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Methanogenic Hydrocarbon Degradation: Evidence from Field and Laboratory Studies

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Key Words

Hydrocarbons · Coal · Oil · Fermentation · Methanogenesis · Methane production rate

Abstract

Microbial transformation of hydrocarbons to methane is an environmentally relevant process taking place in a wide variety of electron acceptor-depleted habitats, from oil reservoirs and coal deposits to contaminated groundwater and deep sediments. Methanogenic hydrocarbon degradation is considered to be a major process in reservoir degradation and one of the main processes responsible for the formation of heavy oil deposits and oil sands. In the absence of external electron acceptors such as oxygen, nitrate, sulfate or Fe(III), fermentation and methanogenesis become the dominant microbial metabolisms. The major end product under these conditions is methane, and the only electron acceptor necessary to sustain the intermediate steps in this process is CO_2, which is itself a net product of the overall reaction. We are summarizing the state of the art and recent advances in methanogenic hydrocarbon degradation research. Both the key microbial groups involved as well as metabolic pathways are described, and we discuss the novel insights into methanogenic hydrocarbon-degrading populations studied in laboratory as well as environmental systems enabled by novel cultivation-based and molecular approaches. Their possible implications on energy resources, bioremediation of contaminated sites, deep-biosphere research, and consequences for atmospheric composition and ultimately climate change are also addressed. © 2016 S. Karger AG, Basel

Introduction

Stable isotope studies carried out in the last two decades demonstrated that large amounts of biogenic methane are formed in oil and coal reservoirs, or contaminated aquifers [Horstad and Larter, 1997; Horstad et al., 1992; Larter, 2006; Thielemann et al., 2004; Weiner and Lovley, 1998]. Oil and coal, which are converted to methane by anaerobic microorganisms in the absence of oxygen (methanogenesis), are most likely the carbon sources for this methane. Microbial degradation of higher hydrocarbons to methane is independent from external electron acceptors besides CO_2, and demands only water as well as small amounts of nutrients and trace elements. Consequently, this methanogenesis provides a suitable model to explain oil biodegradation and gas formation in

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reservoirs without oxidants, like oxygen, nitrate, manganese (IV), iron (III) or sulfate. Reservoir studies suggest that the microbial degradation rates of hydrocarbons to methane at temperatures between 40 and 70°C are in the order of 4–10 kg/m^2/year at the oil-water contact area [Head et al., 2003]. The degrading biota appears to live predominantly at the interface between the oil and the water. Recently, methanogenic Archaea belonging to Methanosarcinales and Methanomicrobiales have been found in water droplets from water-in-oil emulsions in Pitch Lake, Trinidad and Tobago, the biggest asphalt deposit [Meckenstock et al., 2014], suggesting that microhabitats such as these droplets would be enough for in situ methanogenic biodegradation of hydrocarbons to occur.

Until the early 1980s, hydrocarbons were believed to be persistent under anoxic conditions [Atlas, 1981]. In the last few decades, it has been extensively proven that hydrocarbons are biodegradable under strictly anoxic conditions [Dworkin et al., 2006; Fuchs et al., 2011; Gieg et al., 2014; Gray et al., 2010; McInerney et al., 2009; Vogt et al., 2011; Widdel et al., 2010], and there is evidence of ongoing hydrocarbon degradation even in electron-acceptor-depleted environments, such as deep sediments, oil and coal reservoirs or contaminated aquifers, for example, where hydrocarbons represent a significant fraction of the organic matter. In these environments, methanogenesis is the only possible terminal biodegradation process.

Already in the early second half of the 20th century, Bokova [1953] suggested that oil biodegradation linked to methane production could be an important factor in the evolution of oil fields. This was consistent with previous ideas postulated by Kuznetsov [1950], who could isolate bacteria from oil samples but could not demonstrate methanogenic biodegradation of *n*-heptane. Later, Muller [1957] detected methanogenic conversion of longer *n*-alkanes from paraffinic oils. More recently, methanogenic hydrocarbon biodegradation has been identified as one of the main biodegradation processes in subsurface hydrocarbon-dominated environments, such as oil reservoirs [Jiménez et al., 2012; Jones et al., 2008; Milkov, 2010], coal deposits [Green et al., 2008; Krüger et al., 2008; Scott et al., 1994; Zhou et al., 2005] and shales [Krüger et al., 2014]. Biogenic methane represents a considerable amount of the natural gas resources [Rice and Claypool, 1981]. According to estimations by Milkov [2010], in the past, as much as $1{,}883 \times 10^9$ m^3 of methane have potentially been generated by oil biodegradation in bitumen and biodegraded oil reservoirs worldwide through their geological history. Most of the so-produced methane most likely leaked into the atmosphere and ocean, affecting the global carbon cycle and the planetary climate over geological time scales [Milkov, 2010].

Methanogenic hydrocarbon biodegradation has important biotechnological applications. Although it might be unwanted in oil reservoirs, because it decreases oil quality and value, the conversion of entranched oil to methane has been proposed as a method to enhance recovery of carbon in exhausted reservoirs [Gieg et al., 2008]. This transformation is of particular significance, as more than 50% of the original oil cannot be retrieved using conventional technologies [Youssef et al., 2009]. In addition, methanogenic hydrocarbon degradation can contribute to the natural attenuation of hydrocarbon spills in a variety of electron-acceptor-depleted environments, e.g. mineral oil or fuel-contaminated aquifers [Feisthauer et al., 2010, 2012; Gieg et al., 1999], when other electron acceptors such as Fe(III) and sulfate are depleted. Compared to other biodegradation processes, methanogenesis could be easier to sustain, precisely because it does not require any external electron acceptors.

In summary, in recent years, increasing evidence of the occurrence of in situ methanogenic hydrocarbon degradation has been published [Gieg et al., 2010; Gründger et al., 2015; Jiménez et al., 2012; Jones et al., 2008; Milkov, 2011], reflecting its environmental importance. In addition, laboratory experiments and the extensive use of molecular techniques have contributed to gain information about the methanogenic pathways, the conditions affecting methanogenesis and the microorganisms involved. The current review presents a comprehensive overview of the state of the art of methanogenic hydrocarbon degradation with respect to involved microorganisms, metabolic pathways and their environmental distribution and relevance.

