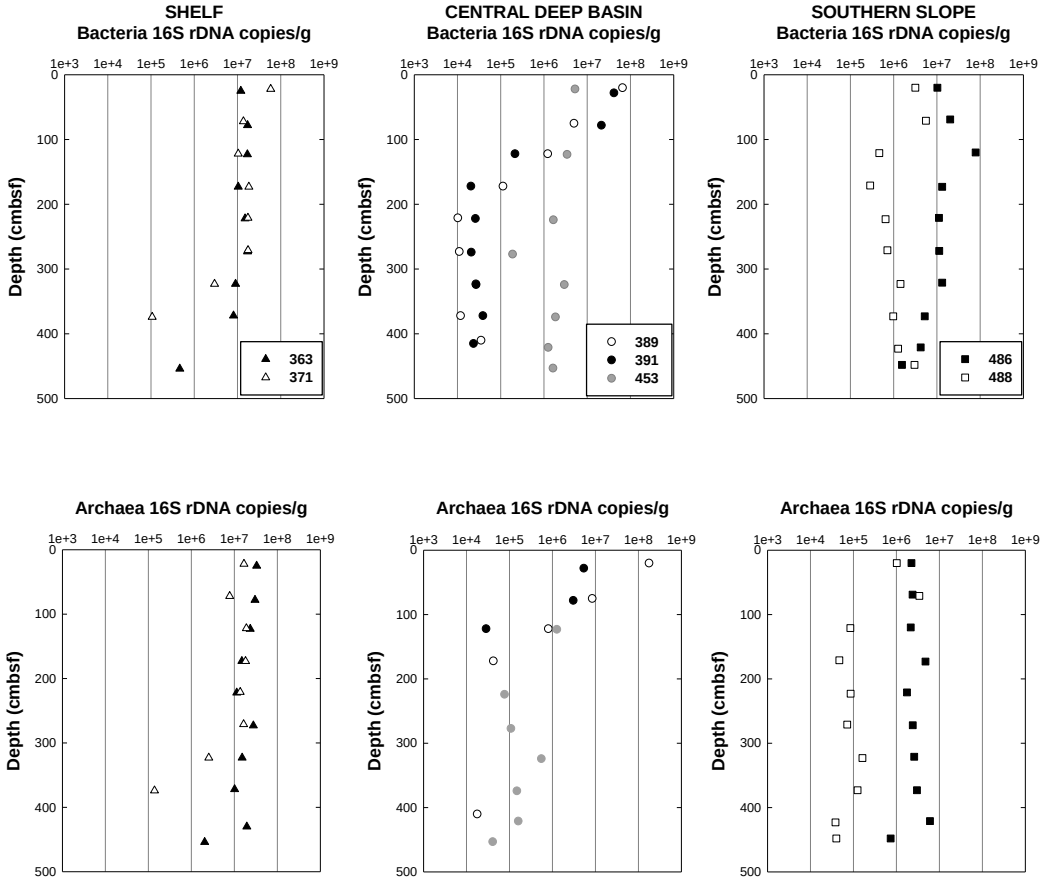

Figure 7.4: Depth profiles of 16S rRNA gene copy numbers per gram of sediment (wet weight) of Bacteria and Archaea for each site under investigation.

7.3.6. Quantification of functional genes and/or physiological groups

Quantification based on the 16S rRNA gene for the bacterial family of the *Geobacteraceae* often able to use Fe^{3+} and Mn^{4+} as terminal electron acceptors (e.g. *Geobacter spp., Desulfuromonas spp.*), was used as a proxy for these well-known metal reducers, which may play an important role in the sediments. The distribution of *Geobacteraceae* 16S rRNA gene copy numbers (Figure 7.5) followed a similar trend like the numbers of total *Bacteria*.

At the shelf sites 363 and 371 16S rRNA gene copy numbers were 10^6 g^{-1} along the depth profiles except in deepest core sections of the profile which showed a decline in numbers. *Geobacteraceae* 16S rRNA gene copy numbers at the Central Deep Basin sites 389 and 391 were highest in the shallowest measured depth of 25 cmbsf, with about 10^7 g^{-1} for site 389 and 10^6 g^{-1} for site 391 but were below detection limits from 125 cmbsf downwards. *Geobacteraceae* 16S rRNA gene copy numbers in site 453 were very low throughout the core. Southern Slope sediments showed very few *Geobacteraceae* 16S rRNA gene copy numbers for both sites. The depth profile of site 486 showed stable copy numbers of 10^5 g^{-1} and site 488 revealed numbers lower than 10^5 g^{-1}.

Q-PCR of the functional genes *dsrA* and *mcrA* was performed to quantify the key functional genes of the environmentally important enzymes dissimilatory sulfite (bi) -reductase (*dsrA*) and the α-subunit of the methyl coenzyme M reductase (*mcrA*). The two enzymes are used as proxies to quantify the physiologically important groups of prokaryotic sulfate-reducers and methanogens, respectively.

