




# Anaerobic microbial communities and their potential for bioenergy production in heavily biodegraded petroleum reservoirs

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## Summary

**Most of the oil in low temperature, non-uplifted reservoirs is biodegraded due to millions of years of microbial activity, including via methanogenesis from crude oil. To evaluate stimulating additional methanogenesis in already heavily biodegraded oil reservoirs, oil sands samples were amended with nutrients and electron acceptors, but oil sands bitumen was the only organic substrate. Methane production was monitored for over 3000 days. Methanogenesis was observed in duplicate microcosms that were unamended, amended with sulfate or that were initially oxidic, however methanogenesis was not observed in nitrate-amended controls. The highest rate of methane production was 0.15  $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ oil d}^{-1}$ , orders of magnitude lower than other reports of methanogenesis from lighter crude oils. Methanogenic *Archaea* and several potential syntrophic**

**bacterial partners were detected following the incubations. GC–MS and FTICR–MS revealed no significant bitumen alteration for any specific compound or compound class, suggesting that the very slow methanogenesis observed was coupled to bitumen biodegradation in an unspecific manner. After 3000 days, methanogenic communities were amended with benzoate resulting in methanogenesis rates that were 110-fold greater. This suggests that oil-to-methane conversion is limited by the recalcitrant nature of oil sands bitumen, not the microbial communities resident in heavy oil reservoirs.**

## Introduction

Despite the need for a significant shift towards renewable energy, it is estimated that the demand for crude oil will increase until 2030 (Smith, 2019). As easily accessible, lower-cost light oil production becomes more limited, production from unconventional sources of fossil fuels, such as heavy oil, has gained momentum in recent years (Gates and Larter, 2014). Heavy oil is commonly the result of microbial biodegradation over geological time-scales (Larter *et al.*, 2003; Fustic *et al.*, 2013), and constitutes the majority of the global oil inventory with a total estimated resource size of 5.6 trillion barrels (Hein *et al.*, 2013). The Athabasca oil sands in western Canada are a mixture of sand and heavily degraded immobile oil commonly referred to as bitumen, and constitute one of the largest deposits of heavy oil in the world. The oil sands are also one of the world's most biodegraded crude oil deposits, ranging between levels 5 and 9 on Peters and Moldovan (PM) scale (Bennett *et al.*, 2006).

Several technologies have been considered and are in place to improve energy production from heavy oil and oil sands (Head and Gray, 2016). The application of such technologies at industrial scale must consider feasibility, scale-up, economic costs and environmental costs. An interesting possibility from an environmental perspective is the use of indigenous microorganisms to convert crude oil to methane (oil-to-gas). Methanogenic degradation of oil appears to be the main degradation process in the

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formation of heavy oil, with estimated rates of up to 4–10 kg m<sup>-2</sup> y<sup>-1</sup> at the oil–water transition zone (Head *et al.*, 2003; Jones *et al.*, 2008), and can take place in various subsurface environments dominated by hydrocarbons (Jiménez *et al.*, 2016 and references therein). This has led to the possibility that this already-methanogenic system may be able to be further stimulated in situ e.g. through providing nutrients to stimulate additional methanogenesis. The produced methane could be used to increase residual oil production by reducing oil viscosity or increasing reservoir pressure (e.g. Gieg *et al.*, 2008; Xia *et al.*, 2016), used for steam generation for recovery of oil sands in deeper reservoirs (Gates and Larter, 2014), or retained and used as a greener fuel, given that methane combustion has a lower carbon footprint per unit energy than oil and coal (Head *et al.*, 2014). Furthermore, since organic carbon degradation coupled to methanogenesis produces CO<sub>2</sub> as well as CH<sub>4</sub>, net carbon emissions from crude oil use can also be reduced by in situ oil-to-gas production, provided only methane is recovered and CO<sub>2</sub> remains sequestered in the subsurface, for example by maintaining slightly alkaline conditions (Head and Gray, 2016).

Conversion of pure hydrocarbons and of crude oil to methane has been demonstrated in several laboratory experiments (Zengler *et al.*, 1999; Jones *et al.*, 2008; Cheng *et al.*, 2014). However, no attempts have been made using a feedstock as heavily biodegraded as oil sands bitumen. Here we present a long-term microcosm experiment with oil sands and its corresponding reservoir formation water as the only sources of carbon and microorganisms, to test the ability of the indigenous microbial community to convert bitumen into methane. Inorganic nutrients and electron acceptors were added in different combinations and microcosms were incubated at room temperature for 3050 days. Microbial communities in the initial formation water, the initial oil sands and in the microcosms at the end of the incubations were characterized, shedding light on the roles of different microbial populations potentially involved in the anaerobic biodegradation of already-biodegraded heavy oil.

## Results and Discussion

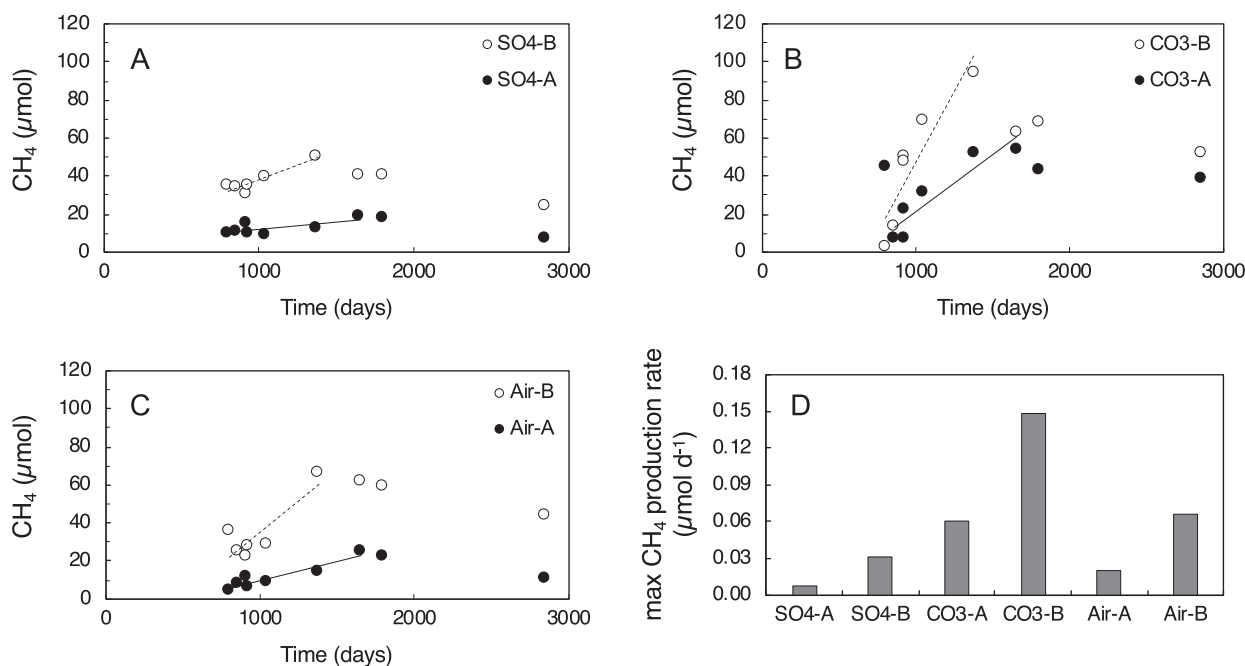
### *Methane production in all microcosms without nitrate*

Microcosms were set up by combining oil sands and formation water from the same oil sands reservoir as the only sources of microbial inoculum and organic carbon. Combining 10 g of oil sands (consisting of approximately 5% water, 10% bitumen and 85% sand) with 75 ml of formation water dramatically increases the oil water contact for anaerobic biodegradation, compared to conditions in situ (Head *et al.*, 2003). Microcosms were amended with

