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LETTERS

Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs

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Biodegradation of crude oil in subsurface petroleum reservoirs has adversely affected the majority of the world's oil, making recovery and refining of that oil more costly[1]. The prevalent occurrence of biodegradation in shallow subsurface petroleum reservoirs[2,3] has been attributed to aerobic bacterial hydrocarbon degradation stimulated by surface recharge of oxygen-bearing meteoric waters[2]. This hypothesis is empirically supported by the likelihood of encountering biodegraded oils at higher levels of degradation in reservoirs near the surface[4,5]. More recent findings, however, suggest that anaerobic degradation processes dominate subsurface sedimentary environments[6], despite slow reaction kinetics and uncertainty as to the actual degradation pathways occurring in oil reservoirs. Here we use laboratory experiments in microcosms monitoring the hydrocarbon composition of degraded oils and generated gases, together with the carbon isotopic compositions of gas and oil samples taken at wellheads and a Rayleigh isotope fractionation box model, to elucidate the probable mechanisms of hydrocarbon degradation in reservoirs. We find that crude-oil hydrocarbon degradation under methanogenic conditions in the laboratory mimics the characteristic sequential removal of compound classes seen in reservoir-degraded petroleum. The initial preferential removal of *n*-alkanes generates close to stoichiometric amounts of methane, principally by hydrogenotrophic methanogenesis. Our data imply a common methanogenic biodegradation mechanism in subsurface degraded oil reservoirs, resulting in consistent patterns of hydrocarbon alteration, and the common association of dry gas with severely degraded oils observed worldwide. Energy recovery from oilfields in the form of methane, based on accelerating natural methanogenic biodegradation, may offer a route to economic production of difficult-to-recover energy from oilfields.

The dominance of anaerobic hydrocarbon degradation, including methanogenesis, in subsurface biodegradation of oil is supported by the lack of sufficiently oxygenated formation waters to oxidize subsurface petroleum[7], the presence of anaerobic microorganisms in formation waters[8,9], the demonstration of anaerobic hydrocarbon degradation in laboratories[10,11] and metabolites of anaerobic hydrocarbon degradation in reservoir degraded petroleum[12]. Nevertheless, although many processes, including reduction of sulphate[10], nitrate[13] or iron[14] and methanogenesis, can be linked to hydrocarbon degradation[11,15], the specific pathway occurring in oil reservoirs remains poorly defined even though the primary controls of reservoir temperature history on biodegradation are well understood[6]. As most degraded oilfields show little evidence of sulphate reduction or carbonate cementation, despite removal of large proportions of petroleum carbon by microbial oxidation, we speculated that methanogenesis is the likeliest fate of most carbon dioxide produced as the terminal oxidation product during biodegradation[6]. Oilfield methane carbon isotopic signatures alone are equivocal in terms of indicating process, so we performed laboratory experiments to monitor the hydrocarbon composition of degraded oils and generated gases to elucidate probable mechanisms of hydrocarbon degradation in reservoirs.

Biodegradation mineralizes up to 60 weight per cent (wt%) of the non-degraded oil[6,16]. The fate of most of the mineralized carbon remains contested because, usually, gases produced from heavily degraded oilfields contain only marginally more CO_2 than gases produced from nearby non-degraded fields[17]. Heavily biodegraded oilfields are typically associated with dry methane-rich gases, possibly enriched in methane from biodegradation of C_2–C_5 alkanes[18] or from methanogenic oil biodegradation[17].

The carbon isotopic composition of methane ($\delta^{13}C_{CH4}$) associated with most subsurface biodegraded oil in marine petroleum systems ranges from −45‰ to −55‰ relative to the Pee Dee Belemnite (PDB) standard[17]; however, complex reservoir filling, degradation and mixing histories usually obscure definitive isotopic signals of origin. In contrast, the $\delta^{13}C_{CO2}$ associated with biodegraded oils is often isotopically very heavy, with values ranging from −25‰ to a maximum of +15‰ to +20‰ ($n = 7$ oilfields), indicative of closed system reduction of carbon dioxide to methane[17,19–21]. The subsurface mass balance of methane production over geological time is hampered by gas loss from shallow leaky biodegraded oilfields, so we examined the molecular systematics of hydrocarbon degradation under methanogenic or sulphate-reducing conditions, those most plausible for subsurface microbial hydrocarbon degradation.