In addition, the *mcrA* of the anaerobic methanotrophs ANME-1 and ANME-2 were specifically targeted and quantified. *dsrA* copy numbers in Shelf sediments were about 10^8 g^{-1} along the depth profile except in deepest core sections which showed $10^7 – 10^6$ g^{-1}. Shelf sediments had the highest *dsrA* copy numbers of all areas. Shelf site 371 showed the maximum *dsrA* copy numbers with 10^9 g^{-1} at 25 cmbsf. In the Central Deep Basin site 389 *dsrA* copy numbers were only detected in the upper 125 cmbsf and with values of 10^8 g^{-1} at 25 cmbsf and decreased to 10^7 g^{-1} at 75 cmbsf and further to 10^6 g^{-1} at 125 cmbsf. Central Deep Basin site 391 showed only amplification in the first 75 cmbsf and *dsrA* copy numbers were around 10^7 g^{-1}. The Central Deep Basin site 453 showed overall stable *dsrA* copy numbers with depth of approximately 10^6 g^{-1}. At the Southern Slope site 486, *dsrA* copy numbers were around 10^7 g^{-1} and stable within the depth profile. At site 488 they were

about 10^6 g^{-1} for the uppermost 75 cmbsf and decreased downwards with *dsrA* copy numbers between 10^5 – 10^6 g^{-1}.

The *mcrA* copy numbers were in general very low in all sediments from all areas. Only the Central Deep Basin site 389 at its uppermost depth showed *mcrA* copy numbers higher than 10^5 g^{-1}. ANME-1 and ANME-2 *mcrA* genes were not detected by Q-PCR in any of the samples.

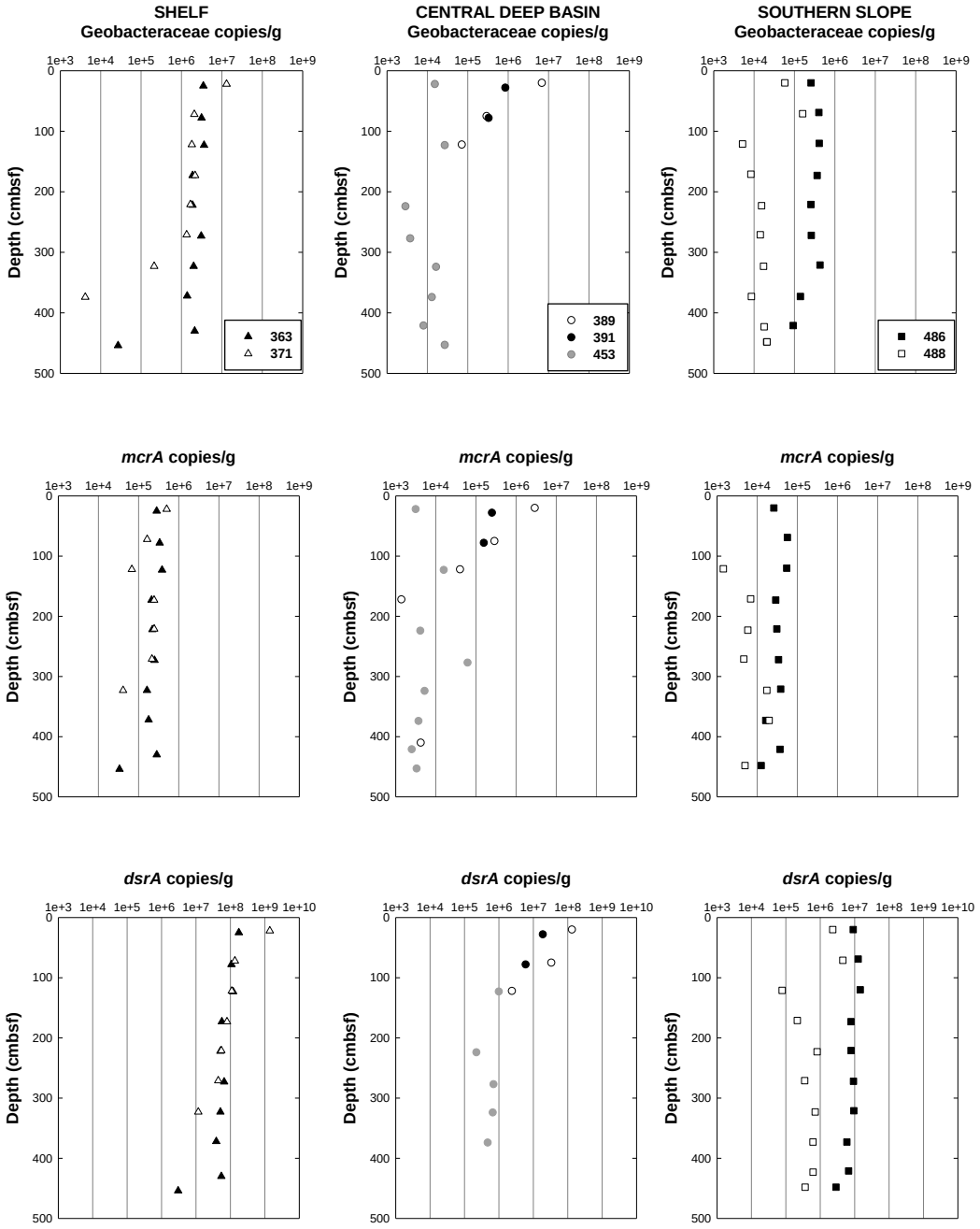

Figure 7.5. Depth profiles of copy numbers of the 16S rRNA gene of the *Geobacteraceae* family and the functional genes *dsrA* and *mcrA* of sulfate-reducing and methane producing microorganisms, respectively.