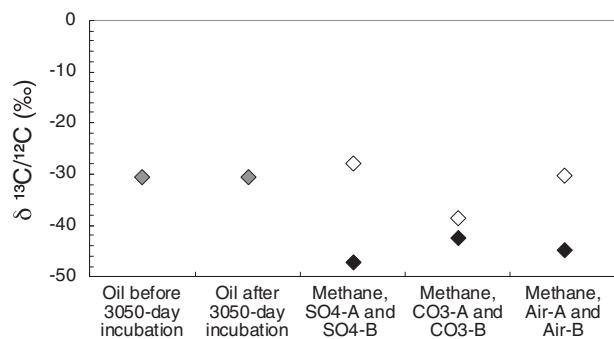
different electron acceptors [nitrate (NO<sub>3</sub>), sulfate (SO<sub>4</sub>), bicarbonate (CO<sub>3</sub>), all three (mix), or oxygen by leaving air in the headspace (air)] and incubated at room temperature for over 3000 days. Analysis of headspace samples after 800 days of incubation revealed that methane had been produced in anoxic microcosms that were amended with sulfate or bicarbonate, and in microcosms that were initially oxic with air in the headspace (Fig. 1). Traces of methane could be detected in the microcosms amended with bicarbonate (CO3-A and CO3-B) after 92 and 102 days (data not shown). After 800 days all of the microcosms under these three experimental conditions (six experimental bottles) had produced methane, to levels ranging between 3 and 45 µmol (Fig. 1A–C). The highest methane concentrations in all microcosms were measured after 1380 or 1653 days of incubation (c. 3.8 and 4.5 years) and varied between 19 and 94 µmol, corresponding to calculated maximum methane production rates between 0.01 and 0.15 µmol d<sup>-1</sup> (Fig. 1D). The amount of methane produced by SO4-B, CO3-A, CO3-B was above the maximum yield (30 µmol) that could be explained by hydrogenotrophic methanogenesis via direct utilization of 5% H<sub>2</sub> included in the headspace of four out of the six methanogenic microcosms (SO4-A, SO4-B, CO3-A and CO3-B). SO4-A produced less than 30 µmol CH<sub>4</sub> during the incubation, yet similar levels were produced by Air-A, which was set up with air in the headspace and then sealed for incubation, i.e., Air-A and Air-B were not amended with any H<sub>2</sub> yet methanogenesis was still observed, indicating it had to be driven by the organic matter (oil sands) that was provided. No methane was detected in the four nitrate-containing microcosms (NO3-A, NO3-B, Mix-A and Mix-B), consistent with nitrate having an inhibitory effect on methanogenesis (Klüber and Conrad, 1998; Siegert *et al.*, 2011).

The isotopic signature of the methane in the microcosms was assessed after 3050 days of incubation. Average δ<sup>13</sup>C values for methane from microcosms amended with sulfate, bicarbonate or air were –37.3‰, –40.4‰ and –37.5‰ respectively, however microcosms differed by 4‰ to 20‰ between duplicates (Fig. 2). Methane δ<sup>13</sup>C values were more negative than that of the oil sands bitumen in four out of six microcosms in which methane was measured. These values reveal a depletion in <sup>13</sup>C relative to the bulk δ<sup>13</sup>C in unincubated oil sands bitumen (Fig. 2). The δ<sup>13</sup>C of all bitumen samples was –30.4‰, consistent with the δ<sup>13</sup>C of Athabasca oil sands being on average –30.7‰ (Marcano *et al.*, 2013). These data suggest that the methane in the microcosms was biogenic and derived from the preferential metabolism of isotopically lighter carbon compounds or moieties in the oil sands bitumen.

To better understand the degradation of oil sands bitumen compounds in these experiments, bitumen chemical



**Fig. 1.** Methane production in duplicate microcosms (open and closed circles) that were (A) anoxic and amended with sulfate, (B) anoxic and amended with extra bicarbonate, or (C) initially oxic with air in the headspace. No methane was detected in parallel anoxic microcosms amended with nitrate or with a combination of nitrate, sulfate and bicarbonate (not shown). Maximum methane production rates (D) were calculated as the slope of the linear regressions (A–C).



**Fig. 2.** Bulk  $\delta^{13}\text{C}$  isotopic signatures of Athabasca oil sands before and after 3050-day incubation (grey diamonds) as well as  $\delta^{13}\text{C}$  isotopic signatures of headspace  $\text{CH}_4$  of methanogenic enrichments that were anoxic and amended with sulfate (SO4-A and -B), bicarbonate (CO3-A and -B) or initially oxic (Air-A and -B). Black diamonds: replicate A; white diamonds: replicate B. Standard deviations of measurements are smaller than the symbol size.

composition was analysed by gas chromatography–mass spectrometry (GC–MS) and Fourier transform ion cyclotron resonance–mass spectrometry (FTICR–MS) to search for degradation of any GC-amenable hydrocarbons and metabolite by-products respectively. Samples were taken at the end of the 3050-day incubations and compared with an unincubated sample of the same oil sands bitumen that had been stored frozen ( $-20^\circ\text{C}$ ) for the same period. Bitumen analysis from the 10 different microcosms did not reveal an obvious compound or compound class that had

been altered or degraded, either in the methanogenic incubations or the nitrate-amended controls (see Fig. S1 in Supporting Information). This result is consistent with biodegradation occurring at very low rates and suggests methanogenesis could have been occurring very slowly in an unspecific manner, possibly explaining variations in methanogenesis rates between replicates.

#### Microbial communities in freshly sampled Athabasca oil sands and formation water

Microbial community analyses were based on a set of 3000 sequences randomly subsampled from each 16S rRNA gene library (i.e., formation water, original oil sands, and each of the 10 microcosms). Prior to subsampling, operational taxonomic units (OTUs) were defined based on 97% sequence identity over approximately 500 nucleotide positions within the V4 and V5 regions of the 16S rRNA gene. Of note, 113 OTUs having a relative abundance  $\geq 0.5\%$  in at least one library were the focus of further downstream analyses. For simplicity, the overview of community composition is presented at the order level (Fig. 3), since in most cases in the amplicon libraries generated in this study the relative abundance for a given order is due to OTUs belonging to a single genus (Table S1 in Supporting Information).

The 16S rRNA gene libraries from the Athabasca formation water comprised 75% *Epsilonproteobacteria*



**Fig. 3.** Relative abundance of taxa at order level in 16S rRNA gene libraries from the inocula (formation water and oil sand, left panel) and from the 3050-day microcosms. Central panel: methanogenic microcosms. Right panel: non-methanogenic microcosms. The size of circles indicates relative abundance of the taxon in the library. Percentages are based on analysis of 3000 reads per library. Orders with relative abundance <1% are grouped as 'other'. Reads that could not be classified beyond phylum level are grouped as 'unknown'. Archaeal orders, as well as bacterial orders that collectively represent > 50% of enriched taxa across all six methanogenic microcosms are highlighted in bold. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

sequences within the order *Campylobacteriales* (all OTUs related to *Sulfuricurvum*, *Sulfurospirillum* and *Arcobacter* spp.; Table S1), 23% *Deltaproteobacteria* sequences within the order *Desulfuromonadales* (OTUs affiliated with *Geobacter* spp.; Table S1 and S2) and 2% sequences from other bacterial lineages (Fig. 3). The low overall diversity and the predominance of *Epsilonproteobacteria* are in agreement with different clone library surveys of the same sample (Hubert *et al.*, 2012). Our previous work used different primer pairs for constructing bacterial and archaeal clone libraries, and revealed that >88% of archaeal sequences were related to *Methanoregula* sp. (JF789587). In the current study, Ion Torrent libraries were made with a primer pair that targets both bacterial and archaeal 16S rRNA genes (F515-R926) with >93% coverage (see Experimental Procedures). Only 3 out of 3052 reads in the formation water library (not subsampled) belong to *Archaea*, and all were identified as *Methanoregula* sp. (OTU0139). This likely represents the same methanogen identified in the previous clone libraries, as the sequences share 205 identical nucleotides within the 206 bp overlapping region of the respective amplicons. The low detection of archaeal sequences in the Ion Torrent library is presumably due to low archaeal abundance in the formation water sample. This is consistent with the predominance of bacteria suggested by intact polar lipids analysis of this formation water (Oldenburg *et al.*, 2009). Dominance of bacteria over archaea has been observed before in other hydrocarbon deposits despite these being methanogenic environments (e.g. Jiménez *et al.*, 2012). Ion Torrent library results are compared with previously published clone library data for this formation water in Table S2.

The microbial community in the oil sand sample, the other part of the inoculum for the microcosm incubations, was dominated by *Clostridiales* (22% of reads; Fig. 3) and within this order mainly by a single *Anaerococcus* OTU representing 14% of all 16S rRNA gene reads (OTU0024; Table S1). Other prominent OTUs belonged to the orders *Burkholderiales* (15%), *Corynebacteriales*, *Lactobacillales*, *Micrococcales* (each at 10% relative abundance), *Pseudomonadales* (9%), *Bacillales* (7%), *Propionibacteriales* (5%) and *Selenomonadales* (1%; Fig. 3). All seven described species within the genus *Anaerococcus* are anaerobic (An *et al.*, 2013), whereas OTUs belonging to the other orders (Table S1) are mostly related to aerobic or facultative anaerobic heterotrophs. High proportions of putative aerobes in heavy, biodegraded oil reservoirs has been reported and described by others (An *et al.*, 2013; Head *et al.*, 2014; Liu *et al.*, 2018).