The effect of (putatively anaerobic) biodegradation on the saturated hydrocarbon composition of crude oils in subsurface reservoirs is well documented, with a common sequence of removal of different compound classes. The most degradable compounds are straight-chain *n*-alkanes, followed by more resistant branched acyclic and monocyclic hydrocarbons, the most resistant polycyclic steroidal and triterpenoidal hydrocarbons[3,22–24], and some aromatic hydrocarbons[22,24]. In biodegraded reservoirs, alkylated naphthalenes and other two- and three-ringed aromatic hydrocarbons are only degraded after significant removal of *n*-alkanes and alteration of acyclic isoprenoids such as pristane and phytane[22,24,25]. In contrast, crude oils degraded under aerobic conditions in the laboratory can show removal of aromatic compounds such as alkylated naphthalenes before the alteration of *n*-alkanes[26]. Townsend *et al.*[27] observed that although the entire *n*-alkane fraction of a weathered Alaskan North Slope crude oil was consumed under both methanogenic and sulphate-reducing conditions in a laboratory microcosm, only the sulphate-reducing systems showed depletion of alkylnaphthalenes after 14 months of incubation. These observations demonstrate

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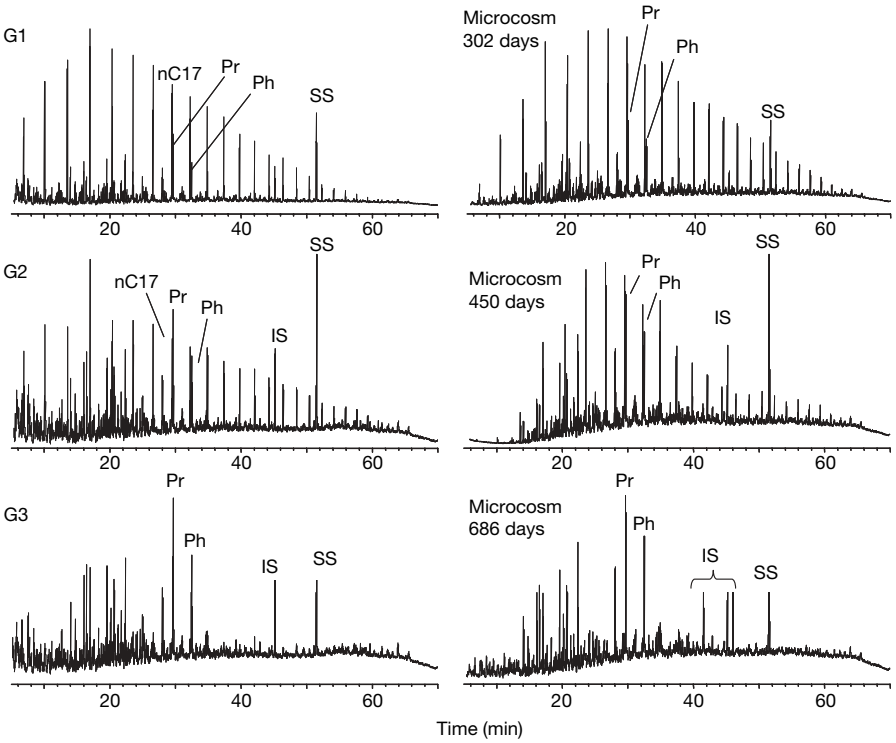