7.4. Discussion

The Baffin Bay is a remote Arctic area ice-covered most of the year and permanently cold sediments, about which nothing was yet known about the sediment microbiology. Here, we present microbial community and biogeochemical data to survey element cycling in different sites of the Baffin Bay sediments. We could show a major role of sulfur and metal cycling and a minor role of the methane cycle. Interestingly, depth profiles of *Archaea* and *Bacteria* showed different trends depending on the location within the Baffin Bay. The Greenlandic shelf sediments contain vital microbial communities adapted to their specific environmental conditions and a range of different substrates and electron acceptors.

7.4.1. Distribution of *Bacteria* and *Archaea*

Overall, in the analyzed sites of the Baffin Bay, archaeal and bacterial 16S rRNA gene copy numbers were similar in each specific site showing no clear predominance of either *Archaea* or *Bacteria*. Similar numbers of *Archaea* and *Bacteria* at a particular site were previously found by numerous Q-PCR-based studies from marine sediments worldwide e.g. sediments from the Porcupine Seabight at the continental margin of Ireland (Webster et al., 2009), in various sediments from the eastern Juan de la Fuca ridge flank (Engelen et al., 2008), in sediments from the forearc basin at the subduction zone along the southeast off Sumatra (Schippers et al., 2010) and in sediments from the Black Sea and the coast off Namibia (Schippers et al., 2012). Our results with arctic sediments support the hypothesis that generally both prokaryotic domains inhabit marine sediments in similar abundances. This is in agreement with Q-PCR data for surface sediments from the Peru continental margin and the Cascadia Margin, but in contrast to deeper sediment layers at these sites, where *Bacteria* have been observed as the predominant domain inhabiting the subsurface (Inagaki et al., 2006b; Schippers and Neretin, 2006).

Overall, archaeal and bacterial numbers decreased with depth in Baffin Bay sediments. This decline differed among the three study areas and was exponential for the Central Deep Basin Area sites 389 and 391. A similar observation was reported by Sahm et al. (1999), who found decreasing cell numbers down to 30 cm depth in sediment cores collected off Svalbard. This is a common trend in marine sediments (Parkes et al., 1994; Parkes et al., 2000; Schippers and Neretin, 2006) which has been related to a decrease in sediment

porosity and progressive mineralization of organic matter with increasing depth (Parkes et al., 2000; Rebata-Landa and Santamarina, 2006).

In our study, this decline in numbers was somehow also associated with the sediment characteristics for Shelf sediments and in Central Deep Basin sites 389 and 391. In these latter sites, core sections deeper than 175 cmbsf, which corresponded with sandy and silty mud sediments, showed very low cell numbers and near-surface sediments of diatomaceous silty muds showed the highest cell numbers of all profiles.

Generally, the total numbers of microorganisms determined in our study was in accordance with results from other studies on Arctic surface sediments collected off Svalbard, using microscopic techniques, like DAPI cell counts and FISH, which had similar contents of organic matter as in the Baffin Bay (Hubert et al., 2010; Ravenschlag et al., 2001; Sahm et al., 1999).

7.4.2. Mn and Fe as important terminal electron acceptors in the Central Deep Basin

In marine sediments, the mineralization of organic matter follows a sequence of oxidants where oxygen is firstly consumed as it yields the highest energy (Froelich et al., 1979). Here, we did not measure oxygen as we focused on the anaerobic mineralization of the organic matter. Once oxygen is depleted, Mn^{+4} and Fe^{+3} become preferable oxidants for the carbon mineralization (D'Hondt et al., 2004; Froelich et al., 1979). In our study, pore water profiles showed the occurrence of the Mn^{+4} and Fe^{+3}-reduction pathways for the carbon mineralization in the Baffin Bay.

This was most pronounced for the Central Deep Basin sites 389 and 391 sediments. Furthermore, high abundances of *Geobacteraceae* in the Baffin Bay sediments indicated the presence of metal reducing microbial key players. Previous studies (Nickel et al., 2008; Vandieken et al., 2006) showed that Mn^{+4} and Fe^{+3}-reduction is the major pathways for carbon mineralization (up to 98 % of the anaerobic organic matter oxidation) in Arctic near-surface sediments, when low organic matter content due to extended seasonally periods of sea cover and the presence of Mn^{+4} and Fe^{+3} occurs.

However, *Geobacteraceae* abundances could not be correlated to Mn^{+2} and Fe^{+2} porewater profiles. Similarly, in a previous study in sediments from Sumatra, *Geobacteraceae* abundances showed no correlation with metal pore water profiles

(Schippers et al., 2010), indicating that other metal reducers not targeted by the primers used in the present study, apart from the genera *Geobacter* or *Desulfuromonas,* may be involved.