As with the formation water sample, no archaeal orders were detected in  $\geq 0.5\%$  relative sequence abundance in

the oil sands library subsampled to 3000 reads. The full non-subsampled oil sands library (5595 sequences) contained only five archaeal reads. One was an unclassified *Thermoplasmatales* and four were associated to a single OTU (OTU1443) affiliated with *Methanoregula*, although not the same *Methanoregula* OTU found in the formation water (OTU0139; data not shown). The oil sands *Methanoregula* OTU1443 shares only 95% nucleotide identity to JF789587 in the 206 bp sequence within the V4-V5 region. Despite low relative abundances, the presence of OTUs corresponding to methanogenic archaea in both the formation water and the oil sands libraries is consistent with both samples being possible sources of methanogens as inocula for the microcosm experiments.

#### Microbial communities in methanogenic microcosms

After > 3000 days of incubation, the microbial community had shifted considerably away from the initial structure in the mixed inoculum (oil sands plus formation water; Table S1 and Fig. S2). ANOSIM tests indicated that there was a significant difference ( $p = 0.004$ ) between the microbial communities enriched in the six methanogenic microcosms and the four nitrate-amended control microcosms. There was no significant difference between the three different methanogenic treatments (sulfate addition vs. bicarbonate addition vs. initially oxic;  $p > 0.3$ ) and between the two nitrate-containing control treatments (i.e. nitrate only vs. nitrate, sulfate and bicarbonate addition;  $p > 0.6$ ).

*Archaea* were enriched in all methanogenic microcosms (Fig. 3), with OTU0032 detected in all six microcosms, representing 0.8%–2.2% of sequences (Table 1). BLAST analysis revealed 100% identity across 206 nucleotides between OTU0032, *Methanosarcina lacustris* and *Methanosarcina subterranea*. Unlike most *Methanosarcina* spp. neither of these closely related methanogens are able to grow on acetate, and *M. subterranea* is not able to grow on  $H_2/CO_2$ . Both are able to use methylated substrates for growth (Simankova *et al.*, 2001; Shimizu *et al.*, 2015). Detecting this methanogen in all six microcosms where methane was observed suggests a common methanogenic process in these experiments. In addition to *Methanosarcina*, a *Methanocorpusculum* OTU (OTU0037) represented 4.8% of sequences in CO3-B (the microcosm with the most methane production) while a *Methanosaeta* (*Methanothrix*) OTU (OTU0072) represented 1.3% in Air-B (Table 1). No archaeal sequences were detected in non-methanogenic control microcosms amended with nitrate (Fig. 3; Table S1). Overall the highest relative abundance of putative methanogens was observed in CO3-B (7.0%; Table 1), the microcosm with the highest level and rate of methane production (Fig. 1).

**Table 1.** Archaeal taxa enriched in 3050-day microcosms.

Order	Family	Genus	OTU	Closest cultivated relative	SO4-A	SO4-B	CO3-A	CO3-B	Air-A	Air-B
<i>Methanosarcinales</i>	<i>Methanosarcinaceae</i>	<i>Methanosarcina</i>	OTU0032	<i>M. lacustris</i> , <i>M. subterranea</i>	0.9%	0.8%	0.9%	2.2%	1.0%	0.8%
	<i>Methanosaetaceae</i>	<i>Methanosaeta</i> ( <i>Methanothrix</i> ) <sup>a</sup>	OTU0072	<i>Methanothrix</i> <i>soehngeni</i>	nd	nd	nd	nd	nd	1.3%
<i>Methanomicrobiales</i>	<i>Methanocorpusculaceae</i>	<i>Methanocorpusculum</i>	OTU0037	<i>Methanocorpusculum</i> <i>sp. MSP</i>	nd	nd	nd	4.8%	nd	nd
<b>Total putative methanogens</b>					<b>0.9%</b>	<b>0.8%</b>	<b>0.9%</b>	<b>7.0%</b>	<b>1.0%</b>	<b>2.1%</b>

Closest cultivated relative determined by RDP SeqMatch. nd = not detected. Only OTUs with  $\geq 0.5\%$  relative abundance in at least one microcosm are listed.

a. See minute 22 of the meeting of the Judicial Commission, 3, 4 and 6 August 2008, Istanbul, Turkey (DOI: 10.1099/ij.s.0.037366-0), which rejects the name *Methanosaeta* and replaces it with *Methanothrix*.

Bacterial communities in methanogenic microcosms (i.e. six libraries sub-sampled to 3000 sequences each, therefore 18,000 sequences in total) were collectively dominated by *Desulfovibrionales* (17%), *Desulfuromonadales* (14%), *Anaerolineales* (11%), *Desulfobacterales* (10%) and *Syntrophobacterales* (6%), i.e., altogether > 50% of the sequence reads in the six methanogenic microcosms (Fig. 3).

*Desulfovibrionales* consisted of *Desulfomicrobium* and *Desulfovibrio* spp. (Table 2). These putative sulfate-reducing bacteria were highly enriched (7%–45% relative sequence abundance) regardless of sulfate amendment (Table 2). Within the *Desulfobacterales*, an OTU affiliated with the genus *Desulfatiferula* was present at > 0.5% relative abundance in SO4-A (Table 2), but not in the microcosms that were not amended with sulfate. Despite the presence of these putative SRB, high concentrations of sulfate were still measured in the microcosms after 3050 days in SO4-A and SO4-B. Sulfate reduction in both of these microcosms was observed in the first 26 days of incubation, with less than 3 mM removal, likely due to the H<sub>2</sub> provided in the headspace (NB: it is unlikely that H<sub>2</sub> supported the methanogenesis observed in SO4 microcosms; Fig. 1), however 20 and 22 mM sulfate was still detected at the end of the incubation period. When the original bitumen-amended microcosms were established, parallel tests for sulfate reduction with lactate amendment were performed, leading to the complete removal of sulfate (10 mM) and lactate (25 mM) in less than 1 month (data not shown), indicating the potential for sulfate reduction within the microbial community in this oil sands reservoir. Despite this, in the two microcosms amended with sulfate where bitumen was the only carbon substrate, methanogenesis was detected in the presence of sulfate and putative SRB. Syntrophic metabolism by these bacteria may have supported methanogenesis. The OTUs that increased in relative sequence abundance were most closely related to *Desulfomicrobium hypogeium*, *Desulfovibrio mexicanus* and

*Desulfatiferula berrensii*. Among these organisms, *D. hypogeium* is not known to grow via fermentation, but it can use thiosulfate or sulfite as electron acceptors in addition to sulfate (Krumholz *et al.*, 1999). *D. mexicanus*, on the other hand, can grow via fermentation of various substrates (Hernandez-Eugenio *et al.*, 2000) and was detected in all six methanogenic microcosms. *D. berrensii* is able to ferment pyruvate and to degrade C<sub>12</sub> to C<sub>20</sub> n-alkenes coupled to sulfate reduction (Hakil *et al.*, 2014).

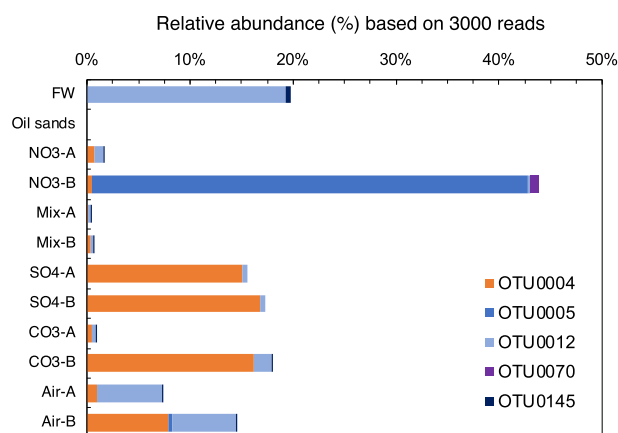
A *Desulfobacterales* OTU affiliated to the genus *Desulfurivibrio* (OTU0006) was detected in very high relative sequence abundance in the bicarbonate-amended microcosms (35% in CO3-A and 10% in CO3-B; Table S1). The only known isolate of this genus, *D. alkaliphilus*, is unable to reduce sulfate. *D. alkaliphilus* is able to grow by the reduction of sulfur compounds or nitrate, by elemental sulfur disproportionation or by performing dissimilatory nitrate reduction to ammonia coupled to sulfide oxidation (Sorokin *et al.*, 2008; Poser *et al.*, 2016; Thorup *et al.*, 2017). OTU0006 was not detected in any of the nitrate-amended controls, thus its predominance in the bicarbonate-amended microcosms may point to other ecophysiological roles for this group.