Figure 1 | Gas chromatograms of total hydrocarbon fractions from field and laboratory degraded oils. Left column, three North Sea (Gullfaks field) oils of different biodegradation levels (G1, G2 and G3). Right column, altered oils from three laboratory methanogenic microcosm experiments showing increasing levels of hydrocarbon biodegradation. Both suites of samples show systematic relative removal of *n*-alkanes relative to the isoprenoid alkanes pristane (Pr) and phytane (Ph). For clarity, peaks for spiked standards (marked IS and SS) have been truncated in samples G3 and the 686-day microcosm extract. Time 0 on the chromatograms, which are displayed from 5 to 70 minutes, corresponds to injection.

that biodegradation conditions do affect the relative rate of removal of different hydrocarbon classes.

The composition of biodegraded petroleum results from biodegradation overprinting the complex charging/filling/spilling/mixing history of the petroleum entering the trap. Thus comparison of global biodegraded oil compositions with those measured from degrading laboratory microcosms using a single starting oil sample is difficult and inappropriate. To assess the reaction pathways of crude-oil degradation under anaerobic conditions in laboratory microcosms we used a non-degraded oil from a North Sea field that shares a common source rock with biodegraded oil from the Gullfaks field. Gullfaks oil has been altered by biodegradation to varying degrees[7] and the field appears to have filled quickly with little evidence of any later charge with non-degraded oil (Fig. 1). Furthermore the reservoir is highly overpressured[28], containing saline formation waters

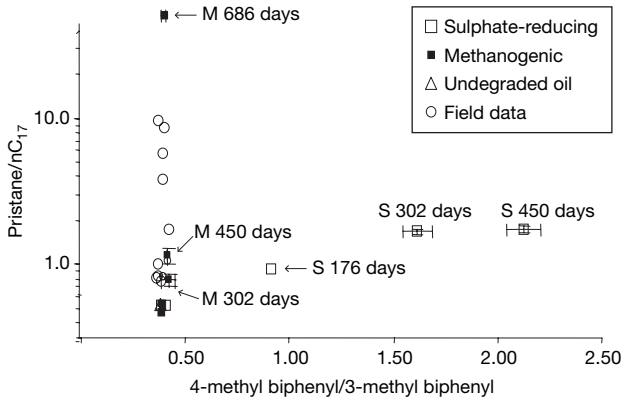

Figure 2 | Plot of the ratios of pristane to n-heptadecane against 4-methylbiphenyl to 3-methylbiphenyl abundances. Samples are from North Sea crude oil degraded in laboratory anaerobic microcosm experiments and from North Sea Gullfaks field crude oil. Error bars for the peak ratios are ±1 standard error ($n = 3$). M refers to laboratory incubations under methanogenic conditions and S refers to incubations under sulphate-reducing conditions. The M 686-day sample had complete removal of nC_{17} and the other *n*-alkanes. The laboratory methanogenic microcosm data and the field data plot along the same biodegradation trajectory with *n*-alkane degradation but no apparent aromatic hydrocarbon degradation, while the sulphate-reducing microcosm data show concomitant degradation of *n*-alkanes and aromatic hydrocarbons.