7.4.3. Sulfate as main terminal electron acceptor in Shelf and Southern Slope sediments

Sulfate is a key terminal electron acceptor for the mineralization of organic matter in marine sediments due to its high concentration in seawater (D'Hondt et al., 2002; Froelich et al., 1979; Jørgensen et al., 2001; Muyzer and Stams, 2008; Schulz and Zabel, 2006). Our pore water depth profiles suggested sulfate-reduction as a major pathway for the carbon mineralization in the Shelf, Southern Slope and Central Deep Basin 453 sites. Sulfate concentrations decrease at the majority of the sites, especially at 453, where it was almost completely reduced towards the bottom of the core. Previous pore water studies at the Baffin Bay ODP site 645 showed pore water sulfate concentrations which depleted only at 35 mbsf (Srivastava et al., 1989).

Correspondingly, potential rates of sulfate-reduction without substrate addition, i.e., based on substrates available in the original sediments, were highest in samples from the sites with highest organic matter content. Potential rates of methane production were significantly lower than rates of sulfate-reduction, indicating that the latter is the dominant process for mineralization in these sediments.

Fitting to this, only very low and steady methane concentrations were found at all sites, indicating that the sulfate methane transition zone (SMTZ) was deeper than the bottom of the cores. The methane concentrations observed were comparable to values found in other normal (i.e., without underlying strong sources like gas hydrates or mud volcanoes) marine sediments, like in the Baltic or North Sea (Iversen and Jørgensen, 1985).

Differences in SRR - or microbial activity more generally - between different sites and depths in marine sediments may be explained by a decreasing reactivity and availability of sediment organic matter during burial (Hubert et al., 2010; Westrich and Berner, 1984). It has been demonstrated that *in situ* sulfate-reduction in Arctic sediments is limited by organic matter availability (Arnosti and Jørgensen, 2006; Arnosti et al., 1998; Kostka et al., 1999; Vandieken et al., 2006). Despite permanently cold temperatures (-2 to +4°C), *in situ* processes in Svalbard sediments are not temperature limited, i.e. metabolic rates are similar

to those measured in warmer shelf sediments at lower latitudes (Arnosti et al., 1998; Kostka et al., 1999; Hubert et al., 2009 & 2010).

Such deep SMTZ in Baffin Bay sediments might be a consequence of the low to intermediate TOC content in its sediments caused by seasonal ice-cover in arctic waters restricting the marine primary production to the summer months. This marine organic matter produced in the photic zone might be quickly mineralized in the water column as sediment carbon isotope values indicated a predominantly terrestrial influence of the organic matter for all our studied sites (values of -25 ‰ have been reported for terrestrial organic matter (Sackett, 1964)). Similarly, Baffin Bay ODP site 645 carbon isotope data reported a terrestrial origin of its organic matter (Srivastava et al., 1989). Moreover, the higher TOC content in the shelf sediments compared the other areas indicated that the vicinity to the shore of Greenland contributes to an increased supply of organic matter. The isotopic data implies that such a terrestrial runoff of organic matter most likely accounts for the majority of the organic matter reaching the seabed in the Baffin Bay.

The distribution of sulfate-reducing microorganisms has previously been studied to elucidate the importance of sulfate-reduction in both the carbon and sulfur cycles in Arctic environments off Svalbard (D'Hondt et al., 2002; Jørgensen, 1982; Muyzer and Stams, 2008). Using FISH, it has been shown in these studies that SRB accounted for up to 70 % of the microorganisms in surface sediments down to 30 cm depth.

Here, we used the *dsrA* gene as proxy for the quantification of sulfate-reducing microorganisms. The disulfite reductase (Dsr) is the enzyme catalyzing the last step in sulfate-reduction (Wagner et al., 1998). Due to its presence in all sulfate-reducing microorganisms, the *dsrA* gene has been used as a biomarker to assess the presence of sulfate-reducers in marine sediments (Kondo et al., 2004). The generally high abundance of *dsrA* in all sediment samples revealed an important role of sulfate-reducing microorganisms in the microbial communities, especially in Shelf sediments and Southern Slope site 486.

This correlates well with the sulfate concentrations in porewater and observed TOC depth profiles. Furthermore, substantial potential sulfate-reduction rates were detected in sediment incubations from sites 363 and 389, and to a lesser extent in Southern Slope 486 sediments. These observations correlate with higher abundances of *dsrA* gene copy numbers, indicating an active *in situ* community of sulfate-reducing microorganisms.

Central Deep Basin site 453 did not show significantly higher *dsrA* numbers compared to the abundances found at the other sites, even though decreasing sulfate concentrations in the porewater point to the occurrence of sulfate-reduction as an important biogeochemical process in its sediments. Similarly, relatively low *dsrA* numbers have been previously observed in sediments despite the fact that sulfate depth profiles showed decreased sulfate concentrations, such as in the Peru margin ODP Leg 201 (D'Hondt et al., 2004; Inagaki et al., 2006b; Schippers and Neretin, 2006), the Porcupine Seabight carbonate mound IODP Leg 307 (Webster et al., 2009) and for microbial communities in the subsurface of two basins with turbidite deposition on the continental slope of the Gulf of Mexico (Nunoura et al., 2009). In general, *dsrA* numbers in the Baffin Bay are higher than previously reported for other habitats such as tidal flats in the German Wadden Sea (Wilms et al., 2007), sediments from the Peru continental margin ODP site 1227 and similarly abundant to the near-surface Peru margin SO147 site 2MC (Blazejak and Schippers, 2011; Schippers and Neretin, 2006).