Methanogenic Hydrocarbon Degradation

Methanogenic hydrocarbon biodegradation occurs in a series of steps and requires close syntrophic associations between fermentative bacteria and methanogenic Archaea [Zengler et al., 1999]. Syntrophic associations within hydrocarbon-degrading cultures have been reviewed recently [Gieg et al., 2014; Sieber et al., 2012]. In this process, fermentative bacteria (e.g. some Clostridia and Proteobacteria) first transform hydrocarbons into smaller molecules such as short-chain fatty acids, alcohols or H_2. The involvement of *Smithella* and other related genera has gained general acceptance [Gray et al., 2011; Tan et al., 2014; Zengler et al., 1999]. Hydrocarbons first need to

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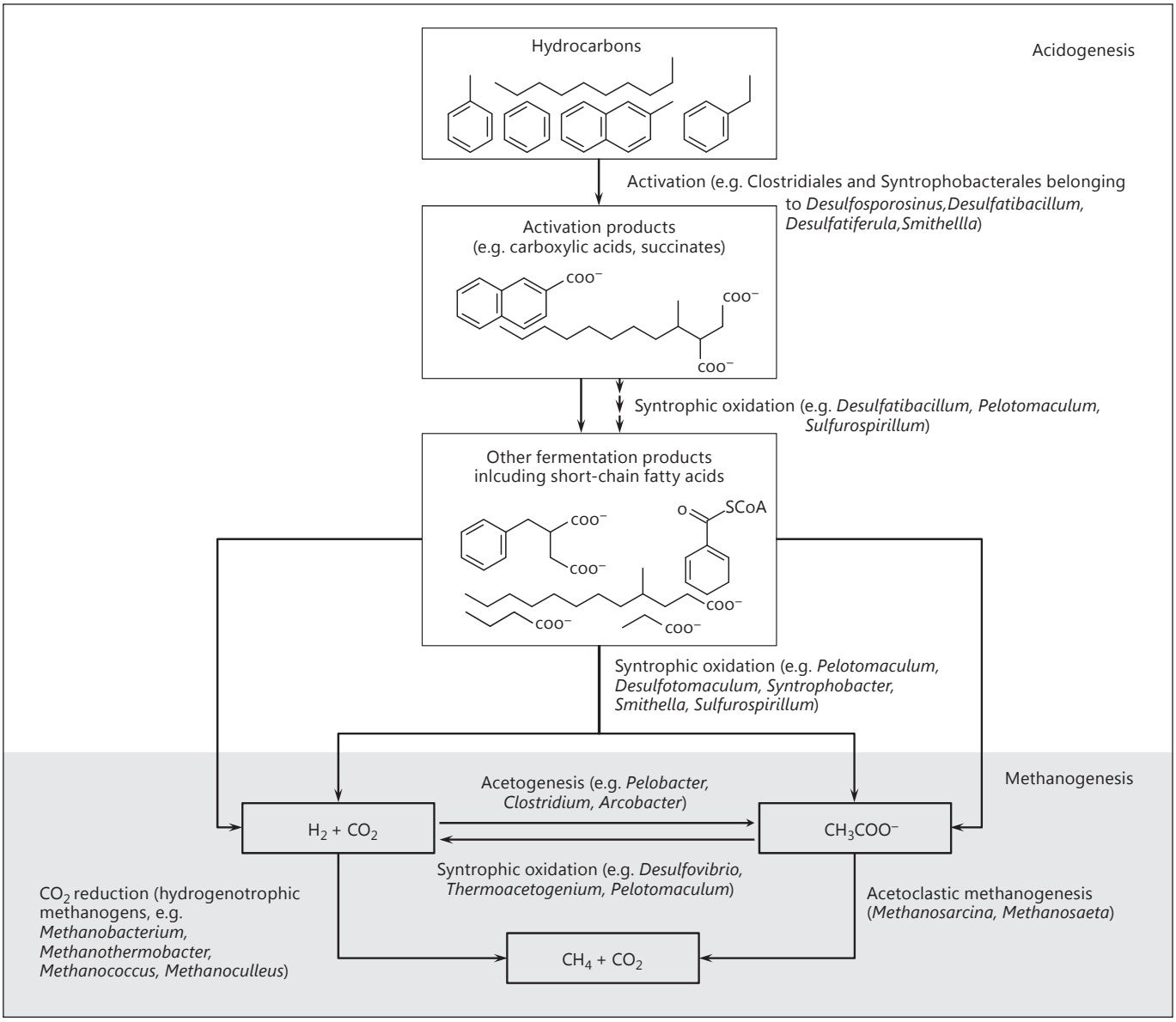

Fig. 1. Schematic diagram of microbial conversion of hydrocarbons to methane.

be activated (e.g. by addition of fumarate) to be further degraded [Heider, 2007; Tan et al., 2014]. Many of the involved reactions are endergonic and only become energetically feasible if the end products (formate, acetate or hydrogen) are kept at relatively low concentrations [McInerney et al., 2008]. According to calculations [Dolfing et al., 2008], the methanogenic transformation of alkanes is possible at hydrogen partial pressures lower than 4×10^{-5} atm. Different groups of methanogenic Archaea, mostly belonging to Methanomicrobia, use those products and transform them to methane and CO_2 through various pathways, mainly CO_2 reduction and acetoclastic methanogenesis (fig. 1).

Hydrogenotrophic microorganisms, e.g. from the genera *Methanobacterium*, *Methanothermobacter*, *Methanocella* and *Methanococcus*, use H_2 as electron donor while reducing CO_2 to methane (equation 1). Interspecies electron transfer through H_2 or formate as electron carriers is a common process. Nevertheless, recent studies reported a direct interspecies electron transfer, in which no electron

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Table 1. Direct and indirect geochemical evidence of biogenic methane in samples collected in field studies

Parameter	Examples	References
Hydrocarbon patterns (e.g. *n*-alkanes and aromatics) consistent with oil biodegradation, heavy oil	Dagang oil field, San Juan, Gippsland and Otway Basins	Aitken et al. 2013; Cai et al., 2015; Head et al., 2003; Jiménez et al., 2012; Jones et al., 2008; Milkov, 2011; Ross et al., 2010; Scott et al., 1994
Heavy $\delta^{13}C\text{-}CO_2$ (> +2‰), and dissolved organic carbon values (> +20‰), indicative of CO_2 reduction to methane	Dagang oil field, San Juan, Gippsland, Otway and Western Siberian Basins, Forest City Basin	Jiménez et al., 2012; Jones et al., 2008; McIntosh et al., 2004, 2008; Milkov, 2010, 2011; Pallasser, 2000; Scott et al., 1994
Relationship between $\delta^{13}C\text{-}CH_4$ and $\delta D\text{-}CH_4$	Forest City Basin, Illinois Basin, Antrim Shale, New Albany Shale	McIntosh et al., 2008; Schoell, 1980; Whiticar, 1999; Whiticar et al., 1986
Relationship between $\delta^{13}C\text{-}CH_4$ and $\delta^{13}C\text{-}CO_2$	Several basins	Milkov, 2011
Linear relationship of δD of CH_4 and H_2O, and fractionation factors, indicative of use of water hydrogen by methanogens to produce methane	Illinois Basin	Martini et al., 1996; McIntosh et al., 2008; Schoell, 1980; Whiticar et al., 1986
Large separations between *n*-alkane homologs (up to +29‰)	Gippsland and Otway Basins	Pallasser, 2000
Dryness, low percentages of C_2^+ hydrocarbons (<2%)	Dagang oil field, Western Siberian Basin	Head et al., 2014; Jiménez et al., 2012; Milkov, 2010, 2011
Relationship between $C_1/(C_2 + C_3)$ and $\delta^{13}C\text{-}CH_4$		
Low acetate concentrations (<1 mM)		
High alkalinity and dissolved organic carbon values (up to 70 mEq/kg)	San Juan Basin, Forest City Basin, Antrim Shale	Formolo et al., 2008; McIntosh et al., 2004., 2008
Ca/Mg ratios (<1.5)	Antrim Shale	McIntosh et al., 2004
Low concentrations of SO_4^{2-} (<10 mM) and other electron acceptors	Dagang oil field, Forest City Basin, Antrim Shale	Jiménez et al., 2012; Martini et al., 1998; McIntosh et al., 2008