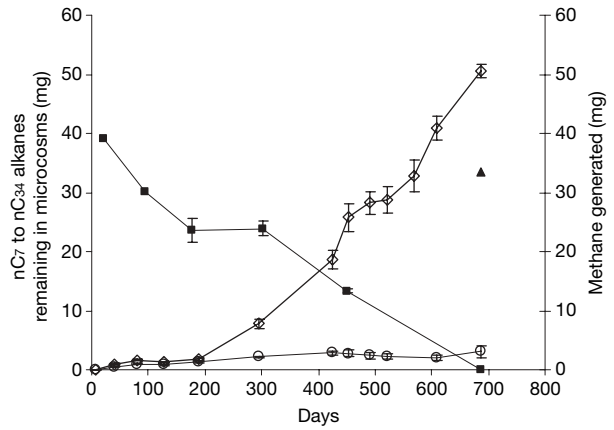

Figure 3 | *n*-alkane depletion and methane production during oil degradation. Loss of C_7–C_{34} *n*-alkanes (squares) and methane production (diamonds) from crude oil incubated under methanogenic conditions in laboratory microcosms (100 ml) for 686 days. The error bars show ±1 standard error ($n = 3$). Methane production from control microcosms without oil (open circles) are also shown for comparison to confirm the conversion of oil hydrocarbons to methane. The triangle represents the theoretical maximum methane production if all the *n*-alkanes in the added oil had been converted to methane with the stoichiometry described in ref. 11.

and thus has probably not received recent meteoric water to stimulate aerobic degradation.

The ratios of easily degradable to more resistant saturated and aromatic hydrocarbons (n-heptadecane/pristane and 4-methylbiphenyl/3-methylbiphenyl) from the Gullfaks oils are similar to those from oil degraded in the laboratory under methanogenic conditions (Fig. 2) with *n*-alkane degradation occurring before significant aromatic hydrocarbon destruction. This is clearly different from oils degraded under sulphate-reducing conditions where aromatic hydrocarbon degradation occurs concomitantly with alkane degradation. Alkylbiphenyl degradation is used here as a proxy for more pervasive aromatic hydrocarbon degradation, because alkylbiphenyls are not lost during sample workup.

Oil alkane conversion to methane in oil-degrading microcosms was confirmed by comparison of the methane yield in control microcosms that were not amended with oil (Fig. 3). Also, negligible amounts of methane with no obvious degradation of the oil *n*-alkanes were detected in microcosms containing an inhibitor of methanogenesis (2-bromethanesulphonic acid, BES). Furthermore, $^{13}CH_4$ enriched gas was produced in microcosms where the oil was spiked (7 atom% of *n*-alkanes) with labelled ^{13}C-hexadecane (Supplementary Fig. 3). The amount of methane produced (Fig. 3) is similar to that expected from methanogenic degradation of *n*-alkanes, using water as the co-reactant, as proposed by refs 11 and 15, though the mass of methane generated exceeded that predicted from the stoichiometric conversion of only *n*-alkanes in the crude oil (Fig. 3). The additional methane was probably from degradation of other hydrocarbons in the oil. We have previously[16,29] shown that the apparent sequential removal of *n*-alkanes and then branched alkanes during biodegradation is illusory and that several classes of compounds are removed in parallel to *n*-alkane removal but at lower rates.

The theoretical methane yield from alkane degradation is reflected in field gas isotope data. Carbon dioxide associated with heavily biodegraded oil reservoirs is highly enriched in ^{13}C, with $\delta^{13}C_{CO2}$ values up to +15 to +20‰ PDB. Figure 4 shows significantly enriched carbon dioxide and depleted methane carbon isotopic signatures from solution gases from heavily degraded oils (Peters and Moldowan[23], PM levels 4 to 5) of the Peace River oil sands area, compared to gases from less degraded oil fields (Gething) and pristine thermogenic gases found west of the Canadian oil sands[30]. Theoretically predicted curves (Fig. 4), represent the gas compositions at various levels of biodegradation (PM levels 1 to 9), assuming methane is derived from acetoclastic methanogenesis and reduction of carbon dioxide, with varying degrees of hydrocarbon conversion. With increasing hydrocarbon conversion, CO_2 becomes isotopically enriched, with methane showing a more complex depletion and enrichment curve.