*Desulfuromonadales* consisted of OTUs most closely related to *Geobacter* spp. Notably, OTU0004 was enriched in most methanogenic microcosms (1%–17%, Fig. 4) and was not detected in the non-methanogenic controls. This OTU is closely related to *Geobacter toluenoxydans* and *Geobacter daltonii*, both known to anaerobically degrade monoaromatic hydrocarbons (Kunapuli *et al.*, 2010; Prakash *et al.*, 2010). These observations, and its high relative abundance in the formation water (Fig. 4, Table S1), are consistent with *Geobacter* OTU0004 possibly being involved in syntrophic methanogenic degradation of components of the oil sands both in situ and in the microcosms. It has recently been shown that some *Geobacter* species are capable of syntrophy via direct interspecies electron transfer (DIET)

**Table 2.** Taxa affiliated with known sulfate-reducing deltaproteobacterial groups enriched in methanogenic microcosms.

Order	Family	Genus	OTU	Closest cultivated relative	SO4-A	SO4-B	CO3-A	CO3-B	Air-A	Air-B
<i>Desulfovibrionales</i>	<i>Desulfomicrobiaceae</i>	<i>Desulfomicrobium</i>	OTU0001, OTU0069	<i>Desulfomicrobium hypogeium</i>	20.2%	40.4%	1.6%	18.6%	nd	nd
	<i>Desulfovibrionaceae</i>	<i>Desulfovibrio</i>	OTU0017	<i>Desulfovibrio mexicanus</i>	2.6%	4.5%	5.5%	5.1%	0.2%	0.7%
<i>Desulfobacterales</i>	<i>Desulfobacteraceae</i>	<i>Desulfatiferula</i>	OTU0071	<i>Desulfatiferula berrensensis</i>	1.4%	0.2%	nd	nd	0.1%	nd
<b>Total putative SRB</b>					<b>24.1%</b>	<b>45.1%</b>	<b>7.1%</b>	<b>23.7%</b>	<b>0.3%</b>	<b>0.7%</b>

Closest cultivated relative determined by RDP SeqMatch. nd = not detected. Only OTUs with  $\geq 0.5\%$  relative abundance in at least one microcosm are listed.



**Fig. 4.** *Geobacter* OTUs found in  $\geq 0.5\%$  relative abundance in the formation water (FW) or oil sands libraries and in at least one of the 3050-day microcosms. OTU0004 is most closely related to *Geobacter toluenoxydans* and *Geobacter daltonii*. All other OTUs are most closely related to *Geobacter bremensis* and *Geobacter bemidjensis*. [Color figure can be viewed at wileyonlinelibrary.com]

to methanogens. DIET has been observed between *Geobacter metallireducens* and *Methanosaeta harundinacea* (Rotaru *et al.*, 2014b) and between *G. metallireducens* and *Methanosarcina barkeri* (Rotaru *et al.*, 2014a), where the *Geobacter* sp. grew on ethanol and transferred electrons via conductive pili to the archaeon. However, the ability of OTU0004 to perform DIET, and the occurrence of this metabolism in our long-term experiments could not be tested in this study. It is worth noting that the *Geobacter* OTUs abundant in the formation water community and non-methanogenic microcosms differed from OTU0004. *Geobacter* sp. OTU0012 represented 19% of the formation water community and 6% of the community in the Air microcosms, and *Geobacter* OTU0005 was highly enriched in the non-methanogenic microcosm NO3-B (42%; Fig. 4). Both are most closely related to *G. bremensis* and *G. bemidjensis*. OTU0070, present in 0.8% relative abundance in NO3-B and OTU0145, 0.5% in

the formation water, are also most closely related to *G. bremensis* and *G. bemidjensis* (Fig. 4).

Other potential syntrophs enriched in all methanogenic treatments included *Smithella* spp. and other *Syntrophobacterales*, which are often associated with methanogenic degradation of crude oil, particularly longer-chain alkanes (Gray *et al.*, 2011; Cheng *et al.*, 2013; Berdugo-Clavijo and Gieg, 2014; Tan *et al.*, 2014; Ding *et al.*, 2015; Jiménez *et al.*, 2016). *Syntrophobacterales* were present in all methanogenic microcosms at relative abundances between 1% and 20% (Fig. 3). *Anaerolineales* also had a high frequency in all methanogenic microcosms (5%–14%), especially in the Air-A and Air-B microcosms (Fig. 3); this group is also commonly found in oil-degrading environments and enrichment cultures (Savage *et al.*, 2010; Sherry *et al.*, 2013; Tan *et al.*, 2015). *Burkholderiales* and *Campylobacterales* were prevalent in Air-A and Air-B microcosms (11%–12%), but were also enriched in non-methanogenic microcosms (Fig. 3).

As the main bacterial taxa detected in the methanogenic microcosms were distinct from the taxa found in non-methanogenic controls that were amended with nitrate, these groups were likely directly or indirectly involved in syntrophic degradation of bitumen leading to methane production. The detection of several of the most prevalent bacterial OTUs in all of the methanogenic microcosms (OTU0004, OTU0008, OTU0012, OTU0014), as well as a methanogen in common to all of them (Table 1; Table 2; Fig. 3; Table S1), suggests that a common mechanism for methanogenesis from bitumen may have been stimulated in these long term experiments.

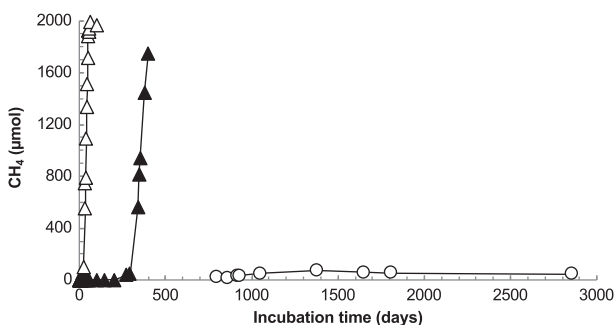
#### *Rapid methanogenesis by oil sands microbial communities in the presence of a readily biodegradable substrate*

Results from the > 3000-day microcosms indicated very low rates of methanogenesis by oil sands microbial communities featuring putative fermentative and syntrophic

networks. To test whether these communities could catalyse more rapid methanogenesis in the presence of a less recalcitrant feedstock, microcosms with the highest methanogenesis rates (CO3-A and CO3-B, Fig. 1) were used to inoculate (5% v/v) synthetic medium amended with 10 mM benzoate as the only additional substrate (see Supplementary Experimental Procedures in Supporting Information for further details). After a lag phase of nearly 300 days, methane was produced at a maximum rate of  $16 \mu\text{mol CH}_4 \text{ d}^{-1}$  (Fig. 5), more than 100-fold faster than the methanogenesis catalysed by the same microbial community with oil sands bitumen as the substrate (Fig. 1D; Fig. 5). This indicated that the low rates of methanogenesis from bitumen were due to the feedstock and not the oil sands microbial community. Comparable rates of methanogenesis, following a shorter lag phase, were observed in parallel positive control incubations of estuarine sediment was amended with benzoate ( $71 \mu\text{mol CH}_4 \text{ d}^{-1}$ ; Fig. 5). The ability of indigenous oil sands microorganisms to rapidly convert a suitable parent compound into methane underscores the potential for biotechnological applications in the subsurface that rely on resident populations instead of on an external inoculum. While augmentation with estuary sediment did enhance methanogenesis, this would not necessarily be the case in a subsurface heavy oil reservoir where any methanogenic consortia (native or augmented) would need to contend with the recalcitrant nature of heavy oil.