shuttle (formate or H_2) is required, between *Geobacter metallireducens* and members of Methanosarcinales, i.e. *Methanosaeta harundinacea* and *Methanosarcina barkeri* [Rotaru et al., 2014a, b]. Acetoclastic methanogens from the genera *Methanosarcina* and *Methanosaeta* utilize acetate as the terminal electron acceptor (equation 2). Acetate can also grow by the conversion of acetate to methane and HCO^{3-} (equation 3) [Dolfing, 2014; Hattori, 2008]. Finally, methylotrophic methanogens, e.g. *Methanosphaera*, *Methanolobus* or *Methanosalsum*, use methylated compounds such as methanol or methylamines instead.

$$CO_2 + 4\,H_2 \rightarrow CH_4 + 2\,H_2O \quad (1)$$
$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (2)$$
$$CH_3COO^- + 2\,H_2O + H^+ \rightarrow 4\,H_2 + 2CO_2 \quad (3)$$

The occurrence of methanogenesis and the predominance of one or another of the methanogenic pathways in the environment may depend on a combination of factors such as temperature, CO_2 concentrations, salinity, pH, availability of electron acceptors and donors and nutrients, porosity and permeability, for example [Dolfing et al., 2008; Kotsyurbenko et al., 2007; Mayumi et al., 2011; Milkov, 2011; Schlegel et al., 2011; Siegert et al., 2011; Waldron et al., 2007]. From the distribution of biodegraded oils worldwide, it seems that reservoirs buried to temperatures of more than 80°C are effectively sterilized with regard to hydrocarbon degraders [Head et al., 2003; Wilhelms et al., 2001]. However, in nutrient-rich environments, such as hydrothermal vents, methanogens can survive at more than 100°C [Takai et al., 2008]. In addition, temperature can select for different methanogenic communities [Blake et al., 2015].

Generally, it has been considered that SO_4^{2-} concentrations above 50 μM cause methanogens to be outcompeted by sulfate-reducing bacteria using the same substrates more efficiently, whereas lower concentrations could even enhance methanogenesis [Siegert et al., 2011].

Regardless of the prevailing pathway, the overall syntrophic reaction yields extremely low Gibbs free energy (around –10 kJ/mol or even less), even below of the predicted minimum increment of energy required for ATP synthesis (15 –20 kJ/mol) [McInerney et al., 2009, and the

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references therein]. Consequently, methanogenic hydrocarbon-degrading microbial communities have typically extremely low growth rates.

Evidence of Microbial Conversion of Hydrocarbons to Methane in situ

Naturally occurring methane includes thermogenic and biogenic gas, which can be identified by distinct isotopic signatures of CH_4 ($\delta^{13}C$ and δD), CO_2 ($\delta^{13}C$) and H_2O (δD) [Whiticar, 1999]. Biogenic methane can also be detected using several other indirect geochemical indicators, including alkalinity, dissolved organic carbon, Ca/Mg ratios, gas dryness or concentrations of CO_2, acetate, SO_4^{2-} or O_2 (table 1) [McIntosh et al., 2008; Rice and Claypool, 1981]. Geochemists differentiate between primary microbial methane, formed by direct decomposition of sedimentary organic matter, and secondary microbial methane, formed during biodegradation of hydrocarbons [Milkov, 2010], either by CO_2 reduction or acetoclastic methanogenesis.

Geological and geochemical studies evidenced the wide distribution of secondary microbial gas in subsurface accumulations from on- and offshore sedimentary basins around the world, including oil reservoirs, coal deposits and shales [Etiope et al., 2009; Jiménez et al., 2012; Jones et al., 2008; Krüger et al., 2008; Martini et al., 2003; Milkov, 2011; Shimizu et al., 2007; Warwick et al., 2008; Zhou et al., 2005]. Production of biogenic methane has been detected in shallow systems such as New Albany and the Upper Devonian Antrim Shales in the Michigan Basin [Shurr and Ridgley, 2002]. Scott et al. [1994] calculated that around 15–30% of the coalbed gas in the San Juan Basin (USA) would derive from the biodegradation of wet-gas components, *n*-alkanes and other organic compounds at relatively low temperatures. Milkov [2010] estimated that a significant part of the shallow dry gas (>99% of methane) in the northern West Siberian Basin (which accounts for 17% of the world's conventional gas endowment) originated from the methanogenic biodegradation of petroleum. Oil legs from this basin were highly degraded and the isotopic signature of CO_2 suggested conversion of 40–70% oil-derived CO_2 to methane [Milkov, 2010].

Bacterial CO_2 reduction is coupled to a kinetic isotope effect, as the lighter carbon stable isotope (^{12}C) is preferentially used [Whiticar, 1999]. This effect produces enrichment in the $\delta^{13}C$ of the remaining pool of CO_2 (with values >0), while the formed product CH_4 becomes lighter (with $\delta^{13}C$-CH_4 sometimes as negative as –110‰ vs. Vienna Pee Dee Belemnite, VPDB) [Milkov, 2011]. 'Heavy' CO_2, enriched in ^{13}C, typically found in strongly biodegraded reservoirs, thus indicates an extensive reduction of CO_2 to methane [Jiménez et al., 2012; Jones et al., 2008; Pallasser, 2000]. In contrast, the hydrogen isotopic discrimination for this pathway is low (δD between –170 and –250‰ relative to the Vienna Standard Mean Ocean Water, VSMOW) compared to other biological methanogenic pathways [Milkov, 2011]. Model studies [Morris et al., 2012] have shown that the concurrence of acetoclastic and hydrogenotrophic methanogenesis can lead to isotopically relatively heavy methane and low discrimination between CO_2 and CH_4, so CO_2 and CH_4 isotopic patterns should be interpreted with caution.