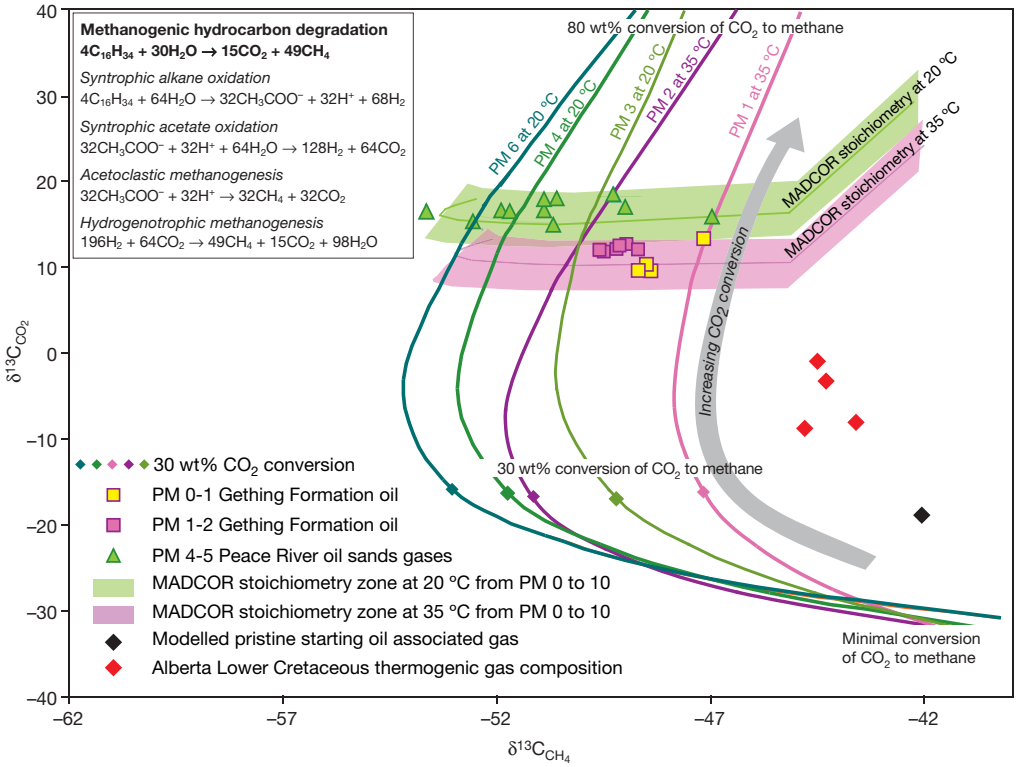

Figure 4 | Measured and modelled methane and carbon dioxide stable carbon isotopic compositions from degraded oil reservoir gases. Carbon isotopic composition of carbon dioxide and methane from gases co-produced with highly degraded oils from the Peace River Oil Sands area of western Canada. Data from gases associated with an equivalent non-biodegraded oil (initial oil) are also shown. The modelled solid lines on the diagram represent theoretically derived trajectories for the evolution of the gas compositions with increasing levels of biodegradation, as reflected by a PM scale equivalent (PM levels 1 to 9). The grey arrow indicates the direction of change in isotopic composition as progressively more CO_2 is reduced to methane. The isotopic composition is calculated assuming closed-Rayleigh fractionation using both hydrogenotrophic and acetoclastic methanogenesis, as described in the Supplementary Information. The region predicted to be occupied by gases produced with the stoichiometry suggested by MADCOR is shaded in green and pink. The different isotopic trajectories show the evolution of gas composition as a function of carbon dioxide conversion to methane when the crude-oil hydrocarbons are degraded to different extents. Two data sets are plotted.The Peace River bitumens (reservoir temperature 20 °C) are typically degraded to PM level 5, suggesting that the +15‰ to +20‰ compositions for the carbon in carbon dioxide reflect the conversion of approximately 60–65 wt% of alkane carbon to methane. The Gething Formation reservoired oils (reservoir temperature 35 °C) are typically degraded to PM levels 1 to 2, suggesting that the +10‰ to +12‰ compositions for the carbon in carbon dioxide again reflect approximately 60–65 wt% of alkane carbon to methane. This is in broad agreement with the conversion of 76 wt% alkane carbon to methane, as indicated by the MADCOR stoichiometry, which (although mechanistically different in detail) is that reported in ref. 11. The coloured boxes indicate the range of isotopic compositions of methane and CO_2 for the two reservoirs, assuming that approximately 80% of the methane is produced from hydrogenotrophic methanogenesis. Observation and modelled prediction broadly agree.