7.4.4. Minor role of the methane cycle in the Baffin Bay

The coenzyme M methyl reductase gene (*mcrA***),** encoding for the key enzyme of methanogenesis and methanotrophy, was used here as a proxy to quantify abundances of methanogenic *Archaea* in the sediment depth profiles low abundances in the Baffin Bay. The low abundances of *mcrA* genes correlated with the low concentrations of methane in the porewater depth profiles. Higher *mcrA* gene copy numbers, which are stable with depth, were found in the Shelf sediments compared to the other study areas, indicating that methanogens are present even though with a relatively low abundance. This methanogenic community is alive as evidenced by the production of methane in the upper-most Shelf sediment microcosms. The still high concentrations of sulfate together with the simultaneous occurrence of high cell numbers of SRB and methanogens in the top sediment layers indicates the availability of non-competitive substrates, like methyl-amines or methyl-thiols (Oremland et al., 1982), for the latter group. In these layers, usually SRB outcompete methanogens due to their much lower threshold for e.g. acetate or hydrogen (Oremland et al., 1982). Only in deeper sediment layers depleted of sulfate, these substrates then become available for methanogens. Interestingly, the microbial communities inhabiting Shelf sediments showed the potential for aliphatic hydrocarbon (hexadecane, hexadecanoic acid)

conversion into methane but not for aromatic hydrocarbons. This is of interest in case that an oil spill occurs in these pristine sediments. Then the autochthonous microbial communities could potentially contribute to oil biodegradation and bioremediation of impacted areas.

Neither aerobic nor sulfate-dependent anaerobic methane oxidation activity was observed in the microcosm experiments. Congruent with the latter, also the Q-PCR assay targeting the mcrA gene of anaerobic methanotrophic Archaea (ANME) did not give evidence for the presence of respective AOM-communities. Also the stable carbon isotopic signature of CH_4 and CO_2 in the porewater gave no indication for methane oxidation.

Altogether, the low *mcrA* numbers, the undetected ANME-1 and ANME-2 methyl coenzyme M reductase gene copies, and the low methane concentrations in the porewater indicate only a minor a role of methanogenesis and methanotrophy, and thus a minor role of the methane cycle in the Baffin Bay. This has also been observed for other marine sites limited in nutrient content (Krüger et al., 2005b).

In conclusion, this comparative study of three different areas within the Baffin Bay revealed that the Shelf sediments are the most active and genetically diverse, which correlate with higher TOC values thus emphasizing the heterotrophic nature of the microbial communities in the subsurface. Knowledge on the element cycles in its sediments and the key microbial players driving them is of great relevance for the future investigation and understanding of important global elemental cycles. In the future, a more exhaustive study on the microbial community structure and diversity with high throughput sequencing analysis, for understanding the changes in marine sediment microbial communities across depth and space within the different areas of the Baffin Bay will be conducted.

ACKNOWLEGDEMENTS

We thank the crew of RV Polarstern and the shipboard scientific party for excellent support during the cruise ARK XXV/3. We particularly thank Thomas Pletsch for help during core sampling on board. Many thanks to Daniela Zoch, Cornelia Struckmeyer, Falk Bratfisch, Sebastian Röther, and Josefine Müller for technical assistance and to Kenneth Wasmund for critical reading of the manuscript. This work was supported by the European Research Council project Microflex, and the German Research Foundation (DFG-SPP1319, grants KR 3311/6-1 and 6-2).

Chapter IIX

Final discussion

8. Final discussion

The focus of this thesis is to show whether and how methanogenic degradation processes of hydrocarbons are performed in different ecosystems. This work makes a comparison between various ecosystems and their inhabiting hydrocarbon-degrading microbial communities distinguished by specific hydrocarbon degradation mechanisms and physiological requirements.

8.1. Methanogenic hydrocarbon degradation processes in coal-rich ecosystems

The knowledge about anaerobic hydrocarbon biodegradation, indigenous methanogenic processes and microbial community composition in lignite-containing coal-bearing basins in Germany (e.g. Beckmann et al., 2011a; Freudenberg et al., 1996; Krüger et al., 2008; Thielemann et al., 2004) as well as worldwide (e.g. Dawson et al., 2012; Flores et al., 2008; Klein et al., 2008; Shimizu et al., 2007; Strąpoć et al., 2011a) is still scarce.