Combining several geochemical indexes allows identifying in situ methanogenic activities and distinct methanogenic pathways, and may be particularly useful when biogenic and thermogenic gases co-occur. For example, Martini et al. [1998] observed $\delta^{13}C$-CH_4 values consistent with a thermogenic or mixed gas (between –56 and –47‰) in the Antrim Shale. However, only the occurrence of microbial transformations could explain the unusually high $\delta^{13}C$ values of CO_2 coproduced with methane (+22‰) and dissolved inorganic carbon in formation waters (+28‰). In a recent study, the isotopic signatures of gases and fluids sampled from a water-flooded oil reservoir in Dagang, PR China, together with the discrimination between CH_4 and CO_2 (32–65‰) and the dryness C1/(C2+C3) and oil gas chromatographic mass spectrometry profiles exhibiting typical biodegradation patterns, i.e. lack of *n*-alkanes or changes in biomarker signatures, indicated an extensive biotransformation of oil to methane [Cai et al., 2015; Jiménez et al., 2012]. Biogenic gas, predominantly deriving from CO_2 reduction, was also predominant in a coal-associated sedimentary basin, as determined by the isotopic signatures ($\delta^{13}C$ and δD) of CO_2 and methane [Gründger et al., 2015]. Biogenic methane production has also been observed in hydrocarbon-contaminated aquifers after spills or leakage of hydrocarbon-containing waste [Feisthauer et al., 2012]. The hydrogen and carbon isotopic compositions were consistent with a predominance of acetoclastic methanogenesis [Feisthauer et al., 2010, 2012].

In vitro Studies

Despite the growing evidence of methanogenic hydrocarbon degradation in situ, until quite recently, microbial transformation of hydrocarbons under methanogen-

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ic conditions had been hardly investigated, although the first reports of methane production by microbial cultures from oil field core materials were published more than 60 years ago [Bokova, 1953; Ekzercev, 1960]. These studies suggested that methane would be formed by the biodegradation of fatty acids present in oil to CO_2 and methane, and from the reduction of CO_2 by H_2. In the last decades, however, the topic has regained scientific attention and many studies attempting to explain the mechanistic aspects and to identify key microorganisms involved have been conducted. The conversion of hydrocarbons to methane has been extensively demonstrated in laboratory experiments (table 2) [Cai et al., 2015; Feisthauer et al., 2010; Gray et al., 2011; Gründger et al., 2015; Jiménez et al., 2012; Jones et al., 2008; Siegert et al., 2011; Tan et al., 2013; Wang et al., 2011; Zengler et al., 1999].

A famous experiment evidencing microbial transformation of *n*-alkanes to methane under strictly anoxic conditions is that of Zengler et al. [1999]. The authors incubated samples in sulfate-free mineral medium inoculated with anoxic ditch sediments spiked with 1.7 mmol *n*-hexadecane, and observed increasing methane formation (up to 4.6 mmol) linked to *n*-hexadecane consumption (0.059 mmol) in 810 days of incubation. The use of a ^{13}C-labeled substrate confirmed that the CH_4 derived from *n*-hexadecane, mainly through acetoclastic methanogenesis. This proved that the complete mineralization of *n*-hexadecane to methane and CO_2 (which the authors proposed to take place in different steps leading to the net reaction described in equation 4) is thermodynamically feasible, even if the energy yield is lower than in other hydrocarbon-degrading reactions [Dolfing et al., 2008; Spormann and Widdel, 2000].

$$4\ C_{16}H_{34} + 30\ H_2O \rightarrow 49\ CH_4 + 15\ CO_2 \quad \Delta G = -1{,}596\ kJ \quad (4)$$

A subsequent study by Feisthauer et al. [2010] determined the stable carbon and hydrogen isotopic signatures of methane, CO_2 and water during microbial formation of methane from *n*-hexadecane. The narrow ranges for the carbon and hydrogen isotopic discrimination between substrate and methane suggested a co-occurrence of acetoclastic and CO_2-reducing methanogenesis.

After the pioneering study by Zengler et al. [1999], biodegradation of *n*-hexadecane under methanogenic conditions has been repeatedly documented [Anderson and Lovley, 2000; Jiménez et al., 2012; Siegert et al., 2011]. In addition, several other alkanes have also proven to be converted to methane and CO_2. This includes short- to medium-length compounds [Siddique et al., 2006, 2011] or long ones, like *n*-C_{28} or *n*-C_{32} (tables 2, 3). Siddique et al. [2006] reported methanogenic biodegradation of short-chain *n*-alkanes by enrichment cultures from oil sands tailings taking place in an unusual sequence (C10 > C8 > C7 > C6). The authors postulate that this might be caused by an increase in the octanol/water partition coefficient *(Kow)* with increasing molecular weight. Another explanation might be a sort of selective transport across cell membranes of the *n*-alkane-degrading microorganisms as proposed by Kim et al. [2002]. Although gaseous *n*-alkanes can also be oxidized under anoxic conditions [see Musat et al., this volume, pp 211–226], their transformation under methanogenic conditions has not been shown yet.

Linear alkanes are the most readily degraded hydrocarbons. This would explain why *n*-alkane-rich light crude oil provides higher methanogenic yields as compared to other complex hydrocarbon substrates (table 3). Enrichment cultures from the Dagang oil reservoir were able to degrade all medium- and long-chained *n*-alkanes (*n*-C_{10} to *n*-C_{36}) from crude oil in less than 200 days, producing methane at a rate of 76 ± 6 μmol/day/g oil added [Cai et al., 2015], paralleling previous observations [Gieg et al., 2008; Jones et al., 2008]. The presence of abundant aliphatic constituents in kerogen-rich Posidonia and Alum shale samples (reflected by high hydrogen index values) determined its potential as methanogenic substrate for methanogenic hydrocarbon-degrading enrichment cultures [Krüger et al., 2014]. In this case, methane production (ranging from 1 to 10^3 nmol/day/g TOC) was influenced by the quality of the TOC and inversely correlated to the thermal maturity of the organic matter.

Recently, methanogenic biodegradation of C_7 and C_8 iso-alkanes (methylhexanes and heptanes) was demonstrated [Abu Laban et al., 2014]. The biodegradation was found to be isomer specific, consistent with some previous in situ observations [Jiménez et al., 2012], and some of the compounds could only be degraded (probably cometabolically) when in iso-alkane mixtures. The presence of putative succinylated iso-alkane metabolites suggested fumarate addition as activation mechanism.