Closed-system Rayleigh isotope fractionation of methanogenic *n*-alkane degradation was modelled using published temperature-dependent carbon isotope fractionation factors, for acetoclastic and hydrogenotrophic methanogenesis (Supplementary Methods). We considered oil biodegraded to different degrees, assessed using mass of hydrocarbon lost. Degradation levels were converted to equivalent PM levels of degradation using data from naturally biodegraded oils. Degrading oil to PM levels of 3 to 5 with ~62.5 wt% CO_2 conversion to methane, based on the stoichiometry of methanogenic alkane degradation[11] (see reaction pathways on Fig. 4), predicts terminal $\delta^{13}C_{CO2}$ values of +15 to +20‰ PDB at 20 °C, which is coincident with CO_2 carbon isotopic compositions of most gases at present-day reservoir conditions in the Peace River oil sands (Fig. 4). The range of $\delta^{13}C_{CH4}$ from −47‰ to −52‰ can be explained by variation in oil charge and leakage of gases at shallow burial depths (Supplementary Information). To predict field data accurately, a combination of acetoclastic and hydrogenotrophic methanogenesis was necessary. The best fit to data was obtained with 75%–92% of the methanogenesis channelled through hydrogenotrophic methanogenesis. This is consistent with the methanogen community composition observed in oil-degrading microcosms that were dominated by hydrogen-oxidizers (86%–87% of clones in archaeal 16S ribosomal RNA gene libraries; Supplementary Table 2).

Thus, highly enriched CO_2 carbon isotopic signatures in heavily degraded oils[18] and laboratory data both suggest that methanogenic hydrocarbon degradation occurs predominantly via syntrophic oxidation of alkanes to acetate and hydrogen. This assumption is corroborated by the dominance of *Syntrophus* sp. in the methanogenic microcosms (Supplementary Fig. 4), similar to those identified in ref. 11. However, about 75% of the carbon must be directly channelled through hydrogenotrophic methanogenesis, implying that the acetate must be oxidized syntrophically to CO_2 and hydrogen before being converted to methane. We refer to this process, which we infer to be the predominant biodegradation process in reservoirs, as methanogenic alkane degradation dominated by CO_2 reduction (MADCOR). The occurrence of syntrophic acetate oxidation in oil-degrading microcosms, which would be required to explain the predominance of CO_2 reduction, was demonstrated using ^{13}C-labelled acetate (Supplementary Information). The effect on $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$ of reducing increasing amounts of CO_2 to methane with hydrogen is illustrated in Fig. 4. We note that field data correspond to the MADCOR stoichiometry, indicating that no external source of hydrogen is required to explain observed gas isotope values or the predominance of methanogenic CO_2 reduction. Thus all hydrogen required is generated during syntrophic alkane oxidation and coupled syntrophic acetate oxidation.

The occurrence of anaerobic degradation of crude oils in subsurface reservoirs under methanogenic conditions explains the consistent hydrocarbon compositional patterns seen in degraded oils worldwide and their association with dry gas accumulations[17,18]. This identification of the pathways inherent in subsurface biodegradation facilitates the engineering of processes to accelerate naturally slow methanogenic biodegradation to recover energy from heavy oilfields as methane, rather than oil. Such processes could enable us to move away from high-CO_2-emitting heavy-oil recovery technologies while maintaining existing infrastructures.