#### Feedstock chemistry determines the potential for methanogenic crude oil biodegradation

The > 3000-day incubation experiments demonstrated that indigenous mesophilic microbial communities in Athabasca oil sands are capable of accessing a limited pool



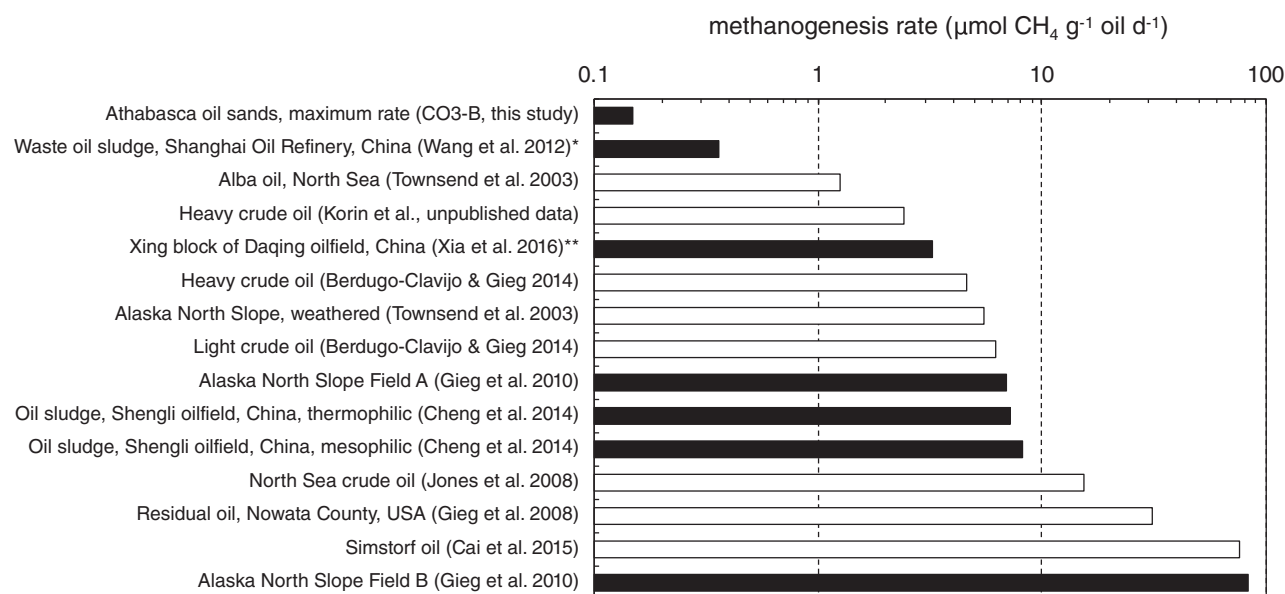
**Fig. 5.** Methane produced in microcosms with oil sands or benzoate as the feedstock. Circles: microcosm with oil sands and formation water as the only carbon substrate and inocula and amended with bicarbonate (cf. microcosm CO3-B; Fig. 1B). Filled triangles: synthetic brackish medium inoculated with CO3-A and CO3-B microcosms after 3050 days of incubation, and containing 10 mM benzoate as carbon source (cf. Fig. S3). Empty triangles: synthetic brackish medium microcosms inoculated with anoxic estuarine sediment and containing 10 mM benzoate as carbon source.

of organic carbon for anaerobic biodegradation and methanogenesis. This is supported by the production of methane in all microcosms other than the nitrate-amended controls (Fig. 1) and by the difference in microbial community composition in methanogenic microcosms compared to non-methanogenic microcosms and the formation water and bitumen used as inocula. The relative abundance of methanogenic *Archaea* increased from <0.1% of sequence reads in the formation water and oil sands inocula to 0.8%–7% in the six methanogenic microcosms (Table 1). Stable isotope data indicated the headspace methane to be biogenic, further supporting the conclusion that methanogenic communities were able to degrade organic matter in the microcosms, facilitating methanogenesis.

As there was no external input of organic carbon besides what was present in the bitumen and formation water, the organic feedstock for methanogenesis must either be compounds present in the bitumen or necromass (dead cells) present in the microcosms. To estimate the potential contribution from the latter, the total amount of carbon in the microbial community needs to be estimated. Braun and colleagues recently quantified amino acids in Baltic Sea sediments and calculated cell-specific carbon content on the order of 19 to 31 fg C cell<sup>-1</sup> (Braun *et al.*, 2016b). The maximum methane production in the oil sands microcosms was between 19 and 94  $\mu\text{mol CH}_4$  (SO4-A and CO3-B respectively, Fig. 1), corresponding to between  $3 \times 10^{11}$  and  $15 \times 10^{11}$  femtograms carbon per microcosm. Using the conservative assumption of 31 fg C cell<sup>-1</sup> (Braun *et al.*, 2016b), and assuming 75% of the organic feedstock is partitioned to methane during methanogenesis (Zengler *et al.*, 1999), between  $1.3$  and  $6.4 \times 10^{10}$  dead cells would be required to provide the carbon required to support the lowest and the highest  $\text{CH}_4$  production values in Fig. 1 respectively. The abundance of cells in both oil sands and formation water has been previously estimated to be between  $10^4$  and  $10^7$  cells g<sup>-1</sup>, with the highest cell densities found at the oil–water transition zone (Bennett *et al.*, 2013). With 10 g of oil sands and 15 ml of unfiltered formation water, and considering the highest cell abundances from other oil sands reservoirs, the microcosms established in this study would have contained at most  $10^8$  cells, roughly two orders of magnitude lower than the amount of necromass needed for anaerobic digestion yielding methane levels observed in our experiments. Conversely, in order for the biomass of  $10^8$  cells to fuel the methanogenesis shown in Fig. 1, a cell-specific carbon content of at least 2280 fg cell<sup>-1</sup> would be required; this is approximately 15 times greater than the cell-specific carbon content of *E. coli* in a densely-grown culture (Braun *et al.*, 2016a).

Methane production in our long-term microcosms can, therefore, be mainly attributed to the biodegradation of organic carbon compounds in already severely



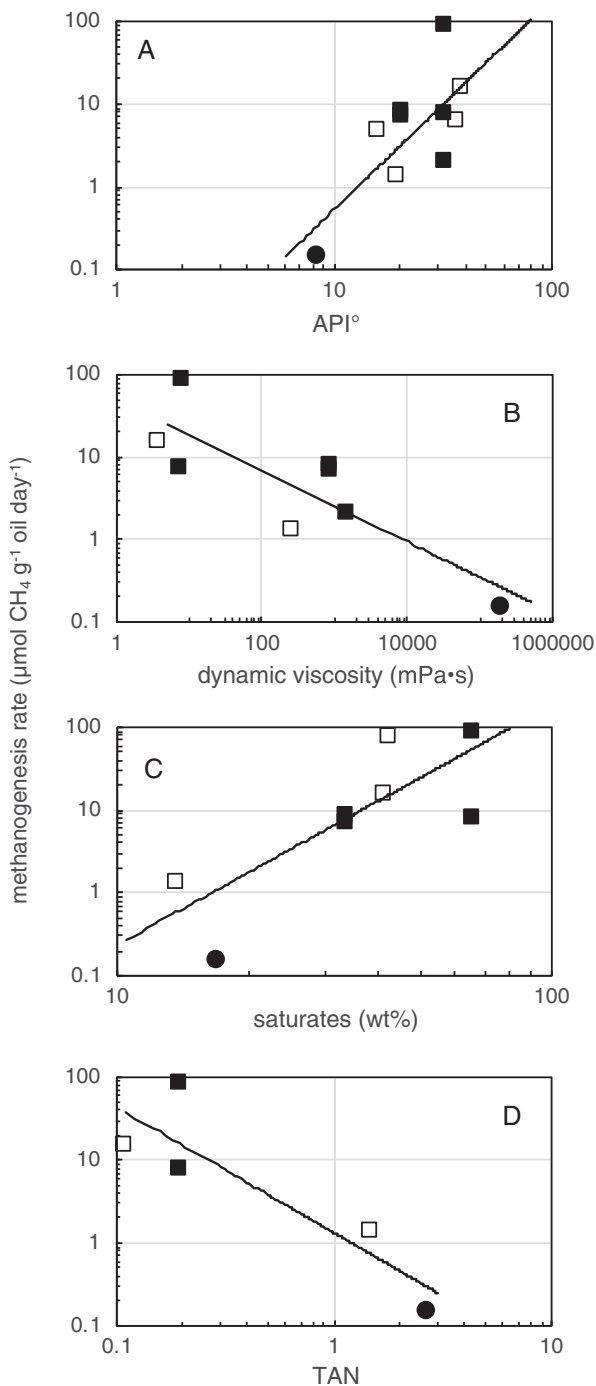


**Fig. 6.** Rates of methanogenesis in microcosms established with different crude oils as feedstocks. Black bars indicate examples where the inoculum and oil are from the same source material. White bars indicate an exogenous inoculum. Rates are maximum values or average values, as reported by each study. Rates are reported as  $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ oil d}^{-1}$  in all cases, with the following exceptions: Wang *et al.* (2012) report  $\mu\text{mol CH}_4 \text{ mL}^{-1} \text{ oil sludge d}^{-1}$  (\*); Xia *et al.* (2016) included  $0.05 \text{ g L}^{-1}$  of both yeast extract and cysteine as exogenous carbon sources (\*\*). For Gieg *et al.* (2010), specific gravity data was used to determine rate per g oil. See Table S3 for more details.