Some aromatic hydrocarbons, such as benzene, toluene, *o*-xylene, ethylbenzene or 2-methylnapthalene, have also proven to be biodegradable under methanogenic conditions [Abu Laban et al., 2015; Edwards and Grbić-Galić, 1994; Feisthauer et al., 2010; Fowler et al., 2012, 2014; Jiménez et al., 2012; Siegert et al., 2011; Sun et al., 2014]. Siegert et al. [2011] observed methane production (up to 58.1 nmol CH_4/ml/day) in enrichment cultures of hydrocarbon-contaminated harbor sediments growing with ethylbenzene. However, no methane was produced when

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Table 2. Overview of methanogenic hydrocarbon-degrading consortia, growing on coal, oil and single hydrocarbons

Substrate	Origin of the inoculum, description of the habitat	Incubation temperature (if available), remarks	Identified taxa (if available)	References[a]
Light oil, heavy oil, *n*-alkanes (*n*-C_{12}–*n*-C_{18})	Plusssee, Germany; Eutrophic lake with stable anoxic hypolimnion; sample depth: 28 m	28° C	–	Eller et al., 2005
Light oil, heavy oil, *n*-alkanes (*n*-C_{12}–*n*-C_{18})	Eckernförde Bay, Germany; Baltic Sea brackish water; sample depth: 28 m	28° C	–	Feisthauer et al., 2010; Siegert et al., 2011
n-hexadecane	Bremen, Germany; freshwater ditch		*Syntrophus, Methanosaeta, Methanoculleus, Methanospirillum*	Zengler et al., 1999
Light oil, heavy oil, shales, coal, timber, *n*-alkanes (*n*-C_{10}–*n*-C_{32})	Kuhgraben, Bremen, Germany; another freshwater ditch; sample depth: 2 m; in situ temperature during sampling: 25° C	28° C, active enrichment on *n*-C_{32}	*Syntrophus, Methanoculleus, Methanospirillum*	Feisthauer et al., 2010; Krüger et al., 2014; Siegert et al., 2011
Light oil, heavy oil, *n*-hexadecane, BTEX	Brazil; sample from intertidal sediments of brackish water mangroves	28° C	–	–
n-hexadecane, ethylbenzene	Zeebrugge Harbor, Belgium; heavy metals and oil-contaminated sediments. Sample depth: 3 m	28° C	–	Siegert et al., 2011
n-hexadecane, BTEX	Weissandt-Gölzau, Germany; gas samples from groundwater aquifer contaminated with crude oil	28° C	–	Feisthauer et al., 2010
Coal, *n*-hexadecane, BTEX	Ruhr Basin, Germany; groundwater and coal-bearing sediments from an open-pit brown coal mine	30° C	*Desulfomonile, Smithella, Desulforhopalus, Desulfatiferula, Pseudomonas, Acetobacterium, Nocardiodaceae, Anaerolineaceae, Deferribacteres*	Gründger et al., 2015
Coal, timber	Ruhr Basin, Germany; timber and coal from a coal mine closed since 1960s; sample depth: 800 m	30° C	*Clostridium, Desulfovibrio, Pelobacter, Burkholderia, Geobacteraceae, Hydrogenophaga, Methanosarcina*	Beckmann et al., 2011; Krüger et al., 2008
Light oil, *n*-hexadecane, toluene	Arctic sediments from the Baltic Sea	4° C	–	Algora et al., 2013, 2015
n-hexadecane	Lusi Mud Vulcano, Sidoarjo village, Northern Java, Indonesia; mud samples from the framing dike of the crater	30° C, very active	–	Mazzini et al., 2007, 2012
n-hexadecane	Paclele Mici volcano, Romania; mud volcano field with naturally occurring oil seepage	30° C	–	Alain et al., 2006; Feisthauer et al., 2010
Light oil, heavy oil, *n*-hexadecane, BTEX, 2-methylnaphthalene	Dagang oil field, PR China; well-head samples from wells from a water-flooded medium- to high-temperature (30–80° C) reservoir	30° C	*Pseudomonas, Smithella, Syntrophorhabdus, Syntrophobacter, Desulfobulbus, Methanosaeta, Methanoculleus, Methanofollis, Thermoplasmata*	Cai et al., 2015; Jiménez et al., 2012, 2015
Oil, *n*-hexadecane, *n*-hexadecanoic acid, *n*-octadecanoic acid	South Platte alluvial aquifer in Weld County near Denver, Colo., USA; gas condensate-contaminated groundwater sediments	21° C	*Clostridium, Desulfobulbus, Desulfatibacillum, Desulfotomaculum, Desulfovibrio, Syntrophus, Methanoculleus, Methanospirillum, Methanosaeta, Methanosarcina*	Gieg et al., 2008; Morris et al., 2012; Townsend et al., 2003
2-Methylnapthalene, 2,6-dimethylnaphthalene	South Platte alluvial aquifer in Weld County; gas condensate-contaminated groundwater sediments	21–23° C	*Clostridium, Desulfobulbus, Desulfovibrio, Methanoculleus, Methanosaeta, Methanolinea*	Berdugo-Clavijo et al., 2012; Gieg et al., 2008; Townsend et al., 2003
Toluene	South Platte alluvial aquifer in Weld County; gas condensate-contaminated groundwater sediments	21° C	*Desulfosporosinus, Syntrophaceae, Desulfovibrionales, Chloroflexi, Spirochaetes, Methanoculleus, Methanolinea, Methanosaeta*	Fowler et al., 2012, 2014; Gieg et al., 1999
Oil	Medicine Hat, Alta., Canada; Glauconitic C low-temperature (30° C) oil field subjected to nitrate injection for souring control	33° C	*Smithella, Pseudomonas, Methanosaeta, Methanoculleus, Methanobacterium*	Berdugo-Clavijo and Gieg, 2014

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Table 2 (continued)