METHODS SUMMARY

Laboratory crude-oil degradation experiments were set up under anoxic conditions in glass 120 ml serum bottle microcosms. Each microcosm comprised a carbonate-buffered brackish medium containing sources of nitrogen and phosphorus, vitamins, and trace minerals, made up with deionized water and sediment inoculum. Sulphate-reducing microcosms were established by the addition of SO_4^{2-}. Methanogenic microcosms were established by the exclusion of added electron acceptors. North Sea crude oil (300 mg) was added to all microcosms except control flasks to determine the extent of methanogenesis in the absence of crude oil. Headspace gas aliquots were periodically removed from the microcosms and analysed for carbon dioxide and methane using gas chromatography-mass spectrometry (GC-MS). After removal of an aliquot for microbiological analyses, the contents of the microcosm bottles from each sampling point were subjected to alkaline saponification and liquid–liquid solvent extraction to obtain the organic soluble fractions. Aliquots of oils or the solvent extracts were separated into saturated and aromatic hydrocarbon fractions, before their analysis by GC-MS.

Bacterial and archaeal 16S ribosomal RNA genes were amplified by polymerase chain reaction (PCR) from microcosm DNA using oligonucleotide primers specific for bacteria and archaea, respectively. Gene fragments were subsequently purified, cloned, sequenced and subjected to comparative analysis to assign phylogenetic identity.

Gas and oil samples were taken at wellheads in sealable metal containers after extensive purging of collection lines with fresh well fluids. Gases were analysed by gas chromatography using thermal conductivity detection to determine composition. Carbon isotopic compositions of carbon dioxide and methane were determined using gas chromatography-isotope ratio mass spectrometry at a commercial laboratory.

Closed-system Rayleigh isotope fractionation was simulated isothermally at reservoir temperatures for the carbon species involved in biodegradation of petroleum via syntrophic alkane oxidation, syntrophic acetate oxidation, acetoclastic methanogenesis and hydrogenotrophic methanogenesis.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Microcosms. Anaerobic microcosms were set up in glass 120-ml serum bottles sealed with butyl rubber stoppers and aluminium crimps (Aldrich). An anaerobic cabinet (Coy Laboratory Products) fitted with an oxygen sensor and with a regulated atmosphere of nitrogen (99%) and hydrogen (1%) (BOC Special Gases) was used in the preparation and incubation of the microcosms. Each microcosm comprised a carbonate-buffered nutrient medium containing sources of nitrogen and phosphorus, vitamins and trace minerals, made up in deionized water, according to the brackish medium of ref. 31 and 10 g of brackish sediment inoculum (from the River Tyne in Newcastle, which preliminary studies had shown were capable of anaerobic hydrocarbon degradation). The total volume of liquid was 100 ml with 20 ml of headspace volume. Sulphate-reducing microcosms were established by the addition of SO_4^{2-}. Methanogenic microcosms were established by the exclusion of added electron acceptors. North Sea crude oil (300 mg) was added to all microcosms except the control flasks used to determine the extent of methanogenesis in the absence of crude oil. Microcosms were prepared in triplicate and control microcosms (that is, where inhibitors or no exogenous electron donors were added, or where microcosms had been subjected to heat treatment) were included. Experimental reproducibility for the different laboratory microcosm experiments are shown with the data in Figs 2 and 3 of the paper.

Hydrocarbon analysis. Headspace gas (10 ml) was removed, periodically, throughout the course of the experiment from all microcosms and injected into evacuated gas tubes. The gas removed was replaced by 10 ml of gas taken from within the purged anaerobic cabinet. Gas samples were analysed for CH_4 using a GC-MS. Peak areas were calibrated using a methane gas standard (BOC) and the reproducibility ($n = 4$) of replicate standard analyses were typically less than 1% relative standard deviation.

After removal of an aliquot of sediment slurry (typically 20 ml) for microbiological analyses, the contents of the microcosm bottles from each sampling point were subjected to alkaline saponification (reflux for 1 h with 1 M KOH in methanol; VWR) followed by acidification (HCl from VWR), Buchner filtration to remove sediment, and liquid–liquid solvent extraction with dichloromethane (DCM from VWR) and methanol to obtain the organic soluble fractions.