biodegraded oil sands bitumen. Methanogenesis coupled to the anaerobic biodegradation of crude oil compounds requires initial activation (Widdel and Rabus, 2001; Agrawal and Gieg, 2013; Aitken *et al.*, 2013; Berdugo-Clavijo and Gieg, 2014; Bian *et al.*, 2015; Toth and Gieg, 2018). In our experiments, this initial degradation of bitumen compounds appears to be the limiting step, based on GC-MS and FTICR-MS revealing no obvious change in specific compounds or compound classes in bitumen incubated for > 3000 days relative to a frozen, unincubated control. Previous work investigating the degradation of similar Athabasca oil sands showed significant bitumen transformation monitored by FTICR-MS under oxic conditions at  $55^\circ\text{C}$  after only 4 months (Wong *et al.*, 2015), whereas little to no bitumen transformation was observed in parallel anoxic incubations (by thermophilic or mesophilic communities). It is nevertheless surprising that, even in incubations lasting more than 8 years presented here, similar measurements did not reveal compositional changes in the bitumen. As such, our results set a benchmark that demonstrates the recalcitrant characteristic of oil sands bitumen as a feedstock for further anaerobic metabolism.

This conclusion is supported by comparing results from the present study with observed methanogenesis rates in other reports of crude oil to methane conversion. The highest rate in our oil sands microcosms ( $0.15 \mu\text{mol CH}_4 \text{ g}^{-1} \text{ oil d}^{-1}$ ; CO3-B) is still 600-fold lower than in other oil-to-methane experiments that

used oils that are far less biodegraded than Athabasca bitumen (Fig. 6). As shown in Fig. 5, transferring the microbial communities from the bitumen-amended, > 3000-day microcosms into fresh medium with benzoate as the carbon source led to methanogenesis rates comparable to rates catalysed by an estuarine microbial community. This shows that the rate of methanogenesis in the oil sands experiments appears not to be limited by the capacity of the system for methanogenesis, but rather by the conversion of recalcitrant organic matter to methanogenic substrates. The > 100-fold greater methanogenesis in the presence of benzoate could also have been promoted by the 20-fold dilution of the oil sands slurry reducing or eliminating unknown inhibitory compounds. To further examine the relationship between feedstock recalcitrance and methanogenesis, rates from selected experiments in Fig. 6 (for which crude oil data are available; Table S3) were plotted as a function of physical and chemical properties of the crude oil feedstock. Methanogenesis rates trend with parent crude oil physical properties (API gravity and dynamic viscosity) and chemical composition (percent saturates and total acid number), with no discernible difference in experiments that added an exogenous inoculum together with the crude oil sample (Fig. 7). These relationships are consistent with the very low metabolic rates recorded in our experiments with severely biodegraded bitumen.



**Fig. 7.** Oil-to-methane rates from selected experiments shown in Fig. 6 for which feedstock crude oil data is available. Methanogenesis rates are plotted as a function of physical (A, B) and chemical (C, D) properties of the crude oils, and are correlated with API gravity (A) and saturated hydrocarbon weight percent (C), and inversely correlated with dynamic viscosity (B) and total acid number (D). Closed symbols denote experiments where the microbial community inoculum and the feedstock oil were from the same source material, with circles representing the results from this study. Open symbols indicate that a different inoculum, other than that present in the crude oil, was added to the experiment. More detailed information for each of the studies plotted is provided in Table S3.

The adaptability of this oil sands microbial community is further demonstrated by the apparent enrichment of SRB in microcosms that received only bicarbonate and no sulfate amendment (CO3-A and CO3-B), as well as the lack of sulfate consumption when sulfate was provided in the medium (SO4-A and SO4-B; c. 3 mM sulfate was removed and > 20 mM sulfate remained at the end of the incubation period). These results suggest that the enriched SRB were involved in a syntrophic metabolism supporting methanogenesis with limited sulfate reduction. This metabolic preference may be associated with the lack of easily degradable substrates, as rapid sulfate reduction was observed in a parallel microcosm amended with 10 mM sulfate and 25 mM lactate as a carbon source. The occurrence of methanogenesis in the presence of high levels of sulfate is an interesting observation that was not anticipated, as sulfate reduction is usually more energetically favourable than methanogenesis. It has been shown that sulfate concentrations below 5 mM can enhance methanogenesis (Siegert *et al.*, 2011), and SRB have been detected in high abundance in methanogenic, low-sulfate oil fields (Jiménez *et al.*, 2012). In the case of enrichments on pure hydrocarbon compounds, the inhibitory effect of sulfate on methanogenesis varies according to the hydrocarbon substrate (Siegert *et al.*, 2011). The methanogenesis observed here in the presence of > 20 mM sulfate over thousands of days is generally unexpected. A similar observation was reported previously for a methanogenic consortium that originated from sediment and groundwater in a gas-condensate-contaminated aquifer in Colorado, USA (Struchtemeyer *et al.*, 2005). The enriched consortium was shown to produce methane from residual oil in a marginal sandstone reservoir core in the presence of 10 mM sulfate (Gieg *et al.*, 2008; c.f. Figure 6). To our knowledge, these are the only reports of methanogenesis via crude oil degradation in the presence of millimolar levels of sulfate, yet many other DNA-based studies indicate high relative abundance of putative SRB in hydrocarbon-degrading microbial communities, which are assumed to perform sulfate reduction (An *et al.*, 2013; He *et al.*, 2013; Lewin *et al.*, 2014). Our study, together with the studies above, highlight the metabolic versatility of SRB and the well-known caveat of metabolic assumptions based solely on 16S rRNA gene data, which may be incorrect or incomplete, and thus should be confirmed with chemical data.

### Conclusion

The present work indicates that microbial oil-to-methane conversion is possible using oil sands bitumen as the parent feedstock for anaerobic microbial consortia in the reservoir, however this does not offer a realistic avenue

for energy production at industrial scale due to the very low rates of methanogenesis that can be sustained. This strategy may be more feasible for residual lighter oils, where it is estimated that an additional production of 5%–10% of the oil mass may be recovered, increasing the lifetime of these reservoirs (Jiménez *et al.*, 2016). Strategies for in situ conversion of oil-to-methane, in addition to being driven by the natural gas market, will thus be determined by conditions specific to given oil field operations (Shelton *et al.*, 2016). In this context, the present work highlights that the extent of pre-existing biodegradation is an important determinant and suggests that heavy oils are poor candidates as feedstocks for further biological conversion by resident microbial communities.

## Experimental procedures

### *Sample collection and preparation of microcosms*

Athabasca oil sands and formation water were sampled at the Muskeg River oil sands mine north of Fort McMurray, Alberta, Canada in 2005 as described elsewhere (Hubert *et al.*, 2012). Oil sands were collected aseptically from an active excavation area and formation water was collected from an associated dewatering well. Samples were stored under anoxic conditions during transport and were kept anoxic during microcosm preparation.