Substrate	Origin of the inoculum, description of the habitat	Incubation temperature (if available), remarks	Identified taxa (if available)	References[a]
n-alkanes (*n*-C_{15}–*n*-C_{20})	Shengli oilfield, PR China; well head samples from a production well at 70° C 2,058 m in depth	37 and 55° C	*Firmicutes, Thermodesulfobiaceae, Thermotogaceae, Nitrospiraceae, Dictyoglomaceae, Archaeoglobales*	Mbadinga et al., 2012; Wang et al., 2011; Zhou et al., 2012
Benzene, toluene	Ferulic-acid-degrading sewage sludge	–	–	Grbić-Galić and Vogel, 1987; Grbić-Galić and Young, 1985
Benzene	Tsuchiura, Japan; lotus field soil	25° C	*Clostridium, Methanoculleus, Methanoregula, Methanosaeta, Thermoplasmata*	Sakai et al., 2009
Benzene	Forested sandy alluvium area in Glen Falls, N.Y., USA; coal tar waste-contaminated sediments	–	*Pseudomonas, Pelomonas, Delftia*	Liou et al., 2008
Oil	Newcastle, UK; Tyne River sediments	–	*Smithella, Marinobacter, Thauera, Methanocalculus, Methanogenium, Methanomicrobiaceae*	Gray et al., 2011; Jones et al., 2008
n-alkanes (*n*C_{14}–*n*C_{18}), BTEX, naphtha	Mildred Lake Settling Basin, Alta., Canada; oil sands tailings ponds	20° C	*Syntrophus, Desulfuromonas, Desulfobacterales, Methanosaeta, Methanoculleus*	Siddique et al., 2006, 2011
n-alkanes (*n*C_6–*n*C_{10}), 2-methyl-pentane, 2-methylcyclopentane	Mildred Lake Settling Basin; Oil sands tailings ponds	28° C	*Peptococcaceae, Anaerolineaceae, Desulfobacteraceae, Smithella, Syntrophus, Methanosaeta, Methanoculleus*	Abu Laban et al., 2014; Siddique et al., 2006; Tan et al., 2013, 2015
Benzene, napthalene, phenanthrene	Baltimore Harbor, Md., USA; Harbor sediments	30° C	*Aquificae, Bacteroidetes, Thermotogae, Clostridia, Pseudomonas, Methanosarcina, Methanoculleus, Methanococcus*	Chang et al., 2005a, b
Phenanthrene, anthracene	Landfield leachate contaminated sediments	20° C	*Methylibium, Legionella, Rhizobiales*	Zhang et al., 2012a, b
n-alkanes (C_{28}–C_{50})	San Diego Bay, Calif., USA; bay sediment	Long-chained *n*-alkanes	*Smithella, Methanoculleus, Methanosaeta*	Marks et al., 2015

[a] Referring to the sampling site, the enrichment culture or both.

Table 3. Methane production rates in methanogenic enrichment cultures originating from freshwater ditch sediments supplemented with different hydrocarbon substrates

Substrate added	CH_4 production rate, nmol CH_4/g TOC/day
Light oil	360–420
Heavy oil	170–250
Lignite	110–150
Hard coal	50–60
Shale (Posidonia)	140–190
PAH (2-methylnaphthalene)	20–50
BTEX (ethylbenzene, toluene)	30–70
Paraffin (*n*-C_{32})	40–70
n-alkanes (C_{12}–C_{18})	350–560

naphthalene was the only substrate added (similar results were later obtained by Berdugo-Clavijo et al. [2012]). The authors concluded that methanogens may not directly participate in the degradation of naphthalene, as previous studies suggested toxicity of this compound for methanogenic microbiota [Sharak Genthner et al., 1997].

However, alkylated naphthalenes can be biodegraded under methanogenic conditions. The conversion of 2-methylnaphthalene to methane by enrichment cultures from Dagang was confirmed in stable isotope tracer experiments by production of ^{13}C-labeled CO_2 and methane from ^{13}C-labeled 2-methylnaphthalene [Jiménez et al., 2012]. Berdugo-Clavijo et al. [2012] reported significant methane production (up to 400 μmol) in crude-oil-degrading enrichment cultures amended with 2-methylnaphthalene or 2,6-dimethylnaphthalene and identified putative metabolites (2-naphthoic acid and 6-methyl-

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2-naphthoic acid) indicating the biodegradation of these substrates.

Methanogenic biodegradation of higher polycyclic aromatic hydrocarbons (PAHs) seems to be energetically feasible as well [Dolfing et al., 2009], and it is likely occurring in situ in highly biodegraded oil reservoirs. However, to date, very few studies have reported biodegradation of tricyclic PAH (phenanthrene or anthracene) under methanogenic conditions [Chang et al., 2005a; Zhang et al., 2012a, b], and the degradation of tetra- or pentacyclic PAHs remains to be demonstrated. Similarly, methanogenic biodegradation of heterocyclic hydrocarbons (like carbazoles) can probably take place in situ, as patterns consistent with biodegradation have been observed [Huang et al., 2003], but there is a general lack of knowledge of the associated mechanisms.

The use of coal as methanogenic substrate has also been demonstrated in laboratory experiments [Harris et al., 2008; Krüger et al., 2008; Orem et al., 2010], although coal becomes more recalcitrant to degradation with increasing thermal maturity [Strąpoć et al., 2011]. Coal contains low-molecular-weight components like hydrocarbons and naphthenic acids, which might partly act as carbon substrates for methanogenesis. Holowenko et al. [2001] observed an increase in methane production with the addition of simple surrogate naphthenic acids (3-cyclohexylpropanoic acid or 4-cyclohexylbutanoic acid) to microcosms that contained a Base Mine Lake fine tailing sample, which the authors attributed to the biodegradation of the lateral chain. However, higher naphthenic acids inhibited the degradation process under these conditions.

In another study, crushed core material from a reservoir was applied to provide a solid surface for the microbial community and nutrients, and enhance their access to sedimentary organic matter and crude oil [Gieg et al., 2008, 2010]. Solid particles (sand, lava or amberlite) were also required to sustain the degrading capacity of a sulfidogenic benzene-degrading consortium [Herrmann et al., 2008], as physiologically active microorganisms tended to grow attached to the surfaces. Solid surfaces may improve substrate availability and biofilm formation, enhancing the biodegradation of hydrocarbons. Nevertheless, microbial consortia can also be successfully shifted to oil-free and solid matrix-free culture media for experiments with labeled and nonlabeled substrates such as hydrocarbons and carboxylic acids [Morris et al., 2012].

Methanogenesis does not require the presence of external electron acceptors, but low concentrations of sulfate and Fe(III) could support methane formation. For example ferrihydrate triggers growth of *Methanosarcina*-related methanogens [Siegert et al., 2011]. However, methanogenesis may be negatively affected by sulfate and nitrate at concentrations of more than 5 and 1 mM, respectively [Siegert et al., 2011].

Microbial Ecology and Molecular Biology of Methanogenic Hydrocarbon Degradation

Hydrocarbon degradation by methanogenic microbial communities has been extensively proven in microcosm studies (see previous section). However, cultivation approaches may result in a biased representation of active or highly abundant microorganisms [Amann et al., 1995] as the biogeochemical conditions in laboratory microcosms usually differ from in situ conditions. Generally, community members in laboratory enrichment cultures and in natural habitats are identified by molecular techniques. The extensive use of metagenomics, metatranscriptomics and metaproteomics aims at circumventing isolation and cultivation biases and provides information about abundant noncultivated taxa of which the functions are often completely unknown [Rappé and Giovannoni, 2003]. These tools can also be used to identify key genes related to the function of methanogenic hydrocarbon-degrading communities, from the peripheral hydrocarbon degradation catabolism, such as *assA* or *bssA*, to the methane-generating pathway, e.g. *mcrA* [Callaghan et al., 2010; Kuntze et al., 2008; Steinberg and Regan, 2009; Tan et al., 2015; von Netzer et al., 2013]. Further information about functional genes for aromatic hydrocarbon degradation, such as those involved in fumarate addition, is summarized by von Netzer et al. [this volume, pp 180–194].