Aliquots (about 50 mg or less) of oils or the solvent extracts from the microcosms were separated into total hydrocarbon fractions using the solid-phase-extraction method[32]. These hydrocarbon fractions were either analysed directly by gas chromatography, or further separated by silver nitrate impregnated silica gel solid-phase-extraction chromatography into saturated and aromatic hydrocarbon fractions[33], before their analyses by GC-MS. *n*-alkane quantitation was achieved by the addition of internal standards (squalane and n-heptadecylcyclohexane, from VWR and Lancaster Synthesis, respectively). Relative response factors for a mixture of *n*-alkanes from C_{12}–C_{32} (VWR) against the internal standards were measured and a calibration curve was established to provide response factors for the *n*-alkanes quantified.

Gas chromatography on the separated hydrocarbon fractions was carried out on a Hewlett Packard 5890 gas chromatograph fitted with a HP-5 coated capillary column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness) and a flame ionization detector. Data acquisition and processing was done using a Thermo LabSystems Atlas chromatography data system. The analytical reproducibility for replicate analyses ($n = 4$) of the C_{12}–C_{35} *n*-alkanes in the oil used was 0.84% relative standard deviation. GC-MS analysis on the hydrocarbons was performed in full scan mode on an Agilent 6890-5973 system and the chromatographic conditions used were the same as those for the GC analyses. Some analyses were also carried out under similar conditions using a Varian 1200 GC-MS-MS system.

Microbial community analysis. DNA extraction (FastDNA Spin Kit for Soil from Q-BIOgene) was performed on microcosm aliquots (10 ml), according to the manufacturer's instructions. Bacterial 16S rRNA genes were amplified using primers pA and pH (ref. 34). Archaeal l6S rRNA genes were amplified using primers Arch46 (ref. 35) and Arch1017 (ref. 36). Cloning of PCR-amplified DNA (~1,500 base pairs for bacteria and ~1,000 bp for archaea) was performed with a TOPO TA cloning kit (Invitrogen). Individual colonies were randomly chosen and amplified by PCR using the vector-specific primers pUCf. (5′-GTT TTC CCA GTC ACG AC-3′) and pUCr (5'-CAG GAA ACA GCT ATG AC-3′). Before sequencing, primers were removed from the amplified inserts with ExoSAP-IT (GE Healthcare), which were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3730xl DNA sequencer (Applied Biosystems). Sequence data was compared to the EMBL Nucleotide Sequence Database at the European Bioinformatics Institute using Fasta3 (ref. 37) to identify the nearest neighbours.

Gas isotope analysis and modelling. Gas and oil samples were taken at wellheads in sealable metal containers after extensive purging of collection lines with fresh well fluids. Gases were analysed by gas chromatography using thermal conductivity detection to determine composition. Carbon isotopic compositions of carbon dioxide and methane were determined using gas chromatography-isotope ratio mass spectrometry at a commercial laboratory. The carbon dioxide isotopic compositions were determined directly after separation of the carbon dioxide using gas chromatography while methane was converted to carbon dioxide online by combustion over copper oxide. Isotopic compositions are reported relative to the PDB standard.

Closed-system Rayleigh isotope fractionation was simulated isothermally at reservoir temperatures for the carbon species involved in biodegradation of petroleum via syntrophic alkane oxidation, syntrophic acetate oxidation, acetoclastic methanogenesis and hydrogenotrophic methanogenesis. The model assumes that after a single initial instantaneous oil charge into the reservoir, there is no influx or leakage from the reservoir of gas of any species and complete instantaneous mixing is assumed. All degradable petroleum mass is given the stoichiometry of *n*-alkanes (C_nH_{2n+2}) with uniform isotopic composition. Temperature-dependent relationships for isotopic fractionation factors for acetoclastic and hydrogenotrophic methanogenesis were developed from the literature for a range of methanogenic taxa, including mesophiles and thermophiles. A more detailed description of the model is given in the Supplementary Information isotopic modelling section.

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