Microcosms were prepared in 158 ml serum bottles and were composed of 10 g oil sands (which contained ca. 1 g bitumen), 15 ml unfiltered formation water as an additional source of microorganisms, and 60 ml 0.2- $\mu$ m filtered formation water. In this way the microcosms were inoculated both with oil sands material as well as with formation water from the oil–water transition zone of the same oil sands reservoir (Hubert *et al.*, 2012). Filtered formation water was amended with the following inorganic nutrients: 0.25 g/l  $\text{NH}_4\text{Cl}$ , 0.2 g/l  $\text{KH}_2\text{PO}_4$  and 1.0 ml/l non-chelated trace element and selenite-tungstate solutions (Widdel and Bak, 1992). Microcosms were prepared in a vinyl anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) that contained a mixed gas atmosphere, creating a headspace of 85%  $\text{N}_2$ , 10%  $\text{CO}_2$  and 5%  $\text{H}_2$ . Besides the oil sands bitumen and 5% hydrogen, no additional carbon source or electron donor was added to the microcosms. Based on an original objective to test different electron accepting processes, a total of 10 microcosms were set up to test five conditions in duplicate: (i) 20 mM nitrate amendment (NO3-A and NO3-B), (ii) 20 mM sulfate amendment (SO4-A and SO4-B) on top of 160  $\mu$ M residual sulfate in the formation water, (iii) 50 mM bicarbonate, in addition to c. 16 mM bicarbonate already present in the formation water (CO3-A and CO3-B), (iv) 20 mM nitrate, 20 mM sulfate and 50 mM bicarbonate (Mix-A, Mix-B) or (v) none of the above (Air-A and Air-B). For the first four treatments (NO3, SO4,

CO3, Mix) the eight bottles were sealed in the anaerobic chamber and received  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  (c. 1 mM) as a reducing agent. For the last treatment (Air), the two bottles (Air-A and Air-B) were sealed with air as the headspace to allow  $\text{O}_2$  to serve as an electron acceptor. As such these last two bottles did not contain  $\text{H}_2$  as a potential electron donor. All bottles received resazurin as a redox indicator (Widdel and Bak, 1992).

Microcosms were incubated at room temperature on a rotary shaker in the dark for 2 years. The headspace was sampled after 0, 12, 26, 92 and 102 days in the first year to assess methane production. For the following > 7 years the microcosms were incubated at room temperature in the dark, without shaking, and the headspace continued being sampled, but less frequently. After approximately 8 years (3050 days) headspace gas in the microcosms was sampled for methane and  $\text{CO}_2$  stable isotope analysis, and the aqueous phase was sampled for DNA extraction and measurement of nitrate, nitrite and sulfate concentrations, as described below. Aliquots were also sampled from CO3-A and CO3-B and transferred into fresh medium for benzoate degradation experiments (Supplementary Experimental Procedures in Supporting Information). After 3550 days, the remaining contents of the microcosms (> 65 g) were used for organic extraction and oil analysis by GC–MS and FT–ICRMS.

### *Measurement of nitrate, nitrite and sulfate concentrations*

Nitrate, nitrite and sulfate concentrations were measured by ion chromatography using a Dionex ICS-1000 with an AS40 auto-sampler. The column was an IonPac AS14A analytical column with the flow rate set to 1 ml/min. The eluent was 8.0 mM  $\text{Na}_2\text{CO}_3$ /1.0 mM  $\text{NaHCO}_3$  solution and the injection loop was 25  $\mu$ l.

### *Methane production in headspace*

For the first 2 years headspace methane (0.2–1 ml) was determined using a Hewlett Packard 5890 gas chromatograph with flame ionization detection (GC-FID) equipped with a stainless steel column (0.049 cm  $\times$  5.49 m) packed with Porapak R 80–100 mesh (Supelco, Oakville, ON, Canada). The injector and the oven temperatures were both 40°C and the detector temperature was 80°C. Helium was used as the carrier gas. Peak areas were determined by a Hewlett Packard 3390A integrator. For subsequent years, headspace gas samples (100  $\mu$ l) were analysed for  $\text{CH}_4$  using a Carlo Erba 5160 GC-FID fitted with a Chrompak Pora plot Q coated fused silica capillary column (30 m  $\times$  0.32 mm) with hydrogen as a carrier gas. The oven (35°C) and injection port (250°C) temperatures were fixed. Methane concentrations were determined periodically with reference to external standard

calibrations (Scientific & Technical Gases, Newcastle-under-Lyme, UK). The volume of gas removed and the concentration of methane were accounted for when calculating the total mass of methane produced in the microcosms. Profiles of methane production as a function of time were examined graphically to determine a linear methane production phase. Maximum methane production rates ( $\mu\text{mol CH}_4 \text{ g}^{-1} \text{ oil d}^{-1}$ ) for individual incubations were calculated from fitted slopes spanning the linear phase (Fig. 1).

#### Stable isotope analysis of bulk oil sands and headspace gas

Analyses of  $\delta^{13}\text{C}$  of bulk unincubated oil sands were done as described elsewhere (Marcano *et al.*, 2013) using Continuous Flow-Elemental Analysis-Isotope Ratio Mass Spectrometry (CF-EA-IRMS) technology based on a Thermo DeltaV+ mass spectrometer interfaced with a Costech 4010 elemental analyser. For isotopic composition of headspace gas from microcosms, triplicate 10 ml gas samples were transferred into evacuated gas tubes. Gas samples were sent to Isodetect GmbH (Leipzig, Germany) for carbon and hydrogen stable isotope analysis. Headspace samples were analysed by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) to determine the carbon and hydrogen isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ,  $^2\text{H}/^1\text{H}$ ) of gas components as described elsewhere (Mulat *et al.*, 2016). Compound-specific stable isotope analysis (CSIA) was performed via (i) manual injection using a gastight syringe into the split/splitless injector of a gas chromatograph (GC, 7890A Series, Agilent Technology), (ii) compound-specific separation of target compounds on a GC column (CP-Porabond Q column,  $50 \text{ m} \times 0.32 \text{ mm} \times 0.5 \mu\text{m}$ ; Varian), (iii) combustion of target compounds to  $\text{CO}_2$  for carbon isotope analysis and pyrolysis of target compounds to  $\text{H}_2$  for hydrogen isotope analysis, and (iv) transfer to an isotope ratio mass spectrometer (MAT 253, Thermo Fisher Scientific) of  $\text{CO}_2$  or  $\text{H}_2$  respectively for carbon and hydrogen isotope analysis. Authentic laboratory standards were used for identification of target compounds and for quality control of CSIA.

Isotope ratios are expressed in the delta notation ( $\delta^{13}\text{C}$ ,  $\delta^2\text{H}$ ) relative to international standards according to Coplen (2011):

$$\delta^{13}\text{C} [\text{‰}] \text{ or } \delta^2\text{H} [\text{‰}] = (R_{\text{sample}} - R_{\text{standard}}) / (R_{\text{standard}}) \quad (1)$$

where  $R_{\text{sample}}$  and  $R_{\text{standard}}$  are the  $^{13}\text{C}/^{12}\text{C}$ - and  $^2\text{H}/^1\text{H}$ -ratios of the sample and the international standard respectively. Vienna Pee Dee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW) were

used as standards for carbon and hydrogen CSIA respectively. Because variations in natural isotope abundance are typically small, the  $\delta^{13}\text{C}$ - and  $\delta^2\text{H}$ -values are reported in per mil [‰]. The reproducibility of CSIA given by the standard deviation of replicate measurements was  $\leq 0.4\text{‰}$  for  $\delta^{13}\text{C}$ -values and  $\leq 5\text{‰}$  for  $\delta^2\text{H}$ -values respectively, which are in agreement with the typical uncertainty for CSIA of gaseous hydrocarbons (Yarnes, 2013), indicating the good performance of CSIA for the samples.

#### Organic matter solvent extraction

After the end of the incubations, microcosms were opened and liquid and solid phases were used for solvent extraction of total organic matter. The original oil sands material that was used as inoculum was stored at  $-20^\circ\text{C}$  after the original sampling at the Muskeg River oil sands mine, and a 10 g sample was removed and processed together with the material from the incubated microcosms. Extraction was performed as follows: microcosm contents (7–8 g oil sands plus remaining water phase) were transferred to a clean steel tube, centrifuged for 15 min at 3000 rpm (1811g rcf) and the liquid phase was decanted into a separating funnel. Of note, 20 ml methanol (MeOH) and 30 ml dichloromethane (DCM) were added to the sand in the centrifuge tube, sonicated for 25 min, centrifuged for 15 min and decanted into a separating funnel. For the original oil sands sample, extraction was initiated with 50 ml MeOH, sonication and centrifugation. Next, for both microcosms and the oil sands sample, 50 ml DCM:MeOH (93:7) was added to the pelleted material in the centrifuge tube, sonicated, centrifuged and decanted as before. The DCM:MeOH extraction was repeated four times in total, until the extracts were clear. Distilled water was added to the separating funnel so that the aqueous phase contained c. 150 ml, and pH was adjusted to 2 with concentrated HCl. After vigorous shaking, funnels were left for at least 30 min to allow separation of organic and aqueous phases. The organic layer was run off into a round bottom flask. Three rounds of 50 ml DCM addition, shaking, 15 min separation and collection of organic phase were performed to complete the extraction. Organic extracts were concentrated by rotary evaporation, re-suspended in DCM and transferred to 10 ml glass vials. Excess DCM was evaporated under a stream of  $\text{N}_2$  until constant weight was obtained.