The analysis of concentrations of the methyl-coenzyme M reductase F430 prosthetic group and its isotopic signatures allows detecting and quantifying methanogenic microorganisms in environmental samples, and simultaneously provides an idea about their substrates [Kaneko et al., 2014; Takano et al., 2013].

Molecular studies have allowed the detection of a variety of microorganisms from oil reservoirs and other hydrocarbon-impacted environments, including aerobes, microaerophilic taxa, fermenters, sulfate reducers and methanogens [An et al., 2013b; Magot et al., 2000], many of which have never been cultivated. The composition of these hydrocarbon-degrading microbial communities seems to be determined by the availability of electron acceptors [Head et al., 2014]. Many of the phylotypes iden-

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tified in methanogenic hydrocarbon-degrading microbial communities from a variety of habitats are similar. Firmicutes and Proteobacteria (particularly Gamma-, Delta- and Epsilonproteobacteria), mostly, but also Bacteroidetes and Spirochaetes are frequently found in coalbeds, oil reservoirs and other hydrocarbon-bearing systems [Gray et al., 2010; Strąpoć et al., 2011].

According to An et al. [2013a], peripheral degradation pathways (hydroxylation, carboxylation and fumarate addition) would be performed by Proteobacteria, Firmicutes and Actinobacteria. Among the Deltaproteobacteria, *Smithella*, *Syntrophus* and other related genera belonging to the Syntrophaceae are often observed in methanogenic microbial communities from hydrocarbon-bearing systems [Johnson et al., 2015; Ramos-Padrón et al., 2011; Shimizu et al., 2007; Siddique et al., 2011] and are frequently enriched in methanogenic cultures (table 2). These organisms seem to be involved in the methanogenic degradation of *n*-alkanes [Zengler et al. 1999; Gray et al. 2011]. Gründger et al. [2015] reported an enrichment of Syntrophaceae (affiliated to the genera *Smithella* and *Desulfomonile*) in methanogenic *n*-hexadecane-degrading cultures from coal-bearing sediments. Moreover, Gray et al. [2011] observed a predominance of *Smithella* in oil-degrading methanogenic enrichment cultures and its exponential growth in parallel to alkane degradation and methane accumulation, whereas *Marinobacter*, a known hydrocarbon degrader, did not participate in the biodegradation. In addition, alkylsuccinate genes *(assA)* closely related to those of *Smithella* spp. were found to be expressed in a methanogenic *n*-octacosane-degrading (n-C_{28}) enrichment culture [Marks et al., 2015].

Several sulfate-reducing hydrocarbon-degrading Deltaproteobacteria, also belonging to the Syntrophobacterales, e.g. *Desulfoglaeba alkanexedens* [Davidova et al., 2006], or Desulfobacterales, e.g. *Desulfatibacillum* spp. [Callaghan et al., 2012; Cravo-Laureau et al., 2005], *Desulfobacula toluolica* [Wöhlbrand et al., 2013], *Desulfotignum toluenicum* [Ommedal and Torsvik, 2007] or *Desulfatiferula olefinivorans* [Cravo-Laureau et al., 2007], have been isolated and described. Some studies have shown the ability of sulfate reducers to grow syntrophically by fermentation when sulfate concentrations are too low. A coculture of the *n*-hexadecane-degrading *Desulfatibacillum alkenivorans* with a hydrogenotrophic methanogen yielded methane [Callaghan et al., 2012]. Also *Desulfatiferula* has been identified in methanogenic enrichment cultures [Gründger et al., 2015]. Sulfate reducers in relative high abundance were detected by quantitative PCR of the *dsrA* genes in the Dagang oil field, which is a low-sulfate environment [Jiménez et al., 2012].

Gammaproteobacteria have also been frequently detected in hydrocarbon-rich environments [Gray et al., 2010]. Particularly, *Pseudomonas* has been found to be abundant in coal-bearing sediments [Gründger et al., 2015; Penner et al., 2010] and in production waters from different oil reservoirs [Cai et al., 2015; Nazina et al., 2006; Ren et al., 2011]. This genus has conventionally been considered aerobic or facultative anaerobic using nitrate as electron acceptor. However, Berdugo-Clavijo and Gieg [2014] observed an enrichment of *Pseudomonas* in a methanogenic oil-degrading enrichment culture, suggesting that this genus may have to grow syntrophically. In addition, molecular analyses (i.e. 454-pyrosequencing and cloning) confirmed that syntrophic (*Smithella* and related taxa) together with other hydrocarbon-degrading (e.g. *Pseudomonas* and *Thauera*) bacteria were among the most represented bacterial phylotypes in methanogenic oil- and 2-methylnaphthalene-degrading enrichment cultures from the Dagang oil field [Jiménez et al., 2015].

Members of Epsilonproteobacteria might be involved in the fermentation of hydrocarbons as well. Acetotrophic and acetogenic *Arcobacter* species, together with fermenters belonging to *Sulfurospirillum,* have often been detected in oil reservoirs or coal deposits, such as coal-bearing sediments from the Ruhr area [Gründger et al., 2015], the Waikato Basin in New Zealand [Fry et al., 2009] or the Pelican Lake oil reservoir in Alberta, Canada [Grabowski et al., 2005a, b], among many others [Gray et al., 2010; Head et al., 2014; Hubert et al., 2012].

Firmicutes belonging to Clostridiales have been detected in several oil reservoirs [Mochimaru et al., 2007], tailing ponds [Ramos-Padrón et al., 2011] and methanogenic enrichment cultures (table 2), and could take part in the fermentation of hydrocarbons. Among them, *Pelotomaculum* species are considered strict syntrophs, as they can only oxidize propionate (an intermediate of hydrocarbon fermentation) when cocultured with a H_2 scavenger. In addition, several *Pelotomaculum*-related phylotypes have been identified as syntrophic benzene degraders [for an overview, see Vogt et al., 2011], so this substrate might also be a niche for Clostridiales.

Archaeal communities are often less abundant than bacteria [Jiménez et al., 2012; Orphan et al., 2000] and usually less diverse, with just a few predominant operational taxonomic units [Schlegel et al., 2011]. Orphan et al. [2000] reported only a minor presence of archaeal clones in 16S rRNA gene clone libraries from production water from a high-temperature oil field in California.