Chapter 3 complemented very well the previous work of Thielemann et al. (2000), both studies focused on the lignite deposits in the Lower Rhine Embayment, next to the Ruhr River Basin, one of the most important mining areas in Germany. Thielemann et al. (2000) calculated methane emission rates from biogenic and thermogenic methane, and documented processes of microbial methane consumption and production as well as abiotic factors which are influence the microbial processes. In the present study, the isotopic composition of CH_4 and CO_2 indicated microbial methane production by acetoclastic methanogenesis. Chapter 3 gives first evidence of the high indigenous microbial diversity of the coal-associated aquifer system and the adjacent ligniteous coal-bearing sediments. With the detailed data set of geochemical analyses, cultivation-based and molecular biological approaches, it was shown that indigenous microbial communities in both ecosystems comprised fermentative, sulfate-, nitrate-, metal-reducing and acetogenic bacteria, as well as acetoclastic and hydrogenotrophic methanogenic archaea. Additionally, relatives affiliated to uncultured *Crenarchaeota* belonging to the *Thermoprotei* subgroup were found in the ligniteous coal-bearing sediments. Similar results showed a study from Krüger et al. (2008) and Beckmann et al. (2011a); (2011b) where both acetoclastic and hydrogenotrophic

methanogens as well as uncultured *Crenarchaeota* were detected in incubations amended with mine timber and hard coal.

The diverse microbial communities enriched from ligniteous coal-bearing sediments were shown to be are able to degrade *in vitro* coal and selected hydrocarbons to methane as the final product. Hitherto, only a limited number of enrichment studies demonstrated the presence of methanogenic archaea and the direct bioconversion of coal to methane (Green et al., 2008; Harris et al., 2008; Krüger et al., 2008; Penner et al., 2010; Thielemann et al., 2004; Ünal et al., 2012; Wawrik et al., 2012). For example, living populations of hydrogenotrophic archaea were reported for coal-mine water collected in the Ruhr River Basin during incubation with hydrogen and fatty acids (Thielemann et al. 2004). Additionally, Harris et al. (2008) incubated low-rank coal under methanogenic conditions and found hydrogenotrophic methanogenesis as dominant methanogenic pathway, as well.

Low-rank coal like lignite and subbituminous coals found in the Lower Rhine Embayment and the Ruhr River Basin are characterized by an high moisture content and high amounts of low-molecular-weight hydrocarbons which are favorable degradable by microorganisms. Thus, these low-rank coals have high potential for coalbed methane exploration (Strąpoć et al., 2011b). A more profound understanding about indigenous coal-associated microbial consortia and methanogenesis processes could help to control and modify *in situ* conditions in order to enhance microbial methane production resulting in increased methane recovery on economic levels.

The here detected microbial community compositions in both groundwater and sediments, and their ability to degrade coal and selected single hydrocarbons substantially increases today´s knowledge about the presence and activity of hydrocarbon-degrading microbial communities *in situ* in coal-associated aquifer systems and the adjacent ligniteous coal-bearing sediments.

8.2. Insights into microbial biodegradation processes in a biodegraded oil reservoir

Oil field microorganisms are subjects of investigation since the beginning of commercial oil production. These investigations focus on the understanding of the transformation of petroleum hydrocarbons by subsurface microbial communities. Todays it

is known that the world's petroleum inventory is dominated by deposits which have been degraded microbially over geological time scales (Roadifer, 1987). Because of that, studies of microbial hydrocarbon degradation strategies are required for the development of microbial technologies for enhanced oil recovery, for countering the effects of biocorrosion of the metal oil field equipment, and to prevent reservoir souring (Nazina et al., 2012). Furthermore, the knowledge of the activity of hydrocarbon-degrading microbial communities and their limits in the different anaerobic ecosystems is central to understanding the global carbon cycle and important processes such as organic matter preservation and subsequent oil formation (Head et al., 2010). Despite of considerably advanced understanding of biodegraded petroleum reservoirs in recent years, the knowledge of the factors which control in-reservoir oil biodegradation, and therefore, the determining microbial processes that led to the biodegradation of crude oil hydrocarbons remains far from being complete (Head et al., 2010).

The results of this thesis get in line with the systematic comparison of the microbial communities associated with biodegraded and non-biodegraded petroleum reservoirs examined in recent years by researchers worldwide.

The Dagang oil field complex located in the Huanghua depression of the Bohai Bay Basin is characterized by mesophilic to thermophilic conditions, relatively low sulfate concentrations, and heavily degraded crude oil. This heavily degraded oil field is an example for the high potential of microbial consortia inhabiting such extreme environment to degrade *n*-alkanes, aromatic fractions of crude oil, specifically alkylbenzenes, alkyltoluenes and light polycyclic aromatic hydrocarbons (PAH), and to use these as energy sources for their survival growth. In this study quantitative analysis of 16S rRNA genes revealed the presence of large numbers of *Bacteria* and *Archaea*, mostly sulfate-reducers and methanogenic archaea. Thermophilic, hydrogenotrophic methanogens related to *Methanobacterium* and relatives of *Methanosarcina* were detected in predominant amounts in the Dagang oil field. These phylogenetic results are complemented with very light $\delta^{13}C$-values of CH_4 and CO_2 indicating that the large methane accumulations originated from hydrogenotrophic methanogenesis.

8.3. Microbial biodegradation of aliphatic and aromatic hydrocarbons under methanogenic conditions

This work shows the successful enrichment of a broad range of novel anaerobic microbial communities growing under methanogenic conditions, from samples of different ecosystems, independent of previous hydrocarbon exposure. These enrichment cultures are amended with selected alkanes and aromatic hydrocarbons as model for oil-related compounds. They are the basis for investigations of the special physiological characteristics, e.g. possible limiting and stimulating factors, and the impact of varying environmental conditions on the conversion of hydrocarbons to methane.