#### Characterization of bitumen extracts

After solid phase extraction (SPE) the saturated and aromatic hydrocarbon fractions of the organic extracts were analysed using GC-MS as described in Bennett *et al.* (2013). Briefly, the hydrocarbon fractions were analysed using a HP-5 coated fused silica capillary column (30 m

length, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies) with helium as the carrier gas and a flow rate of 1 ml/min and splitless injection to an Agilent 6890 GC interfaced to a 5973 quadrupole mass selective detector. The temperature program of the GC was 40°C (held for 5 min) to 325°C at 4°C/min and held at 325°C for 15 min.

In addition, total organic extracts of the original bitumen and bitumen samples following 3050 days of incubation were investigated for changes in the distribution of polar and non-polar compound classes and specifically for possible metabolites using a Bruker Solarix 12 T, Fourier transform ion cyclotron resonance mass spectrometer (12 T FTICR-MS) as described in Oldenburg *et al.* (2014) for general conditions and Oldenburg *et al.* (2017) regarding metabolite identification. Briefly, samples were diluted to a final concentration of 0.25 mg/ml, in a toluene-methanol (1:1 by volume) mixture spiked with 1 ppm reserpine (internal standard). Samples were analysed in electrospray negative (ESI-N) and positive (ESI-P) ion mode as well as by atmospheric pressure photoionization in positive ion mode (APPI-P; krypton lamp at 10.6 eV). 1% of formic acid or ammonium hydroxide (dopants) were added to the sample when analysed in ESI-P and ESI-N respectively. Two hundred scans were collected from each sample at a flow rate of 240 µl/h within a mass range of 156–1400 Da. The mass accuracy was typically below 200 ppb and the resolving power at 400 Da was above 500,000 (M/DMfwhm). The FTICR-MS raw data were processed using CaPA (Aphorist). Ragnarök (Aphorist) data processing software was used for quantitation, visualization and interpretation of the FTICR-MS data.

#### *DNA extraction, amplification and sequencing of 16S rRNA genes*

DNA was extracted simultaneously from the original oil sands sample (inoculum) and from the individual microcosms after 3050 days. The oil sands inoculum was stored at –20°C after collection and removed from the freezer to subsample for DNA extraction. From this oil sands sample, triplicate subsamples of c. 1 g each were removed and DNA was extracted with a PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) using a modified protocol. For DNA extraction from microcosms, contents were homogenized by light shaking and 7 ml of the liquid phase were removed using a 23G needle, placed into sterile centrifuge tubes and stored at –20°C until further processing. Thawed samples were filtered through a sterile hydrophilic nylon membrane (0.20 µm pore size, 25 mm diameter; Millipore, Hertfordshire, UK) held in Swinnex filter holders (Millipore). The filtrate was stored at –20°C for anion chromatography while the

filters were immediately sectioned for DNA extraction using a PowerSoil DNA Extraction Kit. Extraction details for the formation water sample, involving the FastDNA Spin Kit for soil (Q-BIOgene, UK), have been described previously (Hubert *et al.*, 2012). All DNA extracts were subsequently used for 16S rRNA gene amplification and sequenced on an Ion Personal Genome Machine (PGM) platform (Ion Torrent, Life Technologies, Paisley, UK).

PCR amplification of 16S rRNA genes was performed using primers F515 and R926 (Quince *et al.*, 2011), which target regions V4 and V5 of archaeal and bacterial 16S rRNA genes. According to TestPrime (<http://www.arb-silva.de/search/testprime/>; accessed in October 2018) this primer pair covers 93% of archaeal and 95% of bacterial 16S rRNA sequence diversity (SILVA SSU 132 database, RefNR), with one mismatch allowed (Klindworth *et al.*, 2013). Primers were synthesized following the instructions on the Ion Amplicon Library Preparation User Guide, Fusion Method (Life Technologies, publication number 4468326, revision C). The forward primer contained the Ion A adapter, followed by a 12-nucleotide Golay barcode sequence (Hamady *et al.*, 2008), a 4-nucleotide key sequence (TCAG) and the F515 primer sequence. The reverse primer was composed of the trP1 adapter and the R926 primer sequence. Triplicate PCR reactions were performed with the following thermocycling program: initial denaturation at 95°C for 4 min, followed by 25 or 30 cycles of 95°C for 1 min, 55°C for 45 s, 72°C for 1 min and a final elongation step at 72°C for 10 min. PCR products were visible in EtBr-stained agarose gels after 25 cycles for non-methanogenic microcosms and after 30 cycles for methanogenic microcosms. No products were visible from DNA extraction controls or PCR negative controls after at least 35 cycles.

Triplicate PCR products were pooled (7 µl from each reaction) and purified twice with Agencourt AMPure XP (Beckman Coulter, High Wycombe, UK) following the instructions on the Ion Amplicon Library Preparation User Guide, Fusion Method. The only modifications were that the ratio of AMPure XP reagent to sample was 1.1 to 1 instead of 1.8 to 1, and that the products were eluted in nuclease-free water instead of TE buffer. Purity, size (c. 480 bp) and concentration of purified products were verified with a Bioanalyzer 2100 instrument and a High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). Products were diluted to 26 pM and pooled to make an equimolar multiplex library. Templating, enrichment and quantification for Ion Torrent sequencing were performed using the Ion OT 2400 Kit and Ion OneTouch ES system (Life Technologies) according to the manufacturer's instructions (catalogue number 4479878, publication number MAN0007218, revision 2.0). Sequencing was performed on

an Ion PGM (Life Technologies) using 400-bp sequencing kits (catalogue number 4482002, publication number MAN0007242, revision 2.0). The default settings of the Torrent Suite software, version 4.0.2, were used for base calling. A fastq file was generated and further analyses were conducted with mothur (Schloss *et al.*, 2009) software, version 1.35.1. Reads were initially trimmed using trim.seqs with the following parameters: maxambig = 0, maxhomop = 6, bdiffs = 0, pdiffs = 0, minlength = 200, keepfirst = 400, flip = F. Further data processing, including ANOSIM tests, was conducted following the guidelines on [http://mothur.org/wiki/454\\_SOP](http://mothur.org/wiki/454_SOP) (Schloss *et al.*, 2011), accessed in July 2015, with minor modifications. Representative sequences of OTUs, as obtained with mothur's get.oturep function, are available in the Supporting Information (fasta file). Raw amplicon sequence data (fastq format) generated in this study are available through the NCBI Sequence Read Archive (Bio-Project accession number PRJNA509331).

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

#### Appendix S1. Supporting Information.

**Fig. S1.** APPI-P class distribution [A], consolidated DBE distribution [B], and consolidated carbon distribution [C] of unincubated bitumen (oil sands) and bitumen after the 3050-day experiments. No significant changes in compound class, DBE and/or carbon number distribution were observed throughout the sample set.

**Fig. S2.** OTUs found in  $\geq 0.5\%$  relative abundance in either the oil sands or in the formation water (FW) libraries that

were also found in  $\geq 0.5\%$  relative abundance in at least one of the 3050-day microcosms

#### Appendix S2. Supporting Information.

**Table S1** OTU identification and abundance for each 16S rRNA gene library. Only OTUs with relative abundance  $\geq 0.5\%$  are listed for each library. All abundances are based on subsampled libraries (3000 reads). Numbers in parentheses are bootstrap values for the taxonomic assignment.

**Table S2.** OTUs with highest relative sequence abundances in 16S rRNA gene amplicon libraries for Athabasca oil sands formation water. These OTUs represent 100% of bacterial clone libraries B1, B2 and B3 and 71% of the 3000-read Ion Torrent library.

**Table S3.** Chemical and physical properties of various crude oils and their associated methanogenesis rates in oil-to-methane experiments (Cai *et al.*, 2015; Gieg *et al.*, 2010; Gieg *et al.*, 2008; Jones *et al.*, 2008; Cheng *et al.*, 2014; Berdugo-Clavijo and Gieg, 2014; Townsend *et al.*, 2003; Xia *et al.*, 2016; Wang *et al.*, 2012).