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High temperature favors CO_2 reduction versus acetoclastic methanogenesis [Dolfing et al., 2008]. Whereas acetoclastic methanogens have been found in low-temperature reservoirs [Pham et al., 2009], a variety of thermophilic hydrogenotrophic methanogens, like *Methermicoccus shengliensis* [Cheng et al., 2007] or *Methanobacterium thermoaggregans* [Nazina et al., 1995], have been isolated from hydrocarbon-related high-temperature systems. In a recent study, changes in the incubation temperature of Arctic sediments resulted in distinct methanogenic community structures [Blake et al., 2015]. Moreover, the addition of H_2/CO_2, acetate or methanol did not affect methanogenic rates or the microbial structure at 5°C, but favored hydrogenotrophic methanogenesis at 30°C [Blake et al., 2015].

However, as already stated, there are other influencing factors, like salinity, pH or substrate concentrations, so actually, hydrogenotrophic and acetotrophic methanogens may coexist. For example, Zengler et al. [1999] detected *Methanosaeta* species together with *Methanospirillum* and *Methanoculleus* in *n*-hexadecane-degrading enrichment cultures. Similar results were obtained by Berdugo-Clavijo et al. [2012]. An et al. [2013a] identified genes for both hydrogenotrophic and acetotrophic methanogenesis in oil sands tailing ponds. In fact, co-occurrence of both H_2/formate (e.g. *Methanolobus, Methanobacterium, Methanocorpusculum, Methanococcus, Methanoculleus* and *Methanoregula*) and acetate-utilizing (*Methanosarcina* and *Methanosaeta*) methanogens has been reported in several other hydrocarbon-bearing systems such as the Gippsland Basin [Midgley et al., 2010], the Illinois Basin [Strąpoć et al., 2008], the Ishikari coal field [Shimizu et al., 2007] and coalbeds from Alberta [Penner et al., 2010].

Stable isotope probing (SIP) techniques can provide a link between biodegradation processes and specific microbial taxa and can help determine the main methanogenic pathway. They are based on the incorporation of isotopically labelled substrates, such as ^{13}C-hydrocarbons, to cellular biomarkers or biomolecules (lipids, nucleic acids and proteins) and can be used to identify active microorganisms without any prior knowledge of their identity [Radajewski et al., 2000]. The use of SIP for analyzing anaerobic hydrocarbon degradation is summarized by Vogt et al. [this volume, pp 195–210]. Based on DNA-SIP analysis, Beckmann et al. [2011] found predominance of acetoclastic methanogenesis in liquid cultures of hard coal and timber growing with either ^{13}C-acetate or $H_2/^{13}CO_2$. In $H_2/^{13}CO_2$-amended cultures, the substrates were mainly used by acetogens related to *Pelobacter acetylenicus* and *Clostridium* species. Active methanogens utilized acetate instead of the thermodynamically more favorable hydrogen, which could reflect the adaptation of the microbial community to the low H_2 concentrations in coal mines.

In a recent study, Morris et al. [2012] investigated the carbon flow in a model methanogenic community capable of hydrocarbon degradation by using a combination of stable isotope fractionation, protein-based SIP and metaproteomics. Labeling experiments with ^{13}C substrates showed that the proteins of the acetoclastic and hydrogenotrophic methanogens were equally labeled, suggesting acetoclasic and hydrogenotrophic methanogenesis contributed similarly to substrate consumption and thus methanogenesis in this model consortium, indicating complex interactions within the methanogenic and bacterial community.

Future Research Directions

There is a current considerable interest in methanogenic hydrocarbon biodegradation and its application in energy recovery and bioremediation. The combined activity of fermentative and methanogenic microorganisms during conversion of crude oil into methane and CO_2 in the absence of sulfate is widespread in subsurface petroleum reservoirs. This combination of fermentation and methanogenic processes has been proposed as a means to enhance energy recovery from stranded energy assets (i.e. reservoirs where over 70% of the resource can be left in place due to extraction limitations) by stimulating microbial activity. Assuming that light crude oils consist of ~10–15% of *n*-alkanes or BTEX, the microbial degradation of this fraction would convert 50% of these into methane, so approximately additional 5–10% of the total oil mass could be recovered. Thus, the induced conversion of oil or coal into methane thereby increases the production lifetime of these reservoirs.

Furthermore, methanogenic hydrocarbon degradation processes might in the future also be of interest for the bioremediation of contaminated aquifers and other deeper geological systems [Feisthauer et al., 2010], since the respective microorganisms seem to be ubiquitously distributed. Also, no expensive nutrients would be required and no harmful by-products, like H_2S produced under sulfate-reducing conditions, are to be expected. From a biochemical perspective, these unique syntrophic degradation processes hold great promise to detect and further develop novel enzymatic reactions for biotechnological applications.

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During the last years, the availability of new sampling techniques, together with the development of more sensitive single-cell approaches and new-generation sequencing methods, has enabled a deeper knowledge of the extent, function and importance in deep, often methanogenic subsurface environments [Edwards et al., 2012]. In addition, new methodological progress in the cultivation of microorganisms at high pressures and temperatures, resembling in situ conditions of the deep biosphere [Frerichs et al., 2014; Imachi et al., 2011] together with the development of single-cell techniques has allowed the growth of 'unculturable', sometimes also methanogenic, microorganisms in the laboratory. In addition, advances in molecular biology have made it possible to identify biodegradation pathways and the microorganisms involved. SIP techniques or the analysis of metabolites and degradation products by high-resolution mass spectrometry (such as HPLC-MS/MS: High Performance Liquid Chromatography coupled to tandem Mass Spectrometry, FT-ICR-MS: Fourier Transform Ion Cyclotron Resonance Mass Spectrometer/Spectrometry, HPLC-ESI-QTOF: High Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time of Flight Mass Spectrometry) can provide a direct link between the metabolic processes taking place and the microorganisms mainly involved [Jehmlich et al., 2008, 2010; Kaneko et al., 2014; Lenhart et al., 2014; Lueders et al., 2004; Schmidt et al., 2014].

The elucidation of methanogenic hydrocarbon-degrading microbial communities and their degradation pathways has thus made great progress during the past decade, but it is still in its infancy. Biochemistry and physiology of anoxic hydrocarbon degradation in reservoirs and contaminated aquifers are not thoroughly understood, and many questions remain open, e.g. the biodegradation of higher-molecular-weight PAHs or gaseous hydrocarbons, the role of microbial community members of which many have unknown function, the elemental cycling and energy fluxes within the microbial communities, the interactions between environmental factors (pressure, availability of nutrients and trace metals) and regulation of methanogenic communities.

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