The enrichment cultures obtained from eutrophic lake, freshwater, brackish water, mangroves sediments and from sediments of a terrestrial mud volcano were amended with hexadecane. High methane production rates and stable carbon and hydrogen isotopic signatures of methane, carbon dioxide and water demonstrated the microbial transformation of U-^{13}C-hexadecane to CH_4 and CO_2 in the methanogenic enrichment cultures. The physiological characterization of anaerobic microbial communities in the established enrichment cultures showed a broad tolerance compared to environmental parameters such as salinity, temperature, pressure and the effect of electron acceptors. It was found that low sulfate and elevated trace element concentrations stimulates the methanogenesis. Further, phylogenetic examinations showed equally high abundances of *Bacteria* and *Archaea* and a broad diversity of sulfate-reducing bacteria, methanogenic archaea and uncultured *Thermoplasmatales* and *Thaumarchaeota*. The abundance and widespread distribution of uncultured *Archaea* such as *Thaumarchaeota* in the enrichments of the here studied environments indicate that they play important, still unknown roles in the global ecosystem (Dawson et al., 2006).

The comparison of the diversity of the microorganisms between the enrichment cultures on different hydrocarbons showed varying abundances of the detected microorganisms. Enrichment cultures amended with hexadecane mostly showed the presence of both hydrogenotrophic and acetoclastic methanogens, while the latter dominates. Furthermore, the isotopic discrimination for carbon and hydrogen between substrate and methane was calculated, suggesting a coupling of acetoclastic and hydrogenotrophic methanogenic pathways. The isotopic signatures of carboxylic acids showed the highest extent of labeling during microbial degradation of U-^{13}C-hexadecane in

the *n*-C_{17} fatty acid with methyl groups at C-4, presumably indicating the involvement of syntrophic bacteria (*Syntrophus* sp.).

Additionally, more extensive physiological analyses of selected enrichment cultures from two different ecosystems were performed and compared, to determine possible limiting and stimulating factors affecting microbial hydrocarbon degradation processes. The impact of the addition of ferrihydrite, manganese dioxide, nitrate or sulfate as 'competitive' electron acceptors on hydrocarbon-induced methanogenesis in enrichment cultures obtained from hydrocarbon-contaminated harbor mud from the sea port of Zeebrugge (Belgium) and pristine brackish sediment from the Eckernförde Bay (Germany) were studied. Enrichment cultures were amended with hexadecane, ethylbenzene or naphthalene used as oil-related model compounds. Essential for the hexadecane-induced methanogenesis are the concentration and the solubility of the added electron acceptors. Both ferrihydrite and manganese dioxide are insoluble and stimulated the hexadecane-dependent methanogenesis. It could be shown that ferrihydrite triggered the growth of *Methanosarcina*-related methanogens. Furthermore, nitrate in general and high concentrations of sulfate (>5 mM) inhibited methanogenic conversion of hexadecane and ethylbenzene to methane, while lower concentrations of sulfate (<5 mM) or trivalent iron accelerated methanogenesis in aliphatic and aromatic hydrocarbon-degrading communities. In the naphthalene-degrading enrichment cultures amended with sulfate or nitrate as electron acceptor the methanogenesis played a minor role. Thus, methanogens themselves might be directly involved in the degradation of hexadecane and ethylbenzene, but not of naphthalene degradation. Additionally, methane was removed by anaerobic methanotrophy as an accompanying process. It was demonstrated that the enhanced methanogenic alkane degradation is feasible by the addition of ferrihydrite or sulfate in different geological settings. Metal-reducing *Geobacteraceae* and potential sulfate-reducers as well as *Methanosarcina* could be stimulated.

The study of anaerobic microorganism that degrade aliphatic and aromatic hydrocarbons is of interest for the long-term fate of hydrocarbons as globally abundant forms of organic carbon in anoxic sediments, petroleum reservoirs and other subsurface environments, also for hydrocarbon-contaminated deep aquifers, groundwater and the sea floor after oil spills, for the biogeochemical cycling of methane, and for very reactive intermediates derived from the biodegradation of hydrocarbons (Callaghan, 2013; Widdel et

al., 2006). The functional dynamics of microbial communities are largely responsible for the clean-up of hydrocarbons in the environment (Smith et al., 2013). Because of that, the widespread physiological characterization of hydrocarbon-degrading microbial communities of a given ecosystem is of special interest. The identification of metabolic pathways and the directly involved microorganisms lead to a greater understanding on how the microbial consortia are adapting and responding to environmental changes such as an abrupt hydrocarbon contamination event. Hence, as a future extension to the work presented in this thesis, selected active hexadecane-degrading enrichment cultures were used for continuative experiments investigating stable isotope labeling of DNA and proteins to identify the members of the microbial community benefiting from hydrocarbon degradation, and of the methanogenic key players which are directly involved in this process. Cultures were enriched with the substrates hexadecane, hexadecanoic acid, CO_2 and acetate, all ^{13}C-labeled, to study the several steps of the methanogenic degradation pathways. The results of this experiment will be published soon.

Despite a considerable amount of literature on microbial hydrocarbon degradation, until recently knowledge about which organisms are the most important hydrocarbon degraders in the anoxic environment was still lacking (Head et al., 2006). The widespread comparison among the microbial compositions of the novel hydrocarbon-enriched anaerobic communities obtained from different ecosystems and their special physiological characteristics present in this thesis is performed for the first time. This work gives new insights into methanogenic degradation pathways and possible metabolites, fractionation factors, and the carbon flow in selected hydrocarbon-degrading communities.

8.4. Geochemical and microbiological investigations of Northern Baffin Bay sediments

Because of the extreme northern location and the fact that this region is sea-ice covered most of the year the Northern Baffin Bay is only marginally explored. Until today only few geochemical and microbiological studies have been performed (e.g. Galand et al., 2010; Srivastava et al., 1989). This study was the first comprehensive investigation of sediment collected in this region. The resulting data for the geochemistry of porewater and

sediments along transects within the Bay as well as in several meters long sediment cores, and of the parallel molecular biological investigations are unique.

The Northern Baffin Bay is a remote Arctic area restricted in primary production by seasonal ice cover, with presumably low sedimentation rates, carbon content and microbial activity in its sediments. This study focused on a biogeochemical and microbiological comparison of three different sediment sampling sites – the Shelf region, the Central Deep Basin and the Southern Slope. The Shelf sediments revealed a vital microbial community adapted to their specific environmental conditions based on the high organic carbon content from the close Greenlandic continent consists of highest numbers of *Bacteria* and *Archaea* related to sulfate-reducing prokaryotes and methanogens. The Central Deep Basin and Southern Slope sediments contained less organic carbon and overall lower microbial numbers. In general, all three sampling sites showed evidence for sulfate-reducing activities at the sediment surface because sulfate in major electron acceptor in these marine sediments, but low abundances of methanogenic microorganisms and low concentrations of methane in the sediments. Thus, methanogenesis may play only a minor role in the carbon cycle in the Baffin Bay. Additionally, enrichment cultures amended with Shelf sediment and hexadecane or hexadecanoic acid revealed a potential for microbial degradation of aliphatic hydrocarbons. Future investigations of microbial diversity using 16S rDNA gene analysis are necessary. These deep marine sediments harbor numerous novel phylogenetic lineages of *Archaea* and *Bacteria* which are unknown or uncultured so far. The here presented results of geochemistry and of quantities of microbial groups therefore fill a little but important gap in the global investigations of subsurface microbiology, and at the same time indicates the need for further research on Arctic marine sediments.

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Danksagung

Mein spezieller Dank geht an Dr. Martin Krüger und Prof. Dr. Axel Schippers für die Vergabe und Betreuung dieser Dissertation, für die vielen Freiheiten bei der Arbeit mit unseren Kooperationspartnern und für das Vertrauen in meine Arbeit. Ich bedanke mich ebenfalls für die Möglichkeit an zwei überaus lehrreichen, erfolgreichen und beeindruckenden Forschungsausfahrten teilgenommen haben zu dürfen.

Meinen aufrichtigen Dank möchte ich dem gesamten geomikrobiologischen Team an der BGR aussprechen ohne deren Hilfe diese Arbeit unmöglich gewesen wäre. Daniela Zoch, Gudrun Mengel-Jung, Cornelia Struckmeyer und Holger Probst danke ich für die stets engagierte Unterstützung im Labor.

Bei meinen lieben Kollegen Michael Siegert, Janin Frerichs, Simone Gwosdz, Jana Rakoczy, Jana Sitte und Marco Blöthe bedanke ich mich für die freundschaftliche Atmosphäre am Arbeitsplatz, für alle lehrreichen Diskussionen, die fachliche Hilfe und die vielen privaten Gespräche bei und nach der Arbeit.

Herzlich bedanken möchte ich mich bei der Arbeitsgruppe vom IGÖ Neuherberg die mich jedes Mal so unglaublich herzlich aufnahm. Ich danke Frederick von Netzer, Tillmann Lüders und Katrin Hörmann für die produktive Zusammenarbeit, die großartige Unterstützung im Labor während straffer Zeitpläne und für die diversen Fachgespräche bei dem einen oder anderen Maß Bier.

Bei Hans Richnow und seinem Team am UFZ Leipzig möchte ich mich für die sehr erfolgreiche Zusammenarbeit und die ständige Hilfsbereitschaft in allen Isotopenangelegenheiten bedanken.

Mein größter Dank aber geht an meine Familie auf deren emotionale als auch finanzielle Unterstützung ich mich immer verlassen kann und an meine engsten Freunde die mir in dissertationsbedingten Krisen zur Seite standen und auch stets für genügend Ablenkung sorgten. Ich danke der bezaubernden Dr. Jennifer Chow für´s geduldige Korrekturlesen und Gutzureden und Marie für jeden strategischen Rat.

Und Kai, der meine emotionalen Ausbrüche ungepuffert abbekam und geduldig damit umgehen konnte, danke ich außerdem für all die kleinen alltäglichen Sachen die für mich nicht selbstverständlich sind.

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